Doctoral Thesis

Interfacial Electrochemistry of Blood Coagulation Factors
Fundamentals and Applications

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Interfacial electrochemistry of blood coagulation factors: Fundamentals and applications

A thesis submitted to attain the degree of

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presented by

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Blood coagulation factors are proteins involved in a complex cascade of biochemical reactions regulating hemostasis, which is the physiological process by which bleeding is stopped. One of the fundamental steps in hemostasis is the transition from the liquid state of blood to the gel state; transition that we refer to as blood coagulation. This transition is mediated by the polymerization of the blood coagulation factor I (fibrinogen) and consequent formation of fibrin fibers. The polymerization process is triggered by the activation of fibrinogen, mediated by the coagulation factor II (thrombin). Finally, the transglutaminase coagulation factor XIII crosslinks fibrin fibers to chemically and mechanically stabilize the clot.

This thesis constitutes an experimental study of the electrochemical behavior of fibrinogen and coagulation factor XIII at interfaces. First studies on the electrochemical behavior of coagulation factors date the 1960’s and 1970’s. The rapid development of novel techniques in cardiac surgery occurring in those years, led scientists and physicians to study the interaction between blood and its components with artificial surfaces, in an attempt to find minimally thrombogenic implant surfaces. P. N. Sawyer (1926-2014) pioneered the study of the electrochemistry of blood, and demonstrated the influence of surface potentials and electrochemical reactions involving blood coagulation factors in coagulation at interfaces.

In the first part of this thesis, I employed surface-sensitive techniques to study the coagulation of blood at electrified interfaces and the behavior of fibrinogen at cathodic and anodic potentials. The work of Sawyer and co-workers, extensively described in Chapter 1, represents the starting point of my investigation. In particular, using
the quartz crystal microbalance with dissipation monitoring technique, coupled with an electrochemical setup, I first confirmed the reduced coagulation of blood at cathodic potentials. Subsequently, by using surface-sensitive and proteomics techniques, I investigated the nature of the fibrinogen cathodic products and its ability to electropolymerize at anodic potentials. This set of experiments has an impact in the field of bioelectronics, as electrodes are often in contact with blood for stimulation or sensing purposes. Understanding how blood components react at the interface with electrodes is of fundamental interest for their optimal design and operation. Furthermore, several of the aspects related to blood electrochemistry have an impact on the rational design of minimally thrombogenic blood-contacting devices.

In the second part of this thesis, I describe the use of electrochemically generated pH changes at interfaces to control the enzymatic activity of the coagulation factor XIII. Controlling the activity of factor XIII has for long been essential to rationally use this enzyme to crosslink food proteins to form gels. In recent years, researchers have developed synthetic hydrogels based on poly(ethylene glycol) (PEG) that can be crosslinked under physiological and biocompatible conditions via factor XIII. These gels are used in the biomedical field as soft substrates for the culture of cells in three dimensions. Engineering these substrates to mimic as closely as possible the natural tissue environment is an active field of research, and PEG gels are among the most used substrates to achieve this goal. In this work, I show that electrochemically controlling the factor XIII-mediated crosslinking of PEG gels can be used to create structured PEG gels. In particular, by exploiting the pH-dependent activity of this transglutaminase and the ability to locally modify the solution pH close to electrodes, I show the spatial control of the polymerization reaction. Hydrogel patterns on surfaces can be created rapidly and cost-effectively. In addition, the prevention of the crosslinking reaction at gel surface allows the fabrication of crosslinking density gradients that can be used to enhance the gel infiltration of cells seeded topically.
In conclusion, studying the nature and activity of blood coagulation factors at the interface with electrodes opens new perspectives in the development of new blood-contacting devices and in the manufacturing of structured soft materials for biomedical applications.
I fattori della coagulazione del sangue sono proteine implicate in una complessa cascata di reazioni biochimiche volte a regolare l’emostasi, ovvero il processo fisiologico responsabile dell’arresto di un’emorragia. Una tappa fondamentale di questo processo consiste nella transizione dallo stato liquido del sangue allo stato gelificato; transizione alla quale ci riferiamo con il termine di coagulazione del sangue. A mediare questa transizione è la polimerizzazione del fattore coagulante I (fibrinogeno) e la conseguente formazione di fibre di fibrina. Il processo di polimerizzazione viene iniziato a seguito dell’attivazione del fibrinogeno da parte del fattore coagulante II (trombina). Infine, la transglutaminasi fattore coagulante XIII è responsabile del cosiddetto crosslinking (legame covalente incrociato) delle fibre di fibrina, il quale dona stabilità chimica e meccanica al coagulo.

Questa tesi tratta lo studio empirico del comportamento elettrochimico del fibrinogeno e del fattore della coagulazione XIII a varie interfacce. I primi studi concernenti il comportamento elettrochimico dei fattori della coagulazione datano agli anni ’60 e ’70. Il rapido sviluppo nelle tecniche di cardiochirurgia verificatosi in quegli anni, spinse scienziati e medici a studiare l’interazione tra il sangue e le sue componenti con superfici artificiali, nel tentativo di trovare superfici con la minima propensità ad indurre la coagulazione sanguigna. P. N. Sawyer (1926-2014) fu pioniere nello studio dell’elettrochimica del sangue. Egli dimostrò il ruolo di potenziali di superficie e di reazioni elettrochimiche coinvolgenti fattori coagulanti nel processo di coagulazione in superficie.

Nella prima parte di questa tesi, ho utilizzato tecniche sensibili ai cambiamenti di superficie per studiare la coagulazione del sangue ad
interfacce elettrificate e il comportamento del fibrinogeno a potenziali catodici e anodici. Il lavoro di Sawyer e collaboratori costituisce il punto di partenza della mia ricerca. In particolare, ho utilizzato una tecnica denominata “quartz crystal microbalance with dissipation monitoring” in combinazione ad un apparato di misurazione elettrochimica ed ho confermato la ridotta coagulazione del sangue in prossimità di superfici catodiche. In seguito, sempre utilizzando tecniche sensibili a cambiamenti di superfici e tecniche di proteomica, ho investigato la natura dei prodotti catodici del fibrinogeno e la sua capacità ad elettropolimerizzare a potenziali anodici. Questi esperimenti hanno un impatto diretto nel campo della bioelettronica, in quanto elettrodi di vario tipo sono sovente in contatto con il sangue per applicazioni di stimolazione o di monitoraggio e rilevazione biologica. Comprendere le reazioni delle componenti del sangue all’interfaccia con elettrodi è di fondamentale importanza, al fine di ottimizzare il design e l’utilizzo di quest’ultimi. Inoltre, molti aspetti legati all’elettrochimica del sangue hanno un impatto diretto sul design di futuri strumenti ed apparecchiature che, a contatto col sangue, minimizzano la sua coagulazione in superficie.

Nella seconda parte di questa tesi, descrivo l’utilizzo di cambiamenti interfacciali di pH, generati elettrochimicamente, al fine di controllare l’attività enzimatica del fattore della coagulazione XIII. Il controllo dell’attività di questo enzima è da molto tempo essenziale per il suo utilizzo razionale nel legare proteine alimentari e produrre gel. Recentemente, ricercatori hanno sviluppato idrogel sintetici a base di glicole polietilenico (PEG), i quali possono essere formati in condizioni fisiologiche e biocompatibili attraverso l’azione del fattore XIII. QUESTI GEL SONO COMUNEMENTE UTILIZZATI NELL’AMBITO BIOMEDICO COME SUBSTRATI SOFFICI PER LA CULTURA CELLULARE IN TRE DIMENSIONI. L’INGEGNERIA DI TALI SUBSTRATI, VOLTA A RENDERLI IL PIÙ SIMILI POSSIBILE AL NATURALE AMBIENTE TISSULARE UMANO, RAPPRESENTA UN CAMPO DI RICERCA MOLTO ATTIVO. GLI IDROGEL A BASE DI PEG SONO TRA I SUBSTRATI PIÙ USATI PER QUESTO SCOPO. IN QUESTO LAVORO HO DIMOSTRATO CHE L’UTILIZZO DELL’ELETTROCHIMICA PER CONTROLLARE LA POLIMERIZZAZIONE DI GEL DI PEG PERMETTE LA PRODUZIONE DI AMBIENTI DI CULTURA CELLULARE...

In conclusione, lo studio della natura ed attività dei fattori di coagulazione all’interfaccia con elettrodi apre nuove prospettive nello sviluppo di apparecchiature a contatto con il sangue e nella fabbricazione di materiali soffici strutturati per applicazioni biomediche.
1 INTRODUCTION

1.1 ABSTRACT

In this chapter, I provide the reader with a review of the literature in the field of bioelectrochemistry of blood coagulation factors. I review the main fundamental studies involving blood coagulation factors at the electrode interface, which constitute the basis of the work presented in Chapters 3 and 4. To this end, I begin the chapter by introducing the general principles of hemostasis (the physiological process by which bleeding is stopped). I briefly present the blood coagulation factors I (fibrinogen), II (thrombin) and XIII, and their function in the final steps of blood clotting. I subsequently introduce two fundamental principles in the field of interfacial bioelectrochemistry: electron transfer reactions and interfacial pH gradients generated as a result of water electrolysis. Because of the importance of electrochemically generated interfacial pH changes for the work presented in this thesis, the introduction to the blood coagulation factors emphasizes their molecular stability at non-physiological pH.

Chapters 5 and 6 focus on a novel fabrication technique to produce structured hydrogels, which are used as scaffolds for the culture of cells in three-dimensions. This fabrication process is based on the electrochemical control of the blood coagulation factor XIII, used as a hydrogel crosslinking agent. To introduce this topic, I conclude this introduction chapter with a brief overview on the fast-growing field of three-dimensional cell culture, with an emphasis on the fabrication of structured hydrogel scaffolds.
1.2 **BLOOD COAGULATION FACTORS**

1.2.1 General principles of hemostasis

Hemostasis (from Greek heme, blood and stasis, halt) is the physiological process by which bleeding is arrested. Hemostasis is commonly divided in primary hemostasis, secondary hemostasis and fibrinolysis. When a blood vessel is injured, the first physiological response to limit bleeding is vasoconstriction. Subsequently, platelets start adhering to the exposed connective tissue at the site of injury to form a platelet plug (primary hemostasis). In order to reinforce and mechanically stabilize the platelet plug, coagulation of blood occurs. Coagulation is the process by which blood changes from a liquid state to a gel state (secondary hemostasis). Finally, once the vessel integrity has been restored, the blood clot is cleared by a process called fibrinolysis.

Blood coagulation factors are proteins involved in a complex cascade of biochemical reactions regulating hemostasis. In the following paragraphs, I will limit myself to briefly introduce the coagulation factors responsible for the final steps of the coagulation cascade, as they are the main subject of study in this thesis. The reader interested in an extensive description of the important and well documented physiological processes of hemostasis, can refer to a recent book covering molecular, cellular and pathological aspects.[1]

1.2.2 Thrombin-mediated fibrinogen conversion to fibrin

Blood coagulation starts with the conversion of fibrinogen (also factor I and from now abbreviated Fg) to fibrin strands mediated by the serine protease thrombin (also factor II).[2] Fg is a soluble, 340 kDa, rod-shaped protein present in blood plasma at a concentration ranging from 1.5 to 3 mg/mL. It consists of three pairs of chains referred to as α, β and γ chains joined in the central region of the protein (E domain) by disulfide bridges. The amino-terminal of the α chain is located in the E domain and constitutes the fibrinopeptide A (FPA) sequence (Figure 1.1 a). To initiate the conversion to fibrin, the FPA is cleaved by thrombin thus exposing a Fg polymerization site.
(Eₐ), comprising the residue motif Gly-Pro-Arg-Val.[3] This motif has high affinity for a binding pocket located in the lateral regions of the Fg molecule (D domains). This affinity drives the lateral overlapping middle-to-end arrangement of neighboring monomers and the formation of double-stranded fibrin fibrils. The cleavage of the FPA is required for the analogous and subsequent thrombin-mediated cleavage of the N-terminus of the β chain (fibrinopeptide B, FPB), implicated in the lateral aggregation of the fibrin fibrils (Figure 1.1 b).[4, 5] Lateral aggregation of fibrin fibrils results in branched three-dimensional (3D) fiber networks.[6]

1.2.3 Factor XIII-mediated fibrin crosslinking

Factor XIII is a blood plasma transglutaminase (TG) that crosslinks fibrin networks upon activation by thrombin and Ca²⁺ ions (in this thesis the abbreviation FXIII is used for the active and inactive form). Thrombin cleaves off a FXIII activation peptide and then, in presence of Ca²⁺ ions, the inhibitory subunit is released exposing the catalytic site.[7]

As any TG, FXIII catalyzes an acyl transfer reaction. The carboxamide group of a glutamine residue is the acyl donor and a primary amine is the acyl acceptor. In the fibrin(ogen) molecule the substrates of the FXIII are located in the C-terminal region of the γ chains. The γ-dimers are formed by the covalent bond between a lysine of one γ chain and a glutamine of another (Figure 1.1 b).

The role of FXIII-mediated crosslinking in hemostasis is the mechanical stabilization of the clot and the protection from fibrinolysis. From a material perspective, FXIII-mediated crosslinking
gives fibrin gels high elasticity, and particularly the transverse configuration of the covalent bond explains the elastic strain limit.\[8\]

1.2.4 pH-dependent activity of blood coagulation factors

In the following paragraphs, I will briefly review the effect of pH changes on the Fg nature, on the polymerization of Fg and on the FXIII-mediated crosslinking of fibrin. Covering these aspects is relevant for the subsequent description of the electrochemical behavior of blood coagulation factors.

1.2.4.1 Effect of pH on Fg molecular stability

Intuitively, alterations of the molecular nature of Fg can impact its clottability. In 1957, Fitzgerald and co-workers examined the pH, ionic strength and temperature conditions under which the molecular integrity and clottability of Fg are maintained.\[9\] Fg was shown to degrade at highly alkaline pH. When pH values approaching 13 were reached, light scattering examinations
revealed considerable molecular weight reduction, indicating protein fragmentation. In particular, the authors distinguished two fragments of 230 kDa and 14.5 kDa and attributed the fragmentation to the rupture of hydrogen bonds between tyrosine and arginine side chains. Structural and clottability changes could be observed already slightly above pH 11, however with no apparent fragmentation. On the other side, below its isoelectric point (pI, pH 5.5), Fg rapidly starts to denature to form soluble aggregates.[10]

1.2.4.2 Effect of pH on Fg polymerization
Two mechanisms can be identified to describe the effects of pH changes on the Fg conversion into fibrin. The first is the pH-dependent affinity of thrombin for Fg, showing a characteristic bell-shaped curve in the pH range 5-10 with an association peak around pH 8. It was reported that the assembled Fg monomers (also referred to as an intermediate fibrin polymer) are present only above a sharp pH boundary (approximately 6) imposed by the pK\textsubscript{a} of a Fg catalytic histidine group, which has to be deprotonated (thus uncharged) to allow optimal binding of thrombin to Fg.[11-13]

The second mechanism involves Fg electrostatic repulsion with increasing pH as an explanation for the terminated lateral assembly of Fg monomers in alkaline environments.[13] However, this mechanism was later contested by the same authors proposing it, because the length of the intermediate fibrin polymers did not gradually decrease between pH 6 and 10 whereas a sudden shift in the equilibrium towards monomers occurred between pH 10 and 10.2.[12] Di Cera and co-workers later showed that the deprotonation of an N-terminal isoleucine of thrombin yields reduced binding to Fg, providing further insights on the molecular mechanisms responsible for the reduced clotting at alkaline pH.[11]

1.2.4.3 Effect of pH on FXIII-mediated fibrin crosslinking
Knowing the effects of pH (and temperature) on the activity of FXIII has for long been essential to rationally use this enzyme to crosslink food proteins to form gels. The activity of FXIII as a function of pH is described by a bell-shaped curve between pH 6-9, with an optimum at around pH 7.6-7.9.[14]
1.3 PRINCIPLES AND APPLICATIONS IN INTERFACIAL BIOELECTROCHEMISTRY

Bioelectrochemistry is an interdisciplinary field covering various phenomena taking place when biomolecules are found at electrified interfaces. The most studied topics include electron transfer reactions between electrodes and biomolecules and the consequent development of electrochemical biosensors, electrophysiology of biological membranes and bioenergetics. The reader interested to learn about these topics is directed to a recent book edited by R. C. Alkire et al. [15].

Bioelectrochemical interfaces are systems composed of:

- One or more electrode surfaces differing in the electrification mode, in the electrode material(s), in the corrosion properties, in the electrode geometry and micro- or nano-topography;
- An electrolyte that can differ in the concentration of ionic and other inorganic species, in the viscosity, in the temperature and in the flow dynamics;
- A biological entity that can consist of proteins, nucleic acids, lipid membranes and a mixture of these biomolecules. More complex biological systems can consist of living cells and tissues.

In the next paragraphs of this introduction, I will briefly outline the basic concept of electron transfer reaction at biointerfaces which led to the development of electrochemical biosensors. Subsequently, I will introduce an important concept needed to understand the work presented in the next chapters, namely the electrochemically generated pH gradients.

1.3.1 Electron transfer reactions and electrochemical biosensors

Among all biomolecules at the electrode-liquid interface, redox enzymes are the ones that have been most extensively studied. Enzymes are widely used in electrochemical biosensors because of their high selectivity for their substrates (analytes) and because of
the high catalytic activity, resulting in signal amplification. The sensing principle is based on the specific recognition of the analyte by the redox enzyme and the consequent oxidation or reduction of the analyte. Generally, in addition to the redox enzyme, the biosensor system comprises a mobile (e.g. ferrocene and others) or immobile (redox active hydrogels) mediator with the task to shuttle the electrons between the redox enzyme and the electrode, thus enabling the chemical to electronic signal conversion. This type of electron transfer is referred to as mediated electron transfer. Biosensors of the last generation also rely on direct electron transfer from the catalytic redox core of the enzyme to the electrode. However, for the direct electron transfer to occur the distance between the redox core and the electrode should not exceed 10-15 Å. Therefore, the enzyme has to be immobilized on the electrode surface, which limits the number of enzyme molecules participating in the reaction and bear the risk of catalytic activity loss due to denaturation. Even the high proximity to the electrode and the intact catalytic activity might not be sufficient, because the active site of the enzyme is often located within the core of the molecule and shielded by the outer protein shell. So far direct electron transfer was observed for small redox active proteins with an exposed active site towards the protein surface. Smart electron transfer cascades mimicking the ones present in nature, together with nanostructured electrodes are promising strategies to address these challenges. To learn more about past and future amperometric biosensors and find the literature addressing the various aspects mentioned in this short paragraph, the reader is addressed to the book chapter written by Borgmann et al. [16].

1.3.2 Electrochemically generated interfacial pH gradients

1.3.2.1 Generation of interfacial pH gradients
When electrodes are sufficiently polarized to enter the water electrolysis regime, interfacial pH gradients are generated as a result of the following water oxidation and reduction reactions:

At the positively charged anode, water is oxidized:
\[ \text{At the negatively charged cathode, water is reduced:} \]
\[ 4e^- (\text{from cathode}) + 4H_2O (l) \rightarrow 2H_2 (g) + 4OH^- (aq) \]
\[ (E^0= -0.828 \text{ V}; \ E^{0'} = -0.414 \text{ V}) \]

Where \( E^0 \) is the standard electrode potential and \( E^{0'} \) is the standard electrode potential but at pH 7 obtained via the Nernst’s equation.

In this paragraph, I will introduce how electrochemically generated pH gradients can be used to obtain gradients in reactivity and localized control over reactions. This topic was recently reviewed by S. O. Krabbenborg and J. Huskens [17].

The simplest way to create pH gradients is to rely on mass transfer: protons and hydroxide ions diffuse away from the electrode source creating a concentration gradient. This phenomenon is known since two centuries [18] and was studied using colorimetric pH indicators since the 1960’s, when Rand and co-workers visualized interfacial pH changes using a mixture of two pH dye indicators (bromthymol blue and phenocresol purple).[19]

The ability to fabricate microelectrodes of various dimensions and configurations (e.g. microelectrode arrays), together with the ability to freely polarize the electrodes in time, provides spatial and temporal control over interfacial pH changes. Fiedler and colleagues demonstrated the generation of pH gradient patterns on scales ranging from micrometers to several tens of micrometers. Furthermore, the authors showed spatially defined pH clouds moving on the plane of the microelectrode array with a speed of about 1 mm/sec.[20] pH gradients obtained by free diffusion of protons and hydroxide ions can be considered as 3D gradients because the ions diffuse in- and out- of the electrode plane. Consequently, reactions can be locally controlled in the electrolyte...
bulk proximal to the electrodes and on the electrode’s co-planar region adjacent to the electrodes.

