Habilitation Thesis

Engineering of bone and cartilage like tissue at the interface of drug delivery and biomaterials

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Publication Date:
2006

Permanent Link:
https://doi.org/10.3929/ethz-a-005424864

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Engineering of bone and cartilage like tissue at the interface of drug delivery and biomaterials

Habilitation Thesis

presented by
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August 2006
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Preface

This work summarizes our efforts at the interface of drug delivery, the engineering of tissues, and biomaterials. Within that fields, new aspects were identified, such as detailing the use of silk-fibroin scaffolds within the broader context of the engineering of muskuloskeletal and other tissues. Another example is the use of various delivery strategies for biorelevant molecules, such as drug delivery from polymers, drug delivery from genetically engineered cells, or drug delivery from cells transformed by use of viral vectors. These findings expand the available options in the growing field of tissue engineering. During our work, I enjoyed many exciting moments together with my colleagues. My cordial thanks go to David Kaplan and Gordana Vunjak-Novakovic. Without the constant support of my friends, the results wouldn’t have been possible and they always assisted us to make our research run smoothly. I am in particular gratified to Hans Peter Merkle. He offered me the chance to build up my research, provided me the framework in which we could jointly embark towards a new research focus, the work at the interface of drug delivery and tissue engineering. My research team at ETH with Sandra Hofmann, Lorenz Uebersax, Henri Hagenmueller, and Esther Wenk significantly contributed to what is summarized herewithin. This work wouldn’t have been possible without the highly valuable contributions of many other colleagues and friends, more particularly Brigitte von Rechenberg, Ralph Mueller, Robert Langer, Robert Fajardo, and Vassilis Karageorgiou. Finally, I may also thank several sources of funding who have supported our research, including the Alexander von Humboldt Foundation, the “TH Gesuch” of ETH Zurich, the National Science Foundation of the USA, the Association of Orthopaedic Research, the AO Foundation, the BEST Cluster of ETH Zurich, Wyeth Pharma, Novartis Pharma AG, Trudel Inc., Jossi AG, the Swiss National Foundation, and the European Union.
to my parents
Chapter summary

Lorenz Meinel
Tissue engineering, simply described as the combination of biomaterials and cells are cultivated, multiplied, and altered in vitro, is seen in the contemporary international discussion as promising for the production of implantable tissues. The materials are not simply layered with cells, but rather functioned to a three dimensional extracellular matrix, which is in various ways enabled with cellular regulatory signals and is quasi penetrated by the cells. A central goal is, aside from mechanically supportive function, to imitate the functional interplay between cells and their natural extracellular matrix, so important for cell splitting, differentiation, movement, attachment, etc., and finally death. A further goal is, via the introduction of cells, to make use of the unsurpassed synthesis and regulatory function and their capacity for constant renewability. With regard to the respective stage of development, three types of cells are distinguishable: differentiated cells, stem cells (Chapters 2-10), and embryonic stem cells (Chapter 11). The use of differentiated cells is limited, especially because only some types of cells can be multiplied to reach significant numbers, or by means of de-differentiation. However, the field of de-differentiation remains, the efforts of many years notwithstanding, still in its opening phase. The in vitro use of stem cells is considered much more auspicious. Stem cells or progenitor cells are found in almost all adult tissues. These cells are easily accessible and can be expanded to billions of cells in vitro from a single tissue aspirate. Bone marrow in particular is a rich and easily accessible source, e.g. of mesenchymal stem cells.

Some of these complex bio-artificial systems have found their ways in the pre-clincial phases and make just clear how much development has taken place recently. First licenses for clinical use have already been issued in the USA for bio-artifical constructs to replace skin and cartilage.

The following inquiries and reflections summarized in this work are intended to be a contribution to the field of tissue engineering. The task is three fold: firstly to provide a view of the present scientific state at the interface of drug delivery, biomaterials, bioreactors, stem cells, and tissue engineering of mesenchymal tissues (Chapter 1). Secondly, and by employment of multidisciplinary approaches, it is to provide novel ways of controlling the engineering of bone-like and autologous tissues to match complex bone geometries as found within our skeletons and for their functional replacement (Chapters 2-5). Thirdly, approaches of providing regulator molecule supply, as needed for the guidance of directed and selective stem cell differentiation
are provided, by means of protein drug delivery from scaffold materials (Chapters 6),
decorating of scaffolds (Chapter 7), physical adsorption (Chapter 7, 8) or using
adenovirally transformed stem cells, transiently releasing regulatory molecules which
in return trigger their differentiation in an autocrine and paracrine manner (Chapter
9), or employing genetically modified and stably transfected embryonic stem cells,
which continuously release regulatory molecules (Chapter 11). The main target tissue
of this contribution is on the engineering of bone-like tissues from mesenchymal stem
cells (Chapters 2-9) but exemplarily other tissues were engineered such as cartilage-
like tissue (Chapter 10), or neural differentiation of embryonic stem cells (Chapter
11).

In Chapter 1, silk fibroin is introduced as a biopolymer for scaffold manufacture. Silk
fibroin is a particularly interesting biopolymer for scaffold designs, as it is a
biocompatible and mechanically robust biomaterial. Stem cells were successfully used
along with silk fibroin scaffolds for the engineering of bone, cartilage and ligament
tissues. Furthermore, the promising as well as problematic considerations are provided
for the use of human adult stem cells, along with the expectations on the part of the
patients, scientists, and medical professions. The third overview is on bioreactors,
needed for different purposes in tissue engineering such as the homogenous seeding of
cells throughout scaffolds, control of culture conditions including temperature, pH,
nutrients, metabolites, levels of oxygen, the enhancement of molecule transfer between
the cells and the culture medium or to provide physiologically relevant physical
signals, such as interstitial fluid flow within the scaffold, mechanical compression and
shear.

In Chapter 2 we used 3D biomaterial matrices from silk fibroin with controlled pore
diameter and pore interconnectivity, and utilized these to engineer bone starting from
human mesenchymal stem cells (hMSC). Osteogenic differentiation of hMSC seeded
on these scaffolds resulted in extensive mineralization, alkaline phosphatase activity,
and the formation of interconnected trabecular- or cortical-like mineralized networks
as a function of the scaffold design utilized; allowing mineralized features of the tissue
engineered bone to be dictated by the scaffold features used initially in the cell culture
process. This approach to scaffold predictors of tissue structure expands the window of
applications for silk fibroin based biomaterials into the realm of directing the
formation of complex tissue architecture. As a result of slow degradation inherent to
silk fibroin, scaffolds preserved their initial morphology and provided a stable template during the mineralization phase of stem cells progressing through osteogenic differentiation and new extracellular matrix formation. The slow degradation feature also facilitated transport throughout the 3D scaffolds to foster improved homogeneity of new tissue; avoiding regions with decreased cellular density. The ability to direct bone morphology via scaffold design suggests new options in the use of biodegradable scaffolds to control in vitro engineered bone tissue outcomes.

Chapter 3 started off from these findings and extended the control of one architecture on a single scaffold as previously demonstrated (Chapter 2) to the control of different architectures on a single scaffold. This was motivated by the natural constraints, with native bone consisting of different trabecular geometries. This study aimed at engineering different bone geometries with small pores (112-224) µm in diameter on one side of the scaffold, and large pores (400-500) µm in diameter on the other, while keeping scaffold porosities constant among groups. We hypothesized that tissue engineered bone morphology in silk fibroin (SF) implants is pre-determined by the scaffold’s geometry. To test this hypothesis, SF scaffolds with the different pore diameters were prepared and seeded with human mesenchymal stem cells (hMSC). Dynamic cell seeding procedure in spinner flasks was selected for the differentiation studies, as it resulted in equal cell viability and proliferation and better cell distribution throughout the scaffold as visualized by histology and confocal microscopy and compared to static seeding. Differentiation of hMSC in osteogenic cell culture medium in spinner flasks for three and five weeks resulted in increased alkaline phosphatase activity and calcium deposition when compared to control medium. Micro-computed tomography (µCT) detailed the pore geometries of the newly formed tissue and suggested that the morphology of engineered bone tissue was controlled by the underlying scaffold geometry. In ongoing experiments, we detail how these engineered bone-like structures translate into clinical benefits, using rodent defect models.

Chapter 4 aims at elucidating inherent correlations of various parameters of mineralization with time, in an effort to predict the amount of bone-like tissue formation. For that, human mesenchymal stem cells (hMSC) were cultured in osteogenic medium on silk-fibroin (SF) scaffolds, which were visualized and quantified over 44 days in culture using time-lapsed, nondestructive micro-computed tomography (µCT). Each construct was imaged 9 times in situ and images were
superimposed. µCT irradiation did not impact hMSC’s osteogenic performance in terms of DNA deposition, alkaline phosphatase activity and calcium deposition when compared to non exposed control samples. Bone-like tissue formation initiated at day 10 of culture with the deposition of small mineralized clusters which increased throughout the duration of culture. Mineral density increased linearly over time and did not correlate with bone volume. The surface-to-volume ratio of the bone-like tissues asymptotically converged to 26mm⁻¹. In conclusion, although in vitro formation of bone-like tissue started off from clusters, the overall bone volume was not predictable from time or amount of the initial number or size of bone mineralized clusters. A linear increase of mineral density was observed over time, and bone surface-to-volume ratio approximated towards a set value. This data demonstrated the feasibility to qualitatively and quantitatively detail the spatial and temporal mineralization of bone like tissue formation in tissue engineering.

**Chapter 5** details the in vivo relevance of engineered bone-like tissues, using a critical sized, segmental, femoral defect model in rats. Silk fibroin biomaterial scaffolds were evaluated as osteopromotive matrices. Four treatment groups were assessed over eight weeks in vivo: silk scaffolds (SS) with human mesenchymal stem cells (hMSC) that had previously been differentiated along an osteoblastic lineage in vitro (group I; pdHMSC/SS); SS with undifferentiated hMSCs (group II; SS/udHMSC); SS alone (group III; SS); and empty defects (group IV). When hMSCs were cultured in vitro in osteogenic medium for 5 weeks, bone formation was characterized with bimodal peak activities for alkaline phosphatase at 2 and 4 weeks. Calcium deposition started after 1 week and progressively increased to peak at 4 weeks, reaching cumulative levels of deposited calcium at 16 µg per mg scaffold wet weight. *In vivo* osteogenesis was characterized by almost bridged defects with newly formed bone after 8 weeks in group I. Significantly (p<0.01) greater bone volumes were observed with the pdHMSC/SS (group I) implants than with groups II, III or IV. These three groups failed to induce substantial new bone formation and resulted in the ingrowth of cells with fibroblast-like morphology into the defect zone. The implantation of pdHMSC/SS resulted in significantly (p<0.05) greater maximal load and torque when compared to the other treatment regimens. The pdHMSC/SS implants demonstrated osteogenic ability in vitro and capacity to thrive towards the healing of critical size femoral
segmental defects \textit{in vivo}. Thus, these new constructs provide an alternative protein-based biomaterial for load bearing applications.

**Chapter 6** is at the interface of drug delivery, biomaterials, and tissue engineering. The pharmaceutical utility of silk fibroin (SF) materials for drug delivery was investigated. SF films were prepared from aqueous solutions of the fibroin protein polymer and crystallinity was induced and controlled by methanol treatment. Dextrans of different molecular weights, as well as proteins, were physically entrapped into the drug delivery device during processing into films. Drug release kinetics were evaluated as a function of dextran molecular weight, and film crystallinity. Treatment with methanol resulted in an increase in \(\beta\)-sheet structure, an increase in crystallinity and an increase in film surface hydrophobicity determined by FTIR, X-ray and contact angle techniques, respectively. The increase in crystallinity resulted in the sustained release of dextrans of molecular weights ranging from 4 kDa to 40 kDa, whereas for less crystalline films sustained release was confined to the 40 kDa dextran. Protein release from the films was studied with horseradish peroxidase (HRP) and lysozyme (Lys) as model compounds. Enzyme release from the less crystalline films resulted in a biphasic release pattern, characterized by an initial release within the first 36 hours, followed by a lag phase and continuous release between days 3 and 11. No initial burst was observed for films with higher crystallinity and subsequent release patterns followed linear kinetics for HRP, or no substantial release for Lys. In conclusion, SF is an interesting polymer for drug delivery of polysaccharides and bioactive proteins due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered.

**Chapter 7** started off from these principal findings of suitability of silk-fibroin matrices for drug delivery, introducing a novel drug, the growth factor bone morphogenetic protein-2 (BMP-2), which plays a critical role in bone formation and regeneration. Therefore, the ability to immobilize this molecule in certain matrices may be crucial in bone tissue engineering. Using carbodiimide chemistry, BMP-2 was directly immobilized on silk fibroin films. Whereas human bone marrow stromal cells (BMSCs) cultured on unmodified silk fibroin films in the presence of osteogenic stimulants exhibited little if any osteogenesis, the same cells cultured on BMP-2 decorated films in the presence of osteogenic stimulants differentiated into an
osteoblastic lineage as assessed by their significantly elevated alkaline phosphatase activity, calcium deposition and higher transcript levels of collagen type I, bone sialoprotein, osteopontin, osteocalcin, BMP-2 and cbfa1. Using cell culture inserts, it was demonstrated that differentiation was induced by the immobilized protein and not by protein released into the culture medium. Comparison with a similar amount of medium supplemented BMP-2, where no additional protein was added with medium changes, showed that delivery of BMP-2 immobilized on the biomaterial surface was more efficient than soluble delivery. The results illustrate that BMP-2 covalently coupled on silk biomaterial matrices retains biological function in vitro based on the induction of osteogenic markers in seeded BMSCs.

The motivation for Chapter 8 was to demonstrate evidence for clinical relevance of the scaffolds developed in Chapters 6 and 7. Osteopromotive matrices are frequently employed for the local delivery and controlled release of augmentation agents such as BMP-2. Some also provide an osteoconductive scaffold to support new bone growth. In this study, silkworm-derived silk was evaluated as an osteopromotive matrix for healing critical sized mid-femoral segmental defects in nude rats. Four treatment groups were assessed over eight weeks: Silk scaffolds (SS) with recombinant human BMP-2 (rhBMP-2) and human mesenchymal stem cells (HMSC) that had been pre-differentiated along an osteoblastic lineage in vitro (group I; pdHMSC/rhBMP-2/SS); SS with rhBMP-2 and undifferentiated HMSCs (group II; udHMSC/rhBMP-2/SS); SS and rhBMP-2 alone (group III; rhBMP-2/SS); and empty defects (group IV). Bi-weekly radiographs revealed a progressive and similar increase in Group I-III mean defect mineralization through post-operative week (POW) 8. Radiographs, dual energy x-ray absorptiometry, and micro-computed tomography confirmed that Groups I-III exhibited similar substantial and significantly (p<0.05) greater defect mineralization at POW 8 than the unfilled Group IV defects which remained void of bone. However, the maximal torque and load before failure were significantly (p<0.05) less for Groups II and III than for Group I. Histology at POW 8 revealed moderately good bridging of the parent diaphyseal cortices with woven and lamellar bone bridging islands of silk matrix in Groups I and III. Group II defects possessed comparatively less new bone which was most abundant adjacent to the parent bone margins. Elsewhere the silk matrix was more often enveloped by poorly differentiated loose fibrous connective tissue. Group IV defects showed minimal new bone formation. None of the treatment
groups attained the mean mineralization or the mean biomechanical strength of identical defects implanted with SS and pdHMCs alone in a previous study. However, addition of rhBMP-2 to udHMSC/SS or SS prompted substantially more bone than was previously generated using udHMSC/SS or SS alone. These data imply the clinical potential of silk scaffold and rhBMP-2 as composite osteopromotive implants when used alone or with select stem cell populations. Additional studies in larger species are now warranted.

**Chapter 9 and 10** were motivated to circumvent the use of protein drugs prefabricated onto or into the scaffold matrices. **Chapter 9** describes the use of adenovirally transduced cells, which were developed to induce differentiation in an autocrine and paracrine manner, once seeded on the scaffold matrices. **Chapter 10** was - in contrast to all other chapters in which autocrine differentiation was intended for the engineering of tissues - a feasibility study for the use of genetically modified embryonic stem cells on silk fibroin scaffolds to treat diseases, characterized by adenosine deprivation, such as epilepsy.

**Chapter 9** introduces gene therapy as a mode of drug delivery, based on human mesenchymal stem cells (MSC) and silk fibroin biomaterials to study the impact of viral transfection on MSC osteogenic performance in vitro. MSCs were transduced with adenovirus containing a human BMP-2 (Ad-BMP-2) gene at clinically reasonable viral concentrations and cultured for 4 weeks. Controls with nontransfected MSCs, but exposed to exogenous BMP-2 concentrations on an analogous time profile as that secreted by the Ad-BMP-2 group, were compared. Both the Ad-BMP-2 MSC group and the exogenous protein BMP-2 group strongly expressed osteopontin and bone sialoprotein. Cells secreted a matrix that underwent mineralization on the silk fibroin scaffolds, forming clusters of osseous material, as determined by micro-computed tomography. The expression of osteogenic marker proteins and alkaline phosphatase was significantly higher in the Ad-BMP-2 MSC group than in the exogenous protein BMP-2 group, and no significant differences in mineralization were observed in two of the three MSC sources tested. The results demonstrate that transfection resulted in higher levels of expression of osteogenic marker genes, no change in proliferation rate and did not impact the capacity of the cells to calcify tissues on these protein scaffolds. These findings suggest additional options to control differentiation where exogenous additions of growth factors or morphogens can be replaced with transfected MSCs.
Chapter 10 used adenosine kinase deficient (Adk-/-) embryonic stem cells (ESC). These cells, when encapsulated in synthetic polymers, have previously been shown to provide therapeutic adenosine release and transient seizure suppression in epileptic rats. Here we explored the utility of biopolymer-substrates to promote long-term adenosine release from Adk-/- ESCs. Three different substrates were studied: (1) type I collagen (Col-1), (2) silk-fibroin (SF), and (3) poly(L-ornithine) (PO) coated tissue culture plastic. Adk-/- or wild type (wt) ESC-derived glial precursor cells were seeded on the substrates and cultured either in proliferation medium containing growth factors or in differentiation medium devoid of growth factors. In proliferation medium cell proliferation was higher and metabolic activity lower on Col-1 and PO substrates when compared to SF. Cells from both genotypes readily differentiated into astrocytes after growth factor removal on all substrates. Adk-/- cells cultured on biopolymers released significantly more adenosine than their wt counterparts at all developmental stages. Adenosine release was similar on SF and PO substrates and the amounts released from Adk-/- cells (>20 ng/ml) were considered to be of therapeutic relevance. Taken together, these results suggest that silk matrices are particularly suitable biomaterials for ESC encapsulation and for the design of adenosine releasing bioincubators for the treatment of epilepsy.

Chapters 10 and 11 provide examples for tissues beyond bone. As detailed in the previous paragraph, Chapter 10 directed at neural tissues, while Chapter 11 provided evidence that cartilage-like tissue can be engineered. In this chapter, silk fibroin scaffolds were studied as a new biomaterial option for tissue engineered cartilage-like tissue. Human bone marrow derived mesenchymal stem cells (MSC) were seeded on silk, collagen, and cross-linked collagen scaffolds and cultured for 21 days in serum free chondrogenic medium. Cells proliferated more rapidly on the silk fibroin scaffolds when compared to the collagen matrices. The total content of glycosaminoglycan (GAG) deposition was three times higher on silk when compared to collagen scaffolds. GAG deposition coincided with overexpression of type II collagen and aggrecan genes. Cartilage-like tissue was homogeneously distributed throughout the entire silk scaffolds, while on the collagen and cross-linked collagen systems tissue formation was restricted to the outer rim leaving a doughnut appearance. Round or angular-shaped cells resided in deep lacunae in the silk systems and stained positively for type II collagen. The aggregate modulus of the tissue engineered cartilage constructs was
more than two-fold higher when compared to the unseeded silk scaffold controls. These results suggest that silk fibroin scaffolds are suitable biomaterial substrates for autologous cartilage tissue engineering in serum free medium and enable mechanical improvements along with compositional features suitable for durable implants to (re)generate cartilage.

Tissue engineering has a significant point of interest in the public for biotechnological development of autologous tissues. In the case of ultimate success, the lack of human tissues and donor organs in surgical transplantations can be compensated, and thereby the lives of many patients saved or effectively alleviated. The speed with which many discovery projects are realized today is in particular high in the field of tissue engineering. However, the public opinion - at least in part generated by the research field itself - has raised yet unmet expectations to the field, such as the engineering of complex organs, an unmet need until today and most presumably for the next years to come. However, in case the engineering of complex organs should be carried out successfully, tissue engineering would, due to the constant availability of autologous tissues, help to avoid torturous waiting periods and the circumstances of emergency operations, burdensome to patients and physicians alike. With regards to these and similar therapeutic goals, national and international science are expected to endeavour more strongly into the direction of tissue engineered tissues and organs.
Tissue engineering of osteochondral tissues – stem cells, bioreactors, scaffolds, and regulatory molecules
Chapter 1: Review

Introduction

With the progressive aging of the population, the need for functional tissue substitutes is increasing. Reconstruction of osteochondral tissues is required for the treatment of traumatic injuries or other illnesses that affect the skeletal structure (arthrosis, rheumatoid arthritis, osteoarthritis, or congenital malformation). Skeletal deformities due to hypo- or hyperplasia (e.g. gigantism), dysostosis (e.g. Klippel-Feil syndrome), disease (e.g. arthritis), infection (e.g. osteomyelitis), or work (e.g. accident) have a tremendous impact on the appearance, function, psychological and social well being of patients. Risk groups for degenerative joint diseases are e.g. the blue collar population (e.g. mechanically induced microtrauma) as a result of heavy physiological load, the elderly (e.g. physiologic aging with change in cartilage composition), and patients with high body weight (e.g. mechanical) or joint disorders (e.g. hereditary), resulting in an increased risk of osteoarthritis, a substrate on which secondary, inflammatory diseases frequently develop (Vingard et al., 1991). Classical approaches to these problems have, so far, led to unsatisfactory results. Even though a wound healing response is initiated after a traumatic lesion, the substituting tissue does not adhere to the original architecture of the native tissue, leading to loss of its mechanical properties and scar formation (Hunziker, 2002). Organ transplantation and mechanical devices have revolutionized medical practice but have limitations. Full thickness joint defects of articular cartilage for example in the knee, have a chronically poor repair capacity, with a high, associated risk to ultimately lead to total knee replacement. These defects generally affect multiple tissues, the subchondral bone, cartilage, vasculature or tendons. The number of different methods currently under investigation reflects both the inadequacy of existing techniques and the need to more faithfully restore the skeleton (Warren et al., 2003, Fong et al., 2003). Treatment strategies for joint lesions include Pridie drilling to stimulate subchondral bone formation with an intact articular surface (Pridie, 1959), retrograde arthroplastic fillings with spongiosa, transplantation of autologous tissue or the in-vitro expansion of autologous chondrocytes for subsequent transfer into the lesion site (Brittberg et al., 1994). Pridie drilling has limited use once the cartilage surface is severely compromised. Autologous approaches are associated with limitations, such as second site morbidity, additional pain, longer stays in hospitals, increased infection risk and in case of defect filling with chondrocytes, a limited ability for the clinician to contour delicate 3D shapes.
(Einhorn, 1995). Novel methods include conduction (by a scaffold) and induction (by bioactive molecules) of cell migration to repair relatively small defects, and cell transplantation into the defect site (with or without biomaterial) to repair larger defects (Alsberg et al., 2001). Even these methods are inadequate for most parts of the skeleton when complex structures are being replaced. For autologous repair of compromised joints, multiple connective tissues need repair and replacement, tendons, cartilage, bone and osteochondral interfaces. Consequently, an ideal repair mode would provide custom-engineered grafts, by means of directed differentiation of mesenchymal stem cells along multiple lineages on the same implant to achieve a desired structure and functionality. In vitro engineering of functional tissue grafts is emerging as a significant clinical option to address tissue and organ failure. Although significant progress and limited commercialization has been achieved with the transplantation of in vitro expanded autologous cells (Brittberg et al., 1994), in vitro-grown tissue on three dimensional constructs offer the potential advantage of immediate functionality along with the capacity for integration with host tissues. This approach was exemplarily addressed with the in vitro generation of bone and cartilage tissue using (i) autologous mesenchymal stem cells (MSC), (ii) chondro/osteoinductive biomaterial scaffolds, and (iii) bioreactors to generate 3D tissues of clinical significance (Chapters 4-9). Preliminary experiments have demonstrated the successful cartilage and bone formation starting from MSC cultured with turbulent or laminar flow conditions in bioreactors and seeded on a new biomaterial, silk-fibroin (Figure 1) (Meinel et al., 2005a, Meinel et al., 2005b, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004d). Silks provide unique mechanical strength when compared to any other protein material and rival most synthetic high performance fibers, such as Kevlar (Cunniff et al., 1994, Fossey et al., 1991). Thereby, silks provide a mechanically robust biomaterial scaffold, ideal for tissue engineering purposes of osteochondral tissues. Such systems also hold potential for advanced cell culture systems and in vitro studies, to detail tissue development in general and stem cell differentiation in particular under both, normal and pathological conditions, respectively. These in vitro cultures would allow to confine the complex number of factors present in vivo (endocrinologic and immunologic responses) to a well controlled in vitro environment to distinguish the effects of specific signals (cell derived, biochemical and physical) (Freed et al., 1999). These controlled studies can
provide useful, complementary information to that obtained in vivo. The central question to drive tissue morphogenesis along the desired lineages is whether there might be simplifying principles explaining cellular assembly into tissue structures, which can be rationalized, quantified, and translated into a tissue engineering environment (Lauffenburger and Griffith, 2001). More prosaically, we need to understand the language of cellular cross talk resulting in lineage commitment and tissue assembly, to react adequately and rationally e.g. by means of scaffold design, surface structure, and information flow by spatially and temporally controlled growth factor or cytokine exposure. An envisioned scenario of clinically relevant tissue engineering involves the use of adult mesenchymal stem cells obtained from the patient’s bone marrow, biomaterial scaffolds custom-designed to serve as structural and logistic templates of tissue development, growth factor release to drive differentiation, and bioreactors designed to provide precise control over the cellular environment.

**Human mesenchymal stem cells**

Ideally, the patient should be treated with her/his own cells to eliminate concerns associated with the use of allograft cells (e.g., immunorejection, transmission of disease). A possible cell source for engineering osteochondral tissues is adult human bone marrow, containing cells capable both of self-renewal and of differentiation into cells of various mesenchymal lineages including chondrocytes and osteoblasts. These progenitor cells are human mesenchymal stem cells (hMSC) (Pittenger et al., 1999). The identification and later on clinical use of mesenchymal stem cells benefited strongly from previous studies in the embryonic chick limb bud system (Caplan, 1977). Strong differences are present between the two systems, but also certain parallels were identified, between both, the bone marrow and the limb bud. Both systems hold a source of mesenchymal progenitor cells. These cells were identified to be present in small numbers but can readily proliferate and differentiate into chondrocyte-like and osteoblast-like cells, respectively (Caplan, 1991, Bruder et al., 1994, Bruder and Caplan, 1990). One of the first studies utilizing these progenitor cells to form cartilage-like and bone-like tissue was based on isolation, and seeding of these cells on hydroxyapatite-calciumphosphate cubes, which were subsequently implanted
Chapter 1: Review

into syngeneic hosts (Ohgushi et al., 1989, Ohgushi et al., 1990, Ohgushi et al., 1992, Goshima et al., 1991).

Another finding was reported in 1992, when Haynesworth et al. demonstrated that cultured human marrow cells generate bone, but rarely cartilage, when introduced in the same assay system (Haynesworth et al., 1992b). Interestingly, when cells were not loaded into the hydroxyapatite cubes and loaded into the diffusion chambers they did not form bone. These findings clearly demonstrated the importance of the scaffold material the cells grow on, impacting the fate and lineage of differentiation (Haynesworth et al., 1992b, Haynesworth et al., 1992a).

Going back in time, the first report suggesting that stem cells are present in bone marrow was given by Friedenstein (Friedenstein, 1976). His work was based on a description of stromal cells from different species, mouse, guinea pigs, and rabbit. Friedenstein implanted these cells in diffusion chambers in syngeneic host animals and demonstrated their ability to differentiate into different phenotypes starting from the same cell pool (Friedenstein et al., 1970, Friedenstein et al., 1976, Phinney et al., 1999). In analogy to differentiation protocols developed for hematopoietic cells, Owen presented protocols for the differentiation of progenitor cells (Owen, 1988, Owen et al., 1990). These findings were based on the hypothesis that marrow stromal cells can differentiate along the fibroblastic, reticular, adipogenic, osteogenic, and possibly other lineages (Caplan, 1994).

Early isolation protocols describe two methods to obtain mesenchymal stem cells. In the first, cancellous bone from femoral heads was mechanically disrupted and the scattered bone parts were transferred into Petri dishes with serum containing culture medium. For obvious reasons, this method was not ideal for the isolation of progenitor cells, used for the generation of engineered tissues, mainly due to the destructive nature of this protocol (Haynesworth et al., 1992a). In following protocols, bone marrow was aspirated from the iliac crest – requiring minimally invasive methods – resuspended in serum-containing medium, loaded onto a Percoll density gradient, and centrifuged. This protocol was found to result in more potent progenitor cells as compared to those isolated from cancellous bone and was used throughout the following studies. However, both isolation schemes carry over a large portion of nucleated cells most likely of hematopoietic origin, which do not adhere to culture substrates and can, therefore, easily be separated from the anchoring mesenchymal
progenitor cells. The ability of mesenchymal cells to adhere to plastic surfaces is the first and most important isolation step in the selection of human mesenchymal stem cells. These anchored cells identified with established isolation techniques (plastic adhesion, flow cytometry) are still highly heterogeneous. The amount of isolated human mesenchymal stem cells can differ largely between donors but as a general rule, typically 100 – 200 colonies can be expected per 10 cm dish (Friedenstein, 1976, Friedenstein et al., 1970) and have a variable potential for mesenchymal tissue development. However, cells expanded from these colonies have also a different potential to differentiate into target cells (Caplan, 1991, Caplan, 1994). Most laboratories, therefore, have, as an integral part of their research program, procedures in place for the characterization of each batch of isolated cells. Common protocols rely on surface antigen determination and the characterization of the isolated and expanded cell pools to differentiate selectively along different mesenchymal lineages (Meinel et al., 2004c). However, surface antigen determination as a tool to cell sorting is not reliable for mesenchymal stem cell as they lack identified and specific surface antigen markers, in contrast to cells of other origin, such as EMA in epithelial cells, A2B5 in glial cells, PECAM-1 in endothelial cells, and N-CAM in neural cells. That said, recently identified markers of mesenchymal (Gronthos et al., 2003) and bone (Zannettino et al., 2003) progenitors were used to address this problem. The absence of cells associated with lineages that are characterized by a specific marker, therefore, can support an assessment of the purity of the isolated and expanded cell pool. Consequently, we characterized our isolated and expanded hMSC pools by their surface antigen pattern (FACS analysis) and always accompanied these assays by a thorough study on the cell’s capacity to proliferate and differentiate into cartilage or bone tissue in dependency of cell passages prior to their use (Chapters 2-6,9, 11) (Friedenstein, 1976, Friedenstein et al., 1987, Meinel et al., 2004b, Meinel et al., 2005b, Meinel et al., 2004c).
Figure 1: A typical sequence of events for the engineering of bone-like tissue. Scaffold properties (A) including pore geometry here shown for silk-fibroin scaffolds can be controlled within small ranges. In the case of slow degradation rates, as true for silk-fibroin the (B) deposition of bone-like trabeculae, formed by mesenchymal stem cells seeded on these scaffolds and cultured for 4 weeks in osteogenic medium, results in a network with a complex geometry, which is controlled by the design of the scaffold. Implantation of engineered bone results in (C) healing of critical sized cranial defects here demonstrated in comparison to untreated control defects in mice and an apical and caudal view.
**Biomaterials in the specific context of tissue engineering**

Biomaterial scaffolds should be made of biocompatible, biodegradable materials and degrade at a rate matching the rate of tissue deposition (Yannas, 2001). Most studies suggest that a scaffold is essential for promoting orderly tissue regeneration (Caplan et al., 1997). Scaffold structure determines the transport of nutrients, metabolites and regulatory molecules to and from the cells whereas its mechanical properties determine the mechanotransduction at the cellular and tissue levels. Biomaterials that have been explored for use in skeletal tissue repair include biodegradable and non-biodegradable polymers (Attawia et al., 1995, Bennett et al., 1996, Cook et al., 1997, Hollinger et al., 1996, Kim et al., 1997), in some cases in conjunction with the application of regulatory molecules (Callen et al., 1993, Gomi and Davies, 1993, Locklin et al., 1995, Mason et al., 1998, Reddi, 1998, Rogers et al., 1995). Regulatory factors are usually supplemented to culture medium (Chapter 2-5, 6, 9-11) (Lecanda et al., 1997, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004d), and in some cases incorporated into biomaterial scaffolds (Chapter 7,8) (Liu et al., 1992, Laffargue et al., 2000, Massia and Hubbell, 1991, Zheng et al., 1994, Ito et al., 1991, Mann et al., 2001). Tissue engineering of osteochondral grafts generally requires the presence of reparative cells on a structural template, the modulation of cell differentiation and tissue assembly by physical and biochemical regulatory signals.

Scaffolds and scaffold materials play a pivotal role in tissue engineering. A tissue’s nature is critically determined by scaffold composition, architecture and physicochemical properties both in vitro and in vivo. However, today, only limited number of scaffold materials are available, of which again only a small number is commercially available, more particularly for tissue engineering purposes.

Biomaterials are an intense field of research, addressing new, biocompatible materials that are able to induce specific tissue formation. These materials are – in most cases – obtained from three material groups, biodegradable and non-biodegradable polymers and ceramics (Hollinger and Kleinschmidt, 1990, Hollinger, 1993a, Hollinger, 1993b, Hollinger et al., 1996, Hubbell, 1995, Bennett et al., 1996). In tissue engineering, one of the most applied biomaterials are poly(lactide-co-glycolides) and hydroxyapatite matrices. For these materials, proliferation and ingrowth of osteoblast cells has been observed (Attawia et al., 1995). In terms of tissue inductive biomaterials, tethered growth factors (GF) on solid matrices were applied and offered important control of
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the amount and distribution of GFs, while often losing some bioactivity, which, however was still potent to induce tissue responses in the desired way (Chapter 7,8) (Kuhl and Griffith-Cima, 1996, Sofia et al., 2001). Each biomaterial class is associated with certain advantages and disadvantages in terms of manufacture, processing and clinical utility and none of them can meet the full range of required features for the optimal implant. Alternatives might become available using proteins isolated from silk cocoons. Silks are biocompatible if sufficiently pure and free from contaminants. When tissue and cell immunological responses have been observed from silk they have been due to the lack of purity of the material. Silk-fibroin – the structural protein and non glycosylated fibrous protein form silk cocoons is a well characterized and compatible material (Uff et al., 1995). Recent data from a number of groups supported the conclusion that silk fibroins when properly prepared to avoid contaminating proteins are biocompatible and less immunogenic and inflammatory than collagens or polyesters such as PLGA (Uff et al., 1995, Altman et al., 2003, Meinel et al., 2005b). This biocompatibility has been extensively characterized, since silks have been used for decades as sutures in vivo. The initially described adverse immunological reactions prompted the replacement of silkworm silk sutures with nylons ~20 years ago (Soong and Kenyon, 1984). Later, the adverse reactions were ascribed to the presence of residual sericin, a family of glue-like proteins that coat the core silk fibers, and not the silk fibroin itself (Soong and Kenyon, 1984). In addition, thrombogenic responses of polyester fibers were controlled by coating with sericin-free silk fibroin after implantation in vivo (Sakabe et al., 1989.). Santin et al. provided detailed mechanistic studies on foreign body response activations and inflammatory potential of these silks and determined C3 activation, fibrinogen adsorption, and mononuclear cell activation, among other measures in comparison to polystyrene and poly(2-hydroxyethyl methacrylate) (Santin et al., 1999). Silks were equivalent to these model surfaces in terms of the humoral responses related to inflammation, and superior in terms of activation and adhesion of the immunocompetent cells. These studies were corroborated and extended to studies in murine macrophages (Panilaitis et al., 2003). Macrophage TNF levels were followed over 14 days against silk-fibroin fibers (obtained from transgenic animals and, therefore, absolutely devoid of other proteins as found in cocoons), native fibroin fibers with sericin, and sericin alone. Silk fibroin – either genetically obtained or isolated from cocoons - induced a negligible response in
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comparison to collagen fibers, mechanistically supporting the good biocompatibility of properly isolated silk-fibroin.

Protein-based polymers, and in particular silk fibroin, can potentially address the needs for the tissue engineering of osteochondral grafts for several reasons (Chapter 5) (Kaplan et al., 1998). These include the natural role of structural/fibrous proteins such as collagens and silk fibroin – a foreign fibrous protein for mammalian systems - in tissue remodeling. Silk fibroin (SF) is a biocompatible and resorbable material, allowing the ingrowth and re-integration into native tissues. Silk fibroin – due to its transient presence - has minimal negative late phase impact as it is gradually replaced by the surrounding tissues. There is a misconception about the in vivo degradation of SF. According to the U.S. Pharmacopia, an absorbable material is defined as one that loses “most of its tensile strength within 60 days” in vivo. Following this definition, SF is a non-degradable material according to the U.S. Pharmacopia. However, SF is degradable over longer time frames as a function of proteolytic degradation and matrix mechanical fatigue (Horan et al., 2004, Altman et al., 2002a, Altman et al., 2003). It has been demonstrated, that B. mori silkworm silk yarns, incubated in protease or PBS, respectively, substantially fragmented into filaments, linearly lost their diameter with time in protease, had a mass loss greater than 50% after 42 days and lost 50% of their tensile strength after 20 days (Altman et al., 2003).

Perhaps the most distinguishing feature of silk fibroin is its robust mechanical integrity until new tissue is regenerated. Silk fibroin exhibits strength, flexibility and compression properties exceeding all other natural fibers and also rival even synthetic high performance fibers (Altman et al., 2003). In particular for the treatments of bone defects this is important, as a matrix will need to be formed and retain mechanical integrity until the host tissues can provide mechanical function. Initial studies particularly focused on silk-fibroin threads, however, more recently, data became available for scaffolds, prepared form silk fibroin (Nazarov et al., 2004), corroborated by data generated in our own group (in preparation). In these studies, the compression modulus of the scaffolds was evaluated to hold a load that will allow for 1 to 2% strain, the usual constraint matrices are designed for in bone-related applications. when compared to frequently used porous biodegradable polymeric scaffolds often considered in bone-related tissue engineering studies (e.g., polylactic and polylacte/
glycolic acids, collagen) the silk-fibroin porous scaffolds had similar properties at 1 and 2% strain (Nazarov et al., 2004).

Furthermore, silks fill the need for matrices that can be functionalized with cell growth factors or cell-binding – RGD – sequences, using chemistries that result in biocompatible outcome. For example, silk fibroin in film form was chemically coupled with RGD sequences and parathyroid hormone (PTH) and assessed in terms of osteoblastic responses using a human osteosarcoma cell line, Saos-2 (Sofia et al., 2001). As an outcome, RGD coupling onto the films enhanced bone-like responses in terms of increased cell numbers, alkaline phosphatase activity and calcium deposition.

Coupling of the PTH fragment did not result in any responses, when compared to undecorated films. We recently extended these studies to hMSCs using the same types of regenerated silkworm films (Chapters 7,8) (Karageorgiou et al., 2004a, Karageorgiou et al., 2004b). Coupling of bone morphogenic protein (BMP2) resulted in osteogenic differentiation of hMSCs. Recently, we have implanted these films into rat femoral, critical size defects (Chapter 8). Implantation resulted in a complete bridging of defects within 6 weeks post surgery, whereas defects filled with non-decorated silk fibroin scaffolds resulted in very few bone formation at the interface to host bone. These studies demonstrated the feasibility to couple larger proteins in an in vitro and in vivo active form to silk-fibroin substrates.

None of the commercially available materials has the ability to meet the full list of key characteristics as described above for silk fibroin. These involve a precise control of architecture (e.g., porosity), resorption with in reasonable time frames, controlled mechanical properties (e.g., compression and torsion), and possibilities to easily control molecular interactions for cell adhesion and osteoblast stimulation by means of surface decoration in an effort, to maximize tissue repair after implantation. Current synthetic biodegradable matrices carry only limited numbers of available functional groups for chemical coupling for peptide and protein decoration. Furthermore, mechanical and architectural integrity is limited for many materials and confines their spectrum of possible applications, such as when polymer fatigue is an issue. These issues generally arise, when high strength must be combined with reasonable flexibility, or when repeating compression forces must be offset. For example, collagen’s mechanical properties – one of the most commonly used biomaterial in tissue engineering applications - are limited when compared with silk-fibroin and the
rates of collagen degradation are too fast in many instances (Chapter 11). Cross-linking of collagen can in part compensate these issues in vivo, however, adverse cell responses associated to inflammation and spontaneous calcification were observed (van Luyn et al., 1992).

In terms of energy absorbed before break and when compared to other biomaterials, its mechanical properties make silks the strongest natural fibers known, and rivalling the best synthetic high performance fibers, such as Kevlar, in overall performance (Cunniff et al., 1994). Silk fibroin combines high strength with toughness. Furthermore, recent studies detail silk fibroin’s resistance to failure in compression, a feature in which high performance fibers, such as Kevlar were outnumbered. Also, silks are mechanically stable up to 180 °C, as tested in dynamic mechanical evaluations (Cunniff et al., 1994).

**Bioreactors for tissue engineering**

In principle, tissue engineering bioreactors can serve different purposes, among which are the homogenous seeding of cells throughout scaffolds, control of culture conditions including temperature, pH, nutrients, metabolites, levels of oxygen, or the enhancement of molecule transfer between the cells and the culture medium (Chapter 4) (Obradovic et al., 1999, Vunjak-Novakovic et al., 1999, Freed et al., 1988, Wu et al., 1999, Altman et al., 2002b). Furthermore, some bioreactors provide physiologically relevant physical signals, such as interstitial fluid flow within the scaffold, mechanical compression and shear (Freed and Vunjak-Novakovic, 2000).

Ideally, future bioreactors would provide all these interfaces. Bioreactors used for skeletal tissue engineering today include spinner flasks, with tissue constructs fixed in place, with the surrounding medium in a static or by means of a rotating magnetic bar or stirrer, in dynamically mixed medium (Meinel et al., 2004c, Warren et al., 2003), rotating vessels in which constructs are exposed to laminar flow (Freed and Vunjak-Novakovic, 1995, Meinel et al., 2004c), and perfused chambers, in which the medium is either flowing around the construct (Dunkelman et al., 1995, Freed and Vunjak-Novakovic, 1995, Glowacki et al., 1998, Mizuno et al., 2001), or where it is forced through (Pazzano et al., 2000, Meinel et al., 2004c). In general, morphology, molecular composition, and mechanical properties of engineered tissues grown in dynamic environments was better when compared to static culture conditions. It was
hypothesized and modeled, that this was an effect of better mass transfer into and from the scaffold center, resulting in better cell viability and – as a consequence – enhanced tissue formation (Freed and Vunjak-Novakovic, 2000). The engineering of osteochondral grafts requires the generation, support, and maintenance of three different tissue types, cartilage (Chapter 11), bone (Chapters 2-10), and osteochondral transition tissue. Molecule exchange between the medium and the constructs must be anticipated a major problem for bone, a tissue characterized by high oxygen tensions and aerobic conditions, in which glycogen is stored and mitochondria are characterized by a high rate of adenosinetriphosphate (ATP) production. In contrast, chondrocytes are embedded within a cartilaginous matrix, which is rich in sulfated glycosaminoglycans, aggrecan, versican, and type II collagen and characterized by low oxygen tensions. Chondrocytes are in anaerobic states, with diffusional nutritient transport to and metabolite transport from the chondrocytes. This transport is through a relatively dense matrix, which is characterized by a high water content due to water binding capacities of the aggrecans, versicans, and glycosaminoglycans. These physiological observations have direct impact on tissue engineering efforts of cartilage and bone and become more complex, when osteochondral plugs are engineered, i.e. the simultaneous engineering of both tissues on a single scaffold. These cellular constraints directly translate into tissue engineering challenges. Consequently, diffusional limitations into construct centers have severely curtailed efforts to engineer bone, which could be cultured only to thicknesses of approximately 200 µm (Martin et al., 2001a, Martin et al., 2001b, Abukawa et al., 2003, Ishaug et al., 1997). In contrast, this was not a major concern for the engineering of cartilage, an avascular tissue with low cellularity that can be cultured to thicknesses exceeding 5 mm (Freed et al., 1998).

Therefore, overcoming diffusional limitations has been one of the major challenges, since bioreactors were used for tissue engineering purposes (Kellner et al., 2002). In general, the distributions of cells and extracellular matrix (ECM) in engineered tissues are nonuniform. In native tissues, this problem is alleviated by loading-induced interstitial flow of fluid (Garcia et al., 1996). Novel bioreactor strategy and design, aims at mimicking nature’s solution to support supply of cells thriving in deeper tissue regions. We studied the effects of hydrodynamics on engineered tissue using static culture (mass transport by molecular diffusion; no hydrodynamic shear), mixed flasks
(convective mass transport, steady turbulent shear), and perfused cartridges (convective mass transport, dynamic laminar shear) (Meinel et al., 2004c). The cartridge based bioreactors was composed of a chamber, in which constructs were mounted in a support thus blocking the apical and basal parts of the chamber, through which medium was pumped continuously with a speed of 0.2 ml/min. This resulted in an average interstitial velocity of 34 m/s, a hydrodynamic shear of $< 0.05$ dyn/cm$^2$, and a velocity of the smallest turbulent eddy of 0.4cm/s. The spinner flask was mixed at a rate of 50 min$^{-1}$, resulting in an integrated shear force of 8.37s$^{-1}$, a size of the smallest turbulent eddies of 250 µm, and the same velocity of the smallest turbulent eddy of 0.4cm/s as observed for the perfused cartridge. Interestingly, bone tissue formation was more advanced when cultured with convective mass transport and steady turbulent shear (spinner flask) when compared to cultures with convective mass transport and dynamic laminar shear (cartridge). The high interstitial fluid flow and the associated shear forces on cells as present for the cartridge system resulted in the formation of fibrous tissue deposition, which, when cultured in a static environment deposited bone-like tissue (Meinel et al., 2004c). In contrast to nature, where medium flow is organized in a vascular bed and transfer to the cells occurs by diffusion from the vascular compartment into the tissue, in the cartilage experiment, cells were directly exposed to the flow-shear, driving differentiation along the fibrous lineage. It has been shown that fluid flow around constructs positively correlated with tissue specific biomarker deposition, although often reported along with fibrous tissue formation at the outer construct rims, which were exposed to the medium flow (Buschmann et al., 1995, Carver and Heath, 1999, Klein-Nulend et al., 1995b, Klein-Nulend et al., 1995c). However, in a study using non-degradable supports – here a titanium alloy – positive outcome in terms of homogenous bone tissue formation with the absence of fibrous reactions up to flow rates of 1 ml/min were reported (Bancroft et al., 2002). Alternatively, medium supply through interstitial flow should minimize shear forces by slow and intermittent medium flow, induced by mechanical loading devices, gently compressing and relaxing the entire construct. These movements induce a push and pull effect, soaking medium throughout the construct, to minimize spatial gradients in medium composition and avoid nonuniformity in tissue structure and function resulting from diffusional gradients during culture. Essentially, this cell support by
push and pull induced transfer of medium by a mechanical devise follows the same principle nature applies for the support of cells residing in cartilage.

