Doctoral Thesis

Living cell adhesion measured by force spectroscopy

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LIVING CELL ADHESION MEASURED BY FORCE SPECTROSCOPY

a dissertation submitted to

ETH ZURICH

for the degree of

Doctor of Sciences (Dr. sc. ETH Zurich)

presented by

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2010
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Cell adhesion is the binding of a cell to another cell, extracellular matrix or a surface, using cell adhesion molecules. Many of these interactions involve transmembrane integrin receptors. Integrins cluster to provide dynamic links between extracellular and intracellular environments, by bi-directional signaling and stabilization of the focal adhesion points. The interactions involve complex couplings between cell biochemistry, structural mechanics, and surface bonding.

Adhesive interactions play a major role in the development of multicellular organisms, by guiding and anchoring cells into their appropriate locations. Adhesion is also important in the maintenance of the body: changes in the expression or function of cell adhesion molecules are implicated in all steps of tumor progression. Tumor cells are able to loosen their attachment to leave their original location and become lodged at distant tissues (metastasis). Implantology research and tissue engineering are directly focused on cell-surface interactions, since events leading to integration of an implant into bone and to long performance of the device take place at the interface formed between tissue and implant. Modifying the surface of an implant either by providing chemical and/or topographical cues encourages bone cell attachment. Cell adhesion is one way to increase osteointegration and to stabilize the implant. Tissue engineering aims to replace or restore the anatomic structure and function of damaged or missing tissue. Cell adhesion is a crucial parameter in the development of biodegradable scaffolds and cell sheet engineering techniques.
Summary

It is not easy to measure and quantify cell adhesion. Cell adhesion has been investigated using many techniques. The combination of cell biology with force spectroscopy provides a powerful tool for exploring the complexity of cell adhesion. The main objective of this work is to use force spectroscopy to quantify the long-term global adhesion between cells and surfaces and their response to modified surfaces. Atomic force microscopy-based force spectroscopy is capable of resolving individual cell binding events as well as global cell adhesion of living cells under physiological condition. It was used to study and quantify the adhesion of living cells to their growing substrate.

In order to determine if cell adhesion has to be studied independently of cell cycle or not, cell adhesion at different phases was measured. The adhesion of osteosarcoma cells to a glass surface was measured at different phases of the cell cycle. The cells were synchronized in three phases of the cell cycle: G1, S and G2M. Cells in these phases were compared with unsynchronized and native mitotic cells. Individual cells were attached to an atomic force microscope cantilever, brought into brief contact with the glass surface and, then pulled off again. The force-distance curves obtained allowed the work and maximum force of detachment as well as the number, amplitude, and position of discrete unbinding steps to be determined. The properties of the binding proteins present at the cell surface remained similar throughout the cell cycle, including mitosis. Therefore, next cell detachment experiments were allowed to be studied independently of cell cycle.

Long-term cell adhesion involves living adherent cells. The challenge was to find a method of attachment, between cells and atomic force microscope cantilevers, that allows their detachment from the surface. Fibronectin-coated cantilevers were used to detach individual immortalized fibroblasts from their growing substrate. The detachment of living adherent cells by force spectroscopy was tested on a number of chemically functionalized surfaces in order to validate the technique. The forces involved in the adhesion of fibroblasts were quantified. The cells were grown on glass surfaces as well as on surfaces used for cell sheet engineering: glass surfaces coated with polyelectrolyte multilayers (poly-L-lysine and hyaluronic acid) and thermally-responsive poly(N-isopropylacrylamide) brushes. Large differences in cellular adhesion were observed on polyelectrolyte coatings, depending on the number of polyelectrolyte bilayers. On poly(N-isopropylacrylamide)-grafted surfaces, changes of more than an order of magnitude were observed in cell adhesion above and below the lower critical solution temperature. Glass surfaces
patterned with periodic poly(N-isopropylacrylamide) microdomains were also investigated. In this last case, it was shown that cellular adhesion could be reduced while keeping cellular morphology unchanged.

Finally, in order to investigate the potential and limitations of this technique, two important experimental parameters were altered: the topography and the cell type. Immortalized and primary fibroblasts were studied on flat and topographically structured quartz surfaces. Using a fibronectin-coated AFM cantilever, it was possible to detach a large proportion of the immortalized cells from the quartz surfaces. Their adhesion to the quartz surface, and the effects of topography on this adhesion, could be quantified. In contrast, few primary cells were detached under the same experimental conditions. A qualitative analysis of their behavior showed that immortalized fibroblasts adhered less strongly than primary fibroblasts to at least one quartz surface.

The potential and limitations of single cell force spectroscopy in the study of the adhesive properties of cells are discussed. Quantitative data of this nature should open the way for more rigorous investigation and comparison of the influence of different parameters on cell/substrate adhesion.
L’adhérence cellulaire correspond à la liaison d’une cellule à une autre cellule, à la matrice extracellulaire ou à une surface, en utilisant des molécules spécifiques d’adhésion. La plupart de ces interactions impliquent des récepteurs transmembranaires de type intégrines. Les intégrines se regroupent et s’organisent afin d’établir des liens dynamiques entre le milieu extracellulaire et intracellulaire, pour la signalisation moléculaire et la stabilisation des points d’adhésion focaux. Les interactions sont le résultat de couplages complexes entre la biochimie cellulaire, la structure mécanique et les liaisons de surface.

crucial dans le développement des prothèses biodégradables et des feuillets cellulaires.

L’adhésion cellulaire n’est pas facile à mesurer et quantifier. L’adhésion cellulaire a été investiguée par de nombreuses techniques. La combinaison de la biologie et de la spectroscopie de force offre un outil puissant pour l’exploration de la complexité de l’adhésion cellulaire. L’objectif principal de ce travail est d’utiliser la spectroscopie de force pour quantifier les forces globales d’adhésion à long terme entres des cellules et des surfaces, ainsi que leur réponse à des surfaces modifiées. La spectroscopie de force issue de la microscopie à force atomique est capable de mesurer aussi bien des liaisons d’adhésion individuelles que l’adhésion globale de cellules vivantes en condition physiologique. Elle a été utilisée pour étudier et quantifier l’adhésion de cellules vivantes à leur substrat de croissance.

L’adhésion cellulaire a été mesurée à différentes phases du cycle cellulaire afin de déterminer si elle pouvait être étudiée indépendamment, ou non, du cycle cellulaire. L’adhésion entre des cellules provenant d’un ostéo-sarcome et une surface en verre a été mesurée à différente phases du cycle cellulaire. Les cellules ont été synchronisées dans trois phases du cycle cellulaire : G1, S et G2M. Dans ces phases, les cellules ont été comparées avec des cellules non synchronisées et des cellules naturellement en mitose. Des cellules individuelles ont été attachées à un levier de microscope à force atomique, mises en bref contact avec la surface en verre et, ensuite retirées à nouveau. Les courbes de force obtenues en fonction de la distance ont permis de déterminer le travail et la force maximale de détachement aussi bien que le nombre, l’amplitude et la position des étapes de détachement. Les propriétés des protéines d’adhésion présentes à la surface de la cellule sont restées similaires au travers du cycle cellulaire entier, y compris en mitose. Par conséquent, les mesures suivantes de détachement cellulaire ont été étudiées indépendamment du cycle cellulaire.

L’adhésion cellulaire à long terme implique des cellules adhérentes vivantes. Le défi a été de trouver une méthode d’attachement, entre les cellules et les leviers de microscope à force atomique, capable de supporter leur détachement de la surface. Des leviers recouverts de fibronectine ont été utilisés pour détacher des fibroblastes individuels immortalisés de leur surface de croissance. Le détachement de cellules adhérentes vivantes, en utilisant la spectroscopie de force, a été testé sur de nombreuses surfaces fonctionnalisées chimiquement afin de valider la technique. Les forces impliquées dans l’adhésion des fibroblastes ont été quantifiées.
La croissance des cellules a été réalisée sur des surfaces en verre ainsi que sur des surfaces utilisées pour le génie tissulaire : des surfaces en verre fonctionnalisées avec des multi-couches de polyélectrolytes (poly-L-lysine et acide hyaluronique) et avec le polymère thermo-sensible poly(N-isopropylacrylamide). De grandes différences en terme d’adhésion cellulaire ont pu être observées sur les revêtements de polyélectrolytes, en fonction du nombre de bicouches de polyélectrolytes. Concernant les surfaces fonctionnalisées avec le poly(N-isopropylacrylamide), des changements de plus d’un ordre de magnitude ont pu être observés au niveau de l’adhésion cellulaire au-dessus et au-dessous de la température critique inférieure de solution. Des surfaces en verre modifiées avec des micro-domaines de poly(N-isopropylacrylamide) ont également été investiguées. Dans ce dernier cas, il a été montré que l’adhésion cellulaire pouvait être réduite tout en gardant une morphologie cellulaire identique.

Finalement, deux paramètres expérimentaux importants ont été modifiés, la topographie et le type cellulaire, afin d’investir le potentiel et les limites de cette technique. Des cellules immortalisées et primaires ont été étudiées sur des surfaces en quartz plates et avec une topographie. Grâce à l’utilisation d’un levier fonctionnalisé avec de la fibronectine il a été possible de détacher une grande proportion des cellules immortalisées des surfaces en quartz. Leur adhésion aux surfaces en quartz, et les effets de la topographie sur cette adhésion, ont pu être quantifiés. En revanche, seules quelques cellules primaires ont pu être détachées en utilisant les mêmes conditions expérimentales. Une analyse qualitative de leur comportement a montré que les fibroblastes immortalisés adhéraient moins fortement que les fibroblastes primaires sur au moins une surface en quartz.

Résumé
Chapter 1
Introduction

1.1. Cell adhesion

1.1.1. Concept
One prerequisite for the evolution of multicellular organisms was the development of mechanisms by which cells could adhere to one another. Cell adhesion is the binding of a cell to another cell, extracellular matrix or a surface using cell adhesion molecules (CAMs). Many cell adhesion processes occur in two steps. First, single cell adhesion proteins bind to their respective ligands at the cell surface and then the interaction is consolidated by the recruitment of hierarchically assembled protein complexes called focal adhesions (FAs).

1.1.2. At the molecular level
Cell-cell and cell-substrate interactions are mediated through several different families of receptors. In addition to targeting cell adhesion to specific extracellular matrix proteins and ligands on adjacent cells, these receptors influence also many processes in cell regulation, tissue
homeostasis and morphogenesis. Most of the CAMs belong to five families or superfamilies: integrins, immunoglobulins (Ig), cadherins, selectins and homing receptors. These proteins are typically transmembrane receptors that interact either with similar CAMs (homophilic binding) or with different CAMs or the extracellular matrix (heterophilic binding). Integrins are heterophilic molecules that mediate both cell-cell and cell-substratum adhesion. The immunoglobulin superfamily is involved in cell-cell adhesion via homo- and heterophilic interactions. Cadherins are calcium-dependant homophilic cell-cell adhesion proteins. Selectins (or LEC-CAMs) mediate white blood cell/endothelial cell adhesion and finally homing receptors target lymphocytes to specific lymphoid tissues. The CAM family that plays a key role in mediating cell-surface or cell-matrix interactions is the integrin family.

Integrins are a family of cell adhesion receptors that bind to extracellular matrix ligands, cell-surface ligands and soluble ligands. They are transmembrane αβ heterodimers and at least eighteen α and eight β subunits are known in humans, generating twenty-four heterodimers. The RGD sequence (arginine-glycine-aspartic acid) in fibronectin was originally identified as an integrin-binding motif. This sequence and related sequences in extracellular matrix molecules do act as integrin-binding motif. However, integrins also recognize many non-RGD sequences as ligands. As far as ligand specificity is concerned, mammalian integrins can bind to, among others, fibronectin, collagen, laminine, vitronectin, tenascin, thrombospondin, osteopontin or fibrilin. Some integrins are limited to certain cell types or tissues and other integrins are widely distributed. Normally, each cell expresses several types of integrins.

Integrins mediate the attachment of the cell to its surroundings and play a role in cell signalling. They couple the extracellular matrix outside the cell to the cytoskeleton inside the cell. Integrins are the transducers that directly transmit mechanical stress from the extracellular matrix to the cytoskeleton and inversely. This linkage is not only important to stabilize cell adhesion and morphology but is also involved in cell signalling. The signals received through the integrins have been shown to be related to cell growth, proliferation, differentiation, viability and apoptosis. Table 1.1 shows that the deletion of individual genes coding for integrin subunits, by gene knockout in mice, plays a critical role in their morphogenesis. Almost all organs can be affected.
## Table 1.1 Phenotypes of deletions of integrin subunits in the mice

<table>
<thead>
<tr>
<th>Integrin subunit</th>
<th>Viability</th>
<th>Phenotype</th>
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<tbody>
<tr>
<td>α3</td>
<td>perinatal lethal</td>
<td>kidney, lung and skin defects¹¹</td>
</tr>
<tr>
<td>α4</td>
<td>embryonic lethal</td>
<td>placental and heart defects¹²</td>
</tr>
<tr>
<td>α5</td>
<td>embryonic lethal</td>
<td>mesodermal and vascular defects¹³</td>
</tr>
<tr>
<td>α6</td>
<td>perinatal lethal</td>
<td>epidermal detachment and neurogenesis defects¹⁴</td>
</tr>
<tr>
<td>α7</td>
<td>viable</td>
<td>muscular dystrophy¹⁵</td>
</tr>
<tr>
<td>α8</td>
<td>perinatal lethal</td>
<td>kidney defect¹⁶</td>
</tr>
<tr>
<td>αE</td>
<td>viable</td>
<td>skin inflammation¹⁷</td>
</tr>
<tr>
<td>β1</td>
<td>embryonic lethal</td>
<td>failure to gastrulate¹⁸</td>
</tr>
<tr>
<td>β3</td>
<td>viable</td>
<td>platelet defect¹⁹</td>
</tr>
<tr>
<td>β4</td>
<td>perinatal lethal</td>
<td>epidermal detachment²⁰</td>
</tr>
<tr>
<td>β6</td>
<td>viable</td>
<td>inflammation of skin and lungs²¹</td>
</tr>
<tr>
<td>β7</td>
<td>viable</td>
<td>gut-associated lymphocyte defects²²</td>
</tr>
</tbody>
</table>

At the cell level

FAs serve as stabilizers of the attachment of the cell to its surrounding and as regulators of cell signalling. Primary adhesion is dominated by single molecule binding²³. These connections of the integrins with the extracellular matrix or a surface induce intracellular signalling cascades leading to the formation of hierarchically assembled protein complexes called focal adhesions or focal contacts²⁴,²⁵. FAs usually measure a few square microns across and are located along the ventral plasma membrane of adherent cultured cells. Recently, it was shown that layilin, a transmembrane protein, also interacts with the extracellular matrix notably with hyaluronic acid²⁶,²⁷.