By using slightly different electrode characteristics and arrangements, in-plane gradients on the electrode surface can be obtained. Bohn and colleagues connected two electrodes by a gold thin film (< 50 nm in thickness) and passed a current through it. An in-plane potential gradient is generated through the film which the authors used to spatially control the desorption of an alkanethiol monolayer, thus obtaining a 2D gradient of functionality on the electrode surface.[21] This technique was successfully used to create in-plane concentration gradients of adsorbed polymer brushes and proteins (e.g. fibronectin, vascular endothelial growth factor) to study cell adhesion and migration.[22-24] Additionally, Hillier and colleagues applied distinct potential values on different locations on the working electrode to obtain linear pH gradients in the electrode plane.[25]

1.3.2.2 Thin film electrodeposition and electrodissolution

Electrochemically generated pH changes at interfaces are widely used to manufacture surface polymeric coatings to improve biocompatibility, to produce bio-functional and drug-releasing coatings, to immobilize enzymes for sensing purposes and to support cell culture. Various biologically relevant polymers such as chitosan,[26-34] alginate[35] and agarose[36] can be electrodeposited on metal surfaces for such applications. Chitosan is an aminopolysaccharide and is the best-known biological polyelectrolyte to have been electrodeposited. At pH lower than its pK\textsubscript{a} (approx. 6.3), chitosan’s primary amines (NH\textsubscript{3}+) are protonated making it water-soluble. Primary amines are neutralized upon deprotonation at pH above 6.3, leading to chitosan gelation (Figure 1.2). The electrodeposition of this type of biopolymers simply results from interfacial pH changes and does not rely on electron transfer reactions. As a side note, other polymer films, such as poly(ethylene glycol diacrylate) (PEGDA),[37] poly(methyl methacrylate) (PMMA),[38] 2-methacyrloyloxy(ethyl) acetoacetate (PMEA)[39] can be made by direct or indirect reduction at the cathode. Furthermore,
conductive organic films of polyaniline (PANI)[40-42] and polypyrrole (PPy)[43-45] can be electropolymerized by direct anodic oxidation.

Electrochemically generated pH changes at interfaces are not only used to manufacture polymeric coatings, but are also used to trigger their dissolution on demand. Vörös and colleagues electrochemically lowered the interfacial pH to dissolve multi-layered films made of various cationic and anionic polyelectrolyte couples.[46] The dissolution of polyelectrolyte multilayer coatings has been widely employed to detach engineered cell sheets from conductive surfaces for regenerative medicine applications.[47, 48]

1.3.2.3 Effect of interfacial pH gradients on proteins

The effect of electrochemically generated pH gradients on the behavior and nature of proteins is particularly of interest for the fields of protein isoelectric focusing (IEF) and mass spectrometry (MS). IEF is a technique to separate molecules based on their pI and is the first separation step in 2D gel electrophoresis, in which
proteins are first separated by pI and subsequently by molecular weight (MW) by SDS-PAGE. Standard IEF is performed using polyacrylamide gels featuring stable pH gradients. If a protein is located in a region of the gel with a pH value lower than its pI it will be positively charged. Provided that a sufficiently strong electric field is applied, the protein will start to migrate towards the negatively charged cathode until a gel region of pH matching the protein pI is reached. At that point, the protein charge is neutralized and the protein is focused on a sharp band indicating the pI. In electrochemical IEF, proteins can be separated in an aqueous electrolyte in which a stable pH gradient is generated electrochemically.[49]

Protein conformational changes and denaturation resulting from acidification and alkalinisation have been described by A. L. Fink and Y. Goto using circular dichroism (CD).[50, 51] The amount of similar studies on proteins at electrified interfaces are limited. Unfolding of bovine serum albumin and lysozyme induced by electric fields was recently reported using CD,[52] although the authors made no direct link with electrochemically generated pH changes that could have contributed to these observations. In fact, electrolyte acidification is known to induce protein unfolding in the capillary of electrospray mass spectrometers having an influence on the obtained mass spectra.[53] In the field of MS, anodic oxidation was recently proposed as an attractive reagentless and non-enzymatic method to digest proteins. In particular, peptides can be cleaved at tyrosine (Y, Tyr) and tryptophan (W, Trp) residues (sometimes referred to as echem WY-cleavage) and this method was employed with on-line electrospray ionization MS (ESI-MS) detection.[54-56] In general, random protein digestion could occur at the interface with electrodes as a result of acid- and base- catalyzed peptide hydrolysis.
1.4 INTERFACIAL ELECTROCHEMISTRY OF BLOOD COAGULATION FACTORS

After having provided an overview on blood coagulation factors and their biochemical function and on fundamental principles in bioelectrochemistry, I now present an extensive review on the field of bioelectrochemistry of blood coagulation factors.

The interaction of coagulation factors and other blood plasma proteins with various material surfaces is subject of investigation since several decades. The force driving this research is the development of implant surfaces that do not trigger the coagulation of blood.[57-59] Thrombosis on the surface of implants such as catheters, stents, cardiac valves, electrodes and others represents both a medical and an engineering problem. On the medical side, the clot formed on the implant’s surface can occlude a vessel in the vicinity of the implant or even detach causing pulmonary embolism, stroke and other medical conditions with potentially fatal consequences.[60] On the engineering side, the formation of a blood clot on the surface of a device can lead to its premature failure and replacement. For instance, electrodes used in blood for sensing or stimulating purposes might not be able to work as designed if isolated by a thrombus.

In the 1960’s, P. N. Sawyer (Figure 1.3) pioneered the study of the electrochemistry of thrombosis. He demonstrated that the tendency of metals to donate or accept electrons influences the formation of a thrombus in proximity of the metal implant. He subsequently initiated a systematic study of the behavior of blood coagulation factors at the interface with electrodes. Sawyer showed that blood coagulation factors, in particular Fg, were involved in electrochemical reactions at the electrode-liquid interface and demonstrated that these reactions affected the amount of blood clot formed on metal implants.
Over more than two decades, the investigations of P. N. Sawyer, S. Srinivasan and co-workers covered various aspects of the electrochemistry of the vascular tissue, the interaction between metals and blood proteins, and metals and blood cells. Concise reviews of this work were written by Sawyer and Srinivasan [61, 62].

In a set of in vivo experiments, electrodes made of different metals were implanted in the femoral and carotid arteries of dogs. The potential of the inserted electrodes was measured against a standard calomel electrode contained in a beaker, connected to the animal’s vessel via an electrolyte bridge. Forty minutes after implantation the dog was sacrificed and the portion of the vessel containing the electrode was fixed with formalin. The electrode was finally explanted and the amount of thrombus deposited assessed. The results clearly stated that electrodes presenting a negative

potential in blood (magnesium, aluminum and cadmium) were free of deposited thrombi, whereas thrombus deposition was observed on metals presenting a positive potential in blood (copper, nickel, gold and platinum). The authors concluded that the implant’s interfacial potential is at least partly responsible for its thrombogenicity.[63] Furthermore, metals of the thrombogenic group could be made thromboresistant via the application of cathodic potentials. For instance, wires and tubes made of platinum and copper were implanted in the inferior vena cava of dogs and remained free of deposited thrombi for weeks, in contrast to non-polarized controls on which thrombosis immediately occurred (Figure 1.4).[64, 65]

These observations were consistent with early experiments aimed at measuring the potential across the blood vessel wall. The electrode on the luminal side was negatively polarized with respect to the adventitial electrode. Reversing this polarity often resulted in thrombosis.[66] Parallel investigations focused on the interfacial electrochemical behavior of blood coagulation factors and blood cells.

1.4.2 Interfacial electrochemistry of Fg

Fg adsorbs on metal surfaces over a wide range of concentrations and electric potentials. Potentiodynamic and potentiostatic studies revealed that adsorbed Fg is involved in charge transfer reactions at the platinum interface, at highly cathodic and over a wide range of anodic potentials. Ellipsometric experiments combined with potential sweeps showed an increasing Fg coverage with increasing anodic potentials. Sawyer and co-workers measured increased currents at high cathodic potentials when Fg was present in solution, compared to the control (bare electrolyte solution). The authors attributed this observation to electrolytic hydrogenation of the protein by discharged hydrogen at the cathode, and recognized that proteomic studies on the Fg products formed at cathodic potentials
would be beneficial to understand their nature and possibly the reduced thrombogenicity.[67]

More recent studies confirmed some of the aspects reported by Sawyer. Fg adsorption on polarized stainless steel was studied with atomic force microscopy (AFM). The Fg adsorption kinetics was slowed and the surface coverage was reduced at cathodic potentials with respect to anodic potentials.[68] Subsequently, Mallon et al. demonstrated, using AFM and fluorescence microscopy, that Fg pre-adsorbed on a bare gold electrode, desorbed upon the application of highly cathodic potentials.[69] Others previously showed the electrochemical desorption of proteins (mainly BSA) adsorbed on
gold via alkanethiol self-assembled monolayers using electrochemical surface plasmon resonance (ESPR)[70] and electrochemical quartz crystal microbalance (EQCM)[71]. When the proteins are coupled to the electrode surface through alkanethiols, the cathodic desorption of the proteins can be attributed to the reduction of the alkanethiol monolayer and the consequent release of the film. When this is not the case (see ref. [69]), two mechanisms for the electrochemical desorption can be proposed. Assuming that the potential is in the water electrolysis regime, the alkaline pH formed at the electrode-liquid interface increases the negative charge of the protein. Consequently, the electrostatic repulsion between the protein and the negatively charged surface might drive the desorption. In the field of hard surface cleaning, highly alkaline solutions are used in a process known as alkali elution. S. Fukuzaki studied alkali elution of BSA from alumina surfaces.[72] As an alternative process driving the desorption, V. Craig and colleagues described the role of nanobubbles originating from hydrogen evolution at the cathode.[71]

As a second important aspect, Mallon and co-workers reported the degradation of the cathodically desorbed Fg, observed by SDS-PAGE.[69] More advanced proteomic techniques and highly purified Fg should be used in future experiments to confirm this observation and to identify the produced fragments. Based on early description of Fg fragmentation at high pH (see paragraph 1.2.4.1), the electrochemical alkali elution process would explain this observation. Finally, Fg degradation would be a very reasonable explanation for the reduced clottability (increased thrombin time) of the Fg cathodic product obtained by galvanostatic treatment.[73]

Fg is oxidized over a wide range of anodic potentials.[67] G. Stoner examined the structure of the anodized Fg using scanning electron microscopy (SEM) and discovered that Fg electropolymerized in fibrin-like fibers, in absence of thrombin.[74] Anodized Fg resulted in accelerated clotting times when thrombin was added, strongly suggesting a pro-coagulant effect of anodic surfaces.[73] M. Schaldach and co-workers confirmed the oxidative Fg to fibrin
conversion and proposed that thromboresistant semiconductor coatings of stents can be rationally designed by tuning the electronic properties to inhibit the oxidative conversion.[75, 76] This was the rational driving the development of amorphous hydrogen-rich silicon carbide stent coating (a-SiC:H) marketed by Biotronik.[77] Since then, others studied the electronic properties of semiconductor thin film and the relation with non-thrombogenic properties.[78]

1.4.3 Interfacial electrochemistry of thrombin

Although the majority of the studies on the electrochemistry of coagulation factors focused on Fg, there is enough evidence concerning the participation of (pro)thrombin in interfacial electrochemical reactions.

Prothrombin is the inactive circulating form of the enzyme with a MW of approximately 72 kDa. Two sites of prothrombin are cleaved by the activated form of factor X, thus releasing the active thrombin, with a MW of approximately 36 kDa. When prothrombin was incubated 15 min at various constant anodic potentials prior addition to Fg, the clotting time decreased with increasing anodic potential.[79] Later studies by Comtat and colleagues showed that the electrochemical effects on prothrombin depended on the applied voltage. In particular, prothrombin was electrochemically decomposed in the same fragments obtained upon the enzymatic prothrombin-thrombin conversion but only in a relatively narrow anodic window, as shown by SDS-PAGE. If a higher potential was applied, fragments with no apparent catalytic activity were produced.[80] The literature on the electrochemical behavior of other coagulation factor is limited. One study reported the electrochemical activation of surface-bound factor XII (Hageman factor), however I will not review this because of limited information.[81]

In summary, the research work presented in this section supports the fact that coagulation factors respond to interfacial electric potentials and that electrochemical reactions influence the
coagulation of blood at the interface with metal surfaces. At first, some have debated part of these observations. V. L. Gott, a leading cardiosurgeon who contributed with Sawyer in first efforts to standardize the in vivo evaluation of implant thrombogenicity,[82] did not observe the correlation between spontaneous surface potential and thrombogenicity.[83] However, he recognized the beneficial effect of imposing negative potentials to reduce surface thrombogenicity.[84]

1.4.4 Bioelectrochemistry in the development process of vascular devices

The study of the electrochemistry of thrombosis represented an important effort in the development of implant surfaces characterized by reduced thrombogenicity. However, as the extensive research in the field of hemocompatibility over the last fifty years demonstrates, this knowledge has not been sufficient to engineer implant surfaces meeting the increasing requirements. Beside Sawyer work, few other examples of plastic, metal and semiconductor device surfaces maintained at cathodic potentials to reduce thrombosis have been reported.[85, 86] While imposing cathodic potentials to graphite surfaces, Gott realized that even if the connection to the power supply was broken, the surface performed really well. He subsequently discovered that heparin (a highly negatively charged sulphated glycosaminoglycan used as anticoagulant) had bound to graphite via an intermediate layer of the cationic surfactant benzalkonium chloride.[87, 88] Many after him used this strategy effectively to reduce surface thrombogenicity, however the problem of the coating stability was difficult to solve because heparin desorbed in blood.

Besides thrombogenicity, modern surfaces of implants need to deal with desired endothelialization (to make the implant surface more biomimetic), problematic neointimal muscle cell proliferation (causing restenosis in stents), bacterial proliferation (causing catheter-related bloodstream infections), and last but not least the immune activation in response to foreign bodies. To deal with these
complex aspects, various surface properties have been explored and smart coatings engineered, as reviewed by Mani et al. [89]. Among the different strategies, a promising one is to make the surface of the implant invisible and untouchable for the blood and immune system components via so-called ultra-low-fouling polymer films, including poly(carboxybetaine), poly(sulfobetaine) and liquid perfluorocarbons.[90, 91] *In vivo* stability (non-leaching) of the film is critical for the success, and recent studies showed promising results in this respect.[92, 93]

Contemporary ambitions for vascular prosthesis go beyond solving the problems of thrombogenicity and reasonable tolerance from the body. Part of today’s vascular prosthesis aim at replacing and/or regenerating the injured tissue with engineered tissue constructs that can grow with the organism as a part of it. Tissue engineered heart valves are a splendid example. [94, 95] The nature of these constructs depends on the application, but these are generally hybrids of natural materials, living cells and artificial materials, specifically designed to mimic the tissue environment that they will repair or in which they need to operate. In tissue engineering approaches, artificial materials typically have a temporary supporting role and should therefore be resorbed by the body. Sawyer considered the propensity to corrode of metals in his “thromboresistant group” (e.g. magnesium) as a major limitation for applicability. Today, biodegradable metallic and polymeric stents are proposed as a solution to solve problems such as restenosis and late in-stent thrombosis.
1.5 CURRENT TRENDS IN 3D CELL CULTURE

In the second part of this thesis, I will describe how to use interfacial electrochemistry of blood coagulation FXIII to manufacture structured hydrogels, which are commonly used materials in tissue engineering. In the last section of this introduction, I will therefore introduce the field of 3D cell culture and the available platforms used to build tissue engineered constructs and testing platforms.

1.5.1 From 2D to 3D cell culture

The fact that cell monolayers cultivated on glass and plastic flat substrates (2D cell culture systems) poorly represent the physiological conditions of animal tissues is accepted.[96, 97] Already in 1972, T. Elsdale and J. Bard explored the behavior of cells in hydrated fibrous 2D and 3D collagen substrates.[98] Since then, researchers have recognized that in order to reproduce in vivo cell behavior under in vitro settings, a representative model should account for cell-extracellular matrix (ECM) interactions, ECM mechanical properties and architecture, cell-cell communication and interplay between different cell types. Many demonstrations of the higher biological relevance of 3D culture systems over 2D systems are present in literature. I will limit myself to briefly mention two of them. A. Grapin-Botton and colleagues recently reported advanced 3D culture protocols to develop in vitro models of pancreatic development. The authors showed that the 3D environment was necessary to maintain the progenitor expansion, and that the spherical arrangement promoted the formation of hollow structures, with epithelial cells acquiring polarity and other groups of cells differentiating into endocrine and exocrine cells.[99] Acquisition of apical-basal polarity is a fundamental aspect pointing out the difference between 2D and 3D culture. Polarity is desirable for endothelial cells and other type of cells to fulfil specific functions in tissues, but it is not wanted for all type of cells. However, cells are forced to polarize when cultured on flat surfaces, which induces them to deviate from their physiological behavior. As a second example, D. J. Mooney and colleagues cultivated tumor cells in
polymeric scaffolds that recreated the microenvironment of tumors in vivo. The result was that tumor cells acquired angiogenic characteristics, resistance to chemotherapy and high malignant potential which closely recapitulated the in vivo situation.\[100\]

Engineered 3D tissue models are being developed for two fields of application: in vivo tissue replacement/regeneration (regenerative medicine) and highly relevant platforms for biological studies. The engineering approaches to develop 3D models vary depending on the application, yet common platforms are used to achieve the goals. J. W. Haycock edited a book on various methods and approaches used in 3D cell culture.\[96\]

1.5.2 3D cell culture platforms

It is possible to group 3D cell culture platforms in three groups: i) cell cultured in solid porous and fibrous scaffolds, ii) cell cultured in hydrogel scaffolds and iii) scaffold-free aggregated cells.

In the field of 3D cell culture, a scaffold is some sort of material that supports the growth of cells in 3D. In a standard tissue engineering approach, cells are cultivated in the scaffold in vitro (in bioreactors for instance) and the engineered construct may be then implanted in vivo for regenerative purposes. In some cases, a scaffold may be implanted directly in vivo and populated by recruited cells of the recipient tissue. The reader can find an exhaustive coverage of the techniques and materials used to produce tissue scaffolds in the book edited by P. X Ma and J. Elisseff\[101\] and in a review authored by M. M. Stevens and colleagues\[102\]. Solid scaffolds for cell culture consist in porous or fibrous substrates made of various biocompatible polymers and ceramics (or their combination). The technique mostly used in tissue engineering to produce polymeric micro- and nanofibrous scaffolds is electrospinning. It is a scalable technique and adaptable to various synthetic and natural polymers. Electrospinning enables the production of scaffolds with different porosity, fiber dimensions, mechanical properties and biochemical properties.\[103\] In the last years, additive manufacturing techniques have rapidly evolved and stereolithography has been used to
produce scaffolds with a great variety of polymers and composites, with increased freedom in topographic design. D. W. Hutmacher et al. recently reviewed the use of additive manufacturing in tissue engineering.[104] Solid non-fibrous scaffolds include porous bioceramics of calcium phosphate,[105] extensively used in bone tissue engineering, and polymeric foams[106]. The advantages of using polymeric and ceramic scaffolds are the possibility to tune the mechanical, biodegradability and biochemical properties of the constructs. Furthermore, the ability to design macroporous structures facilitates cell infiltration in the construct, diffusion of nutrients and waste, and vascularization. Among the disadvantages, probably the most important to mention is the questioned capability to provide true 3D culture conditions. In particular, experts in the field recognize that if the scaffold’s feature dimensions are large in relation to the cell dimensions (e.g. electrospun scaffolds with diameter in the 10 µm range), ultimately the cell might interact with a flat 2D surface. Much smaller fiber diameters (down to 10-100 nm range) can be obtained by electrospinning, however cells cannot infiltrate these scaffolds because of the reduced porosity, and solutions envisaging in situ formation of the scaffold around the cells are therefore considered. Hydrogels constitute a separate class of scaffolds and are often proposed as candidates to solve this problem of limited three-dimensionality of the cellular environment.[102]

Hydrogels are highly hydrated 3D networks of physically or chemically crosslinked hydrophilic polymers. The high impact of hydrogels in the field of biomedical sciences is proven by the wide use of soft contact lenses made of poly(hydroxyethyl methacrylic)acid[107] and by the use of so-called fibrin-glues in surgical procedures[108], just to mention the most common applications. The current challenge addressed by the community is to engineer hydrogels that mimic the ECM as closely as possible, to direct cell behavior and tissue morphogenesis for tissue engineering applications or in vitro biological studies. There are some important reasons why hydrogels are good candidates to mimic the ECM. First, from a mechanical standpoint, hydrogels can be engineered to match the stiffness of natural tissues, with elastic moduli going from
hundreds of Pa for the brain tissue to hundreds of kPa for collagenous bone. The ability to match the mechanical properties of the mimicked tissue is considered a priority by tissue engineers, since cell differentiation towards specific lineages is directed, among many factors, also by ECM stiffness. Secondly, due to the dense nature of the polymer network, the cell-hydrogel interface closely recapitulates the natural cell-ECM interface. Natural hydrogels are typically made of isolated ECM components, which feature natural binding and degradation sites that trigger complex cellular processes, orchestrating migration, differentiation and tissue morphogenesis. Synthetic hydrogels intrinsically do not possess this ability to interact with cells, but have advantages such as low batch-to-batch variability, high flexibility in tuning the mechanical properties and the possibility to build an artificial matrix through a bottom-up approach with a high control over the cell-hydrogel interface. This bottom-up approach is realized via biochemical modifications of the synthetic hydrogel, aimed at providing bio-functionality (further discussed in the subsequent paragraph 1.5.3). In a very recent review, W. Huck introduces design aspects of hydrogel engineering and provides an exhaustive description of the natural and synthetic materials of which hydrogels can be made, along with their properties.[109] Natural materials for hydrogel scaffolds are mainly fibrin, collagen, gelatin, matrigel and various polysaccharides. Synthetic polymers are mainly poly(acrylamide) (PAAm), PEG, poly(vinyl alcohol) (PVA) and various biocompatible polyesters.