Along with optimized nourishment of cells within a construct, future bioreactors should generate output parameters indicative for the developmental state of the engineered tissue, which can drive a computer algorithm controlling and manipulating culture conditions in an auto-pilot mode, thus optimizing tissue outcome by means of continuous surveillance. Ideally, such a computer assisted bioreactor senses the engineered tissue’s status, modulating input variables accordingly, to maximize the quality and quantity of the outcoming tissue. It is essential to define which parameters define “quality” and “quantity” in this sense, which parameters are accessible to sensors, and can provide important feed-back to the computer system, indicative for the tissue’s state. Generally, sensors exist for more unspecific biochemical parameters, including oxygen tension, CO2 levels, glucose levels and some metabolites (Obradovic et al., 1999). The gold standard to determine quality is bone strength, however by functional, mechanical test (Vunjak-Novakovic et al., 1999, Klein-Nulend et al., 1995a, Athanasiou et al., 2000, Odgaard and Linde, 1991). Such a test gives several measures of bone strength, like ultimate force, ultimate displacement and work to failure. Direct mechanical testing is a straight-forward procedure, but has its limitations; finite element (FE) namely it is a destructive test, indicating that a sample can only be tested once and not be used for implantation afterwards. Furthermore, these tests are prone to errors; where most stem from boundary artifacts (Keaveny et al., 1997, Odgaard and Linde, 1991). Due to the small sample size these tests are even more challenging with tissue engineered bone, where the precision is even lower compared to testing bone of animals. At last, these tests are laborious and due to their destructive nature, do not allow for “on-line” assessment of engineered tissues during culture for later use in patients. Therefore, a non-destructive, high-throughput technique to determine bone strength based on simulations (finite element; FE) and non-destructive imaging (µCT) was developed (Muller et al., 1996a, Muller and Ruegsegger, 1997, Muller and Ruegsegger, 1996, Muller and Ruegsegger, 1995, Muller et al., 1994, Muller et al., 1996b). Within the scope of tissue engineering, these studies can be further developed to predict information about the performance of engineered implants under strains, which normally lead to break or – in other words -
the point of failure can be estimated for each construct, which provides important information for implant selection.

These techniques should be extended to tissue engineering, by developing bioreactors fitting as a whole or in part into an imaging device, allowing continuous surveillance of tissue growth. Coupled with a loading device, structural and mechanical information can be generated at the same time, allowing not only information of the tissue’s state but also about its performance under strain, including predictions of break points as outlined above. We have a bioreactor under development, combining the aforementioned challenges in its design by the integrated use of a horizontal and vertical loading devise and \( \mu \)CT, allowing time-lapsed, image guided quality assessment.

Within the specific context of osteochondral plug engineering, imaging method must be applied to provide both, information on mineralization and information on cartilage deposition. Most studies of engineered tissues have been evaluated by end-point methods of assessment such as histology and biochemical analysis. Given the week-long time for development, a non-destructive method for monitoring tissue development would greatly improve the selection of appropriate culture conditions from the large number of possible biophysical regulators. For cartilage, MRI in general and more specifically a method known as dGEMRIC can be applied. dGEMRIC is an MRI based method for measuring the spatial distribution of glycosaminoglycans (GAG) in cartilage. The dGEMRIC is based on the premise that when the negatively charged MR contrast agent gadolinium diethylenetriamine pentaacetic acid is equilibrated within cartilage, its distributions is inversely related to that of negatively-charged GAG. This relationship has been validated in excised human and bovine cartilage in vitro and in vivo (Bashir, 1999, Bashir et al., 1997, Bashir et al., 1996). We also developed a bioreactor system for cultivation of tissue engineered cartilage in multiple cartridges that are perfused with culture medium within a recirculation loop containing gas exchanger and a fresh medium supply. The cartridges and perfusion loops are compatible with the MRI coils, such that cultured tissues can be monitored on-line without interrupting the cultivation. In a similar system, the development of tissue engineered cartilage was monitored repeatedly for the same samples over of period of up to 8 weeks, and as a function of cultivation conditions (e.g., oxygen tension, flow rate) (Williams et al., 1998). Samples were harvested at timed intervals.
for end-point assessments and these demonstrated a close correspondence between the dGEMRIC measurement, histological and biochemical analyses. These data demonstrate the validity and feasibility of using dGEMRIC to nondestructively monitor GAG production in the cartilaginous component of osteochondral grafts (Williams et al., 1998).

In parallel, we are currently working on employing µCT for on-line imaging of bone in an effort to acquire on line information of tissue growth, which can provide novel input parameters to drive computer-assisted bioreactors. CT is a well established X-ray imaging modality that can acquire images of mineralized tissue with 10 µm resolution (Layton et al., 1988, Feldkamp et al., 1989, Ruegsegger et al., 1996). Some examples of measurements possible with these systems are: bone volume fraction, trabecular thickness, trabecular separation, cortical thickness, marrow cavity diameter (and volume), cortical area, and the cross-sectional properties of bones (Nazarian et al., 2002, Odgaard and Linde, 1991, Alexander, 2001, Bouxsein et al., 2002, Hildebrand et al., 1999). In pilot experiments, we exposed tissue engineered bone – starting off from hMSC, differentiated along the osteogenic lineage in a static environment – to repeated cycles for nondestructive imaging of engineered bone, and directly assessed its 3-dimensional trabecular morphology as a function of time (in preparation). X-ray attenuation of engineered bone-like tissue was translated into measures of bone tissue density, using a set of liquid hydroxyapatite phantoms of known concentrations, as has been described before (Nazarian et al., 2002). In addition of providing information of the osteogenic status of the tissue, the combination of moderate compression or shear forces with µCT based imaging results in a method referred to as Image Guided Failure Assessment (IGFA) - following the aforementioned method of image guided quality assessment combined with on-line monitoring of engineered tissue development (Nazarian et al., 2002). This method – when applied to tissue engineering – will allow to assess a tissue’s status and to predict possible break points for the construct after implantation by applying moderate forces along with imaging the bending of the engineered trabeculae or even the identification of micro-cracks in the engineered tissue. We believe this is in particular of advantage, when it comes to implant selection from a pool of constructs. Image guided failure assessment can quantitatively assess the structure of mineralized tissue specimens and simultaneously visualize a propensity for failure (Figure 2). Finite element analysis has been used to
complement IGFA and evaluate strains and stresses in the individual elements (trabeculae) of bone (Van Rietbergen et al., 1996, Van Rietbergen et al., 1995). This has allowed the development of protocols to predict fracture risk at sites on whole bones using µCT-based calculations of axial, bending, and torsional rigidities (Whealan et al., 2000, von Stechow et al., 2003) by summing the density-weighted area of each pixel by its position relative to the density-weighted centroid (von Stechow et al., 2003). Application of this protocol to clinical trials demonstrated that it had higher accuracy and sensitivity than other structurally-based prediction methods presented in the literature (Whealan et al., 2000). Therefore, the combination of the bioreactor providing horizontal and vertical mechanical stimulation with simultaneous µCT measurements holds promise not only to feed back novel input variables into an algorithm – which again controls culture conditions – but also to provide quality measures on a trabecular level for the selection of optimally engineered implants (Figure 2).

Figure 2: (A) demonstrates the principle of image guided quality assessment. A tissue engineered trabecular tissue is carefully loaded with a low strain, bending the trabeculae without break. This is imaged using µ-CT. (B) Using an algorithm validated for trabecular bone, a miscolour map demonstrates areas with a high propensity for possible breaks. The construct with the lowest propensity will be selected for implantation.
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**Regulatory molecules**

The engineering of osteochondral plugs requires MSC differentiation to be modulated into cartilage or bone in a temporally and spatially controlled sequence. Selective regulatory mechanisms underlying lineage restriction are still a field of intense research, however, commitment and/or differentiation within some of the mesenchymal lineages including osteoblasts and chondroblasts have provided regulatory molecules for this purpose (Aubin and Triffitt, 2002). The challenging part is that these factors are acting in concert and in a delicate balance between observed effect, used dose and kinetics. Furthermore, MSCs are a dynamic cell pool, i.e. developing from a stem cell state through various intermediate-states to the ultimate, final, or adult phenotype. With progression along the lineages, the cells considerably change their transcriptome and surface antigen pattern. Consequently, the cells respond differently when exposed to external signals, as a function of lineage progressions. For example, while many reports document a stimulatory effect of BMPs (bone morphogenic protein) on osteoblasts, a few show inhibitory effects, which may be due to the dependence of the actions of growth factors on the relative stage of differentiation of the target cells. This is also true for TGF-β (transforming growth factor), which has biphasic effects on osteoblasts in vitro, inhibiting early stage progenitors while stimulating matrix production by more mature cells in the lineage, including osteoblasts (Aubin and Liu, 2002). IGF-I (insulin-like growth factor), which has stimulatory effects on osteoblasts in vitro and anabolic effects in vivo, is considered to be the dominant growth-stimulating agent for cartilage (Froesch et al., 1996, Sah et al., 1996, Trippel, 1995). As for BMPs and TGF-β, also IGF-I action on cells and tissues strongly depends on its temporospatial distribution (Meinel et al., 2006). As the nature of the cells is changing during differentiation, temporospatial issues are important for drug delivery of regulatory molecules, to “catch” the cell pool at a certain progenitor state and direct it along the desired lineage. Therefore, temporospatial delivery of regulatory molecules for tissue engineering has to be understood as an attempt to support commitment and/or differentiation of a progenitor cell in a specific state into the adult cell state along the desired lineage.

A possible approach to identify these “optimal” pharmacokinetics and to exploit the full potential of osteo-/chondroinductive growth factors (GF) might again be derived from mimicry of nature. Until now, optimal kinetics have been approximated by trial
and error approaches. Hypothesizing, that a GF is most efficient when its environment is rendered sensitive for its stimuli – e.g. high receptor presentation levels for this GF - in vitro tissue engineering setups can be the tool to start studies in that direction using a simplified environment when compared to in vivo conditions. A possible setup can involve (i) gene expression analysis of the gene of interest (GOI) encoding for the protein intended for application. This analysis of GOI expression – e.g. the gene encoding a GF’s receptor - will be carried out using RNA libraries isolated from preceding tissue engineering experiments. Based on obtained expression data for the GOI, the GF is delivered with pharmacokinetics, matching the expression profile of the GOI. Feasibility studies suggested that this approach can increase the quality and quantity of cartilage-like tissue deposition, starting off from human MSC, insulin-like growth factor I (IGF-I) for basal chondrogenic stimulation and transforming growth factor beta 1 (TGF-1) supplemented into the medium with pharmacokinetics modulating the expression profile of SOX-9, a transcription factor found to be critical for chondrogenic lineage restriction (M Garcia in vitro, L Luginbuehl in vivo, preliminary results) (Bi et al., 1999). Starting off from a well controlled in vitro environment, these considerations – once validated in the tissue engineering setup – can be transferred in vivo. At least for bone tissue, osteoinductive factors have proven their general suitability to promote bone growth, their clinical use is limited and restricted to very few cost intensive non-union cases - approximated costs per conventionally treated non-union defects are $14,000). Therapy costs are approximately 80% due to recombinant production of the drug substance and, consequently, a reduction of doses by e.g. 1/3 while maintaining or even increasing efficacy and safety through precise temporal and spatial delivery, reduces the costs for this therapy and increase market pull. For that, matching pharmacokinetics can lead a way for efficient life cycle management (Reginster, 2002, March and Bachmeier, 1997, Lysaght and O'Loughlin, 2000).
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experimental osteoarthritis by microscopic computed axial tomography.  


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Effect of scaffold design on bone morphology in vitro

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Tissue Engineering, 2006, accepted
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Abstract

Silk fibroin is an important polymer for scaffold designs, forming biocompatible and mechanically robust biomaterials for bone, cartilage and ligament tissue engineering. In the present work, 3D biomaterial matrices were fabricated from silk fibroin with controlled pore diameter and pore interconnectivity, and utilized to engineer bone starting from human mesenchymal stem cells (hMSC). Osteogenic differentiation of hMSC seeded on these scaffolds resulted in extensive mineralization, alkaline phosphatase activity, and the formation of interconnected trabecular- or cortical-like mineralized networks as a function of the scaffold design utilized; allowing mineralized features of the tissue engineered bone to be dictated by the scaffold features used initially in the cell culture process. This approach to scaffold predictors of tissue structure expands the window of applications for silk fibroin based biomaterials into the realm of directing the formation of complex tissue architecture. As a result of slow degradation inherent to silk fibroin, scaffolds preserved their initial morphology and provided a stable template during the mineralization phase of stem cells progressing through osteogenic differentiation and new extracellular matrix formation. The slow degradation feature also facilitated transport throughout the 3D scaffolds to foster improved homogeneity of new tissue; avoiding regions with decreased cellular density. The ability to direct bone morphology via scaffold design suggests new options in the use of biodegradable scaffolds to control in vitro engineered bone tissue outcomes.
Chapter 2: Effect of scaffold design on bone morphology in vitro

Introduction

Scaffolds used for bone tissue engineering have to address biological, mechanical and architectural needs to foster functional tissue outcomes. These factors, along with requirements for biocompatibility and maintenance of a suitable environment for cells to thrive impose an impressive array of challenges and constraints. Optimal porosity, pore interconnectivity and control of pore diameter are just some of the important features that can impact mechanical properties, cell responses and new tissue ingrowth. The impact of porosity and pore diameter were previously detailed for various biomaterials including polyHIPE polymer (Akay et al., 2004), collagen and hydroxyapatite (HA) (Tsuruga et al., 1997) and shown to effect cellular proliferation and osteogenic differentiation (Karageorgiou and Kaplan, 2005). For in vivo applications, the minimum recommended pore size is suggested to be approximately 100 µm (Hulbert et al., 1970) and maximum pore size around 500 m, with larger pores resulting in the formation of fibroblastic tissue (Wake et al., 1994, Karageorgiou and Kaplan, 2005) indicating the relevance of scaffold geometry and lineage restriction. Other considerations for such designs revolve around mechanical properties and biocompatibility. For example, degradable, synthetic polymers such as poly(L-lactic acid) (PLA) and poly(L-lactic-co-glycolic acid) (PLGA) can induce inflammation due to the localized acidity of hydrolysis products and also find difficulty in matching the mechanical properties of native bone (Akay et al., 2004, Athanasiou et al., 1996, Hollinger et al., 1996, Harris et al., 1998, Suh, 1998). Collagens remain problematic due to concerns with bioburdens as well as rapid degradation (DeLustro et al., 1990, Srivastava et al., 1990).

Silks, which represent the strongest natural fibers known, may contribute to this important niche in biomaterial needs due to their remarkable mechanical properties and well documented biocompatibility (Cunniff et al., 1994, Sofia et al., 2001, Meinel et al., 2005, Panilaitis et al., 2003). Recent studies with silk films and fiber matrices have shown a wide range of potential biomedical applications (Altman et al., 2003, Meinel et al., 2004a, Meinel et al., 2004b, Meinel et al., 2004c, Minoura et al., 1995) and detailed the low inflammatory potential both in vitro and in vivo (Meinel et al., 2005). Furthermore, silk fibers have been used for decades in suture formats, thus, there is a long history of biomedical materials experience with this protein polymer (Lange, 1903, Lange, 1907).
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Some current protocols for scaffold preparation start from silk fibroin solutions in organic solvent such as hexafluoroisopropanol (HFIP) or water and frequently use sodium chloride (NaCl) crystals as a porogen to generate the pores in the 3D structures upon processing (Tsukada et al., 1994, Nazarov et al., 2004, Kim et al., 2005). Both the use of organic solvents as well as the salt crystals can potentially restrict the application of silk fibroin based biomaterials one of which would be the embedding of growth factors to support osteo-induction. Some growth factors were reported to be prone to high ionic strength (local remnants of NaCl crystals) or to the presence of some organic solvents (Meinel et al., 2001).

This study presents a process replacing ‘exploratory’ organic solvent with unknown ICH classification by well known organic solvents and water, to minimize possible side effects related to this uncertainty and to further move silk fibroin scaffolds towards clinical application (ICH). Paraffin has been used previously together with PLA and PLGA processing. The use of paraffin is attractive as it allows precise control of pore diameter and morphological features through controlled melting of the paraffin porogen and has demonstrated its use during manufacture and has been found an acceptable raw material (Ma et al., 2003, Chen and Ma, 2004).

In the present study the use of paraffin porogens was integrated into the formation of silk scaffolds and different porogen diameters (100-200 µm, 200-300 µm, 300-400 µm) were considered. Different heat treatments to control the interconnectivity of pores were also considered by careful and controlled melting of paraffin porogen thereby forming different levels of interconnectivity. Osteogenic differentiation of human bone marrow derived mesenchymal stem cells (hMSC) was evaluated on these scaffolds (Table 1) to assess impact on bone-related features, through the use of biochemical assays, mechanical characterization, histology and micro-computed tomography (µCT) imaging. The results suggest that a significant level of control of bone geometries can be achieved in vitro through the ability to manipulate scaffold designs with the methods described herein.
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Materials and Methods

Materials

Paraffin with a melting temperature of 54-56 °C, lithium bromide, Na2CO3, polyethylene glycol (PEG) (MW 6000), formalin, alkaline phosphatase substrate (p-nitro-phenyl-phosphate), ascorbic acid, dexamethasone and β-glycerolphosphate were purchased from Sigma-Aldrich (St. Louis, MO). Potassium bromide was from Merck (Darmstadt, Germany). All antibodies, CD44 (endothelial cells), CD34 (sialomucin/hematopoietic progenitors), CD71 (transferring receptor/proliferating cells), and CD105 (endoglin), anti-CD44, anti-CD14 conjugated with fluoresceine isothiocyanate (CD44-FITC, CD14-FITC), anti-CD31 conjugated with phycoerythrin (CD31-PE), anti-CD34 conjugated with allophycocyanine (CD34-APC), anti-CD71-APC and anti-CD105 with the exception of secondary rat-antimouse IgG-FITC antibody (Neomarkers, Fremont, CA) were purchased from BD Biosciences (Maryland, MD). RPMI1640, Dulbecco’s modified eagle medium (DMEM), fetal bovine serum (FBS), bFGF, Pen-Strep, fungizone, insulin and TGFβ1 were from Gibco (Palo Alto, CA). BMP-2 was kindly provided by Wyeth (Madison, NJ). Papain solution and Matrigel were from BD Bioscience (Maryland, MD). The kit to assess of calcium concentration was from Rolf Greiner BioChemica (Vienna, Austria). Osmium tetroxide was purchased from Electron Microscopy Science (Hatfield, PA). Hexane and N,N-Dimethylformamid was from EGT Chemie AG (Tägerig, Switzerland).

Table 1: Experimental groups

<table>
<thead>
<tr>
<th>Scaffold type</th>
<th>Pore size</th>
<th>Culture system</th>
<th>Medium</th>
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<tbody>
<tr>
<td>37 scaffold</td>
<td>200 - 300 μm</td>
<td>Spinner flask</td>
<td>Control medium</td>
</tr>
<tr>
<td>37 scaffold</td>
<td>200 - 300 μm</td>
<td>Spinner flask</td>
<td>Osteogenic medium</td>
</tr>
<tr>
<td>RT scaffold</td>
<td>200 - 300 μm</td>
<td>Spinner flask</td>
<td>Osteogenic medium</td>
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Preparation of paraffin globules

Paraffin globules were prepared in an emulsion process using water, gelatin and paraffin. Gelatin was dissolved in hot ultra purified water (UPW) (80 °C) at a
concentration of 30 mg/ml and paraffin (melting temperature 54-56 °C) was carefully heated at a concentration of 200 g/l. Phases were emulsified by stirring at 9,000 rpm for 10 minutes and poured into ice-cold water. The solidified paraffin globules were harvested and washed with UPW. After filtering through 12-25 µm pore filter paper (Schleicher & Schuell, Switzerland) and drying, the globules were classified with standard sieves (Retsch, Haan, Germany). Fractions of >500 µm, 400-500 µm, 300-400 µm, 200-300 µm, 100-200 µm were separated and stored at 4 °C.

**Preparation of silk**
Silk fibroin solution was prepared as described previously. Briefly, cocoons from Bombyx mori (Trudel, Zurich, Switzerland), were boiled in 0.02M Na2CO3 solution, rinsed and dissolved in 9M LiBr for 3 h at 55 °C to generate a 10% (w/v) solution. The solution was dialyzed (Pierce, molecular weight cut-off 3,500 g/mol) in 1.5 l of UPW. The UPW was exchanged 5 times in 72 h resulting in a silk fibroin concentration of 3% (w/v).

**Heat treatment of paraffin mold**
The paraffin globules, 1.2 g, were loaded into 20 ml falcon syringes (BD Bioscience, Maryland, MD). The surface was evened by the use of a pistil and the syringes were left at RT or incubated at either 37 °C or 45 °C (Thermocenter, salvis OAKTON Instruments, Vernon Hills, NY) for 50 minutes. After heat treatment the mold was left to cool to RT.

**Preparation and freeze drying of silk scaffolds**
Freshly prepared silk fibroin in UPW was transferred to a dialysis chamber and further dialyzed against a 13.3% (w/v) PEG 6000 solution in UPW overnight resulting in a 20% (w/v) silk fibroin solution. One ml of this solution was soaked in the prepared paraffin mold so that the silk fibroin filled all pores between the porogen. This material was then flash frozen in liquid nitrogen for 1 min. The scaffolds were freeze dried at -30 °C under temperature control for 48h (LYOVAC GT2, FINN-AQUA, Hurth, Germany). Scaffolds were immersed in 90% (v/v) methanol for 30 minutes and then
air dried. The paraffin was extracted in hexane. Hexane was replaced after 12 and 24 h and the leached scaffolds were dried under vacuum for 12 h.

Differential scanning calorimetry (DSC)
For DSC analysis, nitrogen flow was 20 ml/min and baseline optimization was set between 105-250 °C. Calibration was performed with indium (~5 mg). Each sample (1 mg) was encapsulated in an aluminum pan, dried under a nitrogen flow at 105 °C for 10 minutes and then heated at a rate of 10 °C/min up to 250 °C and held at 250 °C for one minute (Magoshi and Magoshi, 1977).

Fourier-transformed infrared spectroscopy (FTIR)
For FTIR, a mortar and pestle was used to grind 5 mg of scaffold that was then mixed with 300 mg potassium bromide, and pressed into a pellet with 10 tons under vacuum for one minute. A 2000 FT-IR Spectrum V3,01 (Perkin Elmer, Boston, MA) was used to analyze the materials.
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*Dissolution study*

Water solubility was assessed using samples of 20 mg each (n=6), placed in 1 ml reaction tubes containing 1 ml of UPW and incubated at 37 °C. For each time point the supernatants were collected and the samples were dried under vacuum in a speed-vac SC110 (Savant Instruments, Farmingdale, NY). The dry weight was assessed over a total of 14 days using an analytical balance (Mettler-Toledo AX205 Deltarange, Greifensee, Switzerland).

*Mechanical testing*

Scaffolds (18 mm in diameter) were cut into cylinders with parallel surfaces (thickness 6-7 mm). Compressive testing between two parallel plated was performed at room temperature using a Zwick 1456 (Zwick Testing Machines Ltd, Kennesaw, GA) for material testing. Preload was set to 2 N and compression speed was 2 mm/minute. The compressive force and travel were graphed and the Young’s modulus was determined using a 2% off set criterion.

*Scanning electron microscopy images (SEM)*

Scaffolds were cut into slices (1-2 mm height), dried, platinum-coated and analyzed by scanning electron microscopy (SEM, Zeiss Leo Gemini 1530, Zeiss, Germany). Head space gas chromatography (GC) for the determination of residual solvents

Residual solvents in silk fibroin scaffolds after the hexane leaching were determined according to the US pharmacopeia (USP, 2006). 350 mg of leached and dried scaffold material was dissolved in 10 ml of 9M Lithium bromide solution. 990 µl of diluted silk fibroin scaffold in LiBr was mixed with 10 µl of N,N- Dimethylformamide containing 0, 250, 500 and 1000 ppm of hexane and sealed in 10 ml headspace vials. The solution was preheated for 10 minutes at 60 °C and 1ml of the gas phase was injected in the gas chromatograph equipped with a flame ionization detector heated at 260°C (HP5890Agilent, Wilmington, DE, USA). GC was carried out using a HP-Fast residual solvents Col 3 column (Agilent, Wilmington, DE, USA). Helium flow rate was set to 6 ml/min, inlet temperature set at 180 °C and splitless time set to 1 min with a split ratio of 1:6. The oven temperature was increased from 40 to 45 °C at 2 °C/min and from 45 to 250°C at 30 °C/min.
Isolation, expansion and characterization of human mesenchymal stem cells (hMSC)

Cell isolation was performed as previously described (Meinel et al., 2004b). Briefly, samples of bone marrow, of a single donor, were diluted in 100 ml of isolation medium (RPMI1640 supplemented with 5% FBS). The cells were centrifuged at 300 x g for 10 minutes. Cells were pelleted and suspended in expansion medium (DMEM, 10% FBS, 1 ng/ml bFGF) and seeded in 75 cm² flasks at a density of 5 x10⁴ cells/cm². The adherent cells were allowed to reach 80% confluence (12–17 days for the first passage, 6 days for later passages). Cells were trypsinized and replated every 6–8 days at 80% confluence. The second passage (P2) cells were used, if not otherwise stated. The hMSCs were characterized with respect to (i) the expression of surface antigens and (ii) the ability to selectively differentiate along the chondrogenic or osteogenic lineages. The expression of the following six surface antigens was characterized by fluorescence-activated cell sorting (FACS; Facscalibur, BD Bioscience, Maryland, MD) analysis, as described before, using the following marker proteins: CD44 (endothelial cells), CD34 (sialomucin/hematopoietic progenitors), CD71 (transferring receptor/proliferating cells), and CD105 (endoglin). Cells were detached with 0.05% (w/v) trypsin, pelleted, and resuspended at a concentration of 1x10⁷ cell/ml. Fifty-microliter aliquots of the cell suspension were incubated (30 min on ice) with 2 µl of each of the following antibodies: anti-CD44 and anti-CD14 conjugated with fluoresceine isothiocyanate (CD44-FITC, CD14-FITC), anti-CD31 conjugated with phycoerythrin (CD31-PE), anti-CD34 conjugated with allophycocyanine (CD34-APC), anti-CD71-APC, and anti-CD105 with a secondary rat-anti mouse IgG-FITC antibody. Cells were washed, suspended in 100 ml of 2% formalin and subjected to FACS analysis. To assess the potential of hMSC for osteogenic differentiation, the cells were cultured in 12 well plates as micromass cultures (5 drops of 15 µl of 2x10⁷ cells/ml per well) in either control medium (DMEM supplemented with 10% FBS, penicillin/streptomycin and Fungizone) or osteogenic medium (control medium supplemented with 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM ß-glycerolphosphate, and 1 µg/ml BMP-2). Medium was exchanged every 2–3 days. After 2 weeks of culture the amounts of calcium were measured, as described before (Meinel et al., 2004b).

Cultivation in bioreactor (spinner flask)
Methanol treated scaffolds, prepared with porogen treated at 37 °C (37-scaffold) and at RT (RT-scaffold), were cut into discs (thickness 1-2 mm) and scaffolds of 12 mm diameter were punched from this material. Matrigel matrix (BD Bioscience, Maryland, MD) was thawed overnight at 4 °C. Cells were trypsinized and cell number was assessed using a Neubauer hemocytometer (Brand GmbH, Wertheim, Germany). Five million cells were aliquoted and resuspended in 20 µl of Matrigel and the cell suspension was seeded on the scaffolds in a Falcon non tissue culture treated 12 well plate (BD Bioscience, Maryland, MD). Scaffolds were incubated for 15 minutes (37 °C and 5% CO2) until gelation of the Matrigel. The seeded scaffolds were then fixed with needles in the spinner between plastic stoppers (3x3 mm squares), which were cut from tubing (Norton Manufacturing Company, Fostoria, OH). The spinner was closed and 150 ml of media was added. For the control, DMEM containing 10% FBS 1% pencillin/streptomycin and 1% fungizone was used. For the differentiation along the osteogenic linage, bone medium was prepared containing DMEM, 10% FBS, 1% pencillin/streptomycin, 1% fungizone, 50 µg/ml ascorbic acid, 10 nM dexamethasone, 10 mM β-glycerol phosphate di-sodium salt hydrate and 1 µg/ml BMP-2. Half of the medium was replaced every 2-3 days with osteogenic replacement medium containing DMEM, 10% FBS, 1% pencillin/streptomycin, 1% fungizone, 100 µg/ml ascorbic acid, 20 nM dexamethasone, 20 mM β-glycerol phosphate di-sodium salt hydrate and 2 µg/ml BMP-2. Total culture time was 4 weeks.

**Biochemical Assays**

Constructs were harvested after 4 weeks. Scaffolds (n=4) were cut into halves, using one half for the assessment of ALP activity and DNA content and the other for quantification of total calcium. The wet weights of the scaffold halves were recorded (Mettler-Toledo® AX205 Deltarange®) and transferred into 2 ml tubes (Fisher Scientific, Pittsburgh, PA, US) for analytical evaluation.

*Alkaline phosphatase (ALP) activity*

For the assessment of ALP, halves of scaffolds (n=4) were incubated with 1 ml 0.2% (v/v) Triton-X100, 5 mM MgCl2 and disintegrated using steel balls and a Minibead-beater (Biospec, Bartlesville, OK). After centrifugation at 300 x g for 10 minutes (4 °C), 80 µl of sample were transferred to a new tube. Alkaline phosphatase (ALP) activity (n=5) was measured using a biochemical assay from (Sigma-Aldrich, Inc.,
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MO, US), based on ALP dependant conversion of p-nitrophenyl phosphate to p-nitrophenol and read spectrophotometrically at 405 nm in a microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

**DNA-Assay**

DNA content (n=4) was measured by the PicoGreen assay (Molecular Probes, Eugene, OR), according to the protocols of the manufacturer. Samples were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 528 nm (Packard instrument, Downes Gore, IL).

**Calcium-Assay**

To measure the amount of calcium, scaffold halves (n=4) were immersed in 1 ml of 5% trichloroacetic acid and disintegrated using steel balls and a Minibead-beater. The debris was re-extracted with another 1 ml and the solutions were combined. Calcium content was determined spectrophotometrically at 575 nm (Microplate reader, Molecular Devices Corporation, Sunnyvale, CA), according to the manufacturer’s protocol.

**Histology**

For histology, constructs (n=4) were fixed in 10% (v/v) neutral-buffered formalin (24 h at 4 °C), dehydrated in graded ethanol solutions, embedded in paraffin, bisected through the center, and cut into 5-µm-thick sections. In order to visualize hMSCs in the scaffold cross sections, H&E staining was carried out after deparrafinization through graded ethanol series. For calcium visualization, von Kossa staining was performed. Briefly, deparaffinized sections were immersed in a 5% silver nitrate solution exposed to a 60 Watt lamp for 1 h. The sections were rinsed 3 times with UPW and fixed in a 5% sodium thiosulfate solution for 5 minutes. After washing, the stained sections were dehydrated with increasing concentration of ethanol and finally rinsed with xylene before the samples were covered with a glass plate.

**X ray Diffraction (XRD) of tissue engineered bone**

XRD patterns of scaffolds after spinner flasks cultivation in osteogenic or control medium were obtained by means of a Stoe STADIP powder diffractometer equipped
with a curved position sensitive detector covering 45 deg. in 2Theta. The radiation source was CuKalpha1 (lambda=1.54059 Angstrom, Ge-monochromator).

**Micro-computed Tomography (µCT)**

µCT was employed for the calculation of scaffold porosity and visualization of morphology of tissue engineered bone.

For assessment of scaffold porosity by µCT scaffolds were stained in 25 mg/ml osmium tetroxide solution for 48 h and left under the hood until completely dried. For the assessment of bone-like tissue morphology, constructs were cultured in spinner flasks, scaffolds were fixed in 10% formalin solution prior to the measurement. Stained scaffolds (n=3) or constructs of tissue engineered bone (n = 4) were analyzed by micro-computed tomography on a micro-CT40 imaging system (Scanco Medical, Bassersdorf, Switzerland) providing an isotropic resolution of 12 µm and 15 µm, respectively. A constrained Gaussian filter was used to partly suppress noise. Stained scaffolds or mineralized tissue were segmented from background using a global thresholding procedure (Ruegsegger et al., 1996). All samples were processed by using the same filter width (1.2) and filter support (1). Quantitative morphometry was performed to calculate the porosity of stained scaffold and bone volume, bone surface and bone surface-to-volume ratio for mineralized tissue, respectively, using direct microstructural bone analysis (Hildebrand et al., 1999). Three-dimensional visualization was generated using in-house software (Muller et al., 1994).

**Statistics**

Statistical data analysis was performed by one-way analysis of variance (ANOVA) and Tukey-HST test for post hoc comparison using Minitab® Release 14 for Windows. p<0.05 was considered statistically significant.
Figure 2: (A) Fourier transformed infrared spectroscopy, and (B) differential scanning calorimetry thermograms from untreated freeze dried scaffolds or scaffolds treated with 90% methanol. (C) Mass loss in% of untreated and methanol treated scaffolds over time.

Results

Characterization of scaffolds
Changes in silk fibroin crystallinity in response to methanol treatment were analyzed by FT-IR (Figure 2A). The treatment with methanol resulted in a absorption at 1232 cm⁻¹ and a visible shoulder at 1262 cm⁻¹ (amide III band shift - assigned to the β-sheet conformation of silk fibroin and absent in untreated scaffolds (Bhat and Nadiger, 1979). From analysis by DSC, a shift in endothermic peaks to higher temperatures was visible after methanol treatment (Figure 2C). Untreated silk fibroin scaffolds showed an endothermic peak at 199.6 °C and methanol treated silk fibroin scaffolds at 213.6
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°C. A brownish coloration and visual evidence of decomposition were observed at these temperatures using a polarization microscope (data not shown), suggesting higher stability of the silk fibroin network after methanol treatment. Mass loss of methanol treated silk fibroin scaffolds in water at 37 °C was 4.9% after 5 days and remained constant until the end of the experiment (14 days), demonstrating the integrity of the scaffold over time, as a prerequisite to provide a stable template for bone-like tissue formation. The mass loss of untreated scaffolds was high during the first two days and kept on until 36% of the mass was lost after 14 days (Figure 2C).

Scanning electron microscope images were taken from scaffolds prepared with 100-200 µm, 200-300 µm and 300-400 µm paraffin globules used as porogen. The treatment of the paraffin globules at room temperature (RT-scaffold), 37 °C (37-scaffold) and 45 °C (45-scaffold), prior to the addition of aqueous 20% silk fibroin solution (w/w) into the paraffin mold, resulted in different pore interconnectivities (Figure 3). Overall distributions revealed an interconnected network for the treatments at 37 °C and 45 °C (Figure 3). Treatments at room temperature did not show apparent interconnectivity, based on qualitative observations. Pore interconnectivity increased for materials based on paraffin treated at 37 °C and 45 °C, resulting in average pore-pore connections between 50-80 µm in diameter for treatments at 37 °C, and 120-200

![Figure 3: Scanning electron microscopy images taken from silk fibroin scaffolds prepared with porogens of different diameters (100-200 µm, 200-300 µm, 300-400 µm). Interconnectivity was controlled by melting of the paraffin porogen at room temperature (RT), 37 °C or 45 °C prior to addition of the silk fibroin solution. Bar length 400 µm for 100 x magnification and 100 µm for 450 x magnification.](image)
µm for treatment at 45 °C, as qualitatively assessed for pore sizes of 200-300 µm. Porosity, as calculated from µCT data, was not significantly increased by the treatment of the porogen at higher temperature but depended on the pore size used (Table 2). Nevertheless, the mechanical properties of the scaffolds were significantly influenced by the treatment of the porogen at different temperatures prior to the addition of the silk fibroin solution, ranging from 0.35 MPa for scaffolds prepared with 45 °C treatment and 1.1 MPa for scaffolds prepared with 37 °C (Figure 4). Significant intergroup differences in Young’s modulus were recorded for the 37 °C porogen treatment when compared to scaffolds resulting from 45 °C porogen treatments (p<0.01) and for 300-400 µm diameter RT-treatment, when compared to 37-scaffolds - (p<0.05), or 45-scaffolds (p<0.01). Absolute values were well below cancellous bone (200 MPa) or cartilage (20 MPa).

Gas chromatography was used to detect traces of residual solvents used in the scaffolding process, such as hexane and methanol. Traces of hexane were 208.93 ± 26.9 ppm. Methanol was below the detection limits.

Figure 4: Compressive strength (Young’s modulus) of scaffolds as a function of porogen diameter (100-200 µm, 200-300 µm, 300-400 µm) and temperature of processing during porogen removal (room temperature (RT), 37 °C, 45 °C) prior to the addition of SF solution (**p<0.01; *p<0.05).

Characterization of isolated mesenchymal stem cells from bone marrow
For the characterization of the bone marrow isolates, cells were investigated for their expression of surface markers by FACS analysis. The expression of the surface antigens CD34 and CD31 was negative, suggesting the absence of hematopoietic progenitor cells and cells of endothelial origin, respectively. Most cells expressed
CD71, a surface antigen commonly present in proliferating cells, and CD44, the transmembrane hyaluronate receptor for osteopontin, ankyrin, and fibronectin. Furthermore, the expression of CD105/endoglin, a putative marker for MSCs and αvβ3, a ligand for osteopontin, were positively expressed. The potential of isolated cells to differentiate into the osteogenic lineage was tested in a micromass culture. The isolated cells treated with osteogenic medium, containing BMP-2, showed a significant deposition of calcium and the activity of alkaline phosphatase was elevated when compared to the control, after 2 weeks of culture (data not shown).

**Figure 5:** Biochemical characterization of silk fibroin constructs seeded with hMSCs after 4 weeks of culture in bioreactors with osteogenic (scaffolds prepared at RT or 37 °C) or control medium, respectively (scaffolds prepared at 37 °C). (A) DNA normalized to mg wet weight of the construct (ng x ml⁻¹ x mg⁻¹)], (B) alkaline phosphatase activity expressed as p-nitrophenol conversion/DNA (µg/ng), and (C) calcium deposition normalized per DNA content (µg/ng) (**p<0.01; *p<0.05).

*Biochemical, histological and micro tomographic evaluation of osteogenic differentiation of hMSC seeded on silk fibroin scaffolds in spinner flasks cultures.*

DNA content in RT and 37-scaffolds, prepared with the identical porogen size (diameter 200-300 µm) was not significant different after cultivation of hMSCs for 4 weeks in osteoinductive medium (Figure 5A). hMSCs cultured on 37°C treated silk scaffolds with the same pore diameter (200-300 µm) in control medium resulted in
significantly higher levels of DNA (p<0.05) when compared to the osteogenic medium.

Alkaline phosphatase (ALP) activity, measured by the conversion of p-nitrophenylphosphate to p-nitrophenol per DNA, was significantly higher for constructs grown in osteogenic when compared to control medium (p<0.05) (Figure 5B). No differences in ALP activity/DNA were observed for scaffolds with different interconnectivities. Calcium deposition per DNA was significantly increased for RT-scaffolds (p<0.05) or 37-scaffolds (p<0.01) when compared to the controls, with no significant differences between these two scaffold geometries (Figure 5B).

<table>
<thead>
<tr>
<th>Control</th>
<th>Osteogenic</th>
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<td>37°C</td>
<td>RT</td>
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</tbody>
</table>

![Figure 6: Histological sections taken from silk fibroin constructs prepared with porogen treated at room temperature (RT-scaffold) (B,D) and 37 °C (37-scaffold; C,E). Constructs were seeded with hMSC and cultured for 4 weeks in osteogenic (RT- and 37-scaffolds; B-E) or control medium (37-scaffolds; A). H&E staining (A-C) and von Kossa staining (D,E); bar length 200 µm for whole images 50 µm for close-ups.](image)

Cellular ingrowth in RT-scaffolds was confined to single pores and prominent at the scaffold surface, whereas complete in-growth was observed in the 37-scaffolds resulting in a dense cellular network after 4 weeks of culture in osteogenic medium (Figure 6B,C). This was corroborated by von Kossa staining (calcium staining) (Figure 6D,E). Mineralization observed on RT-scaffolds (small interconnectivity) was confined to disconnected clusters of bone-like tissue (Figure 6D). Staining of 37-scaffolds resulted in homogenous mineralization of constructs with an interconnected trabecular network of bone-like tissue (Figure 6E).
The calcium staining was confirmed by micro-computed tomography (μCT) showing top view, center-cross view and bottom views of bone-like structures deposited on 37-scaffolds (Figure 7A,B,C) and RT-scaffolds (Figure 7D-F) after 4 weeks culture in bioreactors using osteoinductive medium. The bone formed as observed in the top view of 37-scaffolds; (Figure 7A) was characterized by a trabecular bone-like structure including focally restricted areas of plate-like bone. The bottom view of the scaffolds exhibited the same characteristics (Figure 7C). The center cross-view (Figure 7B) through the scaffold center revealed a gradual transition of trabecular-like bone into non-connected bone clusters from top to center. The top and bottom views of bone-like tissue on the RT-scaffolds showed the formation of plate-like bone (Figure 7D,F). Bone formation was not observed in the center of the construct, with the exception of the small band coinciding with the location of the needle pin, holding the construct in place during culture (Figure 7E). Calculated bone volumes and surfaces revealed no significant difference between the RT-scaffolds and 37-scaffolds (Figure 8A,B). Bone surface to volume ratio was significantly increased for the 37-scaffolds when compared to RT-scaffolds (Figure 8C). BS and BV but not BS/BV are normalized by the wet weight of constructs, a source of additional systemic error, which is circumvented by the measure BS/BV resulting in significant difference.

Table 2: Scaffold porosity (%)

<table>
<thead>
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<th>100 - 200 μm</th>
<th>200 - 300 μm</th>
<th>300 - 400 μm</th>
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<tr>
<td>RT</td>
<td>93.28 ± 7.61</td>
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<td>37°C</td>
<td>88.89 ± 2.28</td>
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<td>93.27 ± 2.32</td>
<td>85.77 ± 1.12</td>
<td>76.85 ± 2.03</td>
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To verify the bone-like nature of the deposited tissue, the scaffolds were characterized by XRD. In spectras obtained from scaffolds cultured with hMSC in osteogenic condition, in contrasts to scaffold cultured in control medium, characteristic 2Theta values of 26° and 40° could be detected corresponding to Miller indices of (0 0 2) and (3 1 0), respectively. Those bands and a additional broader band between 30° and 35°C
fitted the pattern of cortical bone (Bigi et al., 1997) indicating a hydroxyl apatite structure of the tissue engineered bone.

Discussion

Scaffolds used for bone tissue engineering and repair have to fulfill various requirement such as biological, mechanical and architectural aspects. They should be biocompatible, enable the adherence of cells, be slowly degradable and provide a physical support but display highly interconnected pores to allow a homogenous ingrowth of cells, tissue and a sufficient vascularization at the implant site. Therefore, strategies to address these criteria should include on one hand the quest for an ideal biomaterial and on the other hand for a suitable fabrication technology to control the porosity and biomechanical strength of the implant. To expand the available options of silk fibroin scaffolds starting from aqueous silk fibroin solutions, a novel process was used to generate 3D biomaterials with control of pore size and interconnectivity. The pore porosity of our scaffolds was mainly influence by the diameter of the porogen used but did not impact the mechanical structure. The new process circumvents the use of NaCl and the use of non ICH organic solvents (e.g. HFIP) (ICH) during the assembly of the silk fibroin (Figure 1). These scaffolds offer mechanical strength and control over pore size and interconnectivity. This approach adds versatility and options to prior methods of preparing 3D silk scaffolds in which organic solvents of unknown safety, and salt with associated conditions of high ionic strength, were required. hMSCs seeded scaffolds with pore sizes between 200-300 µm exhibited substantially higher interconnectivity of the deposited bone clusters, when the material was prepared at 37 °C when compared to RT. The results suggest that the process for the preparation of aqueous based silk fibroin scaffolds by freeze-drying and the use of paraffin as porogen can direct the morphology of bone-like tissue resulting in deposition of plate-like bone prominent at the surface (RT-scaffold) or an interconnected network of bone trabeculae throughout the construct (37-scaffold). These findings are important as other biodegradable polymeric biomaterial scaffolds do not offer the same level of control of this type of outcome, even aside from the unique mechanical features of silk-based biomaterials. For example, collagen sponges degrade too rapidly to maintain the required template features as we have shown earlier in osteogenic studies (Meinel et al., 2004b). While cross-linking helps in this
regard, a negative impact on the desired biological functions due to cross-linking has been shown (van Luyn et al., 1992). Similarly, PLA systems remain limited due to failure in meeting mechanical demands at the implantation site and acidic by-products as typically released during degradation (Athanasiou et al., 1996, Hollinger et al., 1996).

![Image of bone morphology](image)

**Figure 7**: Morphology of tissue engineered bone on silk fibroin scaffolds seeded with hMSCs after 4 weeks of culture in bioreactors with osteogenic medium as determined by micro-computed tomography (µCT). Porogen diameter was 200-300 μm, and exposed to 37 °C (A-C) or room temperature (RT; D-F) for 50 minutes prior to addition of silk solution. Top view (A,D), center-cross view (B,E) and bottom view (C,F). Bar length = 2mm.

Methanol treatment induced β-sheet crystallinity as indicated by an additional shoulder at 1262 cm⁻¹ when compared to the chromatogram taken from non-methanol treated silk scaffolds. This shoulder is due to a shift of the amide III band and indicative for a conformational shift of silk fibroin into -sheet structure (**Figure 2A**) (Asakura et al., 1985, Mingzhong et al., 1999). The increase in crystallinity for methanol treated scaffolds was reflected in an increase in decomposition temperature as shown in the DSC thermogram (**Figure 2B**). However, for a detailed analysis of changes in crystallinity as a consequence of methanol treatment, further studies should be conducted. FTIR is sensitive to changes in short-range changes – on the functional group level - and less suited for an analysis of supramolecular assembly. Changes in
long range crystallinity of silk fibroin films and fibers as a consequence of methanol treatment have been detailed before using X-ray diffractometry studies (Valluzzi et al., 1999, Valluzzi et al., 2002). The observed changes in crystallinity by both, FTIR and DSC are in line with the substantial decrease in water solubility for methanol treated versus untreated scaffolds, respectively (Figure 2C).

The methanol and hexane content in the scaffolds was assessed by head space gas chromatography. Residual methanol was not present in the scaffold but 208.93 ± 26.9 ppm of hexane were detected. According to ICH limit for residual solvents pharmaceutical products, the permitted daily exposure (PDE) of hexane is 2.9 mg/day corresponding to a daily administration of 10 g product containing 290 ppm of hexane (ICH). Scaffolds prepared according to the presented protocols are well below ICH limits and, therefore, are generally considered as safe for this parameter.

Microarchitectural parameters were related to compressive mechanical properties (Lin et al., 2003). The porosity of scaffolds with different pore sizes (100-200, 200-300,
300-400 µm) and for each treatment regimen (RT, 37 and 45 °C) was identified by µCT (Table 1). The porosity was particularly influenced by the diameter of the porogen among groups manufactured with different porogen diameters but not the mode of temperature treatment, i.e. interconnectivity of the pores within a group manufactured with identical porogen diameters. Therefore, the mechanical properties were mainly influenced by the architecture of the pores and not by the porosity. Heat treatment of the porogen at 45°C transformed the resulting scaffold architecture from separated or connected single pore voids observed at RT and 37°C, respectively, to fused pores forming larger and weaker frameworks (Figure 3). Scaffolds with low pore interconnectivities did not result in improved mechanical properties, with the exception of pore sizes of 300-400 µm, which displayed laminar cracks and structural inhomogeneities after freeze-drying, most likely the result of higher porogen mobility during soaking of the aqueous silk fibroin solution and freezing. Therefore, microarchitectural parameters affect mechanical properties as well as the reproducibility of the production process. The results did not reveal an influence of pore size on the mechanical properties as described in other models (Moroni et al., 2005, Le Huec et al., 1995, Meaney, 1995).

Cells isolated from bone marrow showed an expression of CD105/endoglin (a putative marker of MSCs), and CD71 (a receptor expressed in proliferating cells (Judd et al., 1980, Kuhn et al., 1984)) as well as the lack of expression of CD31 and CD34 (markers for cells that are of endothelial (DeLisser et al., 1993) or hematopoietic origin (DeLisser et al., 1993, Negrin et al., 2000, Spangrude et al., 1988)) that indicated the MSC nature of these cells. Furthermore cells showed the ability to undergo osteogenic differentiation in micromass cultures using osteogenic medium (data not shown).

The formation of bone-like tissue by hMSC cultured in spinner flasks on different scaffold designs was further investigated by micro-computed tomography (µCT) and demonstrated that different morphologies of bone-like tissues can be engineered as a function of scaffold design. This control of geometry extends the design of mechanically stable bone constructs, directing the formation of different bone structures by tissue engineering. The nature of the deposited bone, as a result of scaffold geometry, was further reflected in morphometric analysis (Figure 8A). Bone surface-to-volume ratio was significantly increased in scaffolds prepared with porogen treated at 37 °C, suggesting a shift to trabecular bone-like tissue. This could be a
function of diffusional hindrance for nutrients and gases to enter scaffolds with lower interconnectivity, resulting in impaired deposition of bone and cellular proliferation in more central parts of the constructs, as has been observed for other biomaterials (Ishaug et al., 1997, Martin et al., 2001, Vunjak-Novakovic et al., 1998).