On their cytoplasmic side, FAs are composed of numerous proteins with and without enzymatic activity that play the role of stabilizer elements, transducers, modulators and adaptors. They are also associated with the bundles of actin microfilaments of the cytoskeleton. The main
components of FAs are cytoskeletal proteins such as vinculin, paxillin, tensin, α-actinin, actopaxin or talin that show no enzymatic activity. Kinases, phosphatases, modulators of small GTPases and other enzymes are also very important. Many of these components may be expressed in a cell type specific manner. Figure 1.1 illustrates the complexity of focal adhesions connecting the extracellular matrix via integrins to the actin fibres of the cytoskeleton.28

When cells let go of a surface, they don’t let go of all their integrins.29 A substantial proportion of the integrins is left behind on the substrate as the cell detaches while another fraction is collected into vesicles.30 It has been shown that cells use the endocytic protein clathrin to reabsorb the integrin receptors that attach them to surfaces, instead of simply leaving the molecules behind.31 Cells may use the reabsorbed integrins to build new FAs but this has not yet been demonstrated.
1.1.4. Applications

Both cell-cell and cell matrix adhesions are important in multicellular organisms during morphogenesis and in a variety of basic processes such as proliferation, differentiation, motility, cellular trafficking, tissue architecture or apoptosis\textsuperscript{32}. Cell adhesion plays an essential role in the homeostasis of healthy tissues. Unfortunately, cell adhesion can be disrupted and cause a great variety of diseases.

Some cancers involve mutations in genes for adhesion proteins that result in abnormal cell-cell interactions. Indeed, changes in the expression or function of cell adhesion molecules have been implicated in all steps of tumour progression, including the detachment of tumour cells from the primary site, their intravasation into the blood stream, their extravasation into distant target organs, and the formation of secondary lesions\textsuperscript{33}. Some people have "blistering diseases" that result from inherited molecular defects in genes for adhesion proteins. These diseases involve the formation of a bubble of fluid beneath a thin layer of dead skin. The blisters may start in the deeper layers of the skin and cover widespread areas. Cell adhesion proteins are also important for interactions that allow viruses and bacteria to cause damage to humans. Many pharmaceuticals target the adhesion proteins of viruses and bacteria. Cell adhesion proteins hold synapses together and the regulation of synaptic adhesion is involved in learning and memory. In Alzheimer’s disease, there is abnormal regulation of synaptic cell adhesion. The study of cell-surface interactions allows the indirect study of disturbed cell-cell and cell-matrix interactions in such diseases.

In contrast, implantology research and tissue engineering are directly focussed on cell-surface interactions. A goal in the design of bone and dental implants is the development of devices that induce controlled, guided and rapid healing\textsuperscript{34}. Thus, a better understanding of events at the interface and of the effects that biomaterials have on bone and bone cells is needed. In tissue engineering, cells are placed on or within matrices in order to develop functional substitutes for damaged tissues\textsuperscript{35}.

Cell adhesion has widespread applications. It has been shown that the expression of adhesion molecules on the surface of cells may change in various diseases. In conclusion, cell adhesion is fundamental in cancer progression, implantology research, wound healing and against infection by pathogens.
1.2. Current techniques for cell adhesion studies

Cell adhesion has been investigated using many techniques. Microscope-based techniques provide qualitative cell adhesion measurements whereas mechanical approaches quantify semi-quantitatively or quantitatively cell adhesion. The techniques are not described in chronological order according to their appearance.

1.2.1. Light microscope-based techniques

Light microscopy was used since the earliest examination of cellular structures. Modern light microscopy provides a variety of imaging techniques and also the capability to perturb biological structures in living cells in order to study their functions\textsuperscript{36,37}.

Interference reflection microscopy (IRM) has been used to study the adhesion of cells in contact with planar substrates\textsuperscript{38}. The technique provides information on the separation of the plasma membrane of the substrate. The interference between the reflected light waves from the substrate-medium interface and the plasma membrane-medium interface generate an image with high contrast. IRM allows the visualization of cell-substratum adhesion in living tissue culture cells continuously for long periods of time without the use of fluorescent markers\textsuperscript{39}.

The use of fluorochrome-conjugated antibodies and green fluorescent protein (GFP)-fusion proteins produces high-resolution images of the distribution of binding proteins in cells\textsuperscript{40}. Cells expressing GFP-fusion proteins have been used to determine the compositional dynamics of the cell adhesion structures. However, the comparison of the relative distributions of the labeled components in function of the time is not accurate. This problem was partially solved by a calculation of the pixel by pixel ratio of two fluorescent images\textsuperscript{41,28}. It is particularly suitable for colocalization studies of proteins forming distinct and defined structures such as focal adhesions. This spatiotemporal information is important for structure-function studies in cell adhesion. However, relative change in protein concentration can be estimated but the absolute concentration of the specific adhesion protein cannot be calculated.

Microscope-based-techniques can also be used for the detection of close protein-protein interactions using fluorescence resonance energy transfer (FRET)\textsuperscript{42,43,44}. This is a process involving the radiationless transfer of energy from a donor fluorophore to an appropriately positioned acceptor fluorophore. FRET can occur when the emission spectrum of a donor
fluorophore significantly overlaps the absorption spectrum of an acceptor and when they are in favourable mutual orientation. This technique allows the determination of molecular associations of adhesive components such as the details of the fibronectin fibril assembly process\textsuperscript{43}. Alternatively, photobleaching techniques have been used to measure the transport of molecules in living cells or at their surface\textsuperscript{45,46}. The fluorescence of defined regions of the cell was destroyed and the recovery of fluorescence into those regions reflected the type of transport such as diffusion or flow. It was used to study the trafficking of cytoskeletal proteins\textsuperscript{47}. The exchange of $\alpha$-actinin between the cytoplasmic pool and the focal adhesions was observed. The advantage of the light-directed technique is the temporal control in the molecular perturbation when compared to genetic approaches.

In summary, light microscopy-based techniques can be used \textit{in situ} to study molecular interactions involved in cell adhesion and dynamics. Specific molecular perturbation in cellular structures can also be induced. However, cell adhesion is never measured quantitatively. Although light microscope-based techniques are limited to qualitative images, they are essential for the implementation of mechanical approaches to measure cell adhesion.

\subsection*{1.2.2. Mechanical approaches for cell population studies}

The mechanical behaviour of a living cell cannot be characterized simply in terms of fixed properties, as the cell structure is a dynamic system that adapts to its local mechanochemical environment\textsuperscript{48}. An large number of experimental tools have been developed to measure and apply forces and displacements to probe entire cells as well as individual molecules. Some tools allow the detection of forces down to the pN regime and displacements down to the Å regime.

In the past, studies were focussed on the mechanical response or mechanical manipulation of entire cell populations\textsuperscript{49}. A direct manipulation of the substrate to which cells adhere provided a means of mechanical manipulation\textsuperscript{50}. This approach was used in an attempt to impose cyclic deformation representative of \textit{in vivo} conditions for cell adhesion studies. Strains were imposed and measured via low-resolution displacement sensors and global forces were calculated from substrate stiffness.

An alternative of providing mechanical stimuli to a cell population through substrate manipulation was done by controlling the chemical composition. Responses of cells to
mechanical properties of the adhesion substrate were examined by varying the concentration of one component or the degree of crosslinking in polymeric gels. The forces imposed by the cells were not quantified.

Techniques based on force-application measurements have also been developed in order to detach the cells from their growing substrate. Cells were allowed to adhere on substrates that were placed in a centrifuge (Fig. 1.2A). The centrifugal (normal) force allowed the detachment of cells and the absolute strength of adhesion varied in function of the cell type. The centrifugal assay was restricted to comparative cell adhesion measurements. The ratio of cells between the top and the bottom of the chamber of centrifugation was quantified at a particular spin speed but no information was obtained at the single cell level.

![Fig. 1.2. Schematic of a centrifugal assay for cell adhesion studies.](image)

### 1.2.3. Mechanical approaches for single cell studies

Numerous mechanical approaches for single cells have been used to study the interaction between cells and substrates. Extensive uses of flexible substrates were used for the detection and the measurement of traction forces exerted by cells including embedded particle tracking, micropatterned substrates and microfabricated substrates.

Marker beads were embedded within a polymeric substrate in order to characterize the traction forces by two-dimensional displacements of the beads (Fig. 1.3A). The displacement of the beads was measured optically and the corresponding force was calculated via the experimentally
determination of the stiffness of the substrate. Bead-displacement values were translated into a traction force map using specialized algorithms. In addition, polyacrylamide gels with variable degrees of cross-linking allowed the modification of the rigidity of the substrate\textsuperscript{55,56} (Fig. 1.3B).

Micropatterned substrates were fabricated to allow pointwise control of cell adhesion and traction measurement through displacement of the pattern features\textsuperscript{57,51,58}. Alternatively, micro-fabricated arrays containing force-sensing elements were developed\textsuperscript{59,60,61} (Fig. 1.3C). Silicon substrates were prepared with flexible cantilevers of known bending stiffness with small pads to grow the cells. The forces exerted by cells on these pads were directly computed by the deflection of the cantilevers. Otherwise, silicon elastomeric substrates, that were micropatterned to give a regular array of surface indentations, were also used to measure the surface distortion of the micropattern caused by cells\textsuperscript{62}.

Fig. 1.3. (A) Schematic of the embedded particle tracking assay reporting cell tractions as displacements of beads firmly anchored in the film\textsuperscript{54}. (B) Polyacrylamide-based traction assay after mapping the magnitude of stress in different colors (dyn/cm\textsuperscript{2})\textsuperscript{55}. (C) Schematic of a force sensitive substrate with an attached cell on the pads of the flexible cantilevers\textsuperscript{61}.

Traction forces analysis represents a powerful tool that can easily be combined with light microscope-based techniques to allow experimentation at high spatial and temporal resolution. There are a number of measurements that are resulting in increasingly accurate estimates of traction forces in order to correlate the stresses with the adhesive structures. Basically, traction forces are generated by actomyosin interactions and actin polymerization whereas adhesion
forces by focal adhesions. Cell traction forces and adhesion forces can be correlated. However, none of these approaches quantifies the adhesion forces, or only in a semi-quantitative way via traction forces. Finally, the resolution of the forces and displacements of these techniques (10\(^{-9}\) N and 10\(^{-7}\) m) limits the measurements to the single cell level, rather than to the single molecule.

The micropipette aspiration technique was originally developed to study time-dependent deformation of living individual cells subjected to extracellular pressure. Cells were allowed to spread for varying amounts of time on glass surfaces and then their free extremity was aspirated into a micropipette at given pressure levels (Fig. 1.4A). Applied aspiration pressure ranged from 0.1-1000 Pa, with resolution of 0.1 Pa. Displacement of the cell membrane was tracked by light microscopy with a claimed resolution of ± 25 nm. Then dual pipette assays were used to pull cells apart and quantify the strength of cadherin-dependent cell-cell adhesion. Cells were manipulated in suspension, an approach that eliminates matrix-mediated signalling and bypasses the initiation of intercellular adhesion through filopodial activities occurring during adhesion on a substrate. Alternatively, one of the two cells could adhere on a surface. The weak point of this technique is the indirect measurement of the adhesion force via the measurement of the aspiration pressure and the cell deformation in the micropipette. Computational models of the cell deformation differ in their interpretation and the cell deformation can also interfere in the adhesion properties. Although the instrumentation is not commercially available, the technique has been used successfully for cell-cell interactions.

In contrast to static cell adhesion assays, hydrodynamic shear stress was used to analyze the immobilization and release of non-adherent cells on surfaces (Fig. 1.4B). Parallel and radial flow chambers as well as capillaries were used to control the hydrodynamic stress level. The problem is the development of hydrodynamic shear methods in which there is a clear dose-response relationship between the number and strength of the adhesion bonds and the measured hydrodynamic parameter. The application of a hydrodynamic flow is very useful in analysing catch bond interactions. A catch bond is a bond that becomes stronger or longer lived when subjected to tensile mechanical force. This adhesion assay was used to mimic the conditions in the blood vessel under which leucocytes adhere to vascular endothelial cells. The indirect computation of the adhesion forces via the hydrodynamic forces involves laborious mathematical models. Moreover, this technique is limited to short-term adhesion. However, it is useful to simulate what occurs in blood or lymph vessels.
Optical tweezers employ light to trap and manipulate cells (Fig. 1.5A). The basic physical principle underlying optical tweezers is the radiation pressure exerted by light when colliding with matter\textsuperscript{75}. For transparent materials, light passes through the material but changes direction upon entering and leaving the material by refraction. A fraction of the light momentum is imparted onto the refracted material. When a laser beam is focussed tightly by a strong lens, the effective radiation pressure pulls refractive objects towards the focus and holds it there: an optical trap. If the laser focus is moved through space, the trapped object is forced to follow. When the refractive object is pulled away from the laser focus by an external force, the object will scatter the trapping light, yielding a slight deflection of the ongoing laser light. This allows for three-dimensional tracking of the position or the force on the trapped object to sub-nanometer or sub-picoNewton accuracy. Several experimental approaches developed to investigate single cell mechanics are based on the controlled displacement of dielectric objects that are either attached to the cell membrane or placed inside the cell. Optical tweezers have been used to study, for example, short-term adhesion of cells to fibronectin\textsuperscript{76,77}. One drawback is the potential damage to the cell via radiation. Photo-damage may influence the results because such irradiation levels were observed to induce a loss of filopodia and a general inhibition of motility\textsuperscript{78}. Moreover, optical tweezers are limited to the detection of forces in the pN regime and cannot be therefore employed for the investigation of the global cell adhesion.
A similar approach to the optical trap is the magnetic trap. Magnetic tweezers that use a magnetic field gradient can exert and measure forces on magnetic particles coupled to cells (Fig. 1.5B). The coupling of the magnetic beads to cells was achieved by phagocytosis or covalent linkage\textsuperscript{79,80}. Magnetic tweezers have been used to measure integrin interactions in initial cellular adhesion processes\textsuperscript{81}. Two advantages of this approach over optical tweezers are that out-of-plane rotations and thus torque can be applied, and the elimination of possible damage to the cell via radiation. A limitation of this technique is that the magnetic susceptibility of the magnetic beads varies widely, so that these experiments can be difficult to calibrate accurately. Magnetic tweezers provide great sensitivity but the maximal vertical force of 200 pN that can be applied greatly restricts their use in studying global cell adhesion, which requires forces in the nN regime. Optical and magnetic tweezers are unique tools for force microscopy at the cellular level and the non-contact nature of both techniques removes complicating surface interactions. The low force regime available with optical and magnetic tweezers means that the nN regime (and higher) can only be investigated using contact probe methods.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{tweezers.png}
\caption{(A) Schematic of optical tweezers\textsuperscript{48} and (B) magnetic tweezers\textsuperscript{81} used for cell adhesion studies.}
\end{figure}

Thus, other techniques such as the microneedle technique were developed to apply and quantify large forces (in the nN regime) for global cell adhesion investigations. The instrument applies a mechanical force normal to the cell/substrate interface via a cantilevered probe. The position of the probe is monitored within an inverted optical microscope via a position-sensitive photodiode.
and the force is calculated from this deflection. Fine glass needles were employed to quantify the forces needed to displace single adherent cells.\textsuperscript{82} This cytodetachment technique is restricted to horizontal displacements: only shear stress can be applied instead of normal stress for cell detachment. Therefore, there is no direct measurement of the normal (vertical) adhesion and the risk to perforate the cell is not negligible.