Scaffold-free 3D cell culture approaches rely on the ability of isolated cells to form compact aggregates (so-called microtissues or spheroids) and planar cell sheets (not covered in this paragraph), when the adhesion to a solid substrate is prevented. Cells adhere to each other via the secreted ECM proteins and cell-cell contacts. Since more than two decades, cell spheroids represent a model to study the interaction between tumors and their microenvironment [110] and to study early processes in embryonic development[111]. Current research and development efforts are focusing on i) fabricating arrays of microtissues with high reproducibility and throughput for applications in high throughput toxicology screenings
and tissue engineering; ii) implementing microtissues in microfluidic devices for so-called organ-on-a-chip or even body-on-a-chip (multiple interacting organs) applications. Current techniques widely used to produce cell spheroids are the hanging drop technology (in which cells are suspended in a hanging drop of medium and aggregate on the bottom region of the drop due to gravity[111, 112]); and the sedimentation of cells in wells which are micromolded in non-fouling substrates such as agarose[113] and PEG[114]. Once successfully fabricated, spheroids are used as organ models for screening applications. As an example among many, Fayad et al. used a spheroid tumor model to screen for apoptosis-inducing chemical compounds. Interestingly, the screen led to the identification of 11 hit compounds, which were not identified as hits in the 2D monolayer screening.[115] To model the complex physiological mechanisms involved in the body, A. Hierlemann and co-workers connected hanging drops containing spheroids of different cell types through a microfluidic device in order to account for inter-organ metabolic communication.[116] Once fabricated, spheroids can be precisely placed next to each other to allow tissue fusion. Therefore, spheroids are proposed as building blocks for more complex organ models additively manufactured.[117, 118]

1.5.3 PEG hydrogels and FXIII-mediated crosslinking

Part of the work presented in this thesis focuses on a particular PEG-based hydrogel platform for 3D cell culture; in which crosslinking is mediated by the coagulation factor XIII. I briefly introduce this platform in the next paragraphs.

Building up an artificial tissue model starting from a synthetic hydrogel is a fascinating strategy because it offers great design control over the mechanical properties and over the biofunctionality (cell-matrix interactions), while minimizing problems typically linked to natural polymers including batch-to-batch variation, immunogenicity and pathogen transmission. Thanks to the minimal intrinsic interaction between cells and non-fouling synthetic polymers such as PEG, this bottom-up approach is particularly interesting when designing culture platforms used to investigate the
role of specific cell-matrix interactions on cell behavior and tissue morphogenesis. However, providing artificial ECM with the functionality required to support and instruct cells in 3D culture is a difficult task and required a considerable engineering work in the past years, as reviewed by M. P. Lutolf and J. A. Hubbell[119].

In vivo, cells enzymatically degrade ECM components via matrix metalloproteinases (MMPs) and serine proteases (e.g. plasmin) to migrate and invade tissues and remodel the ECM.[120] This local degradation is of fundamental importance when the porosity is low and the ECM represents a physical barrier to migration. Cell permissive protease-sensitive PEG hydrogels have been developed via copolymerization of the PEG monomers with enzymatically degradable peptides[121] and by grafting PEG monomers with proteins containing cleavable sites prior photopolymerization[122].

In order to confer biological functions to otherwise inert PEG hydrogels, various chemistries have been used to incorporate peptides and proteins into the polymer matrix. An exhaustive description goes beyond the scope of this introduction. The reader is addressed to a recent review by J. Zhu[123].

In this thesis I focus on PEG hydrogels enzymatically crosslinked via transglutamination by the coagulation FXIII (for transglutamination by FXIII see paragraph 1.2.3). J. J. Sperinde and L. G. Griffith first reported the TG-mediated crosslinking of Gln-functionalized PEG monomers and a Lys-containing polydipeptide to form hydrogels proposed to be formed in mild conditions and in presence of living cells.[124] More recently, M. Ehrbar et al. designed star-shaped PEG monomers terminally functionalized (via Michael addition) with Gln- and Lys-containing peptides, hence crosslinkable via transglutamination (Figure 1.5).[125] The advantage of this crosslinking system is twofold: i) crosslinking occurs at physiological conditions and is compatible with the presence of cells or tissues; ii) following the same crosslinking scheme, peptide and proteins can be selectively incorporated into the hydrogel without affecting their structure and functionality. Gln-functionalized RGD adhesion motif[126] and Gln-functionalized vascular endothelial growth
factor (VEGF)[125] were successfully incorporated in MMP-sensitive PEG hydrogels. Furthermore, the FXIII-mediated crosslinking of PEG can be triggered upon temperature stimulation, as shown by P. B. Messersmith and co-workers.[127] In particular, Ca\(^{2+}\) ions, which act as cofactor of FXIII, were encapsulated in temperature-sensitive liposomes and released when the body temperature was reached, thus starting gelation in situ.

1.5.4 Structured hydrogels with heterogeneous architectures

Hydrogels constitute a versatile and robust platform to cultivate cells in 3D. However, experts recognize that much still has to be done to reproduce the complexity characterizing the ECM of human tissues. In particular, artificial tissue models should mimic the heterogeneous and highly-defined distribution of cells and biomolecules, which finally enables complex human tissue functions.[128]

Hydrogel fabrication techniques progressed rapidly in the last years. At the forefront there are photochemistry-based patterning technologies (photopatterning), holding great promise to provide researchers with hydrogels featuring complex architectures and increased functionalities.[129] J. L. West and co-workers used two-
photon laser scanning ultraviolet (UV) irradiation, to locally incorporate an adhesive peptide in enzymatically degradable PEG hydrogels, and direct the migration of human dermal fibroblasts exclusively on the patterned track.[130] K. S. Anseth and co-workers presented an orthogonal UV-induced thiol-ene click chemistry to photopattern PEG gels.[131] M. S. Shoichet and co-workers demonstrated simultaneous patterning of multiple growth factors in 3D agarose hydrogels, by photochemically uncaging sulfhydryl groups that subsequently react with maleimide-functionalized biomolecules. The authors used this technique to promote neural precursor cell migration along a photopatterned gradient of stem cell differentiation factor sonic hedgehog.[132] M. P. Lutolf and colleagues used photoirradiation to locally expose a substrate of the coagulation FXIII in PEG hydrogels, and subsequently enzymatically

Figure 1.6. “Concept of light-controlled enzymatic biomolecule patterning of hydrogels. a) A photolabile, caged, and therefore inactive enzymatic peptide substrate is covalently incorporated into PEG hydrogels and can be activated by light. b) Localized cleavage of the cage by controlled light exposure from a confocal laser allows reactivation of the enzyme substrate. c) Enzyme-catalyzed (here: the transglutaminase factor XIII) reaction of the uncaged substrate with a counter-reactive substrate on a biomolecule of interest allows covalent biomolecule tethering in a highly localized, user-defined pattern.” Figure and caption reproduced from ref. [133] with the permission of Nature Publishing Group.
incorporate adhesive peptides as well as sensitive proteins such as platelet-derived growth factor (Figure 1.6) [133]

Another use of photochemistry is to degrade photolabile hydrogels to modify locally the mechanical properties of hydrogels and even create cavities to direct migrations.[134, 135] Additionally, hydrogel photodegradation can be used to produce crosslinking density gradients used to study cell response.[136] Finally, local degradation of hydrogels to which biomolecules are linked, results in biomolecule release in the microenvironment. This local release could be used to generate gradients of bioactive moieties.[137]

Other techniques have been explored to provide increased complexity to hydrogel-based cell culture platforms, including template removal manufacturing to create artificial vessels[138, 139] and microfluidic devices to establish morphogen gradients directing cell fate[140].

1.6 REFERENCES


This doctoral thesis is motivated by the desire to understand how
the nature and functionality of blood coagulation factors are
affected in the proximity of an electrode surface, and by the
fascinating idea of electrochemically manipulating the activity of
coaulation factors for various applications in the biomedical field.
These applications include the development of implant surfaces
resistant to thrombus deposition and the fast-growing field of
hydrogel fabrication for 3D cell culture and tissue engineering.

In Chapter 3 and Chapter 4, I present two studies on the
electrochemical behavior of fibrinogen (Fg). In Chapter 5 and 6, I
present the use of interfacial electrochemistry to control locally the
coaulation factor XIII (FXIII)-mediated crosslinking of poly(ethylene
glycol) (PEG) hydrogels.

The pioneering work of P. N. Sawyer represents the starting point of
my research presented in Chapter 3. As introduced in the previous
chapter, Sawyer investigated the electrochemical behavior of Fg,
and reported that this protein is involved in charge transfer reactions
at high cathodic potentials and over a wide range of anodic
potentials. With increasing cathodic potentials, Fg coverage on
electrodes is reduced, as indicated by ellipsometric studies. More
recent investigations pointed towards the electrochemical
desorption of Fg from the surface. Sawyer hypothesized that
electrochemical reactions involving Fg at highly cathodic potentials
might result in partial protein degradation, with a direct
consequence on its ability to clot. He stated that a molecular analysis
of the Fg cathodic product(s) might shine light on the molecular
mechanisms leading to decreased thrombus formation on
cathodically polarized surfaces. In Chapter 3, I used the
electrochemical quartz crystal microbalance technique (EQCM) to study for the first time blood plasma coagulation at cathodic potentials. I subsequently studied with the same technique the electrochemical desorption of Fg from a gold cathode as function of the applied current density. Finally, we analyzed the Fg cathodic products with mass spectrometry.

In Chapter 4, I use the EQCM-D to monitor in real-time the anodic polymerization of Fg as a function of the applied potential. Fg is known to oxidize over a wide range of anodic potentials, which induces its polymerization to form fibrin-like fibers. Additionally, Fg is also known to precipitate at acidic pH, and for this reason, it was suggested that Fg precipitates on the anode surface as a result of the interfacial acidification of the electrolyte. In order to investigate the role of interfacial pH changes in the polymerization/precipitation of Fg at the anode, we performed a potentiodynamic study using the EQCM-D in non-buffered and buffered electrolyte.

In Chapter 5, I study the electrochemical control of the FXIII coagulation activity. As described in the Introduction, FXIII has a pH-dependent activity with an optimum at 7.6-7.9. By electrochemically generating interfacial pH gradients, I show that it is possible to enter or exit the activity window and therefore locally promote or inhibit the FXIII-mediated crosslinking of PEG hydrogels. This novel technique can be used to fabricate biological microenvironments featuring relatively complex architectures.

Based on the same effect, in Chapter 6, electrochemically generated gradients are used to fabricate hydrogels featuring surface crosslinking density gradients. It is known that when cells are topically seeded on the surface of PEG hydrogels (but not only), they tend to grow on the surface plane in a 2D monolayer, without invading the bulk (3D culture). Consequently, cells generally must be encapsulated in the hydrogel during the polymerization process. This might have two important disadvantages: i) cells are present in culture when the hydrogel is processed and structured (for example via photopatterning techniques), potentially leading to unwanted cell stimulation; ii) hydrogel assembly and cell encapsulation is highly
impractical for high-throughput screening applications. In this chapter, I introduce surface density gradients as an efficient strategy to enhance penetration into the hydrogel bulk, of cells topically seeded.

In summary, understanding how blood coagulation factors react at the interface with electrodes is of interest to reduce surface thrombosis and to spatio-temporally control biochemical reactions, here used to manufacture structured hydrogels for cell culture applications.
3 COAGULATION AT THE BLOOD–ELECTRODE INTERFACE: THE ROLE OF ELECTROCHEMICAL DESORPTION AND DEGRADATION OF FIBRINOGEN†

3.1 ABSTRACT

The influence of electrochemistry on the coagulation of blood on metal surfaces was demonstrated several decades ago. In particular, the application of cathodic currents resulted in reduced surface thrombogenicity, but no molecular mechanism has been so far proposed to explain this observation. In this article we used for the first time the quartz crystal microbalance with dissipation monitoring technique coupled with an electrochemical setup (EQCM-D) to study thrombosis at the blood–electrode interface. We confirmed the reduced thrombus deposition at the cathode, and we subsequently studied the effect of cathodic currents on adsorbed fibrinogen (Fg). Using EQCM and mass spectrometry, we found that upon applying currents Fg desorbed from the electrode and was electrochemically degraded. In particular, we show that the flexible N-terminus of the α-chain, containing an important polymerization site, was cleaved from the protein, thus affecting its clottability. Our work proposes a molecular mechanism that at least partially explains how cathodic currents reduce thrombosis at the blood–electrode interface and is a relevant contribution to the rational development

of medical devices with reduced thrombus formation on their surface.

3.2 INTRODUCTION

Over the past decades a considerable effort has been made to develop surfaces with minimally thrombogenic properties for endovascular devices such as stents, catheters, filters, sensors, and many others. The formation of a thrombus resulting from the contact of blood with the implant’s surface represents a risk from a medical perspective (thromboembolism) and from an engineering perspective (implant failure).[1, 2] The major difficulty that scientists and engineers face in the development of “more hemocompatible” blood-contacting devices is the limited understanding of the phenomenon of contact activation of blood coagulation and the lack of a paradigm capable of explaining all empirical observations.[3] Consequently, there is little or no consensus concerning the parameters to optimize in designing better surfaces.

The leading opinion states that protein adsorption at the implant’s surface is the first step of a long series of events, including platelet adhesion and activation, finally leading to thrombosis.[4-6] Regardless of what strategy is pursued to reduce blood coagulation on a surface, it is crucial to determine the effect of the surface treatment on the adsorbed plasma protein layer. The understanding of this phenomenon is necessary, though possibly not sufficient, to rationally design and develop blood-contacting devices.

Various strategies have been used to control surface coagulation including coating the surface with protein-resistant polymers,[7-9] active molecules such as anticoagulants[10, 11] or fibrinolysis promoters,[12-15] endothelial cells[16, 17] or polymers mimicking their membrane,[18, 19] and last but not least inorganic thin films conferring various surface properties as reviewed by Mani and colleagues[20]. A strategy not comprised in this list was proposed by P. N. Sawyer more than 40 years ago and consisted in cathodically polarizing the implant’s surface. Sawyer’s pioneering work demonstrated that thrombogenic metals had reduced clotting at
their surface upon application of cathodic potentials \textit{in vivo}.\cite{21, 22} In contrast, anodic surfaces were associated with accelerated thrombus formation.\cite{23} The authors suggested that the electrochemical reactions occurring at the electrode surface involving the plasma protein fibrinogen (Fg) could partially explain these observations. A large part of the studies on hemocompatibility focused on Fg because it constitutes the building block of the polymeric scaffold of a blood clot (fibrin), because of its high capacity to activate platelets, and because surfaces precoated with Fg, among other proteins, showed increased surface-induced thrombogenesis.\cite{24-26}

At anodic potentials Fg is electropolymerized to form fibrin-like fibers, and this reaction might cause an acceleration of the coagulation process.\cite{21, 27} At the cathode Fg was recently shown by fluorescence and atomic force microscopy to desorb from the electrode surface.\cite{28} Sawyer recognized that an in-depth investigation of the nature of the Fg reduction products was necessary in order to understand what is the mechanism behind the reduced thrombus formation at cathodic potentials.\cite{21}

In this article, we used for the first time the quartz crystal microbalance with dissipation monitoring technique coupled with an electrochemical setup (EQCM-D) to semiquantitatively study thrombosis at the cathode–blood plasma interface. We confirmed Sawyer’s observation and found that Fg desorbs upon the application of cathodic currents in a current density-dependent manner. We also demonstrated using EQCM and mass spectrometry (MS) that the alkaline environment in proximity of the cathode causes the degradation of Fg. In particular, we found a low molecular weight (MW) Fg degradation product containing an important polymerization site (EA located at the \(\alpha\)-chain N-terminus). The degradation of the \(\alpha\)-chain N-terminus explains the impaired clottability of the cathodically desorbed Fg and the reduced thrombosis at the cathode’s surface.
3.3 EXPERIMENTAL SECTION

3.3.1 Materials

Frozen anticoagulated human citrated blood plasma (CBP; ACD stabilized) was purchased from Blutspende Zürich (Zurich, Switzerland), and 1 mL aliquots were stored at −80 °C until use. Human fibrinogen (Fg; plasminogen, von Willebrand factor and fibronectin depleted, code FIB3) was purchased from Enzyme Research Laboratories Ltd. (Swansea, UK). Fg was dissolved during 4 h at 37 °C at a concentration of 50 mg/mL in phosphate-buffered saline 1X (PBS; pH 7.4, no CaCl$_2$ and no MgCl$_2$, Life Technologies Ltd., Paisley, UK, code 10010-015), and 50 μL aliquots were stored at −80 °C until use. Thrombin (>2000 u/mg, code T6884), sodium chloride (NaCl; 99.8%, code 71380), ethanol (C$_2$H$_6$O; 99.8%, code 02854), 2-propanol (C$_3$H$_8$O; 99.8%, code 34965), calcium chloride (CaCl$_2$; anhydrous, 96.0%, code C5670), trifluoroacetic acid (TFA, CF$_3$COOH; 99%, code T62200), and glutaraldehyde (CH$_2$(CH$_2$CHO)$_2$; 8% in H$_2$O, code G7526) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN, CH$_3$CN; hypergrade, code 100029) was purchased from Merck Millipore (Billerica, MA). MALDI-MS matrix α-cyano-4-hydroxycinnamic acid (HCCA, C$_{10}$H$_7$NO$_3$; code 8201344) and MALDI-MS targets (MTP 384 TF AnchorChip target plate 800 μm) were purchased from Bruker Daltonics GmbH (Bremen, Germany). Deconex 12PA-x cleaner was purchased from Borer Chemie AG (Zuchwil, Switzerland). Cleaner “Cobas Integra” was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Electrochemical experiments were performed using an Autolab PGSTAT 302N potentiostat–galvanostat and potential–current signals were monitored with a computer interface NOVA v1.9 (Metrohm Autolab, Utrecht, The Netherlands). Quartz crystal microbalance with dissipation monitoring (QCM-D) experiments were performed on a Q-Sense E4 (data acquired using Q-Soft software v2.5.15) using gold-coated sensors (100 nm gold layer thickness, 14 mm diameter, code QSX301) (Q-Sense, Västra Frölunda, Sweden). A custom-made transparent electrochemical QCM (EQCM) module (capacity 200 μL) was developed in house to directly inject the sample on the sensing
surface and to clearly monitor eventual formation of gas bubbles during electrochemical experiments. The working electrode (WE) consisted of the gold-coated sensor and the counter/reference electrode (CE/RE) of a platinum wire (Alfa Aesar, Ward Hill, MA). The cell incorporated a cellulose acetate membrane (500 MWCO, Harvard Apparatus, Holliston, MA) separating the CE/RE and the WE (Appendix A, Figure A.4).

3.3.2 Methods

3.3.2.1 Cleaning Procedures
Gold-coated sensors were reutilized and cleaned before every experiment as follows: the surface was gently wiped with a 100% polypropylene tissue soaked in Cobas cleaner (10 v/v % in Milli-Q water) and rinsed with Cobas cleaner. Subsequently, the surfaces were thoroughly rinsed with 2-propanol and Milli-Q water and dried using a nitrogen stream. Immediately before starting the experiment, the surface was oxygen plasma cleaned (1 min, 30 W) (PDC-32G, Harrick Plasma, Ithaca, NY). The EQCM-D modules were cleaned before and after the experiment with Deconex cleaner (4% v/v in Milli-Q water), rinsed with Milli-Q water, and finally dried using a nitrogen stream.

3.3.2.2 EQCM-D: Blood Plasma Coagulation on a Gold Cathode
After 15 min thawing at 37 °C, 200 μL of CBP was injected in the EQCM-D module maintained at 37 °C, and a baseline was acquired for approximately 10–15 min. Subsequently, the gold-coated sensor was cathodically polarized by applying a square pulse current in galvanostatic mode (I = −1 mA/cm², where I is the pulse amplitude; τ_{ON} = 1 s where τ_{ON} is the pulse width and τ_{OFF} = 5 s where τ_{OFF} is the inactive time). The control sample was left at open circuit potential (OCP). CBP was recalcified in order to trigger the coagulation process by injecting 20 μL of CaCl₂ (100 mM). The coagulation process was monitored via changes in frequency and dissipation.