In summary, a new fabrication protocol for the preparation of silk fibroin scaffolds was identified that circumvents the use of organic solvents at concentrations not generally recognized as safe (ICH) and provides control of mechanical properties, pore size and interconnectivity. The geometry of the engineered bone, as a result of calcium deposition during osteogenic differentiation of hMSCs, was guided or templated by the scaffold design. This relationship resulted in more plate-like or trabecular-like geometries and degrees of cellular penetration into the scaffold centers correlated to the scaffold features used in the cell cultivation process. Future studies will focus on combining the mechanical advantages of silk fibroin scaffolds with drug delivery through co-encapsulation of growth factors to impact cell proliferation and differentiation in vitro and/or in vivo (Karageorgiou and Kaplan, 2005, Lee, 2000, Murphy et al., 2000, Whitaker et al., 2001, Holland et al., 2005, Hile et al., 2000).

Acknowledgement

We thank Trudel (Zurich, Switzerland) for silk cocoons, Wyeth Biopharmaceuticals (Andover, MA) for BMP-2 supply and the group of Prof. Altorfer (ETH Zurich) for access to their DSC equipment. We further thank Prof. Amadò and Thomas Schärer (ILW, ETH Zurich) for the access and the help with the GC analysis and Chunmei Li (IPW, ETH Zurich) for the help with the XRD. Financial support was from the AO Foundation (AO Biotechnology Research Grant 2003), Association for Orthopaedic Research, ETH Zurich (TH 26.04-1), the US National Science Foundation (Grant Nr. 0436490) and the National Institute of Health (P41 - EB002520) is gratefully acknowledged.

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Chapter 2: Effect of scaffold design on bone morphology in vitro


Control of in vitro engineered bone geometries using mesenchymal stem cells and silk scaffolds

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Tissue Engineering, 2006, accepted
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Abstract

Natural bone consists of cortical and trabecular geometries. This study aims at engineering different bone geometries with small pores (112-224) µm in diameter on one side of the scaffold, and large pores (400-500) µm in diameter on the other, while keeping scaffold porosities constant among groups. We hypothesized that tissue engineered bone morphology in silk fibroin (SF) implants is pre-determined by the scaffold’s geometry. To test this hypothesis, SF scaffolds with the different pore diameters were prepared and seeded with human mesenchymal stem cells (hMSC). Dynamic cell seeding procedure in spinner flasks was selected for the differentiation studies, as it resulted in equal cell viability and proliferation and better cell distribution throughout the scaffold as visualized by histology and confocal microscopy and compared to static seeding. Differentiation of hMSC in osteogenic cell culture medium in spinner flasks for three and five weeks resulted in increased alkaline phosphatase activity and calcium deposition when compared to control medium. Micro-computed tomography (µCT) detailed the pore geometries of the newly formed tissue and suggested that the morphology of engineered bone tissue was controlled by the underlying scaffold geometry.
Introduction

The next generation of tissue engineered musculoskeletal tissues aims at mimicking physiological tissue geometry and function by generating more complex and structurally organized implants. This is particularly important for bone, a complex tissue consisting of sponge-like morphology with characteristic geometries and porosities along with solid compact bone in varying proportions (Sikavitsas et al., 2001). In the specific context of bone tissue engineering, input variables comprise the selection of cells, spatial and temporal control of growth factor presentation, scaffold frameworks for cell growth and differentiation as well as integration with the host tissue at the implantation site (Karageorgiou and Kaplan, 2005, Maniatopoulos et al., 1988, Meinel et al., 2005a, Meinel et al., 2004c, Meinel et al., 2004e, Meinel et al., 2004g, Tabata, 2000, Vacanti and Langer, 1999, Vunjak-Novakovic and Freed, 1998, Zeltinger et al., 2001, Gauthier et al., 2005) Little work has been devoted to controlling the architecture of the engineered bone (Hutmacher, 2000, Gauthier et al., 2005). This study aims at demonstrating the feasibility of controlling scaffold geometries on a single scaffold. Engineering of bone may require design strategies to more closely mimic the anatomical organization of scaffold structures and tissue matrix. Importantly, changes in a scaffold’s structure may impact cellular performances due to differences in the mechanical environment as well as differences in diffusion of gases, nutrient, and metabolites (Detamor and Athanasiou, 2003).

Silk fibroin (SF) is a biocompatible (Altman et al., 2003, Panilaitis et al., 2003, Meinel et al., 2005b), enzymatically degradable (Horan et al., 2004, Arai et al., 2004) material, which can be processed into water insoluble implants using biomimetic approaches derived from the silkworm and spider silk spinning process. This allows for a process with minimal use of organic solvents (Meinel et al., 2005a, Panilaitis et al., 2003, Soong and Kenyon, 1984) SF is different from silk – which constitutes a mix of various proteins including SF and sericin - from the silkworm Bombyx mori, which has been used as clinical sutures for decades, and in textile production for centuries (Lange, 1907, Lange, 1903, Halsted, 1892). Adverse biological responses to silk sutures were the result of residual contaminations with sericin, the glue-like protein holding the SF fibers together. Sericin can be extracted from SF to overcome this problem (Altman et al., 2003, Meinel et al., 2005b, Panilaitis et al., 2003, Santin et al., 1999). It has been shown that purified, i.e. sericin free SF is as biocompatible as
collagen and PLGA (Meinel et al., 2005b, Altman et al., 2003, Panilaitis et al., 2003). Perhaps the most distinguishing feature of SF when compared to other biomaterials such as collagens or synthetic materials such as polyesters is the wide range of mechanical properties that can be achieved, allowing adaptation to the requirements at the implantation site (Sofía et al., 2001, Altman et al., 2003, Meinel et al., 2004d, Meinel et al., 2004e, Meinel et al., 2004g, Gosline et al., 1986, Nazarov et al., 2004, Minoura et al., 1995, Servoli et al., 2005). This includes the possibility to match the mechanical properties, the shape of the scaffolds or the conformation of SF. Therefore, SF may fill an important niche in scaffold functionality, addressing considerable needs in musculoskeletal tissue repair.

Previous studies with SF lead to the assumption that the structure of tissue engineered bone – starting off from human mesenchymal stem cells (hMSC) seeded SF scaffolds in osteogenic medium and harvested in bioreactors – can be controlled by SF scaffold structure (Meinel et al., 2005a, Meinel et al., 2004g). Building from these observations, the present study aimed at engineering various pore sizes and in consequence different engineered bone geometries on a single SF scaffold. Mechanically stable SF 3D scaffolds with separate domains of different pore diameters ranging from 100 to 500 µm on one single scaffold were produced to provide a structural framework for cells to grow on. Initially, the impact of scaffold pore size on cell number and viability was assessed. This was followed by an investigation of the calcification process of human mesenchymal stem cells in osteogenic culture medium and a visualization of the engineered bone morphologies. Our goal was to control bone morphology through scaffold design and to provide an implant that mimics tissue macrostructure at the implantation sites.
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Materials and Methods

Materials

Fetal bovine serum (FBS), RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), basic fibroblast growth factor (bFGF), transforming growth factor-ß1 (TGF-ß1), penicillin and streptomycin (Pen-Strep), Fungizone, nonessential amino acids (NEAA, consisting of 8.9 mg/L L-alanine, 13.21 mg/L L-asparagine, 13.3 mg/L L-aspartic acid, 14.7 mg/L L-glutamic acid, 7.5 mg/L glycine, 11.5 mg/L L-proline, 10.5 mg/L L-serine), and trypsin were from Gibco (Carlsbad, CA). L-Ascorbic acid 2-phosphate, dexamethasone, β-glycerophosphate and 3-(4,5-dimethylidiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were from Sigma (St. Louis, MO). All other substances were of analytical or pharmaceutical grade and obtained from Sigma. Silkworm cocoons were kindly supplied by Trudel Silk Inc (Zurich, Switzerland). Bone morphogenic protein 2 (BMP-2) was kindly supplied by Wyeth Biopharmaceuticals (Andover, MA).

SF scaffold preparation and characterization

SF scaffolds were prepared as previously described (Nazarov et al., 2004, Sofia et al., 2001). In short, cocoons from Bombyx mori were boiled for 1 hour in an aqueous solution of 0.02M Na2CO3 and rinsed with water to extract sericin. Purified SF was solubilized in 9M LiBr solution and dialyzed (Pierce, MWCO 3500 g/mol) against water for 2.5 days, lyophilized and redissolved in hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) SF solution. NaCl crystals with particle diameters of either 112-224 μm, 400-500 μm or both was weighed in a Teflon container and SF/HFIP solution was added at a ratio of 20:1 (NaCl/SF). HFIP was allowed to evaporate for 2 days and NaCl/SF blocks were immersed in 90% (v/v) methanol for 30 minutes to induce a conformational transition to β-sheet domains (Nazarov et al., 2004). Blocks were removed, dried and NaCl was leached by incubation in water for 2 days, resulting in scaffolds with approximately 98% porosity (Nazarov et al., 2004). Disk shaped scaffolds (8 mm diameter, 2 mm thick) were prepared using a dermal punch (Miltey, Lake Success, NY), and autoclaved at 121ºC for 15 min.
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**Scaffold porosity**

Scaffold porosity was evaluated by comparison of the overall scaffold volume versus the true volumes of the silk material of the specific scaffolds, which were evaluated by means of a gas pycnometer (Accu Pic 1330, Micromeritics, Mönchengladbach, Germany).

**Scanning electron microscopy (SEM)**

Pore distribution of dry and platinum-coated scaffolds was characterized using scanning electron microscopy (SEM, Zeiss Leo Gemini 1530, Cambridge, UK).

**Cell isolation, expansion and characterization**

Human mesenchymal stem cells (hMSC) were isolated by cell adhesion to tissue culture plastic from 25 cm³ whole bone marrow obtained from Clonetics (Santa Rosa, CA). 10 mL aliquots of bone marrow were diluted in 100 mL of isolation medium (RPMI 1640 supplemented with 5% FBS). Cells were pelleted, resuspended in expansion medium (DMEM, 10% FBS, Pen-Strep, Fungizone, NEAA and 1 ng/mL bFGF) and seeded in 175 cm² flasks at a density of 5x10⁴ cells/cm². Adherent cells were allowed to reach approximately 80% confluence (12-17 days for the first passage). Cells were trypsinized and replated every 6-8 days at approximately 80% confluence. The second passage (P2) of the cells was used if not otherwise stated.

To confirm their mesenchymal character, the cellswere characterized with respect to (i) the expression of surface antigens and (ii) the ability to selectively differentiate into chondrogenic or osteogenic lineages in response to environmental stimuli, as previously described (Meinel et al., 2005a, Meinel et al., 2004a, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2005b, Meinel et al., 2004e, Meinel et al., 2004f, Meinel et al., 2004g, Meinel et al., 2004h).

**Scaffold seeding**

Two different seeding strategies were evaluated in respect to cell attachment and proliferation capacity on SF scaffolds with small, large, or mixed pore sizes in a spinner flask bioreactor: (i) dynamic seeding and (ii) static seeding:
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Dynamic seeding

For dynamic seeding (Vunjak-Novakovic and Freed, 1998), 5x10^6 P2 hMSC per scaffold were suspended in a total of 150 mL control medium (DMEM, 10% FBS, Pen-Strep, Fungizone) and directly added into the spinner flask with 8 scaffolds, threaded onto 4 needles embedded in the stoppers of the spinner flask (2 scaffolds per needle) (Meinel et al., 2005a, Vunjak-Novakovic and Freed, 1998, Vunjak-Novakovic et al., 1999). The side arm caps were loosened to permit gas exchange and the cell suspension was stirred with a magnetic stirrer at 60 rpm and incubated for 24 h in an incubator (37°C, 5% CO2).

Static seeding

Static seeding was performed as previously described (Meinel et al., 2004c, Meinel et al., 2004g). Briefly, P2 hMSC were suspended in liquid BD Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA) at a concentration of 5x10^6 cells per scaffold in 20 µl Matrigel while working on ice to prevent gelation. The cell suspension was seeded onto prewetted scaffolds and placed in an incubator (37°C, 5% CO2) for 15 min to allow gel hardening. For cultivation in spinner flasks, these cell-seeded SF scaffolds were threaded onto needles as described for the dynamic seeding method. Flasks were filled with 150 mL control medium and placed in a humidified incubator (37°C, 5% CO2), with the side arm caps loosened to permit gas exchange and stirred with a magnetic bar at 60 rpm for 24 h.

Cell Assessments

Cell attachment and viability was verified by an epifluorescence staining using the LiveDead® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR). Briefly, seeded scaffolds were washed three times with PBS at 37°C, then incubated in a mixture of 4 µM ethidium homodimer-1 and 2 µM calcein AM in PBS for 30 minutes in a humidified incubator (37°C, 5% CO2). Fluorescent images from cross-sections were obtained immediately using a confocal laser scanning microscope (CLSM, Zeiss 410 inverted microscope, Zurich, Switzerland) equipped with Argon (488 nm) and
HeNe (543 nm) lasers; 2-dimensional multichannel image processing was performed using the IMARIS software (Bitplane AG, Switzerland).

Cell proliferation was determined by DNA analysis. After seeding, the constructs were blotted on a clean paper towel and wet weights were noted. Scaffolds were cut into halves, weighed and disintegrated using steel balls and a Minibead-beater (Biospec, Bartlesville, OK) in 1 mL 0.2% Triton X-100 and 5 mM MgCl2 solution. DNA content was measured using the PicoGreen Assay (Molecular Probes, Eugene, OR), according to the protocol of the manufacturer. Samples (n=4-5) were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Metabolic activity of the cells was evaluated using a MTT assay. Constructs (n = 4-5) were transferred to 2-mL plastic tubes, 1 mL serum free DMEM supplemented with 0.5 g/L MTT was added and incubated in the dark at 37°C and 5% CO2 for 2 h. Tubes were centrifuged for 10 min at 2000g and the supernatant was aspirated. For solubilisation, 1 mL of 0.04 M HCl in 2-propanol was added and constructs were disintegrated using steel balls and a Minibead-beater (Biospec, Bartlesville, OK). Tubes were centrifuged at 2000g and absorption of the supernatant was measured spectrophotometrically at 570 nm.

**Osteogenic differentiation of hMSC in spinner flasks**

P2 hMSC were seeded on SF scaffolds with small and large pores on one construct using the dynamic seeding method (see above). For cultivation in spinner flasks under osteogenic conditions (DMEM, 10% FBS, Pen-Strep, fungizone, 0.5 μg/mL L-ascorbic 2-phosphate, 10 nM dexamethasone, 10 mM β-glycerophosphate, 1 μg/mL BMP-2), medium with double concentrations of L-ascorbic acid 2-phosphate, dexamethasone, β-glycerophosphate and BMP-2 was replaced at a rate of 50% every second or third day for a total of 5 weeks.

**Biochemical analyses**

After 5 weeks of cultivation in bioreactors, scaffolds were blotted on a clean paper towel and their wet weight was noted. After cutting into halves, scaffolds (n=5) were weighed again and disintegrated using steel balls and a Minibead-beater (Biospec, Bartlesville, OK) in 1 mL 5% trichloroacetic acid in water. After a second extraction
with 1 mL 5% trichloroacetic acid in water for 30 minutes and allocation of the samples, calcium content was measured spectrophotometrically at 575 nm following the reaction with o-cresolphthalein complexone according to the manufacturer's protocol (Sigma, St. Louis, MO). For alkaline phosphatase (AP) activity and DNA analysis, 5 halves were disintegrated using steel balls and a Minibead-beater in 0.2% Triton X-100 and 5 mM MgCl2 solutions. AP activity was measured using a biochemical assay from Sigma (St. Louis, MO), based on conversion of p-nitrophenyl phosphate to p-nitrophenol which was measured spectrophotometrically at 405 nm. DNA content was measured as described in the seeding strategies section.

Histology

For histology, constructs were fixed in 10% neutral buffered formalin (24 h at 4°C), dehydrated in graded ethanol solutions and embedded in paraffin, bisected through the center, cut into 5 µm thick sections and mounted on SuperFrost microscope slides (Microm International AG, Volketswil, Switzerland). For a general overview, sections were stained with hematoxylin and eosin (H&E). To identify mineralization, a staining according to von Kossa in 5% AgNO3 for 1 h, exposed to a 60 W bulb, was performed.

Micro-computed tomography (µCT)

Mixed pore size SF constructs were analyzed after three and five weeks of osteogenic culture on a µCT 40 imaging system (Scanco Medical, Bassersdorf, Switzerland) providing an isotropic resolution of 10 µm. A constrained Gaussian filter was used to partly suppress noise. Mineralized tissue was segmented from non-mineralized tissue using a global thresholding procedure (Ruegsegger et al., 1996). All samples were thresholded by using the same filter width (1.2), filter support (1), and threshold (150; in permille of maximal image grey value, corresponding to an attenuation coefficient of 1.2 cm⁻¹). Quantitative morphometry was performed to assess bone surface-to-volume ratio of the entire construct as well as of the parts of the scaffold with small silk pores and with large silk pores for direct comparison using direct microstructural bone analysis (Hildebrand et al., 1999). Three-dimensional visualizations were generated using in-house software (Muller et al., 1994).
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Statistical analysis

All quantitative data are presented as means ± standard deviation. Differences in porosity between scaffolds with different pore sizes were investigated using one-way ANOVA. The seeding strategies and histomorphometry were analyzed by a general linear model (GLM) approach. Osteogenic differentiation after three or five weeks culture or at five weeks compared to the control were evaluated by Student's t-test. Differences between groups of p = 0.05 were considered statistically significant, and p = 0.01 were considered highly significant.

Figure 1: SEM images of silk scaffolds (A-C) and a µCT image of a cynomolgus monkey (L1) vertebra (D). (A) SF scaffold with a pore diameter between 112 and 224 µm, (B) SF scaffold with a pore diameter between 400 and 500 µm, (C) SF scaffold with a transition from small (112-224 µm) to large (400-500 µm) pore diameters. (D) Cynomolgus monkey L1 vertebra showing one example of natural occurrence of pore size transitions. Scale bar length: 500 µm.

Results

Scaffold structure and effects of seeding

Using a salt leaching method with sieved NaCl crystals, scaffold pore diameters were controlled from 112 µm to 500 µm as determined by SEM (Figure 1). Scaffolds with either small pores (112-224 µm, Figure 1A), large pores (400-500 µm, Figure 1B), or with pore diameters of 112-224 µm on one side and 400-500 µm on the other side of the scaffold - referred to as mixed pores in this publication - (Figure 1C) were fabricated. The porosity of the scaffolds was statistically equal and ranged between 93
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to 96% for all types of scaffolds used in this study and was, therefore, not dependent on the pore size (data not shown).

Both dynamic and static seeding were evaluated in terms of numbers of total and metabolically active cells on SF scaffolds as a function of scaffold geometry (Figures 2, 3). Static seeding resulted in significantly higher amounts of DNA per mg wet weight than the dynamic seeding method (p < 0.01), whereas no difference was observed among the different pore sizes (Figure 2A). Both seeding methods showed comparable cellular activity per DNA (Figure 2B). Upon dynamic seeding, cells had a more globular cell structure and attached closely to the SF lattice (Figure 3A-C, G-I), whereas after static seeding, cells embedded in Matrigel were located in the void spaces (Figure 3D-F, K-M).

Figure 2: Cell proliferation and activity 24 hours after seeding on scaffolds with different pore sizes: small pores (112–224 µm diameter), large pores (400–500 µm diameter), mixed pores (112–224 µm diameter on one half, 400–500 µm diameter on the other side of one scaffold) by two different strategies: static seeding and dynamic seeding. (A) Cell number on scaffolds (ng/mL DNA per mg wet weight). (B) Cell activity on scaffolds (units MTT per ng/mL DNA). Data are shown as average +/- standard deviation, n=3. Asterisks indicate significant difference from the other group (** p < 0.01).

Engineering of bone pore geometries

After 3 and 5 weeks of cultivation in spinner flasks in osteogenic medium, AP activity increased significantly (p < 0.01), i.e. from 96.6 ± 5 g converted p-nitrophenol per g DNA to 146.7 ± 14 g/g, whereas hMSC cultured in control medium for 5 weeks showed only 28 ± 10 g p-nitrophenol per g DNA (Figure 4). Mineralization was significantly increased, as indicated by a deposition of calcium of 355.9 ± 274 g per g DNA after 3 weeks and 1458 ± 422 g/g DNA after 5 weeks. No mineralization was observed in hMSC cultured in control medium (Figure 4). Mineralization of cells
grown in osteogenic medium was also demonstrated by histological evaluation (Figures 5D, E) whereas no mineralization was observed under control conditions (Figure 5F).

![Figure 3: hMSC on scaffolds with different pore sizes as visualized by CLSM (A-F) and histology (G-L) 24 hours after seeding: small pores (A, D, G, J; 112–224 µm diameter), large pores (B, E, H, K; 400-500 µm diameter), mixed pores (C, F, I, L; 112-224 µm diameter on one side of the scaffold, and 400-500 µm diameter on the other). Two different seeding strategies were applied: dynamic seeding (A-C, G-I) and static seeding (D-F, J-L). Scale bar lengths are 200 µm for CLSM images, 400 µm for histological pictures and 20 µm for the inserts.](image_url)

Images taken with μCT of hMSC-seeded mixed pore scaffolds grown under osteogenic conditions for 3 weeks demonstrated the formation of bone tissue with variable geometries (Figure 6), whereas in control medium no calcium deposition was found.
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(data not shown). High intra-group variation was observed with some scaffolds showing interconnected calcified structures (e.g. Figure 6 A,D,G), whereas in others, mineralization is limited to clusters with poor bone formation at the interfaces between large and small pores, respectively (Figure 6 C,F,I). The characterization of bone-like architectures was based on histomorphometric analysis. A high variability was observed between the individual scaffolds (n=5). For example, total bone surface (of mixed pore size scaffolds) at three weeks of culture ranged from 93 to 757 mm² with a mean surface of 461 mm² and a relative standard deviation of 53% and from 440 to 924 mm² with a mean surface of 486 mm² and a relative standard deviation of 51% after five weeks of culture. Total bone volume ranged from 1.1 to 11.2 mm³ with an average volume of 6.4 mm³ and a relative standard deviation of 58% at three weeks and from 5.5 to 20 mm³ with an average volume of 9.2 mm³ and a relative standard deviation of 66% at five weeks. The bone surface-to-volume ratio decreased significantly (p = 0.003) between three and five weeks in mixed pores constructs. For analysis of bone-like tissue formation on the different pore diameter regions on a single scaffold, constructs were divided and cylindrical pieces of the construct half with small silk pores and the half with large silk pores, respectively, were histomorphometrically analysed. For the small pore sections the surface-to-volume ratio decreased from 85.4 ± 11 mm⁻¹ at three weeks to 79 ± 13 mm⁻¹ at five weeks of culture, for the large section a decrease from 73.5 ± 7 mm⁻¹ at three weeks to 64.3 ± 10 mm⁻¹ at five weeks was observed. This indicated the formation of different bone morphologies on the two sections. After three and five weeks, the smaller pore sections showed a significantly higher bone surface-to-volume ratios than sections with the large pores, although scaffold porosity – but not pore size – were equal for the small pore and large pore sections, respectively (p = 0.002; data not shown).

Discussion

To mimic natural bone morphologies consisting of cortical and trabecular domains (Figure 1D), the goal of this study was to engineer both structures on a single scaffold. Recent studies led to the assumption that the morphology of tissue engineered bone using hMSCs seeded on SF scaffolds in osteogenic medium was guided by the structure of the scaffold framework (Meinel et al., 2005a, Zeltinger et al., 2001). The results of the present study corroborated these findings and demonstrated the
feasibility to address complex structural shapes within a single scaffold. Two sets of scaffolds were generated with (i) homogenous pore size distributions and a diameter of 112-224 µm or 400-500 µm, respectively, and (ii) SF scaffolds with two pore sizes on a single scaffold: with 112-224 µm and 400-500 µm pore diameters at either scaffold side, respectively. These templates were seeded with hMSCs and cultured in osteogenic medium in spinner flask type bioreactors for up to 5 weeks.

**Figure 4:** Biochemical parameters of osteogenic differentiation of hMSC cultured on SF scaffolds with mixed pores in spinner flasks cultured in osteogenic (black bars) or control conditions (grey bars). Data were collected after 3 and 5 (osteogenic group) or 5 weeks (control group), respectively, showing alkaline phosphatase activity (left) and calcium deposition (right), both per DNA content.

All scaffolds require cell seeding in a tissue culture vessel or a bioreactor for homogenous cell distribution (Burg et al., 2000, Vunjak-Novakovic and Freed, 1998, Vunjak-Novakovic et al., 2004). Two different seeding strategies – static and dynamic seeding - were evaluated in terms of cell viability and cell distribution. It has been shown that a high seeding density could improve the structural stability and biochemical composition of the engineered tissues from chondrocytes (Vunjak-Novakovic et al., 1998) and cardiac muscle cells (Carrier et al., 1999). Cell numbers below a critical minimum resulted in the formation of fibrous tissue (Temenoff and Mikos, 2000). Static seeding resulted in higher cell numbers for both dead and viable cells per scaffold than dynamic seeding. Through active adhesion to the scaffold, dynamic seeding restricted cell deposition to viable cells whereas static seeding led to the deposition of all cells that were passively embedded in the gel matrix - irrespective of their viability. Both seeding strategies were equivalent in terms of cell viability and activity (Figure 2B). Overall, the dynamic seeding strategy was selected for the experiments circumventing the deposition of significant numbers of dead cells, which
were previously reported to adversely impact cellular differentiation (Vukicevic et al., 1995).

Many studies have shown the importance of porosity, pore size and geometry of scaffolds for tissue engineering outcomes (Gauthier et al., 2005, Ma et al., 2003, Zeltinger et al., 2001, Tampieri et al., 2001, Tampieri et al., 2005). The presented data demonstrate the versatility of SF scaffolds to allow the manufacture of not just various pore sizes in general but on one single implant to meet the intrinsic geometric requirements at the implantation site. This was been previously shown for hydroxyapatite scaffolds (Tampieri et al., 2001, Tampieri et al., 2005). For SF scaffolds the geometry of bone formation was pre-determined by scaffold structure. Smaller pore sizes resulted in a higher surface-to-volume ratio of the engineered bone-like tissue when compared to sections with higher pores. This may allow the engineering of bone pore size gradients in vitro while keeping the porosity of the scaffolds at a constant high level of more than 90%, which is preferential for tissue engineering purposes (Ma et al., 2003).

Apart from detailing controlled engineering of bone architectures, this work showed limitations of the current approaches. This is demonstrated by the variability of bone
tissue formation, in particular at the transition zones from small to large pores, respectively. Possible scenarios to overcome these limitations include the improvements in scaffold design by generating a smooth pore gradient rather than an abrupt change in pore size, as engineered for the scaffold’s manufactured for this study. Furthermore, the use of bioreactors can facilitate nutrient/metabolite exchange throughout the scaffold or induce mechanical stimulation for bone formation, respectively.

Figure 6: Three representative µCT images of tissue engineered bone on mixed pore scaffolds. Magnifications of the transition zone of the pores (A-F), top view (D-F); and tilted sections (G-I). Scale bar lengths are 1 mm (A-C) and 2 mm (D-I).

At this point, it is difficult to predict how this translates into an in vivo environment. A potential challenge could be the ingrowth of other tissues as a function of pore size and porosity with a direct impact on cell survival implant integration into the host tissues. For example, BMP-2 induced bone formation has been demonstrated to be affected by the pore geometry in vivo. Comparison of porous hydroxyapatite blocks with honeycomb-shaped hydroxyapatite scaffolds and with porous particulate hydroxyapatite systems showed that tubular and curved geometries of the blocks hindered the penetration of mesenchymal cells and capillaries into the scaffold, which was not the case with honeycomb-shaped scaffolds and particulate systems. Bone formation occurred through endochondral ossification in honeycomb-shaped scaffolds and not through direct ossification as in porous blocks or particulate systems (Jin et al.,
2004, Jin and Kaplan, 2003). In similar studies with hydroxyapatite and growth factor loaded scaffolds with small (90-120 µm) and large (350 µm) pores, subcutaneous implantation in rats resulted in initial chondrogenesis and subsequent formation of bone tissue for smaller pore scaffolds and direct bone formation with no cartilage intermediate for larger pores (Kuboki et al., 2001). This indicates the relevance of in vivo data for biodegradable and protein based biomaterials as demonstrated in this study, to investigate more specifically the vascularization and the type of tissue and how it has been formed after implantation.

Future studies are planned to evaluate the in vivo effects of bone engineered with a trabecular network, mimicking the replaced host tissue at the implantation site. Readouts will include host-tissue integration, type and quality of formed tissue, vascularization and clinical benefit.

Acknowledgments

We thank Helene Rüedlinger for her assistance in cell culture and Trudel Inc. (Zurich, Switzerland) for the supply of silk cocoons. Financial support was from the Association for Orthopaedic Research, from the NIH (EB003210-01, EB002520, RO1 DE016525-01) and the ETH Zurich (TH Gesuch) and is greatly appreciated.

References

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Noninvasively monitoring and quantifying the engineering of bone-like tissue from human mesenchymal stem cells cultured on silk scaffolds

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Abstract

The formation of bone-like tissue from human mesenchymal stem cells (hMSC) cultured in osteogenic medium on silk-fibroin (SF) scaffolds was imaged and quantified over 44 days in culture using time-lapsed, nondestructive micro-computed tomography (µCT). Each construct was imaged 9 times in situ and images were superimposed. µCT irradiation did not impact the osteogenic performance of hMSCs based on DNA deposition, alkaline phosphatase activity and calcium deposition when compared to non exposed control samples. Bone-like tissue formation initiated at day 10 of culture with the deposition of small mineralized clusters which increased throughout the duration of culture. Mineral density increased linearly over time and did not correlate with bone volume. The surface-to-volume ratio of the bone-like tissues asymptotically converged to 26mm$^{-1}$. In conclusion, although in vitro formation of bone-like tissue started from mineralized clusters, the overall bone volume was not predictable from time or amount of the initial number or size of bone mineralized clusters. A linear increase of mineral density was observed over time, and bone surface-to-volume ratio approximated towards a set value. These data demonstrate the feasibility of qualitatively and quantitatively detailing the spatial and temporal mineralization of bone like tissue formation in tissue engineering.
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Introduction

Bone tissue engineering is a field of increasing interests in orthopedics (Alsberg et al., 2001, Meinel et al., 2004c, Vunjak-Novakovic et al., 2005). In particular the use of in vitro engineered bone tissues offer the opportunity to substitute malfunctioning, injured, or diseased tissue in patients suffering e.g. from degenerative diseases or tumors. Tissue engineering of skeletal tissues generally requires at least three elements: (i) 3-dimensional scaffolds, (ii) cells, and (iii) bioreactors with appropriate media, to form tissues like bone, cartilage or even osteochondral plugs (Meinel et al., 2004c, Vunjak-Novakovic et al., 2005, Meinel et al., 2005, Meinel et al., 2004b, Demarteau et al., 2003, Meinel et al., 2004a). Engineered constructs have the potential to replace compromised or missing host tissue or to serve for extended in vitro studies of bone formation in well controlled settings.

Mesenchymal stem cells (MSC), derived from human bone marrow, are a well known cell source to engineer autologous bone tissues. Human MSC (hMSC) can be expanded to large cell numbers in an undifferentiated state and when exposed to the appropriate extrinsic signals, selectively differentiate along mesenchymal lineages, including cartilage and bone (Pittenger et al., 1999). Scaffolds prepared from silk-fibroin (SF), have shown promising results as substrates for in vitro culture of bone-like tissue. SF scaffolds demonstrated equivalent or better biocompatibility than type I collagen scaffolds, and a distinguishing mechanical strength, and mechanical integrity when compared to other commonly used degradable polymeric biomaterials (Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2005). Furthermore, the degradation of SF scaffolds is slow, a prerequisite for the present study aimed at imaging and quantifying bone-like tissue formation on a stable, yet degradable polymeric system.

Faster degradation rate as observed for type I collagen would impact the ordered formation of bone-like tissues and therefore the demonstration of feasibility for time lapsed bone visualization sought in the present study, due to disintegration of the substrate during cell growth and tissue formation.

At present, assessments of in vitro engineered tissue quality and quantity rely on destructive methods, thereby terminating cultures and necessitating multiple samples for adequate statistical analysis. Obvious requirements also hinders the understanding of dynamic tissue formation processes for individual specimens and information is generally taken from pooled samples forming a treatment group (Washburn et al.,
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2004, Sikavitsas et al., 2005, Meinel et al., 2004b, Meinel et al., 2004c). Future clinical supply scenarios will require quality assessment of the selected implant, rather than retrospective data obtained from destructive analysis or from pooled samples as is currently the state of the art. Automated, well controlled bioreactors can potentially be coupled to non-invasive imaging setups, thereby providing the necessary information of tissue quality and formation events during culture for each individual implants (Martin et al., 2004).

The goal of this study was to monitor the development of bone-like tissue, engineered from human MSC, in situ by the integral use of µCT. Novel illustrations of growing bone-like tissue and detailed, individual quantification and variable correlation concerning tissue morphology and mineral density of the constructs offer new insights into the formation of engineered bone-like tissue from hMSCs and on SF scaffolds. In the future, this or similar imaging techniques should facilitate the selection of implants from a pool of autologous engineered implants based on qualitative and quantitative yet non-destructive imaging regimens.

Materials and Methods

Materials

Cocoons from Bombyx mori were kindly provided by Trudel, Zurich, Switzerland. Bone morphogenetic protein-2 (BMP-2) was kindly provided by Wyeth, Madison, NJ. Sodium Carbonate (Na2CO3), Lithium Bromide (LiBr), 1,1,1,3,3,3-Hexa-fluoro-isopropanol (HFIP), and Triton X-100 were from Fluka, Buchs, Switzerland. Bone marrow was from Cambrex, Walkersville, MD. Ascorbic acid-2-phosphate, dexamethasone, β-glycerolphosphate, and alkaline phosphatase (AP) assay were from Sigma-Aldrich, St. Louis, MO. Trichloroacetic acid was from Haenseler, Herisau, Switzerland. Calcium quantification assay was from Rolf Greiner Biochemica (# G210117), Flacht, Germany. Antibodies CD31-PE, CD34-APC, CD44-FITC, CD105-FITC, aVb1-FITC, Matrigel, and flow cytometer (FACScalibur) were from BD Bioscience, San Jose, CA. Dialysis Cassette, 3500 MWCO was from Pierce, Woburn, MA. Lyophilizer was from Christ, Osterode, Germany. Sieving tower was from Retsch, Arlesheim, Switzerland. Tissue culture flasks were from Nunc, Roskilde, Denmark. 12-well plates were from TPP, Trasadingen, Switzerland. Micro-computer
tomograph (µCT 40) was from Scanco Medical, Bassersdorf, Switzerland. Minibead-beater was from Biospec Products, Bartlesville, OK. All other substances were obtained from Invitrogen, Carlsbad, CA.

Scaffold production

Purified silkworm fibroin was prepared and formed into porous scaffolds as previously described (Nazarov et al., 2004, Hofmann et al., 2006). In brief, cocoons from B. mori were boiled for 1 h in an aqueous solution of 0.02 M Na2CO3, and rinsed with water to extract sericin and other contaminating proteins. Purified silk fibroin was solubilized in 9 M LiBr solution and dialyzed against water. The ultra pure water was exchanged 5 times in 72 h resulting in silk fibroin concentration of 3% (w/v). The SF solution was lyophilized until all water evaporated and afterwards dissolved in HFIP resulting in a 17% (w/v) solution. Granular NaCl was characterized for size using a sieving tower. The range of sizes of the employed porogen NaCl was 224-300 microns. For each scaffold, NaCl was weighed in a Teflon container and silk fibroin/HFIP solution was added at a ratio of 20:1 (NaCl/silk fibroin). HFIP was allowed to evaporate for 2 days and the NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 min to induce a protein conformational transition to β-sheet (Nazarov et al., 2004). The blocks were removed, dried, and NaCl was extracted in water for 2 days. Disk-shaped scaffolds (5 mm diameter, 1 mm thick) were prepared using a dermal punch and autoclaved.

Cell isolation and expansion

Cell isolation was performed similar as previously described (Meinel et al., 2004b). Briefly, samples of bone marrow were diluted in Dulbecco’s modified eagle medium (DMEM). The cells were centrifuged at 300 x g for 10 minutes. Cells were pelleted and suspended in expansion medium (DMEM, 10% fetal bovine serum (FBS), 1% nonessential amino acids (NEAA), 1 ng/ml basic fibroblast growth factor (bFGF)) and seeded in 175 cm2 flasks at a density of 5x104 cells/cm2. The adherent cells were allowed to reach 80% confluence (12–17 days for the first passage). Cells were trypsinized and replated every 6–8 days at 80% confluence. The second passage (P2) cells were used for tissue culture.
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The hMSC were characterized with respect to (i) the expression of surface antigens and (ii) the ability to selectively differentiate along the chondrogenic and osteogenic lineages. The expression of the following five surface antigens was characterized by flow cytometry analysis, as described previously (Meinel et al., 2004b) CD31 (PECAM-1/endothelial cells), CD34 (sialomucin/hematopoietic precursors), CD44 (hyaluronic acid receptor/hematopoietic cells and MSC), CD105 (endogelin/endothelial cells and MSC), and VI (immunocells and MSC). To assess the potential of hMSC for osteogenic and chondrogenic differentiation, the cells were cultured in 12-well plates as micromass cultures (5 drops of 15 µl of 2×10^7 cells/ml per well) in either control medium (DMEM supplemented with 10% FBS, penicillin-streptomycin, Fungizone, 50 µg/ml ascorbic acid-2-phosphate, and 10 nM dexamethasone) or osteogenic medium (control medium supplemented 10 mM β-glycerolphosphate, and 1 µg/ml BMP-2) or chondrogenic medium (control medium supplemented with 1% NEAA, 5 µg/ml insulin, and 5 ng/ml transforming growth factor beta (TGF-1)). Medium was exchanged 3-times per week. After 2 weeks of culture the amounts of DNA, AP activity, glycosaminoglycans (GAG) and calcium were measured, as described previously (Figure 1)(Meinel et al., 2004b).

**Tissue culture**

For cultivation on SF scaffolds (5 mm diameter × 1 mm thick disks), P2 hMSC were suspended in liquid Matrigel (106 cells per scaffold in 10 l Matrigel) while working on ice to prevent gelation, and the suspension was seeded onto prewetted scaffolds (overnight incubation of scaffold in DMEM). Seeded constructs (in culture dishes, without added medium) were placed in an incubator at 37°C for 15 min to allow gel hardening before placement in the culture vials. Custom culture vials were used to host the constructs during culture. These vials enabled tissue culture due to their biocompatibility; while also allowing sterile µCT of the constructs in a fixed position. Like that the constructs were cultured in the incubator and could be taken out of the incubator and transferred to the µCT for imaging without disturbing the scaffold systems. Due to the maintained sterility, the constructs could be returned to the incubator after each µCT scan without terminating the culture. Five constructs were placed in each vial. The vials were filled with 5 ml of
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osteogenic medium as detailed above. The vials were placed in a humidified incubator with gas exchange allowing position. For imaging, the vials were locked to their closed position for approximately 1 hour. Medium was replaced completely 3-times per week for 44 days of cultivation.

Micro-computed tomography

In situ μCT of the constructs was started at day 7 of the culture and until day 28 of the culture, images were taken twice a week. After this time frame, additional images were taken at days 37 and day 44. For imaging, the constructs were outside the incubator for approximately 1 hour. Constructs were analyzed on a μCT imaging system providing an isotropic resolution of 36 microns. Energy was set to 40 kVp, integration time was 200 ms, 2-fold frame averaging was chosen. A constrained Gaussian filter was used to partly suppress noise. Mineralized tissue was segmented from nonmineralized tissue using a global thresholding procedure (Ruegsegger et al., 1996). All samples were processed by using the same filter width (1.2), filter support (1), and threshold (20.7% of maximal image grey value, corresponds to an attenuation coefficient of 1.7 cm⁻¹). Quantitative morphometry was performed to assess bone volume (BV) and bone surface-to-volume ratio (BS/BV) using direct microstructural bone analysis (Hildebrand et al., 1999). For the assessment of mineral density, the grey value images were correlated to calibrated grey values of hydroxyapatite phantoms – provided by the μCT manufacturer. The μCT was calibrated with respect to hydroxyapatite density on a weekly basis. Therefore, a grey value in an image could be directly be correlated to a hydroxyapatite density value. Mineral density was determined within the defined BV only.

Three-dimensional visualizations were generated using in-house software (Müller and Ruegsegger, 1997, Müller et al., 1994). To visually follow the growth of the bone-like tissue, the subsequence of images was superimposed and volumes imaged at different times were illustrated by different colors and degrees of transparency.

Biochemical analysis

After 28 days of culture and 7 μCT scans samples from the imaged group and samples from a non-imaged control group were biochemically assessed for the effect of the
\( \mu \text{CT} \) irradiation on the tissue. For determination of DNA content, 10 constructs per group were disintegrated in 0.1% Triton X-100 solution using steel balls and a Minibead-beater. The content of each group was transferred to a new tube and centrifuged at 3000 x g for 10 min at 4°C. The resulting supernatant was used for the PicoGreen DNA quantification assay, according to the manufacturer’s protocol. The same aliquots were used to measure the AP activity using a commercially available assay based on conversion of p-nitrophenyl phosphate to p-nitrophenol. For determination of calcium content, 10 constructs per group were disintegrated in 0.5 ml 5% trichloroacetic acid using steel balls and a Minibead-beater. The content of each group was transferred to a new tube and centrifuged at 3000 x g for 10 min at 4°C. The resulting supernatant was again washed with trichloroacetic acid. The assay allowed spectrophotometric assessment following the reaction with o-cresolphthalein according to the manufacturer’s protocol.

**Statistical analysis**

Data is presented as mean ± stdev and statistical analysis of data was performed by Student t-test using Microsoft Excel. Values of \( p = 0.05 \) were considered significant, and \( p = 0.01 \) were considered highly significant.

**Results**

**Characterization of human mesenchymal stem cells**

Surface antigen expression demonstrated that the cells were positive for CD105 (hyaluronate receptor, a putative marker for mesenchymal stem cells), integrin alpha-5/beta-1 (fibronectin receptor) (Figure 1B, C) and, to a lesser extent, expression of CD44 (transmembrane hyaluronate receptor for osteopontin, ankyrin, and fibronectin). Negative expression of surface antigens for CD31 and CD34 suggested the absence of cells of endothelial or hematopoietic origin (Figure 1A, C).

Culture-expanded hMSC maintained their ability to selectively undergo chondrogenic and osteogenic differentiation, as assessed biochemically (Figure 1D-F). Micro-mass cultures of P2 and P3 hMSC cultured for 2 weeks in osteogenic medium showed higher degrees of AP activity, whereas cells cultured in chondrogenic medium did not
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(Figure 1D). Calcium deposition was observed in osteogenic medium, but not in control medium (Figure 1E). GAG accumulation was strikingly higher in chondrogenic medium when compared to control medium (Figure 1F). The amounts of calcium, AP activity, and GAG in micro-mass cultures of P2 and P3 cells were comparable. Importantly, cultures of hMSC deposited chondrogenic markers only when cultured in chondrogenic medium and osteogenic markers only when cultured in osteogenic medium.

![Figure 1: Characterization of human mesenchymal stem cells. (A) No expression of surface marker CD34. (B) Expression of surface marker CD105. (C) Expression of surface markers CD 31, CD44, vI. (D) GAG deposition of P2 and P3 cells cultured in control and chondrogenic medium, respectively. (E) Alkaline phosphatase activity of P2 and P3 cells cultured in osteogenic and chondrogenic medium, respectively. (F) Calcium deposition of P2 and P3 cells cultured in control and osteogenic medium, respectively.](image)

Effect of µCT imaging on cell performance

DNA content per scaffold of the non-irradiated control group (94.6 ± 50.3 µg, n=10) and the irradiated group (87.9 ± 15.5 µg, n=10) were not significantly different after 28 days of culture and 7 repetitive µCT based imaging cycles. No significant irradiation effect was found for alkaline phosphatase activity per DNA (AP activity, 0.38 ± 0.19 and 0.42 ± 0.09, respectively; n=10) or calcium deposition per scaffold (235.4 ± 77.5 µg and 272.7 ± 100.2 µg, respectively; n=10).
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**Bone volume**

Trabecular-like bone formation was observed with µCT (Figure 3). BV increased over time with high standard deviations starting off at 0.04 ± 0.04 mm³ at day 10 up to 3.8 ± 2.5 mm³ at day 44 (Figure 2A). For pooled samples, BV as a function of time followed an exponential pattern, with \( BV(t) = 0.0046e^{0.226t\text{[day]}} \) (\( R^2 = 96.8\% \)) up to day 24, followed by a linear deposition up to day 44 with \( BV(t) = (0.15t\text{[day]} - 2.7) \) (\( R^2 = 99.6\% \)). Individual samples followed different mineralization trends over time, ranging from strictly linear patterns (\( R^2 = 99.6\% \)) to exponential pattern \( R^2 = 96.4 \) over 44 days (Figure 3B).

BV was visualized in superimposed maps as a function of time (BV after 14, 28 and 44 days are displayed in blue, green, and red, respectively (Figure 4)). Mineralization heterogeneity was corroborated and BV after 44 days in culture was found to be non-predictable from BV at early time points (Figure 4). Bone-like tissue formation originated from the outer scaffold rims and proceeded, starting off from these early clusters towards the scaffold center. Over time continued formation of new bone-like tissue clusters was observed as indicated by red spots (end point day 44) in the cross and side view (Figure 4).

![Figure 2](image.png)

*Figure 2: Pooled data (n=11) on bone volume over time (A) and on bone surface-to-volume ratio over time (B) displaying mean values and standard deviation.*

**Bone surface-to-volume ratio**

A constant decrease of the mean bone surface-to-volume ratio (BS/BV) was observed over time (from 80.0 mm⁻¹ at day 10 to 26.1 mm⁻¹ at day 44) (Figure 2B). BS/BV...
standard deviation declined over time, from ±64% at day 10 to ±21% at day 44. Evidence was provided that all samples regardless of the initial BS/BV value asymptotically resulted in a value of approximately 26 mm⁻¹ (Figure 3B).

**Mineral density**

Mineral density over time increased linearly over time ($R^2 = 98\%$) following $\text{Mineral Density}(t) = 138 + 0.8 \times t[\text{day}]$ (Figure 5A). No correlation was found for mineral density as a function of BV at day 44 (Figure 5B).

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**Figure 3:** Individual sample data showing 3D top view images of four representative samples at days 10, 17, 28, and 44 (A), as imaged using µCT. Corresponding bone volume per final bone volume over time (B) and corresponding bone surface-to-volume ratio over time (C).
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**Discussion**

The goal of this study was to detail in vitro bone-like tissue formation by in situ visualization and quantification, starting from the integrated use of hMSC, SF scaffolds, and µCT imaging. Furthermore, developing µCT imaging as a safe and integral tool for the qualitative and quantitative evaluation of bone implants, using tissue engineering approaches was a goal for the study. Furthermore, this work was motivated by the need to provide feasibility data leading towards the development of an imaging interface, which, when integrated into future bioreactor systems, can provide image-guided input parameters, which when fed into algorithms can guide the continuous adaptation of medium composition, physical and chemical stimuli in an automated and feed-back mode to optimize the engineering of bone implants.

The surface antigen pattern of expanded cells isolated from human bone marrow were typical for hMSC, and demonstrated the capacity to selectively differentiate along osteogenic and chondrogenic lineages, respectively (Figure 1)(Meinel et al., 2004b, Pittenger et al., 1999). Based on this pattern of surface antigen expression and selective differentiation this cell isolate was referred to as human mesenchymal stem cells (hMSCs) in this study.