\textbf{Fig. 1.6. (A) Fine glass microneedle\textsuperscript{82} for cell adhesion studies.}

In summary, several methods have been developed for measuring the forces that cells exert on their substrate, mainly involving the use of deformable substrates. These methods provide quantitative evaluations of the forces, and some combine this with visualization of the adhesion sites.\textsuperscript{83,84} However, their capacity to compare the forces applied at the focal adhesions of a cell is limited. The micropipette assay can be used for simultaneous measurement of separation force between two cells and detection of fluorescent proteins involved in adhesion. However, the separation force is not monitored in function of the distance of separation because the position of the micropipette is controlled by a micromanipulator. Force-distance curves cannot be obtained. Moreover, this instrumentation is not commercially available and therefore, it is difficult to establish controls that demonstrate the specificity of the molecular interaction being studied. Hydrodynamic flow assays are especially useful for cells in suspension such as blood cells. Optical and magnetic tweezers are very sensitive but they are restricted to the detection of low forces (pN regime) while global cell adhesion requires the detection of high forces (nN regime). Finally, the microneedle technique doesn’t have a direct measurement of the vertical adhesion.
1.3. Atomic force microscopy for cell adhesion studies

One of the most promising techniques for the quantification of the adhesion forces is force spectroscopy, an AFM-based method (Atomic Force Microscopy). The AFM was originally developed for high-resolution imaging but it has also become a powerful tool to manipulate biomolecules and cells in a very wide force range. The AFM allows direct force and displacement measurements from the single cell down to the single molecule level. Force spectroscopy can be performed in numerous commercially available instruments.

1.3.1. Principle

The atomic force microscope is a scanning probe microscope that was developed in 1986. The AFM can be used to study non-conducting materials, such as insulators and semi-conductors, as well as electrical conductors. The AFM can operate under ultra high vacuum, ambient air conditions or in liquid, which is highly useful for biological systems.

The AFM uses a flexible cantilever as a spring to measure the force between the tip of the cantilever and the sample. The basic principle is that the local attractive or repulsive force between the tip and the sample is converted into a deflection of the cantilever (Fig. 1.7A). The deflection of the cantilever is detected via a laser beam that is reflected from the back of the cantilever onto a detector. A small change in the deflection angle of the cantilever is converted into a large deflection in the position of the reflected spot. Most AFMs use a photo-sensitive detector (PSD) that is composed of four quadrants, so that the laser spot deflection can be determined precisely in two directions.

Originally, the AFM was developed for high-resolution imaging. The detection system measures the cantilever deflection when the tip is moved over a surface by a scanning system. Different imaging modes are available: constant height imaging consists of measuring the deflection signal without changing the height of the cantilever. This is not the most common solution because the force applied by the cantilever depends on the deflection, so higher parts of the sample will experience higher applied forces. In contrast, it is much more common to use constant force imaging. In this mode, a feedback loop monitors the cantilever response and adjusts the cantilever (or sample) height to take account of the changes in surface height. Thus, all parts of the probed sample should experience the same force.
Several forces typically contribute to the deflection of an AFM cantilever, the most common being relatively weak, attractive van der Waals forces as well as electrostatic repulsive forces. The general dependence of these forces upon the distance between the tip and the sample is shown schematically in figure 1.7B. When the tip is less than a few Angströms from the sample surface, the interatomic force between the cantilever and the sample is predominantly repulsive, due to the overlap of electron clouds associated with atoms in the tip with those at the sample surface. In contrast to this contact regime, there is the non-contact regime where the tip is somewhere between ten to a hundred Angströms from the sample surface. In this case, the interatomic force between the tip and the sample is attractive as a result of long-range attractive van der Waals interactions.

Fig. 1.7. Schematic diagram of (A) an atomic force microscope and (B) the tip-sample separation forces\(^6\).

### 1.3.2. Force spectroscopy mode

AFM is also a powerful tool for sensitive force measurements\(^7\). Basic force spectroscopy curves are obtained with a cantilever in air approaching a hard and incompressible surface (such as glass) (Fig. 1.8). Initially, the cantilever approaches the surface and the forces are too small to give a measurable deflection. At some point, the attractive forces overcome the cantilever spring constant and the tip jumps into contact with the surface. Then, the tip remains on the surface and the separation between the piezo and the sample decreases further causing a deflection of the cantilever. During the retraction of the cantilever from the surface, the tip remains in contact with
the surface due to adhesion. At some point, the force from the cantilever will be sufficient to overcome the adhesion and the tip will break free.

Fig. 1.8. Schematic force curve on a hard surface with (A) approach and (B) retraction curves.

For a soft and compressible biological sample in liquid, the approach force curve shows a gradual increase in force without the sharp onset of the interactions seen in air (Fig. 1.9A). It is often difficult to define a single point where the tip and sample come into "contact" since the initial compression of the surface causes a very little deflection of the cantilever. Forces curves are obtained directly from the simple relationship between the force and the cantilever deflection, Hooke’s law:

\[ F = -k \, d_c, \]

where

- \( F \): force
- \( k \): spring constant of the cantilever
- \( d_c \): deflection of the cantilever

The interpretation of a force curve relies almost entirely on established force laws. These force laws describe force as a function of the probe-sample separation distance rather than as a function of the z-piezo position. Thus, the force curves must be transformed into descriptions of force as a function of distance. However, current AFMs do not have an independent measure of the probe-sample distance. Instead, the transformation to distance is achieved by subtracting the cantilever deflection from the z-piezo movement. A corrected force curve is called a force–distance curve (Fig. 1.9B).
Fig. 1.9. Schematics of (A) an uncorrected force curve and (B) a corrected force curve (force-distance curve)\textsuperscript{90}. At the beginning of the curve (i), the tip is far from the sample and there is no interaction and no cantilever deflection. The approach brings the tip closer to the sample, and long- and short-range tip-sample interactions cause the cantilever to deflect (ii). When the tip contacts the surface, the stage movement and cantilever deflection become coupled (iii). The retracting curve displays hysteresis due to a variety of tip-sample interactions.

1.3.3. Single bio-molecule force spectroscopy

Force spectroscopy can be used to examine rupture forces of molecular bonds and ligand-receptor interactions, unfolding and folding of various proteins in a stepwise process, extraction of a protein from a membrane, DNA mechanics or polysaccharide stretching\textsuperscript{91,92,93,94,91,95}. Such an approach to measuring forces is unique in that components of a single unbinding or unfolding event can be directly monitored. The flexibility of the AFM is such that force spectroscopy can be conducted on samples ranging from individual membrane proteins to complex and heterogeneous whole cells in their native environment. At the cell level, the bond rupture events represent a combination of the unbinding of ligand-receptor complexes and the disruption of non-specific binding events.

Adhesion forces between individual ligand-receptor pairs were already quantified more than fifteen years ago using force spectroscopy on biotin and avidin\textsuperscript{96}. This ligand-receptor pair was chosen as a model system because of its high affinity and the availability of thermodynamic and structural data on it. The bond rupture forces were reported for fixed pulling velocities and spring constants. Further details about the interaction potential of the biotin-avidin pair were revealed by
dynamic force spectroscopy\textsuperscript{97}. Similar experiments were reported on the protein A-IgG bond\textsuperscript{98,99}. The analysis and interpretation of these experiments can be complex, since bond dissociation is a non-equilibrium dynamical process\textsuperscript{100}. Bonds in cell adhesion are therefore being continually created, loaded over some period of time and then failing. They will fail under any level of pulling force if held for sufficient time\textsuperscript{101}. The kinetics of the anchoring process during the adsorption of fibrinogen molecules on silica surfaces was for example explored\textsuperscript{102}.

In single molecule unfolding experiments, the force applied to a single protein acts as a denaturant leading to complete unfolding of its three-dimensional structure\textsuperscript{103}. In an early study, Rief and co-workers applied single molecule force spectroscopy to the giant muscle protein titin which consists of repeats of globular immunoglobulin and fibronectin domains\textsuperscript{104,105}. The continuous mechanical extension of the protein resulted in the subsequent unfolding of the globular domains allowing the unfolding force and pathway of each domain to be detected. Similarly, individual bacteriorhodopsin molecules were unfolded and extracted from purple membrane patches from Halobacterium\textsuperscript{106,107,108}. Force spectroscopy was also employed to unfold single sodium-proton antiporters of Escherichia membrane patches. Some fundamental differences have to be considered when the forced unfolding by mechanical manipulation is compared with the classical experiment in which a protein is unfolded by means of chemical denaturants or by exposure to heat\textsuperscript{109}. As mentioned for receptor-ligand pairs, the unfolding force of proteins depends also on the force loading rate\textsuperscript{110}. Secondly, mechanically forced unfolding may proceed via a different path along the protein’s configurational energy landscape than the thermal process\textsuperscript{111}.

DNA molecule mechanics has also been investigated. Sequence-dependent mechanical properties of DNA have been measured by stretching individual DNA double strands attached between a surface and an AFM tip\textsuperscript{112} (Fig. 1.10). The forces needed to open the double strands showed variations between 10 and 20 pN on a length scale of 10 bases.
Fig. 1.10. Complementary DNA oligonucleotides are chemically attached to an AFM tip and a glass surface. As the tip is brought in contact with the surface a double strand forms, which can subsequently be unfolded upon retraction of the tip\textsuperscript{113}.

Single molecule force spectroscopy has also been used to study DNA-protein interactions\textsuperscript{114}. Overstretched structures of DNA were shown to be stabilized by a protein. Alternatively, direct force measurements were performed to mechanically unbind a transcriptional regulator protein that binds to promoter regions in a gene cluster\textsuperscript{115,116}.

The AFM has been used to stretch individual molecules of polysaccharides. Deflections in the force extension curves for certain polysaccharide molecules were shown to be due to conversion of the polysaccharide rings in the chain from the energetically favoured “chair” conformation to the longer “boat” conformation\textsuperscript{117,118}. Dextran molecules were also manipulated and probed by vertical stretching revealing details of the molecular basis of the mechanical properties of the polysaccharide that could not have been obtained otherwise. At low forces, the deformation of dextran was found to be dominated by entropic forces and at elevated forces the strand elongation was governed by a twist of bond angles\textsuperscript{119}.
Force spectroscopy has rapidly evolved and is now on the verge of becoming a standard technique for the structural and functional investigations of bio-molecules\textsuperscript{120,121}. It allows the characterization of interactions that stabilize functional membrane proteins as well as those that destabilize them, leading to malfunction and misfolding. This new tool has more recently been used to investigate bio-molecules in their native environment using entire living cells as probes: single cell force spectroscopy.

### 1.3.4. Single cell force spectroscopy (SCFS)

Living single cell force spectroscopy allows the adhesion of cells and single adhesion proteins to be studied under near-physiological conditions\textsuperscript{122}. The advantage of using living cells rather than single proteins is that the receptors probed are under physiological conditions and that they can interact with the cytoskeleton or other components of the cell. It also ensures the correct orientation, surface density and posttranslational modification of the proteins.

The first single cell force spectroscopy experiment to investigate cell adhesion \textit{in vivo} at the single molecule level was reported by Benoit and co-workers\textsuperscript{123} in 2000. It should be noted that previously force spectroscopy had been widely used for cell mechanics measurements such as estimations of Young’s moduli\textsuperscript{124,125} (more details in section 2.7.2). Benoit’s measurements were focussed on the interaction of the glycoprotein contact site A (csA) between two eukaryotic cells of Dictyostelium. CsA is expressed in aggregating cells of Dictyostelium which are engaged in the development of a multicellular organism. Using a light microscope, a Dictyostelium cell was captured on the end of a tipless AFM cantilever. The end of the cantilever was functionalized with a lectin to give a firm attachment of the cell to the cantilever. A target cell was positioned underneath the cantilever-mounted cell and was approached until a repulsive contact force was established. This contact force was held constant for a predefined time interval to allow the establishment of cell adhesion. Upon retraction of the cantilever, the force was recorded as a function of the distance that the cantilever was moved until contact between the cells were broken (Fig. 1.11). A de-adhesion force of 23 pN was quantified which may be due to discrete molecular entities.
Fig. 1.11. Force spectroscopy of adhesion between individual Dictyostelium cells\textsuperscript{123}.

The use of living cells as probes to study receptor-ligand interactions has been carried out using many cell types. Several studies have focussed on the characterization of integrin interactions with their ligands on a surface. The unbinding forces of integrins with fibronectin\textsuperscript{126,127}, collagen\textsuperscript{128}, V-CAM\textsuperscript{129,130} (V: vascular), I-CAM\textsuperscript{131,132,133} (I: intercellular) and I-CAM\textsuperscript{134} have all been determined. The integrin-fibronectin adhesion forces obtained range from 35 to 80 pN. The wide range of values obtained might indicate that the strength of the receptor-ligand interaction depends on the experimental conditions. Indeed, it has been reported that α5β1-integrin-fibronectin adhesion force on K562 cells varied depending on the force-loading rate from 40 to 140 pN\textsuperscript{135}. The unfolding and detachment of fibronectin from surface may also interfere. Other possible influences on the forces measured include non-specific binding of fibronectin to the cell, extraction of integrins from the cell membrane, temperature, or changes in cell properties such as membrane fluidity that could result in altered integrin mobility. However, there is close agreement about the scale of forces required to rupture the bond between integrins and their ligands. Unfortunately, experimental conditions have not been standardized and are often not sufficiently documented to allow rigorous comparisons between experiments.