3.3.2.3 Scanning Electron Microscopy (SEM)
Following the monitoring of CBP coagulation on a cathodically polarized gold surface using EQCM-D, the gold-coated sensors were
removed from the EQCM-D modules and rinsed 3 times in PBS. Subsequently, the sensors were fixed in a 3% v/v solution of glutaraldehyde in PBS for 30 min at room temperature. Sample dehydration in a graded series of ethanol (from 30% to 95% v/v) and drying over the critical point of CO2 using a critical point dryer followed (CPD 030, Bal-Tec AG, Balzers, Liechtenstein). Dried samples were subsequently sputter-coated with a 5–10 nm layer of platinum (SCD500, Bal-Tec AG, Balzers, Liechtenstein). Finally, surfaces were imaged with a Supra 50 VP scanning electron microscope (Zeiss AG, Oberkochen, Germany) at 10 kV using secondary electron signals with a magnification between 1 and 10 000.

3.3.2.4 **EQCM-D: Fg Electrochemical Desorption Studies**
Fg was thawed 15 min at 37 °C and diluted in NaCl (100 mM) to a final concentration of 1 mg/mL. After acquisition of a baseline in NaCl (100 mM) for 10–15 min, 200 μL of Fg (1 mg/mL) was injected in the EQCM module maintained at 37 °C, and the adsorption of Fg on the gold surface was monitored. Once saturation of adsorbed Fg was reached, the chamber was rinsed 3 times by injecting 200 μL of NaCl (100 mM). Subsequently, the gold-coated sensor was cathodically polarized in galvanostatic mode by applying a constant current of I = [−1000, −100, −80, −50, −10] μA/cm² or a square pulse current of I = −1 mA/cm² and τ_ON = [0.5, 1, 5] s and τ_OFF = 5 s. The frequency and dissipation signals were monitored for approximately 15 min.

3.3.2.5 **EQCM-D: Fg Degradation Studies and Fg Clottability**
Fg was adsorbed on the gold surface as described in the previous paragraph. No rinsing steps were performed after the adsorption to have high Fg concentration (1 mg/mL) in the bulk. The gold surface was cathodically polarized (I = −1 mA/cm², dc) for 10 min. The control sample was left at OCP. Subsequently, the samples were collected from the EQCM module. In a first experiment, the collected samples were reinjected on clean gold crystals at OCP after baseline acquisition in NaCl (100 mM). To test for the Fg clottability, 20 μL of CaCl₂ (100 mM) was injected after baseline acquisition and 20 μL of
thrombin (120 u/mL in PBS) was injected after saturation of Fg adsorption. In a second experiment, the collected samples were subjected to centrifugal filtration through a 30 kDa MWCO membrane following provider’s instructions (Amicon Ultra-0.5 mL for protein purification, Merck Millipore, Billerica, MA). The filtrates were subsequently reinjected on clean gold crystals at OCP after baseline acquisition in NaCl (100 mM).

3.3.2.6 MALDI-TOF MS and MALDI-TOFTOF MS
The filtrates obtained by centrifugal filtration through a 30 kDa MWCO membrane (see previous paragraph) were concentrated by vacuum centrifugation (Savant SpeedVac SC110 equipped with RH40-11 rotor, Thermo Fisher Scientific Inc., Waltham, MA) for approximately 40 min. Subsequently, the concentrated samples were further concentrated and desalted using ZipTipC18 pipet tips (Merck Millipore, Billerica, MA) and eluted in 2 μL of a 1:1 solution of H₂O:ACN with 0.1% TFA. 1 μL of the eluted samples was spotted on the MALDI target followed by on-plate mixing with 1 μL of a 0.7 mg/mL HCCA matrix solution in 1:1 of H₂O:ACN with 0.1% TFA. Measurements were performed on the UltrafleXtreme spectrometer (Bruker Daltonics, Bremen, Germany). Protein fingerprints in the 500–5000 Da mass range were acquired, and peptides were analyzed via reflectron type measurement.

3.3.2.7 Data Analysis
QCM-D data were analyzed with Matlab 7.12.0 (The Math Works Inc., Natick, MA). The normalized frequency response was obtained by dividing the desorption curve by the adsorption saturation value. Only the third overtones are shown for sake of clarity. EQCM curves were acquired at least 3 times (single measurements and averages are shown). MS data were acquired using the FlexControl3.3 software, and the MS-MS spectra were matched to peptide sequences generated via in silico digestion (no enzyme, threshold 0.8–1.2) using the Sequence Editor of BioTools3.2 (Bruker Dalctonics, Bremen, Germany). The MS spectra were measured on four independent replicate samples without showing variations in the dominant peaks.
3.4 RESULTS AND DISCUSSION

3.4.1 EQCM and SEM: Blood plasma coagulation was reduced at the cathode surface

The QCM-D technique has been used in the past to study blood coagulation at the surface of various materials.[29-31] Here we used the QCM-D technique coupled with an electrochemical setup (EQCM-D) to semiquantitatively study the coagulation of human CBP on a cathodically polarized gold surface (Figure 3.1). After acquisition of a stable baseline in CBP, we recorded frequency and dissipation changes upon CBP recalcification. On the gold surface at OCP, we observed a 240 Hz frequency drop occurring approximately 15 min after the injection of CaCl₂ (Figure 3.1a). Simultaneously, we observed a $120 \times 10^{-6}$ increase in dissipation (Figure 3.1b). As previously described, these shifts are indicative of the formation of a blood clot on the sensor surface.[29, 30]

The application of a pulsed cathodic current ($I = -1 \text{ mA/cm}^2$, $\tau_{\text{ON}} = 1 \text{ s}$, $\tau_{\text{OFF}} = 5 \text{ s}$) prior recalcification immediately caused a progressive increase of the frequency signal (Figure 3.1a), suggesting the desorption of plasma proteins from the surface, as later confirmed in Figure 2. We can exclude that this shift was due to permanent modifications of the gold substrate (e.g., delamination) because the same electric pulses applied in 100 mM NaCl did not cause a comparable frequency change (Supporting Information Figure 3.7). Approximately 15 min after the injection of CaCl₂ the frequency increase reversed probably because of the adsorption of fibrin fibers, resulting in a final 25 Hz net frequency drop (Figure 3.1a). The corresponding increase in dissipation was in the order of $50 \times 10^{-6}$ (Figure 3.1b). The observed frequency and dissipation changes were approximately 10% and 40% of the signal shifts observed for the control surface, respectively.
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Figure 3.1. Citrated blood plasma (CBP) coagulation at the cathode’s surface studied with EQCM-D and SEM. After baseline acquisition in CBP we applied cathodic pulses and subsequently injected CaCl₂ to trigger coagulation. The frequency (a) and the dissipation (b) signals showed reduced plasma coagulation upon the application of a pulsed cathodic current. (c) A photograph of the gold-coated sensors was taken (side view) after the experiment and showed a thick blood clot deposited onto the control surface at OCP (left side) while the polarized surface was clot-free (right side). The sensors were finally imaged at the SEM showing a densely packed fibrin network deposited onto the control surface (d) and a few isolated fibrin strands deposited onto the cathodically polarized surface (e).

Figure 3.1c shows the gold-coated crystals after the CBP recalcification experiment. The cathodically polarized surface appeared clean while the control surface was covered by a thick blood clot. SEM inspection revealed a homogeneous coverage of the control surface by a dense fibrin network (Figure 3.1d) and only isolated clusters of fibrin strands on the cathodically polarized gold surface (Figure 3.1e).

In summary, we showed that the coagulation of human CBP on a cathodically polarized gold surface was reduced compared to the coagulation on a non-polarized control surface. This result
supported previous work done by P. N. Sawyer and colleagues, who demonstrated the influence of the interfacial potential of metal prostheses on their thrombogenicity and showed that metal surfaces maintained at cathodic potentials are characterized by an increased thromboresistance in vivo.[23] Efforts to understand the mechanisms responsible for this effect led researchers in the past to focus on the interfacial electrochemical reactions involving plasma proteins, in particular Fg.[21] The frequency increase observed immediately after the cathodic current onset in the CBP recalcification experiment (Figure 3.1a) led us to hypothesize that protein desorption from the cathode surface might partially explain the reduced deposition of fibrin fibers.

3.4.2 EQCM: Fg electrochemical desorption from a gold cathode as a function of current density

Figure 3.2a shows the 120 Hz frequency drop caused by the adsorption of Fg (1 mg/mL in 100 mM NaCl) on the gold surface and the subsequent signal recovery once we applied the cathodic dc (I = −1 mA/cm², for potential values refer to the Supporting Information Table 3.1). The recovery of the frequency signal to values close to the baseline in NaCl indicated the almost complete desorption of Fg upon the application of the cathodic current. Figure 3.2b shows the desorption curves obtained at different dc current densities. Figure 3.2c shows the desorption curves obtained using a square wave pulse current of −1 mA/cm² in I for different duty cycles. Our results showed that the desorption rate increased with increasing current density and increasing duty cycle. We observed an initial drop in frequency following the onset of the cathodic current (most likely due to current-induced protein conformational changes on the surface) and a subsequent frequency increase if we applied a minimum current density of −80 μA/cm² (dc). Within 300 s, 80% of the Fg desorbed when the highest current density (i.e., −1 mA/cm² dc) was applied but only less than 20% removal was observed at −80 μA/cm². Interestingly, the average current density calculated for the lowest tested duty cycle (τ_{ON} = 0.5 s, τ_{OFF} = 5 s) is approximately −90 μA/cm², and the respective desorption curve is bounded by the
Figure 3.2. Dependency of Fg electrochemical desorption on current density. (a) After baseline acquisition in NaCl, Fg was adsorbed onto the gold surface, and once saturation was reached the chamber was rinsed with NaCl. Subsequently, a cathodic current was applied (~1 mA/cm², dc) and once the frequency signal recovered and stabilized close to baseline levels the current was switched off and the chamber rinsed with NaCl. (b) Fg desorption curves obtained with constant cathodic currents of various I. (c) Fg desorption curves obtained with pulsed cathodic current of I = −1 mA/cm² and various duty cycles. In (b, c) the bold curves are the averages of the single trials (dashed curves).

curves obtained at dc current densities −80 and −100 μA/cm² (Supporting Information Figure 3.7), suggesting that the average
current density is the parameter determining the desorption rate of Fg.

Our EQCM experiments supported the previous work of Mallon and co-workers showing Fg electrochemical desorption from a gold cathode using fluorescence and atomic force microscopy.[28] Additionally, our experiments showed the kinetics of the electrochemical desorption of Fg for different dc current densities and for a varying square pulse design. Fg desorption might explain previous ellipsometry and capacitance measurements revealing the decrease in Fg coverage with increased cathodic potentials.[21, 28]

3.4.3 EQCM: Readsoption of electrochemically desorbed Fg revealed Fg degradation

The elucidation of the nature of the electrochemically desorbed Fg might provide us with important insights about the reduced clot deposition at the cathode.[21] We therefore injected Fg in the EQCM chamber, monitored the adsorption, and without rinsing applied a cathodic dc (I = −1 mA/cm²). The same process was performed for the control, only no current was applied. We performed desorption experiments in the presence of high Fg bulk concentration in order to obtain large amounts of electrochemically treated Fg for the subsequent analysis. The apparent desorption rate of Fg measured with the EQCM decreased if Fg remained in the bulk probably because both adsorption and desorption take place (not shown).

After 10 min of electrochemical desorption we retrieved the content of the chamber and injected it into another QCM chamber containing a clean gold-coated crystal at OCP. Figure 3.3a shows the adsorption curves of the electrochemically desorbed Fg and the Fg that did not undergo electrochemical desorption. The readsoption of the electrochemically desorbed Fg resulted in a 30 Hz frequency shift whereas the readsoption of the Fg control resulted in a 100 Hz frequency shift comparable to the shift normally obtained for the first adsorption. Because the concentration of the two samples initially injected was the same and the chambers were not rinsed during the electrochemical experiment, the decreased frequency
drop was due to the reduced MW of the species present in the electrochemically treated sample. We ensured that the cellulose acetate membrane separating the electrodes was not Fg permeable after the exposure to the electric currents, thus excluding Fg dilution.
Figure 3.3b shows the adsorption curves of the electrochemically desorbed and the native Fg after centrifugal filtration through a 30 kDa filter. As expected, the injection of the filtrate of the Fg control resulted in no frequency shift because the three Fg chains all have MWs larger than 30 kDa. In contrast, we observed a 10 Hz frequency shift for the electrochemically desorbed Fg, indicating the presence of species with a MW lower than the membrane cutoff but large enough to be observed with the QCM.

In summary, these results indicated that the electrochemically desorbed Fg was degraded, and standard proteomics techniques were subsequently used to further investigate the nature of the desorbed products.

3.4.4 MALDI-TOF MS: Mass determination of the sub-30 kDa Fg degradation products

Figure 3.4 shows the mass spectra of the sub-30 kDa filtrates obtained from the electrochemical desorption of Fg described above (section 3.4.3). The NaCl blank sample did not show any peak in the 800–5000 Da range (Figure 3.4a). This control was relevant to exclude the presence of contaminants in the solvents and possibly coming from the leaching of the setup components during the electrochemical experiment. The native Fg control showed three dominant peaks at 1465.7, 1536.7, and 1616.7 Da (Figure 3.4b). The Fibrinopeptide A (FPA) has a mass of 1536.7 Da, and the peaks at 1465.7 and 1616.7 Da correspond to the FPA without the N-terminal Ala-20 and the phosphorylated FPA (phosphoserine present at position 22), respectively (the amino acid numeration in the text comprises the signaling peptide not present in the mature protein). Each Fg α-chain contains an N-terminal FPA which is cleaved by the enzyme thrombin to expose a polymerization site ($E_A$). This process starts the fibrillization process.[24] Interestingly, in the QCM experiment we did not observe the presence of the FPA (Figure 3b), possibly because of its too low concentration (MALDI samples were
subjected to several preconcentration steps) or because of its too low MW.

In addition to the FPA peaks, the electrochemically desorbed Fg presented a dominant peak at 2752.3 Da (Figure 3.4c). Because this peak was not present in the control samples, we concluded that this was a Fg degradation product of the electrochemical desorption. We further investigated the nature of these species using MALDI-TOF TOF MS.
MALDI-TOFTOF MS: The Fg degradation product contained the α-Chain N-Terminus polymerization site \( E_A \)

Using MALDI-TOFTOF MS, we determined that the peak at 2752.3 Da observed in the electrochemically desorbed Fg sample (Figure 3.4c) consisted of a 27 amino acid fragment of the mobile Fg α-chain N-terminus comprising the FPA (starting with Ala-20) and extending to the Ala-46 (Supporting Information Table 3.2 and Figure 3.9). This result suggested that a cleavage between the Ala-46 and the Cys-47 occurred upon the electrochemical desorption of Fg. Additionally, in this 2.7 kDa fragment we found the α-chain polymerization site EA Gly-Pro-Arg-Val (36–39). Finally, we confirmed that the peak at 1536.7 Da is the FPA (Supporting Information Figure 3.10 and Table 3.3).

Fg electrochemical degradation was previously reported using SDS-PAGE.[28] However, the low purity of the previously used Fg did not allow the identification of the low-MW degradation products observed in our study. Furthermore, the lack of an anodic and cathodic compartment separation (acetate cellulose membrane in our study) resulted in the inability to separately elucidate the nature of the anodically electropolymerized Fg and the cathodically degraded Fg.[21] This limitation is important for two reasons. First, a liquid chromatography–MS study showed that protein fragmentation can result from anodic oxidation.[32] Second, the anodic polymerization and the cathodic degradation of Fg have opposite effects on interfacial thrombosis, the first accelerating it[21, 33] and the second reducing it.

QCM-D: Electrochemical degradation of Fg resulted in impaired clotability

We addressed the functionality of the electrochemically degraded Fg. Figure 3.5 shows the clottability of the electrochemically desorbed Fg versus the clottability of the native Fg measured using the QCM-D. After the injection of the electrochemically desorbed Fg in the chamber (as described in section 3.4.3), thrombin was added
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To trigger the polymerization. The polymerization of Fg resulted in a 200 Hz frequency drop and a simultaneous $18 \times 10^{-6}$ dissipation increase. In contrast, the frequency and dissipation signals did not

Figure 3.5. Clottability of electrochemically desorbed Fg. Electrochemically desorbed Fg was readsorbed onto a clean gold surface (OCP) after baseline acquisition in NaCl and CaCl$_2$. Once saturation was reached thrombin was injected to trigger the polymerization of Fg. The frequency signal (a) and the dissipation (b) signals did not change upon injection of thrombin for the electrochemically desorbed Fg. In contrast, the frequency and dissipation shifts observed for the native Fg control indicated Fg polymerization.
shift in response to the injection of thrombin into the electrochemically desorbed Fg solution, suggesting that the electrochemically desorbed Fg lost its clottability.

Our QCM-D and MS results allowed us to attribute the impaired clottability of Fg and the reduced clot deposition at the cathode surface at least partially to the electrochemical cleavage of the N-terminal flexible sequence of the α-chain (2.7 kDa) containing the polymerization site EA (as schematically represented in Figure 3.6).

Figure 3.6. Schematic representation of the Fg molecule with the flexible N-terminus of the α-chain starting with the FPA and containing the polymerization site EA. Only one of the two α-chain N-termini is represented for better clarity. Upon electrochemical desorption from the cathode, the flexible N-terminus is cleaved. The 3D representation was adapted from Kollman et al.,[40] and the mobile α-chain N-terminus was drawn manually because its 3D coordinates have not been determined.
3.4.7 Possible mechanisms leading to the electrochemical desorption and degradation of Fg

Our final experiments were aimed at clarifying the mechanism(s) responsible for the electrochemical desorption of Fg from the cathode surface and for its degradation. Here we discuss three mechanisms that are likely to be involved in our observations. First, P. N. Sawyer and co-workers showed in previous potentiostatic and potentiodynamic studies of the interface between a Fg solution and a platinum electrode that the presence of Fg catalyzed the hydrogen evolution reaction at cathodic potentials (as reproduced in Supporting Information Figure 3.12). This was attributed to the electrolytic hydrogenation of Fg,[21, 34] which can lead to structural changes in the protein. We have limited information concerning site-specific electrocatalytic hydrogenation of proteins and peptides. The catalytic hydrogenation of aromatic amino acids is described in the literature. In particular, the indolyl group is sensitive to hydrogenolysis and this makes Tryptophan vulnerable to alteration due to hydrogenation.[35] However, our present work does not allow us to state if and which aromatic amino acids are involved in the observed Fg structural changes. Second, the alkaline environment forming in the proximity of the cathode due to electrolysis of water can result in alkali peptide hydrolysis. Previous work of our group addressed theoretically and experimentally the extent of pH changes resulting from electrolysis of water in the proximity of an indium tin oxide electrode.[36] To investigate the effect of an extreme alkaline environment on the Fg structure, here we prepared Fg solutions in 100 mM NaCl and added 200 mM NaOH. After overnight incubation at room temperature we neutralized the solution and performed centrifugal filtration through a 30 kDa MWCO membrane to analyze the filtrate using the QCM as previously done in Figure 3.3b. The adsorption of the alkali treated Fg filtrate resulted in a 10 Hz frequency shift and supported the hypothesis of alkali peptide hydrolysis as a possible mechanism explaining the electrochemical desorption and degradation of Fg (Supporting Information Figure 3.11). The easy accessibility of the flexible α-chain N-terminus region to the aggressive environment
might make it especially susceptible to cleavage. However other Fg regions not identified in our work might be degraded. It is important to mention that interfacial pH changes affect the structure and function of other blood coagulation factors not studied in this work. As most of enzymes, thrombin and factor XIII show pH-dependent activity,[37, 38] and we recently demonstrated that the activity of factor XIII is indeed changed close to the electrode surface.[39] We thus consider the electrochemical degradation of Fg and the consequent loss of Fg clottability to be an important mechanism explaining the reduced coagulation at the cathode, but not the only one.

Finally, to investigate if the formation of reactive oxygen species was involved in the desorption process, we performed the desorption experiment in oxygen-depleted atmosphere. We did not observe changes in the frequency signal compared to previous experiments in air (Supporting Information Figure 3.13), suggesting negligible contribution of such reactive groups in the degradation process.

3.5 CONCLUSIONS

Electrodes might contact blood in various situations. Examples include, but are not limited to, their use in vivo as stimulators and their in vivo and in vitro uses as sensors for substances present in blood. Understanding the interfacial thrombosis mechanism and the electrochemical reactions involving plasma proteins at the electrode surface is of fundamental importance to improve the design of electrodes in contact with blood and the way these are operated. Our understanding of the role of cathodic currents in reducing interfacial thrombosis considerably increased. We found that Fg electrochemically desorbs from the cathode and loses an important polymerization site located at one of its termini, affecting its ability to clot. This is most likely due to the local alkali peptide hydrolysis induced by the electrode, but more experiments have to be designed to provide stronger evidence on the direct or indirect electrochemical reaction(s) responsible for this observation. The conversion from Fg to fibrin and the fibrin deposition on the surface
are fundamental steps leading to thrombosis. However, future studies should focus on the electrochemical reactions involving other fundamental blood coagulation factors. In fact, a large part of this study was carried out using solutions of purified Fg; therefore, the validity of our conclusion in blood plasma, a solution containing hundreds of different proteins competing for the surface, is somewhat limited. For these reasons, the results presented here should be used with caution when comparisons with different systems (both *in vivo* and *in vitro*) will be made.