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Figure 4 Superimposed 3D bone volumes of 3 representative samples illustrating the progression of bone-like tissue deposition: Top views (A-C) and side views on corresponding center slices (D-F); blue = bone volume (BV) at day 14, green = BV at day 28, red = BV at day 44, as imaged using micro-computed tomography.
The impact of irradiation on osteogenic cell performance was assessed. Effects of ionizing irradiation on gene expression (e.g. Telomerase, c-myc, c-jun, IL-6) and DNA strand breaks in human cells was reported from doses in the order of 0.1 mGy or larger and after 1-14 days (Neuhof et al., 2001, Enomoto et al., 2003, Hallahan et al., 1996, Beetz et al., 1997, Rogakou et al., 1999). Depending on exposure time, µCT as used in the present study exposes specimens to X-ray doses in the order of 0.1-1 Gy (data not shown) and, therefore, risk is present. The biochemical evaluations completed here are insufficient to assess this risk e.g. of malignant transformations (Ritman, 2004). Furthermore, multiple exposures as used in this study further enhance potential pathological cell alterations. We confined the biochemical assessment to cellular osteogenic performance, when exposed to X-ray. In this study, cellular proliferation and calcium deposition were not influenced after 7 µCT cycles, a finding corroborating previous reports (Cartmell et al., 2004). This goal of this study was not to demonstrate that the integral use of µ-CT can be generally recognized as a safe tool. Eventual use of µ-CT or the assessment of implants must be accompanied by genotox studies and other toxicological evaluations. However, the biochemical data suggested that an analysis of temporal and spatial mineralization events was possible without inducing alterations in osteogenic patterns, an encouraging first step in this process.

Figure 5: Mean and standard deviation of mineral density (t) (n=11, A) and correlation between mineral density and bone volume (BV) at day 44 (R² = 0.15, B).

Trabecular-like bone structures were tissue engineered (Figure 3, 4). Pooled data illustrated a steady increase of average BV over time, as intuitively expected and corroborating previous reports (Figure 2A) (Cartmell et al., 2004). Large standard deviations were observed, although particular care was taken to standardize the experimental steps by employing the same operator, same batch of scaffolds, same
course of cell seeding, cell number, medium supply, and imaging. When the data on samples per group were pooled, the high variations were not clearly detected. However, the individual assessment of samples over time avoiding destructive endpoint data collection revealed the different mineralization patterns for each sample. To our knowledge, this study presents for the first time detailed views on the progression of bone-like tissue formation of individual samples (Figure 3B). Based on this novel approach, the heterogeneous mineralization among samples was detected, resulting in a lack of correlation between BV and time of culture.

Compared to biochemical assays on calcium deposition from similar cultures, µCT-determined BV data showed larger standard deviation (Figure 2A) (Meinel et al., 2004c, Kim et al., 2005). In biochemical calcium assays the calcium content of the entire constructs is quantified, including remaining medium in the scaffolds and calcium in low-mineralized areas, which would not be detected by µCT due to thresholding. Consequently, data on mineralization collected by µCT actually reflect the portion of mineralization that resulted in µ-CT detectable signals. Therefore, the higher variation in mineralization as determined by µ-CT when compared to less variable measurements using biochemical calcium assays (e.g. (Meinel et al., 2004b, Meinel et al., 2004c, Li et al., 2006, Bancroft et al., 2002) might reflect the heterogeneous formation of clusters with dense mineralization but not necessarily overall calcium deposition.

Bone-like tissue growth was visualized by superimposing µCT images taken from three consecutive time points (Figure 4). Bridging of bone components and, therefore, structure development was observed with mineralization mainly at the periphery of the scaffolds – as reported elsewhere for static cultures (Cartmell et al., 2004, Ishaug et al., 1997), whereas other samples demonstrated progressive ingrowth into the scaffold. This variation might be explained by different diffusion access for nutrition and gas, or inhibited cell migration to the center among samples. Diffusion could have been blocked in some cases by the initial scaffold geometry or cellular sheets occluding the pore’s interconnectivity (Cartmell et al., 2004, Ishaug-Riley et al., 1998) and might not necessarily reflect differences in the osteogenic potential but rather differences in scaffold homogeneity. Image-guided optimization can develop into a useful tool to improve protocols resulting in the homogenous formation of bone-like tissue. In spite of the heterogeneous tissue formation process among the samples, evidence was
demonstrated that neither initial BV or the initial number of mineralized clusters were not predictive for overall mineralization after 44 days of culture (Figure 2 and 3). Mean mineral density determined within the defined BV increased linearly between day 10 and day 44 (Figure 5A) demonstrating the continuous growth of the tissue. The deviations in mineral density between the samples were small and mineral density correlated linearly with time. However, a comparison of mineral density to BV revealed no correlation (Figure 5B), suggesting that high BV did not necessarily result in high mineralization levels or vice versa.

Mean bone surface-to-volume ratio (BS/BV) dropped throughout the culture period (from 80.0 mm\(^{-1}\) to 26.1 mm\(^{-1}\), Figure 2B). An explanation is the deposition of several small seeding sites at the beginning. With progression of tissue culture, mineralized clusters converged, forming less, larger volumes and, therefore, decreasing their surface-to-volume ratio. The decreasing deviation in BS/BV over time indicated the aim for a certain bone surface-to-volume ratio. This was corroborated by the course of the BS/BV ratio for individual samples (Figure 3C). These samples all starting with different BS/BV at day 10 resulted in a common value of approximately 26 mm\(^{-1}\). Further studies are needed to detail the absolute value of 26 mm\(^{-1}\), and the variation dependent on issues such as scaffold properties, medium composition, donor or chemical and physical stimulation.

The results of this study demonstrated the feasibility of monitoring and quantifying the formation of in vitro engineered bone-like tissue from hMSC by the integral use of \(\mu\)CT. Trabecular-like bone was grown and visualized in situ. In the future, individual monitoring of morphology and mineralization over time should enable feed-back loops with controlled bioreactors and the generation of homogeneously mineralized implants. Furthermore, this process can result in advanced test systems, detailing drug action on tissue performance. However, detailed studies on the genotoxicological potential for the described setting are needed, before embarking into and \(\mu\)-CT based and image guided future of bone tissue engineering.

### Acknowledgments

Financial support from ETH Zürich (TH Gesuch) and the Association for Orthopedic Research (AFOR) is greatly appreciated. We thank Dr. Martin Stauber for help in AVS
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illustration, Trudel Inc. for silk cocoons, and Wyeth Biopharmaceuticals for BMP-2 supply.

References


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Chapter 4: Monitoring and quantifying engineered bone-like tissue


Silk based Biomaterials to heal critical sized femur defects

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Bone, 2006, accepted
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Abstract

Bone auto- and allo-grafts have inherent drawbacks, therefore the treatment of non-unions and critical size defects in load bearing long bones would benefit from the use of osteopromotive biodegradable, biocompatible and mechanically durable matrices to enhance migration or delivery of cell populations and/or morphogens/cytokines. Silk fibroin biomaterial scaffolds were evaluated as osteopromotive matrices in critical sized mid-femoral segmental defects in nude rats. Four treatment groups were assessed over eight weeks in vivo: silk scaffolds (SS) with human mesenchymal stem cells (hMSC) that had previously been differentiated along an osteoblastic lineage in vitro (group I; pdHMSC/SS); SS with undifferentiated hMSCs (group II; SS/udHMSC); SS alone (group III; SS); and empty defects (group IV). When hMSCs were cultured in vitro in osteogenic medium for 5 weeks, bone formation was characterized with bimodal peak activities for alkaline phosphatase at 2 and 4 weeks. Calcium deposition started after 1 week and progressively increased to peak at 4 weeks, reaching cumulative levels of deposited calcium at 16 \( \mu \text{g} \) per mg scaffold wet weight. In vivo osteogenesis was characterized by almost bridged defects with newly formed bone after 8 weeks in group I. Significantly (p<0.01) greater bone volumes were observed with the pdHMSC/SS (group I) implants than with groups II, III or IV. These three groups failed to induce substantial new bone formation and resulted in the ingrowth of cells with fibroblast-like morphology into the defect zone. The implantation of pdHMSC/SS resulted in significantly (p<0.05) greater maximal load and torque when compared to the other treatment regimens. The pdHMSC/SS implants demonstrated osteogenic ability in vitro and capacity to thrive towards the healing of critical size femoral segmental defects in vivo. Thus, these new constructs provide an alternative protein-based biomaterial for load bearing applications.
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Introduction

The clinical need for bone replacement is expected to increase, due in part to the substantial rise in the elderly population of the western world (Reginster, 2002, Lysaght and Reyes, 2001). Currently, close to 1 million bone grafts are performed each year for the purpose of skeletal augmentation (McAndrew et al., 1988, Heuer et al., 1992, Cornell and Lane, 1998, Hollinger et al., 1996, Suh, 1998). Autografts remain the clinical gold standard, driving bone repair by providing host cells, growth factors, and a template for bone regeneration. Unfortunately, donor site morbidity, cosmetic concerns, and prolonged hospitalization are just a few of the drawbacks of this procedure (Einhorn, 1995). Similarly, the use of allografts presents risks due to immune rejection and disease transmission (Horowitz and Friedlaender, 1987, Bos et al., 1983). Orthopaedic surgeons would therefore benefit from the introduction of osteopromotive scaffolds that could be used to enhance migration and/or delivery of osteogenic cells and cytokines. These materials should combine essential characteristics including biocompatibility, porosity, and appropriate mechanical properties (Hollinger and Leong, 1996, Albrektsson and Johansson, 2001, DeLustro et al., 1990, Luginbuehl et al., 2004).

The use of mechanically robust 3D scaffolds offers additional options to more traditional fixation or cast methods to regenerate damaged bone tissue. A major advantage of a mechanically robust matrix is that it is less likely to undergo deformation caused by adjacent tissues (i.e. muscle) at the implant site. This deformation can be a problem, leading to the implant becoming compressed within the defect or even displaced beyond the borders of the defect site, giving rise to the potential for heterotopic bone. Several studies have shown that hMSCs can differentiate along an osteogenic lineage and form three-dimensional bone-like tissue but also detail limitations. Some scaffolds (e.g., calcium phosphate) are characterized with a slow degradation (Ohgushi et al., 1992), while others degrade too fast (Petite et al., 2000). Polymeric scaffolds used for bone tissue engineering, such as poly(lactic-co-glycolic acid) or poly-L-lactic acid can induce inflammation due to the acidity of their hydrolysis products (Athanasiou et al., 1996). Moreover, matching mechanical properties of native bone remains an issue with most polyesters (Harris et al., 1998, Suh, 1998). Therefore, there is a need to identify alternate biomaterials to overcome
these limitations and meet the challenging combination of biological, mechanical, and degradation features for bone tissue engineering.

Silk fibroin based porous biomaterial scaffolds may contribute to this important niche in biomaterials applications. These scaffolds have low immunogenicity when properly separated from immunogenic and glycosylated proteins (Rossitch et al., 1987, Santin et al., 1999, Panilaitis et al., 2003, Meinel et al., 2005b). Furthermore, they can be produced in a variety including electrospun nets, three dimensional scaffolds, or tendon-like structures (Sofia et al., 2001, Halsted, 1892, Asakura and Kaplan, 1994, Altman et al., 2002, Jin and Kaplan, 2003, Nazarov et al., 2004, Kim et al., 2004). Silks represent the strongest and toughest naturally occurring polymer materials and recently developed production processes enable the formation of silk biomaterials with decreased content of crystalline $\beta$-sheets (Jin and Kaplan, 2003). This allows for the maintenance of desirable mechanical properties, but more closely regulates the degradation/resorption rates, which can range from weeks to years.

Silk biomaterials have recently been shown to be suitable substrates for the \textit{in vitro} engineering of bone-like and cartilage-like tissues derived from human mesenchymal stem cells (hMSC) (Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004d, Meinel et al., 2005a). Interestingly, the geometry of the deposited bone could be reproducibly controlled as a function of scaffold design, shifting bone structures between trabecular-like bone tissue and more plate-like or cortical-like bone (Meinel et al., 2005a). The results of this research has demonstrated that silk fibroin scaffolds promoted bone formation in critical sized cranial defects in mice when seeded with hMSCs pre-differentiated in osteogenic medium for 5 weeks (pdHMSC/SS). Bone formation occurred to a much lesser extent when undifferentiated hMSCs (udHMSC) were seeded on the silk scaffolds and minimal bone formation occurred when unseeded silk scaffolds were implanted alone (Meinel et al., 2005a).

In the present study we build on prior studies with nonloaded cranial defects by implanting seeded and unseeded silk scaffolds in load bearing segmental defects in rat femurs. Starting off from detailing the isolated and expanded cell pool used for seeding of the silk scaffolds, and a characterization of the events leading to \textit{in vitro} formation of tissue engineered bone, the goal of this study was to characterize the healing of the implanted femoral segmental defects after implantation of this system by histology and...
immunohistochemistry, micro-computed tomography, morphometric quantification of bone formation, and mechanical characterization.

Materials and Methods

Materials

Fetal Bovine Serum (FBS), RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), penicillin and streptomycin (Pen-Strep), Fungizone, nonessential amino acids (NEAA, consisting of 8.9 mg/L L-alanine, 13.21 mg/L L-asparagine, 13.3 mg/L L-aspartic acid, 14.7 mg/L L-glutamic acid, 7.5 mg/L glycine, 11.5 mg/L L-proline, 10.5 mg/L L-serine), and trypsin were from Gibco (Carlsbad, CA). Ascorbic acid-2-phosphate, Histopaque-1077, insulin, and dexamethasone were from Sigma (St. Louis, MO). 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxy-succinimide (NHS) were purchased from Pierce (Rockford, IL). All other substances were obtained from Sigma. Silkworm cocoons were kindly supplied by M. Tsukada, Institute of Sericulture, Tsukuba, Japan, and Marion Goldsmith, University of Rhode Island. BMP-2 was kindly supplied by Wyeth Biopharmaceuticals, Andover, MA.

Scaffold preparation and characterization

Silk fibroin scaffolds were prepared as described previously (Meinel et al., 2004a, Meinel et al., 2004b, Meinel et al., 2004c). Cocoons from Bombyx mori were boiled for 1 hour in an aqueous solution of 0.02M Na₂CO₃ and rinsed with water to extract sericin. Purified silk was solubilized in 9M LiBr solution and dialyzed (Pierce, MWCO 2000 g/mol) first against PBS for 1 day and then against 0.1M 2-(N-morpholino)ethanesulfonic acid buffer (MES), 0.5M NaCl, pH 6, for 1 day. To attach RGD sequences, silk fibroin solution was coupled with GRGDS peptide as previously described (Nazarov et al., 2004). Briefly, the COOH groups on silk were first activated by reaction with EDC/NHS for 15 min at room temperature. To quench the EDC, 70 µl/ml β-mercaptoethanol was added. The solution was then incubated with 0.5 g/l peptide for 2 h at room temperature. The reaction was stopped with 10 mM
hydroxylamine. Purified silk fibroin was dialyzed against 0.1M MES, pH 4.5-5, for 1 day, lyophilized and redissolved in hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) solution. Granular NaCl (diameter 200-300 µm) was weighed in a Teflon container and silk/HFIP solution was added at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 2 days and NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 minutes to induce a protein conformational transition to β-sheet (Nazarov et al., 2004). Blocks were removed, dried and NaCl was extracted by incubation in water for 2 days. Disk shaped scaffolds (8 mm diameter, 2 mm thick) were prepared using a dermal punch (Miltey, Lake Success, NY), and autoclaved at 121ºC for 15 min; autoclaving does not alter the structure or shape of the scaffolds.

Cell isolation, expansion and characterization

Human mesenchymal stem cells (hMSC) were isolated by density gradient centrifugation from 25 cm³ whole bone marrow obtained from Clonetics (Santa Rosa, CA). 10 ml aliquots of bone marrow were diluted in 100 ml of isolation medium (RPMI 1640 supplemented with 5% FBS). 20 ml aliquots of this suspension were overlaid onto a poly-sucrose gradient (δ = 1.077 g/cm³, Histopaque, Sigma, St. Louis, MO) and centrifuged at 800xg for 30 minutes at room temperature. The cell layer was carefully removed, washed in 10 ml isolation medium, pelleted at 300xg and the contaminating red blood cells were lysed in 5 ml of Pure-Gene Lysis solution. Cells were pelleted and suspended in expansion medium (DMEM, 10% FBS, Pen-Strep, Fungizone, NEAA and 1 ng/ml bFGF) and seeded in 175 cm² flasks at a density of 5x10⁴ cells/cm². The adherent cells were allowed to reach approximately 80% confluence (12-17 days for the first passage). Cells were trypsinized and replated every 6-8 days at approximately 80% confluence. The 2nd passage (P2) of the cells were used if not otherwise stated.

hMSC were characterized with respect to (a) the expression of surface antigens and (b) the ability to selectively differentiate into chondrogenic or osteogenic lineages in response to environmental stimuli, as follows. The expression of the following six surface antigens: CD14 (lipopolysaccharide receptor), CD31 (PECAM-1/endothelial cells), CD34 (sialomucin/hematopoietic progenitors), CD44 (hyaluronate receptor), CD71 (transferrin receptor/proliferating cells), and CD105 (endoglin) was
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characterized by Fluorescence Activated Cell Sorting (FACS) analysis, as in our previous studies (Meinel et al., 2004b, Meinel et al., 2004c). Cells were detached with 0.05% (w/v) trypsin, pelleted and resuspended at a concentration of 1x10^7 cells/ml. Fifty µl aliquots of the cell suspension were incubated (30 min on ice) with 2 µl of each of the following antibodies: anti-CD14 and anti-CD44 conjugated with fluoresceine isothiocyanate (CD14-FITC, CD44-FITC), anti-CD31 conjugated with phycoerythin (CD31-PE), anti-CD34 and anti-CD71 conjugated with allophycocyanine (CD34-APC, CD71-APC), and anti-CD105 with a secondary rat-anti mouse IgG-FITC antibody. Cells were washed, suspended in 100 µl of 2% formalin, and subjected to FACS analysis.

To assess the potential of hMSC for osteogenic or chondrogenic differentiation, the cells were cultured in pellets in either control medium (DMEM supplemented with 10% FBS, Pen-Strep and Fungizone), chondrogenic medium (control medium supplemented with 0.1 mM NEAA, 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 5 µg/ml insulin, 5 ng/ml TGF-β1) or osteogenic medium (control medium supplemented with 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM β-glycerolphosphate, and 1 µg/ml BMP-2). Cells were isolated from monolayers by trypsin and washed in PBS. Aliquots containing 2x10^5 cells were centrifuged at 300xg in 2 ml conical tubes and allowed to form compact cell pellets over 24 hours in an incubator (5% CO₂, 37°C). Medium was changed every 2-3 days. After 4 weeks of culture, pellets were washed twice in PBS, fixed in 10% neutral buffered formalin (24 hours at 4°C), embedded in paraffin and sectioned (5 µm thick). Sections were stained for general evaluation (haematoxilin and eosin, H&E), the presence of glycosaminoglycan (GAG, safranin O/fast green), and mineralized tissue (according to von Kossa in 5% AgNO₃ for 1 hour, exposed to a 60 Watt bulb and counterstained with fast red).

**Tissue culture**

For cultivation on scaffolds, P2 hMSC were suspended in liquid BD Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA) at a concentration of 1x10^6 cells per scaffold in 10 µl Matrigel while working on ice to prevent gelation. The cell suspension was seeded onto prewetted scaffolds and placed in an incubator (37°C,
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5% CO₂) for 15 min to allow gel hardening. For cultivation in spinner flasks, these cell-seeded silk scaffolds were threaded onto needles embedded in the stoppers of the spinner flask (2 scaffolds on 4 needles per flask) as previously described (Vunjak-Novakovic et al., 1999, Vunjak-Novakovic et al., 1998). Flasks were filled with 150 ml osteogenic medium (see above) and placed in a humidified incubator (37°C, 5% CO₂), with the side arm caps loosened to permit gas exchange and stirred with a magnetic bar at 60 rpm (Meinel et al., 2004c). Medium was replaced at a rate of 50% every 2-3 days for 5 weeks of cultivation.

Biochemical analysis

After 5 weeks of cultivation in bioreactors, the tissue was punched into 5 mm diameter disks using a dermal punch (Miltey, Lake Success, NY). These scaffolds (n=5) were weighed and disintegrated using steel balls and a Minibead-beater (Biospec, Bartlesville, OK) in 0.5 ml 5% trichloroacetic acid in water. After a second extraction with 0.5 ml 5% trichloroacetic acid in water for 30 minutes and combination of the samples, calcium content was measured spectrophotometrically at 575 nm following the reaction with o-cresolphthalein complexone according to the manufacturer's protocol (Sigma, St. Louis, MO). For DNA analysis, 3–4 punched scaffolds were weighed, disintegrated using steel balls and a Minibead-beater in 0.2% Triton X-100 and 5 mM MgCl₂ solutions. DNA content was measured using the PicoGreen assay (Molecular Probes, Eugene, OR), according to the protocol of the manufacturer. Briefly, aliquots of the solutions prepared from the samples were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Alkaline phosphatase activity (AP activity) was measured from aliquots as prepared for the DNA assay, using a biochemical assay from Sigma (St. Louis, MO), based on conversion of p-nitrophenyl phosphate to p-nitrophenol which was measured spectrophotometrically at 405 nm.

Operative Procedure

An established, critically sized, femoral defect rat model was used in this study (Einhorn et al., 1984). All procedures were approved by the operative institution’s Animal Care and Use Committee. Adult, male athymic T-cell deficient RH-rnu rats
weighing 325 – 400 g were maintained under general anesthesia using isofluorane and oxygen delivered by mask. The animals received intramuscular injections of procaine penicillin antibiotic (200,000 IU/kg), once immediately prior to surgery and then once daily for 3 days. Buprenorphine (0.05 mg/kg) analgesic was administered subcutaneously once pre-operatively and then 3 times daily for approximately 48 hours. Subsequent administration was based on the rat’s disposition and was supplemented where indicated by the administration of subcutaneous ketoprofen (5 mg/kg once daily). The anesthetized rats were placed in lateral recumbency and the uppermost hindlimb was shaved and aseptically prepared for surgery, prior to being draped. An approximately 25 mm long skin incision running the full length of the femur was created approximately 2 mm cranial to the bone. The subcutaneous tissues and facia were incised, the biceps femoris muscle was retracted posteriorly and the vastus muscle was retracted anteriorly. Self retaining retractors were used to expose the full length of the femur. A four-hole pre-drilled aluminum external fixator was placed over the lateral aspect of the exposed femur and used as a template to ensure appropriate positioning of four bicortical 0.9-mm diameter drill holes, which were created in the proximal (2) and distal (2) metaphysis of the femur. Four 1.1-mm external diameter self tapping threaded pins were then placed transcortically such that the pins extended approximately 0.5 mm beyond the trans cortex. Four small stab incisions were placed through the skin immediately caudal to the incision on the lateral aspect of the limb allowing it to be placed over the pins. The external fixator was then placed over the pins and secured to them with miniscrews. A high speed dental drill was used to carefully create a 5-mm long full thickness osteoperiosteal critical size defect in the mid-diaphysis which was subsequently packed with the test implant. Size 4-0 suture was used to appose the biceps femoris and vastus muscles around the defect, helping retain the implant in place, and a 4-0 subcuticular suture was used to close the subcutaneous space and skin.

Rats were euthanized 56 days after treatment. Both femora of each animal were harvested and either immediately frozen for biomechanical testing and micro-computed tomography (µCT) evaluation or placed in 4°C 4% paraformaldehyde for histological and immunohistochemical evaluation.

The rats were randomly assigned to one of four treatment groups (Table 1). Group I (6 rats) received pre-differentiated hMSC on silk scaffolds (pdHMSC/SS), group II (6
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Rats) received silk scaffolds seeded with undifferentiated hMSC (udHMSC/SS), group III (6 rats) received unseeded silk scaffolds (SS), and in group IV, defects were left empty (control). Bone healing was monitored by weekly radiographs. There are some serious complications associated with this model operating procedure, specifically, peri-anesthetic death (maybe related to rat strain), fracture of the parent bone during the technically very demanding fixation procedure or shortly thereafter, and pin tract infection that usually manageable with cleaning and occasionally antibiotics. We encountered 15 euthanasias or deaths out of 54 prior to 8 weeks after operation, most of them occurred around the time of surgery.

Histology and Immunohistochemistry

Fixed and decalcified (12% EDTA, pH 7.0) femur explants were dehydrated in graded ethanol solutions and xylene, embedded in paraffin and cut into 3 µm-thick sections. For a general overview, sections were stained with H&E. Immunohistochemical studies were performed for bone sialoprotein (BSP) and collagen type I using a rabbit anti-bone sialoprotein II polyclonal antibody (Chemicon, Temecula, CA) and mouse anti-collagen type I monoclonal antibody (Sigma, St. Louis, MA), respectively. For collagen 1 antibody, sections were preincubated with 0.05% Pronase (Dako Cytomation, Baar, Switzerland) for 10 minutes and blocked two times 10 minutes with 3% H₂O₂ and with serum-free protein block (Dako Cytomation, Baar, Switzerland). The primary antibody was added to the slides at a dilution of 1:100 overnight and after washing, an anti-mouse-peroxidase labeled polymer (EnVision® System, K4001 Dako Cytomation, Baar, Switzerland) was applied for 30 minutes at room temperature. Samples were developed with an AEC Substrate Kit (Zymed Laboratories Inc., San Francisco, CA) under a light microscope. For BSP, the antibody was diluted 1:400 and the same procedure was followed except that there was no pronase pretreatment and the secondary antibody was an anti-rabbit peroxidase labeled polymer (EnVision® System K4003 Dako Cytomation, Baar, Switzerland).

MicroCT Imaging and Mechanical testing

Femoral defects were scanned using 55 kVp, 250 ms integration time, and 0.030 mm voxel side lengths (isotropic) on a microCT-40 system (Scanco Medical, Bassersdorf,
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Specimens were immersed in saline to homogenize the background image and provide better contrast. The distance between the threaded pins was consistent and this regularity aided in the scanning procedure. MicroCT scans spanned the entire distance between the pins proximally and distally adjacent to the critical defect. After image reconstruction, volumes of interest (VOIs) were created for every specimen. VOIs were drawn contoured to the external boundary of the bone, beginning ten image slices distal to the fixator pin proximal to the defect and ending ten image slices proximal to the pin distal to the defect. VOIs were created only in regions where bone was present; in unhealed specimens the gaps were excluded from the VOIs. Thus, morphometric analyses represented the geometry and bone morphology of the mineralized tissue in the region. Prior to morphometric analysis, a constrained Gaussian filter was used to suppress noise in the image (0.7 filter width) and images were thresholded. A previously described (Meinel et al., 2005a) adaptive, iterative threshold protocol was used that determined the threshold based on the characteristics of the image's histogram. Performance tests (precision, accuracy, and stability of iterative algorithm) of this threshold approach on a variety of image types have proven it to be robust. After thresholding, the total bone volume (BV), bone volume fraction (BV/TV), and average bone thickness (Th) were quantified. Bone volume was calculated by counting the total number of bone voxel and multiplying by their known volume. The bone volume fraction was calculated as the ratio of the bone voxel to the total number of voxel (bone and non-bone voxel after thresholding). Bone thickness was measured in three-dimensions in accordance with the method established by Hildebrand and Ruegsegger (Hildebrand et al., 1999).

Both ends of specimens were embedded in blocks of polymethylmethacrylate (PMMA) to provide gripping points for torsional testing. The specimens were kept wet during the PMMA exothermic curing process to avoid drying. Mechanical testing was performed using a torsion testing system that applies a force couple via cables attached to a pulley fixed to the top of a cylindrical bone specimen such that the axis of rotation of the pulley is coincident with the geometric neutral axis of the cylindrical bone specimen. A module containing a roller clutch, an XY sliding surface and coupling cables was mounted to a MTS200 screw axis load frame (MTS, Saint Paul, MN, USA) for the application of torque. The module maintains the prescribed torque on the specimen when it is removed from the load frame. Torsional testing was performed.
using a strain rate of 5 rad/min, and the data was collected with a LabVIEW (National Instruments, Austin, TX) acquisition system. Maximum torsional displacement (rad) and load (N) and maximum torque (Nm) were measured for each specimen. Additionally, torsional stiffness (N.m/rad) was calculated from the slope fit to the linear portion of the torsional load displacement curve.

Statistical analysis

For statistical significance, samples were evaluated using a student t-test as well as ANOVA where appropriate. ANOVA was followed by a post-hoc assessment using the Tukey HSD method. Differences were considered significant when equal or less than \( p=0.05 \).

Results

Cell characteristics and bioreactor culture

The cells were adherent to tissue culture plastic after about 6 hours and showed a fibroblast-like morphology (Figure 1A). Their surface antigen pattern was CD105\(^+\) (Figure 1B), CD71\(^+\) and CD34\(^-\) (Figure 1C) and CD44\(^+\), CD14\(^-\), CD31\(^-\) (data not shown). After 5 weeks of pellet culture in chondrogenic medium, the cells deposited an intensely red staining matrix with safranin O (Figure 1D) indicating deposition of extracellular glycosaminoglycans. The culture in osteogenic medium resulted in the formation of a black staining matrix in a von Kossa staining (Figure 1E) noting for mineralized extracellular matrix. In control medium, no positive safranin O or von Kossa staining was observed (data not shown).

When cells were seeded on silk fibroin scaffolds (SS) and cultured in spinner flask bioreactors with osteogenic medium for 5 weeks, alkaline phosphatase activity followed an undulating pattern, with significantly higher activity after 2 and 4 weeks, when compared to weeks 1 and 3 (Figure 2A; \( p<0.05 \) or \( <0.01 \)). Mineralization determined by calcium assay started between weeks 1 and 2 (\( p<0.01 \)) and continued to increase between weeks 3 and 4 (\( p<0.01 \)). No increase in mineralization was observed between weeks 4 and 5 (Figure 2B).
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Table 1: Description of the four treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>I</td>
<td>pdHMSC/SS</td>
<td>Human mesenchymal stem cells seeded on silk scaffolds and differentiated under osteogenic conditions for 5 weeks in spinner flasks prior to implantation</td>
</tr>
<tr>
<td>II</td>
<td>udHMSC/SS</td>
<td>Undifferentiated human mesenchymal stem cells seeded on silk scaffolds 1 day prior to implantation</td>
</tr>
<tr>
<td>III</td>
<td>SS</td>
<td>Silk scaffolds without cells</td>
</tr>
<tr>
<td>IV</td>
<td>control</td>
<td>No implant – empty defects</td>
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In vivo studies

The implantation of pdHMSC/SS (group I, Table 1) into critical size femoral defects resulted in good integration of the implant with the fracture ends at 8 weeks post surgery. Substantial new bone formation (25 – 40%) was observed, with more cortical bone in the outer implant zones and more inter-connected, woven bone in the centers, as shown with H&E staining (Figure 3A). Positive staining for bone sialoprotein (BSP) and collagen type I (data not shown) was found in the newly formed matrix of actively bone forming sites. Aside from long cells with fibroblastic morphology and stranded connective tissue, the formation of new woven bone was observed (Figure 3A insert). No apparent signs of silk scaffold degradation were observed, as indicated by the smooth lattice surfaces, although foreign body cells were present (Figure 3A).

The implantation of udHMSC/SS (group II, Table 1) resulted in the formation of a fibroblast tissue throughout the implantation zone (Figure 3B), with some few capillaries randomly dispersed through the implant’s lattice (Figure 3B insert). Bone formation was mainly observed at the defect margins with the parent bone but not at the center of the defect. Moderate positive staining for BSP and type I collagen was mainly found at the interface between the defect border and the newly formed bone as well as few and randomly clusters distributed throughout the matrix (data not shown). A higher number of foreign body cells than in group I were present in the defect zone, but the SS seemed well embedded in the cell- and extracellular matrix-rich environment.

Defects treated with unseeded scaffolds SS (group III, Table 1) developed a dense cellular network in the vicinity of the defect borders and appositional to the scaffold
lattice (Figure 3C). Substantially fewer cells were found in the defect center, and scaffold interaction with the host tissue was weaker as observed in groups I and II and as indicated by lower cell numbers and void spaces at the host-implant interface. (Figure 3C). Immunohistochemistry showed positive staining for both, BSP and type I collagen within the matrix and cells with a fibroblastic morphology within the scaffold center, whereas loosely connected cells with a connective-tissue-like morphology stained positive only for type I collagen but not BSP. The small amounts of new woven bone stained positive only for BSP.

Tissue formation in the empty defects (group IV, Table 1) was confined to the defect margins with parent bone, with some clusters of non-bone forming cells found in more distal regions (Figure 3D and insert). These clusters did stain only faintly for BSP and positive for type I collagen, respectively.

Figure 1: Characterization of human mesenchymal stem cells. (A) Cells had a fibroblast-like morphology, and presented the (B) CD105 and (C) CD71, but not the CD34 antigen on their surfaces, respectively. Pellet culture in chondrogenic medium (D) resulted in the deposition of an intensively red staining matrix with safranin O. Pellets cultured in osteogenic medium and stained with von Kossa, displayed black staining. Magnifications (A) 40x and 100x (insert), (D, E) bar length = 100 μm.
The morphology of the formed bone was evaluated using μ-CT (Figure 4). In general, defects treated with pdHMSC/SS (group I) were spanned by cortical-like bone in the outer zones and a cancellous-like but irregularly and loosely connected bone tissue within the defect center. The defects in three of the six rats were completely bridged with no apparent cracks, two by small in the center of the implant and along the line of the stacked implant discs, and one by large cracks spanning throughout the defect zone. The implantation of udHMSC/SS (group II) resulted in bone formation at the defect margins with parent bone and some minor formation within the defect center, however no bridging of the defect was observed. Finally, no bone formation was apparent for defects treated with unseeded SS (group III) or empty control defects (group IV).

Figure 2: (A) Alkaline phosphatase activity and (B) calcium deposition over time from silk scaffolds cultured in bioreactors for 5 weeks (** p<0.01, * p<0.05; n = 5 per group).

Morphometric analysis based on the tomographic evaluations quantified the qualitative observations previously detailed. Significantly more bone volume was observed in group I when compared to all other treatment groups (p<0.01; Figure 5A). The ratio of bone volume to total volume, as an indicator of the relative amount of formed bone,
was significantly higher for group I treated defects when compared to all other groups (p<0.01; **Figure 5B**). The thickness of the bone formed was consistent amongst groups, with apparently high standard deviations (data not shown).

**Figure 3:** Histological cross sections taken from rat critical size femoral defects 8 weeks after surgery, (A) pdHMSC on silk scaffolds, (B) silk scaffolds seeded with udHMSC, (C) unseeded silk scaffolds, and (D) untreated (control) defects. Magnification 50x, inserts 100x. Asterisks indicate silk scaffold, arrows the fracture ends, arrowheads newly formed bone within the defect.

The defects were mechanically characterized (**Figure 6**). The maximal load before failure was significantly higher for group I when compared to groups II (p<0.05) and III (p<0.01). The implantation of udHMSC/SS (group II) resulted in significantly higher maximal load levels when compared to group III (p<0.01). Similarly, the maximal torque was higher for pdHMSC/SS (group I) when compared to group II (p<0.05) and III (p<0.01), respectively. Maximal torque was significantly higher for
group II when compared to the SS (group III) (p<0.01). The total stiffness of the defects was higher for the pdHMSC/SS (group I) when compared to group III (p<0.01), but no significant differences were observed between groups I and II or between groups II and III.

Figure 4: Micro-computed tomography from three representative rat critical size femoral defects 8 weeks after surgery; pdHMSC grown five weeks on silk scaffolds (group I), silk scaffolds seeded with udHMSC (group II), unseeded silk scaffolds (group III), and untreated (control) defects (group IV). The dotted box approximately indicates the original defect zone.
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Discussion

The goal of the present study was to evaluate a new protein-based biomaterial scaffold system with mechanically robust and durable properties upon implantation into long bone and critically sized femoral defects. A tissue engineered trabecular bone-like tissue was generated from pdHMSC/SS (group I, Table 1) which were pre-cultured in osteogenic medium for five weeks. These implants were inserted into the defect site and osteogenesis when compared to defects treated with udHMSC seeded silk scaffolds, those not pre-cultured in osteogenic medium (group II, Table 1), unseeded silk scaffolds (group III, Table 1), and untreated control defects (empty, group IV, Table 1).

Figure 5: Morphometric analysis of new bone formation from rat critical size femoral defects 8 weeks after surgery, and treated with pdHMSC grown for five weeks on silk scaffolds (group I), silk scaffolds seeded with udHMSC (group II), unseeded silk scaffolds (group III), and untreated (control) defects (group IV). (A) Bone volume per defect, and (B) bone volume normalized to the trabecular volume (** p<0.01, * p<0.05).
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Silk fibroin, the structural, hydrophobic, and approximately 370 kDa protein isolated from the cocoon of *Bombyx mori* (Dewair et al., 1985, Craig et al., 1999, Inoue et al., 2000, Tanaka et al., 1999) has emerged as a novel substrate for engineering biomaterials, has proven to be biocompatible (Meinel et al., 2005b, Meinel et al., 2005a), offers distinguishing mechanical properties when compared to other naturally derived or degradable polymeric biomaterials (Cunniff et al., 1994), and exhibits low inflammatory response (Meinel et al., 2005b, Panilaitis et al., 2003).

Stem cells are believed to be one of the most interesting cell sources for tissue engineering, particularly for the (re)generation of bone tissue. This is due to characteristics inherent in stem cells such as (i) a high proliferation capacity, (ii) ability for self-renewal, (iii) multilineage differentiation and, therefore, potential for the repair of various tissues (Service, 2000, Jaiswal et al., 1997, Aubin, 1998, Bruder et al., 1994, Friedenstein et al., 1987). The cells used in this study were characterized as stem cells based on their surface markers as well as the multipotent and selective differentiation outcomes shown to bone or cartilage. Silk-fibroin scaffolds seeded with hMSC and cultured in osteogenic medium in spinner flask bioreactors generated bone-like biochemical responses as typically observed for mesenchymal cells on other biomaterials (Choong et al., 1993, Meinel et al., 2004d, Meinel et al., 2004c). The high degree of mineralization observed, beginning between weeks one and two post-seeding is evidence of the strong osteogenic commitment of the hMSC on silk scaffolds and in the presence of BMP-2 (one of the osteogenic media components).

Substantial differences were observed in vivo in bone healing between pdHMSC/SS (group I), udHMSC/SS (group II) and the SS (group III). The implantation of pdHMSC/SS into the defect resulted in substantial and homogenous bridging of the majority of defects (Figures 3, 4), significantly (p<0.01) higher bone volumes and bone volumes per trabecular volume (which indicates a shift from trabecular to cortical bone) and a substantial increase in mechanical properties when compared to the other groups. The premineralization of the silk scaffolds appears to be important in overall bone restoration in vivo. Thus, future studies should also address the relative contribution of mineralization on silk fibroin scaffolds related to osteoconductivity with and without the presence of HMSCs during implantation. Although bone formation within the defect zone was restricted to the defect margins, the udHMSC seeded scaffolds (group II) resulted in significantly (p<0.01) higher maximal load and torque
when compared to unseeded SS (group III). This may be due to the formation of a dense cellular and matrix-rich network supported by the silk scaffolds between the host bone and the scaffold center, fixing the stacked scaffold disks in place and, thereby, resulting in a more stabilized mechanical environment. However, the mechanical data was presented to provide a relative strength profile across study groups. This must be noted as the femurs were fixed before measurements, instead of most biomechanical studies. We have not tested, if the fixation altered the mechanical properties of bone. Therefore, one should be careful to compare the presented data to normal unfixed rat bones, since only the relative strength of specimens across different groups is of consequence in this study. As part of the morphometric analysis of the engineered bone-like tissue, we evaluated the bone thickness using an algorithm validated for trabecular bone (data not shown). However, the repair side consisted of complex, inhomogenous structures other than trabecular bone (Figure 3). The validation for the algorithm used was not validated for the type of tissue formed in the defect site. Consistently, our data showed a large variation. The validation of algorithms provided should be carefully evaluated for the intended application.

The gold standard of bone replacement is autograft bone or bone that has been harvested from another anatomic location in the same individual and then inserted into the fracture zone (Einhorn, 1995, Johnson et al., 1988). The advantage of this approach is the combined transplantation of autologous cells, scaffold material, and growth factors stored within the matrix (Eiselt et al., 1998, Nade and Burwell, 1977). However, harvesting autologous bone is associated with second site morbidity, and hampered by insufficient amounts of collected bone substance to fill the entire defect (Einhorn and Lee, 2001). Apart from other options such as the use of allograft bone, which is associated with the risk of disease transmission or immune rejection, metals and ceramics are used to treat larger defects (Kokubo et al., 2004). Metals provide instant mechanical stability but are associated with poor overall integration and can fail because of infection or due to fatigue loading (Suh, 1998). Ceramics generally have very low tensile strength, are brittle and can not be used in locations of significant torsion such as long bones (Dennis et al., 1992, McAndrew et al., 1988, Heuer et al., 1992, Klein et al., 1984, Driessen et al., 1982). Alternatively, biomaterials such as type I collagen base implants are frequently used, but have limitations related to
rapid biodegradation or – when stabilized through cross-linking – foreign body responses and uncontrolled calcification (Nimni et al., 1987, Srivastava et al., 1990).

**Figure 6:** Mechanical features of rat critical size femoral defects 8 weeks after surgery, and treated with pdHMSC for five weeks on silk scaffolds (group I), silk scaffolds seeded with udHMSC (group II), and unseeded silk scaffolds (group III). (A) Maximal load before break, (B) maximal torque, and (C) torsional stiffness (**p < 0.01, *p < 0.05).
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The combined use of an organic template based on silk fibroin and hMSC predifferentiated in bioreactors with osteogenic medium (group I) provides an alternative route for future treatment scenarios of larger femoral defects. In this study, the filling of the defect was achieved by stacking five silk scaffolds, each of which was individually engineered, to result in good bone integration. In vivo bone formation resulted in a good formation of bone between the stacked disks and the fracture ends, respectively. However, the engineering of a pdHMSC/SS using a single block of silk instead of staggered disks should further improve the mechanical properties, integrity, and safety. However this is currently challenging due to limitations in nutrient- and gas transport during predifferentiation in vitro. Bone is a highly vascularized and metabolically active tissue and especially prone to low oxygen tensions, quickly resulting in necrotic responses. These limitations curtail current tissue engineering approaches, resulting in bone thicknesses limited to approximately 500 µm to 1 mm (Vunjak-Novakovic et al., 2005, Meinel et al., 2004d). Future studies aiming at increased mass and gas transport in the scaffolds when cultured in vitro are important and could be addressed through novel designs for the bioreactors, scaffolds, and medium used in the process (e.g. by addition of oxygen binding molecules mimicking the function of hemoglobin (Vunjak-Novakovic et al., 2005)).

Tissue engineering can provide adequate bone replacement resulting in full recovery of the patient (Langer and Vacanti, 1993). The results of the present study indicate that the implantation of tissue-engineered bone on silk scaffolds was suitable for this application because it leads the way to bridging of critical size defects with newly formed functional bone. This was also reflected in mechanical properties, with group I exceeding all other groups in terms of maximal load before failure, maximal torque, and torsional stiffness. Although bone formation was limited in groups II and III, some mechanical integrity was achieved due to the presence of the silk and some fixation due to the invasion of fibrous tissues into the implant materials. The osteoinduction achieved in group I rats resulted in complete bridging of femoral, segmental critically sized defects with a callus on the outside and around 30% newly formed woven bone tissue inside the defect. These findings encourage the continued investigation of potential use of silks as a mechanically stable and robust scaffold for tissue engineered constructs and load bearing bones. Future studies should be conducted to validate the effectiveness of this procedure in larger animals.
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Acknowledgements

This work was supported by the German Alexander von Humboldt Foundation (Feodor-Lynen fellowship to LM), the National Institutes of Health (DE13405-04 and EB002520 to DK), and the National Science Foundation (DMR-0090384 to DK) and the association for orthopaedic research (ETH Zurich, TH Gesuch). We thank Katie Zlinszky for technical assistance in immunohistological stainings and Nipun Patel for help with micro-CT as well as the mechanical evaluations.

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Silk fibroin as an organic polymer for controlled drug delivery

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Abstract

The pharmaceutical utility of silk fibroin (SF) materials for drug delivery was investigated. SF films were prepared from aqueous solutions of the fibroin protein polymer and crystallinity was induced and controlled by methanol treatment. Dextrans of different molecular weights, as well as proteins, were physically entrapped into the drug delivery device during processing into films. Drug release kinetics were evaluated as a function of dextran molecular weight, and film crystallinity. Treatment with methanol resulted in an increase in β-sheet structure, an increase in crystallinity and an increase in film surface hydrophobicity determined by FTIR, X-ray and contact angle techniques, respectively. The increase in crystallinity resulted in the sustained release of dextrans of molecular weights ranging from 4 kDa to 40 kDa, whereas for less crystalline films sustained release was confined to the 40 kDa dextran. Protein release from the films was studied with horseradish peroxidase (HRP) and lysozyme (Lys) as model compounds. Enzyme release from the less crystalline films resulted in a biphasic release pattern, characterized by an initial release within the first 36 hours, followed by a lag phase and continuous release between days 3 and 11. No initial burst was observed for films with higher crystallinity and subsequent release patterns followed linear kinetics for HRP, or no substantial release for Lys. In conclusion, SF is an interesting polymer for drug delivery of polysaccharides and bioactive proteins due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered.
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**Introduction**

Silks belong to a group of high molecular weight organic polymers characterized by repetitive hydrophobic and hydrophilic peptide sequences (Valluzzi et al., 2002). Due to the highly repetitive primary domains, fibrous proteins and especially silk fibroin (SF) assemble into regular structures during materials formation and can be considered as Nature’s equivalents to synthetic block copolymers (Chen et al., 2002, Valluzzi et al., 2002, van Beek et al., 2002, Vollrath et al., 2001, Wilson et al., 2000). Silks are naturally produced by spiders or insects, such as *Nephila clavipes* and *Bombyx mori*, respectively (Knight and Vollrath, 2002, Vollrath and Knight, 2001). The primary sequence of SFs have achieved wide evolutionary adaptation to such diverse needs as spinning underwater nets to trap air for underwater breathing, lifelines, and prey capture, common features associated with the formation of robust and stable material structures. The repetitive organization and the presence of high contents of short side chain amino acids, glycine, serine, and alanine have been preserved in these protein polymer systems (Altman et al., 2003).

During the spinning process, several motifs in the silk form crystalline β-sheet stacks by hydrogen bonding and hydrophobic interactions, forming the basis for the tensile strength and toughness of the material (Altman et al., 2003, Altman et al., 2002b). This assembly process starts from highly concentrated silk solutions either in vivo or emulated in vitro. The protein assembly process in vivo is initiated by extraction of water, changes in salt concentration and finally triggered by mechanical stress or chain alignment during fiber spinning. This process has been biomimetically transferred into in vitro environments, providing the basis for the fabrication of silk scaffolds as implant materials (Jin and Kaplan, 2003). These silk-based biomaterials have interesting mechanical, morphological and structural properties that may fill important niches in biomaterial applications, in particular for the musculoskeletal system due to the robust mechanical properties (Meinel et al., 2004a, Meinel et al., 2005, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004e).

The present study relates to the understanding of silk fibroin processing and control of structure development (β-sheet content as a reflection of degree of crystallinity) towards utility as a controlled release delivery matrix. The effect of the molecular weight of the compound to be delivered on the release kinetics from SF matrices was investigated within the context of control of crystalline content during processing.
Furthermore, model proteins (enzymes) were formulated into the SF polymer films and their release pattern was followed with potency tests in order to extend the potential utility of this new controlled release device with bioactive molecules. The ability to formulate and control structural features of this family of proteins in an all aqueous process suggests that sensitive biologicals can be incorporated into these matrices without significant loss of biological activity.

**Figure 1:** AFM images of silk film surfaces either treated with 90% methanol (A, C) or left untreated (B, D). (A, B): bar length 2.5 μm, (B, D): bar length 0.5 μm.

### Materials and Methods

#### 2.1. Materials

Fluorescein isothiocyanate-dextran with molecular weights of 4, 10, 20 and 40 kDa, respectively, horseradish peroxidase (HRP; EC 1.11.1.7.), lysozyme (Lys; EC 3.2.1.17), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), bovine serum albumin (BSA), *Micrococcus luteus*, 0.01 M phosphate-buffered saline (PBS: 2.7 mM potassium chloride, 0.137 M sodium chloride, pH 7.4) and o-phenylenediamine Fast Kit (OPD-Fast Kit; 0.4 mg/ml in 0.05 M phosphate-citrate buffer containing 0.4 mg/ml urea hydrogen peroxide) were from Sigma-Aldrich (Buchs, Switzerland), H₂O₂ was from Haenseler (Herisau, Switzerland). Purified polyclonal rabbit anti-chicken lysozyme antibody (80 mg/ml) and purified goat anti-
rabbit IgG horseradish-peroxidase-linked antibody (2.0 mg/ml) were obtained from Acris Antibodies GmbH (Hiddenhausen, Germany). Silk was kindly supplied by Trudel Silk Inc. (Zurich, Switzerland). All other substances were of analytical or pharmaceutical grade and obtained from Sigma-Aldrich.