Several CAMs mediate only cell-cell adhesion such as cadherins and selectins. It has proved difficult to characterize individual cell-cell adhesion events given the heterogeneous and complex nature of cell surfaces. AFM-based force spectroscopy is capable of resolving such individual cell-cell binding events but is limited in these kind of applications due to insufficient effective pulling distances\textsuperscript{136}. Long range force spectroscopy has been made possible by the development
of two AFM instruments by Asylum Research (USA) and JPK Instruments (Germany) with extended vertical ranges of 40 and 100 µm respectively. Using these instruments, homophilic and heterophilic binding capabilities of E-cadherin and N-cadherin have been compared\textsuperscript{137}. Individual E- and N-cadherin displayed different biochemical and biophysical properties. The kinetic of cadherin pairwise bonds formation was also probed\textsuperscript{138}. Similarly, it has been shown that the visually distinct rolling patterns of leukocytes over selectin substrates are a consequence of the kinetic and mechanical properties of the participating adhesive groups\textsuperscript{139}.

The use of living cells to study global cell adhesion has been poorly investigated. It is fundamental to distinguish affinity-based from avidity-based studies. The affinity describes the strength of a single bond between an individual receptor on the surface of a cell and its ligand on another cell or a surface. The avidity describes the global adhesion between two cells or between a cell and a surface. The affinity of all the individual receptors to their ligands control the overall adhesiveness (avidity) and for that reason, it is also important to investigate the global cell adhesion. The interpretation of global cell adhesion is much more complex because avidity depends on a multitude of biochemical and biophysical parameters. There is certainly the possibility to extrapolate multiple-bond avidity to evaluate single-bond affinity but the assumptions are not realistic. Avidity may depend on the density and affinity of the receptors for their ligands as well as on their spatial arrangement and orientation. The surface area with the probed cell, the duration of contact, the local geometry and stiffness of the plasma membrane, and the rate of surface diffusion of cell receptors play also important roles\textsuperscript{140}.

Short-term global cell adhesion has been used to compare the global cell adhesion properties of wild-type embryonic cells from mutant embryonic cells, that carry a loss-of-function mutation in a gene involved in gastrulation movements\textsuperscript{141,142}. These findings suggested that the Wnt11 gene is needed for the adhesion of zebrafish mesendodermal progenitor cells to fibronectin during gastrulation.

Finally, long-term global cell adhesion has never been successfully reported in the literature. It has been partially explored in a PhD thesis\textsuperscript{143}. Fibroblasts were allowed to adhere to substrates and then detached with a functionalized cantilever. The fraction of the probed cells that were successfully detached was not sufficient (less than forty percent) to quantify significantly the cellular adhesion forces.
1.4. SCFS configurations

Cell-surface interactions can be investigated using SCFS in several configurations\textsuperscript{[144,145,94,91]}. Figure 1.12 summarizes the feasible SCFS configurations. Firstly, the contact time of the interaction between a cell and a surface can be either short (I-IV) or long (V-VIII). In this thesis, short-term adhesion is defined as contact times from seconds to minutes whereas long-term adhesion is defined as contact times from hours to days. Therefore, short-term adhesion measurements involve cells showing a round morphology in contrast to long-term adhesion where cells have time to spread and to react to the surface. Secondly, the interaction surface that is studied can be either the substrate or the cantilever. Finally, the cell can either stay on its initial surface or be captured and transferred to the opposite surface. The study of cell-surface interactions can be categorized in eight configurations:

(I) Short-term adhesion measurements have been widely investigated for receptor-ligand interactions using living cells as probes with the cell on the cantilever\textsuperscript{[129,135,128,134,132,130,133]}. To date, global short-term adhesion has been poorly quantified\textsuperscript{[141,142]}.  

(II) The transfer of a cell from the surface to the cantilever is used to perform short-term adhesion measurements with the cell on the cantilever (configuration I).  

(III) Short-term adhesion measurements have also been widely investigated for receptor-ligand interactions using living cells as probes with the cell on the substrate\textsuperscript{[126,131,127]}.  

(IV) The transfer of a cell from the cantilever to the substrate can happen during a prolonged contact time between the cell and the substrate.  

(V) In contrast, long-term adhesion measurements have been very little explored. Long-term measurements with adherent cells on the substrate were restricted to the analysis of the mechanical properties of cells\textsuperscript{[124,125]}.  

...
Fig. 1.12. Single cell force spectroscopy configurations for cell-surface interactions.
The detachment of living single adherent cells has never been successfully reported in the literature. It was partially explored in a PhD thesis but only a small fraction of the probed cells were successfully detached from their growing substrate. This fraction was not sufficient to quantify the cellular adhesion forces.

Finally, the direct growth of cells on the cantilevers for long-term adhesion in order to quantify their adhesion forces has not been reported.

1.5. Dissertation objectives

The main objective of this work is to use force spectroscopy to quantify the long-term global adhesion between cells and surfaces and their response to modified surfaces. Long-term adhesion measurements are important to allow the cells to spread and their adhesion proteins to be renewed in order to react to their growing substrate.

Many techniques have been proposed for the quantification of cell adhesion. AFM-based force spectroscopy has been shown to be a versatile tool allowing the quantification of receptor-ligand interactions in living cells.

The quantification of long-term cell adhesion involves adherent cells. The first objective of this work is to determine if the cell cycle influences cell adhesion or not. Cells in interphase all show the same morphology under the microscope and therefore cannot be selected as a function of their progression through the distinct phases of the cell cycle.

The quantification of long-term adhesion requires the detachment of adherent cells from their culture substrate. The challenge is to find a method of attachment, between cells and atomic force microscope cantilevers, that allows their detachment from the surface. The establishment of the attachment has to be quick in order to avoid cell adhesion modifications.

The next step is to validate the technique for the detection of the effects of surface modification on cell adhesion. The application of the technique in tissue engineering and implantology is particularly interesting because both research fields are directly focused on cell-surface
interactions. The objective is to determine if long-term adhesion measurements might be useful in the development of surfaces for tissue engineering. Long-term cell adhesion forces are quantified on chemically modified surfaces used in cell sheet engineering in order to correlate the results with the qualitative effects already reported.

In order to investigate the sensitivity and limitations of the detachment of adherent cells by force spectroscopy, different experimental parameters can be altered. Surface topography is easily tunable and its effects on cell adhesion have been widely studied. Another objective therefore is to analyze the effects of topographical surface modifications on cell adhesion and to evaluate the possible contribution to bone implant applications. Here cell type is especially important since both immortalized and primary cells have been studied and very different results have been found for different cell lines and primary cells. An objective therefore is to compare the detachment of different cell types in order to determine the potential of the technique in this respect.

1.6. Organization of the dissertation

The dissertation starts with one chapter describing the materials and the experimental techniques used in the next four chapters focussed on cell adhesion measurements.

Chapter 3 describes the short-term adhesion of Saos-2 human osteosarcoma cells to a glass surface during the cell cycle. This first experimental chapter examines the effects of the different phases of the cell cycle on cell adhesion in order to determine if cell adhesion has to be studied independently of cell cycle or not. The cells were synchronized in three phases of the cell cycle, trypsinized to release them from the culture surface and captured on a cantilever. Cell adhesion was compared with unsynchronized cells. Short-term adhesion was used to study the adhesion proteins already exposed at the cell surface in each phase of the cell cycle without influence of the glass surface.

Chapter 4 focuses on long-term adhesion measurements with adherent cells. The challenge is to find a method of attachment, between cells and atomic force microscope cantilevers, that allows their detachment from the surface. Long-term adhesion on a glass surface was used and several cantilever functionalizations were tested. Saos-2 osteoblasts were replaced by immortalized 3T3 and primary mouse fibroblasts.
Chapter 5 presents the quantification of 3T3 cell adhesion to chemically modified glass surfaces used for cell sheet engineering. This chapter studies the effects of surfaces modifications on cell adhesion in order to validate the technique. The cells were grown on different polyelectrolyte multilayers and on poly(N-isopropylacrylamide) films before their detachment.

Chapter 6 presents the quantification of 3T3 and primary fibroblasts to topographically modified quartz surfaces in order to investigate the sensitivity, the potential and limitations of the technique. Two cell types were grown on different topographies and their properties of detachment were compared.

Finally, the last chapter recaps the objectives in their context and summarizes the main conclusions of the dissertation with an outlook for future work.
Chapter 2 Materials and Methods

The materials and experimental techniques used in this thesis are described in this chapter. In some cases more detailed explanations about the specific experiments are included in the individual chapters.
2.1. **Substrate preparation**

The substrates used were either glass or quartz surfaces.

2.1.1. **Cleaning protocol**

Glass coverslips (Milian, Switzerland) and quartz wafers (Si-Mat, Germany) were cleaned in Piranha solution (H$_2$SO$_4$/H$_2$O$_2$) (4: 1)$_{v/v}$ for 10 min at 120 °C. The substrates were rinsed in flowing water (MilliQ 185 plus, Millipore AG, Switzerland) and dried in a nitrogen flow. Piranha solution reacts violently with all organics and should be handled with care.

2.1.2. **Surface modification with polyelectrolytes**

The cleaned glass coverslips were treated with an oxygen plasma for 2 min. Poly-L-lysine hydrobromide (PLL, Mw ~15 - 30 kDa, Sigma, Switzerland) and hyaluronic acid sodium salt from bovine vitreous humor (HA, Mw ~300 kDa, Sigma, Switzerland) were used at a concentration of 0.5 mg/ml in buffer solution. The buffer solution was 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) with 150 mM NaCl, pH adjusted to 7.4 with NaOH.

The polyelectrolyte multilayers were built up on the substrates by spray deposition, using a custom-made automated spraying system. Four parallel Paasche VLS airbrushes (Paasche Airbrush Co., IL, USA) connected to a compressor with pressure control allowed for the spraying of each polyelectrolyte or buffer solution at a constant pressure of 1 bar, with one airbrush for each different solution. A Lego Mindstorms NXT programmable robotics kit (Lego Group, Denmark) was used to fabricate a spraying device with programmable spraying time, pause time and sequence order. The cleaned substrates were placed on a sample holder allowing for drainage of the solutions, fixed at a distance of 19 ± 2 cm from the airbrush aperture and tilted at 45°. Each solution was sprayed according to the following sequence: 5 s polyelectrolyte spraying, 15 s pause, 5 s buffer spraying, 5 s pause. In this way, the deposition of each bilayer required one minute. The substrates were rinsed and conserved in the buffer solution.

*The surface modification with polyelectrolytes was done by O. Guillaume-Gentil at ETH Zurich (Switzerland).*
2.1.3. Preparation of PNIPAM-grafted surfaces

A thin layer of chromium (2 nm) and another of gold (20 nm) were deposited on the cleaned glass coverslips by evaporation (Auto 306 Turbo, Edwards, France). A self-assembled monolayer of 11-mercaptoundecanoic acid (SAM-COOH) was prepared on the gold surface by immersion of the coverslips in a 2 mM ethanolic solution of 11-mercaptoundecanoic acid (Sigma, MO, USA) overnight. Finally, the samples were sonicated for 30 s in ethanol and dried in a nitrogen stream. Amine-terminated poly(N-isopropylacrylamide) (NH2-PNIPAM) with an average molecular weight of 41 000 g/mol (Polymer Source, Canada was grafted under melt onto anhydride reactive SAMs (SAM-ANH). The activated anhydride groups were obtained by immersion of the SAM-COOH in a solution of dimethylformamide containing 0.1 M of trifluoroacetic acid (Sigma, MO, USA) and 0.2 M of triethylamine (Sigma, MO, USA) for 20 min followed by several washing steps in dimethylformamide. A PNIPAM film was spin-coated from a 1% w/v ethanolic solution onto the reactive SAMs at 3000 rpm for 20 s. The substrates were then placed in an oven and annealed at 150 °C for 5 h. The PNIPAM-grafted surfaces were finally rinsed in ethanol for 1 h and in deionized water overnight to eliminate the excess of polymer.

2.1.4. Preparation of patterned PNIPAM-grafted surfaces

Patterned PNIPAM-grafted surfaces were obtained using the same method except that the uniform layer of gold was replaced by a pattern of squares of gold. The squares, 3 x 3 µm, with an inter-space of 6 µm were obtained by classical photolithography and lift-off. The height of the gold structures was 20 nm.

2.1.5. Micro-fabrication of low aspect-ratio pillars in quartz*

Low aspect-ratio (270nm high) quartz pillars were fabricated using a modified procedure previously reported for silicon. In a first step, a polymer blend thin film was deposited on the four inch quartz wafers. Poly(methyl methacrylate) (PMMA, Mw=106 kDa, Polymer Standard Services, Germany) and polystyrene (PS, Mw=101 kDa, Polymer Standard Services, Germany) were dissolved in dioxane at a concentration of 15 mg/mL to give stock solutions. These were mixed to obtain a PS/PMMA ratio of (30: 70) w/w. The polymer blend solution was then spin-
coated onto the substrate under a controlled atmosphere (T °C=21 °C and RH=35 %) using a two step spin-coating procedure: 2 s at 500 rpm followed by 60 s at 2000 rpm. The resulting thin film was structured in two phases, one of which (PS) was removed by rinsing the sample in cyclohexane to reveal the quartz surface underlying the PS areas.

In the second step a durable etch mask was fabricated from the thin polymer film. The polymer-coated surface was first exposed to an oxygen plasma (O₂, 50 sccm, 0.05 torr, 15 W) for 90 s using an RIE plasma (Plasmalab 80plus, Oxford Instruments, United-Kingdom) to remove any residual PS layer and expose the quartz substrate. A 10 nm thick chromium layer was then deposited on the sample using a thermal evaporator (Lab 600H, Leybold Optics, Germany). The remaining PMMA phase and its chromium layer were removed by sonication in acetone.

The last step was the etching of the quartz, which was carried out in a deep reactive ion etcher (DRIE) (AMS 200, Alcatel, France) using fluorine chemistry. After etching, the samples were dipped for 5 min in a chromium etchant CR-7, then cleaned in SC-1 solution (H₂O/NH₄OH/H₂O₂) (5: 1: 1) v/v for 10 min at 70 °C and finally exposed to an oxygen plasma (RIE, O₂, 100 sccm, 0.2 torr, 100 W) for 1 h.