3.6 **Acknowledgments**

The authors thank Stephen Wheeler (IBT-ETH Zurich workshop) for manufacturing the custom-made QCM modules. SEM was performed at the Center of Microscopy and Image Analysis (ZMB, ETH and University of Zurich). B.R.S. thanks Dr. Andres Kaech and Klaus Marquardt for teaching and support. The authors thank Prof. Quan Jason Cheng (University of California, Riverside) for discussions. MS was performed at the Functional Genomics Center Zurich (FGCZ, University and ETH Zurich), and the staff is acknowledged for teaching and support. This work was financially supported by ETH and University Zurich.
3.7 SUPPORTING INFORMATION

3.7.1 Figures

Figure 3.7. Frequency signal measured in 100 mM NaCl upon the application of a cathodic square wave pulsed current ($I = -1 \text{ mA/cm}^2$, $\tau_{\text{ON}} = 1 \text{ sec}$, $\tau_{\text{OFF}} = 5 \text{ sec}$).

Figure 3.8. Dependency of Fg electrochemical desorption on current intensity (see Figure 3.2).
Figure 3.9. MALDI-TOFTOF MS spectrum of the 2752.3 Da precursor.
Figure 3.10. MALDI-TOFTOF MS spectrum of the 1536.7 Da precursor.
Figure 3.11. Cyclic voltammogram of the gold-coated QCM crystal in 100 mM NaCl before and after addition of Fg (1 mg/mL). The potential was swept for 15 minutes at the corresponding condition and then recorded. The platinum counter electrode and the Ag/AgCl pseudo-reference were separated from the gold working electrode by the acetate cellulose membrane impermeable to Fg.

Figure 3.12. Adsorption of the filtrate of native Fg incubated overnight at room temperature with 0.2 M NaOH and without NaOH. After incubation of the Fg in 0.2 M NaOH, the solution was neutralized by adding HCl. Centrifugal filtration was subsequently performed through 30 kDa MWCO membranes as done in Fig. 3. The baseline was acquired in 0.1 M (0 M NaOH) and 50 µL of the Fg filtrates were injected. After stabilization of the frequency signal, the chambers were rinsed 3X with 0.1 M NaCl (0 M NaOH). The shaded blue areas show the frequency change due to temperature stabilization.
Figure 3.13. Electrochemical desorption experiment in Oxygen-depleted atmosphere. 1 mg/mL Fg solution was prepared in 100 mM NaCl previously de-aired in vacuum for 2 hours and bubbled with a Nitrogen gun for 10 minutes. The electrochemical desorption experiment of Figure 3.2 (-1mA/cm², dc) was repeated. The Q-Sense E4 chamber was placed in a plastic bag and was perfused with a constant Nitrogen flow starting 15 minutes before the experiment and during the desorption.

3.7.2 Tables

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Table 3.1. Potential measured after 2 min of application of the minimal and maximal current density (dc) used in this study. The potential was stable and measured in a 3-electrode setup against a pseudo-reference Ag/AgCl. The platinum counter electrode and the Ag/AgCl pseudo-reference were separated by the gold working electrode from the acetate cellulose membrane.
3 COAGULATION AT THE BLOOD-ELECTRODE INTERFACE: THE ROLE OF ELECTROCHEMICAL DESORPTION AND DEGRADATION OF FIBRINOGEN

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Table 3.2. MALDI-TOFTOF MS of the electrochemically generated Fg degradation product. Theoretical masses of the fragmented 2752.3 Da peptide are shown. The experimental values matching the theoretical fragmentation values are marked in bold. The 2752.3 Da precursor was identified as the mobile N-terminus of the Fg α-chain starting with the FPA and terminating with the Ala-46. Importantly, this sequence includes the Fg α-chain EA constituted of the Gly-Pro-Arg-Val (36-39) motif. The spectra of the 2752.3 Da and of the 1536.7 Da precursors are shown in Suppl. Figures S4 and S5. The amino acid numeration starts at position 20 (N-term) because it includes the signalling peptide (1-20) not present in the mature protein.
Table 3.3. MALDI-TOF MS of the Fibrinopeptide A (FPA). Theoretical masses of the fragmented 1536.7 Da peptide are shown. The experimental values matching the theoretical fragmentation values are marked in bold. The amino acid numeration starts at position 20 (N-term) because it includes the signaling peptide (1-20) not present in the mature protein.

3.8 References


4 ANODIC POLYMERIZATION OF FIBRINOGEN: AN ELECTROCHEMICAL QUARTZ CRYSTAL MICROBALANCE STUDY

4.1 ABSTRACT

Fibrinogen (Fg) was previously shown to oxidize over a wide range of anodic potentials and the oxidation process is considered the mechanism leading to its electropolymerization to form fibrin-like fibers. First, we used SEM to confirm the formation of anodic fibrin. We subsequently used for the first time the electrochemical quartz crystal microbalance with dissipation monitoring (EQCM-D) technique to monitor in real-time the formation of anodic fibrin as function of the applied anodic potential. We used buffered and non-buffered electrolytes to investigate the role of the interfacial electrolyte acidification in the polymerization process. This chapter presents a set of preliminary experiments and several of the aspects presented here require future investigation to formulate solid conclusions.
4.2 INTRODUCTION

Potentiostatic and potentiodynamic studies of Fg in saline solution at the interface with a platinum electrode revealed its participation to charge transfer reactions over a wide range of anodic potentials. In particular, currents measured at anodic potentials increased when Fg was present in solution even at small concentrations compared to the measurement in saline. This effect was more evident for higher and physiological Fg concentrations ([1] and Chapter 1). The anodic reaction of Fg was related to the previously observed Fg electropolymorization leading to the formation of fibrin-like fibers deposited at the anode surface.[2] The interfacial oxidation of Fg was proposed as a mechanism responsible for its activation and fibrillization at conducting or semi-conducting surfaces. As shown by reversed phase high performance liquid chromatography, this mechanism involves the anodic cleavage of Fg peptides. In contrast to the thrombin-mediated specific peptide cleavage and activation, the anodic peptide cleavage appeared non-specific, with possible consequences on the nature of the fibrin fibers formed.[3]

The molecular stability of Fg is affected by pH changes (see Chapter 1). Below its pl (5.5), Fg denatures and start to rapidly agglomerate.[4] It is therefore appropriate to question whether the electropolymorization of Fg is a consequence of a direct oxidative process of the protein or is a consequence of the interfacial acidification of the electrolyte resulting from water oxidation. This matter was already discussed at the time when the anodic polymerization was first described.[5]

Here we first confirmed using SEM the formation of fibrin-like fibers at the anode surface. We subsequently used the EQCM technique to study Fg oxidation and real-time fibrin deposition at anodic potentials. We performed experiments in buffered and non-buffered electrolyte in an attempt to isolate the effect of the interfacial acidification in the electrodeposition process.
4.3 EXPERIMENTAL SECTION

4.3.1 Materials

Information concerning the materials used in this chapter, including Fg, EQCM sensors and instrumentation, is provided in Section 3.3.1. Sodium chloride (NaCl; 99.8%, code 71380) was purchased from Sigma-Aldrich (St. Louis, MO). Tris(hydroxymethyl)-aminomethane (Tris base, C₄H₁₁NO₃, code A2264,0500) was purchased from AppliChem, Germany.

4.3.2 Methods

Information concerning the cleaning procedures of the EQCM crystals is provided in the paragraph 3.3.2.1. Preparation of the electrode surfaces for SEM inspection was performed as described in paragraph 3.3.2.3.

The potentiodynamic EQCM-D experiment was carried out as follows. A baseline was acquired in 100 mM NaCl and 50 mM Tris buffer at OCP for at least 5 min. The gold electrode was then anodically polarized from the OCP to 500 mV (vs Ag/AgCl pseudo-reference electrode) within 5 min. The anodic potential was maintained constant for 5 min and then decreased to 0 mV within 5 min. This first potential cycle was performed in order to measure the frequency and dissipation response of the bare gold crystal to the potential change. Fg (3 mg/mL in 100 mM NaCl and in 50 mM Tris buffer) was injected in the EQCM-D module and the frequency and dissipation changes, due to Fg adsorption, were monitored for approximately 30 min at OCP. The potentiodynamic protocol was repeated in presence of Fg. The first cycle reached a maximal anodic potential of 500 mV within 5 min (as previously done in NaCl and Tris) whereas the second cycle reached a maximal anodic potential of 700 mV within the same time. The frequency and dissipation signals were recorded in order to detect Fg anodic polymerization.
4.4 RESULTS AND DISCUSSIONS

4.4.1 SEM inspection revealed fibrin-like fibers at the anode surface

Figure 4.1 shows SEM micrographs of gold electrode surfaces incubated for 30 min with Fg (3 mg/mL in 100 mM NaCl). In Figure 4.1a, the surface was left at OCP, whereas in Figure 4.1b the electrode was anodized (+800 mV vs Ag/AgCl). The anodic potential produced fibrous structures deposited on the surface. The fibrous aggregates were heterogeneous in size and had a nodular appearance. The distribution of the electrodeposited fibers over the electrode surface was homogeneous. The SEM inspection provided us with an impression of the electrodeposited fibers in the dry state. Therefore, in a separate set of experiments which is not presented here, we monitored their formation on thin transparent anodized
gold or indium tin oxide films with an optical microscope. The fibers appeared anchored at the surface of the electrode at one extremity only, with the remaining portion of the fiber free in solution. The film thus appeared to us as a “nodular fibrin brush coating”.

The morphology of the fibers produced at the anode is different from the morphology of natural fibrin assembled upon thrombin activation. In the SEM micrographs depicting natural fibrin fibers, presented in the previous chapter (Figure 3.1d and e), we could not observe the nodular character of the strands which characterized the anodic fibrin. Studies based on targeting specific sites of the fibrin molecule with antibodies should be designed to investigate the differences between natural and anodic fibrin. It would be equally interesting to study the adhesion of platelets on anodic fibrin fibers to acquire information on the preserved natural functionality.

4.4.2 Potentiodynamic EQCM-D study

In Figure 4.2 we present a potentiodynamic study performed in combination with QCM-D. This experiment was designed to monitor in real time the deposition of the anodic fibrin and possibly identify a potential threshold above which the process is triggered. Additionally, we conducted this experiment in buffered and non-buffered electrolyte in an attempt to gain information on the role of the acidification of the electrolyte close to the anode in the electropolymerization process. Figure 4.2a shows the applied potential vs time. The experiment started with the acquisition of baseline frequency (Figure 4.2b) and dissipation (Figure 4.2c) signals, with the gold electrode at OCP in the electrolyte containing no Fg. The first anodic cycle was performed in order to measure the frequency and dissipation response upon anodization in absence of Fg. In particular, gradually increasing and maintaining the potential at 500 mV did not affect the baseline signals. Finally, we gradually returned the potential to 0 mV. We subsequently injected Fg (3 mg/mL) and after 30 min, we observed a frequency drop in the range of 120-150 Hz and dissipation increase in the range of 5-8 × 10⁻⁶.
When we gradually increased the potential to 500 mV in presence of Fg, we observed only a minor decrease in frequency (< 10 Hz) and no dissipation increase. In contrast, when we reached 700 mV in the second anodic cycle, we observed a clear drop in frequency and dissipation increase, regardless of the presence of buffer in the electrolyte. However, the magnitude of the changes in the signals that we observed in absence of buffer in the electrolyte was considerably higher. In particular, we measured a frequency drop

Figure 4.2. Potentiodynamic EQCM-D experiment revealing Fg anodic polymerization. a) Applied potential vs time. Frequency (b) and dissipation (c) signals were monitored to detect Fg adsorption and anodic polymerization.
upon anodization at 700 mV of approximately 520 Hz in NaCl and of approximately 40 Hz in Tris buffer. Analogously, the dissipation increase was approximately $35 \times 10^{-6}$ in NaCl compared to $2.5 \times 10^{-6}$ in Tris buffer. Increasing the potential to 700 mV in absence of Fg did not result in similar frequency decrease and dissipation increase. In contrast, approaching 700 mV in absence of Fg induced a positive drift in frequency (10 Hz over the first 15 min), suggesting the slow corrosion of the gold film (not shown here). These results revealed two important aspects of the anodic deposition of Fg. The first aspect concerns the presence of a minimum anodic potential (here between 500-700 mV vs Ag/AgCl) in order to observe anodic fibrin formation with the EQCM-D technique. It is possible that this process occurs at less anodic potentials and we could not observe it on the EQCM-D. SEM inspection of electrodes anodized at different potentials is needed to confirm the potential threshold deduced with the EQCM-D. The second important aspect revealed by this experiment is the role of the buffered electrolyte. The reduced frequency and dissipation changes observed in the potentiodynamic experiment in Tris buffer compared to NaCl, suggested less formation of anodic fibrin. If we consider that Fg anodic polymerization is the result of the electrolyte acidification when the potential triggers water electrolysis, one explanation for our observation might be the reduced acidification of the electrolyte in Tris buffer compared to saline as the cause for the reduced anodic fibrin deposition. Fg is known to denature and aggregate at low pH.[4] However, it is not possible to certainly attribute the observed difference to the reduced acidification of the electrolyte, because the Tris base might have interfered with the anodic polymerization in other ways that we currently ignore. It would be therefore necessary to repeat this experiment with several buffered electrolytes to see if the reduction of frequency and dissipation is always observed when acidification of the electrolyte is reduced.

Fg was shown to oxidize in NaCl on platinum electrodes[1] and also in Tris buffer on SnO$_2$ electrodes[3]. In accordance with these observations, we report in Figure 4.3 the anodic current measured during the anodic sweep from OCP to 600 mV in presence and
absence of Fg. We do observe an oxidative current over a wide range of anodic potentials introduced by Fg. However, this measure provided different results when parameters such as the electrode used and polarization rate were changed. An informative experiment to be carried out in the future should measure the anodic current in presence of Fg and in presence of another protein adsorbed on the electrode (e.g. BSA). The comparison might be facilitated by similar capacitive currents which could make easier the identification of the oxidative current specific to Fg. Our experiments did not challenge the previous observations of the Fg oxidation and consequent polymerization but strongly suggested that the electrolyte acidification, might at least accelerate or enhance the aggregation of Fg on the surface of the anode.

4.5 CONCLUSIONS

We confirmed by SEM inspection the formation of fibrin-like fibers depositing at anodized interfaces and we found the morphology of these fibrous aggregates to be different from previously imaged natural fibrin on similar surfaces. Subsequently, we used for the first time the EQCM-D technique to study the anodic polymerization of Fg. We demonstrated that it is possible to measure the anodic polymerization in real-time and found that substantial frequency and dissipation changes, indicating polymerization, occurred when
the potential reached the range between 500 and 700 mV, but did not occur at less anodic potentials. Our EQCM-D experiment highlighted the importance of the electrolyte acidification at the anode as a possible driving force for the polymerization. Several of the aspects presented in this chapter require further investigation and appropriate experiments were suggested.

4.6 References


5 ELECTROCHEMICAL CONTROL OF THE ENZYMATIC POLYMERIZATION OF PEG HYDROGELS: FORMATION OF SPATIALLY CONTROLLED BIOLOGICAL MICROENVIRONMENTS

5.1 ABSTRACT

Control of pH gradient profile at the electrode–electrolyte interfaces allows the control of the enzymatic PEG-hydrogel polymerization. By tuning the solution pH, buffer capacity, and the applied current, the extent of the local inhibition and confinement of the Factor XIII-mediated polymerization of PEG are controlled. This technology opens new perspectives for the production of 3D-structured biological microenvironments.

5.2 INTRODUCTION

Three-dimensional (3D) engineered tissues are largely desirable to allow physiological studies and the development of regenerative therapies. Most of the recent attempts to build tissue mimetics were based on the culture of cells in extracellular matrix (ECM) hydrogels. In contrast to the inherent biological properties of natural hydrogels, synthetic hydrogels such as poly(ethylene glycol) (PEG) have no intrinsic interaction with biological systems, hence don’t trigger – nor are affected by biological processes. However, the ability to precisely tailor the physical properties and the molecular architecture together with the selective introduction of biological functional molecules, namely proteolytic sites, cell adhesion sites and other biological cues makes PEG hydrogels an ideal platform for the emulation of naturally occurring ECM.[1-3]

We recently introduced modular-designed PEG hydrogels, which are polymerized by a Factor XIII (FXIII)-mediated transglutamination (TG) reaction and thus are referred to as TG-PEG hydrogels.[4] During the FXIII-mediated crosslinking of the Glutamine-PEG (Gln-PEG) and the Lysine-PEG (Lys-PEG) precursors, various Gln- and Lys-tagged peptides and proteins can be covalently bound to the forming matrix.[5, 6] The enzymatic crosslinking reaction allows the site specific, orthogonal integration of bioactive molecules under physiological conditions to provide various functionalities.[7-9]

Although the ability to chemically functionalize PEG hydrogels represented a major achievement, controlling the spatial chemical composition is necessary to engineer synthetic hydrogels mimicking the natural ECM.[10-13] Techniques including casting,[14, 15] additive manufacturing (e.g. printing and layer-by-layer deposition),[16, 17] photo-patterning[10, 18, 19] and microfluidics[20, 21] are amongst the techniques successfully used to produce 3D structured hydrogels with spatial control of the morphogenetic cues.

One of the main challenges in the additive manufacturing of soft hydrogels remains the optimization of the depositing material’s
Electrochemical polymerization (e.g. oxidative polymerization) has the considerable advantage of being inherently independent of the polymer mechanical properties and was successfully used to locally control the polymerization of polyaniline,[23, 24] polypyrroles,[25] polyacrylates[26] and other electroactive polymers[27, 28] for applications spanning from organic electronics to organic film deposition on biomedical implants. Here we describe for the first time the use of electrochemistry to control TG-PEG hydrogel
polymerization, resulting in spatially controlled microenvironments containing stably integrated biological cues.

Like most chemical and enzymatic reactions, the FXIII-mediated transglutamination is pH-dependent and occurs with highest efficiency at pH 8, while at more acidic or basic conditions it is damped (Figure 5.1a). Upon the application of an electric current the electrolysis of water results in a local pH decrease at the anode-electrolyte interface and in a local pH increase at the cathode-electrolyte interface (Figure 5.1b).

Consequently, we show that the extent of the region where the polymerization could be inhibited or confined depend on the applied current density, pH and buffer capacity of the precursor solution in proximity of an electrode. We used this concept to engineer TG-PEG microenvironments characterized by a defined architecture and by the controlled spatial distribution of chemical moieties that can be used to instruct cells in culture. We demonstrate that the formed microenvironments can serve as provisional cell-permissive matrices, which provide graded biological cues, much like natural ECM. The complexity of the patterns obtained with this method is mainly limited by the ability to design appropriate electrode configurations.

5.3 RESULTS AND DISCUSSIONS

To perform all the experiments described here we designed polydimethylsiloxane (PDMS) molds accommodating up to three Tungsten electrode wires into which we casted the TG-PEG precursor solution (Figure 5.1c). To demonstrate that the hydrogel polymerization is inhibited at the anode, due to the local reduction of pH, FITC-tagged Lys substrates (Lys-FITC) was admixed to TG-PEG hydrogel precursors and formulated at pH 7.6. Confocal fluorescence microscopy images of carefully washed hydrogels demonstrated that the hydrogel polymerization was inhibited in proximity of the anode as indicated by the reduced fluorescence intensity. The extent of the inhibition region increased with an increasing current density and with a decreasing buffer concentration (Figure 5.2).
Figure 5.2. Inhibition of TG-PEG polymerization by electrochemistry. Confocal fluorescence images of FITC-tagged TG-PEG hydrogels prepared with precursor solutions containing 50 mM Tris (pH 7.6) and current densities varying from 0 to 8 μA/mm² applied to the anode (left side of images; a–e) and corresponding fluorescence intensity profiles (i). f–h) Hydrogels prepared with 10 mM Tris (pH 7.6) with current densities varying from 0 to 2 μA/mm² and comparison of the intensity profiles at different buffer concentrations and current densities (j). Representative images and intensity profile curves are shown.
Confocal fluorescence images of the FITC-tagged hydrogels prepared with a Tris concentration of 50 mM and current density increasing from 0 to 8 µA/mm² are shown in Figure 5.2a-e. Figure 5.2f-h shows images of hydrogels prepared with a Tris concentration of 10 mM and increasing current density from 0 to 2 µA/mm². A quantification of the fluorescence intensity is presented in Figure 5.2i-j. In particular, at 50 mM Tris we did not observe a decrease in fluorescence intensity in proximity of the anode when we applied 500 nA/mm² (Figure 5.2b). In contrast, a decrease in fluorescence was already visible at 500 nA/mm² when we decreased the Tris concentration to 10 mM (Figure 5.2g). At 50 mM Tris we measured a maximal extent of the visible inhibition region of approximately 200 µm when we applied 8 µA/mm² (Figure 5.2e). We approached this value using only 2 µA/mm² when we decreased the Tris concentration to 10 mM (Figure 5.2h). These observations correlate with studies describing the pH profile at the electrolyte-electrode interface being highly affected by the applied current density and buffer concentration.[29, 30]

To confirm that the pH drop in proximity of the anode accounted for the inhibition of the TG-PEG polymerization we prepared hydrogels at pH 5 and pH 11. Under both conditions the TG-PEG hydrogels did not polymerize (Figure 5.3a and d). However, when we applied 5 µA/mm² we observed the polymerization locally taking place in proximity of the cathode for the hydrogel prepared at pH 5 (Figure 5.3c) and in proximity of the anode for the hydrogel prepared at pH 11 (Figure 5.3e). The extent of the polymerization region was approximately 100 and 200 µm at the anode and at the cathode, respectively. The choice of the working solutions’ pH was made to be symmetrically distant from the FXIII optimum and intuitively the polymerization region would extend equally at the anode and at the cathode. However, we do not know whether the enzyme activity decreases symmetrically at acid and basic pH. The importance of this experiment is twofold: first it showed the ability to spatially confine the polymerization in proximity of the electrode. Secondly, it demonstrated that the inhibition of the polymerization observed in Figure 5.2 was not due to a quenching effect of the FITC caused by
the electric current and/or pH change nor due to electrophoretic mobility of molecules rather to the blockage of the enzymatic function of FXIII. Overall, these results indicated that the FXIII activity can be actively inhibited or activated by electrochemical modulation of the local pH.