2.2. Film preparation

Cocoons from *Bombyx mori* (Linne, 1758) were boiled 2 times for 1 hour in an aqueous solution of 0.02 M Na₂CO₃ and rinsed with water as previously described (Sofia et al., 2001). Purified silk was solubilized in 9 M aqueous LiBr solution and dialyzed (Pierce, Woburn, MA; MWCO 3500 g/mol) against water for 2.5 days. Fibroin concentration was determined after evaporation of water overnight and using an analytical balance (Mettler, Greifensee, Switzerland). The concentration was adjusted to 5% (w/v). One hundred and fifty μl of the silk fibroin solution was transferred into polystyrene 96-well plates (for release study; Nunc, Wohlen, Switzerland) or onto flat Teflon surfaces (for physical characterization) and dried at 37°C and 500 mbar overnight. Dried films were treated with 300 μl of (i) 90% (v/v) methanol in H₂O or (ii) H₂O for 30 minutes, respectively or (iii), left untreated.

2.3. Film characterization

2.3.1. Atomic force microscopy (AFM)

Surface analysis of silk fibroin films, 6 mg each and either treated with 90% methanol (v/v) or left untreated, was performed in contact mode using a Nanoscope IIIa (Digital Instruments, San Diego, CA) with oxide-sharpened Si₃N₄ tips mounted on triangular cantilevers with spring constants of 0.58 N/m (specified by the manufacturer). Images were taken in air and flattened and plane-fitted as required.

2.3.2. Fourier-transform infrared spectroscopy (FTIR)

Compound-loaded and unloaded (empty) films were prepared and either methanol treated or left untreated as described before. The structure of the various films was analyzed by FTIR on a Bruker Equinox 55 Spectrometer equipped with a MIRacle™ attenuated total reflection (ATR) Ge crystall cell in reflection mode. Background
measurements were taken twice with an empty cell and subtracted from the sample readings.

2.3.3. Wide angle X-ray scattering (WAXS)

Real-time wide angle X-ray scattering studies were performed at beamline X27C of the National Synchrotron Light Source (NSLS; Brookhaven National Laboratory, NY). Intensity data were collected at room temperature with films encapsulated in Kapton™ tape. Monochromatic X-radiation with a wavelength of \( \lambda = 0.15 \) nm was used. The data were collected in transmission mode using two one-dimensional position sensitive wire detectors. The scattering vectors, \( q = 4 \sin \theta / \lambda \), with \( \theta \) as the half-scattering angle) were calibrated using sodellite and silicon reference powders for WAXS. Scans were collected for 3 minutes over an angular range from \( 2 \theta = 10-30^\circ \). Intensity data were corrected to account for detector linearity, background scattering, sample absorption, and changes in incident beam intensity. Due to the detector geometry, the range of angles from \( 2 \theta = 1-7^\circ \) was not accessible at NSLS. Therefore, room temperature WAXS studies were performed using a conventional sealed tube X-ray source having \( \lambda = 0.15 \) nm. A Phillips PW1830 X-ray generator and optically encoded diffractometer were used to investigate the range of scattering angles at which the OMS typically shows its gallery spacing, i.e. from \( 2 \theta = 2-7^\circ \). Films were examined in \( 0/20 \) reflection mode, using a step scan interval of 0.01° with 2.4 s/step; d-spacings, obtained from Bragg's Law (\( n \lambda = 2d \sin \theta \); where \( \lambda \) is the wavelength of the beam of X-rays and is equal to 0.15 nm, \( \theta \) is the angle of incidence in degrees, and \( d \) is the spacing between atomic planes and is given here in nm).

2.3.4. Contact angle

Static contact angle measurements were performed on dry films (n=3) that were either treated with 90% (v/v) methanol or untreated at ambient temperature using a goniometer (NRL C, ramé-hart inc., Mountain Lakes, NJ). Ultrapure water droplets were used with a drop volume of approximately 30 \( \mu \)l.

2.4. Drug load and release

The silk fibroin solution (5% w/v) was mixed with a drug solution in either PBS (10 mg/ml; FITC-dextrans) or PBS + 0.1% (m/v) BSA (10 mg/ml; HRP, Lys) at a ratio of 7.5:1 (v/v), respectively. One-hundred and fifty \( \mu \)l per well of this mixture or 150 \( \mu \)l
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Silk fibroin solution (5% w/v) was pipetted into polystyrene 96-well plates. Films were dried overnight (37°C; 500 mbar) and treated with methanol or water as described above. The supernatant was collected and the solvent was evaporated using a speedvac concentrator (sc110, Savant, Fisher Scientific, Wohlen, Switzerland). Residues were redissolved in 300 µl PBS (FITC-dextrans) or PBS + 0.1% (m/v) BSA (HRP, Lys) and the compound content in the supernatant was measured as described later. The films were dried again at 37°C and 500 mbar overnight. For release studies, 300 µl of release medium was added to each well and the plates were incubated at 25°C for 23-28 days. At each time point the whole medium was collected for measurement and replaced by fresh release medium (Table 1).

2.4.1. Size exclusion chromatography (SEC) of FITC-dextrans
FITC-dextrans were assayed by SEC-chromatography using a Merck system (Duebendorf, Switzerland), consisting of an autosampler (Merck Hitachi AS), a computer interface (Merck Hitachi A6000), a pump (Merck Hitachi L6200A) and a fluorescence detector (Merck Hitachi F-1050). Separation was performed on a Superdex® 200 HR 10/30 column (Amersham Biosciences Europe, Otelfingen, Switzerland) at 4°C with 50 nM phosphate buffer (pH 7.4) at a flow rate of 0.5 ml/min. FITC-dextran fluorescence was detected with an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

2.4.2. HRP activity assay
The enzyme solutions were diluted in a solution which consisted of 0.5% (v/v) Triton-X 100 and 0.25% (w/v) BSA in 40 mM potassium phosphate buffer at pH 6.8. Ten µl samples or standards were added to 200 µl freshly prepared substrate solution (0.5 mg/ml ABTS and 0.03% (w/v) H₂O₂ in 100 mM citrate buffer at pH 4.1) and the absorption was read at 405 nm at 25°C using a spectrophotometer (Cary 300; Palo Alto, CA).

2.4.3. Lys activity assay
Lys activity was determined by turbidity measurements with Micrococcus luteus cell suspension in 50 mM potassium phosphate buffer at pH 7. The cells were mortared and suspended in 50 mM potassium phosphate buffer until the absorption was in the range of 0.7-0.9 as measured at 450 nm. Twenty-five µl lysozyme solution was added to 1.5 ml cell suspension and the decrease in absorption (450 nm) was read for 5 minutes.
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The maximum linear rates for samples, standards and blank were obtained and the activity of the enzyme was calculated.

2.4.4. ELISA for lys quantification

For quantification of released Lys protein, an indirect ELISA procedure modified from Vidal et al. was performed. Incubation was at room temperature (25°C) on a rotating microplate shaker (IKA Labortechnik, Staufen, Germany). Flat-bottom 96-well polystyrene microtiter plates (Nalge Nunc, Hereford, UK) were washed with PBS between every step of the assay and 4 times at each step (100 µl/well except after blocking: 200 µl/well). Reactant volumes were 50 µl/well. Microplates were initially coated for 2 h with Lys in PBS and nonspecific sites were blocked for 1 h using a solution of 3% BSA (w/v) in PBS. Polyclonal rabbit anti-chicken lysozyme antibody and goat anti-rabbit IgG horseradish peroxidase-linked antibody in 1% BSA-TPBS (1% (w/v) BSA-PBS plus 0.05% (w/v) Tween 20) were subsequently added and incubated for 2 hours. The substrate (OPD at 0.4 mg/ml in 0.05 M phosphate-citrate buffer containing 0.4 mg/ml urea hydrogen peroxide) for peroxidase was placed in the wells for 15 minutes. The reaction was stopped by the addition of 50 µl 1 M H2SO4. Absorbance was read at 540 nm using a microplate reader (Molecular Devices, Bucher Biotec AG, Basel, Switzerland).

2.4.5. Gel filtration chromatography (GFC) for Lysozyme

Lys was assayed for aggregation and degradation products by GFC-chromatography using a Merck system (Duebendorf, Switzerland), consisting of an autosampler (Merck Hitachi AS), a computer interface (Merck Hitachi A6000), a pump (Merck Hitachi L6200A) and a UV/VIS detector (Merck Hitachi L-4250). Separation was performed on a Shodex protein KW804 column (Infochroma AG, Zug, Switzerland) at 25°C with 50 mM phosphate buffer with 0.3 M sodium chloride (pH 7.5) at a flow rate of 1.0 ml/min. Lys absorption was detected at 274 nm.

2.4.6. Protein adsorption to silk films

Three hundred µl protein solution (HRP: 1 g/ml; Lys: 10 mg/ml) in PBS containing 0.1% BSA was added to either methanol treated or non-methanol treated films and incubated at 25°C for 24 hours. Adsorption of the proteins to the silk films was determined by measuring the reduction of protein activity in the supernatant.
2.5. Statistical analysis

For statistical significance, samples were evaluated using a student t-test as well as ANOVA where appropriate. ANOVA was followed by a post-hoc assessment using the Tukey HSD method. Differences were considered significant when equal or less than $p=0.05$.

**Figure 2:** Physicochemical characterization of silk films either untreated or treated with water or 90% methanol. (A) FTIR spectra of methanol or water treated silk films; determination of crystallinity by XRD of methanol treated (B) and water treated (C) films; determination of hydrophility/hydrophobicity of the film surface by contact angle measurements of methanol treated (D) and non-methanol treated (E) films over time.
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Results

3.1. Surface characterization and physicochemical analysis of silk films

Silk film morphology was assessed by atomic force microscopy before and after methanol treatment (Figure 1). Methanol treatment resulted in rougher surfaces when compared to non-methanol treated films and the formation of globular structures, which were not found in native (i.e. not methanol treated) films. FTIR structural analysis of methanol treated films showed an N-H bending vibration bond (amide II) intensity shift from 1540 cm\(^{-1}\) to 1535 cm\(^{-1}\) when compared to native films (Figure 2A). Similarly, methanol treatment resulted in additional shoulders at 1630 cm\(^{-1}\) (amide I) and 1265 cm\(^{-1}\) (amide III). The water treated silk films for the most part lack the peaks for secondary structure at 1695, 1627 and 1520 cm\(^{-1}\), however, it seems as though with the addition of FD20 and FD40, the FTIR structures start showing a more pronounced shoulder or peak at ~1627 and 1515 cm\(^{-1}\) indicating an increase in β-sheet conformation for those films. These data were corroborated by wide-angle X-ray scattering (WAXS) also used in the material characterization (Figure 2B, C). With WAXS, silk crystallinity was monitored by calculating the intersheet \(d\)-spacing distances from the X-ray plots. Methanol treatment resulted in a shift of the major peak (20 = 20°; Figure 2B, C) from 4.4 to 4.3 Å, indicating a shift from silk I to silk II structure. For comparison with our data, representative silk I and silk II model structures that had been taken by X-ray and electron diffraction, infrared spectroscopy, nuclear magnetic resonance, and raman spectroscopy were collected from literature and resulted in three predictions of silk I structure models, the Crankshaft model (Lotz and Keith, 1971), the out-of-register model (Fossey et al., 1991) and the repeated β-turn type II model. Silk II structure is commonly described by antiparallel β-sheets (Asakura et al., 1985, Marsh et al., 1955). More peaks corresponding to reported silk II β-sheet crystalline structures were present in the methanol treated films including the shift in the position of the major peak at 20=20°, indicating a shift from silk I to the more dense silk II structure (data not shown).

Film surface hydrophobicity of methanol treated silk films showed a significantly higher contact angle than for non-methanol treated films at each time-point (p<0.001; Figure 2D, E). Contact angles remained stable for methanol treated films (Figure 2D)
whereas a significant drop over time was observed for non-methanol treated films 
(p<0.05 or p<0.01; Figure 2E).

Figure 3: Cumulative release of different molecular masses of FITC-dextrans from 
silk fibroin films treated with H_2O (A) or 90% methanol (B).

3.2. Compound release

The release of fluorescently labelled dextran (FD) with molecular weights ranging 
from 4 kDa (FD4) to 40 kDa (FD40), respectively, was evaluated as a function of 
methanol treatment using HPLC quantification (Figure 3, Table 1). In water treated 
films, FDs with molecular weights from 4 to 20 kDa showed a burst release after 8 
hours of 60.7 +/-30%, 47.9 +/- 20% and 50 +/- 32%, respectively. Later than 3 days, 
FD4-release ceased almost completely (77 +/- 6% after 28 days), while FD10 and 
FD20 showed a continuous release pattern up to 94.0 +/- 0.2% and 90.2 +/- 1.7% after
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28 days, respectively. In contrast, the release of the larger molecule FD40 was retarded and showed an initial release of only 17.9 +/- 15% followed by a continuous release to up to 40.9 +/- 3% after 28 days (Figure 3A). Methanol treatment of the drug loaded silk films resulted in a strong and molecular weight dependent retardation of the release of all FDs. Burst releases of FD4, FD10, FD20 and FD40 were 58.4 +/- 4%, 26.3 +/- 19%, 7.7 +/- 52% and 8.4 +/- 55%, respectively. With an increase in molecule size, a reduction of the incline was observed for the first 10 days of release, levelling off after 14 days for all molecules (Figure 3B).

Table 1. Physicochemical properties of model drugs

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight [kDa]</th>
<th>Release medium</th>
<th>Radius [nm]</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD4</td>
<td>4</td>
<td>PBS</td>
<td>1.4 \textsuperscript{a}</td>
<td>-</td>
</tr>
<tr>
<td>FD10</td>
<td>10</td>
<td>PBS</td>
<td>2.3 \textsuperscript{a}</td>
<td>-</td>
</tr>
<tr>
<td>FD20</td>
<td>20</td>
<td>PBS</td>
<td>3.3 \textsuperscript{a}</td>
<td>-</td>
</tr>
<tr>
<td>FD40</td>
<td>40</td>
<td>PBS</td>
<td>4.5 \textsuperscript{a}</td>
<td>-</td>
</tr>
<tr>
<td>HRP type VI-A</td>
<td>44</td>
<td>PBS + 0.1% BSA</td>
<td>3 \textsuperscript{b}</td>
<td>6-9 \textsuperscript{c}</td>
</tr>
<tr>
<td>Lys</td>
<td>14.3</td>
<td>a</td>
<td>1.8 \textsuperscript{b}</td>
<td>11.35</td>
</tr>
</tbody>
</table>

\textsuperscript{a}. approximate Stoke's radii given by the supplier
\textsuperscript{b}. Solute radius was calculated from aqueous diffusivities found in the literature using the Stokes-Einstein equation.
\textsuperscript{c}. depending on the isoenzyme

Further studies detailed the efficacy of silk fibroin films as delivery vehicles for bioactive compounds, using HRP and Lys as model drugs (Figures 4, 5, Table 1), based on bioactivity assessments. A discontinuous release from non-methanol treated silk films was observed for HRP, characterized by an initial burst of 7.4 +/- 0.3 µg, followed by a lag phase of two days and a continuous release from days 3 to 8. At later timepoints, no more release of bioactive HRP was measured (Figure 4A). HRP release from methanol treated films started at day 5 and continued until day 23, with a total release of 25.2 +/- 3.0 µg HRP (Figure 4A). The affinity of HRP to the silk films was measured upon incubation of non-methanol treated and methanol treated silk films with the model drug for 24 hours. HRP demonstrated strong affinity to the silk fibroin.
surface, but no significant differences were observed for the two treatments (Figure 4B).

**Figure 4: Interaction of horseradish peroxidase with silk films: (A) Cumulative release of HRP from silk films treated with either 90% methanol or H₂O, respectively. (B) Adsorption of HRP from an aqueous solution to a silk fibroin surface either treated with 90% methanol or non-methanol treated.**

Bioactive Lys release from non-methanol treated silk films was characterized by an initial burst of about 21.3 +/- 12.9 µg, followed by a short lag phase and a continuous release between days 3 and 8 (Figure 5A). After 9 days of release, no bioactive Lys was released. No bioactive Lys was detected from methanol treated films throughout the study time (Figure 5A). However, ELISA measurements of the amount of released protein by ELISA using a polyclonal Lys antibody showed a release from methanol treated silk films, starting at day 8 with a continuous and linear release pattern thereafter and – although at a lower level - parallel to the release from non-methanol treated films (Figure 5A). The released Lys was further analyzed to detail the cause of
the positive ELISA and negative potency results, using qualitative GFC-HPLC studies. Clearly, by-products of lower size (degraded) and higher size (aggregates) of Lys were released in the incubation medium and their presence increased with time for both, methanol (Figure 5C) and non-methanol treated (Figure 5D) films. An apparent silk peak was observed at early timepoints and for the water treated - and better water soluble - silk films when compared to the methanol treated ones, and overlap was observed for the silk peak and the Lys peak (Figure 5B, D). This observed degradation phenomenon correlates with measurements of weight loss of non-methanol treated silk films in PBS (data not shown). The adsorption of Lys was slightly higher for methanol treated as observed to non methanol treated films. but not significant (p=0.08; Figure 5E).

Discussion

Silk fibroin is isolated from cocoons of the silkworm, *B. mori*. This protein has recently found growing interest as a biomaterial for musculoskeletal implants, including substrates for tissue engineered cartilage, bone, and ligaments (Altman et al., 2002a, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004d, Meinel et al., 2004e). Several studies detail the advantages of SF based materials, including the directed differentiation of human mesenchymal stem cells (MSC) into different tissues and excellent biocompatibility (Meinel et al., 2005, Panilaitis et al., 2003, Santin et al., 1999). Furthermore, the mechanical properties of SF in fiber form, rivaling high performance fibers such as Kevlar in terms of energy adsorbed before the fiber breaks, distinguish this organic polymer from other naturally occurring alternatives, such as collagen (Cunniff et al., 1994).

MSC differentiate selectively along different lineages including cartilage and bone, when exposed to specific proteins - e.g. growth factors and cytokines. Ideally, a scaffold material provides a substrate in which cells can thrive and receive stimuli such as through protein release to guide the differentiation process of cells. Therefore, this study evaluated SF as a delivery vehicle for the sustained release of compounds and enzymes as model drugs to further expand the material options available with SF in the important niche of controlled release. Earlier studies have demonstrated the use of silk fibroin carriers for enzyme immobilization as needed for the preparation of biosensors (Demura et al., 1992, Tsukada et al., 1994). These studies detailed the protective
properties of silk fibroin matrices for several proteins including staphylococcal protein A (Kikuchi et al., 1999), alkaline phosphatase (Demura et al., 1992), and various other proteins and peptides (Asakura and Kaplan, 1994, Mitsui et al., 1989). Ultimately, this type of materials system could lead to a novel mechanically useful implant material, stabilizing and releasing proteins that guide the differentiation process of cells in a directed fashion through controlled drug delivery.

Figure 5: Interaction of lysozyme with silk films: (A) Cumulative release of Lys from silk films treated with either 90% methanol or H₂O, respectively. Data are presented as amount of protein released as measured with ELISA (open symbols) or amount of protein released as calculated from the activity measurements (black symbols). (B, C, D) SE-HPLC measurements showing: silk, Lys and a mixture of both in release buffer as controls (B), released molecules out of silk films treated with 90% methanol (C) or water (D) after 1, 9 and 23 days, respectively. (E) Adsorption of Lys from an aqueous solution to a silk fibroin surface either treated with 90% methanol or non-methanol treated.
Methanol treatment of the silk films resulted in physicochemical changes with the formation of globular surface structures which were absent on surfaces from water treated films (Figure 1). Further, methanol treatment induced a shift to higher amounts of crystalline β-sheets structures (Figure 2A, B) and resulted in higher hydrophobicity as indicated by contact angle measurements (Figure 2D, E). The different crystalline states were detected by FTIR spectra, resulting in a typical shift for amide III bond stretching (1235 and 1265 cm⁻¹) and changes in the amide V bond regions. In particular the shoulder in the amide III band region is indicative for a conformational shift of silk fibroin into β-sheet structure, as described before (Asakura et al., 1985). Changes in silk assembly as a result from a silk I to a silk II conformational change were further detailed by wide angle X-ray scattering (WAXS) before and after methanol treatment. The assembly of silk fibers in the silk I conformation state was described by three different models, obtained through measurements of d-spacing data from WAXS, the Crankshaft, β-turn type II (Fossey et al., 1991) and the out of register model, respectively. Assembly in silk structures with a silk II conformational state and as a result of methanol treatment was presented by Marsh and Asakura. A comparison of d-spacing from WAXS spectra and taken from water treated (mainly silk-I conformation) films in this study (extracted from Figure 2B, C), fitted best with the out-of-register model of silk assembly, which was subject to substantial changes after methanol treatment, resulting in structures of a silk-II conformational state and as previously described (Asakura et al., 1997, Marsh et al., 1955).

For evaluation of drug size/molecular weight impact on release patterns, dextrans with increasing sizes and molecular weights were used. Dextrans with molecular weights of 2 to 10 kDa arrange in expandable coils, whereas dextrans with molecular weights exceeding 10 kDa organize in branched structures. The higher the molecular weight, the larger was the retention of the drug, more specifically for methanol treated films (Figure 3). These retentions were a result of the above described changes in physicochemical properties, characterized by an increase of crystalline β-sheets and a concomitant decrease in water solubility (Bini et al., 2004, Jin and Kaplan, 2003, Lazaris et al., 2002), ultimately resulting in more sustained release kinetics.

In the present study, crystallinity was induced by methanol treatment (Jin and Kaplan, 2003, Kaplan et al., 1998, Valluzzi et al., 2002, Wilson et al., 2000), which can be detrimental to the bioactivity of a drug, in particular for protein drugs (Pace et al.,...
These detrimental methanol effects were observed for lysozyme by comparative experiments of drug release into the supernatant using an ELISA and a potency assay (Figure 5). Lys release from water treated films resulted in similar release profiles as measured by the two different assays (potency versus presence of antigen), whereas a substantial loss in bioactivity was determined for drugs released from methanol treated films. Therefore, silk scaffolds failed to protect lysozyme potency after methanol treatment. Interestingly, HRP entrapment into methanol treated films resulted in a release of bioactive HRP (Figure 4), whereas complete loss of potency resulted from methanol supplementation to aqueous HRP solutions (data not shown). Based on these findings, a selective protection for protein drugs can be postulated for silk biopolymers. Future studies are needed detailing the mechanistic principles of protection by silks.

Drug release kinetics were further influenced by the drug’s nature, as can be exemplarily seen with a comparison of HRP (Stoke's radius of approximately 3 nm) and FD20 (Stoke's radius of 3.3 nm). Apart from similarities in diameters, both HRP and FD20 resulted in completely different release patterns. Therefore, easy predictions of drug release kinetics from silks are difficult and call for a clear need to individually assess a drug’s retention within the biopolymer.

The experiments highlight the strength but also the limitations of using silk fibroin as a polymer for drug delivery. The motivation of this study was to combine the excellent biomaterial properties of silks - biocompatibility, mechanical integrity, and biodegradation – with drug delivery options. We believe a general feasibility of this approach can be postulated from the presented work, although detrimental effects of processing steps involving the use of methanol on drug potency need to be addressed for each individual drug intended to be delivered. Further, adsorption to silk surfaces can significantly – and again as a result of individual drug properties – change drug release kinetics. Current experiments in our lab aim at using repetitive cycles of water vapor exposition instead of methanol treatment to induce changes in silk crystallinity, to by-pass the detrimental effects of methanol. Preliminary own results and supporting data from literature (Kim et al., 2005) suggest the feasibility of this replacement, while maintaining suitable drug delivery kinetics similar to the ones shown for methanol treated films.
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An envisioned scenario for the above introduced system in tissue engineering would be the use of drug releasing SF biomaterials to guide the differentiation of MSC into musculoskeletal tissues. A close monitoring of drug potency and aggregation phenomena is required to optimize such systems. These systems offer preservation of sensitive protein activity, allow for a controlled and sustained delivery of proteins, along with control of scaffold structure and morphology. When these features are considered along with the remarkable mechanical properties of these proteins in materials, intriguing options for new biomaterial utility for this family of proteins begin to emerge.

Acknowledgements

We thank Prof. Peggy Cebe and Xiao Hu for their help in XRD data acquisition at the XY synchrotron (Brookhaven, MD), and Trudel Inc. (Zurich Switzerland) for cocoon supply. Financial support from NIH (EB00252 and EB003210), the NSF (DMR) and the Association for Orthopedic Research (AFOR) is greatly appreciated.

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Bone morphogenetic protein–2 decorated silk fibroin films induce osteogenic differentiation of human bone marrow stromal cells

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Journal for Biomedical and Material Research, 2004, 71 (3), p528-537
Abstract

Bone morphogenetic protein-2 (BMP-2) plays a critical role in bone formation and regeneration. Therefore, the ability to immobilize this molecule in certain matrices may be crucial in bone tissue engineering. Using carbodiimide chemistry, BMP-2 was directly immobilized on silk fibroin films. Whereas human bone marrow stromal cells (BMSCs) cultured on unmodified silk fibroin films in the presence of osteogenic stimulants exhibited little if any osteogenesis, the same cells cultured on BMP-2 decorated films in the presence of osteogenic stimulants differentiated into an osteoblastic lineage as assessed by their significantly elevated alkaline phosphatase activity, calcium deposition and higher transcript levels of collagen type I, bone sialoprotein, osteopontin, osteocalcin, BMP-2 and cbfa1. Using cell culture inserts, it was demonstrated that differentiation was induced by the immobilized protein and not by protein released into the culture medium. Comparison with a similar amount of medium supplemented BMP-2, where no additional protein was added with medium changes, showed that delivery of BMP-2 immobilized on the biomaterial surface was more efficient than soluble delivery. The results illustrate that BMP-2 covalently coupled on silk biomaterial matrices retains biological function in vitro based on the induction of osteogenic markers in seeded BMSCs.
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Introduction

Bone formation is one of the most widely explored areas of tissue engineering. A variety of biomaterials have been used as scaffolds for bone growth in vitro and in vivo, ranging from inorganics like metals and ceramics to natural polymers like collagen. Osteogenic factors, and particularly members of the transforming growth factor β (TGF-β) superfamily, play a key role in bone formation and repair (Wozney and Rosen, 1998). Cloning and expression of bone morphogenetic proteins (BMPs) has stimulated research on osteogenesis. BMP-2 has been shown to be one of the most potent members of the BMP family for the induction of bone formation in vivo and has been studied using a variety of delivery systems including collagen (Hollinger et al., 1998), porous hydroxyapatite composites (Noshi et al., 2001), Si-Ca-P xerogels (Santos et al., 1998) and poly-L-lactic acid scaffolds (Kim et al., 1998). Mesenchymal stem cells have been transduced with an adenoviral vector carrying the BMP-2 gene; this induced their differentiation in vitro and bone formation in vivo (Cheng et al., 2001). In addition, the majority of the in vitro studies in which BMP-2 has been used involved supplementation of the medium with BMP-2 in soluble form (Thies et al., 1992, Cheng et al., 1998, Prabhakar et al., 1998, Blum et al., 2001).

Covalent binding of small molecules, and especially peptides containing the RGD sequence, has been widely explored on alginates (Rowley and Mooney, 2002), biomimetic coated titanium (Verrier et al., 2002), poly(methyl methacrylate) (Kantlehner et al., 2000), borosilicate glass (Dee et al., 1999) and poly(ethylene) glycol-modified polyurethane films (Lin et al., 2001). Although RGD peptides have a beneficial outcome for the attachment and proliferation of several cell lines, the class of signaling factors known as cytokines that mediate complex cellular events, such as bone tissue formation, would also be useful.

Functionalizing a biomaterial surface with cytokines would provide spatial control and moderation of release profiles. This approach can provide a useful therapeutic tool for medical applications due to enhanced control of location, concentration and release kinetics compared to soluble delivery. The immobilization of these factors, however, must be accomplished with retention of biological function.

Successful direct coupling of larger proteins has been achieved with transforming growth factor-β1 (TGF-β1) on collagen gels by the addition of an auxiliary collagen binding domain (WREPSFMALS) (Andrades et al., 1999). The result was better
access of the protein to the cells, probably due to a longer half-life and slower release, resulting in more potency than TGF-β1 supplied directly in medium (Andrades et al., 1999). For osteogenic applications, bone morphogenetic protein-4 (BMP-4) (Puleo et al., 2002) and BMP-2 (Jennissen et al., 1999) have been covalently bound to titanium alloys, while oligopeptides derived from BMP-2 (Suzuki et al., 2000, Saito et al., 2003) and BMP-7 (Kirkwood et al., 2003) have been immobilized on alginate hydrogels and glass, respectively. In these studies immobilized proteins and peptides induced elevated alkaline phosphatase activity in short-term cultures of the C3H10T1/2 (Puleo et al., 2002) and MC3T3-E1 cell lines (Wiemann et al., 2002), mineralization in primary rat calvarial osteoblasts (Kirkwood et al., 2003) and ectopic bone formation in rats (Suzuki et al., 2000, Saito et al., 2003). The use of a poly(ethylene) glycol spacer can help overcome loss of biological activity, due to interactions between the growth factor and the biomaterial matrix. This was found with transforming growth factor-β2 on fibrillar collagen (Bentz et al., 1998) and horseradish peroxidase on three-dimensional collagen matrices (Chen et al., 2002).

Previously, modified parathyroid hormone and a pentapeptide containing the RGD sequence were coupled to silk fibroin films using carbodiimide chemistry and an increase in osteogenic activity was observed by the osteoblast-like cell line Saos-2 (Sofia et al., 2001). Silks are attractive biomaterials for bone tissue engineering because of their biocompatibility (Santin et al., 1999, Altman et al., 2003) and excellent mechanical properties (Gosline et al., 1986). In the present study the properties of silk fibroin were further enhanced by decorating the surface of films with covalently bound BMP-2. Human bone marrow stromal cells (BMSCs), cultured in medium supplemented with osteogenic stimulants, were used to assess the biological activity of immobilized BMP-2 and to emphasize the potential utility in bone-tissue engineering. Alkaline phosphatase activity, calcium deposition, and transcript levels related to bone specific markers, were present at significantly higher levels than in the controls, which included adsorbed BMP-2 and unmodified fibroin. Cell culture inserts were also used to determine if the observed differentiation response by the BMSCs was due to the immobilized protein or to the protein released from the films into the cell culture medium. Finally, the effectiveness of the immobilized BMP-2 in terms of stimulation of osteogenic differentiation when compared to a range of soluble concentrations of the same cytokine.
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Materials and Methods

Materials

BMP-2 and I\textsuperscript{125}–BMP-2 were generously supplied from Wyeth (Andover, MA). Bovine serum, Dulbecco's Modified Eagle Medium (DMEM), basic fibroblast growth factor (bFGF), Pen-Strep, Fungizone, non essential amino acids, trypsin were from Gibco (Carlsbad, CA). Ascorbic acid phosphate, dexamethasone, \( \beta \)-glycerophosphate, Histopaque-1077 were from Sigma (St. Louis, MO). Cell culture plates and inserts were from Becton Dickinson (Franklin Lakes, NJ). All real time PCR reagents, including assay kits for human bone sialoprotein, human osteopontin, human BMP-2 and human cbfa-1, as well as synthesized primers and probes for GAPDH, collagen I, and osteocalcin, were from Applied Biosystems (Foster City, CA). All other substances were of analytical or pharmaceutical grade and obtained from Sigma. Silkworm cocoons were kindly supplied by M. Tsukada (Institute of Sericulture, Tsukuba, Japan).

Film preparation and decoration

Silk fibroin from \textit{B. mori} silkworm cocoons was extracted and films were cast in tissue culture plates as described elsewhere (Sofia et al., 2001). BMP-2, supplied in a formulation buffer, was dissolved using dialysis cassettes (Pierce, Woburn, MA; MWCO 3500) against 0.5 M arginine, 5 mM glutamic acid, pH 3.8 for 1 day, 0.5 M arginine, 0.1M MES (Pierce, Woburn, MA), 0.5 M NaCl, pH 6 for another day and 0.1M MES, 0.5 M NaCl, pH 6 (coupling buffer) for 2 days. The purpose of this dialysis procedure was to solubilize BMP-2 in the coupling buffer while removing the additives from the formulation buffer. Protein precipitated during dialysis, but with consecutive centrifugations and re-suspensions in coupling buffer almost 80% of the original BMP-2 was recovered. The activity of the protein was tested by measuring the alkaline phosphatase activity of the W-20 mouse stromal cell line at Wyeth (Thies et al., 1992, Blum et al., 2001).

Films were hydrated for 30 min with coupling buffer and COOH groups were activated by reaction with 0.5 mg/ml 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC) / 0.7 mg/ml N-hydroxysuccinimide (NHS) (Pierce, Woburn,
MA) solution in coupling buffer for 15 minutes at room temperature (Sofia et al., 2001). The films were rinsed and contacted with 0.1 mg/ml BMP-2 in coupling buffer for 2 hours at room temperature on a shaker. At the end of the reaction the protein solution was removed, films were rinsed 3 times with coupling buffer (the 2nd time they were left for 10 min on a shaker), 2 times with water and finally air-dried. Control films with adsorbed BMP-2 were prepared the same way but by omitting the EDC/NHS activation step. Films were sterilized with 70% ethanol for 30 min and then dried in a tissue culture hood.

Quantification using $^{125}$I-BMP-2

In experiments with radiolabeled BMP-2 a small amount of the radioactive protein ($0.92 \mu$Ci/$\mu$g) was mixed with the non-labeled protein (ratio 1:85 or 1:170 depending on the experiment) and the same coupling procedure was followed as described above. Sodium dodecyl laurylsulfate 0.1% (w/v) was used to remove any non-specifically bound protein for 30 min. Films were re-solubilized in 9M LiBr for 30 min at 37°C and the radioactivity was measured with a LKB-Wallac CliniGamma 1272 (Wallac, Finland) gamma counter.

Human bone marrow stromal cell isolation and expansion

Total bone marrow from two male donors under 25 years old (25 cm$^3$, Clonetics, Santa Rosa, CA.) was diluted in 100 ml of isolation medium (5% FBS in RPMI 1640 medium). Cells were separated by density gradient centrifugation. Briefly, 20 ml aliquots of bone marrow suspension were overlaid onto a poly-sucrose gradient ($\delta=1,077$ g/cm$^3$, Histopaque, Sigma, St. Louis, MO) and centrifuged at 800 x g for 30 min at room temperature. The opaque cell layer was carefully removed, washed in 10 ml isolation medium, pelleted and contaminating red blood cells were lysed in 5 ml of Pure-Gene Lysis solution. Cells were pelleted and suspended in expansion medium (DMEM, 10% FBS, 1 ng/ml bFGF, 0.1 mM non-essential amino acids, 100 U/ml penicillin, 100 mg/l streptomycin and 0.5 $\mu$g/ml fungizone) and seeded in 75 cm$^2$ flasks at a density of $5\times10^4$ cells/cm$^2$. The adherent cells were allowed to reach 80%
confluence (12-17 days for the first passage). Cells were trypsinized, suspended in FBS containing 9% DMSO, stored in liquid N₂, replated and used for the experiments.

*Tissue culture*

For cultivation on films, BMSCs were seeded \((5 \times 10^3 \text{ cells/cm}^2)\) onto pre-wetted films (DMEM, 1 hr) in 12-well tissue culture plates. Osteogenic medium was DMEM supplemented with 10% FBS, 50 µg/ml ascorbic acid-2-phosphate, 10 nm dexamethasone, 7 mM β-glycerophosphate, 100 U/ml penicillin, 100 mg/l streptomycin and 0.5 µg/ml fungizone. Half of the medium was replaced twice per week.

The same cell culture conditions were applied for experiments with cell culture inserts. BMSCs from the second donor were cultured in cell culture inserts, placed above the silk fibroin films with coupled or adsorbed BMP-2. The same inserts were placed above BMSCs cultured on these films to check for release of the protein due to the presence of the cells (Scheme 1). In order to maximize medium and BMP-2 exchange two holes (5 mm each) were additionally formed on the sides of each insert.

For comparison with soluble BMP-2, BMSCs from the second donor were seeded in 24-well plates and 0.35 ml of osteogenic medium containing 0 (for coupled BMP-2), 0.1, 1, 10 and 100 µg/ml BMP-2 were supplied to the cells. Every 5 days osteogenic medium without BMP-2 was added to the cells, while no medium was removed from the wells.

*Scheme 1:* Illustration of the experimental set-up with cell culture inserts.
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Biochemical analysis and calcein and phalloidin labelling

Cells were lysed in 0.2% (w/v) Triton X-100 and 5mM MgCl₂ and alkaline phosphatase activity was measured using a biochemical assay from Sigma (St. Louis, MO). The assay was based on the conversion of p-nitrophenyl phosphate to p-nitrophenol, which was measured colorimetrically at 405 nm. The volume that was left was used for total DNA content determination. DNA content was measured using the PicoGreen assay (Molecular Probes, Eugene, OR), according to the protocol of the manufacturer. Samples were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Calcium was extracted twice with 0.5 ml 5% trichloroacetic acid. Calcium content was determined by a colorimetric assay using o-cresolphthalein complexone (Sigma, St. Louis, MO). The calcium complex was measured spectrophotometrically at 575 nm. Calcein and phalloidin labelling were performed as previously described (Sofia et al., 2001). Cells were visualized with a Zeiss microscope.

RNA isolation, real-time-reverse transcription polymerase chain reaction (real time RT-PCR)

Cells were lysed in Trizol, transferred to 1.5 ml tubes, centrifuged at 12000 × g for 10 minutes and the supernatant was transferred to a new tube. Chloroform (200 µl) was added to the solution and incubated for 5 minutes at room temperature. Tubes were again centrifuged at 12000 × g for 15 minutes and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasy mini spin column (Qiagen, Hilden, Germany). The RNA was washed and eluted according to the manufacturer’s protocol. The RNA samples were reverse transcribed into cDNA using oligo (dT)-selection according to the manufacturer’s protocol (Superscript Preamplification System, Life Technologies, Gaithersburg, MD). Transcript levels were quantified using the ABI Prism 7000 Real Time PCR system (Applied Biosystems, Foster City, CA). PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, and then 50 cycles at 95°C for 15s, and 1 min at 60°C. The expression data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The GAPDH probe was labelled at the 5’ end with fluorescent dye VIC and with the quencher dye TAMRA at the 3’ end. Primer
sequences for the human GAPDH gene were: Forward primer 5’-ATG GGG AAG GTG AAG GTC G-3’, reverse primer 5’-TAA AAG CCC TGG TGA CC-3’, probe 5’-CGC CCA ATA CGA CCA AAT CCG TTG AC-3’. The collagen I probe was labelled at the 5’ end with fluorescent dye 6FAM and with the quencher dye TAMRA at the 3’ end. Primer sequences for the human collagen I gene were: Forward primer 5’-CAG CCG CTT CAC CTA CAG C-3’, reverse primer 5’-TTT TGT ATT CAA TCA CTG TCT TGC C-3’, probe 5’-CCG GTG TGA CTC GTG CAG CCA TC-3’. The osteocalcin probe was labelled at the 5’ end with fluorescent dye 6FAM and with the quencher dye TAMRA at the 3’ end. Primer sequences for the osteocalcin gene were: Forward primer 5’-GAA GCC CAG CGG TGC A-3’, reverse primer 5’-CAC TAC CTC GCT GCC CTC C-3’, probe 5’-TGG ACA CAA AGG CTG CAC CTT TGC T-3’.

**Figure 1:** A) BMP-2 surface density on silk fibroin films for coupled (black columns) and adsorbed (white columns) BMP-2. B) BMP-2 surface density during cell culture for coupled () and adsorbed () BMP-2. Data are represented as the average ± standard deviation (* = p < 0.05 and ** = p < 0.01).
Figure 2: Alkaline phosphatase activity of BMSCs from two different donors (A and B) for BMP-2 coupled on silk fibroin (black columns), BMP-2 adsorbed on silk fibroin (white columns), and unmodified silk fibroin (grey columns). Data are represented as the average ± standard deviation (* and # = p<0.05 and ** and ## = p<0.01, asterisks denote significantly higher values and pounds significantly lower values).

Statistical analysis

For standard deviation analysis N=4 wells were dedicated to each assay. Statistical analysis of data was performed by one-way ANOVA with Tukey HSD post-hoc tests for comparisons between films with coupled BMP-2 and control groups and p values less than 0.05 were considered statistically significant.

Figure 3: Calcium deposition of BMSCs from 1st (black columns) and 2nd (white columns) donor after 4 weeks in culture for BMP-2 coupled on silk fibroin, BMP-2 adsorbed on silk fibroin, and unmodified silk fibroin. Data are represented as the average ± standard deviation (* = p<0.05 and ** = p<0.01).
Results

Activity of BMP-2 re-dissolved in the coupling buffer

The W-20 mouse stromal cell line was used to determine if BMP-2 was still active after precipitation that occurred during dialysis and solubilization in coupling buffer. The specific activity of the sample ($5.1 \pm 0.18$ units/mg of BMP-2) was similar to the specific activity of the internal control ($4.5 \pm 0.07$ units/mg of BMP-2), thus the procedure developed to prepare the BMP-2 for the coupling reactions preserved the majority of the biological activity.

**Figure 4:** Calcein (A, B, C) and phalloidin (D, E, F) labeled BMSCs after 4 weeks in culture for BMP-2 coupled on silk fibroin (A, D), BMP-2 adsorbed on silk fibroin (B, E), and unmodified silk fibroin (C, F). Magnification is 100X.

Quantification of immobilized BMP-2

The amount of BMP-2 immobilized on the film’s surface is shown in Figure 1A. Initially, there is no significant difference between the coupled and the adsorbed (no coupling agents) BMP-2, however treatment with 0.1% (w/v) SDS removed most of the adsorbed protein resulting in $31.3 \pm 0.86$ ng/cm$^2$ surface density for immobilized BMP-2. For cell culture, films were prepared without SDS treatment and almost 90% of the adsorbed protein was removed within 4 weeks with 70% being removed the first week of culture (Figure 1B). On the other hand, 50% of the amount of BMP-2 was
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retained by the end of the culture on the silk fibroin films where the coupling agents EDC and NHS had been used.

**Figure 5:** Transcript levels by real time RT-PCR for bone markers from BMSCs from 1st (A, C, E, G, I, K) and 2nd (B, D, F, H, J, L) donor for BMP-2 coupled on silk fibroin (black columns), BMP-2 adsorbed on silk fibroin (white columns), and unmodified silk fibroin (grey columns). Data are shown on a logarithmic scale relative to the expression of the respective gene in BMSCs at 0 weeks and represented as the average ± standard deviation (* and # = p<0.05 and ** and ## = p<0.01, asterisks denote significantly higher values and pounds significantly lower values).
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Differentiation of BMSCs on BMP-2 decorated silk fibroin films

BMSCs from two different donors were cultured for 4 weeks on unmodified silk fibroin films, silk fibroin films with adsorbed BMP-2 and films with covalently bound BMP-2. BMSCs growing on films where BMP-2 had been chemically immobilized showed significantly higher alkaline phosphatase activity at all timepoints for the second donor (Figure 2B) and at 2 and 4 weeks for the first donor (Figure 2A). In the beginning of the culture (week 1) adsorbed BMP-2 induced a significant increase in alkaline phosphatase activity compared to unmodified films for both donors. This difference was retained at the end of the culture only for the second donor. Overall amounts as well as temporal activity were different for the two donors with the first one showing higher activity at all timepoints and the second showing an increase in activity with time.

No calcium was biochemically detected at 1 and 2 weeks for BMSCs from both donors (data not shown). After 4 weeks in culture calcium deposition for coupled BMP-2 was more than threefold higher than adsorbed BMP-2 for both donors and sevenfold and fivefold higher than unmodified films for the first and second donor, respectively (Figure 3). Adsorbed BMP-2 had an effect on mineralization as well, since more mineral was deposited than on the unmodified films. Overall amounts of calcium were similar for both donors in the respective groups. Visualization of the mineralization with calcein, a fluorescent dye that binds calcium phosphate, further supported the biochemical data. No calcium deposition could be seen at 1 and 2 weeks of culture (data not shown). At 4 weeks BMSCs were confluent in all groups (Figure 4D-F) and films with covalently bound BMP-2 showed a homogenous distribution of mineral deposition (Figure 4A), while no fluorescence could be detected from the controls groups (Figure 4B-C).

In addition, several mRNA transcripts levels were upregulated for BMSCs grown on silk fibroin films where BMP-2 had been immobilized (Figure 5). Specifically, when compared to control films this effect was more prominent for bone sialoprotein (Figure 5B), osteopontin (Figure 5C), osteocalcin (Figure 5D) and BMP-2 (Figure 5E). Expression of bone sialoprotein (Figure 5B) and BMP-2 (Figure 5E) for BMSCs cultured on films where BMP-2 was adsorbed showed a consistent upregulation for both donors when compared with unmodified films. A similar pattern in expression for both donors could be observed for bone sialoprotein (Figure 5B), osteopontin (Figure
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5C) and osteocalcin (Figure 5D), where levels increased with time. On the contrary, transcript levels of BMP-2 decreased with time and this trend was the same for both donors (Figure 5E).

![Figure 6: Alkaline phosphatase activity (A) and calcium deposition (B) of BMSCs in inserts above decorated films (white columns), inserts above BMSCs cultured on decorated film (grey columns), and on decorated films (black columns) after 4 weeks of culture. Data are represented as the average ± standard deviation (* and # = \( p<0.05 \) and ** and ## = \( p<0.01 \), asterisks denote significantly higher values and pounds significantly lower values).]

Mechanism and efficacy of coupled BMP-2-induced differentiation

In order to investigate if the differentiation induced by coupled BMP-2 was due to immobilized protein on the film or to the protein released into the culture medium via hydrolysis of the amide bond formed by the carbodiimide chemistry, BMSCs were cultured in cell culture inserts, placed above silk fibroin films with coupled and adsorbed BMP-2. These inserts have a porous membrane bottom that supports cell growth and allows exchange of medium with the surrounding well.

After 4 weeks in culture BMSCs cultured on films where BMP-2 was immobilized showed the highest alkaline phosphatase activity (Figure 6A) and calcium deposition (Figure 6B) (both values were not statistically different than those illustrated in the independent experiment of Figure 2B and Figure 3) compared to both types of inserts (inserts placed above fibroin films with and without BMSCs). Therefore it can be concluded that BMP-2 that was released into the culture medium did not significantly contribute to the differentiation of the BMSCs and the bound protein in the local
environment of the cells induced the osteogenic response. No statistical difference in alkaline phosphatase activity could be seen in BMSCs cultured in inserts that were co-cultured with films where BMP-2 was immobilized, regardless of whether these films were seeded with cells or not (Figure 6A). In contrast, for films where BMP-2 had been adsorbed the highest alkaline phosphatase activity was observed for BMSCs cultured in inserts that were co-cultured with films seeded with cells. A similar trend could be seen in mineralization but in this case with the films where BMP-2 had been immobilized. BMSCs in inserts that were co-cultured with films where BMP-2 had been immobilized and seeded with cells showed a statistically higher mineralization level based on calcium deposition than inserts co-cultured with non-seeded films (Figure 6B). These results suggest that factors that were released from the differentiating cells had a stronger impact on osteogenesis than BMP-2 released from the films.

Since BMP-2 is usually delivered in soluble form in the culture medium for in vitro applications, a comparison with various concentrations of soluble BMP-2 was performed in order to assess how effective the immobilized form of delivery was in terms of comparable “soluble BMP-2 concentration”. However, there were two differences in the usual procedure of experiments where BMP-2 is supplied in the culture medium in order to mimic the conditions under which the covalently bound
protein was delivered. First, although soluble BMP-2 was continuously supplied fresh with every medium change, in this experiment the soluble protein was supplied only at the beginning, in order to simulate more precisely the fact that coupled BMP-2 was supplied at the beginning of the culture and no additional BMP-2 was added during the rest of the culture. Additionally, there was no medium removed (and subsequently no BMP-2 removed) throughout the culture period in an effort to minimize protein dilution; fresh medium was added every 5 days. Increasing BMP-2 concentrations elicited a dose dependent response on both BMSCs alkaline phosphatase activity (Figure 7A) and calcium deposition (Figure 7B) that reached a plateau. Immobilized protein resulted in almost 20% of the maximum alkaline phosphatase activity at 2 weeks (Figure 7A) and over 25% of the maximum calcium deposition at 4 weeks (Figure 7B).