Prior to cell seeding, the quartz surfaces were activated with an oxygen plasma (Harrick plasma, NY, USA) for 10 min and then incubated in a solution of 0.01 % poly-L-lysine (MW = 150-300 kDa, Sigma, MO, USA) for 30 min to promote cell attachment.

### 2.1.6. Micro-fabrication of high aspect-ratio pillars in quartz*

The above procedure could be used to fabricate quartz pillars up to 270 nm high. However, further etching resulted in mask failure. For this reason, an alternative procedure was developed for higher aspect-ratio structures. A 200 nm layer of amorphous silicon (a-Si) was first deposited on the quartz by means of LPCVD (Centrotherm Furnace, Germany). The chromium etch mask was then fabricated on top of the a-Si layer as described above. The chromium layer was used as an intermediate etch mask that was transferred into the a-Si layer using DRIE. The good etch selectivity and the relatively thick (200 nm) a-Si mask, allowed the fabrication of deeper structures in the quartz. After fabrication, the cleaning procedure was the same as for 270

*The microfabrication of pillars in quartz was done by N. Blondiaux & M. Klein at CSEM Neuchâtel (Switzerland).
nm pillars except that the samples were also exposed to KOH after the chromium etchant step in order to remove the a-Si layer.

2.1.7. Sterilization protocol

The surfaces were sterilized with ethanol before their use in cell culture experiments. The surfaces were successively immersed in 70% ethanol for 10 s, absolute ethanol for 10 s and rinsed thrice in sterile deionised water.

2.2. Cell culture

Three cell types were used including immortalized human osteoblasts, immortalized mouse fibroblasts and primary mouse fibroblasts.

2.2.1. Saos-2 osteosarcoma cells

The human osteosarcoma cell line, Saos-2, was obtained from American Type Culture Collection (Manassas, VA, USA) and was maintained in continuous culture in McCoy’s 5A medium supplemented with 10% heat-inactivated standardized fetal bovine serum (Biochrom AG, Germany), 50 units/ml of penicillin (Sigma, MO, USA), 50 µg/ml of streptomycin (Sigma, MO, USA) and 1.5 mM of L-glutamine (Sigma, MO, USA) at 37°C in a humidified 5% CO₂ atmosphere.

2.2.2. 3T3 mouse fibroblasts

The mouse fibroblast cell line, 3T3, was obtained from Marinpharm GmbH (Luckenwalde, Germany) and was maintained in continuous culture in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated standardized fetal bovine serum (FBS, Biochrom AG, Germany), 50 units/ml of penicillin (Sigma, MO, USA), 50 µg/ml of streptomycin (Sigma, MO, USA), 1.5 mM of L-glutamine (Sigma, MO, USA) and 1% of non-essential amino acids (Bioconcept, Switzerland) at 37 °C in a humidified 5% CO₂ atmosphere.
2.2.3. Primary embryonic mouse fibroblasts

Primary mouse embryonic fibroblasts (PMEF) were isolated from embryos derived from C57 mothers between 12 and 14 days of gestation. Embryos were cut into small pieces and incubated with a solution of 2.5 g/l trypsin and 0.38 g/l of ethylenediaminetetraacetic acid (EDTA) for 20 min at 37 °C under agitation after removing extracellular tissue, head and liver. Trypsin was inactivated by addition of DMEM containing 10% FCS and cells were purified by successive steps of filtration and centrifugation. Cells were maintained in DMEM supplemented with 10% heat-inactivated standardized fetal bovine serum (FBS, Biochrom AG, Germany), 50 units/ml of penicillin (Sigma, MO, USA), 50 µg/ml of streptomycin (Sigma, MO, USA) at 37 °C in a humidified 5% CO₂ atmosphere.

2.2.4. Cell cycle synchronization

Saos-2 cells were grown to about 60% confluency. Cell cycle arrest in late G₁ phase was induced using mimosine (Sigma, MO, USA) synchronization. Mimosine was added to a final concentration of 0.4 mM and the cells were incubated for 48 h.

Cell cycle arrest in late S phase was induced using thymidine (Sigma, MO, USA) synchronization. Thymidine was added to a final concentration of 4 mM and the cells were incubated for 48 h. Cells were then washed twice in PBS and incubated with regular medium for 8 h.

Cell cycle arrest in G₂M phase was induced using nocodazole (Sigma, MO, USA) synchronization. Nocodazole-treated cells enter mitosis where they are blocked since they cannot form metaphase spindles. Nocodazole was added to a final concentration of 0.6 µg/ml and the cells were incubated for 48 h. Cells were then purified by gentle shaking and by pipetting them off the dish.

Native mitotic cells were also studied. These were obtained by agitating unsynchronized cultures of cells to detach mitotic cells from the polystyrene culture surface and then pipetting off the resulting cells.

2.2.5. Cell proliferation assay

Cells were incubated for 18 h in complete medium before adding Alamar Blue (dilution 1: 10,
Invitrogen, CA, USA) for 6 h. Aliquots were transferred to a spectrofluorimeter (Infinite M200, Tecan, Switzerland) and the fluorescence intensity ($\lambda_{ex}$ = 530 nm and $\lambda_{em}$ = 590 nm) was measured. The remaining Alamar Blue was removed and replaced with fresh complete medium for similar measurements at 2, 3 and 4 days.

2.2.6. Cell seeding

Cells were detached from the culture dishes by incubating with a solution of 2.5 g/l trypsin and 0.38 g/l of EDTA for 5 min. The dissociated cells were seeded on the surfaces following the regular conditions of cultivation. Cell detachment experiments were performed in regular medium supplemented with 25mM HEPES at 37 °C in a temperature-controlled chamber.

G2M synchronized and native mitotic cells were already partially or completely detached from the culture dishes but were also exposed to trypsin under identical conditions.

2.3. Instrumentation

2.3.1. Atomic force microscope (AFM)

The atomic force microscope (AFM) is one of the family of scanning probe microscopes and was invented in 1986. It is widely used in biological applications. The AFM uses a flexible cantilever as a type of spring to measure the force between the tip and the sample. The attractive or repulsive force between the tip and the sample is converted into a deflection of the cantilever. This cantilever deflection is detected with a laser beam that is reflected from the back of the cantilever onto a detector. A small change in the deflection angle of the cantilever is converted to a measurable large deflection in the position of the reflected spot on the photodiode-detector. Piezoelectric elements are used to control the position of the cantilever.

AFM is particularly suited for biological applications because the samples can be probed in physiological conditions. There is no need for staining or coating. The AFM is best known for its high-resolution imaging capabilities but it is also a powerful tool for sensitive measurements in force spectroscopy mode. In this mode, the base of the cantilever is moved in the vertical direction towards the surface using the piezo and then retracted again.
A Nanowizard® II BioAFM (JPK Instruments, Germany) was used. It has been developed for life science research reaching from cell biology and biophysics to surface science and biomedicine. The Nanowizard® was mounted on an Axiovert 200 inverted optical microscope (Carl Zeiss, Germany) (Fig. 2.1). A CellHesion® module expended the vertical travel range of the Nanowizard® to 100 µm by integrating a precise sample lift mechanism into the AFM stage. An incubation chamber, the BioCell™ (JPK Instruments, Germany), maintained the sample at the desired temperature (Fig. 2.2).

The atomic force microscope was mainly used in force spectroscopy mode to study cell/surface interactions at the both cell and molecule levels.

Fig. 2.1. Picture of the Nanowizard® II life science setup mounted on an inverted optical microscope. The stage and AFM head are shown sketched separately. The micropositioners to move the sample, represented by a red glass slide, are surrounded in red (A) and the micropositioners to move the AFM head are surrounded in blue (B).
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2.3.2. Fluorescence activated cell sorter (FACS)

Flow cytometry is a technology that simultaneously measures and analyzes multiple physical characteristics of single cells flowing in a fluid stream through a beam of light. Size, granularity and fluorescence intensity are measured. These characteristics are determined using an optoelectronic system that records how the cells scatter the incident laser light and emit fluorescence.

A flow cytometer is composed of three systems including fluidics, optics and electronics. The fluidics system transports the cells in a stream to the laser beam for exposure. The optics system consists of lasers to illuminate the cells in the sample stream and optical filters to direct the resulting light signals to the appropriate detectors. The electronics system converts the detected light signals into electronic signals that are processed by the computer (Fig. 2.3). Some instruments are equipped with an electronics system capable of sorting the cells as a function of their characteristics.
A FACSARia cell sorter (Becton Dickinson, CA, USA) was used to determine the relative cellular DNA content during the various phases of the cell cycle using the fluorescent dye propidium iodide. The incorporated amount of dye was proportional to the amount of DNA. Three distinct phases were determined including the G1, S and G2M phases. G2 and M phases have an identical DNA content and cannot be distinguished.

### 2.3.3. Confocal microscope

Confocal microscopy offers several advantages over conventional optical microscopy: the depth of field is controlled and all the out of focus structures are suppressed at image formation. The confocal approach uses spatial filtering to eliminate out-of-focus light in specimens that are thicker than the focus plane. The confocal microscope offers also the ability to collect serial optical sections from thick specimens. The cells were imaged with the confocal Leica TCS SP5 (Leica Microsystems, Germany).

### 2.3.4. Scanning electron microscope (SEM)

A scanning electron microscope (SEM) is a microscope that uses electrons instead of light to form an image. The image is produced in vacuum by probing the specimen with a high-energy focused electron beam. The incident electron beam loses some energy at each point on the specimen that is converted into emission of secondary electrons, back-scattered electrons, characteristic X-rays, light and heat. The display maps the varying intensity of any of these
signals to generate the image.

The samples were imaged with the XL-30 ESEM (Royal Philips, Netherlands) using secondary electrons since they are the most sensitive to topography.

2.4. Force-distance curves

Throughout this work “short-term adhesion” was defined as short cell-substrate contact time \( t_{\text{contact}} = 1 \text{s} \). The cell was captured on the cantilever before the measurement. In contrast, “long-term adhesion” was used for long cell-substrate contact time \( t_{\text{contact}} = 24 \text{ h} \). In this case, the cell was grown on the substrate and then detached with the cantilever.

2.4.1. Cantilevers for short-term adhesion

AFM cantilevers were functionalized with concanavalin A using a protocol adapted from Wojcikiewicz et al\(^{149}\). 500µm long tipless silicon cantilevers (Arrow TL1, Nanoworld, Switzerland) with a nominal spring constant of 0.03 N/m were used for all short-term adhesion measurements. Cantilevers were cleaned in an oxygen plasma (Harrick plasma, NY, USA) for 5 min, and then incubated overnight at 37°C in a solution of 0.6 mg/ml biotinamidocaproyl-labeled bovine serum albumin (Sigma, MO, USA) in 100 mM NaHCO\(_3\), pH 8.6. The cantilevers were then rinsed twice in PBS, incubated in a solution of 0.6 mg/ml streptavidin (Sigma, MO, USA) in PBS at pH 7.3 for 30 min. After two further rinses in PBS, they were finally incubated in a solution of 0.6 mg/ml biotin-labeled concanavalin A (Sigma, MO, USA) in PBS for 60 min. Cantilevers were calibrated in water before each measurement using the thermal fluctuation method.

2.4.2. Cell capture for short-term adhesion

Using the AFM in force capture mode, the extremity of a concanavalin A-decorated cantilever was precisely positioned above one cell. An approach step brought cell and cantilever into contact for 2 s. This was immediately followed by a retraction step to remove the cell from the surface. The cell was then left undisturbed on the cantilever for 15 min to ensure strong adhesion between cell and cantilever.
2.4.3. **Short-term adhesion measurements**

Force distance curves were acquired with an approaching and retracting speed of 5 µm/s using the maximum z-range of 100 µm. The cell was approached towards the surface until a repulsive force of 900 pN was reached. It was left in contact with the surface for 1 s before the retract step was started. There was a pause of 60 s at the maximal retract distance between each measurement (Fig. 2.4).

![Fig. 2.4. Schematic illustrating a short-term adhesion measurement with a contact time of 1 s between the cell and the substrate.](image)

For each cell the first and last force distance curves were compared to ensure that there were no gross differences due to cell damage. To ensure that the cell-surface contact areas were similar for all measurements, the largest and smallest cells of each population – in total no more than 20% of the population – were excluded from measurements.

2.4.4. **Cantilevers for long-term adhesion**

AFM cantilevers were functionalized with fibronectin to promote cell adhesion\(^{143}\). 130 µm long silicon tipless cantilevers (NSC12, Mikromasch, Estonia) with a rectangular shape and a nominal spring constant of 4.5 N/m were used for all long-term adhesion measurements. Cantilevers were cleaned in piranha solution at 120°C for 10 min and activated with an oxygen plasma (Harrick Plasma, NY, USA) for 3 min. The cantilevers were then incubated in a solution containing 94 % acidic methanol (1 mM acetic acid in methanol), 5 % water and 1 % (3-aminopropyl)-triethoxysilane (Sigma, MO, USA) for 30 min. The cantilevers were extensively rinsed in methanol and dried before incubation in a solution of 1 % glutaraldehyde for 1 min. They were finally rinsed in water and incubated in a solution of 10 µg/ml fibronectin (Sigma, MO, USA) for 1 h. The cantilevers were kept in the solution of fibronectin. Cantilevers were calibrated in water before each measurement using the thermal fluctuation method\(^{150}\).
2.4.5. **Long-term adhesion measurements**

The extremity of a fibronectin-decorated cantilever was precisely positioned above the targeted adherent cell. To ensure that the cell-cantilever contact area was similar for all measurements, representative cells of comparable size were selected for each condition. The cantilever was approached towards the cell until a repulsive force of 1.5 nN was reached. It was left in contact with the cell in constant height for 30 min before the retract step was started. Force distance curves were acquired with an approaching and retracting speed of 0.5 µm/s using the maximum z-range of 100 µm. A new cell sample and a new cantilever were used before each measurement (Fig. 2.5).

![Fig. 2.5. Schematic illustrating a long-term adhesion measurement on an adherent cell with a contact time of 30 min between the cantilever and the cell.](image)

2.4.6. **Cantilevers for indentation**

Cell elasticity measurements were performed with the same cantilevers as for short-term adhesion but without functionalization. Arrow TL1 tipless cantilevers (Nanoworld, Switzerland) were send to Novascan to be modified by gluing glass spheres (radius = 5 µm, Novascan, IA, USA) to the end of each cantilever to give well-defined indenters\(^\text{151}\) (Fig. 2.6).