Next, we demonstrated that the ability to spatially control the polymerization of TG-PEG can be successfully used to create spatially defined biological microenvironments. The formation of microchannels in 3D hydrogels remains an important challenge in the development of in vitro scaffolds. Chrobak and colleagues addressed this challenge by placing a stainless steel microneedle prior gel polymerization and by subsequently removing it to form a microchannel.[31] Our initial attempts to use metal wires as
templates to create microchannels in TG-PEG hydrogels often resulted in the disruption of the latter because of the too strong adhesion to the metal surface (Figure 5.6). We therefore anodically polarized template tungsten wires and as a result of the local inhibition of the polymerization at the anode’s interface we could create channels without mechanically stressing the hydrogel. We quantified the mechanical stress induced to the hydrogel during the tungsten needle removal by suspending 20 μm fluorescent microparticles in the hydrogel (Figure 5.4). By tracking the microparticle displacement as a function of the applied current density (Figure 5.4d), we found that an anodic current density of 2
µA/mm² was insufficient whereas 5 µA/mm² drastically suppressed bead displacement and the consequent damage to the hydrogel structure. Importantly, the production of channels could be even performed in the presence of cells without affecting their viability (Figure 5.7).

To demonstrate the versatility and potential of the described method we combined the local inhibition and the confined polymerization of TG-PEG to create a structured biological microenvironment. We sequentially polymerized a pH 5 gel functionalized with a chemical compound of choice (i.e. FITC or interleukine-4 (IL-4)) around the cathode by applying -5 µA/mm² (Figure 5.5a). After thorough washing of the unbound hydrogel precursors and growth factors (Figure 5.5b) the PDMS mold was backfilled with another Alexa 561-labelled TG-PEG hydrogel (pH 7.6) (Figure 5.5c), in which a channel was created as described above (Figure 5.5d). Various cell types ranging from bone marrow derived MSCs (Figure 5.5h), preosteoblasts or fibroblasts (not shown) could be delivered to the channel and invade the surrounding environment (Figure 5.5i and Figure 5.8). Additionally, HEK cells engineered to sense and report IL-4 by expressing a YFP signal, and placed in a microenvironment containing a local IL-4 repository, responded in a spatial specific manner (Figure 5.5j-m). In particular, the number of IL-4 sensitive cells expressing high levels of YFP (Figure 5.5k) over the number of transfected cells (mCherry positive cells, Figure 5.5j), increased at the vicinity of the IL-4-incorporated gel (Figure 5.5m). Taken together, the ability of cells to invade electrochemically engineered substrates and to sense the locally immobilized molecular cues demonstrated the potential of this approach to generate 3D structured substrates emulating the ECM-mediated presentation of growth factor or cytokine gradients.

In conclusion, by electrochemically controlling the enzymatic cross-linking, we have successfully established a platform to produce 3D patterned PEG hydrogels which can serve as cell-instructive microenvironments to build tissue-models. Based on the remarkable 3D microstructures realized in the field of organic electronics we
expect that significant improvements in the spatial complexity and resolution will be achieved.\[25, 32\] Additionally, the electrochemical control of the enzymatic polymerization is a versatile technique combining additive and preventive manufacturing (inhibition of polymerization). Indeed, the decreased adhesion of the hydrogel to the electrode surface described in this work can be used to improve the reproducibility of template removal-based techniques.\[31, 33\] Thus, electrochemically controlling the enzymatic polymerization of TG-PEG hydrogels has the potential to become a precise, inexpensive and automatized tool for 3D patterning of cells and cell-instructive cues in hydrogels.
Figure 5.5. Electrochemical control of gel polymerization can be used to form complex, locally functionalized 3D microenvironments. a–d) Simplified schemes depicting the production of an engineered microenvironment: FITC or IL-4 containing gel precursors at pH 5 were cast in a PDMS mould (Scheme 1) and locally polymerized around a cathode applying −5 μA/mm². After polymerization and removal of the unpolymerized precursors by rinsing (b), a second gel precursor solution functionalized with Alexa 561 (pH 7.6) was poured in the mould and an anodic current of 5 μA/mm² was applied to a second electrode during polymerization (c), resulting in a microenvironment constituted, after electrode removal, of a channel for cell delivery and a locally biofunctionalized region (d). Confocal microscopic images of the resulting microenvironment (g) with FITC-incorporated region (e) and a channel formed in the Alexa 561 gel (f). Mesenchymal stem cells (MSCs) were perfused in the channel (h) and invaded the surrounding environment (i, picture taken at day 7). IL4-sensing HEK cells, constitutively expressing mCherry (j), expressed higher YFP levels (k) in proximity of the IL-4-incorporating gel. Bright field image is shown to locate the electrode (l). m) Ratio of YFP/mCherry-expressing cells as function of the distance from the electrode (values represent means and standard deviation of three experiments).
5.4 EXPERIMENTAL SECTION

5.4.1 Preparation of the PDMS frames

Polydimethylsiloxane (PDMS) frames were made as follows: the silicon elastomer and the curing agent (Sylgard 184, Dow Corning Corporation, USA) were mixed (10:1 in mass) at 2000 rpm for 3 min in a ARE-250 mixer (Thinky Corporation, Japan). The mixture was subsequently poured into poly(methyl methacrylate) (PMMA) molds, where 500 µm in diameter stainless steel wires were positioned to create the holes for the future electrodes. The mixture was subsequently degassed for 30 min in a vacuum chamber and baked for 4 h at 60 °C. The stainless steel wires and the PDMS forms were removed from the PMMA molds, rinsed with isopropanol (IPA), oxygen plasma cleaned (1 min at 300 W, Plasma-System 100, Technics Plasma GmbH, Germany) and finally pressed onto microscope glass cover slips. Straightened Tungsten wires (W, 500 µm in diameter, Advent Research Materials Ltd, UK) were inserted in the PDMS forms and connected to a potentiogalvanostat in a two electrode setup (PGU-10V-1A-IMP-S and ECMwin computer interface, Elektroniklabor Peter Schrems, Germany).

5.4.2 Production of PEG precursors

Eight-arm PEG precursors containing the pending Factor XIII activated (FXIIIa) substrate peptides glutamine acceptor substrate (n-PEG-Gln) or lysine donor substrate containing a MMP-sensitive linker (n-PEG-MMP-sensitive-Lys) were produced and characterized as described elsewhere.[6] In brief, eight-arm PEG mol. wt. 40000 was purchased from Nektar (Huntsville, AL, USA). Divinyl sulfone was purchased from Aldrich (Buchs, Switzerland). PEG vinylsulfone (PEG-VS) was produced and characterized as described elsewhere.[34] The FXIIIa substrate peptides H-NQEQVSPL-ERCG-NH2 (TG-Gln) Ac-FKGG-GPQGIWGQ-ERCG-NH2 (MMP-sensitive-Lys), and the adhesion ligand Ac-GCYRDGSPG-NH2 (TG-Gln-RGD) were obtained from NeoMPS (Strasbourg, France) (immunograde, C18-purified, HPLC analysis: > 90%). The NQEQVSPL cassette corresponds to the FXIIIa substrate site in α2-plasmin inhibitor,[35] the FKGG cassette
to an optimized FXIIIa substrate site,[36] and the ERCG cassette to the vinylsulfone-reactive Cysteine[37]. In separate vials TG-Gln, TG-MMP-sensitive-Lys were added to PEG-VS in 1.2-fold molar excess over VS groups and allowed to react in 0.3 M triethanolamine (pH 8.0) at 37°C for 2 h. The products were dialyzed (Snake Skin, MWCO 10K, PIERCE, Rockford, IL, USA) against ultrapure water for 3 days at 4°C. After dialysis, the salt-free products (8-PEG-MMP-sensitive-Lys and 8-PEG-Gln, respectively) were lyophilized.

5.4.3 PEG hydrogel preparation

1mL of FXIIIa (200 U/mL, Fibrogammin, CSL Behring, Switzerland) was activated with 100 μL of thrombin (20 U/mL, Sigma-Aldrich, Switzerland) for 30 min at 37°C. Small aliquots of activated FXIIIa were stored at -80°C for further use. Hydrogels with a final dry mass content of 1.5% were prepared by stoichiometrically balanced ([Lys]/[Gln] = 1) precursor solutions of n-PEG-Gln and n-PEG-MMP-sensitive-Lys in Tris-Buffer (Tris) with varying molarity and pH, containing 50 mM calcium chloride (CaCl₂). Lys-FITC, TG-Alexa 561, Gln-RGD or combinations were added to the precursor solution prior to initiation of cross-linking by 10 U/mL thrombin-activated FXIIIa and vigorous mixing.

5.4.4 Electrochemical control of TG-PEG polymerization

To study the effect of electrochemistry on TG-PEG polymerization a 60 μL solution composed of TG-PEG, Tris (50 mM or 10 mM, pH 5, 7.6 or 11), CaCl₂ and a fluorescent agent such as Lys-FITC, TG-Alexa 561 or fluorescent polystyrene beads (Fluoresbrite Plain YG 20 micron microspheres, Polyscience Inc.) was mixed with the FXIIIa. The mixture was immediately poured in the PDMS frame. The polymerization of the TG-PEG was allowed to progress during 6 minutes in presence of a dc current applied in galvanostatic mode. The current density was varied in a range between 0 and 8 μA/mm².

5.4.5 Confocal laser scanning microscopy (CLSM)

The TG-PEG hydrogel-electrode interfaces were imaged using a LSM 510 confocal laser scanning microscope (Carl Zeiss AG, Germany).
The focal plane was adjusted to obtain the maximal section of the tungsten wire (500 μm). The FITC was detected upon excitation at 490 nm with 0.7% laser power, and emission band-pass filter 505–550 nm. Alexa 561 was detected upon excitation at 515 nm and with emission band-pass filter 575–615 nm. The intensity profiles were obtained over a 500 μm × 500 μm field of view by setting the minimum intensity as the average intensity of the electrode and by normalizing the values over the average intensity of the distal 200 μm (maximal intensity). At least three samples per condition were analyzed and two images per electrode were acquired.

5.4.6 Quantification of hydrogel displacement upon electrode removal

To study the effect of electrochemistry on the adhesion of the Tungsten wire from the TG-PEG hydrogel upon removal and on the subsequent formation of a microchannel, the displacement of 20 μm polystyrene particles dispersed in the hydrogel was tracked using a Leica fluorescence microscope (BM550B, Leica Microsystems, Germany). The TG-PEG hydrogel was prepared as described above and the Tungsten wires were manually pulled out of the gel. Images were recorded every 100 ms and the particles were detected upon excitation at 488 nm. The particle trajectories were calculated using an Image J script previously described.[38]

5.4.7 Cell invasion assay

After channel formation, a solution of 10⁶/mL human bone marrow derived mesenchymal stem cells (MSCs) in serum free DMEM/F-12 + GlutaMAX™ (Gibco Life Technologies, cat. no. 31331-028) supplemented with 1% (v/v) penicillin/streptomycin solution (P/S, Gibco Life Technologies, cat. no. 15140-122) was perfused into the channel. Gels were subsequently placed in medium supplemented with human platelet-derived growth factor BB (PDGF-BB, 10 ng/ml, Peprotech, cat. no. 100-14B) and kept in culture for 7 days. Bright field images were acquired with a ZEISS Axiovert 200M inverted microscope.
5.4.8 Cell IL4 response assay

HEK-IL4 reporter cells were produced as described previously [9]. In brief, HEK 293T cells were transfected with pHW40 (PSTAT6-eYFP-pA) and the constitutive expression vector STAT6 (obtained from Open Biosystems, Huntsville, AL, Clone ID 5530399). A constitutive mCherry expression plasmid (pMK47) was used as internal control. For the 3D IL4 response assay, $10^6$/mL reporter cells were resuspended in TG-PEG precursor solution and cultured for 24 hours in DMEM/F-12 + GlutaMAX™ supplemented with 10% (v/v) fetal calf serum (FCS, Gibco Life Technologies, cat. no. 10500) and 1% (v/v) P/S. Fluorescent and brightfield images were acquired with a LEICA DMI6000 B inverted microscope.

As a measure of IL-4 responding cells, the ratio of cells expressing YFP over cells expressing mCherry was determined with ImageJ. In particular, when a microenvironment was produced with a IL-4 functionalized area around the electrode, the IL-4 response in 600 µm wide regions was measured, and the mean and standard deviation calculated out of 3 independent experiments.

5.5 ACKNOWLEDGMENTS

This work was supported by the Centre for Clinical Research University Hospital and University of Zurich, the Swiss National Foundation grant numbers CR2313_143766/1, 310034A_141051/1, and ETH Zurich. The authors thank Stephen Wheeler (IBT Toolshop, ETH Zurich) for manufacturing the PMMA molds and the technical support. The authors also thank Prof. Wilfried Weber (University of Freiburg, DE) for providing plasmid pMK47 and Prof. Ivan Martin (University Hospital Basel, CH) for providing human bone-marrow-derived mesenchymal stem cells.
5.6 **SUPPLEMENTARY INFORMATION**

![Supplementary Image](image)

Figure 5.6. Brightfield images of channels formed in PEG hydrogels by template removal of tungsten wires without anodic polarization. Example of a) intact channel and b) disrupted gel.

![Supplementary Image](image)

Figure 5.7. Live/Dead assay on MSCs suspended in the gel during channel production with 0 (a) and 5 µA/mm² (b). No difference in cell viability was observed, as almost no red (dead) cells were visible. For the Live/Dead assay, $10^6$/mL human bone marrow derived mesenchymal stem cells (MSCs) were resuspended in TG-PEG precursor solution. The solution was polymerized with and without anodic polarization prior to wire removal. Live/Dead assay (Sigma Aldrich, Switzerland) was carried out according to the manufacturer’s protocol.
Figure 5.8. Brightfield images of MSCs perfused in the channel (a) and consequent invasion of the surrounding environment after 2 (b), 4 (c) and 7 days (d).
REFERENCES


5 Electrochemical control of the enzymatic polymerization of PEG hydrogels: Formation of spatially controlled biological microenvironments


6 DENSITY GRADIENTS AT HYDROGEL INTERFACES FOR ENHANCED CELL PENETRATION§

6.1 ABSTRACT

We report that stiffness gradients facilitate infiltration of cells through otherwise cell-impermeable hydrogel interfaces. By enabling the separation of hydrogel manufacturing and cell seeding, and by improving cell colonization of additively manufactured hydrogel elements, interfacial density gradients present a promising strategy to progress in the creation of 3D tissue models.

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6.2 INTRODUCTION

Hydrogels have been extensively developed over the past decades for emulating the natural extracellular matrix (ECM).[1-5] The ability to independently control the physical and biochemical properties of hydrogels makes these cell culture platforms capable of supporting the growth and differentiation of a wide range of cell types and tissues.[6-8] By spatially controlling the incorporation of bioactive molecules especially using photopatterning techniques, it is possible to partially recapitulate the complex and heterogeneous architecture of human tissues and thus create better tissue mimics.[9-14] For example, Shoichet and co-workers produced 3D protein motives in agarose hydrogels to promote and direct cell growth and neurite extension.[9] Furthermore, Lutolf and colleagues directed the migration of human mesenchymal stem cells by spatio-temporally controlling the incorporation of growth factors in 3D hydrogels.[11] Because of long reagent incubation times and extensive washing steps, the production of such patterned gels can last up to several days.[9, 10] During this time, undesired interactions can take place between the soluble bioactive molecules (used to create the pattern) and the cells cultured in the hydrogel, possibly affecting them in an uncontrolled manner.

A strategy to overcome uncontrolled and unwanted cell alterations during these procedures is to seed cells after scaffold fabrication and patterning. However, cells seeded on the surface of a hydrogel do not – or very poorly – invade the matrix.[9, 15] This phenomenon occurs also for hydrogels optimized to be permissive to embedded cells, indicating that the surface boundary represents a barrier to cell invasion of the bulk. Anseth and co-workers previously highlighted how cell migration in a hydrogel is arrested by abrupt increases in crosslinking density.[16] Such an abrupt change in density could also be the cause of the impaired cell penetration across the surface of the gel. Therefore, we assumed that a hydrogel featuring a gradual increase in crosslinking density from the surface towards the bulk would facilitate the penetration of cells.
In this work, we describe for the first time that density gradients can be used for enabling cell penetration through hydrogel interfaces. These findings open new perspectives for tissue engineering as they enable the separation of hydrogel manufacturing processes from the cell seeding. This is of particular interest for creating patterned hydrogels that can be stored and into which cells can be added at any time upon need. To demonstrate this concept we produced hydrogels with various interface density gradients and characterized their density by fluorescence microscopy and elasticity by colloidal probe force spectroscopy. Mesenchymal stem cells (MSCs) seeded on these engineered interfaces could penetrate few hundreds of micrometers into the bulk of the hydrogel within days, whereas they formed a monolayer on conventional hydrogels.

6.3 Results and Discussions

In this study, we used RGD-functionalized MMP-sensitive poly(ethylene glycol) (PEG) hydrogels enzymatically crosslinked by transglutamination.[17] Since this reaction is pH-dependent, acidic gradients generated in the vicinity of an anodized electrode can be exploited to locally inhibit the polymerization.[18] Consequently, we produced hydrogels with surface density gradients by casting the PEG precursor solution in a custom made PDMS chamber covered with a gold electrode during polymerization (Figure 6.1a), anodized with different current densities (samples referred to as 0.1µA/mm²-hydrogel and 1µA/mm²-hydrogel). As a first control, we prepared the gel in absence of the electrode, as conventionally done (from here referred to as Air-hydrogel). To account for the possible water evaporation at the surface of the gel, as a second control, we covered the gel precursor with a gold surface without imposing any electric current during polymerization (from here referred to as No current-hydrogel, Figure 6.1b). Because of the strong adhesion of the PEG hydrogel to gold and Teflon, the removal of any of these surfaces after polymerization resulted in the disruption of the gel. Therefore we applied a non-adhesive PLL-g-PEG layer (that does not contain the peptidic crosslinking substrate) to the gold surface (S. I Figure 6.5).[19]
To characterize the crosslinking density profiles of the electrochemically engineered hydrogel surfaces, FITC-tagged Lys substrates (Lys-FITC) were admixed to the PEG hydrogel precursors. Since the fluorescent dye is covalently incorporated into the PEG matrix by the same crosslinking reactions, the fluorescence signal is indicative of the crosslinking density.[20] We inspected sections perpendicular to the hydrogel surface by confocal laser scanning microscopy (CLSM, (Figure 6.1c and d). The surface boundaries produced in presence of anodic currents showed fluorescence intensity gradient profiles which reached a plateau approximately 250 µm (0.1 µA/mm²-hydrogel) and 500 µm (1 µA/mm²-hydrogel) from the surface. These observations correlate with our previous study describing that gel polymerization is inhibited at the anode-liquid interface in a current-dependent manner, due to the longer acidic gradient produced with increasing anodic currents.[18] The
6 Density gradients at hydrogel interfaces for enhanced cell penetration

No current-hydrogel showed a homogenous signal intensity throughout the gel thickness, while the Air-hydrogel had a peak of fluorescence within the 50 µm vicinity of the surface. This interfacial increase of crosslinking density was presumably due to water evaporation during polymerization and consequent local increase of monomer concentration.