Discussion

BMP-2 plays a critical role in bone regeneration in vitro and in vivo. In the present study we investigated the immobilization of BMP-2 on fibroin, a natural biocompatible and biodegradable protein polymer, in an effort to stimulate osteogenic differentiation directly from the scaffold, as opposed to the delivery of soluble BMP-2 in the medium. We hypothesized that direct coupling on the surface of the material would retain the biological activity of the molecule. To test this hypothesis we used BMSCs than maintain their undifferentiated character through ex vivo expansion and have the ability to differentiate into multiple mesenchymal lineages like bone, cartilage or fat (Pittenger et al., 1999, Meinel et al., 2004). These cells, when cultured on unmodified silk fibroin films in the presence of osteogenic stimulants, exhibited little if any osteogenesis.

Carbodiimide chemistry was used for the covalent immobilization of BMP-2 on the silk fibroin films resulting in a surface density of approximately 30 ng/cm². Using the same chemistry the GRGDS peptide and parathyroid hormone (PTH) 1-34 amino acids were previously covalently bound on silk fibrin films at surfaces densities of 24 and 20 pmol/cm², respectively (Sofia et al., 2001). Although the surface density of BMP-2 was significantly lower (~1 pmol/cm²), the fact that both GRGDS and PTH1-34 are smaller peptides with a less complex protein conformation compared to the BMP-2
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dimer can explain this difference. A higher surface density of 60-100 ng/cm² BMP-2 was achieved on silane decorated titanium plates activated with carbonyldiimidazole (Jennissen et al., 1999), but in this case the number of available coupling sites was higher as opposed to the available –COOH groups from fibroin. All radioactivity measured during the quantification of BMP-2 corresponded only to labeled protein, as control experiments showed that the binding of free $^{125}$I on silk fibroin was insignificant (data not shown).

Although there were concerns that direct coupling might interfere with the active site of BMP-2 or may render it inaccessible to cells and therefore reduce bioactivity, the increased alkaline phosphatase activity, mineralization and bone-characteristic transcript levels clearly demonstrated that the biological activity of BMP-2 was not compromised by this coupling procedure and that immobilized BMP-2 was able to induce differentiation of BMSCs into the osteogenic lineage. Direct immobilization of BMP-2 using heat-fixation on titanium, stainless steel, nitrocellulose and glass also resulted in retention of the protein’s bioactivity (Wiemann et al., 2002) and BMP-2-derived oligopeptides that were immobilized on alginate hydrogels using carbodiimide chemistry resulted in ectopic bone formation when implanted in rats (Suzuki et al., 2000, Saito et al., 2003). Other members of the BMP family, BMP-4 and BMP-7-derived peptides, have been successfully immobilized using the same chemistry on a titanium alloy (Puleo et al., 2002) and glass coverslips (Kirkwood et al., 2003), respectively, either directly or via silane coupling. Nevertheless only Kirkwood et al. maintained a long-term culture (up to 3 weeks) of primary rat calvarial cells to observe mineralization (Kirkwood et al., 2003), while other studies only demonstrated elevated alkaline phosphatase activity after 3 days in culture of mice cell lines (Wiemann et al., 2002, Puleo et al., 2002). In the present study the culture was extended to 4 weeks and, most importantly, adult BMSCs were used, emphasizing the potential use of BMP-2 decorated silk fibroin for therapeutic tissue engineering applications.

BMSCs cultured on collagen films in osteogenic medium supplemented with 1 µg/ml BMP-2 formed clusters as shown by calcein labeling, demonstrating more mature mineralization (Meinel et al., 2004). In this latter case, however, BMP-2 was supplied fresh into the medium twice a week. Bentz et al. have shown that tethering TGF-β2 to fibrillar collagen via polyethylene glycol had a stabilizing effect on the growth factor and may improve shelf-life when compared to the free molecule (Bentz et al., 1998). In
the present system the biological effect of immobilized BMP-2 when compared to the maximum osteogenic result that could be achieved with the free protein under the constraint that no additional free BMP-2 was added into the culture. There is a dose response to increasing BMP-2 concentrations (van den Dolder et al., 2003). Alkaline phosphatase activity and calcium deposition increased with increased BMP-2 concentration and reached a plateau for 1 to 100 µg/ml. At this point it should be noted that concentrations of 10 and 100 µg/ml far exceed the physiological dose range of a local regulatory factor (Thies et al., 1992) and were used only for the determination of a maximum osteogenic effect. The immobilized BMP-2 induced 20% of the maximum alkaline phosphatase activity at 2 weeks and 25% of the maximum calcium deposition at 4 weeks. However, a direct comparison with any of the three concentrations cannot be made, since the lowest concentration necessary to induce the maximum osteogenic result has not been determined. A comparison can be made with the 0.1 µg/ml concentration (35 ng of free BMP-2 per well). The immobilized BMP-2 was almost two times higher (~30 ng/cm² or ~60 ng per well) but it induced more than a sixfold increase in alkaline phosphatase activity at 2 weeks and almost 28 times more mineralization at 4 weeks. This comparison shows that delivery of BMP-2 immobilized on the biomaterial surface is more efficient than soluble delivery, perhaps because of the higher concentration at the local environment of the cells or slower degradation of immobilized BMP-2 by reducing the rate of unfolding or access by proteases, as has been routinely reported for immobilized enzymes (Itoyama et al., 1994, Pereira et al., 2001, Gouda and Abdel-Naby, 2002). It has been demonstrated that TGF-β2, another member of the TGF superfamily, completely lost activity after 1 week of incubation at 37 °C in vitro (Bentz et al., 1998). Furthermore, the hypothesis that the free protein was susceptible to more rapid degradation was supported by the outcome of the experiment with the cell culture inserts. Covalently immobilized BMP-2 induced differentiation, a response that was not due to protein released into the medium from either the coupled or adsorbed systems.
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Acknowledgements

We thank the NIH (DE13405/04) for support of this study, Tom Porter and Chris Mahoney (Wyeth) for help with BMP-2 protocols and for conducting the W-20 bioassay, Gloria Gronowicz (Orthopaedic Surgery, University of Connecticut Health Center) for help with protocols for biochemical assays and technical discussions, and Jim Schwob and Peggy Harris (Department of Anatomy and Cellular Biology, Tufts University School of Medicine) for use of their laboratory for $^{125}$I-BMP-2 experiments.

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BMP-silk composite matrices heal critically sized femoral defects

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Abstract

Clinical drawbacks of bone grafting prompt the search for alternative bone augmentation technologies such as use of growth and differentiation factors, gene therapy, and cell therapy. Osteopromotive matrices are frequently employed for the local delivery and controlled release of these augmentation agents. Some matrices also provide an osteoconductive scaffold to support new bone growth. In this study, silkworm-derived silk fibroin was evaluated as an osteopromotive matrix for healing critical sized mid-femoral segmental defects in nude rats. Four treatment groups were assessed over eight weeks: Silk scaffolds (SS) with recombinant human BMP-2 (rhBMP-2) and human mesenchymal stem cells (HMSC) that had been pre-differentiated along an osteoblastic lineage ex vivo (group I; pdHMSC/rhBMP-2/SS); SS with rhBMP-2 and undifferentiated HMSCs (group II; udHMSC/rhBMP-2/SS); SS and rhBMP-2 alone (group III; rhBMP-2/SS); and empty defects (group IV). Bi-weekly radiographs revealed a progressive and similar increase in Group I-III mean defect mineralization through post-operative week (POW) 8. Radiographs, dual energy x-ray absorptiometry, and micro-computed tomography confirmed that Groups I-III exhibited similar substantial and significantly (p<0.05) greater defect mineralization at POW 8 than the unfilled Group IV defects which remained void of bone. However, the maximal torque and load before failure were significantly (p<0.05) less for Groups II and III than for Group I. Histology at POW 8 revealed moderately good bridging of the parent diaphyseal cortices with woven and lamellar bone bridging islands of silk matrix in Groups I and III. Group II defects possessed comparatively less new bone which was most abundant adjacent to the parent bone margins. Elsewhere the silk matrix was more often enveloped by poorly differentiated loose fibrous connective tissue. Group IV defects showed minimal new bone formation. None of the treatment groups attained the mean mineralization or the mean biomechanical strength of identical defects implanted with SS and pdHMCs alone in a previous study. However, addition of rhBMP-2 to udHMSC/SS or SS prompted substantially more bone than was previously generated using udHMSC/SS or SS alone. These data imply the clinical potential of silk scaffolds and rhBMP-2 as composite osteopromotive implants when used alone or with select stem cell populations. Additional studies in larger species are now warranted.
Introduction

Refinements in anesthetic and surgical techniques, implant design and application, and peri-operative management have significantly improved the treatment of complex fractures and other skeletal defects caused by trauma, disease, developmental deformity and tumor resection. Nonetheless, an unfavorable wound environment, suboptimal surgical technique, or biomechanical instability, can all lead to delayed or non-union. Under these circumstances, bone grafts have been the principal means of augmenting or accelerating bone regeneration, and as many as 1 million per year are now carried out in North America (Niklason, 2000).

Unfortunately, significant drawbacks are associated with the use of this approach such as: The need for additional anesthetic time or personnel for graft harvesting; an insufficient quantity of graft, limited access to donor sites, loss of osteogenic cells, donor site pain or hemorrhage, and predisposition of the donor bone to failure. Vascularized autografts are technically demanding and allografts and xenografts carry the hazards of immune-mediated rejection, graft sequestration, and transmission of infection between donor and host. Also, bone banks are costly to maintain (Kirker-Head, 2000).

Accordingly, investigators have been pursuing alternative bone augmentation technologies and several novel approaches to bone regeneration now hold promise for clinical application, among them the use of bone growth and differentiation factors (Kirker-Head et al., 1998), gene therapy (Meinel et al., 2006b), and cell therapy (Meinel et al., 2004c, Meinel et al., 2004d, Meinel et al., 2006b). In each instance, osteopromotive matrices are frequently employed for the local delivery and controlled release of the augmentation agents, some of which also provide an osteoconductive physical scaffold for neo-vascularization and neo-osteogenesis (Johnson et al., 1988, Zellin et al., 1996, Linde and Hedner, 1995, Niklason, 2000).

A wide range of organic and inorganic materials have been tested as osteopromotive matrices both in vitro and in vivo (Zellin et al., 1996), among them Bombyx mori silkworm silk fibroin (Meinel et al., 2005a, Meinel et al., 2004a, Meinel et al., 2006b, Meinel et al., 2005b, Meinel et al., 2004b, Meinel et al., 2004c, Meinel et al., 2004d, Sofia et al., 2001). Our ability to genetically, physically, and chemically manipulate B. mori silk allows us to generate silk matrices that are biocompatible, mechanically strong, stable at physiological temperatures, and predictably degradable (Altman et al.,
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2003, Asakura et al., 2001, Asakura and Kaplan, 1994). Studies imply their osteopromotive potential, particularly when combined with a responsive population of stem cells and/or osteoinductive cytokines: When critical size cranial defects in mice were implanted with silk scaffolds (SS) and a population of human mesenchymal stem cells (HMSCs) pre-differentiated along an osteoblastic lineage (pdHMSC/SS), substantial bone regeneration occurred (Meinel et al., 2005a). When a similar pdHMSC/SS was implanted in critical size weight-bearing femoral defects in rats, bone regeneration was correspondingly prominent and the defects progressed towards healing (Meinel et al., 2005a) {Meinel, 2005 #1340}. In both defect models, however, bone regeneration was much less substantial when the silk matrix was implanted alone (SS) or with undifferentiated HMSCs (udHMSC/SS) (Meinel et al., 2006a, Meinel et al., 2005a). Using a murine cranial defect model, we subsequently demonstrated that these latter limited osteogenic responses could be effectively enhanced by pre-loading the SS or udHMSC/SS implants immediately prior to implantation with a supraphysiological dose of recombinant human BMP-2 (rhBMP-2), a potent osteoinductive cytokine (Karageorgiou et al., 2006).

In this study we used a rat critical size femoral defect model to determine if a single application of a supraphysiological quantity of rhBMP-2 could enhance the osteogenic response to SS implanted alone or with udHMSCs or pdHMSCs. Three treatment groups and one control group were assessed: i) silk, rhBMP-2 and pre-differentiated HMSCs (pdHMSC/rhBMP-2/SS); ii) silk, rhBMP-2 and undifferentiated HMSCs (udHMSC/rhBMP-2/SS); iii) silk and rhBMP-2 alone (rhBMP-2/SS); and a no implant control. A series of radiographic, absorptiometric, micro-computed tomographic, biomechanical, and histological analyses were employed to generate the data.

Materials and methods

Experimental design
Critical size femoral defects were surgically created in the right hind limb of 24 rats. At the time of surgery (post-operative week [POW] 0) rats were randomly assigned to one of four defect treatment groups (Table 1): Group I (n=6) received rhBMP-2 loaded silk scaffolds which had been seeded with undifferentiated HMSCs and then incubated in osteogenic medium for 4 weeks prior to implantation (pdHMSC/rhBMP-2/SS);
group II (6 rats) received rhBMP-2 loaded silk scaffolds seeded with undifferentiated HMSCs one day prior to implantation (udHMSC/rhBMP-2/SS); group III (6 rats) received rhBMP-2 loaded silk scaffolds devoid of cells (rhBMP-2/SS); and in group IV (n=6), defects were left empty (control). Radiographs of the operated limb were completed at post-operative weeks (POW) 0, 2, 4, 6, and 8. Animals were sacrificed at POW 8 and femora underwent dual energy x-ray absorptiometry (DEXA) analyses. The operated femora were then frozen for biomechanical testing and micro-computed tomography (µCT) or placed in 4°C 4% paraformaldehyde for histological and immunohistochemical evaluation.

Materials
Silkworm cocoons were kindly supplied by M. Tsukada, Institute of Sericulture, Tsukuba, Japan, and Marion Goldsmith, University of Rhode Island. Recombinant human BMP-2 was kindly supplied by Wyeth Biopharmaceuticals, Andover, MA. Dulbecco's Modified Eagle Medium (DMEM), RPMI 1640 medium, Fetal Bovine Serum (FBS), basic fibroblast growth factor (bFGF), penicillin and streptomycin (Pen-Strep), Fungizone, trypsin and nonessential amino acids (NEAA, consisting of 8.9 mg/L L-alanine, 13.21 mg/L L-asparagine, 13.3 mg/L L-aspartic acid, 14.7 mg/L L-glutamic acid, 7.5 mg/L glycine, 11.5 mg/L L-proline, 10.5 mg/L L-serine) were from Gibco (Carlsbad, CA). Histopaque-1077, Ascorbic acid-2-phosphate and dexamethasone were from Sigma (St. Louis, MO). All other substances were obtained from Sigma.

Scaffolds
Extraction and purification of silk fibroin from B. mori silkworm cocoons has been previously described (Meinel et al., 2004c), as has silk scaffold preparation (Tsukada et al., 1994, Kim et al., 2005). Briefly, following purification, the silk was lyophilized and redissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) solution. Granular NaCl was added to the silk/HFIP solution at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 3 days and NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 minutes to induce a protein conformational transition to an insoluble -sheet structure (Meinel et al., 2004c). Blocks were removed, dried and...
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NaCl was extracted by incubation in water for 2 days. Disk shaped scaffolds (8 mm diameter, 2 mm thick) were created using a dermal punch and autoclaved at 121ºC for 15 minutes. Recombinant human BMP-2, supplied in a formulation buffer, was dialyzed against 0.5 M arginine, 5 mM glutamic acid, at a pH 3.8 for 1 day, 0.5 M arginine and Dulbecco’s Phosphate Buffered Saline (D-PBS) for another day, and D-PBS for 2 days. The protein solution was sterilized with 0.22 um syringe filters (Millipore, Billerica, MA). Scaffolds were transferred into 10 ml syringes (Becton Dickinson, Franklin Lakes, NJ) and contacted with 1.5 ml of 0.05 mg/ml rhBMP-2 in D-PBS solution for 6 hours. The procedure was carried out aseptically. Characterization of BMP-2 load was described before (Karageorgiou et al., 2006).

Cell isolation, expansion and characterization

Cell preparation has been previously described (Caplan, 1991, Prockop et al., 2001, Jones et al., 2002, Majumdar et al., 2000). Briefly, HMSCs were isolated by density gradient centrifugation (800g; 30 minutes) of whole bone marrow (Clonetics, Santa Rosa, CA) diluted (1:10 v/v) in isolation medium (RPMI 1640 supplemented with 5% FBS) over a poly-sucrose gradient (1.077 g/cm³, Histopaque, Sigma, St. Louis, MO). The cell layer was removed, washed in isolation medium, pelleted at 300g and contaminating red blood cells were eliminated using Pure-Gene RBC Lysis solution (Gentra Systems Inc, Minneapolis, MN). Cells were then suspended in expansion medium (DMEM, 10% FBS, Pen-Strep, Fungizone, NEAA and 1 ng/ml bFGF) and seeded in flasks at a density of 5x10⁴ cells/cm². Adherent cells were allowed to reach approximately 80% confluence, requiring 12-17 days for the first passage before being replated.

For preparation of the Group I implants (pdHMSC/rhBMP-2/SS), second passage HMSCs were suspended in liquid BD Matrigel Basement Membrane Matrix (BD Biosciences, San Jose, CA) before being seeded onto silk scaffolds loaded with 2.5 (2.4 ± 0.14 [SD]) µg rhBMP-2 (Karageorgiou et al., 2006). The composite implants were then placed in spinner flasks as previously described (Meinel et al., 2004c, Meinel et al., 2004d)\{Meinel, 2004 #844; Meinel, 2004 #975\} for 4 weeks prior to surgical implantation. The flasks were filled with osteogenic medium (DMEM supplemented with 10% FBS, Pen-Strep and Fungizone supplemented with 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM b-glycerolphosphate) which,
in combination with the rhBMP-2, prompted pre-differentiation of the HMSCs along an osteoblastic lineage (pdHMSCs). Flasks were placed unsealed in a humidified incubator (37°C, 5% CO2) which permitted gas exchange and stirred with a magnetic bar. Medium (but not rhBMP-2) was replaced at a rate of 50% every 2-3 days for the 4 weeks of cultivation (Meinel et al., 2006a). Group II implants (udHMSC/rhBMP-2/SS) were prepared by seeding second passage HMSCs onto silk scaffolds loaded with 2.5 (2.4 ± 0.14 [SD]) µg rhBMP-2 on the day immediately prior to surgical implantation.

Operative Procedure
A critical size, femoral defect rat model was used in this study. All procedures were approved by the operative institution’s Animal Care and Use Committee. Briefly, adult, male athymic T-cell deficient RH-rnu rats weighing 325 – 400 g were placed under general anesthesia and maintained using inhalant isofluorane and oxygen. The rats were positioned in lateral recumbency and the uppermost hindlimb was aseptically prepared for surgery. An approximately 25 mm long skin incision was created over the femur and underlying soft tissues were retracted to reveal the bone. One pair of 1.1-mm external diameter self tapping threaded pins were placed transcortically in a latero-medial plane in the proximal femur and a second pair were similarly placed in the distal femur, leaving the mid-diaphysis readily accessible. The pin tips extended 0.5-mm beyond the trans cortex. Four small stab incisions were made in the skin, allowing it to be re-seated over the pins and an external fixator bar was secured to all 4 pins with miniscrews. A high speed dental drill was used to carefully create a 5-mm long full thickness osteo-periosteal critical size defect in the mid-diaphysis which was subsequently filled with the test implant. Overlying soft tissues were then routinely closed.

Radiography
Standardized digital radiographs (Kodak Directview Version 5.2, Eastman Kodak, Rochester, NY) of the operated femurs were completed immediately post-implantation (POW 0) and then every second week (POW 2, 4, 6, and 8) for the duration of the study. Defect mineralization was semi-quantitatively graded by 2 radiologists blinded to the treatment groups, using a standardized scale (1. trace radiodense material in
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defect; 2. flocculent radiodensity and incomplete bridging of the defect; 3. bridging of the defect at 1+ location; 4. bridging of the defect at cis and trans cortices, parent cortex visible; 5. one cortex obscured by new bone; 6. bridging of the defect by uniform new bone, cut ends of cortex not seen).

_Dual energy x-ray absorptiometry_
Immediately post-sacrifice (POW 8), a region of interest centered within the femoral defect of the operated femora was subject to DEXA analysis (QDR 4500 Acclaim Elite, Hologic, Inc, MA) as a means of quantifying regenerate bone mineral content (BMC) (g). A sub-region high resolution software algorithm was employed.

_Micro-computed tomography (μCT)_
As a means of assessing defect 3-D bone regeneration, operated femora were analyzed at POW 8 using a μCT40 imaging system (Scanco Medical, Bassersdorf, Switzerland) using the following scan parameters: 20 mm field of view, 55 kVp x-ray energy setting, 1024 reconstruction matrix, slice thickness 0.02 mm, and a 250 millisecond integration time. Mineralized tissue was segmented from non-mineralized tissue using a global thresholding procedure with a value approximating 1.20 g/cm3 (150 on μCT) (25% lower than 1.6 g/cm3 which is the mineral density of healthy human compact bone (Sofía et al., 2001).

_Mechanical testing_
Both ends of the femora undergoing mechanical testing were embedded in polymethylmethacrylate (PMMA). A novel torsional testing system was employed such that the axis of rotation of the torsional forces correlated with the geometric neutral axis of the cylindrical bone specimens. Torque was applied using a strain rate of 5 rad/min via a MTS200 screw axis load frame (MTS, Saint Paul, MN, USA). Data were collected with a LabVIEW (National Instruments, Austin, TX) acquisition system. Maximum torsional displacement (rad) and load (N) and maximum torque (Nm) were measured for each specimen. Additionally, torsional stiffness (Nm/rad) was calculated from the slope fit to the linear portion of the torsional load displacement curve (Meinel et al., 2006a).
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**Histology and Immunohistochemistry**

Fixed and decalcified (12% EDTA, pH 7.0) femoral explants were dehydrated in graded ethanol solutions and xylene, embedded in paraffin and cut into 3 µm-thick sections. For a general overview, sections were stained with hematoxylin and eosin. Immunohistochemical studies were performed for bone sialoprotein (BSP) and collagen type I using a rabbit anti-bone sialoprotein II polyclonal antibody (Chemicon, Temecula, CA) and mouse anti-collagen type I monoclonal antibody (Sigma, St. Louis, MA) respectively, as previously described (Meinel et al., 2006a).

**Statistical analysis**

For statistical significance, samples were evaluated using a student t-test as well as ANOVA where appropriate. Error bars always represent the standard deviation of groups. ANOVA was followed by a post-hoc assessment using the Tukey HSD method. Differences were considered significant when equal or less than p=0.05.

**Results**

**Radiographic data**

The semi-quantitative assessment of defect mineralization by 2 radiologists blinded to the treatments revealed a progressive and similar increase in mean defect mineralization from POW 0 through POW 8 for treatment Groups I (pdHMSc/rhBMP-2/SS), II (udHMSc/rhBMP-2/SS), and III (rhBMP-2/SS). At POW 8 these 3 groups’ radiographic scores reflected bridging of the defect with new bone traversing between proximal and distal components of the cis- and/or trans-cortices. The parent bone cortices, however, were still well defined. Group I-III radiographic scores were significantly (p<0.05) greater than the unfilled control Group IV score at POW 8. Group IV defects reflected only the presence of trace mineralization (**Figure 1**). No other significant differences were observed between groups.

**Dual energy x-ray absorptiometry (DEXA) data**

Quantitative DEXA data confirmed that Groups I through III had similar and substantial mean defect Bone Mineral Content at POW 8, which was significantly
greater (P<.05) than that of the unfilled Group IV defect (Figure 2). No other significant differences were observed between groups.

**Figure 1:** Radiographic assessment of defect mineralization. Radiographs were completed immediately post-implantation (POW 0) and then every second week (POW 2, 4, 6, and 8). Defect mineralization was semi-quantitatively graded using the following standardized scale: 1. trace radiodense material in defect; 2. flocculent radiodensity and incomplete bridging of the defect; 3. bridging of the defect at 1+ location; 4. bridging of the defect at cis and trans cortices, parent cortex visible; 5. one cortex obscured by new bone; 6. bridging of the defect by uniform new bone, cut ends of cortex not seen.

**Micro CT data**

At POW 8 the morphology of the formed bone within the defect was evaluated using µ-CT (Figure 3A). Group IV defects showed little evidence of bone regeneration with the parent cortices at the defect margins remodeling to form a rounded cap characteristic of non-union. Groups I-III all demonstrated variable bridging of the defect. Group II defects more commonly exhibited large voids within the regenerated bone. In Groups I-III, bone was cortical like towards the periphery of the defect but more cancellous or sponge-like towards the defect’s center. Several defects in Groups I-III had narrow cracks propagating across the full thickness of the regenerated bone. In all cases, new bone was limited to the immediate defect or a distance extending no more than approximately 2-mm beyond the parent diaphyseal diameter.

Similar defect bone volumes were observed in Groups I through III at POW 8, each of which was significantly (P<.05) greater than those measured in the unfilled Group IV defects (Figure 3B). The ratio of bone volume to trabecular bone volume, which reflects the geometry of the formed bone, was also substantially but not significantly greater in Groups I-III than in Group IV. The thickness of the trabecular bone plates was, however, consistent amongst groups. No other significant differences were observed between groups.
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Figure 2: Regenerate bone mineral content (BMC) acquired through DEXA analysis of the operated limb immediately post-sacrifice (POW 8).

Biomechanical testing data

The defects of Groups I-III were mechanically characterized following sacrifice (Figure 4). The maximal load before failure was significantly (p<0.05) greater for Group I than for Groups II or III. Similarly, the maximal torque was significantly (p<0.05) higher for Group I (pdHMSc/rhBMP-2/SS) than for Groups II and III. No other significant differences were observed between groups.

Histology

Histological analyses at POW 8 revealed moderately good integration of the parent diaphyseal cortices with new bone in Groups I (Figure 5A, 5B) and III (Figure 5E, 5F). In both groups the defects were characterized by the presence of a continuum of woven and lamellar bone surrounding islands of silk matrix more centrally with elements of cortical bone more peripherally. Subjectively, the extent of trabecular bone in Group I defects (Figure 5A, 5B) was moderately less than that noted in Group III (Figure 5E, 5F) defects. Group II defects revealed comparatively less new bone formation than either Groups I and III and that present was principally found adjacent to the parent bone margins (Figure 5C, 5D). The Group II silk matrix was often diffusely enveloped by poorly differentiated loose fibrous connective tissue (Figure 5C, 5D). While few in number, there were moderately more cartilage elements towards the center of the defect in Group III than in Groups I or II. In Groups I and III, variable staining for bone sialoprotein (BSP) and collagen type I was found in the newly formed matrix at sites of active bone formation. Staining for BSP and collagen type I was less prominent (data not shown). Group IV defects showed minimal evidence of new bone formation, the defect being filled with fibrous connective tissue and muscle bellies extending from adjacent soft tissues (Figure 5G, 5H). These tissues did not stain positively for BSP or type I collagen (data not shown).
Figure 3: (A) Micro-computed tomography from three representative rat critical size femoral defects for each treatment group 8 weeks after surgery. The dotted box approximately indicates the original defect zone, which was of same size for all groups.
Figure 3(B) Morphometric analysis of new bone formation from rat critical size femoral defects 8 weeks after surgery, and treated with pdHMSC grown for five weeks on rhBMP-2 loaded silk scaffolds (group I), rhBMP-2 loaded silk scaffolds seeded with udHMSC (group II), unseeded rhBMP-2 loaded silk scaffolds (group III), and untreated (control) defects (group IV). (A) Bone volume per defect, (B) bone volume normalized to the trabecular volume, and (C) trabecular thickness (**p<0.05).

Discussion

The goal of this study was to assess the ability of rhBMP-2 to augment the osteogenic response to a novel silk fibroin-derived biomaterial when implanted alone or in combination with HMSC populations in a critical size rat femoral defect. Using several analytical tools, we demonstrated prominent bone formation and defect healing in rats
implanted with silk scaffolds loaded with rhBMP-2 and seeded with HMSCs and then expanded in osteogenic culture media for 4 weeks prior to implantation (Group I; pdHMSC/rhBMP-2/SS). While boney defect bridging also occurred when silk scaffold was loaded with rhBMP-2 and seeded with HMSCs one day prior to implantation (Group II; udHMSC/rhBMP-2/SS), the biomechanical integrity of the regenerated bone was significantly less as was the case when silk scaffold was loaded only with rhBMP-2 (Group III: rhBMP-2/SS).

The impetus for this study was a prior investigation in which we used the same animal model and investigative techniques to assess bone formation following implantation of silk scaffold alone (SS), SS with undifferentiated HMSCs (udHMSC/SS), or SS with pre-differentiated HMSCs (pdHMSC/SS) (Meinel et al., 2006a). While substantial new bone growth was prompted by pdHMSC/SS along with defect healing, implantation of udHMSC/SS or SS alone yielded comparatively little bone regeneration (Meinel et al., 2006a). These data implied the clinical potential of SS as an osteopromotive biomaterial for pdHMSC populations, but an additional impetus was apparently required for bone formation to occur with SS implanted alone or with more naïve udMHSC populations.

Since another previously published study had demonstrated that the peri-operative addition of a supraphysiological dose of rhBMP-2 to SS or udHMSC/SS could prompt boney healing of a murine critical size cranial defect (Karageorgiou et al., 2006) it was appropriate to repeat the study here using the rat femoral defect model (An and Friedman, 1999). The latter exposes the implant to an environment which differs from the cranial defect in several respects including the nature of the biomechanical load, the type and extent of enveloping soft tissues, the propensity of the defect to heal (Karageorgiou et al., 2006) and, quite possibly, local influences associated with the fact that, developmentally, long bone formation is endochondral while cranial bone formation is intramembranous.

The benefits of peri-operatively combining rhBMP-2 with SS alone or in combination with HMSCs could be several-fold. Potentially, we may enhance the healing response already demonstrated using pdHMSC/SS. Also, if defect healing could be achieved by peri-operatively adding rhBMP-2 to udHMSC/SS, the laborious (4 weeks) and expensive ex-vivo pre-differentiation of the HMSCs along an osteogenic lineage could be avoided. Finally, if satisfactory healing could be achieved using peri-operative
rhBMP-2 and SS alone, it might be possible to augment bone healing in acute injuries without waiting to generate ex vivo a responsive stem cell population.

Recombinant human BMP-2 has been well documented as a potent osteoinductive cytokine in a wide range of species including non-human primates and man (Kirker-Head, 2000, Pecina et al., 2001, Solheim, 1998, Elima, 1993). Although the recombinant protein was first developed in the late 1980’s, it was not until 2001 that it received regulatory approval for clinical use and then only as a bone augmentation agent for a select group of long bone fractures and spinal fusions. Much of this delay can be attributed to the difficulty in identifying an appropriate matrix for the protein’s local delivery (Solheim, 1998, Elima, 1993). Presently, a Type I bovine-derived absorbable collagen sponge (ACS) is used clinically but it has drawbacks, for example, minimal load-bearing capacity, sub-optimal handling characteristics on wetting, and rhBMP-2 retention that is easily influenced by small differences in pH, anion concentration, cross-linking and ACS mass (Friess et al., 1999). Using silk fibroin as a biomaterial offers several advantages over ACS including its more substantial mechanical strength (Meinel et al., 2006a), the ability to produce it in a variety of physical formats (e.g., electrospun nets, gels, films and three dimensional scaffolds) which help facilitate in vivo use (Meinel et al., 2006a), and a biodegradability that can be readily manipulated. Additionally, it is readily biocompatible and it has a low inflammatory response once rendered devoid of immunogenic and glycosylated proteins (Meinel et al., 2006a). Finally, there are no known bioburdens associated with
this protein-based biomaterial and there is a long history of human in vivo use of the same protein in suture format.

Figure 5: Histological cross sections taken from rat critical size femoral defects 8 weeks after surgery and stained with H&E; (A-B) pdHMSC on rhBMP-2 loaded silk scaffolds (Group I), (C-D) rhBMP-2 loaded silk scaffolds seeded with udHMSC (Group II), (E-F) unseeded rhBMP-2 loaded silk scaffolds (Group III), and (G-H) untreated (control) defects (Group IV). Scale bar 500 µm (A, C, E, G) and 100 µm (B, D, F, H).

Although no statistical analyses were used to compare our results with those generated in a previous study (Meinel et al., 2006a), the data reveal that while Group I (pdHMSC/rhBMP-2/SS) femurs possessed greater (P<.05) biomechanical integrity at POW 8 than either Group II (udHMSC/rhBMP-2/SS) or Group III (rhBMP-2/SS) femurs, their mean maximal load (10.82+/-1.58 N) and mean maximal torque (1.78+/-0.53 N) were marginally less than those of defects treated with pdHMSC/SS in the
prior study (mean maximal load: 11.73+/−1.42 N; mean maximal torque: 2.08+/−0.49 N) (10). The apparent inability of rhBMP-2 to significantly enhance the osteogenic response to implantation of pdHMSC/SS can be explained by the different pre-implantation preparation of the implants used in this study and that previously reported (11). In the study reported here the Group I pre-differentiated HMSCs (pdHMSC) evolved in response to a single 2.5 µg application of rhBMP-2 which was adsorbed onto the silk scaffolds one day prior to cell seeding. The composite implants (HMSCs, rhBMP-2 and silk scaffolds) were then incubated for 4 weeks in spinner flasks prior to implantation in the bone defects. During that time no additional BMP-2 was supplied with the culture medium. Conversely, the HMSCs seeded on the silk scaffold in the earlier study (Meinel et al., 2006a) were exposed to a comparatively greater quantity of rhBMP-2 in the expansion media (1 µg/ml with a total of 250 µg viz 2.5 µg). Furthermore, in that study 250 mg of fresh BMP-2 were supplied twice per week with every medium change (Meinel et al., 2006a), while only the media but not the rhBMP-2 were refreshed in the study reported here. Consequently, we might expect that the HMSCs’ total exposure to rhBMP-2 during the differentiation and cell expansion process would be greater in the earlier study (Meinel et al., 2006a) and hence the cells would be more likely to have committed towards an osteoblastic phenotype capable of optimally generating bone before their implantation in vivo.

The DEXA and radiographic data indicated that the bone mineral content (BMC) of Groups I-III defects were equivalent and significantly greater than the BMC of unimplanted Group IV rats. This finding was complemented by our tomographic data which identified a similar pattern. Although not statistically analyzed, a comparison of our tomographic data with that generated in a prior study (Meinel et al., 2006a) reveals that the peri-operative (1 day pre-implantation) addition of rhBMP-2 to udHMSC/SS (Group II) and SS (Group III) implants substantially increases mean bone volume/defect (udHMSC/rhBMP-2/SS viz udHMSC/SS = 163.26±28.05 viz 46.78±0.92 mm3; rhBMP-2/SS viz SS = 133.82 ±14.19 viz 63.65±2.09 mm3) as well as mean bone volume/trabecular bone volume (udHMSC/rhBMP-2/SS viz udHMSC/SS = 83.97±1.59 viz 19.99±2.91 N; rhBMP-2/SS viz SS = 70.51±9.48 viz 28.92±5.73 N). The single time addition of rhBMP-2 in groups I-III failed, however, to increase the mean bone volume/defect to that level previously achieved following pdHMSC/SS implantation (207.41+/−13.94 mm3). Again, this likely reflects the greater exposure of
HMSCs to rhBMP-2 in the previously reported study (Meinel et al., 2006a) and hence their greater degree of osteoblastic differentiation.

The data confirm that rhBMP-2 can be combined with SS to singularly prompt neo-osteogenesis and, in this model, moderate defect healing. Under these circumstances the SS was an effective osteoconductive matrix, enabling rhBMP-2’s potent osteoinductive up-regulation of the local pluripotent, osteoprogenitor and osteoblastic cell populations. The fact that peri-operative addition of rhBMP-2 to silk scaffold (Group III) prompted substantial bone formation compared with that generated following implantation of SS only (10) implies the successful and effective SS-mediated delivery of rhBMP-2 to the defect site in this study. While the biomechanical strength of the resultant bone at POW 8 was significantly less than that generated using pdHMSC/rhBMP-2/SS implants, the data imply some basis for using rhBMP-2/SS implants in acutely injured patients who would be adversely effected by delaying surgery while a responsive cell population was generated. Further, since there was no significant difference between Groups II and III data in this study, there is apparently no basis for supplementing rhBMP-2/SS implants with udHMSCs, effectively avoiding the potential risks associated with the ex vivo culture of HMSCs. It should be noted however, that when silk scaffold was seeded with udHMSCs in the absence of rhBMP-2 (Meinel et al., 2006a), the resultant biomechanical properties of the newly formed bone were significantly greater than those achieved with the implantation of silk scaffold alone (Meinel et al., 2006a). Together these data imply that while udHMSCs and rhBMP-2 can both singularly augment osteogenesis in response to implantation of a silk scaffold biomatrix (i.e. udHMSC/SS) or rhBMP-2/SS), their effects are not summative when implanted together (i.e. udHMSC/rhBMP-2/SS).

In summary, the data support the use of rhBMP-2 in this rodent long bone defect model for the purposes of augmenting the osteogenic response to silk scaffolds implanted alone or in combination with responsive stem cell populations. Additional studies using larger animal species (e.g., dogs, sheep or goats) which will represent a more clinically pertinent challenge for the implants are now warranted.

Acknowledgements

We thank the NIH Tissue Engineering Resource Center (DK), the NSF (DK, LM) for support of this research, Professor Hans Peter Merkle of the Department of Chemistry
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and Applied Biosciences, ETH Zurich, and Dr Brigitte von Rechenberg of the Musculoskeletal Research Unit, Equine Hospital, University of Zurich.

References


Chapter 8: BMP-silk matrices


Osteogenesis by human mesenchymal stem cells cultured on silk biomaterials: Comparison of adenovirus mediated gene transfer and protein delivery of BMP-2

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Biomaterials, 2006, accepted
Abstract

Bone tissue engineering, gene therapy based on human mesenchymal stem cells (MSC) and silk fibroin biomaterials were combined to study the impact of viral transfection on MSC osteogenic performance in vitro. MSCs were transduced with adenovirus containing a human BMP-2 (Ad-BMP-2) gene at clinically reasonable viral concentrations and cultured for 4 weeks. Controls with nontransfected MSCs, but exposed to exogenous BMP-2 concentrations on an analogous time profile as that secreted by the Ad-BMP-2 group, were compared. Both the Ad-BMP-2 MSC group and the exogenous protein BMP-2 group strongly expressed osteopontin and bone sialoprotein. Cells secreted a matrix that underwent mineralization on the silk fibroin scaffolds, forming clusters of osseous material, as determined by micro-computed tomography. The expression of osteogenic marker proteins and alkaline phosphatase was significantly higher in the Ad-BMP-2 MSC group than in the exogenous protein BMP-2 group, and no significant differences in mineralization were observed in two of the three MSC sources tested. The results demonstrate that transfection resulted in higher levels of expression of osteogenic marker genes, no change in proliferation rate and did not impact the capacity of the cells to calcify tissues on these protein scaffolds. These findings suggest additional options to control differentiation where exogenous additions of growth factors or morphogens can be replaced with transfected MSCs.
Chapter 9: Comparison of adenovirus mediated gene transfer and protein delivery

Introduction

The feasibility of using silk scaffolds in combination with human mesenchymal stem cells (MSC) to form bone ex vivo has been recently demonstrated (Meinel et al., 2004a, Meinel et al., 2004d, Meinel et al., 2004f, Karageorgiou et al., 2004). These studies were performed with high concentrations of recombinant bone morphogenetic protein 2 (BMP-2), an osteoinductive protein known to stimulate the differentiation of MSC along the osteogenic lineage (Katagiri et al., 1990, Bruder et al., 1997, Rickard et al., 1994). The limitation of this otherwise successful approach was the high cost of the growth factor. Alternatively, gene transfer would allow the engineering of cells to secrete BMP-2 while maintaining the capacity to differentiate into cells, actively depositing mineralized tissues (Dragoo et al., 2003). This pilot study was performed to evaluate the efficiency and impact of viral transduction on MSC’s differentiation and osteogenic performances on silk scaffolds.

The type of carrier employed for MSC is important for osteogenic differentiation. It was found that the ability of human MSC to form bone is dependent on the type of biomaterial, such as hydroxyapatite (Mankani et al., 2001). We selected silk fibroin as a biomaterial because it combines slow biodegradability, excellent mechanical properties, and biocompatibility. Recent studies with silks have advanced the fundamental understanding of this group of proteins and expanded their application for tissue engineering (Meinel et al., 2004d, Meinel et al., 2005, Asakura and Kaplan, 1994, Altman et al., 2003, Sofia et al., 2001). Silk protein scaffolds were shown to support tissue engineering of bone in vitro (Altman et al., 2003), and they can potentially support bone repair in situ. Native silk fibers are the strongest natural fibers known, and rival the best synthetic high performance fibers, such as Kevlar, in overall performance (energy absorbed to break) (Cunniff et al., 1994). This mechanical integrity makes this material interesting for orthopaedic applications, in particular in the context of MSC tissue engineering, where robust materials are required to form bone.

It has been demonstrated that MSC engineered to secrete BMP-2 not only show paracrine effects on the host mesenchymal tissue but also autocrine effects supporting their own differentiation along the osteogenic lineage in vivo. The transfected MSC showed an increased osteogenic potential over non-MSC cells, that also expressed
BMP-2 (Gazit et al., 1999) but were shown less effective in terms bone nodule number than mesenchymal precursor cells isolated from fat tissues (Dragoo et al., 2003). The purpose of this study was to (1) evaluate the correlation between virus concentration and transfection efficiency, (2) to assess the impact of viral transfection on MSC proliferation and osteogenic differentiation using viral concentrations considered to be safe and (3) to evaluate the feasibility of viral transfection for tissue engineering purposes on silk fibroin scaffolds in comparison to the use of exogenous BMP-2. To approximate culture conditions of the adenovirally transfected group and the BMP-2 group, initially the time concentration profile of BMP-2 as a consequence of secretion by adenovirally transduced MSCs was monitored. The experiment was repeated using untransfected MSCs exposed to the same time concentration profile of BMP-2 generated by supplementing the growth factor to the culture medium. Both groups – with very similar time concentration profiles of BMP-2, either as a consequence of adenoviral transfection or supplementation of BMP-2 to the culture medium - were compared in ex vivo cell culture experiments for proliferative and osteogenic performance on silk scaffolds.

Materials and Methods

2.1. Materials

Bovine serum, RPMI 1640 medium, Dulbecco's Modified Eagle Medium (DMEM), basic fibroblast growth factor (bFGF), transforming growth factor-B1 (TGF-B1) (R&D Systems, Minneapolis, MN), Pen-Strep, Fungizone, non-essential amino acids and trypsin were from Gibco (Carlsbad, CA). Ascorbic acid phosphate, Histopaque-1077, insulin, dexamethasone and β-glycerolphosphate were from Sigma (St. Louis, MO). All other chemicals were of analytical or pharmaceutical grade and obtained from Sigma. Silkworm cocoons were kindly supplied by M. Tsukada (Institute of Sericulture, Tsukuba, Japan) and Marion Goldsmith (University of Rhode Island, Cranston, RI). BMP-2 was a generous gift from Wyeth Biopharmaceuticals, Andover, MA (Thomas Porter).
2.2. Fabrication of silk scaffolds

Porous 3D silk fibroin sponge-like scaffolds were prepared as previously reported (Nazarov et al., 2004, Meinel et al., 2004c, Meinel et al., 2004d). Briefly, cocoons from Bombyx mori (Linne, 1758) were boiled for 1 hour in an aqueous solution of 0.02M Na2CO3, and rinsed with water to extract sericins. Purified silk was solubilized in 9M LiBr solution and dialyzed (Pierce, Woburn, MA; MWCO 3500 g/mol) against water for 1 day and again against 0.1M MES, 0.5 M NaCl, pH 6 buffer for another day (Sofia et al., 2001). Silk solutions were dialyzed against water for 2 days and redissolved in hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) solution. Granular NaCl was sieved to obtain particle sizes ranging from 200–325 µm, weighed in a Teflon container and silk/HFIP solution was added at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 2 days and NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 minutes to induce a protein conformational transition to beta-sheets. The silk blocks were removed, dried, and the NaCl was extracted in water for 2 days. Disk shaped scaffolds (5 mm diameter, 2 mm thick) were prepared using a dermal punch (Miltey, Lake Success, NY), and autoclaved at 121°C for 15 minutes.

Table 1: Experimental groups for transduction efficiency and osteogenic response

<table>
<thead>
<tr>
<th>Group</th>
<th>Abbreviation in text</th>
<th>Medium</th>
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</thead>
<tbody>
<tr>
<td>Adenoviral transfer of BMP-2</td>
<td>Ad-BMP-2</td>
<td>osteogenic</td>
<td>3</td>
</tr>
<tr>
<td>Supplementation with BMP-2</td>
<td>BMP-2</td>
<td>osteogenic + BMP-2</td>
<td>3</td>
</tr>
<tr>
<td>Adenoviral transfer of GFP</td>
<td>Ad-GFP</td>
<td>osteogenic</td>
<td>2</td>
</tr>
<tr>
<td>Adenoviral transfer of GFP plus</td>
<td>Ad-GFP plus BMP-2</td>
<td>osteogenic + BMP-2</td>
<td>1</td>
</tr>
<tr>
<td>supplementation with BMP-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No adenovirus, No BMP-2 supplementation</td>
<td>control</td>
<td>osteogenic</td>
<td>3</td>
</tr>
</tbody>
</table>

2.3. Isolation, expansion and characterization of MSC

Total human bone marrow (25 cm3, Clonetics, Santa Rosa, CA.) from three donors was processed individually and diluted in 100 ml of isolation medium (5% FBS in RPMI 1640 medium). Cells were separated by density gradient centrifugation. Briefly,
20 ml aliquots of bone marrow suspension were overlaid onto 15 ml of a poly-sucrose gradient (=1,077 g/cm3, Histopaque, Sigma, St. Louis, MO) and centrifuged at 800xg for 30 min at room temperature. The cell layer was carefully removed, washed in 10 ml isolation medium, pelleted and contaminating red blood cells were lysed in 5 ml of RBC lysis buffer (Gentra Systems, Minneapolis, MN) solution. Cells were pelleted and suspended in expansion medium (DMEM, 10% FBS, 1 ng/ml bFGF) and seeded in 75 cm2 flasks at a density of 5x104 cells/cm2. The adherent cells were allowed to reach approximately 80% confluence (12-17 days for the first passage). Cells were trypsinized, replated and passage 2 (P2) cells (80% confluence after 6-8 days), were used for the experiments. The isolated and expanded cells were characterized as previously described (Meinel et al., 2004d). These cells were characterized by their surface antigen expression pattern and their ability to undergo chondrogenic and osteogenic differentiation (Meinel et al., 2004d, Meinel et al., 2004c, Meinel et al., 2004f). The cell surfaces were positive for CD105 (endoglin) and negative for markers of hematopoietic and endothelial origin. In presence of chondrogenic and osteogenic medium, the cells selectively formed cartilage- and bone-like tissue, consistent with the properties of MSC.

2.4. Transduction of cells with adenovirus expressing BMP-2

MSC (about 80% confluence at passage 2) were transduced with a replication-deficient, first generation adenoviral vector carrying the human BMP-2 cDNA (Ad-BMP-2) or a control vector carrying green fluorescent protein cDNA (Ad-GFP). The construction of Ad-BMP-2 was described earlier (Pascher et al., 2004). Virus was amplified in 293 cells and purified by standard CsCl banding techniques. MSC were transduced with the adenovirus at confluence. Ad-GFP or Ad-BMP-2 viruses were added to the MSC at a multiplicity of infection (MOI) of 25, suspended in FBS-free DMEM medium. Flasks were rotated every 20 minutes and the virus was aspirated after 2 hours of incubation. Cells were washed three times with DMEM.

2.5. Effectiveness of gene transfer

FACS analysis was performed to characterize the effectiveness of gene transfer based on MOI. MSC were transduced with Ad-GFP at 1, 3, 10, 30, 100, 300, and 1000 MOI
and incubated for 2 hours. Cells were cultured for 48 hours in DMEM supplemented with 10% FBS, Pen-Strep and Fungizone in a 6 well plates (BD Biosciences). MSC were detached with 0.05% (w/v) trypsin, pelleted, washed three times in PBS, and resuspended at a concentration of 1x10^7 cell/ml in 100 µl of 2% formalin for FACS analysis. FACS analysis was carried out using a BD FACScalibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ).