![Fig. 2.6. TL-1 tipless cantilever modified by Novascan with a 10 µm sphere attached (SiO\(_2\)).](image)
2.4.7. Elasticity measurements

Force distance curves were acquired with an approach speed of 2.5 µm/s to minimize hydrodynamic effects. A maximum applied force of 200 pN resulted in a maximal indentation of 300 nm for the softest samples. There was a pause of 60 s at the maximal retract distance between each measurement. Measurements were performed only on thicker regions of the cell.

2.5. AFM imaging*

Polyelectrolyte layers were imaged on a Nanowizard I BioAFM (JPK Instruments, Germany) using CSC38/noAl cantilevers (Mikromasch, Estonia) in intermittent-contact (fluid) mode.

2.6. Cell preparation

2.6.1. FACS

Cells were harvested by trypsinization, washed in PBS, collected by centrifugation and fixed in cooled 70% ethanol for 1h. Cells were collected by centrifugation once more and suspended in PBS containing 40 µg/ml propidium iodide (Sigma, MO, USA), 0.015% v/v Nonidet P40 substitute (Sigma, MO, USA) and 100 µg/ml RNase A (Sigma, MO, USA). The DNA content was analyzed after at least 30 min incubation on ice.

2.6.2. Confocal microscopy

Cells were washed with cold PBS and fixed for 20 min in PBS containing 4% paraformaldehyde. They were then rinsed twice with PBS and incubated for 10 min in a solution of 0.1 M glycine in PBS. After two further rinses in PBS, the cells were incubated in a blocking solution composed of 0.05% saponine and 0.2% bovine serum albumin in PBS, for 20 min. Cells were stained for actin filaments with AlexaFluor 488 Phalloidin (dilution 1:40, Sigma, MO, USA) for 2 h at room temperature under agitation. The cells were finally washed twice in blocking solution, in PBS and in water. A mounting medium was used to preserve fluorescence.

*The AFM images were obtained by O. Guillaume-Gentil at ETH Zurich (Switzerland).
2.6.3. **SEM**

Cells were rinsed with PBS and fixed in a solution of 2.5 % glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4 overnight. After this incubation, the cells were dehydrated in a series of ethanol/water mixtures: 20 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 %, 100 % ethanol (5 min each incubation), followed by drying in air. The cells were observed after sputtering with gold.

2.7. **Data processing and statistical analysis**

2.7.1. **FACS**

For each cell population, 15,000 cells were analyzed. Each synchronization experiment was repeated at least 5 times. Data acquisition was performed with the FACSDiva software (Becton Dickinson, CA, USA) and further analysis of the data was carried out with the WinMDI software (J. Trotter, Scripps Research Institute, CA, USA).

2.7.2. **Analysis of force-distance curves**

After completion of a force-distance cycle, the measured changes in piezo z-position and the corresponding deflection signal in newtons were saved by the Scanning Probe Microscopy software (JPK Instruments, Germany). Force-distance curves were inspected before they were included in the data analysis. They were discarded if the baseline was too short to ensure that the cell was completely detached from the surface during cantilever retraction. The baseline was linear in all curves analyzed. Acoustic noise, mechanical interference, microscopic objects or other cells floating in the medium may cause unwanted cantilever deflection, which results in undulating baselines.

For short-term adhesion, data analysis was carried out using ORIGIN 7.5 (OriginLab, MA, USA). The force-distance curves were complex, usually containing a maximal force peak and several force steps either preceded by a force plateau or not. The parameters analyzed were the maximum force of detachment; the number, position and amplitude of the discrete detachment steps observed along the retraction curve; and the work required to detach the cell from the substrate. The work of detachment was obtained by integrating the adhesion forces over the
distance travelled by the cantilever. For each cell population, 10 force distance curves were obtained from 10 different cells, giving 100 measurements. Each measurement provided one maximum force of detachment, one work of detachment and several positions and amplitudes of the discrete detachment steps.

For long-term adhesion, data analysis was carried out using the Image Processing software (JPK Instruments, Germany). The force-distance curves were simple and contained one single maximal force peak. A number of mechanical parameters related to cell detachment were determined from the force-distance curves including the maximum force, work and distance of detachment. For each condition, 1 force distance curve was obtained from each of 20 different cells, giving 20 independent measurements. Each measurement provided one maximum force, one work and one distance of detachment. The percentage of cells that detached from the surface was determined by optically examining the end of the AFM cantilever after each measurement.

For elasticity measurements, data analysis was carried out using the Image Processing software (JPK Instruments, Germany). The software gave the possibility to derive the Young’s modulus from force curves running through several steps. All operations were applied to the approach curve. The first step of the processing was to remove any offset or tilt from the curve. The contact point was calculated automatically. Then the software calculated the indentation depth by taking the difference between the piezo movement and the cantilever vertical deflection in units of length. Finally the curve was ready to be fitted to the Hertz model to derive the Young’s modulus. The formula was adapted for parabolic indenter (Fig. 2.7)\textsuperscript{153}. For each condition, 5 force distance curves were obtained from each of 20 different cells, giving 100 measurements.

The Hertz model assumes linear elastic behaviour as well as homogeneity of the sample. However, most biological materials are neither homogeneous nor absolutely elastic. The viscous nature of the cell means that the energy delivered by the indenter is not completely given back by a cell but dissipates. The variability of the viscous behaviour becomes visible if different indentation velocities are tested\textsuperscript{154,155}. Each cell type has its own relaxation time. Moreover, the Hertz model is only valid for small indentations (a maximum of 10% of the cell height) where the substrate doesn’t influence the measurements and where the geometry of the indentation matches the geometry of the indenter. The Hertz model is a simple model to have a number in order to quantify the stiffness of the cells.
Chapter 2 Materials and Methods

Fig. 2.7. The Young’s modulus can be fitted using the Hertzian model for parabolic indenter. The model assumes infinite thickness and linear elasticity of the sample.

2.7.3. Statistical analysis

The forces, the work and the distances of detachment of the cells were quantified as described in the previous section. The mean of the values together with its standard error was taken as characteristic for each mechanical parameter of detachment.

For short-term adhesion, several force distances were obtained from each cell. Therefore, not all the data were independent: results from any one cell were dependent, while data compared between cells were independent. For this reason, the median value of each parameter was determined separately for each cell. The mean of these median values together with its standard error was then taken as characteristic for each condition.
The data obtained were analyzed with the statistical analysis and data analysis software S-PLUS 8.0 (Insightful, Switzerland). The two-sample T-test was used to test whether or not two population means, m₁ and m₂, are equal. The samples are assumed to come from Gaussian (normal) distributions. If it was not the case, logarithmic transformations giving normal distributions of the mechanical parameters were analyzed. A two-tailed test was used, with a null hypothesis H₀ that is the logical counterpart, mutually exclusive and exhaustive, to an alternative hypothesis H₁. Depending on the outcome of the test, the null hypothesis is either rejected or retained. Rejection of the null hypothesis logically leads to acceptance of the alternative hypothesis and retention of the null hypothesis leads to an inability to accept the alternative hypothesis; H₀: m₁ – m₂ = 0 and H₁: m₁ – m₂ ≠ 0. The Bonferroni correction was used for multiple comparisons to account for the inflation of the Type I-error.

For all statistical tests, a p-value <0.05 was considered significant. The p-value is the probability of obtaining a test statistic at least as extreme as the one that is actually observed, assuming that the null hypothesis is true. The smaller the p-value, the more strongly the test rejects the null hypothesis, that is, the hypothesis being tested.
In order to determine if cell adhesion has to be studied independently of cell cycle or not, cell adhesion at different phases was measured. In this chapter, force spectroscopy was used to measure the adhesion of Saos-2 cells to a glass surface at different phases of the cell cycle.

The cantilevers were functionalized for cell capture in order to perform short-term adhesion measurements.

The cells were synchronized in 3 phases of the cell cycle: G₁, S and G₂M. Cells in these phases were compared with unsynchronized and native mitotic cells. Individual cells were attached to an AFM cantilever, brought into brief contact with the glass surface and then pulled off again. The force-distance curves obtained allowed the work and maximum force of detachment as well as the number, amplitude and position of discrete unbinding steps to be determined.

‡Part of this chapter was published in Measuring cell adhesion forces during the cell cycle by force spectroscopy. Weder G., Vörös J., Giazzon M., Matthey N., Heinzelmann H. and Liley M. *Biointerphases*, 2009, 4(2), 27-34.
3.1. Introduction

3.1.1. The cell cycle

The eukaryotic cell cycle is divided into four phases. The two most dramatic events are when the nucleus divides, a process called mitosis, and when the cell splits into two cells, a process called cytokinesis. These two processes constitute the M phase (M = mitosis) of the cell cycle. The period between the M phase is called interphase, during which the cell grows in size. The interphase is divided into the remaining three phases of the cell cycle: G1, S and G2. During the S phase (S = synthesis), the cell replicates its DNA. The G1 phase (G = gap) is the interval between the completion of the M phase and the beginning of the S phase. The G2 phase is the interval between the end of the S phase and the beginning of the M phase.

Cell cycle can be analyzed by fluorescence labeling of the DNA of cells in suspension and using a fluorescence activated cell sorter (FACS). G1 cells have one copy of DNA and have therefore 1X fluorescence intensity. Cells in G2M phase have two copies of DNA and have therefore 2X fluorescence intensity. Since the cells in S phase synthesize DNA, they have fluorescence values between the 1X and 2X populations.

Fig. 3.1. (A) The four successive phases of a standard eukaryotic cell cycle. (B) The relative amount of DNA per cell during the cell cycle.
3.1.2. Adhesion of mitotic cells

For most adherent cells in vitro, dramatic changes in cell morphology take place during the cell cycle\textsuperscript{158}. For most of the cell cycle, the cells are spread over the surface of the substrate, then, at the beginning of mitosis, they round up and lose most of their contact to the substrate in preparation for cytokinesis. Mitotic rounding up is associated with changes in the cytoskeleton, plasma membrane and cell volume, while adhesion of the cell to the substrate is greatly reduced\textsuperscript{159}. However, the underlying mechanisms of these changes are poorly understood.

Adherent cells attach to their surroundings via focal adhesions. These large protein complexes consist of a variety of cytoskeletal and cytoskeletal-associated proteins providing the primary stabilizing force for the attachment of cultured cells as well as initiation sites for actin stress fibres\textsuperscript{160,161}. There have been many studies of the role of individual adhesion proteins in the rounding up of mitotic cells\textsuperscript{162}. These studies show that various phosphorylation events of cytoskeletal\textsuperscript{163} and focal adhesion proteins\textsuperscript{164,165,166,167,168,169} contribute to the pre-mitotic disassembly of focal adhesions\textsuperscript{170}, the deconstruction of the actin cytoskeleton of the interphase\textsuperscript{171} and the construction of a new mitotic cytoskeleton\textsuperscript{172}. However, despite this dissociation and deconstruction, the mitotic cell remains attached to the substrate. There is direct contact between the plasma membrane and the substrate\textsuperscript{173}. In addition, there are a number of retraction fibres, thin actin filaments, that anchor the cell to the substrate via focal adhesions. Thus some adhesive elements remain during mitosis.

Not only the cytoskeleton, but also the plasma membrane is remodelled during mitosis. Rounding up of the cell involves a reduction of the total area of plasma membrane, which has been recently found to take place via normal endocytosis of the plasma membrane and a reduction in the recycling of internalized membranes\textsuperscript{174}.

3.1.3. Short-term adhesion measurements

A single cell was attached to an AFM cantilever, brought briefly into contact with a glass substrate, and then pulled off the substrate\textsuperscript{175} (Fig. 3.2A). Measurement of the cantilever deflection during this process allowed the forces between cell and surface to be quantified at each moment and represented in force distance curves (Fig. 3.2B). Several different mechanical parameters were extracted from these curves\textsuperscript{152} including the maximum force of detachment, the
displacement needed to completely remove the cell from the substrate and the work of detachment. Not only global cellular parameters were studied: it was also possible to identify and analyze individual unbinding events, discrete steps in the force distance curve that occur on the release of individual proteins or protein complexes from the surface\(^{176}\).

Fig. 3.2. (A) Schematic of a single-cell force spectroscopy measurement, and (B) a force-distance curve acquired during approach (I-II in blue) and retracting steps (III-IV in orange). In the initial phase of the approach there is no contact between the cell and the surface (I). Then the cell is pressed onto the surface until a pre-set maximal force is attained. During this phase the elastic response of the cell can be observed (II). The position of the cantilever is held constant for a given contact time. Information on different mechanical parameters can be obtained from the retraction: the work of detachment, the number, amplitude and position of the unbinding events corresponding to single proteins or protein complexes and, finally, the maximal force needed to detach the cell from the surface (III). In the last step, there is no physical contact between cell and surface (IV).

With this approach, the results obtained reflected the composition and organization of the plasma membrane, for example, concerning the presence, activity and organization of adhesion proteins. However, the short contact time – one second - between cell and substrate means that no information could be gained about how the cell responds to the surface on a longer time scale, thus excluding the effects of, for example, changes in protein expression.
3.2. Cell cycle synchronization

3.2.1. \( G_1, S \) and \( G_2M \)

Saos-2 cells were synchronized in 3 phases of the cell cycle, \( G_1, S \) and \( G_2M \). Unsynchronized cell populations showed the expected distribution of 56% of cells in the \( G_1 \) phase, 21% in the \( S \) phase and 23% in the \( G_2M \) phase. Good synchronization was obtained in both the \( G_1 \) phase with 78% of cells in \( G_1 \) and in the \( G_2M \) phase with 80% of cells in \( G_2M \). \( S \) phase synchronization was limited to 62% due to differences in resumption of the cell cycle for individual cells after release from the thymidine synchronization (Fig. 3.3). A more specific synchronizing agent to synchronize the cells in the intermediate \( S \) phase of DNA replication was not found.

Fig. 3.3. DNA content analysis of the cell cycle measured by flow cytometry of SaOs-2 cells in the presence of synchronizing agents: (A) unsynchronized, (B) mimosine as a \( G_1 \) synchronizing agent, (S) thymidine as a \( S \) synchronizing agent and, (D) nocodazole as a \( G_2M \) synchronizing agent.
3.2.2. Native mitotic cells

When studying synchronized cells, it is important to be aware that the synchronizing agent may affect several properties of the cell; nocodazole, for example, inhibits the polymerization of the microtubules. In order to exclude the possibility that any differences observed between G2M cells and unsynchronized cells were an unexpected artifact due to the use of nocodazole, native mitotic cells were also investigated. Native mitotic cells were obtained from unsynchronized cell cultures and were separated from cells in other phases of the cell cycle thanks to their reduced adhesion to the culture surface. Gentle agitation of the medium or culture support resulted in their release from the surface into the culture medium while other cells remained firmly attached.