Because fluorescence measurements only provide insights into the crosslinking variations within one sample, we used colloidal probe force spectroscopy to compare the rigidity of the different hydrogel surfaces (Figure 6.2). We acquired force-distance curves by indenting beads dispersed on the gel surfaces using the FluidFM,[21] an atomic force microscope equipped with cantilevers having an embedded fluidic channel. Reversibly fixing beads to the cantilever via its in-built microfluidic channel combines two advantages in probing the gel rigidity: the possibility of using beads of large dimensions (here 50 µm in diameter), enabling a deeper exploration of the gel; and the use of a fresh colloid for each indentation, thus avoiding history effects due to probe contamination.[22] The Air-hydrogel presented the most rigid surface: we reached the maximal

Figure 6.2. Mechanical characterization of the hydrogel boundaries using colloidal force spectroscopy. Force-distance curves were measured by indenting 50 µm polystyrene beads deposited on the surface of the hydrogels. Three force-distance curves of independent replicates are presented for each condition.
force (500 nN) at a penetration of 3.7 ± 0.3 µm only. The No current-
hydrogel presented a softer surface compared to the Air-hydrogel,
reaching 500 nN with a penetration of 10.6 ± 0.6 µm. This
observation is in accordance with the fluorescence microscopy
inspection, which revealed the presence of a shell of higher
crosslinking density in the Air-hydrogel. The surfaces of the
hydrogels prepared electrochemically were considerably softer and
this effect increased with increasing current densities. In particular,
the force-distance curves of the 0.1 µA/mm²-hydrogel reached a
force of 50 ± 21 nN at the maximal penetration depth (50 µm) and
for the 1 µA/mm²-hydrogel a force of 19 ± 12 nN at 50 µm.

The maximal indentation depth was not sufficient to probe the
entire extent of the gradient, reaching few hundreds of micrometers
according to the fluorescence microscopy inspection. Therefore, to
probe the rigidity of the hydrogel bulk and compare it to the surface
rigidity, we sectioned the gels perpendicularly to the surface and
indented i) as close as possible to the surface (referred to as surface)
and ii) at least 500 µm away from it (referred to as bulk) (S. I. Figure
6.6a). For all measured conditions, the bulk rigidity was comparable,
indicating that the electrochemical treatment did not affect the bulk
properties of the matrix. Additionally, this set of experiments
confirmed that i) the No current-hydrogel was homogenous in
rigidity, ii) the Air-hydrogel showed a more rigid surface than the
bulk and iii) the 0.1 µA/mm²-hydrogel had a softer surface than the
bulk (S. I. Figure 6.6b).

To assess the ability of cells to cross the above described hydrogel
interfaces, we seeded MSCs on top of the engineered gel surfaces
and inspected the cell distribution within the first 150 µm of the
hydrogel after 1 and 3 days in culture using CLSM (Figure 6.3). The
gel formulation chosen for this study was previously shown to
support migration of embedded cells[20] and this was confirmed for
the culture conditions used in this work (S. I. Figure 6.7). Cells
seeded on both Air- and No current-hydrogels grew in 2D forming a
monolayer. The average cell fraction at the surface of these
hydrogels (first 25 µm) was larger than 85 % at day 1 and larger than

110
65% at day 3 (Figure 6.3a-f). In contrast, when placed on the electrochemically engineered hydrogel surfaces, cells were able to penetrate into the bulk. The average cell fraction at the surface of the 0.1 µA/mm²-hydrogel decreased to 62% at day 1 and 51% at day 3 (Figure 6.3g-i, not significant). Furthermore, the penetration depth increased with increasing current density and we observed a significant reduction of the average cell fraction at the surface of the 1 µA/mm²-hydrogel to 18% and 22% at day 1 and 3, respectively (comparison shown in S. I. Figure 6.8). In particular, cells could penetrate more than 150 µm into the gels prepared with the highest current density after 1 day already (Figure 6.3j and l). The cell
distribution after 3 days was quite homogenous within the first 150 µm from the gel surface (Figure 6.3k and l). These results show that a crosslinking density gradient perpendicular to the hydrogel surface enhanced cell penetration of cells seeded on the surfaces. Interestingly, homogeneously reducing the rigidity of the gels was not sufficient to enhance cell penetration. In fact, even the softest gels that we are able to produce (0.8% PEG), did not allow for cell penetration from the surface into the bulk of the hydrogel after 3 days (S. I. Figure 6.9). Within 3 days, the cells migrating through the gradient have not yet reached bulk rigidity comparable to the Air- and No current-hydrogels one. However, the fact that cells entered the 3D environment together with their ability to migrate when embedded in the Air-hydrogel (S. I. Figure 6.7), strongly suggests that invasion of the gel will continue. Cells are known to move from soft to stiff regions, by a phenomenon referred to as durotaxis,[23] also in 3D hydrogels[24]. However, it was shown that sharp increases in stiffness prevent durotaxis and that cells migrate backwards or align along the soft-stiff interface.[16] Taken together, these studies indicate that a gradual – and not sharp – increase of stiffness favors durotaxis, and cell penetration across a hydrogel surface.

Hydrogel permeability to cells or tissue is an important challenge in the development of functional scaffolds for tissue engineering and many strategies have already been explored. For instance, Wylie et al. observed that on similarly RGD-functionalized hydrogels neuronal precursor cell infiltration was very limited, not exceeding 20 µm after 14 days. The authors enhanced cell penetration up to 85 µm after 2 weeks by creating a gradient of SHH (Sonic HedgeHog) spanning from the surface into the bulk of the hydrogel using photopatterning. Despite its elegance, this approach requires long manufacturing times.[9] Creating macro-pores in hydrogels is an alternative strategy to overcome limited cell infiltration (reviewed in [25]). However, while including macro-pores has been shown to effectively improve cell or tissue infiltration in a variety of both natural hydrogels, i.e. collagen,[26] gelatin,[27] and synthetic hydrogels including PEG,[15, 28] this technique alters the bulk properties of the constructs and provides little or no spatial control over the
In contrast, the interfacial density gradients proposed here promote cell infiltration while preserving desired bulk properties. To test whether the electrochemical treatment produced porous structures in the gel, we poured fluorescent microbeads (3 and 20 µm in diameter) on the surface of 1 µA/mm²-hydrogels. After 1 hour of sedimentation, the microbeads did not infiltrate in the hydrogel bulk, suggesting the lack of pores larger than 3 µm (S. I. Figure 6.10). Surface density gradients are broadly applicable to most commonly used hydrogel systems, not only because of the versatility of the electrochemical processing, but also because of other available gel processing techniques that could be adapted to this end. These techniques include surface irradiation of photodegradable[30-32] or photolinkable gels[33, 34] and microfluidics-based approaches[35]. These techniques have been used to study cell mechanobiology on 2D density gradients in the gel surface plane.[36, 37] However, we are not aware of depth gradients manufactured to enhance cell infiltration into hydrogels.

Sharp hydrogel boundaries represent barriers for cell migration not only at the liquid-gel interface but also at the interface between individually added elements of constructs produced by additive manufacturing. While it was repeatedly shown that cell and matrix components could be precisely deposited forming heterogeneously organized and viable constructs resembling native tissues,[38-40] the interface between the assembled elements was so far mostly overlooked and the question of how cells sense and respond to this interface remains elusive. Bordeleau et al. are among the few addressing this issue: by sequentially polymerizing adjacent cell-containing collagen gels varying in density, they showed that cells never migrated from a soft gel to a stiffer one, and could only rarely migrate from a stiff gel to a softer one.[41] This observation indicates that also for additive manufacturing, well-defined gel boundaries represent a barrier to cell invasion. To investigate whether density gradients could enable cell migration across the interface between two gels, we produced an Air-hydrogel and a 1 µA/mm²-hydrogel (Lys-FITC-labelled), on top of which a second gel (Gln-Alexa 561-labelled) containing MSCs was polymerized (Figure 6.4a).
Interestingly, after 3 days of culture, cells could not cross the interface of the Air-hydrogel, but invaded the electrochemically engineered gel (Figure 6.4b-d). This final experiment indicates that the approach described here is beneficial to reduce cell compartmentalization in individual hydrogel elements.

6.4 CONCLUSIONS

In conclusion, in this work we demonstrate that manufacturing gels with density gradients is a promising approach to address the challenge of cell penetration through hydrogel interfaces. These surface gradients can be produced via rapid and cost-effective procedures compatible with all commonly used hydrogels and hold great promise for enabling topical cell seeding on processed hydrogels as well as cell migration through the interface of additively manufactured hydrogels.
6.5 EXPERIMENTAL SECTION

6.5.1 Preparation of the PDMS chambers

Polydimethylsiloxane (PDMS) chambers were fabricated as follows: the silicon elastomer and the curing agent (Sylgard 184, Dow Corning Corporation, USA) were mixed (10:1 in mass) at 2000 rpm for 3 min in an ARE-250 mixer (Thinky Corporation, Japan). The mixture was subsequently poured into poly(methyl methacrylate) (PMMA) molds, where a 500 µm diameter stainless steel wire was positioned to create the holes for the future counter electrode. The mixture was subsequently degassed for 30 min in a vacuum chamber and baked for 4 h at 60 °C. The stainless steel wire and the PDMS form were removed from the PMMA molds and subsequently rinsed with isopropanol (IPA) and MilliQ water.

6.5.2 Preparation of PEG hydrogels

Metalloprotease (MMP)-sensitive transglutamination (TG)-PEG hydrogels were prepared as described previously.[42] In brief, 40 kDa eight-arm PEG precursors containing the pending activated factor XIII (FXIIIa) substrate peptides glutamine acceptor (n-PEG-Gln) or lysine donor with an additional MMP-sensitive linker (n-PEG-MMPsensitive-Lys) were mixed 1:1 stoichiometrically (final dry mass content 1.7%, unless otherwise specified) in Tris-Buffer (50 mM, pH 7.6) containing 50 mM calcium chloride. Lys-FITC (1.6 µM), Gln-Alexa 561 (1.6 µM), Gln-RGD (50 µM) were added to the precursor solution prior initiation of cross-linking by 10 U/mL thrombin-activated FXIII (FXIIIa) and vigorous mixing. Lys-FITC was added to gel precursor for the evaluation of the density gradient profiles. For cell penetration studies, Gln-RGD was added to all gel formulations. Additionally, to study cell penetration across the gel-gel interface, Lys-FITC was added to the bottom gel and Gln-Alexa 561 was added to the top gel.

6.5.3 Electrochemical control of PEG polymerization

The precursor mixture was immediately poured into the PDMS chamber (4 mm length, 5.5 mm width and 2 mm thickness)
accommodating a platinum wire (0.5 mm in diameter, Alfa Aesar, Ward Hill, USA) used as auxiliary electrode. Cobalt-chromium disks (15 mm in diameter and 0.8 mm in thickness) evaporated with 10 nm chromium and 200 nm gold were used as working electrode to be placed on top of the PDMS chamber. The polymerization of the PEG was allowed to progress during 8 minutes in presence of a direct anodic current applied in galvanostatic mode. The current density was +0.1 µA/mm² or +1 µA/mm².

6.5.4 Confocal laser scanning microscopy (CLSM) of hydrogels

FITC-labelled hydrogels were cut perpendicularly to the hydrogel surface with a razor blade and kept in Tris-Buffer (50 mM, pH 7.6). The sections were imaged using an LSM 510 confocal laser scanning microscope (Carl Zeiss, Germany). The FITC was detected upon excitation at 490 nm (0.7% laser line attenuator transmission) with an emission band-pass filter of 505-550 nm. The pinhole section was set to 30 µm and the focal plane was selected to maximize the intensity over the field of view. The intensity profiles were obtained by setting the minimum intensity as the intensity above the gel surface and by normalizing the values by the intensity measured at 800 µm from the surface.

6.5.5 Colloidal probe force spectroscopy

Force-distance measurements were performed in Tris-Buffer using a commercial FluidFM system (Cytosurge, Switzerland).[21] For long-range force spectroscopy, the system includes a motorized microscope stage. The setup was mounted on an Observer.Z1 optical microscope (Carl Zeiss, Germany). All devices were controlled by the CYui FluidFM software. Tipless FluidFM cantilevers with 8 µm apertures (Cytosurge, Switzerland) were used after oxygen plasma cleaning (2 min, Harrick PDC-32G 18 W). Prior to the force measurements, the cantilever spring constant was calculated from the thermal spectrum (in the range of 1.56-2 N/m).[43] After filling the cantilever with buffer solution, the optical lever sensitivity in
liquid was determined from a deflection-distance curve on a rigid glass surface.

A droplet of approximately 60 μL polystyrene colloids (50 μm in diameter, $1.8 \times 10^5$ particles/mL, Micromod, Germany, cat. no. 01-00-504) was deposited on the gel surface to be probed. The colloids were left to sediment for 10 min and the gel was immersed in Tris Buffer (50 mM, pH 7.6). A bead of interest was selected and a first approach with a setpoint of approximately 5 nN was performed on the bead, while applying an underpressure of 700 mbar. When the probe was approached, the colloid was immobilized on the aperture due to the underpressure, avoiding lateral movement of the colloid during the indentation. Force-distance measurements were performed at an indentation speed of 100 nm/s until either the maximal deflection of 9.5 V or the maximal piezo range of 50 μm was reached. Curves acquired at higher indentation speeds (500 nm/s and 1 μm/s) did not significantly vary. After each indentation, the bead was released by applying an overpressure of 1 bar and the next bead was selected. For each sample, 3 force-distance curves were recorded and 3 samples per condition were probed.

6.5.6  Estimation of the elastic modulus

Elastic moduli were estimated by fitting the force-distance curves over a 5 μm indentation using the Hertz model:

$$F = \frac{4}{3} \frac{E}{1 - \nu^2} \sqrt{R\delta^3}$$

where $F$ is the applied force, $\nu$ is the Poisson’s ration (equal to 0.5), $E$ is the elastic modulus, $R$ is the radius of the colloidal probe (equal to 25 μm) and $\delta$ is the sample indentation. The estimation of the elastic modulus was performed only on force-distance curves measured on hydrogel sections cut perpendicularly to the surface because the Hertz model is not appropriate to describe the indentation of surface density gradients.
6.5.7 Cell culture

Human-derived bone marrow MSCs, isolated as described elsewhere,[44] were cultured in minimal essential medium alpha (MEMalpha, Gibco Life Technologies, cat. no. 22571-020) supplemented with 10% (v/v) fetal calf serum (FCS, Gibco Life Technologies, cat. no. 10500), 1% (v/v) penicillin/streptomycin solution (Gibco Life Technologies, cat. no. 15140-122), 5 ng/mL FGF-2 (Peprotech, cat. no. 100-18B) and 50 nM PDGF (Peprotech, cat. no. 100-14B).

6.5.8 Gel penetration

MSCs were seeded on the hydrogel surface by depositing a 50 µL droplet of medium containing 200'000 cells/mL. The cells were left to sediment on the gel surface for 30 min. Subsequently, the gel was completely covered by medium. MSCs were kept in culture for 1 or 3 days. At each time point, samples were fixed with 4 % paraformaldehyde, rinsed three times and kept in PBS until staining.

6.5.9 Penetration across the gel-gel interface

Cell-containing gel precursors were poured on top of hydrogels produced with an engineered surface and left to polymerize during 8 min. The assembled constructs were subsequently placed in culture for 1 or 3 days. At each time point, samples were fixed with 4 % paraformaldehyde, rinsed three times and kept in PBS until staining.

6.5.10 CLSM of cells in hydrogels

Permeabilization was performed for 30 min at room temperature with 0.1 % Triton X-100 in PBS followed by 2 washing steps with PBS. For f-actin staining, samples were incubated overnight at 4 °C with Alexa 633-labeled phalloidin (Molecular Probes, cat. no. A22284). Afterwards, samples were washed 3 times with PBS before analysis with CLSM. The PEG hydrogels and cells were imaged using an SP5 confocal laser scanning microscope (Leica, Germany). At least 3
6.5.11 Infiltration quantification

Stacks (125 × 2 µm) acquired by CLSM were reconstructed in 3D, and a side projection was performed. The FITC channel was used to determine the gel surface and the Alexa-633 channel was used to determine the position of cells in the gel cross-section. A threshold was applied to the Alexa-633 channel images, which were subsequently cleaned (noise removal) and segmented into 25 µm thick regions starting from the gel surface. The amount of positive pixels was quantified in each region as a representation of the cell number. The ratio of cells in each section was calculated as a percentage of the overall amount of cells in the sample. The values represent mean values ± standard deviation of at least 3 gels per time point, in which at least 3 regions were analyzed. T-test and two-way ANOVA (time and treatment) followed by post-hoc multicomparison analysis (Tukey-Cramer range) were performed on data representing the cell infiltration at the hydrogel surface (first 25 µm). Significant difference (p<0.05) is indicated with an asterisk.

6.6 Acknowledgements

This work was supported by the Centre for Clinical Research University Hospital and University of Zurich, and ETH Zurich. We thank Stephen Wheeler (IBT workshop, ETH Zurich) for manufacturing the PMMA molds and the technical support. We thank Martin Lanz for the evaporated gold surfaces and Prof. Ivan Martin (University Hospital Basel, CH) for providing human bone marrow derived mesenchymal stem cells.
6.7 **SUPPLEMENTARY INFORMATION**

Figure 6.5. Removal of the covering surface and effect on the hydrogel integrity. The removal of the non-polarized covering gold electrode and of a covering Teflon disk resulted in the disruption of the hydrogel because of the too strong adhesion between the gel and the cover (a and d). The application of an anodic current (0.1 µA/mm²) or the application of an anti-adhesive PLL-g-PEG coating to the covering gold surface enabled the removal without hydrogel disruption (b and c). The use of anodic current to decrease the adhesion of PEG hydrogels to metal surfaces was previously reported.[18]
6 Density gradients at hydrogel interfaces for enhanced cell penetration

Figure 6.6. Mechanical characterization of the hydrogel cross-sections. a) Schematic representation of the experimental setup: Hydrogels were cut perpendicularly to the surface and flipped (cut showing upwards). 50 µm beads were poured on the sections and used to probe the mechanical properties of the hydrogel at different positions: within the 50 µm vicinity of the surface (surface) and at least 500 µm from the surface (bulk). b) Representative force-displacement curves confirmed that all hydrogels had similar bulk rigidities with estimated elastic moduli ranging from 1.6 to 2.1 kPa. By comparing bulk and surface force-displacement curves, we confirmed that the No current-hydrogel had a homogenous rigidity (1.7 kPa), whereas the Air-hydrogel had a more rigid surface compared to the bulk (3.5 kPa), and the 0.1 µA/mm²-hydrogel a softer surface compared to the bulk (0.1 kPa). For the thickness of the gel used here, the large extent of the gradient obtained at 1 µA/mm² made the hydrogel difficult to section without disrupting it. For this reason, we do not present this condition in this figure.

Figure 6.7. Cell migration within the hydrogel. MSCs were embedded in PEG hydrogels (final concentration: $0.5 \times 10^6$ mL$^{-1}$) and imaged every 20 min for 24 hours (Leica DMI6000 B). Cell migration was followed for 24 hours using the manual tracking plugin in ImageJ. 30 tracked cells had an average migration of $226 \pm 44$ µm in 24h.
Human derived bone marrow MSCs seeded on Air-hydrogels with lower PEG densities (0.8%). a), c), are 3D reconstruction of 250 µm thick stacks (consisting of 50 images acquired every 5 µm for the 0.8% condition and of 125 images every 2 µm for the 1.7% condition) acquired by LSCM 3 days after seeding; cells formed a 2D sheet on the gel regardless of the rigidity. Cell morphology (top view, right column) show that cells responded to the gel rigidity by spreading more on more rigid gels, as shown in previous studies.\[36\] e) Force-distance curves measured by indenting 50 µm polystyrene beads deposited on the surface of the hydrogels show that the gels produced with lower PEG density were indeed softer than the 1.7% gel commonly used. These gel surfaces were nonetheless still considerably more rigid than the electrochemically prepared hydrogel surfaces.

Figure 6.8. Two-way ANOVA followed by post-hoc multicomparison test (Tukey-Cramer critical range) of the average cell fraction present at the surface of the hydrogels (first 25 µm). The asterisk indicates significant difference between the groups (p<0.05). The entire cell distribution across the hydrogel is presented in Figure 6.3.
6.8 REFERENCES


Figure 6.10. Fluorescent microbeads on 1 µA/mm²-hydrogel surface. 3 µm (a) and 20 µm diameter (b) fluorescent beads (Fluoresbrite Plain YG, Polyscience Inc.) were deposited on the hydrogel surface and left to sediment for 1 hour. Their distribution was assessed by acquiring 150 µm thick Z-stacks of the hydrogel surface using a SP5 confocal laser scanning microscope (Leica, Germany).