2.6. Scaffold seeding

MSC were suspended at a concentration of 4x10^7 cells/ml DMEM and 40 µl aliquots of the suspension (1.6x10^6 cells) were seeded on each scaffold (corresponding to an initial seeding density of 20x10^6 cells/cm^3 scaffold volume). Seeded scaffolds were incubated at 37°C and 5% CO2 for 2 hours to allow the cells to attach and 10 µl of DMEM was added carefully every 30 minutes to prevent the seeded scaffold from drying out. The seeded scaffolds were transferred into 6 well, untreated polystyrene plates (BD Biosciences, Franklin Lakes, NJ) and 4 ml of osteogenic medium (DMEM supplemented with 10% FBS, Pen-Strep, Fungizone, 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone and 7 mM b-glycerolphosphate). Medium was changed every other day for a period of 4 weeks.

2.7. Cultivation of tissue constructs

The effectiveness of gene transfer was quantified using MSC transduced with Ad-GFP. Based on measured gene transfer efficiencies, a MOI = 25 was chosen for the subsequent experiments testing the osteogenic differentiation, using Ad-BMP-2 (Table 1). To test the osteogenic differentiation of MSC, four different experimental groups were formed (Table 1). MSC were either transduced with adenoviruses carrying Ad-BMP-2 or Ad-GFP. Non-transduced and Ad-GFP transduced MSC were cultured in osteogenic medium supplemented with BMP-2 according to the concentration – time profiles measured in the Ad-BMP-2 group. Non-transduced cells receiving osteogenic medium without BMP-2 supplementation served as controls, as the goal of this publication was to investigate into the direct influence of BMP-2, either produced by the cells themselves or added to the culture medium. Seeded constructs were cultured for 4 weeks and samples were harvested at weekly intervals.
2.8. Secretion of BMP-2 following adenoviral transduction

Medium samples from MSC transduced with 25 MOI of Ad-BMP-2 (n = 5 per group and time point), MSC exposed to supplemented BMP-2 and cells grown in osteogenic medium (n = 5 for the Ad-BMP-2 group and time point and n=3 for the other groups and time point) were collected every other day and the concentration of BMP-2 was measured using a human BMP-2 ELISA kit using a standard curve (r^2>0.98) according to the manufacturers instructions (R&D Systems, Minneapolis, MN).

2.9. Biochemical analyses

For all analyses, constructs were disintegrated after 1, 2, 3, and 4 weeks of culture and after 2 and 4 weeks for the Ad-GFP group in 0.1% Triton X-100 solution using steel balls except for calcium quantification when constructs were disrupted in 0.1 trichloroacetic acid (5% w/v). Scaffolds were disintegrated in repetitive cycles (5-6) using a Minibead beater (Biospec, Bartlesville, OK) at 25 kHz and 10 seconds per cycle, with tubes kept on ice between the cycles. DNA content (n = 5-6) was measured using the PicoGreen assay (Molecular Probes, Eugene, OR), according to the protocol of the manufacturer. Samples were measured fluorometrically at an excitation wavelength of 480 nm and an emission wavelength of 528 nm. Alkaline phosphatase (ALP) activity (n = 5-6) was measured using a biochemical assay from Sigma (St. Louis, MO), based on conversion of p-nitrophenyl phosphate to p-nitrophenol, which was measured spectrophotometrically at 410 nm. For total calcium content, samples (n=5-6) were extracted twice with 0.5 ml 5% trichloroacetic acid. Calcium content was determined by a colorimetric assay using o-cresolphthalein complexone (Sigma, St. Louis, MO). The concentration of the calcium complex was measured spectrophotometrically at 575 nm.

2.10. RNA isolation, and reverse transcriptase real-time polymerase chain reaction (RT-PCR)

Constructs were disintegrated after 1, 2, 3, and 4 weeks of culture in Trizol reagent (Gibco, Carlsbad, CA) using steel balls (baked at 300°C for 4 hours) and the Minibead beater (Biospec, Bartlesville, OK) at 25 kHz and 10 seconds per cycle, with tubes kept...
on ice between the cycles (n = 5-6). The suspension was vortexed and extracted with chloroform. The aqueous phase was transferred and isopropanol was added. RNA was collected using the QuiAmp DNA mini kit according to the manufacturer's protocol (Quiagen, Hilden, Germany). The RNA samples were reverse transcribed using oligo (dT)-selection (Superscript Preamplification System, Life Technologies, Gaithersburg, MD). Osteopontin, bone sialoprotein, BMP-2, Osteocalcin and the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) expression were quantified using the ABI Prism 7000 real time system (Applied Biosystems, Foster City, CA). PCR reaction conditions were 2 min at 50°C, 10 min at 95°C, and then 50 cycles at 95°C for 15s, and 60°C for 1 min. The expression data were normalized to the expression of GAPDH. Primer sequences for the human GAPDH gene were (5`-3\'): Forward primer ATG GGG AAG GTG AAG GTC G, reverse primer TAA AAG CCC TGG TGA CC, probe CGC CCA ATA CGA CCA AAT CC G TTG AC. Primers and probes for osteopontin, bone sialoprotein, and BMP-2 were purchased from Applied Biosciences (Foster City, CA).

2.11. Histology

Constructs were embedded in paraffin and 5 µm sections were taken. Sections were stained with von Kossa (1% AgNO3 under a 60 W bulb for 15 minutes until initial light brown deposits were visible by eye) to assess mineralization, and with haematoxylin & eosin (H&E), for general evaluation.

2.12. Microcomputerized tomography (µ-CT)

For the visualization of bone distribution, scaffolds (n=5 per group) were weighed, and analyzed using a µCT40 imaging system (Scanco Medical, Bassersdorf, Switzerland). The specimens were scanned with the following settings: 55 kVp, 250 ms integration time, and 16 mm voxel side lengths. Cylindrical regions (6 mm in diameter 2mm high) were selected for analysis.
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2.13. Statistics

Statistics were performed by one-way analysis of variance (ANOVA) and Tukey-HST procedure for post hoc comparison. p<0.05 was considered statistically significant.

Figure 1. Effectiveness of gene transfer into human MSC determined after 48 hours by FACS analysis. Cells were transduced with Ad-GFP at 1, 3, 10, 30, 100, 300, and 1000 MOI. A. FACS analysis of MSC after transduction with various MOIs of Ad-GFP, B. Fraction of GFP positive cells as a function of MOI.

Results

3.1. Adenoviral transduction efficiency

The primary cells isolated from the human bone marrow are referred to as MSC, based on our previous characterization of these cells with respect to the expression of surface markers and their ability for selective differentiation (Meinel et al., 2004b, Meinel et al., 2004d). The effectiveness of gene transfer to passage 2 (P2) MSC was tested with Ad-GFP at MOI of 1, 3, 10, 30, 100, 300, and 1000, and an incubation time of 2 hours. After 48 hours in culture, the number of fluorescent cells was determined by FACS analysis and found to correlate with the MOI (Table 1, Figure 1A). The effectiveness of transduction was an exponential function of MOI, with the best cure fit following the two-parameter equation with $y = a\{1-e(-bx)\}$, $r^2>0.99$ (Figure 1B).
3.2. BMP-2 secretion in culture medium

Based on considerations of immunogenicity observed with MOIs exceeding 50 (Harvey et al., 1999, Chirmule et al., 1999) and the effectiveness of transduction of MSC at low MOI, as demonstrated (Figure 1A, B), we selected an MOI of 25 for the transduction of MSC with Ad-BMP-2. The over-expression of BMP-2 was associated with the synthesis and secretion of BMP-2 protein (Figure 2). BMP-2 levels measured 18 ng/ml at day 3, peaked at day 5 to about 40 ng/ml BMP-2, and declined to undetectable levels by 15-20 days of culture. Non-transduced cells showed no detectable BMP-2-release into the medium. In series of experiments, the same BMP-2 concentrations as a result of BMP-2 release by the Ad-BMP-2 group – measured by ELISA - were achieved in the non-transduced group by supplementing culture medium with recombinant BMP-2 (Figure 2). The equivalence of total BMP-2 produced (Ad-BMP-2) or supplemented (BMP-2) was confirmed by measuring the ratio of the areas under the curves for the two groups, which were 1.01 ± 0.01 (Ad-BMP-2: BMP-2 group).
3.3. Biochemical, transcriptional, and histological characterization of osteogenic differentiation

Human MSC were seeded onto silk scaffolds with a pore diameter range of 200–325 µm and all seeded scaffolds were cultured in osteogenic medium. The cells were either transduced with Ad-BMP-2 or Ad-GFP, or BMP-2 protein was added at concentrations similar to those measured in medium conditioned by the Ad-BMP-2 group (Table 1). Cell concentration (measured as the amount of DNA per unit construct wet weight) decreased from week 1 to week 3 of culture, and then returned to the initial level. No significant differences were observed between the groups at any
measured time point (Figure 3A). The bioactivity of transduced and supplemented BMP-2 (Ad-BMP-2 and BMP-2 groups) was evidenced by markedly increased alkaline phosphatase activity and calcium deposition when compared to controls (Ad-GFP and non-transduced control) (Figure 3B and C). Alkaline phosphatase (ALP) activities at days 3 and 5 were significantly higher for the Ad-BMP-2 and the BMP-2 and the Ad-GFP group when compared to the control group (p<0.05). In the Ad-BMP-2 and the BMP-2 group, ALP activity was increasing after 1 and 2 weeks of cultivation (p<0.05 when compared to Ad-GFP and control), a finding consistent with the transient response preceding tissue mineralization during osteogenic differentiation (Puleo, 1997). The ALP activity of the non-transduced control group increased slightly but not significantly after 2 weeks and remained at the same level at weeks 3 and 4. After 3 and 4 weeks, no statistic differences for ALP activity were observed among the groups (Figure 3B).

Mineralization started between weeks 1 and 3 of cultivation, resulting in significant within-group differences for the Ad-BMP-2 group and the BMP-2 group between weeks 2 and 3 (p<0.05), and remained at this level after 3 weeks (Figure 3C). This pattern of mineralization was observed for two out of the three donors tested. A comparative experiment, using MSCs transfected with Ad-GFP and exposed to the same BMP-2 profile as the BMP-2 group (Figure 2), resulted in the same time-mineralization profile as observed for the Ad-BMP-2 group and the BMP-2 group (e.g. comparison Ad-GFP plus BMP-2 group when compared to BMP-2 group for donor #3 in µg calcium per mg wet weight: week 1: 2.44±2.77 µg/mg versus 3.96±2.72 µg/mg; week 2: 4.91±1.08 µg/mg versus 3.43±1.66 µg/mg; week 3: 7.67±3.09 µg/mg versus 6.97±2.42 µg/mg; week 4: 6.51±0.26 µg/mg versus 6.33±0.91 µg/mg). For the exception, MSCs deposited significantly more calcium in response to BMP-2 after 4 weeks, when compared to adenovirally transduced MSCs (p<0.01; data not shown).

As expected, compared to control groups, MSC transduction with Ad-BMP-2 resulted in a 2500 fold over-expression of BMP-2 after 1 week, which significantly dropped to levels of 500 fold after 2 and 3 weeks (p<0.05), and again by an order of magnitude at week 4 when compared to BMP-2 gene expression by non-transduced cells prior to seeding (p<0.05, Figure 4C). Significant differences were observed in mRNA levels of bone matrix proteins associated with bone mineralization (Gorski, 1992). Both the Ad-BMP-2 and BMP-2 groups expressed osteopontin and bone sialoprotein at levels that
were significantly higher at all time points than those measured for the control group (p<0.01), whereas osteocalcin levels were equal to control levels throughout weeks 1 – 3, then raised significantly to expression levels that were higher than the control (p<0.01; Fig 4). During weeks 1 – 4, expression levels of osteopontin remained constant in the BMP-2 group, while they further increased in the Ad-BMP-2 group between weeks 1 and 3. The resulting expression levels of osteopontin were significantly higher in the Ad-BMP-2 group when compared to the BMP-2 group (p<0.05; Figure 4A). Bone sialoprotein expression was comparable in all groups during weeks 1-2 of culture, and significantly higher in weeks 3-4 for the Ad-BMP-2 and the BMP-2 group when compared to the control (p<0.05). Furthermore, bone sialoprotein expression continued to increase in the Ad-BMP-2 group, and was significantly higher at weeks 3 and 4 when compared to all other groups (p<0.05; Figure 4B). Osteocalcin expression in the Ad-BMP-2 and the BMP-2 raised significantly between weeks 3 and 4 compared to the control (p<0.01) that stayed at a constant level throughout the culture time. The Ad-BMP-2 group showed a higher increase than the BMP-2 group, resulting in significantly higher expression levels compared to both the control and the BMP-2 group (p<0.01; Figure 4D). The expression of the BMP type 1B receptor gene (BMPR1B) was below the detection limit for all groups and at all time points (data not shown). The expression of the BMP type II receptor gene (BMPR2) was not significantly different between groups (data not shown).

Histology results indicated no visible scaffold degradation over the 4 weeks of culture (Figure 5), consistent with the findings of former studies. Cells filled the void areas between the silk lattice within 2 weeks and this matrix became more granular after 4 weeks as seen in the H&E staining. The cells were in close contact with silk (Figure 5 A-C;G-I) and they displayed an osteoblast-like morphology as seen in higher magnifications. Calcium deposition was detected in the void areas and at the surfaces of the silk lattice, covering the scaffold with a continuous layer of tissue after 2 weeks (Figure 5 E,F). By week 4, dark brown staining for calcification was observed for both, the BMP-2 and the Ad-BMP-2 group (Figure 5 K,L). No calcification was observed in the control group (Figure 5 D,J) cultured in medium without BMP-2, or upon transduction with Ad-GFP (data not shown).
Qualitative tomographic evaluation demonstrated that the deposition of mineralized tissue was more prominent at the scaffold peripheries and surfaces with approximately the same thickness of the rim in the BMP-2 and the Ad-BMP-2 groups (Figure 6). The Ad-GFP group as well as the control group showed clearly less formation of calcified tissue than Ad-BMP-2 transduced cells (data not shown).

**Figure 4.** Gene expression of (A) osteopontin (OP), (B) bone sialoprotein (BSP), (C) bone morphogenetic protein 2 (BMP-2) and (D) Osteocalcin (Oc) in MSC relative to the expression of MSC quantified before seeding on scaffolds, all values normalized to GAPDH. Seeded cells were cultured in osteogenic medium (control) and either transduced with adenovirus (Ad-BMP-2) or cultured with BMP-2 protein (BMP-2) supplementation to the medium at concentrations adjusted to the concentrations as secreted and measured in the Ad-BMP-2 group (* p<0.05, ** p< 0.01, n=4-5).

**Discussion**

Several studies have demonstrated that bone formation at skeletal and non-skeletal sites can be induced by the implantation of mesenchymal stromal cells transduced with Ad-BMP-2 (Lieberman et al., 1999, Lieberman et al., 1998, Cheng et al., 2001). However, tissue engineering studies using in vitro techniques and mechanically robust
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scaffolds such as silk, and comparing the performance of either adenovirally transfected or untransfected MSCs with both groups being exposed to similar time – concentration profiles of BMP-2 have not been reported. This study utilized a tissue engineering approach to interrogate the effects of BMP-2 on osteogenesis in human MSC cultured on silk scaffolds. Our objectives were to assess transient secretion of BMP-2 by human MSCs transduced with Ad-BMP-2, to provide the same amounts of BMP-2 protein by supplementation of culture medium, and to compare osteogenesis for the two modes of BMP-2 delivery on the molecular, cellular and engineered tissue levels. The three-dimensional cultivation of human MSC on silk scaffolds can thus provide a controllable tissue model for studies of MSC differentiation in response to a variety of genetic and exogenous signals.

In this study a MOI of 25 was chosen, although higher transgene expression was observed for higher adenovirus concentrations (Figure 1). The selection was based on evidence from former studies, linking the key limitations of adenoviral gene therapy such to: (i) immunogenicity in vivo, (ii) deleterious effects upon long transduction times, and (iii) unclear fate of the adenovirus to high MOIs, which could be at least in part, circumvented by selecting MOIs below 50 (Harvey et al., 1999, Chirmule et al., 1999). Other studies used an MOI of 100 for the transfection of MSC and observed minimal signs of apoptosis, as determined by antigen expression studies (Dragoo et al., 2003). The relatively low doses of BMP-2 achieved with a MOI of 25 - up to 40 ng/ml at day 5 – resulted in the deposition of bone-like tissue adjacent to the silk lattice and also within the void spaces (Figure 5).

In earlier studies working with non-transduced cells but constant BMP-2 concentrations of 1 µg/ml, we observed stronger mineralization adjacent to the silk and less prominent effects in the void areas (Meinel et al., 2004a, Meinel et al., 2004d, Meinel et al., 2004e, Meinel et al., 2004f). Therefore, in these former studies the formation of a highly interconnected trabecular-like bone tissue was observed, an outcome most likely related to the higher BMP-2 concentrations since all other parameters including the cell source were kept constant (Meinel et al., 2004f, Meinel et al., 2004d). To meet the constraints reported with higher MOI inducing inflammatory reactions (Harvey et al., 1999, Chirmule et al., 1999), we selected an 25 MOI as a baseline for comparing gene delivery and protein delivery, following our goal to compare two modes of delivery rather than optimizing bone formation. Former studies
using MSCs transfected with 100 MOI in culture plate (two dimensional) resulted in 5 times lower concentrations of secreted BMP-2 when compared to the outcomes reported here for three dimensional culture, yet osteogenic response were reported when implanted intramuscularly in mice (Dragoo et al., 2003). As a consequence, advanced outcomes of the system presented here might be anticipated due to the higher BMP-2-secretory activity of these cells.

**Figure 5.** Light microscopy of construct cross sections after 2 weeks (A – F) or 4 weeks (G – L) of cultivation in osteogenic medium (B,C,E,F,H,I,K,L) or control medium (A,D,G,J). MSC were either transduced with Ad-BMP-2 (center column) or
exposed to BMP-2 concentrations as secreted and measured for the Ad-BMP-2 transduced cells (right column) or cultivated in control medium (left column). Sections were stained with H&E (A-C;G-I) or with von Kossa (D-F;J-L). Bar length is 100 µm.

The transduction of human MSC with Ad-BMP-2 resulted in significant expression of BMP-2, with transcript increases between 2500-fold (week 1) and 50-fold (week 4), over the baseline levels measured prior to transduction (Figure 4C). Compared to that, secretion of BMP-2 protein was transient, peaking at day 5 and rapidly decreasing thereafter (Figure 2). These profiles measured for human MSC are consistent with the corresponding data reported for mouse cell lines (W20-17, A549) (Lieberman et al., 1998, Olmsted et al., 2001), suggesting that the attenuation of BMP-2 secretion is a general phenomenon across different species and cell sources when adenoviruses as used here are utilized. A potential explanation for the discrepancy between transcript level and protein secretion may be found in the regulation of processing steps for the BMP precursor protein. It has been speculated that the protease involved in cleaving the BMP-2 precursor may be regulated during cell differentiation, thereby impacting the amount of secreted and active BMP-2 (Olmsted et al., 2001).

This study is the first demonstration of an integrated use of human MSC, adenovirus transduction, and silk biomaterials in an in vitro system designed to engineer bone. An envisioned application involves directed differentiation of human MSC grown on structurally and mechanically stable silk scaffolds into a variety of orthopaedic tissues, by provision of specific combinations of growth factors. The results of the present study support this concept for the specific case of bone formation in response to BMP-2, and suggests that under the conditions studied, the gene transfer of Ad-BMP-2 is as effective as direct BMP-2 protein supplementation to culture medium on silk scaffolds. Mineralization observed using Ad-GFP transfected cells exposed to the same BMP-2 time-concentration profile (Figure 2 for profile) as in the BMP-2 group resulted in similar and statistically non-distinguishable profiles as the Ad-BMP-2 or the BMP-2 group, respectively. This raises the suggestion that the viral transfection process itself, regardless of the transfected gene, does not impair the performance of MSC undergoing osteogenic differentiation to deposit bone. However, in one out of three donors tested, mineralization was significantly less for the adenovirally transduced cells when compared to the BMP-2 group, suggesting that donor specificity might have a substantial effect on the success of virally dependent strategies.
Bone formation in vivo was classified into three phases, an initial proliferation and matrix deposition, matrix maturation, and finally, matrix mineralization phase (Owen et al., 1990). It is, however, questionable how these phases are reflected in ex vivo tissue engineering approaches, and most certainly the temporal sequence of phases differs from in vivo events. However, certain biochemical and cell-biological events are essential for the bone formation process, either in vivo or ex vivo and therefore, the term “phases” is also used for the purpose to describe the state of the differentiating cell pool in this tissue engineering approach. The second (matrix maturation) phase is characterized by early osteogenic markers such as ALP. ALP is a membrane bound enzyme that is abundant in early bone formation and increased ALP levels were correlated with increased bone formation histomorphometrically (Ljunghall and Lindh, 1989). Other studies were not able to demonstrate a direct correlation on the amount of bone that will be formed in response to alkaline phosphatase levels (Puleo, 1997). Therefore, it has to be taken into account, that ALP activity is not a completely reliable marker for mineralization on its own. Osteopontin (OP) is another non-specific early bone marker that is expressed bimodally, with an early peak during the proliferative phase and another after initial mineralization (Yao et al., 1994) and OP was linked with pre-osteoblastic cell stages (Prince et al., 1987, Mark et al., 1987). Gene expression levels for OP were identical for the Ad-BMP-2 group and the BMP-2 protein group after 1 and 2 weeks but significantly higher for the Ad-BMP-2 group after 3 and 4 weeks. Similar results were measured for bone siaoloprotein (BSP) gene expression, a late bone marker first produced by differentiated osteoblasts forming bone. BSP was shown to bind collagen (Fujisawa et al., 1995), nucleate hydroxyapatite formation in vitro (Goldberg et al., 1996), and has been isolated in cells actively mineralizing a type I collagen matrix (Chen et al., 1992). Osteocalcin (OC), the most specific marker for osteoblast maturation, showed an increase in expression over the control group in both the Ad-BMP-2 and BMP-2 group after 3 weeks. The relatively late onset of both BSP and OC expression results from the time lag that is needed for the MSC to differentiate along the lineage and confirms the presence of a mature stage of osteoblasts and mineralization within the tissue. The extent of mineralization was not different between the BMP-2 group and Ad-BMP-2 group for 2 out of three cell sources tested (Figure 3C, Figure 5). Comparative side experiments with Ad-GFP, exposed to the same BMP-2 levels as the BMP-2 group, also resulted in the same mineralization rates,
suggesting good tolerance of the adenoviral transduction process on osteogenic performance.

**Figure 6.** Micro-computer tomography images of three representative constructs cultured in osteogenic medium for 4 weeks. Cells were either transduced with Ad-BMP-2 (A – C) or non-transduced cells were exposed to BMP-2 at concentrations adjusted to the levels secreted and measured for the transduced cells (E – F). Bar length = 1 mm.

In conclusion, this study corroborates other studies demonstrating that human MSC can be effectively transduced with adenoviruses at low MOI, resulting in the transient synthesis and secretion of biologically active BMP-2. Transient exposure to BMP-2 induced strong osteogenic responses in both the adenoviral transduced and non-transduced cells, as reflected in expression of osteopontin, BSP and alkaline phosphatase activity, with higher values for the adenovirally transduced cells. Viral transduction, independent of the carried gene, did not impact osteogenic performance of MSCs in two out of three cell sources, whereas for one donor, virally transduced cells deposited significantly less calcium when compared to untransfected cells exposed to BMP-2 protein. This raised evidence that the success of osteogenic therapies using viral vectors may be in part patient dependent. As a result, the adenoviral approach increases the cell's capacity to express osteogenic markers, does
not interfere with the cell's capacity to proliferate, or the cell's capacity to deposit calcified tissues at low adenoviral concentrations.

Acknowledgements

We thank Elvire Gouze for support with the FACS analysis. This work was supported by the German Alexander von Humboldt Foundation (Feodor-Lynen fellowship to LM), the Association for Orthopaedic Research, and the National Institutes of Health (DE13405-04, NIH AR46563-01A2 to DK, NIH P41 EB002520 to DK, and NIH AR050243-01 to CE). The authors thank Trudel Inc. (Zurich, Switzerland) for providing B.mori cocoons.

References


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The support of adenosine release from adenosine kinase deficient embryonic stem cells by silk substrates

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Biomaterials, 2006, accepted
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Abstract

Adenosine kinase deficient (Adk-/-) embryonic stem cells (ESC) encapsulated in synthetic polymers have previously been shown to provide therapeutic adenosine release and transient seizure suppression in epileptic rats. Here we explored the utility of biopolymer-substrates to promote long-term adenosine release from Adk-/- ESCs. Three different substrates were studied: (1) type I collagen (Col-1), (2) silk-fibroin (SF), and (3) poly(L-ornithine) (PO) coated tissue culture plastic. Adk-/- or wild type (wt) ESC-derived glial precursor cells were seeded on the substrates and cultured either in proliferation medium containing growth factors or in differentiation medium devoid of growth factors. In proliferation medium cell proliferation was higher and metabolic activity lower on Col-1 and PO substrates when compared to SF. Cells from both genotypes readily differentiated into astrocytes after growth factor removal on all substrates. Adk-/- cells cultured on biopolymers released significantly more adenosine than their wt counterparts at all developmental stages. Adenosine release was similar on SF and PO substrates and the amounts released from Adk-/- cells (>20 ng/ml) were considered to be of therapeutic relevance. Taken together, these results suggest that silk matrices are particularly suitable biomaterials for ESC encapsulation and for the design of adenosine releasing bioincubators for the treatment of epilepsy.
Introduction

The purine ribonucleoside adenosine is an important modulator of central nervous system functions, exerting primarily inhibitory effects on neuronal activity and suppressing seizure activity in various rodent models of epilepsy (Dunwiddie and Masino, 2001, Boison, 2005, Gouder et al., 2003). However, traditional methods for the administration of adenosine to epileptic patients are limited by severe systemic side effects ranging from a decrease of heart rate, blood pressure, and body temperature to sedation (Dunwiddie, 1999). Recently, new strategies have been developed aiming at the local administration of adenosine by intracerebral implantation of cells engineered to release adenosine (Guttinger et al., 2005, Boison, 2005). Since self-renewing, totipotent embryonic stem cells (ESC) may provide a virtually unlimited donor source for implantation, mouse ESCs were genetically engineered to lack the main adenosine-metabolizing enzyme, adenosine kinase (Adk), causing the cells to release significant amounts of adenosine (Fedele et al., 2004). These Adk deficient (Adk-/-) ESCs were subjected to a glial differentiation protocol and the glial precursors could be maintained under proliferative culture conditions for several passages (>20). When allowed to differentiate, Adk-/- glial precursors were shown to mature into primarily astrocytes (Fedele et al., 2004). When encapsulated and implanted into the brain ventricle of epileptic, electrically kindled rats, the adenosine release from the Adk-/- glial precursors was effective in suppressing seizures for 5-7 days after capsule implantation at which time the cells died and seizures resumed (Guttinger et al., 2005). While these studies demonstrated the effectiveness of local adenosine application for the treatment of epilepsy, they do not offer a therapeutic solution due to disadvantages associated with the material selected for encapsulation, polysulfone derivatives. One main disadvantage is the persistence of the biomaterial which may impact the viability of the encapsulated cells by causing immunogenic reactions and by eliminating activity dependent support from the host cells (Wenz et al., 1990). Furthermore, cells did not adhere to the polysulfone matrix. To address some of these challenges, protein-based biomaterials with variations related to rates of degradation were investigated in the present study. Adk-/- glial precursor growth, differentiation, and adenosine release were evaluated on fast degrading type I collagen (Col-1) substrates and slow degrading silk-fibroin (SF) substrates in comparison to poly(L-ornithine) coated tissue culture plastic surfaces, routinely used for culture of these cells.
Collagens have a longstanding tradition as biomaterial substrates for nervous tissue formation in two- (Hauw et al., 1972) and three-dimensional cell culture (Satou et al., 1986). Nerve guidance tubes based on collagen or collagen/agarose polymer blends have been used to repair segments in rat distal peripheral nerve increasing the percentage of axons crossing the anastomosis (Hinckley et al., 1991, Chen and Liu, 1994). However, challenges remain, mainly due to the lack of mechanical integrity and rapid degradation rates of collagens. Tubes prepared from untreated collagen were shown to separate into different layers and stenosis of the lumen occurred early after implantation, a problem which could be partly solved by cross-linking the collagen thus stabilizing the tubes (Itoh et al., 2002). However, cross-linking of collagen is associated with unwanted inflammatory potential, spontaneous and uncontrolled calcification and remaining issues concerning the rapid biodegradation within 8 weeks (Srivastava et al., 1990b, Srivastava et al., 1990a, Collins et al., 1991).

Silk, which is a mixture of mainly silk fibroin (SF) and sericin, has an extensive history in clinical application as a suture material. Unwanted immune reactions to silk sutures observed early on in their application were assigned to the presence of sericin. However, in vitro and in vivo evaluations of pure SF films, scaffolds, or fibers demonstrated excellent biocompatibility, while maintaining the distinguishing mechanical features of silks when compared to most synthetic and all biopolymers known (Soong and Kenyon, 1984, Panilaitis et al., 2003, Meinel et al., 2005). The performance of SF might be improved by the addition of cell-binding domains, such as the RGD domain, to increase the release of bioengineered products from terminally differentiated cells, such as interferon-beta release from fibroblasts (Higuchi et al., 2003). However, the use of unmodified SF, which does not contain cell-binding domains and after methanol treatment predominantly forms beta-sheets, might be advantageous for the maintenance of defined differentiation pathways of stem cells or precursor cells. Therefore, unmodified SF should be a particularly suitable substrate for cells in which differentiation needs to be tightly controlled by exogenous cues. In addition to the biocompatibility of SF and its robust mechanical stability, the slow and complete biodegradation of the material in vivo is an important characteristic. This is relevant in light of the estimated time frame for function of bioincubators (release matrices) in epilepsy of 6 – 12 months, and based on previous findings detailing
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Biodegradation at other anatomic sites than the brain (Meinel et al., 2005, Altman et al., 2003, Meinel et al., 2004a).

The present study addresses the need for novel biomaterial options as a basis for bioincubator manufacture for clinically applicable treatments of epilepsy. Both protein biomaterials tested here, Col-1 and SF, appeared to be suitable materials as substrates for Adk-/- ESC derived glial precursor cells regarding cellular adherence, metabolic activity, differentiation and adenosine-release. Initial studies of nutrient transport through the silk fibroin membranes, as a prerequisite for survival of encapsulated cells, is also provided to further substantiate the unique applicability of this biomaterial system for applications in the treatment of epilepsy.

Materials and Methods

2.1 Materials

All cell culture materials including medium, medium supplements and growth factors were purchased from Invitrogen (Carlsbad, CA, USA) unless otherwise noted. The ATP Bioluminescent assay kit, the MTT assay kit, poly(L-ornithine) hydrobromide (mol wt 30,000-70,000), Triton-X, paraformaldehyde, and EDTA were purchased from Sigma-Aldrich (St. Louis, MO, USA). All primary antibodies used were obtained from Chemicon International (Temecula, CA, USA). Donkey Anti-Mouse Cy3 was purchased from Jackson Immuno Research Laboratories (West Grove, PA, USA). The PicoGreen assay kit was obtained from Molecular Probes Europe BV (Leiden, The Netherlands). In addition, Collagen I (Calf Skin,) and D(+) Glucose anhydrous were purchased from Sigma-Aldrich (St. Louis, MO, USA). D[1-14C]-Glucose aqueous solution containing 3% ethanol was purchased from Amersham Life Science CFA349, B73L01 (2GBq/mmol) and Ultima Gold from Perkin Elmer (Boston, MA, USA).

2.2 Preparation of silk films

Silk fibroin was prepared as described previously. Briefly, cocoons derived from Bombyx mori (Trudel Inc. Zurich, Switzerland) were boiled in 0.02 M Na2CO3, rinsed and dissolved in 9M LiBr overnight at 37°C to generate a 10% (w/v) solution. This solution was dialyzed (Pierce, Rockford, IL; MWCO 2000 g/mol) to give a 3%
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(w/v) solution. The solution was then sterile filtered through a 0.2 µm filter into 22 mm diameter Teflon molds. The films were dried overnight and subsequently treated with 90% methanol (v/v) for 30 min to induce crystallization and insolubility to the biomaterial matrix in the aqueous culture medium.

2.3 Preparation of collagen films

Calf-skin collagen type 1 (Col-1) films were prepared in 12-well non tissue culture treated polystyrene microtiter plates by pipetting 1 ml of 0.5 g/l collagen (in 0.1% v/v acetic acid) into the wells. Films were allowed to dry overnight and were then neutralized by applying 2 ml of 0.1 M Na2HPO4 in each well for 1 h and the wells were then washed twice with PBS before cells were seeded. For the immunostaining, glass coverslips were coated twice with 400 µl of collagen solution and sterilized by ethylene oxide after drying.

2.4 Preparation of poly(L-ornithine) coated plastic

Tissue culture grade poly(L-ornithine) hydrobromide (30 – 70 kDa) was used as an aqueous solution at a final concentration of 100 µg/ml. A sufficient volume of the diluted solution was used to cover the bottom of the cell culture vessels (flasks and Petri dishes) and the vessels were incubated for at least 2 hours at 37°C. Immediately before plating the cells, the PO was rinsed from the surface with two washes of PBS and one final wash of DMEM.

2.5 Contact angle measurement

To measure the hydrophobicity of the biopolymer membranes, static contact angle measurements were performed on dry SF or Col-1 films and on PO-coated tissue culture plastic (n=3) at ambient temperature using a goniometer (NRL C, Ramé-Hart Inc., Mountain Lakes, NJ, USA). Ultra-pure water droplets were used with a drop volume of approximately 30 µl.

2.6 Cell culture and differentiation
Wt and Adk-/- murine ESCs (Fedele et al., 2004) were stepwise differentiated to the glial precursor stage as previously described (Brustle et al., 1999). Glial precursor cells were routinely cultured on poly(L-ornithine) coated dishes in N3 medium, a 1:1 mixture of DMEM with Ham's F12 supplemented with insulin (25 g/ml), human apo-transferrin (100 g/ml), progesterone (20 nM), putrescine (100 M), sodium selenite (30 nM), penicillin (100 U/ml), and streptomycin (100 g/ml). In addition, laminin (1 g/ml) was added when plating the cells. To keep the cells in a proliferative state, the growth factors, FGF2 (10 ng/ml) and EGF (20 ng/ml), were added daily and this medium was changed every 48 hours. Frozen stocks of undifferentiated glial precursor cells could be kept in liquid nitrogen. To induce the differentiation of glial precursor cells, fresh N3 medium was added without growth factors. Growth factor free medium was replaced every 48 hours. After 4-5 days of growth factor withdrawal, the cells had differentiated as was evidenced by immunostaining for GFAP, a marker of mature astrocytes (see below).

To prepare glial precursor cells for seeding onto different biopolymers, cells were thawed and plated onto PO-coated, 25 cm2 tissue culture flasks in N3 medium plus growth factors and maintained in a proliferative state until approximately 90% confluent. At this point, the cells were washed 3 times with PBS, scraped off the plastic in PBS plus 1% DNAse, centrifuged and replated on 3 PO-coated 25cm2 flasks in N3 medium plus growth factors. Once the 3 flasks were about 90% confluent the cells were again scraped off, counted and plated onto the appropriate substrate (silk, collagen, or PO) in 12-well plates at a concentration of 3x 105 cells per ml.

2.7 MTT assay to determine cell viability and metabolism

ESC derived glial precursor cells were seeded in replicas of 5 in a concentration of 1.5x105 cells/cm2 on the different substrates and incubated until measurement. At 48 hours and 96 hours the proliferation of the cells on the different substrates was assessed using a 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was dissolved in PBS for a final concentration of 5 mg/ml and sterile filtered. The MTT solution was diluted in DMEM medium to a final concentration of 0.5 mg/ml before use. The culture medium was removed from the cells and replaced by 2 ml MTT (0.5 mg/ml in DMEM medium). After 2 hours of incubation at 37°C and
5% CO2, the solution was removed and 1 ml of acidic isopropylac acid (500 ml Isopropylac acid + 3.5 ml 6M HCl) was added to the culture to dissolve the crystals that had formed. After 10 minutes incubation at room temperature, the tubes were centrifuged (300 g, 1 min, 4°C) and 100 µl of the supernatant were transferred to a 96 well plate. The absorbance was assessed at 570 nm and the background (630 nm) was subtracted using a Thermomax microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

2.8 PicoGreen assay to determine cell number

The PicoGreen assay quantifies double-stranded DNA in solution using a fluorescent nucleic acid stain. A defined concentration of DNA (100 g/ml) was diluted in Tris-EDTA (TE) assay buffer to provide a standard curve of 0–1000 ng/ml DNA. ESC derived glial precursor cells (n = 5) were seeded in a concentration of 1.5 x 105 cells/cm² on the appropriate substrates and maintained in N3 medium plus growth factors. After 48 and 96 hours the supernatant was removed and cell samples were disrupted by addition of 1 ml lysis buffer [Triton-X (0.2%) + 5mM MgCl2] to each well with incubation in the dark for 48 h. The lysate was then transferred to a tube and centrifuged at 3000 g for 10 min at RT. Subsequently, 41 ml of the supernatant were transferred to a new tube and 166.6 µl of diluted PicoGreen solution (PicoGreen stock solution in dimethylsulfoxide; diluted 200 times in TE assay buffer) were added. After vortexing for 3 minutes, 100 µl of the solution were transferred to a 96-well plate. Plates were then incubated for 5 minutes in the dark. Standards/samples were excited at 485 nm and the fluorescence emission intensity measured at 530 nm in a FluoroCount™ plate reader (Packard instrument, Downes Gore, IL).

2.9 Immunofluorescence staining for the assessment of the differentiation of glial precursor cells

ESC derived glial precursor cells were plated in a concentration of 1.5 x 105 cells/cm² on glass cover slips coated with PO or collagen or on silk films in a 12-well plate. After culturing the cells for 96 hours in N3 medium with growth factors the differentiation was induced by 5 days of growth factor withdrawal. Cells were then
fixed with 4% paraformaldehyde. The following primary antibodies were used: anti-mouse GFAP (1:10,000) to stain astrocytes, anti-mouse O4 (1:50) to stain oligodendrocytes, and anti-mouse III tubulin (1:1000) to stain early neurons. Donkey anti-mouse Cy3 (1:500) was used as the secondary antibody. To stain the nucleus of all the cells in the culture, Hoechst 33258 (1:10,000) was used. Immunofluorescence analyses were performed using a Zeiss fluorescence microscope (Karl Zeiss AG, Jena, Germany) with Openlab imaging. Images were acquired with a resolution of 512 × 512 pixels.

2.10 Quantification of adenosine release

Single cell suspensions of ESC derived glial precursors were plated at a density of 1.5 x 10^5 cells/cm² onto the different substrates in 12-well plates (n = 6 wells per substrate) and maintained under culture conditions supporting proliferation at 37°C under 5% CO2. After 48 hours and 96 hours the medium was exchanged, and 2 hours thereafter 150 l of the medium were collected from each well and stored at –20°C for subsequent analysis. After the 96 hours samples were taken, the medium was changed to N3 medium without growth factors to induce the differentiation of the cells. On the fifth day after growth factor withdrawal, the medium was changed and 2 hours later 150 l samples of the medium were collected. Adenosine was quantified using an enzyme-coupled bioluminescence assay as previously described (Huber et al., 2001).

2.11 Permeation of 14C-d-glucose through silk fibroin films

Silk fibroin films were prepared from 8% (w/w) aqueous silk fibroin solutions, casted on Teflon surfaces and carefully transferred to a diffusion chamber (Crown Glass Co., Somerville, NJ, USA) (n = 4). The receiver chamber was filled with 3 ml PBS and 3 ml of glucose solution (4.5 mg/ml) in PBS containing 1 ml of 14C-d-glucose (2 GBq/mmol) was added to the donor chamber. Samples (3 ml) were taken at the receiver side every day for two days. One ml of sample was mixed with 6 ml of Ultimate Gold and measured in a Beta Counter (Packard Instruments, Downers Grove, IL, USA).
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2.12 Statistical analysis

Statistical data analysis was performed by one-way analysis of variance (ANOVA) and Tukey-Kramer procedure for post hoc comparison. \( p<0.05 \) was considered statistically significant.

![Figure 1: Contact angle measurement on non tissue culture treated plastic (Plastic), poly(L-ornithine) coated plastic (PO), collagen I (Col-1) and silk fibroin (SF) films (** \( p<0.01 \)).](image)

Results

3.1 Hydrophobicity of poly(L-ornithine), collagen, and silk fibroin substrates.

The hydrophobicity of non-coated, non tissue culture treated plastic when compared to that of PO-coated tissue culture plastic, Col-1 and SF by contact angle measurements (Figure 1). PO coated tissue culture plastic was significantly (\( p<0.01 \)) less hydrophobic (contact angle of \( 43 \pm 1.41^\circ \)) than non-coated, non tissue culture treated plastic (contact angle of \( 77 \pm 0.84^\circ \)). The resulting contact angle was significantly lower than the contact angle measured on Col-1 (\( 58 \pm 0.73^\circ \)) and SF (\( 52 \pm 0.89^\circ \)).

3.2 Adherence and morphology of proliferating wt and Adk-/- ESC derived glial precursor cells on SF, Col-1, and PO substrates.

Wt and Adk-/- glial precursor cells were plated on SF, Col-1, and PO coated tissue culture plastic (\( n=5 \), for each combination). After 24 hours, both wt and Adk-/- glial
precursor cells adhered to SF and Col-1 to the same degree as that observed in control, PO-coated, wells. Cellular morphology was comparable on all three substrates with the cells spreading homogeneously on the surfaces. After 48 and 96 hours, glial precursor cells of both genotypes grown on SF and Col-1 covered approximately 30 - 40% or 80 - 90% of the available surface, respectively, whereas the density of cells grown on PO was slightly higher at both time points as assessed qualitatively (Figure 2). Cellular morphologies did not differ between SF, Col-1, and PO substrates, time points (48 and 96 hours post seeding) or genotypes (wt or Adk-/-) - and there were no signs of cell death.

Figure 2: Adherence of Adk-/- (-/-) and wt ES derived glial precursor cells on poly(L-ornithine) (PO), collagen I (Col-I) and silk fibroin (SF) after 48 h and 96 h. Scale bar = 20 µm.

3.3 Metabolic activity and proliferation of Adk-/- ESC derived glial precursor cells cultured on PO, Col-I, and SF

To evaluate the influence of different substrates on the metabolic activity of undifferentiated glial precursor cells, Adk-/- glial precursor cells were plated (n=5) on SF, Col-1 and PO. Metabolic conversion of MTT was measured, using the MTT assay and comparing relative units of absorbance (rUMTT) (Figure 3A). 48 hours after plating on SF, Adk-/- cells showed a conversion of 0.066 ± 0.019 rUMTT. This value
was significantly lower than those obtained from cells cultured on Col-1 (0.116 ± 0.039 rUMTT) and PO (0.186 ± 0.009 rUMTT). MTT conversion measured after 96 hours were as follows: SF: 0.628 ± 0.152 rUMTT; Col-1: 0.333 ± 0.042 rUMTT; PO: 0.404 ± 0.068 rUMTT. On all substrates cells showed a significant (p<0.01) increase of absorbance after 96 hours when compared to 48 hours of culture. This increase was most evident in Adk-/- cell cultures on SF, increasing almost 10-fold (48h: 0.066 ± 0.019 rUMTT; 96h: 0.628 ± 0.152 rUMTT). Compared to cells on PO and Col-1, MTT conversion of glial precursor cells on SF was significantly higher (p<0.05 or p<0.01, respectively) after 96 hours of culture.

Figure 3: Metabolism and DNA content of Adk-/- (-/-) ES derived glial precursor cell cultures on poly(L-ornithine) (PO), collagen 1 (Col-1) and silk fibroin (SF) after 48 h and 96 h (*p<0.05, **p<0.01).
To evaluate changes in cell numbers across substrates and time in culture, DNA content of the cell cultures was assessed with the PicoGreen assay (Figure 3B). After 48 hours, the DNA content in Adk-/- glial precursor cells was similar on all substrates (SF: $0.557 \pm 0.135$ ng; Col-1: $0.421 \pm 0.037$ ng; PO: $0.479 \pm 0.0433$ ng). DNA content of Adk-/- cell cultures on PO and, to a lesser extent, on Col-1 increased significantly (**p<0.01) at 96 hours (Col-1: $4.669 \pm 2.323$ ng; PO: $9.086 \pm 6.474$ ng) when compared to the 48 hour time point. However, on SF, the Adk-/- cell numbers remained essentially the same at 96 hours ($0.755 \pm 0.175$ ng).

To assess the metabolic activity irrespective of cell viability, the conversion of MTT (rUMTT) was normalized according to the DNA content of the cultures (rUMTT/ng DNA). The normalized metabolic activity of Adk-/- cells was significantly increased (p<0.01) on the SF films at 96 hours ($0.831 \pm 0.396$ rUMTT/ng DNA) when compared to 48 hours ($0.119 \pm 0.064$ rUMTT/ng DNA). The normalized metabolic activity of Adk-/- glial precursor cells grown on Col-1 and on PO decreased significantly (p<0.01) at 96 hours (Col-1: $0.071 \pm 0.039$ rUMTT/ng DNA; PO: $0.044 \pm 0.039$ rUMTT/ng DNA) when compared to 48 hours (Col-1: $0.276 \pm 0.116$ rUMTT/ng DNA; PO: $0.387 \pm 0.055$ rUMTT/ng DNA) of culture, a time point when the normalized metabolic activity of the cells grown on Col-1 and PO was slightly higher than that of the cells grown on SF ($0.119 \pm 0.064$ rUMTT/ng DNA). Adk-/- glial precursor cells cultured on SF for 96 hours showed a significantly (p<0.01) higher normalized metabolic rate when compared to the respective cells cultured for 96 hours on Col-1 or PO surfaces (Figure 3C).

3.4 Differentiation of wt and Adk-/- ESC derived glial precursor cells on SF and Col-1.

To determine the impact of SF and Col-1 on stem cell derived glial precursor differentiation in comparison to that on PO, cells of both genotypes were plated on the different substrates (n = 5), differentiated by growth factor withdrawal and immunostained with markers indicative of astrocytes (GFAP), oligodendrocytes (O4) and early neurons (III-tubulin). The majority of wt and Adk-/- glial precursor cells cultured on the PO-coated tissue culture plastic stained positive for GFAP (Figure 4A). Wt and Adk-/- cells cultured on Col-1 (Figure 4B) and SF (Figure 4C) also
stained almost exclusively for GFAP. Minimal or no positive staining was observed for O4 and III-tubulin (data not shown).

3.5 Adenosine release from wt and Adk-/- ESC derived glial precursor cells cultured on SF and PO.

To determine the influence of SF on adenosine release from Adk-/- cells, a bioluminescence assay was used. Wt and Adk-/- cells were cultured on SF and PO (n = 6) and samples of medium were taken 2 hours after medium change. Overall, Adk-/- cells on SF released significantly more adenosine than the respective wt cells on all
substrates. This was comparable to the adenosine release from cells cultured on PO (Figure 5). Adenosine release from Adk-/- cells cultured for 48 hours was 43.9 ± 13.9 ng/ml on SF and 48.1 ± 11.0 ng/ml on PO compared to 10.5 ± 3.1 ng/ml and 10 ± 0.7 ng/ml from the respective wt cells (p<0.01). At the 96 hour time point, Adk-/- cells cultured on SF released 68.6 ± 12.0 ng/ml adenosine and Adk-/- cells on PO released 74.0 ± 9.7 ng/ml adenosine when compared to the respective wt cells, which released 23.5 ± 4.5 ng/ml adenosine on SF and 10.5 ± 2.3 ng/ml adenosine on PO (p<0.01). After 5 days of withdrawal from growth factors allowing the cells to differentiate into glial cells, Adk-/- cells on SF released 44.3 ± 12.7 ng/ml adenosine while Adk-/- cells on PO released 40.2 ± 6.1 ng/ml adenosine. The respective wt cells after 5 days of differentiation released significantly less adenosine, 10.5 ± 7.6 ng/ml on SF and 11.7 ± 3.1 ng/ml on PO (p<0.01).