3.3. Short-term adhesion

3.3.1. Presence of adhesion proteins throughout the cell cycle

First experiments carried out with the Saos-2 cells were used to select the experimental parameters for the short-term adhesion measurements. An applied force of 900 pN was chosen and a contact time of 1 s was selected to avoid very strong cell-surface interactions that might result in a failure to totally detach the cell from the surface even at a retraction distance of 100 µm.

Using these experimental parameters, typical force distance curves obtained for each of the synchronized cell populations are shown in figure 3.4. Qualitatively, the curves look similar, with discrete unbinding events indicating the presence of adhesion-protein complexes at the cell membrane in each phase of the cell cycle. A statistical analysis of these events was carried out, comparing the number of events per curve, the amplitude of the events and the position of the events along the retraction curve for the three synchronized cell populations and for a population of unsynchronized cells.
Fig. 3.4. Typical force distance curves showing the detachment of G₁, S and G₂M synchronized human osteoblasts from a glass surface (contact for 1 s with an applied force of 900 pN). The step-like features in the curves correspond to the release of adhesion proteins from the glass and can be observed in all three phases of the cell cycle.

Histograms showing the number of unbinding events are shown in figure 3.5. In all cell populations, there were between 4 and 23 unbinding events per curve, reflecting the number of adhesion protein complexes that bound to the surface. Comparing the mean numbers of unbinding events for the different populations, the values obtained are 8.9 and 12 in interphase (G₁ and S), while in the two M-phase populations (G₂M and native mitotic) the means are 11.3 and 10.9. For the unsynchronized cells, which are expected to be approximately 80% in interphase (since made up of 56% G₁ and 21% S), 12.6 is obtained. The number of unbinding events in interphase and metaphase are broadly similar: there is no large decrease in unbinding events that might be associated with a loss of adhesion in M-phase (Table 3.1).
Fig. 3.5. Histograms showing the number of unbinding events of human osteoblasts on a glass surface after an applied force of 900pN for 1s. The number of unbinding events was measured for unsynchronized cells and synchronized cells in G\textsubscript{1}, S and G\textsubscript{2}M phases. Between 4 and 23 unbinding events were observed in each force distance curve independently of the cell cycle phase.

<table>
<thead>
<tr>
<th></th>
<th>Number of unbinding events</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (Median) ±SE</td>
</tr>
<tr>
<td>Unsynchronized</td>
<td>12.6 ±0.5</td>
</tr>
<tr>
<td>G\textsubscript{1} phase</td>
<td>8.9 ±0.4</td>
</tr>
<tr>
<td>S phase</td>
<td>12 ±1</td>
</tr>
<tr>
<td>G\textsubscript{2}M phase</td>
<td>11.3 ±0.5</td>
</tr>
<tr>
<td>Native mitotic</td>
<td>10.9 ±0.8</td>
</tr>
</tbody>
</table>

Table 3.1. Comparison of the number of unbinding events for 5 conditions: unsynchronized cells, G\textsubscript{1}, S, G\textsubscript{2}M synchronized cells and native mitotic cells (SE: standard error).
3.3.2. Position of the unbinding events

The positions (distance from the surface) of the unbinding events in the force distance curves were also analyzed. Clear differences were observed between the G2M phase and the other populations, while differences between unsynchronized, G1 and S phases were not significant. A statistical analysis of the number of events before and after 30 µm displacement of the cantilever is shown in table 3.2.

- For unsynchronized cells, 78% of unbinding events occur within the first 30 µm and 22% in the remaining distance. Similar values were obtained for cells in G1 and S.
- For cells in G2M, 93% of unbinding events occur within the first 30 µm and 7% in the remaining 70 µm of travel.
- For native mitotic cells, 95% of unbinding events occur within the first 30 µm and 5% in the remaining 70 µm of travel.

Clearly, cells in G1 and S can be stretched further before the cell is released from the surface than cells in G2M or native mitotic cells.

<table>
<thead>
<tr>
<th></th>
<th>Position of unbinding events</th>
<th>≤ 30µm (%)</th>
<th>&gt; 30µm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsynchronized</td>
<td></td>
<td>78</td>
<td>22</td>
</tr>
<tr>
<td>G1 phase</td>
<td></td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>S phase</td>
<td></td>
<td>79</td>
<td>21</td>
</tr>
<tr>
<td>G2M phase</td>
<td></td>
<td>93</td>
<td>7</td>
</tr>
<tr>
<td>Native mitotic</td>
<td></td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

*Table 3.2. Comparison of the distribution of the position of unbinding events between 5 conditions: unsynchronized cells and G1, S, G2M synchronized cells and native mitotic cells.*

3.3.3. Adhesion per protein complex in the different cell cycle phases

The amplitudes of the unbinding events are very similar in all the different conditions, with mean values between 84 and 108 pN (Table 3.3). The size of these events suggests that they correspond to the rupture or release of either individual proteins or small protein complexes from the glass surface.
Comparing the values obtained for interphase (G₁ and S) with M-phase (G₂M and native mitotic), 86 and 84 pN respectively for the interphase populations and 108 and 96 pN for the M-phase populations are observed. In contrast, the unsynchronized population, which is expected to be approximately 80% in interphase, gives a value of 104 pN.

The similarity of the unbinding forces obtained for all phases of the cell cycle is perhaps surprising: there are numerous reports of the phosphorylation of individual proteins associated with the cytoskeleton and with focal adhesions, which might lead one to expect differences (i.e. a reduction) in the binding ability of the proteins exposed at the cell membrane.

<table>
<thead>
<tr>
<th>Amplitude of unbinding events</th>
<th>Mean (Median) (pN)</th>
<th>±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsynchronized</td>
<td>104</td>
<td>17</td>
</tr>
<tr>
<td>G₁ phase</td>
<td>86</td>
<td>6</td>
</tr>
<tr>
<td>S phase</td>
<td>84</td>
<td>13</td>
</tr>
<tr>
<td>G₂M phase</td>
<td>108</td>
<td>5</td>
</tr>
<tr>
<td>Native mitotic</td>
<td>96</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 3.3. Comparison of the amplitude of the unbinding events for 5 conditions: unsynchronized cells, G₁, S, G₂M synchronized cells and native mitotic cells (SE: standard error).

### 3.3.4. Maximum force of detachment

The values obtained for the means of the maximum forces of detachment are similar for all phases of the cell cycle: the values, between 0.88 and 1.2 nN, are shown in table 3.4. Comparing interphase and M-phase, 0.88 and 0.99 nN for G₁ and S populations and 1.2 nN for both G₂M and native mitotic cells were obtained. The unsynchronized population gives a value of 1.2 nN. The maximum force of detachment is not strictly an independent parameter as it is related to the number of unbinding events and their amplitude. It is, however, not simply the product of the number of unbinding events and their amplitude due to contraction and/or relaxation of the cell during the measurement, particularly the first few microns of retraction. The values obtained for the unsynchronized cell population are compatible with those obtained for each of the phases individually. The only statistically significant differences are between the distributions of the G₁-phase and mitotic cells.
### Table 3.4. Comparison of the maximum force of detachment for 5 conditions: unsynchronized cells, G\textsubscript{1}, S, G\textsubscript{2M} synchronized cells and native mitotic cells (SE: standard error).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (Median) (nN)</th>
<th>±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsynchronized</td>
<td>1.2</td>
<td>0.1</td>
</tr>
<tr>
<td>G\textsubscript{1}</td>
<td>0.88</td>
<td>0.03</td>
</tr>
<tr>
<td>S phase</td>
<td>0.99</td>
<td>0.09</td>
</tr>
<tr>
<td>G\textsubscript{2M}</td>
<td>1.2</td>
<td>0.06</td>
</tr>
<tr>
<td>Native mitotic</td>
<td>1.2</td>
<td>0.06</td>
</tr>
</tbody>
</table>

### 3.3.5. Work of detachment

The work of detachment – similarly to the maximum force of detachment – is not strictly an independent parameter as it is related to the amplitude and number of unbinding events and their position during retraction of the cantilever. The mean works of detachment are similar for the unsynchronized and synchronized cells with values between 1.9 and 0.94 x 10\textsuperscript{-14} J (Table 3.5). In interphase, the values obtained are 1.4 and 1.7 x 10\textsuperscript{-14} J for G\textsubscript{1} and S populations. In M-phase the values are 1.1 and 0.94 x 10\textsuperscript{-14} J for G\textsubscript{2M} and native mitotic. 1.9 x 10\textsuperscript{-14} J was obtained for unsynchronized cells. The only statistically significant differences are between the distributions of the unsynchronized and mitotic cells.

### Table 3.5. Comparison of the work of detachment for 5 conditions: unsynchronized cells, G\textsubscript{1}, S, G\textsubscript{2M} synchronized cells and native mitotic cells (SE: standard error).

<table>
<thead>
<tr>
<th>Condition</th>
<th>Mean (Median) (J)</th>
<th>±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsynchronized</td>
<td>1.9 x 10\textsuperscript{-14}</td>
<td>0.2 x 10\textsuperscript{-14}</td>
</tr>
<tr>
<td>G\textsubscript{1}</td>
<td>1.4 x 10\textsuperscript{-14}</td>
<td>0.2 x 10\textsuperscript{-14}</td>
</tr>
<tr>
<td>S phase</td>
<td>1.7 x 10\textsuperscript{-14}</td>
<td>0.2 x 10\textsuperscript{-14}</td>
</tr>
<tr>
<td>G\textsubscript{2M}</td>
<td>1.1 x 10\textsuperscript{-14}</td>
<td>0.1 x 10\textsuperscript{-14}</td>
</tr>
<tr>
<td>Native mitotic</td>
<td>0.94 x 10\textsuperscript{-14}</td>
<td>0.2 x 10\textsuperscript{-14}</td>
</tr>
</tbody>
</table>
3.3.6. Higher stiffness of mitotic cells

The analysis of the positions of the unbinding events suggested that G2M and native mitotic cells were stiffer than cells in the other phases. The increased stiffness of mitotic cells has been reported by other authors\textsuperscript{177,124} and is associated with a reorganization of the cytoskeleton (Fig. 3.6).

This was verified for Saos-2 cells using force spectroscopy to obtain an estimate of the Young’s modulus of the cells. For these measurements, the cells were adherent on a glass surface and a round glass sphere attached to an AFM cantilever was brought into contact with them. Given the size of the glass sphere and the forces applied during the indentation process it was possible to calculate a value for the Young’s modulus using a Hertzian model (section 2.7.2). The indentation is the difference between the piezo movement and the cantilever vertical deflection in units of length (Fig. 3.7).
Values for the Young’s modulus of individual G2M synchronized cells and individual adherent unsynchronized cells are shown in figure 3.8. Typically, the Young’s moduli of the G2M synchronized cells (mean value 1.4 kPa) are higher than those of the unsynchronized cells (mean value 0.4 kPa) confirming the increase of the cell stiffness in mitosis. The data obtained are within reported values for the Young’s modulus of living cells, (between 0.1 and 10 kPa)\textsuperscript{178,179}. It is difficult to make close comparisons with literature values, given the range of experimental parameters that influence the values obtained (e.g. geometry of the indenter, depth of indentation, speed of measurement). However, studies on osteosarcoma cell lines and on primary human bone cells including osteoblasts give very similar Young’s moduli: between 1 and 2 kPa for MG-63 cells\textsuperscript{180}, from 0.7 to 2.6 kPa for primary human bone cells and MG-6335, and from 2.1 to 8.8 kPa for Saos-2 cells\textsuperscript{181}.

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**Fig. 3.7.** Force indentation curves derived from (A) unsynchronized and (B) G2M synchronized Saos-2 cells. The applied force of 200 pN resulted in an indentation of 210 nm for the unsynchronized cells and of 90 nm for the G2M synchronized cells.
3.4. Discussion

3.4.1. Cell adhesion between M-phase and interphase

The experiments described here show that binding proteins and/or protein complexes at the cell membrane remain similar both in number and in their binding properties during all the phases of the cell cycle. Indeed, while some differences are observed in the surface binding properties of the cells, the variations are, in all cases, less than a factor of two and the lowest values are not associated with the M phase.

A detailed statistical analysis of the data was used to determine the probability that each measured parameter of each group of cells is the same (the p-value). Table 3.6 shows that the statistical differences (see white dots on table 3.6) existing between two cell populations are not associated with the M phase. However, the statistical analysis cannot determine whether or not the ten cells selected for analysis are representative of the entire cell population under this condition. It is almost certainly this limitation that results in some unexpected data points (see white dots). Despite the difficulty to draw a conclusion for small differences between cell populations, cell adhesion related to M-phase was not different from those related to interphase.
Chapter 3 Cell adhesion during the cell cycle

<table>
<thead>
<tr>
<th>Number of unbinding events</th>
<th>Unsyncr</th>
<th>G1 phase</th>
<th>S phase</th>
<th>G2M phase</th>
<th>Work of detachment</th>
<th>Unsyncr</th>
<th>G1 phase</th>
<th>S phase</th>
<th>G2M phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unsynchronized</td>
<td></td>
<td>● ○</td>
<td>● ● ● ○</td>
<td>● ● ● ○ ○</td>
<td>● ○ ● ● ● ● ●</td>
<td></td>
<td>● ○ ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ● ●</td>
</tr>
<tr>
<td>G1 phase</td>
<td>● ○ ● ●</td>
<td>● ○ ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ○ ● ● ● ● ●</td>
<td>● ○ ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
</tr>
<tr>
<td>S phase</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
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<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
</tr>
<tr>
<td>G2M phase</td>
<td>● ● ● ●</td>
<td>● ○ ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
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<td>● ● ● ●</td>
<td>● ● ● ●</td>
</tr>
<tr>
<td>Native mitotic</td>
<td>● ● ● ●</td>
<td>● ○ ● ●</td>
<td>● ● ● ●</td>
<td>● ● ● ●</td>
<td>● ○ ● ● ● ● ●</td>
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<td>● ● ● ●</td>
<td>● ● ● ●</td>
</tr>
</tbody>
</table>

Table 3.6. Comparison of the number and amplitude of unbinding events, and the maximum force and work of detachment for 5 conditions: unsynchronized cells, G1, S, G2M synchronized cells and native mitotic cells. Data were compared using the two sample T-test with Bonferroni correction (p-value 0.05). The black dots indicate that the cell populations are not statistically significantly different (p-value ≥0.05) and the white dots indicate that the cell populations are statistically significantly different (p-value <0.05).