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References appearing in the experimental section and supplementary information and not in the article main text:


In this thesis, I covered fundamental aspects of the interfacial electrochemistry of blood coagulation factors, in particular of fibrinogen and coagulation factor XIII (FXIII), and used the acquired knowledge for applications in the broad field of biomedical engineering. In this final chapter, I summarize the most important findings and provide the reader with personal considerations concerning possible directions to take in future research and development activities.
7.1 Cathodically polarized blood-contacting devices

In the initial part of this thesis, I presented a study on the behavior of Fibrinogen (Fg) at the interface with cathodically polarized gold electrodes. The reason for my interest in studying this subject came from the fundamental work of P. N. Sawyer from the 1960’s to the 1980’s. Sawyer and co-workers were among the firsts recognizing the importance of surface potentials and electrochemistry in determining the propensity of an implant to trigger thrombosis on its surface (see Section 1.4). In the beginning of my doctoral work, I adapted the electrochemical quartz crystal microbalance with dissipation monitoring technique (EQCM-D) to study thrombosis at the blood-metal electrode interface, in vitro and in a semi-quantitative manner. The QCM-D technique had been used before to study blood coagulation mostly on various polymeric substrates. For the first time I used it in combination with an electrochemical setup to study the electrochemistry of thrombosis. I could confirm the results of Sawyer and colleagues, stating that cathodically polarizing a gold electrode caused less thrombus to deposit on its surface. The study subsequently focused on Fg, which was found to electrochemically desorb from the electrode and degrade at cathodic potentials. Electrochemical desorption and degradation of Fg was hypothesized by Sawyer and Srinivasan, who emphasized the importance to undertake a study of the nature of the Fg cathodic product, since this could have revealed molecular reasons accounting for the reduced thrombogenicity at the cathode. I therefore used the EQCM to study the electrochemical desorption of Fg as function of the cathodic current density and showed a faster desorption with increasing current. The cathodically treated Fg had a compromised ability to clot. I used MS and identified one cathodic Fg degradation product which comprised an important polymerization site (see Chapter 3) explaining the observations.

This work leaves us with a few interesting open questions:
i) Is the Fg electrochemical degradation mainly limited to its N-terminal region of the α-chain, as found in our study, or is it a process involving the entire protein? To address this question, future experiments should include the acquisition of mass spectra of the Fg cathodic products over different mass ranges. Preliminary experiments in this regard, in which I separated the Fg cathodic product in its 3 chains, by breaking the disulfide bridges prior MS analysis, showed that the α-chain peak is the one which is mostly decreased in intensity and that new peaks appeared between 10 and 20 kDa. The experiment was not conclusive and should be reproduced. However, since we believe the degradation to be the results of alkali peptide hydrolysis, we expect the process to be rather unspecific and dependent on the time during which Fg has been in contact with the electrode.

ii) Is the cathodic polarization a valid strategy to reduce blood coagulation on surfaces? The answer probably depends on the device in question and its application. If the requirement of the surface is to remain clean from protein deposits and not much of interest is given to the nature of the surrounding (including blood and other tissues), electrochemistry is a cheap and effective strategy. Instruments, such as catheters and others, exploiting this concept are described.[1] Solvent-less cleaning via electrochemical alkali protein elution and degradation remains a strategy to consider for devices that have special cleaning requirements (e.g. difficult access to the dirty surface). In contrast, when it is critical that tissues (blood or others) in contact with the implant are minimally affected by its presence, electrochemistry might constitute an excessively invasive technique. To provide a more satisfactory answer, new studies should be specifically aimed at investigating the effect of electrochemistry on the behavior of blood and immune cells and tissue integrity. In this respect, stable non-fouling implant coatings recently developed by others (see introduction paragraph 1.4.4), are probably the most convenient strategy to keep surfaces clean without affecting the neighboring tissues.
iii) Is electrochemical thrombolysis (destruction of blood clot) possible? A fascinating idea that I investigated but not extensively pursued during this work concerned the possibility to develop filters to block travelling blood clots and then destroy them electrochemically. Vena cava metal filters are used in patients at risk of pulmonary embolism presenting complications with anticoagulation therapy.[2] These filters are placed in the vena cava to block emboli leaving the legs and possibly reaching vital organs. Combining filtration and thrombolysis should be possible, because of the possibility to electrochemically degrade proteins. In vitro preliminary experiments that I performed (not reported here) and another set of in vitro experiments present in literature [3] show that electrochemical thrombolysis is possible. However, the same considerations made in the previous point, regarding the effect of electrochemistry on the neighboring environment (blood or other tissues) apply, making this strategy difficult to be seen applied in vivo.

7.2 ANODIC POLYMERIZATION OF FG

Fg takes part to charge transfer reactions over a wide range of anodic potentials, leading to its electropolymerization at the anode surface (see paragraph 1.4.2). In chapter 4, I used the EQCM technique to study in real-time the anodic polymerization of Fg. Our results confirmed the presence of a minimum anodic potential to trigger the anodic polymerization and suggested that the polymerization might be the result of the electrolyte acidification at high anodic potentials. More experiments should be designed to confirm this aspect, as suggested in Chapter 4.

H. Schubert and co-workers used the anodic polymerization of Fg to co-deposit epithelial cells in fibrin-like fibers on gold microelectrodes. Fibroblast deposition and Fg electropolymerization occurred locally, on the anode microstructure only.[4] Locally and dynamically controlling the electropolymerization of Fg is a fascinating strategy to spatially control cell adhesion and cell growth. In a set of planned experiments, we will deposit Fg molecules in proximity of an anodized surface using a small-volume dispenser. This would allow
us to freely draw tracks of fibrin-like fibers to guide the growth of several cell types.

Future experiments should be designed to study the nature and properties of the electropolymerized Fg. In particular, the question of whether the anodic fibrin has similar structure and function of the natural fibrin, should be addressed using electron and atom force microscopy as well as directed antibody-antigen assays.

### 7.3 Electrochemical Polymerization and Biofunctionalization of PEG Hydrogels

In Chapter 5 and in the Appendix B, I presented the use of electrochemically generated pH gradients at the electrode interface to locally control the activity of the coagulation FXIII. FXIII has a bell-shaped activity as function of pH, with a maximum around pH 7.6-7.9 (see paragraph 1.2.4.3). Therefore, using electrochemistry it is possible to spatially and temporally control the FXIII-mediated crosslinking of poly(ethylene glycol) (PEG) hydrogels, to manufactured complex biological microenvironments.

We demonstrated the ability to create hydrogel patterns on gold electrodes (see Appendix B) in the millimeter range. In the future, it is necessary to explore the lower dimension limit of this technique and particularly study the feasibility to electropolymerize hydrogel patterns in the microscale.

Additionally, the technique presented here can be directly applied to bio-functionalize hydrogels. Because any bio-molecule (containing the TG substrate) can be incorporated in polymerized TG-PEG hydrogels, it is possible to create planar functionality patterns on hydrogels, with spatial and temporal control. An example would be a PEG hydrogel conventionally formed on top of a gold surface featuring an electrode grid. To incorporate a desired biomolecule in a particular region of the gel, one would simply cathodically polarize the underneath electrode. This procedure could be sequentially repeated to create dynamic patterns. The
current limitation is the fact that the hydrogel bulk pH has to be approximately 5.5, meaning that the compatibility with living cells encapsulated in the gel is not guaranteed and would have to be tested. An alternative would be to have the hydrogel in a slightly alkaline solution and activate the local polymerization using anodic currents. Whether cells would resist these mild acidic and alkaline temporary conditions has to be tested for each application.

7.4 HYDROGEL SURFACE DENSITY GRADIENTS

By exploiting electrochemically generated acidity gradients and the pH-dependent activity of FXIII, we manufactured hydrogels with crosslinking density gradients at their surface (Chapter 6). Surface density gradients are of particular interest to promote infiltration into the hydrogel bulk of cells seeded on the hydrogel surface.

Our current efforts are aimed at developing a hydrogel-based 3D culture platform for cell-based compound screening, which is fully compatible with industry-standard automated equipment. This platform has to satisfy important requirements in terms of format, versatility and robotic handling. Cell-based screenings are generally performed in microtiter well plates (96 and 384 wells per plate). In standard screenings, cells are automatically dispensed on the flat polystyrene or glass surface of the microtiter plates and cultured in 2D. Dispensing cells in an analogous manner on PEG hydrogels contained in the wells of microtiter plates, would result in cells growing on the hydrogel surface in 2D monolayer. The alternative of encapsulating the cells while assembling the hydrogel in situ is not practical, potentially difficult to automate and would ultimately reduce the screening throughput. For this reason we are developing a microtiter plate of standard format, which contains PEG hydrogels featuring surface density gradients that allow cells topically dispensed to invade the bulk of the gel. This novel plate will combine the versatility of 3D cell culturing in PEG hydrogels with the automation compatibility requested by the industry.
7.5 ELECTROCHEMICALLY CONTROLLING THE ACTIVITY OF OTHER ENZYMES

Because every enzyme shows a characteristic pH-dependent activity, I can envisage to use electrochemically generated pH gradients to control a wide range of different biochemical reactions. In experiments not presented in this thesis, we attempted to control the degradation activity of gastric serine proteases close to anodically polarized electrodes. The results were indeed promising, however the difficulties arise when the enzyme is irreversibly inactivated at the pH for which no activation is desired.

The advances in protein engineering might even lead to engineered proteins with dual enzymatic activity and where the activity depends on the pH. This would allow to spatially and temporally control which biochemical reaction is taking place in the reactor, using a cheap and easy to use electronic setup.

7.6 REFERENCES


In this appendix, I present the design of two electrochemical quartz crystal microbalance (EQCM) modules used in my research. The first design consists of a transparent module to perform standard EQCM measurements (Figure A.1a). The design is based on the commercial open module 401 developed by Q-Sense (Biolin Scientific Holding, Sweden). This module is composed of three parts:

- The bottom part, is the contact unit of the commercial QEM401 electrochemical module (Q-Sense, Sweden) (represented in the bottom of Figure A.1b).
- The mid part, is a plastic (PMMA or PTFE) or metallic (stainless steel or titanium) frame, used as a liquid chamber (represented in the middle of Figure A.1b and in the technical drawing in Figure A.2). The chamber was designed to have a volume of 150 µL.
- The top part, is a transparent plastic (PMMA) lid incorporating the platinum ring as a counter electrode and if needed a silver/silver chloride ring as a pseudo-reference electrode (represented in the top Figure A.1b and in the technical drawing in Figure A.3).

The advantages of this design are:

- The presence and formation of gas bubbles can be seen through the transparent lid and easily removed if needed. This is important when filling the chamber to avoid signal drifts (the signal stabilization in this module takes less than 10 minutes if loaded without bubbles). In addition, monitoring gas evolution might be important during EQCM
experiments, since bubble formation can influence the frequency and dissipation signals.

- It is possible to access the chamber with regular pipette tips, avoiding the use of tubing systems. This is an advantage when low sample volumes are used.
- Without the lid it can be used as a regular open module 401.
Figure A.2. Technical drawing of the mid part liquid chamber.
Figure A.3. Technical drawing of the transparent plastic lid, incorporating an O-ring and the platinum counter electrode ring. The lid is mounted on the mid part (Figure A.2) by 4 screws positioned in the lid’s corners, not indicated on this drawing.
The second module is designed to perform EQCM experiments in which the cathodic and the anodic compartments need to be separated by a membrane. In particular, the membrane used in this thesis was a cellulose acetate dialysis membrane with MWCO of 500 Da, therefore impermeable to Fg. The module consists of the bottom and middle parts of the first module design (Figure A.1b, bottom and middle) and a newly designed top part (Figure A.4). This new top part consists of two plastic (PMMA) components. The first component (Figure A.5) supports the membrane, a sealing O-ring and incorporates the inlet and outlet. The second component has a cylindrical shape (Figure A.6) and incorporates the counter and reference electrodes and seals the O-ring to the membrane when placed inside the first component in Figure A.5.

Figure A.4. Schematic of the cross-section of the EQCM module with membrane separator (not in scale). White circles indicate sealing O-rings.
Figure A.5. Technical drawing of the top part of the EQCM module with membrane separator. This component is mounted on the mid part liquid chamber (Figure A.1b) and supports a cellulose acetate membrane and sealing O-rings.
Figure A.6. Technical drawings of the inner cylinder of the EQCM module with membrane separator. This cylinder supports the counter electrode (platinum ring) and features an opening on the top side to accommodate a reference electrode. It is designed to fit in the component presented in the Figure A.5 and seals the membrane and O-ring, to separate the anodic and cathodic compartments.
B.1 Abstract

This section is an appendix to Chapter 5. Here, we present an electrochemical quartz crystal microbalance with dissipation monitoring (EQCM-D) study on the electrochemical control of the enzymatic polymerization of poly(ethylene glycol) (PEG). We first demonstrated the role of coagulation factor XIII (FXIII) in the electrochemical polymerization. We subsequently used the EQCM-D technique to optimize the acidity level of the PEG precursor solution for the controlled polymerization at the cathode surface. If the solution is too acid, the FXIII might become irreversibly inactive or the raise of pH at the cathode interface might not be large enough to activate the enzyme. In contrast, if the precursor solution is not acid enough, the solution might polymerize in absence of electric current, making the process not spatially controllable. Once we established the optimal pH conditions, we demonstrated our ability to polymerize PEG hydrogels locally on planar gold electrodes to create patterned hydrogel films.
B.2 EQCM-D: FXIII-MEDIATED ELECTROCHEMICAL POLYMERIZATION OF PEG HYDROGELS

We prepared PEG precursor solutions (pH 6) containing and not containing FXIII. Regardless of the presence of FXIII in solution, the precursors remained in a liquid state for at least 1 hour time. The precursor solution lacking FXIII obviously could not polymerize due to the absence of the crosslinker. The solution containing FXIII remained liquid because of the solution pH (pH 6), which is more acidic than the optimal pH of the FXIII and thus considerably slowed down the linking reaction.

We injected both precursor solutions in the EQCM-D module and waited for the frequency and dissipation signals to stabilize. We subsequently applied a cathodic dc current for 30 min. Finally, we rinsed the chamber and recorded the drop in dissipation signal (Figure B.1). The magnitude of the dissipation drop measured upon rinsing indicated the amount of gel remaining on the electrode surface. A small dissipation drop corresponded to an electrode covered by a hydrogel film and a large dissipation drop indicated effective rinsing of the unpolymerized precursor. The precursor containing FXIII showed a dissipation drop of about $4 \times 10^{-6}$, while the precursor containing FXIII showed a dissipation drop of about $21 \times 10^{-6}$. When we removed the electrodes from the EQCM-D module, we observed that the electrode in contact with the precursor not containing FXIII was relatively clean compared to the electrode in contact with the precursor containing FXIII, which was covered by a thick hydrogel film. This result indicated that the electrochemical polymerization is indeed caused by the activation of the FXIII and is not the result of the physical polymerization/precipitation of PEG on the electrode. The electrochemical physical deposition of polymers such as chitosan and other polysaccharides has been widely studied and I presented it to the reader in the Section 1.3.2.2 of this thesis.

Using EQCM-D, We investigated above which pH value gelification of the FXIII-containing precursor spontaneously occurred (i.e. without electrochemistry). In all experiments showed below the reported pH
corresponds to the final pH of the hydrogel precursor solution as measured prior addition of FXIII. Figure B.2a shows dissipation drops measured upon rinsing of the precursor solutions left in the EQCM-D chamber for 30 min at OCP. In particular, for the precursor at pH 3.8 and 5.5, the dissipation signal rapidly dropped upon rinsing (22 \times 10^{-6}) and stabilized within 15 min. For the precursor at pH 6.0, the dissipation drop, measured 15 min after rinsing, was smaller compared to the other conditions (16 \times 10^{-6}), and stabilized after 1 hour. This result indicated that a pH value close to 6 is the upper limit in order to avoid spontaneous PEG polymerization under these conditions.

We subsequently repeated this experiment by subjecting the PEG precursors to a dc cathodic current of -50 µA/cm² and investigated the maximal precursor acidity for which electropolymerization still occurred (Figure B.2b). For the precursors at pH 5.5 and 6.0, the dissipation drop measured upon rinsing was approximately 2 \times 10^{-6}, indicating that electropolymerization occurred. In contrast, for the precursor at pH 3.8, the dissipation drop was approximately
and indicated that polymerization did not occur or occurred less than in the other two conditions. We can propose two explanations for this observation. First, the excessive acidity of the precursor solution might have caused denaturation of the enzyme and its irreversible loss of function. Alternatively, the cathodic current used in this experiment was not high enough to generate the pH increase necessary to reach the activation window in the most acidic precursor. We would need supplementary experiments to

Figure B.2. a) Polymerization of PEG hydrogels prepared at pH = [3.8, 5.5, 6.0] in absence of current. After injection, the PEG precursors were left at OCP for 30 min. b) Electrochemical polymerization of PEG hydrogels prepared at pH = [3.8, 5.5, 6.0] subjected to constant cathodic current of -50µA/cm². The signal indicates the dissipation change upon rinsing.
provide a clear mechanism. However, this experiment indicated that a precursor pH value of 5.5 would be optimal in order to electropolymerize PEG hydrogels in spatially controlled manner. At pH above 5.5 the PEG precursor might polymerize even in absence of electric current, making the process not specific, and at pH below 5.5 the precursor might not polymerize even by applying electrochemistry.

Finally, using the same procedure described above, we investigated the minimal density current needed to electropolymerize PEG on flat gold electrodes. We used a PEG precursor with pH 5.5 and we
applied 0, -20 and -50 µA/cm² (Figure B.3). We observed the expected trend, following which more hydrogel deposited at the electrode surface with increasing polarization. The minimum current density tested for which we electropolymerized PEG in the EQCM-D, at these particular conditions, was -50 µA/cm², as indicated by the minimal drop in dissipation upon rinsing.

B.3 PATTERNED HYDROGELS ON GOLD SURFACES

We performed a proof-of-principle experiment aimed at demonstrating the feasibility to spatially control the polymerization
of PEG hydrogels on patterned gold electrodes. Using standard lithography, we created a non-polarizable region in the center of the planar gold electrode. This region was surrounded by a cathodically polarizable concentric circular region (Figure B.4a). In the control experiment we used the standard non-patterned gold electrode (Figure B.4b). Based on the experiments described in the previous paragraphs, the PEG precursor will polymerize on the cathodically polarized surface only. In addition to this, it is important to account for the geometry and position of the counter electrode, which in our setup consisted of a platinum ring concentrically placed 0.8 mm above the planar gold working electrode. The platinum counter electrode acted as an anode and locally acidified the precursor, thus inhibiting the polymerization. Therefore, the expected pattern of electropolymerized PEG hydrogel consisted of two concentric rings of PEG (Figure B.4a and b). By using FITC-labelled PEG hydrogel, we demonstrated using standard fluorescence microscopy the fabrication of such patterns (Figure B.4c and d). The reason for the unclear definition of the inner fluorescence circle in the Figure B.4c is probably the non-precise concentric positioning of the counter electrode.

B.4 EXPERIMENTAL SECTION

The PEG gel formulation is introduced in the Section 5.4. To produce hydrogel precursor solutions of various final pH, the pH of Tris buffer (50 mM) solutions was adjusted according to Table B.1. The pH of the PEG formulation was always measured prior addition of FXIII and, if necessary, the pH was adjusted to the desired value by adding low volumes (μL range) of NaOH 0.6M or HCl 0.4M. The accurate pH measurement in small volumes of hydrogel precursor solutions was done using a Micro-Combination pH Electrode (MI-415, Microelectrodes, Inc. USA).
Data concerning the QCM crystals used in this set of experiments can be found in Section 3.3.1, as well as cleaning protocols. The EQCM-D setup and the potentiostat-galvanostat used here are described in Section 3.3. Patterns on 200 nm-thick evaporated gold films were created by standard etching procedures of the gold film to expose the silica substrate (Martin Lanz, ETH Zurich). The electrodeposition of fluorescent PEG hydrogels on the gold surfaces was assessed with an upright fluorescence microscope (BM550B, Leica Microsystems, Germany).

The electropolymerization of PEG hydrogels was studied using the EQCM-D technique as follows. Baseline frequency and dissipation signals were acquired after the EQCM-D module was filled with Tris buffer (50 mM, buffer pH matched the pH of the hydrogel formulation to be injected). The PEG precursor solutions (150 μL) were subsequently injected, thus replacing the Tris buffer in the chamber. The frequency and dissipation signals stabilized over 10 min. The dc cathodic current was subsequently applied during 30 min. Ten minutes after switching off the cathodic current, the EQCM-D module was rinsed with 3 x 150 μL Tris buffer (pH matching the pH of the precursor solution in the chamber). The dissipation response upon rinsing was recorded and used as an indication of the extent of the PEG polymerization on the gold surface.

### B.5 Acknowledgments

The experiments presented in this appendix were designed in collaboration with and performed by Ms. Xueying Mao (Semester project in the Biomedical Engineering Master’s, spring 2014).

<table>
<thead>
<tr>
<th>Tris Buffer pH</th>
<th>Final pH of PEG Hydrogels</th>
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<tbody>
<tr>
<td>2.0</td>
<td>3.8</td>
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<tr>
<td>2.4</td>
<td>5.5</td>
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<tr>
<td>2.6</td>
<td>6.0</td>
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Table B.1. Correspondence between the pH of the Tris buffer used in the PEG precursor formulation and final formulation pH (prior addition of FXIII).