3.6 Glucose permeation through silk films

To evaluate nutrient transport through the silk films, we measured the diffusion of radioactively tagged glucose through the films. The initial concentration of glucose in the donor chamber was set at 4.5 mg/ml, which was equivalent to the glucose concentration in the cell culture medium (DMEM). The rate of glucose diffusing through the silk films was constant over 48 h. The flux per unit surface, as calculated by linear regression, was 8.6 µg mm-2 h-1 (Figure 6).

Discussion

Local cell-mediated adenosine release has been demonstrated to represent an effective strategy to suppress seizures in the rat kindling model of epilepsy (Huber et al., 2001, Guttinger et al., 2005). In these previous studies, cells were engineered to release adenosine based on a genetic disruption of adenosine kinase (Adk-/-). The cells were encapsulated into a semipermeable, synthetic polyethersulfone membrane and implanted into the lateral brain ventricle of kindled rats. While this approach proved that paracrine cell-mediated adenosine release into the lateral ventricle was an efficacious treatment of epilepsy, the therapeutic effect did not persist because of limited cell viability within the polymer capsules. Furthermore, the system faced limitations due to the persistent nature of polysulfone, demanding a second surgical
intervention if the bioreactor had to be replaced. New approaches in cell therapy must consider the long-term maintenance of cells on a biopolymeric substrate, which exhibits mechanical strength, biocompatibility, slow degradation, little or no immune or inflammatory response from the host tissue, and allow for cell adherence to the biomaterial matrix.

Collagens have already proven their compatibility in nervous tissue (Hauw et al., 1972, Satou et al., 1986, Hinckley et al., 1991, Chen and Liu, 1994). However, they exhibit weak mechanical stability, fast biodegradation and may not be stable enough to maintain cell cultures in vivo. Silk fibroin (SF) may offer an alternative to fill the niche due to its distinguished mechanical properties and optimal biodegradation times, adjustable between 6–12 months or more (Altman et al., 2003). In addition, cells adhere directly to SF matrices and inflammatory reactions were less when compared to collagens or synthetic polymers (Panilaitis et al., 2003, Meinel et al., 2005, Meinel et al., 2004b, Sofia et al., 2001). Here we compared silk fibroin (SF) and fast degrading collagen (Col-1) with poly(L-ornithine) (PO) coated tissue culture plastic as substrates.
for Adk-/- embryonic stem cell (ESC) derived glial precursor cell culture in an effort to determine the feasibility of such substrates for clinically relevant epilepsy treatment. While the hydrophobicity of PO coated tissue culture plastic appeared to be significantly lower than non-coated plastic, it was comparable to that of SF and Col-1 (Figure 1). Therefore, in terms of hydrophilic interactions with the cells, SF and Col-1 appear equally capable compared to the traditional substrate used for cell culture, PO. Corroborating these findings, we observed that both Adk-/- and control (wt) ESC derived glial precursor cells cultured on SF and Col-1 appeared to adhere and spread on the substrate to a similar degree as that observed on PO (Figure 2). Therefore, SF and Col-1 are able to support the initial adherence and culture of ESC derived glial precursor cells.

![Glucose permeation through silk films over 2 days. Glucose flux was 8.6 µg mm⁻² h⁻¹.](image)

Figure 6: Glucose permeation through silk films over 2 days. Glucose flux was 8.6 µg mm⁻² h⁻¹.

To assess relative metabolic activity and proliferation of cells attached to various substrates we used the MTT assay (Berridge et al., 2005, Pabbruwe et al., 2005). In order to compare the metabolic activity of cells under the various conditions irrespective of the number or viability of the cells, we performed a Pico Green DNA assay to normalize the metabolic activity to DNA content of the cultures. Thus, the relative metabolic activity [rUMTT] divided by DNA amount [ng DNA] allowed us to
assess the normalized metabolic activity of the cells [rUMTT/ng DNA], which takes into account the possible different proliferation rates and viability of the cells during the time period of assessment.

Our results demonstrate that both the relative (Figure 3A) as well as the normalized (Figure 3C) metabolic activity of the cells grown on Col-1 and PO was slightly higher than that of the cells grown on SF for 48 hours. Since the relative metabolic activity can be indicative of cell proliferation, we observe a corresponding increase in cell number at 96 hours compared to 48 hours on Col-1 and PO. Such an increase in cell number was not observed on SF at the 96 hour time point compared to the 48 hour time point; therefore, the normalized metabolic activity of the cells increased significantly at this later time point. The normalized metabolic activity further revealed the dramatic increase in metabolism of cells grown on SF at 96 hours compared to 48 hours and compared to the other substrates at the 96-hour time point. This indicates that there is a slight delay in cells grown on SF to reach their maximal metabolic activity and we would expect the number of cells on SF to increase accordingly after the 96 hour time point.

Despite an initial delay in cell proliferation of cultures grown on SF, cells on all substrates differentiated into astrocytes after growth factor withdrawal for 5 days (Figure 4), as indicated by the positive stain for GFAP, a marker of astrocytes, and the phenotypic change indicative of astrocytic differentiation. This suggests that both Col-1 and SF supported the capacity of the glial precursor cells to differentiate into astrocytes to the same extent as that observed on PO coated tissue culture plastic surfaces. Since our SF membranes are mainly in beta-sheet conformation and do not contain any cell binding domains, such as RGD domains, cell differentiation processes were not expected to be influenced by the film itself, a prediction that was confirmed here. This is an important finding since the differentiation of ES cell derived glial progenitor cells into astrocytes needs to be controlled by exogenous cues (Fedele et al., 2004, Brustle et al., 1999). As determined previously for terminally differentiated cells (Higuchi et al., 2003), the inclusion of cell binding domains or of Pronectin-F or Pronectin-L into the silk membrane may help to augment the release of bioengineered products.

To assess whether SF supports the release of adenosine in the absence of such cell binding domains, adenosine release was determined from Adk-/- glial precursor cells
grown on SF and PO. Adk-/- cells cultured on SF for 48 and 96 hours and after 5 days of differentiation released the same amount of adenosine as the respective Adk-/- cells cultured on PO. Thus, the inclusion of cell binding domains is not necessary to promote cell mediated adenosine release. In contrast to our previous study, here we observed that differentiated cells do not release significantly more adenosine than proliferating cells (Fedele et al., 2004). One difference between the two experiments is the time period the cells were in culture before sampling the medium. In the previous study adenosine release was measured 24 hours after plating of glial precursor cells on PO, and glial differentiation was initiated 24 hours after plating. Therefore, the delayed sampling of adenosine performed in the present study resulted in higher levels of adenosine release from the proliferating cells when compared to the differentiated cells. Since in the present study the actual number of cells growing on SF could not be determined, cell counts were replaced by DNA quantification with the PicoGreen assay. Therefore, it is difficult to compare the current release data with that of our previous study, where the release was normalized according to cell numbers.

Glucose permeability studies demonstrated a high glucose flux through the SF films used in the present work. Recent diffusion studies, including small molecular drugs and oxygen permeation through silk fibroin membranes (Chen and Minoura, 1994), further support the quality of silk fibroin and its possible application as a material for bioincubators suitable for cell based drug delivery.

The results of this study demonstrate that the differentiation of adenosine kinase deficient ES cell derived glial precursor cells into astrocytes was efficient on silk fibroin. We demonstrated that silk fibroin supports the release of adenosine from the differentiated Adk-/- astrocytes. We conclude that silk fibroin constitutes a suitable substrate for the directed differentiation of ES cell derived progeny and for the cell-mediated therapeutic release of adenosine. Thus, future developments might allow the construction of silk- and Adk-/- stem cell based bioincubators for the treatment of partial epilepsies.
Acknowledgements

We thank Trudel Inc. (Zurich, Switzerland) for providing the silk cocoons. Financial support was from the Association for Orthopaedic Research, from ETH Zurich (TH project), the US National Science Foundation (grant # 0436490), the NIH grant P41 (EB002520), the NIH grant R01 NS047622-01A2, the Good Samaritan Hospital Foundation, the Swiss National Science Foundation grant # 3100A0-100841, and the NCCR on Neural Plasticity and Repair.

References


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Cartilage-like tissue engineering using silk scaffolds and mesenchymal stem cells

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Tissue Engineering, 2006, accepted
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Abstract

Silk fibroin scaffolds were studied as a new biomaterial option for tissue engineered cartilage-like tissue. Human bone marrow derived mesenchymal stem cells (MSC) were seeded on silk, collagen, and cross-linked collagen scaffolds and cultured for 21 days in serum free chondrogenic medium. Cells proliferated more rapidly on the silk fibroin scaffolds when compared to the collagen matrices. The total content of glycosaminoglycan (GAG) deposition was three times higher on silk when compared to collagen scaffolds. GAG deposition coincided with overexpression of type II collagen and aggrecan genes. Cartilage-like tissue was homogeneously distributed throughout the entire silk scaffolds, while on the collagen and cross-linked collagen systems tissue formation was restricted to the outer rim leaving a doughnut appearance. Round or angular-shaped cells resided in deep lacunae in the silk systems and stained positively for type II collagen. The aggregate modulus of the tissue engineered cartilage constructs was more than two-fold higher when compared to the unseeded silk scaffold controls. These results suggest that silk fibroin scaffolds are suitable biomaterial substrates for autologous cartilage tissue engineering in serum free medium and enable mechanical improvements along with compositional features suitable for durable implants to (re)generate cartilage.
Introduction

Cartilaginous tissues have an intrinsic lack of regenerative potential, rendering it a target for tissue engineering approaches. Most engineering concepts are aimed at the (re)generation of hyaline cartilage, which covers the subchondral bone of diarthrodial joints (Poole et al., 2001). Articular cartilage damage is common due to fatigue in the aging population, young athletes and others, often resulting in defects exceeding 1 cm in diameter. Tissue engineering aims to restore this compromised function through the use of cells in combination with scaffolds to guide tissue repair. Osteoarthritis resulting from cartilage damage is problematic in the elderly and approximately 25% of the population over 60 has significant pain and disability caused by osteoarthritis (Decade, 2000). The degeneration of cartilage associated with this disease results in enormous economic costs and causes the most problems to populations after retirement age (Decade, 2000). Current treatment protocols for osteoarthritis, such as chondral shaving (McCarroll et al., 1983), abrasion arthroplasty (Menche et al., 1996), subchondral drilling (Pridie, 1959), chondrogenic cell and chondrocyte transplantation, or autologous tissue grafting (Wakitani et al., 1994, Brittberg et al., 1994) have limitations because of suboptimal integration with the defect zone and limited availability of autologous donor material.

A potential cell source for the tissue engineering of autologous cartilage are mesenchymal stem cells (MSCs), thereby potentially addressing cartilage breakdown by avoiding autologous grafting techniques. These cells proliferate well in culture while maintaining their capacity to differentiate along the chondrogenic, osteogenic or other mesenchymal pathways under appropriate culture conditions (Friedenstein, 1976, Friedenstein et al., 1987, Caplan, 1994, Mackay et al., 1998). For successful tissue engineering of cartilage, a mechanically robust and biocompatible biomaterial is needed on which MSCs undergo chondrogenic differentiation and form three-dimensional cartilage-like tissue. In particular, polyesters have been used such as poly(lactic-co-glycolic acid) or poly(lactic acid) (Caterson et al., 2001, Martin et al., 2001). However, these materials are problematic as (i) they can induce inflammation due to elevated acidity caused by polymer hydrolysis, (ii) processing difficulties may lead to inconsistent biodegradation rates and tissue response profiles, and difficulties in matching mechanical properties remain an issue (Athanasiou et al., 1996, Hollinger et al., 1996, Harris et al., 1998, Suh, 1998). Many hydrogel based systems, such as
those based on alginites, are compromised by a large gap between the mechanical features of a rather soft matrix and the need for mechanical robustness of the in vitro generated chondrogenic tissue. Recently, silks have been demonstrated to offer a new option to fill the niche. Silk scaffolds provide mechanical properties superior to collagen or hydrogels, and foster the deposition of cartilage in a homogenous and robust fashion due to the slower biodegradation at comparable and biocompatibility of these fibroin scaffolds when compared to collagen scaffolds (Meinel et al., 2004b). The purpose of the present study was to further develop the use of silk fibroin as a substrate for stem cell based cartilage tissue engineering towards clinical applications. Importantly, tissue formation was studied under serum-free conditions. This strategy was expected to minimize the risk of disease transmission and increase the level of control of serum composition. The present results were tracked by assessing transcription, protein-expression, and mechanical features.

Materials and Methods

Materials

Ascorbic acid-2-phosphate, Histopaque-1077, bovine insulin and dexamethasone were from Sigma (St. Louis, MO). BDTM ITS+ Premix was obtained from Becton Dickinson (Allschwil, Switzerland) and papain from Worthington Biochemical Corporation (Allschwil, Switzerland). Fetal bovine serum (FBS), Dulbecco's minimum essential medium (DMEM), Roswell park memorial institute medium (RPMI-1640), basic fibroblast growth factor (bFGF), transforming growth factor-b1 (TGF-b1), penicillin-streptomycin, fungizone, nonessential amino acids (NEAA, consisting of 8.9 mg/L L-alanine, 13.21 mg/L L-asparagine, 13.3 mg/L L-aspartic acis, 14.7 mg/L L-glutamic acid, 7.5 mg/L glycine, 11.5 mg/L L-proline, 10.5 mg/L L-serine) and trypsin were purchased from Gibco (Carlsbad, CA). BMP-2 was kindly supplied by Wyeth (Andover, MA). Silkworm cocoons were generously donated by M. Tsukada (Institute of Sericulture, Tsukuba, Japan) and Marion Goldsmith (University of Rhode Island). All other substances were of analytical or pharmaceutical grade and obtained from Sigma (St. Louis, MO).
Human bone marrow stromal cell isolation, expansion, and characterization

Total bone marrow (25 cm³, Clonetics, Santa Rosa, CA) was diluted in 100 ml of isolation medium (5% FBS in RPMI-1640 medium). Cells were separated by density gradient centrifugation. Briefly, 20 ml aliquots of bone marrow suspensions were overlaid onto a polysucrose gradient (d=1.077 g/cm³, Histopaque, Sigma, St.Louis, MO) and centrifuged at 800xg for 30 minutes at room temperature. The cell layer was carefully removed, washed in 10 ml isolation medium and pelleted. Cells were resuspended in expansion medium (DMEM, 10% FBS, 1 ng/ml bFGF, 1% NEAA) and seeded in 75 cm² flasks at a density of 5x10⁴ cells/cm². The adherent cells were allowed to reach approximately 80% confluence (12-17 days for the first passage). Cells were trypsinized, replated and passage 2 (P2) cells (80% confluence after 6-8 days), were used for the experiments.

hMSCs were characterized with respect to (a) the expression of surface antigens and (b) the ability to selectively differentiate into chondrogenic or osteogenic lineages in response to environmental stimuli, as follows. The expression of the following six surface antigens: CD14 (lipopolysaccharide receptor), CD31 (PECAM-1/endothelial cells), CD34 (sialomucin/hematopoietic progenitors), CD44 (hyaluronate receptor), CD71 (transferrin receptor/proliferating cells), and CD105 (endoglin) were characterized by Fluorescence Activated Cell Sorting (FACS) analysis, as in our previous studies (Meinel et al., 2004b, Meinel et al., 2004c). Cells were detached with 0.05% (w/v) trypsin, pelleted and resuspended at a concentration of 1x10⁷ cell/ml. Fifty µl aliquots of the cell suspension were incubated (30 min on ice) with 2 µl of each of the following antibodies: anti-CD44 and anti-CD14 conjugated with fluorescein isothiocyanate (CD44-FITC, CD14-FITC), anti-CD31 conjugated with phycoerythrin (CD31-PE), anti-CD34 and anti-CD71 conjugated with allophycocyanine (CD34-APC, CD71-APC), and anti-CD105 with a secondary goat-anti mouse IgG-FITC antibody. Cells were washed, suspended in 100 µl of 2% formalin, and subjected to FACS analysis.

To assess the potential of hMSCs for osteogenic and chondrogenic differentiation, the cells were cultured in pellets in control medium (DMEM supplemented with 10% FBS, Pen-Strep and Fungizone), or chondrogenic medium (control medium supplemented with 1% NEAA, 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 5 µg/ml insulin, 5 ng/ml TGF-b1) or osteogenic medium (control
medium supplemented with 50 µg/ml ascorbic acid-2-phosphate, 10 nM dexamethasone, 7 mM b-glycerolphosphate, and 1 µg/ml BMP-2). Cells were isolated from monolayers by trypsin and washed in PBS. Aliquots containing 2x10^5 cells were centrifuged at 300xg in 2 ml conical tubes and allowed to form compact cell pellets over 24 hours in an incubator (5% CO2, 37°C). Medium was changed every 2-3 days. After 4 weeks of culture, pellets were washed twice in PBS, fixed in 10% neutral buffered formalin (24 h at 4°C), embedded in paraffin and sectioned (5 µm thick). Sections were stained for general evaluation (haematoxilin and eosin, H&E), the presence of glycosaminoglycan (GAG with safranin O/fast green), and mineralized tissue (according to von Kossa in 5% AgNO3 for 1 hour, exposed to a 60 Watt bulb and counterstained with fast red). In addition, the amounts of GAG and calcium were measured as described below using n = 5-6 pellets per sample.

Silk scaffold preparation

Cocoons from Bombyx mori (Linne, 1785) were boiled 2 times for 1 hour in an aqueous solution of 0.02 M Na2CO3 and rinsed with water to extract the sericins, as previously described (Meinel et al., 2004a, Meinel et al., 2004d). Purified silk was dissolved in 9 M LiBr solution and dialyzed (Pierce, molecular weight cut off 2000 g/mol) against PBS for 2 days. The silk solution was lyophilized and resolved in hexafluoro-2-propanol (HFIP) to obtain a 17% (w/v) solution. Sieved granular NaCl with a diameter between 212 and 300 mm was weighed in a Teflon container and silk/HFIP solution was added at a ratio of 20:1 (NaCl/silk). HFIP was allowed to evaporate for 2 days and NaCl/silk blocks were immersed in 90% (v/v) methanol for 30 minutes. Blocks were removed, dried and NaCl was extracted in water for 2 days. Disk shaped scaffolds (5 mm diameter, 2 mm thick) were prepared using a dermal punch (Miltey, Lake Success, NY) and autoclaved.

Collagen and crosslinked-collagen scaffolds preparation

Collagen scaffolds were punched from sheets of Ultrafoam (collagen hemostat obtained from Davol, Cranston, RI). Ultrafoam is water-insoluble, partial HCl salt of purified bovine dermal (corium) collagen formed as a sponge with large (at the order
of 100 µm) interconnected pores. Discs had dry dimensions of 5 mm diameter x 2 mm thickness.

To reduce the rate of degradation, collagen scaffolds were cross-linked using the 1-ethyl-3-(3-dimethylaminopropyl) carboadiimide hydrochloride/N-hydroxy succinimide (EDC/NHS) chemistry previously established for collagen films and fibers (Sofia et al., 2001) and used in our previous work to modify Ultrafoam scaffolds for tissue engineering applications (Meinel et al., 2004c). This method of chemical cross-linking was shown to decrease the rate of degradation of collagen by two orders of magnitude (Sofia et al., 2001). Briefly, collagen scaffolds were incubated for 30 min in a MES buffer (0.1 M of 4-Morpholinoethanesulfonic acid MES, 0.5 M NaCl, pH 6). The cross-linking reaction was carried out using a solution of 1.713 g EDC and 0.415 g NHS in 100 ml 0.1 M MES buffer, using 100 ml solution per gram of collagen. The reaction was allowed to proceed for 4 h under gentle shaking and was stopped by washing for 2 h with 0.1 M Na2HPO4, and scaffolds were extensively rinsed with water (4 times for 30 min). The entire procedure was carried out aseptically.

Scaffold characterization

Porosity of unseeded scaffolds was determined by helium gas pycnometry using a Micrometrics Accupyc 1330 device (Micromeritics, GmbH, Moenchengladbach, Germany). The measurement on the vacuum-dried scaffolds was performed in quintuplicate. The microstructure of the surface of dry and platinum-coated scaffolds was characterized using scanning electron microscopy (SEM, Zeiss Leo Gemini 1530).

Tissue culture

Passage 2 mesenchymal stem cells (1x10^6 cells) were suspended in 20 ml DMEM and the suspension was seeded onto prewetted scaffolds. Seeded scaffolds were placed in 24-well cell culture plates (non tissue culture treated, Becton Dickinson, Allschwil, Switzerland) and incubated at 37°C for 2 hours to allow cell attachment. To keep the constructs wet, 10 µl DMEM was added every 15 minutes. Subsequently, 1 ml FBS-free chondrogenic medium was added per well. Chondrogenic medium was DMEM supplemented with Pen-Strep, Fungizone, 100 nM dexamethasone, 50 mg/ml ascorbic acid-2-phosphate, 40 mg/ml proline, 6.25 mg/ml ITSTM, 6.25 mg/ml bovine insulin
and 10 ng/ml TGF-b1. Medium was exchanged every 2 days for a total culture period of 21 days.

**Biochemical analysis, histology and immunohistochemistry**

Constructs were cultured for 21 days in chondrogenic medium and processed for biochemical analysis, histology and immunohistochemistry. For DNA analysis, n=3 constructs per group and time points (3, 6, 9, 12, 15, 18, 21 days) were disintegrated using steel balls and a MiniBead beater (Biospec, Bartlesville, OK). DNA content was measured fluorometrically using the PicoGreen assay (Molecular Probes, Basel, Switzerland), according to the protocol of the manufacturer (excitation wavelength of 480 nm; emission wavelength of 528 nm). To measure the amount of GAG, samples (n=3) per group and time points (3, 6, 9, 12, 15, 18, 21 days) were digested for 16 hours with 1 ml 500 U/ml papain solution in buffer (0.1 M disodium hydrogen phosphate, 0.01 M EDTA disodium salt, 14.4 mM L-cysteine) at 60°C. GAG content was determined spectrophotometrically (Cary 50, Varian, Zug, Switzerland) at 525 nm following binding to the dimethylmethylen blue dye (Farndale et al., 1986) using chondroitin sulphate as standard. To measure the amount of calcium in cell pellets, samples (n=3-4 per group) were extracted twice with 0.5 ml 5% trichloroacetic acid. Calcium content was measured spectrophotometrically at 575 nm following the reaction with o-cresolphthalein complexone according to the manufacturer's protocol (Sigma).

For histology, constructs were fixed in 10% neutral buffered formalin (24 h at 4°C), dehydrated in graded ethanol solutions, embedded in paraffin, bisected through the center, cut into 5 mm thick sections and mounted on SuperFrost microscope slides (Microm International AG, Volketswil, Switzerland). To stain for GAG, sections were treated with eosin for 1 minute, fast green for 5 minutes, and 0.2% aqueous safranin-O solution for 5 minutes, rinsed with distilled water, dehydrated through xylene, mounted and placed under a coverslip. To immunostain for type II collagen, a monoclonal antibody against type II collagen (2B1.5, dilution 1:100, Neomarkers, P.H. Stehelin & Cie AG, Basel, Switzerland) was used. Paraffin embedded tissue sections were deparaffinized through a series of graded alcohols and treated with protease II for 16 minutes. The primary antibody was added to each slide and the slide was incubated
for 32 minutes at room temperature in a humidified chamber. The secondary antibody (horseradish peroxidase) was then applied and developed according to the manufacturer's protocol (DAKO Corporation, Carpinteria, CA).

**RNA isolation and Real-Time Reverse Transcription Polymerase Chain Reaction (Real-Time RT-PCR)**

Fresh constructs (n=5-6) per group and time point (0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 21 days) were transferred into 2 ml plastic tubes containing 1 ml Trizol (Invitrogen AG, Basel, Switzerland) and 2 steel balls per construct. Scaffolds were disintegrated using steel balls and a MiniBead beater. Tubes were centrifuged at 12,000g for 10 minutes and the supernatant was transferred to a new tube. Chloroform, 190 ml, was added to the solution, vortexed and incubated for 5 minutes at room temperature. Tubes were again centrifuged at 12,000xg for 15 minutes and the upper aqueous phase was transferred to a new tube. One volume of 70% ethanol (v/v) was added and applied to an RNeasy minispin column (Qiagen, Hombrechtikon, Switzerland). The RNA was washed and eluted according to the manufacturer's protocol.

The RNA samples were reverse transcribed in cDNA using oligo (dT)-selection according to the manufacturer's protocol (SuperScriptTM First-Strand Synthesis System for RT-PCR, Invitrogen AG, Basel, Switzerland). Collagen type II and aggrecan gene expression was quantified using the ABI Prism 7000 Real Time PCR system (Applied Biosystems, Rotkreuz, Switzerland). PCR reaction conditions were 2 minutes at 50°C, 10 minutes at 95°C, 50 cycles at 95°C for 15 seconds, and 1 minute at 60°C. The data were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). Probes were labeled at the 5' end with fluorescent dye FAM (VIC for GAPDH) and with the quencher dye TAMRA at the 3' end. Primer sequences for the human GAPDH gene were: Forward primer 5'-ATG, GGG AAG GTG AAG GTC G-3'; Reverse primer 5'-TAA AAG CCC TGG TGA CC-3'; Probe 5'-CGC CCA ATA CGA CCA CCA AAT CCG TTG TTG AC-3'. Primers and probes for human collagen type II and human aggrecan were purchased from Applied Biosystems (Assay on Demand, #Hs00156568_m1 and #Hs00153936_m1, respectively).
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Mechanical testing and analysis

Specimens for mechanical testing were punched from the central regions of the engineered cartilage tissue on silk scaffolds and on silk scaffolds cultured under the same conditions but without cells using a biopsy punch (Stiefel Laboratorium, Germany). The cylindrical plugs were tested in confined compression using a mechanical testing system (EnduraTec ELF 3200, Bose Corporation, Minnetonka, MN) with a 22 N low force cell (Model 31, Honeywell Sensotec, Columbus, OH). Each specimen was transferred into a smooth confining chamber (diameter 3.62 mm) and loaded with a porous sintered filter (diameter 3.54 mm, pore size 45 mm, porosity 45%, Schunk Sintermetalltechnik, Germany) in PBS. The indentation site was the top layer of the engineered cartilage specimen. Thickness of the samples was measured after equilibration under a tare load of 0.02 N. Strains of 5%, 10% and 15% were applied in a stepwise manner at a rate of $V = 1$ mm/s with 1800s relaxation after each step. The aggregate modulus HA was calculated from the slope of the best linear fit of the equilibrium stress plotted against the applied strain.

Statistical analysis

For evaluation of statistical significance, samples were evaluated using a student t-test as well as ANOVA where appropriate. ANOVA was followed by a post-hoc assessment using the Tukey HSD method. Differences were considered significant when equal or less than $p=0.05$ using SPSS software (Chicago, IL).
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**Results**

*Scaffold properties and stem cell characteristics*

Scaffolds were prepared from bovine Type I collagen, native and cross-linked, and from silk fibroin, isolated from B. mori cocoons. All scaffolds had a porosity of approximately 95%, with the collagen scaffold exceeding those of the silk (p<0.05) and the cross-linked collagen (p<0.01; **Figure 1A**) scaffolds. The structure of the scaffolds prepared from the different biomaterials was similar, with the silk scaffolds having a more sheet like and spongy structure when compared to the collagens (**Figure 1 B,C**).
The mesenchymal stem cells used in this study demonstrated proliferative potential when analyzed over 5 passages, with approximately a 14 fold increase in biomass per passage determined by DNA assay. Cellular surface antigen expression patterns were CD14+, CD31-, CD34-, CD44+, CD71+, and CD105+, suggesting the presence of mesenchymal cells and demonstrating the absence of other cells of hematopoietic or endothelial origin. In addition, the cells differentiated selectively into cartilage and bone depositing cells in a pellet culture and in response to TGF-b1/insulin and BMP-2, respectively (data not shown). Due to their proliferative capacity, their antigen surface expression pattern and their potential to differentiate along the chondrogenic and osteogenic lineages, respectively, these cells are referred to as human mesenchymal stem cells (hMSC) in this study.

![Figure 2: Biochemical data of hMSC grown for 21 days on silk, crosslinked collagen and collagen scaffolds. Cell numbers indicated as amount of DNA per scaffold (A), and deposition of glycosaminoglycans expressed as chondroitin sulphate per scaffold (B).](image)

**Proliferation and chondrogenic differentiation**

MSC proliferation rate was high within the first 9 days of culture on silk and cross-linked collagen scaffolds, but significantly lower on the uncrosslinked collagen at day 6 (p<0.05) and insignificant at day 9. Cells continued to proliferate on the silk scaffolds although with a lower rate. Total DNA per scaffold was significantly higher on silks at day 15 when compared to the native and cross-linked collagens (p<0.01) and at days 13 (p<0.05), 17 (p<0.01), and 21 (p<0.05) when compared to the native...
collagen (Figure 1A). Cross-linked collagen and silk scaffolds retained their wet weight, whereas the wet weight of native collagen scaffolds was reduced to approximately 50% of the initial wet weight during the 21 days of culture (data not shown).

The MSCs, differentiating along the chondrogenic lineage, deposited sulfated glycosaminoglycans (GAG), a major component of the cartilage matrix, on all three biomaterials. GAG deposition was first observed at day 7, with a maximum deposition rate between days 7 to 12, after which GAG deposition leveled off. GAG deposition was significantly higher on silk when compared to the native and cross-linked collagens at day 7 (p<0.05) and at all later time points (p<0.01), with the exception of day 9 (insignificantly higher; Figure 1B).

Cartilage-like tissue deposition was corroborated at the transcriptome level and characterized by over-expression of type II collagen by MSCs on all three biomaterials. In general, collagen type II expression followed a biphasic expression pattern, with a small peak at day 7 on silk scaffolds and a second and larger peak at day 17 on silk and collagen. Relative peak levels of overexpression ranged from 50,000- to 150,000-fold when compared to MSCs at day 0 of culture and no significant differences were found among groups at any time point (Figure 3A). Aggrecan transcript overexpression was induced at days 7 and 9 followed by lower induction at day 19 on all biomaterials. No significant differences were found among groups at any time point, and relative peak levels of aggrecan transcript overexpression ranged from 40 to 80 fold when compared to MSCs at day 0 of culture (Figure 3B).
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The deposition of a negatively charged matrix, determined by safranin O staining and characteristic for chondrogenic tissues, was observed on all biomaterials, with cells residing in intensively staining lacunae as observed in cross sections of the scaffolds (Figure 4 A-C). GAG deposition was more pronounced at the scaffold rim for the native and cross-linked collagen but spanned throughout the silk scaffolds with a homogenous deposition throughout the scaffold diameter. Advanced scaffold degradation was observed for the native collagen scaffolds but not for the silks and cross-linked collagens, respectively.

![Silk](image1.png) ![Crosslinked Collagen](image2.png) ![Collagen](image3.png)

**Figure 4**: Production and localization of extracellular matrix components by hMSC on silk (A, D), crosslinked collagen (B, E) or collagen (C, F) scaffolds. Staining of sulphated GAG (safranin-O; A, B, C) and type II collagen (D, E, F). Scale bars 200 mm.

The chondrogenic nature of the deposited cartilage was corroborated by the deposition of type II collagen – the major collagenous component of chondrogenic matrices. Positive staining for type II collagen was in particular found in the lacunae and around the cells (Figure 4 D-F). Tissue deposition resulted in an increase in overall scaffold thickness after 21 days of culture on the silk scaffolds (p<0.05; Figure 5A). The elastic modulus increased from 0.02 MPa for the unseeded silk scaffolds after 21 days of incubation in culture medium to 0.04 MPa for the tissue engineered cartilage on silk scaffolds after 21 days of culture (p<0.05; Figure 5B). The mechanical properties of collagens scaffolds were not assessed due to advanced biodegradation which resulted...
in shrinkage and disintegration. Constructs based on cross-linked collagen scaffolds were assessed but demonstrated large within-group variability; excluding conclusions in terms of mechanical properties for this material (data not shown).

Discussion

Cartilage-like tissue was engineered starting from MSCs and highly porous and structurally stable silk fibroin, with cross-linked collagen scaffolds as well as noncrosslinked collagen scaffolds for comparison. All differentiation studies were run in serum free media. Homogenous cartilage-like tissue, characterized by GAG deposition (Figure 2B, 4 A-C), type II collagen deposition (Figure 4 D-F), and aggrecan and type II collagen mRNA overexpression (Figure 3), was formed throughout the silk scaffolds. In contrast, cartilage formation on the native and cross-linked collagens was restricted to the borders of the scaffolds, and more specifically noted for the native collagen which was substantially degraded throughout culture.

For clinical application of tissue engineered cartilage, it is imperative that in vitro culture protocols should be devoid of animal or human products, to preclude from potential contamination with pathogens or related bioburdens. Furthermore, avoidance of culture medium additives reduces variability within the culture milieu due to variations in serum batches, therefore, providing more stringent quality control of tissue outcomes. Finally, the adherence of serum pathogens, as possible contaminants in serum-containing media, to cell or scaffold surfaces may increase immunogenicity of implanted tissue engineered systems (Heng et al., 2004). Therefore, for differentiation of the MSCs, serum containing media were replaced by completely characterized and serum-free media in this study. Generally, serum-free media results in a lower mitotic index, apoptotic responses, and poor adhesion to surfaces for MSCs (Wong and Tuan, 1993). Despite these findings in micromass cultures, the use of serum-depleted medium in this study resulted in better proliferation rates of the MSCs in differentiation medium on silk but not on collagen scaffolds, respectively, as described in earlier studies using medium supplemented with serum (Meinel et al., 2004b). These data suggest that the nature of the substrate is important in terms of interactions with media components, and this interaction can significantly impact tissue-related outcomes. In the present study, the combination of silk fibroin scaffolds with serum free medium was superior to the collagen based materials, either cultured
in presence or absence of serum, in terms of cartilage-related outcomes. In principle, such differences could stem from variations in surface area between silk and collagen based scaffolds. However, this is unlikely for the scaffolds prepared in the present study, as they share similar surface morphologies and porosities (Figure 1).

![Figure 5: Thickness (A) and aggregate modulus (B) of unseeded silk scaffolds and tissue-engineered cartilage-like tissue after 21 days in culture medium. (p<0.05)](image)

Similarly, the deposition of GAG was an order of magnitude higher on both collagens and silk in serum free media when compared to medium supplemented with serum, in accordance with earlier reports (Barry et al., 2001, Barry, 2003). The progression of GAG deposition coincided with strong overexpression of type II collagen and aggrecan. No differences were observed between groups, mainly due to the large standard deviations (Figure 3). In the presence of serum-containing media, no differences in chondrogenic potential were observed for silk or cross-linked collagen scaffolds, respectively (Wong and Tuan, 1993). In contrast, using serum-free medium, chondrogenic performance was significantly improved, approximately 3-fold, on silk when compared to cross-linked collagen. Similar findings in the case of both proliferation and chondrogenic differentiation have been described for scaffolds prepared from poly(lactic acid) and poly(glycolic acid) when compared to collagen scaffolds (Freed et al., 1993). It is well established from insights into extracellular matrix function, that biomaterial surfaces may have a substantial effect on the action of growth factors on cellular performance. For example, type II collagen has been shown to affect TGF-b impact on chondrocytes enhanced by non-denatured type II collagen while heat-inactivated type II collagen had no effect (Qi and Scully, 1998, Qi and
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Scully, 1997). The data reported in this study suggest that chondrogenic effects mediated by TGF-b/insulin are significantly stronger on silk when compared to collagen and cross-linked collagen scaffolds, respectively, a substrate difference that was not evident in the presence of 10% serum in earlier studies, possibly due to the presence of adsorbed serum proteins on the different biomaterial surfaces. Another important consideration for clinical applications is the biocompatibility of the implanted construct. Silks were initially associated with adverse immunological reactions in sutures used for cataract surgery. These early findings prompted the replacement of silkworm silk sutures with nylon ~20 years ago (Soong and Kenyon, 1984). However, these adverse reactions to silks were due to the presence of residual sericin, a family of glue-like proteins that coat the core silk fibers, and not the fibers (fibroin) (Altman et al., 2003, Panilaitis et al., 2003). Implantation of properly purified silk fibroin films into muscular pouches of rats, provoked less immunogenic responses than polylactide films and were equivalent to collagen films (Meinel et al., 2005). These differences were recently corroborated in a detailed study of the inflammatory potential of silks based on C3 activation, fibrinogen adsorption, and mononuclear cell activation, - among other measures - in comparison to polystyrene and poly(2-hydroxyethyl methacrylate). The silks were not different from these model surfaces in terms of humoral responses related to inflammation, and the degree of activation and adhesion of the immunocompetent cells was less of a problem (Santin et al., 1999). Our data, both in vitro (Santin et al., 1999) and in vivo (Meinel et al., 2005), illustrate that silkworm silk is at least as biocompatible as other biomaterials in use today and tested in this study.

Tissue engineered implants for cartilage repair must have adequate mechanical integrity to withstand implantation procedures including handling, as well as the mechanical forces typically experienced at joint surfaces. Typically, aggregate moduli of native hyaline cartilage are reported to range from 0.53–1.82 MPa as a function of harvest location and species (Athanasiou et al., 1991). The aggregate modulus of the tissue engineered cartilage reported here is one order of magnitude lower. Future studies are aimed at closing this gap through the use of rotating bioreactors to facilitate nutrient, gas, and metabolite exchange to increase the levels of new ECM production, as well as the use of mechanical stimuli during culture with more advanced bioreactors (Vunjak-Novakovic et al., 2002).
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In conclusion, this study demonstrates that cartilage-like tissue can be engineered on silk scaffolds under serum free and clinically acceptable conditions, forming an implant rich in GAG, type II collagen and aggrecan, and resulting in mechanical properties that offer a path toward similar features found in native hyaline cartilage. These findings expand the available options for biopolymer implant materials by a durable and mechanically robust option as a substrate for tissue engineering of cartilage and the treatment of cartilage defects in vivo.

Acknowledgments

This work was supported by the ETH Zurich (TH Gesuch), Association for Orthopaedic Research (AFOR), and the NIH (NIH P41 EB002520).

References


Conclusions and outlook

Lorenz Meinel
Biotechnological development - as also demonstrated in these studies - has successfully employed autologous cells for the generation of in vitro engineered tissues. This success required a combination of detailed knowledge from different research fields, such as material sciences, cell biology, immunology, pharmaceutics, or chemical engineering.

The efforts presented here, at the interface of biomaterials, drug delivery and cell biology, were motivated by insufficiently met needs in the field of tissue engineering. At the biomaterial edge, these resulted from the introduction of a new protein biomaterial - silk fibroin. The novelty of the material resulted in a series of efforts to meet specifications related to biocompatibility, biodegradability, and drug stability, which was either adsorbed to the silk-fibroin surfaces, encapsulated within the silk-fibroin or covalently bound to the silk-fibroin surfaces, respectively. The ultimate intended application in man narrowed our choices to organic solvents during manufacture, listed in the International Conference of Harmonization (ICH) guidelines with acceptable limits for residual solvents as stated in this document. These limits are generally recognized as safe (GRAS). As a result, we provided manufacturing protocols, which circumvented the previous use of not listed solvents for silk-fibroin implant manufacture as used in previous protocols - e.g. hexafluoropropionate - and presented a mainly water based process. This protocol became possible by understanding and mimicking aspects of silk spinning in spiders or insects, which naturally are restricted to water as a solvent. At this end, some challenges must still be met. For example, the control of porosity, interconnectivity, and pore diameters, was controlled by use of paraffin as a porogen, and hexane as a leaching solvent. We could not entirely remove the hexane from the scaffold but reduce it to limits far below concentrations GRAS according to the ICH limits (Chapter 2). In conclusion, this work outlined a path for the development of silk-fibroin based implants with methods and materials complying with clinical demands and complying with most governmental guidelines.

From these and previous studies we had evidence, that the scaffold structure determined the geometry of the engineered bone-like tissue. Consequently, we designed silk-fibroin scaffolds, with different pore diameters on the same implant. In conclusion, this allowed for the controlled engineering of bone-like geometries over a wide range of pore diameters as a prerequisite to adapt engineered implant structure to
anatomical constraints at implantation sites. This work is based on the hypothesis, that adapting engineered bone geometries to the anatomical constraints at the implantation sites, translates into a clinical benefit. Therefore, future in vivo studies can start off from Chapters 2 and 3 to approach these questions using the provided implant materials. Possible read-outs should include the vascularization and cell ingrowth as a function of pore design for both, the host bone at the implantation site and the implant. We are currently addressing this hypothesis in a murine cranial defect model to evaluate e.g. if engineered structures retain after implantation or e.g. to which extent, pre-engineered structures are subject to bone remodeling along with detailing vascularization, fibrous tissue formation, foreign body reaction, or the presence of neutrophils and lymphocytes (unpublished data).

Quality assessment of individual implants prior to insertion into defect sites is a prerequisite for clinical use as pooled information is of limited interest for the selection of an implant. Therefore, destructive end point assessments are not compatible with the clinical demand of implant selection. Therefore, we became interested in non-destructive imaging methods, which allow the visualization and quality assessments of individual implants using μ-CT. A feasibility study is demonstrated in Chapter 4. This research area is still in its infancy in our laboratory. We have recently gained speed in this area with a novel designed bioreactor, which combines on line monitoring with a stimulation device. The device induces torsional or compressive movements and by means of pressure sensitive elements can measure the resistance of the implant over time (experimental data pending). The beauty of this system - which is under investigation at present - stems from the possibility, to image the bending of individually engineered trabecula under mechanical compression. This principally allows for predictions of possible weak points in the implant. We believe this might direct towards novel methods for quality assessments, needed for an adequate level of analysis, prior to implant selection and insertion. However, the safety risks of this analysis - the imaging is based on μ-CT, i.e. X-rays - are not evaluated at present and, therefore, one should balance our expectations with a proper perspective until e.g. genotox safety is demonstrated.

Another focus in our laboratory - apart from the work on biomaterials - was at the interface to drug delivery. Drug delivery, when defined as a controlled method to achieve spatially and locally controlled levels of drugs, can be achieved by various
means, for example from reservoirs (Chapter 6, 7), or from cells, either virally transformed cells providing transient regulatory molecule release (Chapter 9) or stably transfected cells (Chapter 10), providing a continuous release of regulatory molecules. All these options were successfully implemented into our settings and demonstrated the wide utility of silk-fibroin materials as substrates for tissue engineering, and drug delivery.

The materials and drug delivery systems were - at least in part - evaluated for their in vivo relevance. We consistently confine our in vivo evaluations to two species and a single breed within the species - rat and mouse - and two defect models, which are both critical sized; a cranial defect model in mice and a mid diaphyseal, segmental defect model in rats with external fixation. Chapters 5 and 8 evaluated the in vivo relevance for silk-fibroin scaffolds (SS) alone, SS freshly seeded with stem cells, SS with tissue engineered bone, and SS with encapsulated bone morphogenic protein 2 (BMP-2), among other groups. Interestingly, the seeding with stem cells did not increase the osteogenic response upon implantation, rendering this approach questionable for clinical use. It seems, that seeding with these osteogenic progenitor cells and insertion of these constructs into a defect site is insufficient to trigger osteogenic differentiation of the cells. In contrast, the missing osteogenic responses is alarming as they can ultimately lead to fibrous tissue formation. In contrast, tissue engineered bone always resulted in a substantial healing of the defect sites and, therefore, remains an option for a clinically viable scenario. Interestingly, mineralization in response to implantation of BMP-decorated implant materials was in the same range as for tissue engineered bone.

This interesting finding directs towards studies to compare the performance of tissue engineered bone versus BMP-2 decorated implants when implanted into defect sites. These pending studies are planned using silk-fibroin scaffolds, covered with hydroxyapatite layers, in which BMP-2 will be entrapped and slowly released when compared to hydroxyapatite covers without BMP-2 and scaffolds with entrapped BMP-2.

As outlined, bone and cartilage are complex tissues, whose formation is triggered by numerous growth factors and cytokines as well as interaction with other cells (osteoclasts, osteocytes, osteoblasts, etc.). For the moment, a duplication of at least parts of this complex regulatory environment is most likely the most challenging aspect to engineer tissues of higher similarity as compared to their natural model.
Although advances in the understanding of bone biology have been made, their translation into applied settings is difficult. Also, further knowledge is required to understand how the growth factors interact in concert with cells and how target pathways can be selectively activated or inactivated. Similarly, a better understanding of cellular migration events into defect sites will help to work towards decorated scaffolds that home cells selectively into defect sites.

Besides protein-based cytokines and growth factors, small chemical compounds should be evaluated to promote cell proliferation and differentiation. From a stability standpoint, these compounds promise prolonged shelf lives, and tend to be less prone to chemical and physical degradation when compared to proteins and peptides. This is in particular needed, as after implantation the compounds are exposed to elevated temperatures further compromising stability when compared to storage at 2-8°C. Furthermore, proteases can degrade biologicals. First evidence for the feasibility of this approach comes from chemicals with known morphogenic and teratogenic properties, such as retinoic acid (a vitamin A derivative), triggering neurogenic differentiation or embryonic stem cell differentiation into cardiomyocytes. Another example is dexamethasone, a steroid that has been demonstrated to facilitate osteogenic differentiation of mesenchymal cells. However, due to their teratogenic potential and lack of safe application, these compounds are more likely suitable for in vitro applications. Future work may find specific chemicals that specifically target pathways to selectively drive differentiation along desired pathways possibly opening this research direction for human use.

Another promising avenue to direct cellular differentiation is the application of physical stimuli. The physical trigger can come from mechanical devices, or magnetic fields in presence of e.g. ferric particles. Osteogenic differentiation has been demonstrated to be positively influenced in presence of mechanical stimulations and resulted in up regulations of osteogenic marker genes. Application of heat and, therefore, up-regulation of heat shock protein expression has been demonstrated to support cellular differentiation. Heat shock proteins are involved in glial differentiation, neural plate induction in embryonic development, and erythroid differentiation. However, these stimuli lack specificity when randomly applied and the use of this method is more in a conceptual phase at present.
Although a large number of studies detailing adult or embryonic differentiation is present, this particular field of research is still more in its infancy, when it comes to its transfer into directed and selective differentiation of cells in tissue engineering. The control of differentiation might be enhanced, when various techniques such as mechanical stimulation, induced differentiation by biologicals, and others are used in combination. In their natural milieu, all these factors are present and involve multiple signaling pathways which commit cell differentiation much more efficiently. The transfer of these insights into the application in tissue engineering holds promise to synergistically improve differentiation.

One of the more difficult challenges, however, might be the development of safe and acceptable culture conditions from a patients and health authority perspective. The transplantation of stem cell loaded implants must rely on well defined culture conditions in chemically defined environments. Ideally, these implants can be grown in environments which are devoid of contact with any material of animal or human origin - of course apart from the autologous cells. This level of control would provide the prerequisite for a clinical application of tissue engineered implants within larger scale clinical settings. Substantial further effort will be needed to achieve this goal.
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*Books and book chapters*


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*Selected oral presentations*

Meinel L. Drug delivery from biopolymers, NIH panel session, August 4, 2005, Cambridge, MA

Meinel L. Characterization of mesenchymal stem cells, SSB Meeting, July 1, 2005, Davos, Switzerland

Meinel L. Tissue engineering of osteochondral plugs using human mesenchymal stem cells and silk scaffolds, June 25 – 27, 2004, Belgrade, Serbia and Montenegro

Meinel L. Silks for (re)-generation of musculoskeletal tissues, Group Seminar Pharmaceutics, June 15, 2004, Regensburg, Germany

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mesenchymal stem cells cultured on collagen scaffold, ACS National Meeting 2004, March 28 – April 1, Anaheim, CA
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Meinel L. Controlled local delivery of IGF I induces new bone formation. 3rd World Meeting on Pharmaceutics and Biopharmaceutics Pharmaceutical technology, April 3-6, 2000, Berlin, Germany
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Teaching experience

2004-present
Drug Formulation and Delivery (Master in Human Biology University of Zürich)
  - Introductory course in drug delivery and tissue engineering

Tissue Engineering and Drug Delivery (Master in Biomedical Engineering Program, ETH Zurich)

Pharmaceutical Technology (Institut für Pharmazeutische Wissenschaften, ETH Zurich)
  - Lectures in drug delivery, tissue engineering, and drug delivery kinetics
  - Seminar to the “Praktikum Arzneiformenlehre”
  - Practical courses in solid dosage forms

1999-2001
Assistant in Pharmaceutical Technology (Institut für Pharmazeutische Wissenschaften, ETH Zurich)
  - Practical courses in solid and liquid dosage forms
## Curriculum Vitae

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