3.4.2. Statistics

Force spectroscopy is inherently a single cell technique: values for different mechanical properties are determined individually for each cell of each population studied. Single cell analyses allow us to study not only the average properties of a sample (an ensemble of cells), but also the variation and heterogeneity within the sample. While this gives access to qualitatively new information, it also comes with its disadvantages. In our case, the statistical analysis was made significantly more complicated: for each cell condition ten cells were selected for study – this relatively small number was chosen because of the lengthy preparation required for measurements on each individual cell - and ten force distance measurements were carried out per cell. This method gave a dataset that was a mix of dependent and independent results. In general, the analysis required that the data for each cell (dependent results) were analyzed first to obtain a value characteristic of that cell. The values for each cell in the sample were then analyzed as independent data points to give a value that was representative of the sample as a whole.
3.4.3. The use of trypsin

A significant limitation of this study is the use of trypsin to detach the cells from the culture surface before fixing them to the cantilever. Trypsinization clearly damages at least some of the binding proteins exposed at the cell surface and so may well influence results in a study of these proteins. The effects have tried to be minimized by using a short, reproducible trypsinization on all samples and allowing the cells to recover for 30 minutes before measurement. Studies by other authors have shown that, at least in some cases, trypsinization leaves the binding functions of surface proteins largely intact\textsuperscript{128,130}. SDS-PAGE analyses also showed no effect of the trypsinization on either \(\alpha_2\) integrin or \(\beta_1\) integrins\textsuperscript{28}. While trypsinization may limit the work to the observation of relatively large effects, it is believed to have no influence on the conclusions of this study.

3.4.4. The short contact time

Finally, the short contact time between cell and surface during this study limits the analysis to a study of proteins and protein complexes that are already present at the cell membrane and to the gross mechanical properties of the cell. There is insufficient time for the cell to react significantly to the presence of the surface, for example, by changes in the cytoskeleton or in protein expression. A more complete study of cell surface interactions requires much longer contact times between cell and surface. However, increased contact times between SaOs-2 cells and glass result in increased cell/surface adhesion. The results are either a rupture of the cell as cantilever and surface are separated, or alternatively, the cell does not rupture, but remains attached to both surface and cantilever even at separations of 100 microns.

3.5. Conclusion

Single cell force spectroscopy was used to study the adhesive and mechanical properties of SaOs-2 cells in culture. The study focused in particular on the changes in these properties between interphase, when cells are well spread and firmly attached to culture surface and M-phase, when the cells round up and are much more loosely attached to the surface. The differences in cell/surface attachment are striking: in M-phase SaOs-2 cells may be removed by shaking culture vessels while in interphase attempts to pull SaOs-2 cells off the surface may result in cell rupture.
Because of the short time of contact between cell and surface, the investigation is relevant to cell surface properties and cell stiffness. The experiments described here show that binding proteins and/or protein complexes at the cell membrane remain similar both in number and in their binding properties (amplitude of the unbinding events) during interphase and M phase. While some differences can be observed in the mean values obtained in the different phases, the differences are, in all cases, less than a factor of 2 and the extreme values are not associated with the G2M or native mitotic (M) phase. No large reduction was observed either in number or in amplitude of the unbinding events that might contribute to the loss of adhesion of M-phase cells. This may be surprising given the numerous studies showing the phosphorylation of proteins associated with focal adhesions during mitosis.

A second clear conclusion can be drawn from the position of the unbinding events. As the cell was stretched during detachment from the surface, unbinding events took place at a smaller extension for the mitotic/G2M cells than for cells in other phases. These cells cannot be stretched as far before they are released from the surface. This is due to an increased stiffness of the cell caused by a reorganization of the cytoskeleton during mitosis as also observed by other researchers in the field.

The maximum force of detachment and the work of detachment were also studied for all the cells. These two parameters are not strictly independent as they are related to the number and amplitude of unbinding events and to their position. Here, again the differences observed between M phase and interphase were relatively small compared to the differences between cellular adhesion during the cell cycle.
Long-term cell adhesion involves adherent cells. The challenge is to establish an attachment between cells and AFM cantilevers strong enough to allow their detachment. In this chapter, AFM cantilevers were functionalized for long-term adhesion measurements in order to detach living adherent 3T3 fibroblasts.

The cantilevers were prepared with 4 different functionalizations: concanavalin A, fibronectin, poly-L-lysine and zirconium. 3T3 mouse fibroblasts were allowed to grow for one day on a glass surface. Then the cantilevers were pressed onto the adherent fibroblasts for a given contact time and withdrawn. The functionalization showing the highest percentage of cell detachment was selected for the rest of this work.
4.1. Introduction

4.1.1. Long-term adhesion

The use of force spectroscopy permits the study of adhesion of living cells to be studied. In this thesis, “short-term adhesion” is defined as short cell-substrate contact times, from seconds to minutes, whereas “long-term adhesion” is defined as long cell-substrate contact times, from hours to days. Long-term adhesion allows the cells to form strong adhesion complexes that involves a large number of receptors bindings cooperatively. Long-term adhesion measurements characterize the cell-surface interaction because the cells can spread, react to the surface and their adhesion proteins can be renewed. That is the reason why the main objective of this work is to detach living single adherent cells in order to quantify their global adhesion forces.

Other authors have reported that single cell force spectroscopy is currently restricted to short contact times that range from milliseconds to about twenty minutes\(^{152}\). This restriction was observed using single cells captured on an AFM cantilever and the main limitation was the loss of the cell from the cantilever at prolonged cell/surface contact times i.e. exceeding one hour (Fig. 4.1). A single cell was captured on a coated AFM cantilever and allowed to settle on the cantilever for approximately fifteen minutes away from the surface. Then the cell was pressed onto the substrate for more than one hour. At long contact times, the adhesion between the cantilever and the cell was weaker than that between the substrate and the cell. Using concanavalin A to immobilize the cell on the cantilever, forces of up to 50 nN were measured before the cells detached from the cantilever. This force was, however, dependent on cell type. In addition, the authors noticed that the almost unavoidable thermal and mechanical drifts in AFM complicate those experiments that exceeded thirty minutes.

![Fig. 4.1. Schematics illustrating a cell captured on an AFM cantilever for (A) a short cell-substrate contact time and (B) a long cell-substrate contact time exceeding one hour: the interaction cell-cantilever is weaker than the interaction cell-substrate and there is a loss of the cell from the cantilever.](image)
One possible solution is to first seed the cell on the surface and then to detach it with functionalized AFM cantilever (Fig. 4.2). In this way, the cell can be incubated for the desired time on a specific surface under physiological condition without mechanical stress by the cantilever on it. In this approach, the challenge is to establish a quick adhesion between cells and AFM cantilevers capable of supporting their detachment. The establishment of the connection cell-cantilever has to be fast. This is to avoid a disturbance of the adhesion properties of the cell on the specific surface, and to limit problems related to the thermal and mechanical drifts at the system. Another advantage of seeding cells on the surface is the elimination of the use of trypsin. During the long contact time between cell and substrate, cellular adhesion proteins are renewed.

Fig. 4.2. Schematic illustrating a long-term adhesion measurement. The cell is first incubated on the surface for a long cell-substrate contact time and then detached with an AFM functionalized cantilever. The interaction cell-cantilever is stronger than the interaction cell-substrate and the cell is detached from the surface.

### 4.1.2. Cantilever functionalization for cell detachment

In order to successfully detach cells, the adhesion between cell and cantilever has to be stronger that between cell and substrate. A wide variety of molecules exposed at the cell surface such as sugars, proteins or lipids are candidates to establish this strong adhesion.

Several cantilever functionalizations have been used by other authors for cell capture. Lectin-decorated cantilevers, especially with concanavalin A and wheat germ agglutinin, have been widely used to capture several cell types. Lectins are sugar-binding proteins that interact with the glycoproteins exposed at the cell surface. Cantilevers have also been decorated with fibronectin that interacts with integrins that are the major adhesion receptors that mediate cell-substrate adhesion. Alternatively, a charge-based approach has been reported to allow capture of cells on a cantilever array (several cantilevers in parallel). The
cantilevers were functionalized with poly-L-lysine and then the cells were added in a low conductivity medium. The biotin-streptavidin system has been used to capture cells on cantilevers\textsuperscript{140,137}. Cell surface molecules were biotinylated while the cantilever was decorated with streptavidin. Finally, antibody-antigen interactions have been used for specific cell capture. Jurkat cells, an immortalized line of T lymphocytes, were coupled to AFM cantilevers using an anti-leucosialin functionalization\textsuperscript{129}. All these cantilever functionalizations have been shown to be efficient for cell capture in order to perform short-term adhesion measurements. In contrast, cantilever functionalization for long-term adhesion with cell detachment is much more demanding due to the necessity for a stronger attachment of the cell to the substrate.

In order to determine which cantilever functionalization was the most suitable for cell detachment, three different cantilever functionalizations, commonly used for cell capture, were systematically tested. The cantilever functionalizations targeted various molecules exposed at the cell surface. Concanavalin A was selected to bind sugars, fibronectin to bind proteins and poly-L-lysine to interact with charges.

In addition, a zirconium cantilever functionalization was tested to interact with the lipids of the cell membrane. Indeed, tetravalent metal ions such as Zr\textsuperscript{4+} have been shown to interact strongly with phosphates and phosphonates\textsuperscript{186}. Recently, the affinity between the metal ion and phosphates/phosphonates was exploited in the bioanalytical field\textsuperscript{187}: capillary columns\textsuperscript{188} and porous wafers\textsuperscript{189} were coated with zirconium phosphonate for phosphopeptide enrichment for mass spectroscopy. Zirconium coatings on AFM cantilevers have never been used for cell capture or cell detachment.

### 4.2. Detachment of adherent cells

#### 4.2.1. Percentage of cell detachment

Single adherent cells were detached from glass surfaces using different AFM cantilever functionalizations. 3T3 mouse fibroblasts were grown for 24 hours on a flat glass surface. The glass sample was placed in a temperature-controlled fluidic chamber and a single adherent cell was selected. The functionalized-cantilever was positioned above the cell and gently pressed on it...
until a force of 1.5 nN was attained (Fig 4.3A). The cantilever was left in contact for 30 minutes to allow the cell to interact and bind actively with the functionalized cantilever. Finally, the cantilever was withdrawn until the cell detached either from the surface or from the cantilever. For each condition, one force distance curve was obtained from each of twenty different cells. The cantilevers were coated with concanavalin A, fibronectin, poly-L-lysine or zirconium.

During this process, the forces exerted on the cell were continuously recorded and the values were represented in a force distance curve (Fig 4.3B). The first qualitative information that was obtained was the percentage of cells detached from the glass surface. The maximum force, corresponding to the maximum deflection of the cantilever, and the work of detachment were quantified for the cells successfully detached from the surface.

![Figure 4.3](image)

Fig. 4.3. (A) Schematic of the detachment of a single adherent cell and, (B) a corresponding force-distance curve. The cantilever is pressed onto the adherent cell until a preset maximal force of 1.5 nN is attained. The position of the cantilever is held constant for 30 min before it is withdrawn. A force-distance curve is recorded indicating the maximum force and work of detachment. On both panels, orange defines the approach and turquoise defines the retraction.
The percentages of cell detachment from the glass surface were between zero and seventy-five percent for all cantilever functionalizations. Fifteen percent of cells were detached using the concanavalin A-functionalized cantilevers, seventy-five percent with the fibronectin, thirty-five percent with the poly-L-lysine and zero percent with the zirconium. Native cantilevers without functionalization didn’t allow the detachment of any cells.

<table>
<thead>
<tr>
<th>Cantilever functionalization</th>
<th>Percentage of cell detachment (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>15</td>
</tr>
<tr>
<td>Fibronectin</td>
<td>75</td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>35</td>
</tr>
<tr>
<td>Zirconium</td>
<td>0</td>
</tr>
<tr>
<td>Native silicon</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4.1 Comparison of the percentages of detachment of 3T3 cells detached from glass surfaces using four cantilever functionalizations: concanavalin A, fibronectin, poly-L-lysine and zirconium.

### 4.2.2. Maximum force of detachment

The maximum forces of detachment of the cells detached with the different functionalized cantilevers were not statistically different. The maximum forces were quantified only for the cells that detached from the glass surface. The mean values for the maximum forces of detachment were 170 nN with the concanavalin A, 230 nN with the fibronectin and 190 nN with the poly-L-lysine (Fig. 4.4).

### 4.2.3. Work of detachment

The works of detachment – similarly to the maximum forces of detachment – were similar. The mean values for the work of detachment were 2.5 pJ with the concanavalin A, 3.3 pJ with the fibronectin and 2 pJ with the poly-L-lysine (Fig. 4.5). The works of detachment showed no statistically significant differences.
Fig. 4.4. Histogram showing the distribution of the maximum forces of detachment of 3T3 cells that detached from glass surface using cantilevers functionalized with concanavalin A, fibronectin and poly-L-lysine.

Fig. 4.5. Histogram showing the distribution of the works of detachment of 3T3 cells that detached from glass surface using cantilevers functionalized with concanavalin A, fibronectin and poly-L-lysine.
4.3. Discussion

4.3.1. Selection of the cantilever functionalization

The results described in the previous sections demonstrate that it is possible to detach living 3T3 fibroblasts from the surface on which they are growing after 24 hours of culture using functionalized AFM cantilevers. A functionalization of the AFM cantilever is necessary to achieve a strong adhesion between cell and cantilever.

A functionalization of fibronectin on the cantilever and 30 minutes contact between cell and cantilever provided a sufficiently strong cell/cantilever adhesion to detach seventy-five percent of 3T3 cells from glass surfaces. In contrast, a cantilever functionalization of concanavalin A allowed the detachment of only fifteen percent of cells. A cantilever functionalization of poly-L-lysine allowed an intermediate percentage of thirty-five percent. The fibronectin was found to be the best cantilever functionalization for the detachment of adherent 3T3 fibroblasts and was selected for the rest of this work.

The selection of the cantilever functionalization depends on both the cell type and the SCFS configuration. For example, fibronectin does not induce spreading in HeLa cells\textsuperscript{190} and their formation of focal adhesions is not fibronectin-dependent\textsuperscript{191}. However, while the cantilever functionalization is crucial to promote the attachment of the cell to the cantilever, it may also influence the adhesion of the cell to the substrate. For this reason, an interaction with the sugars exposed at the cell surface, instead of the adhesion proteins, may be advantageous for some SCFS configurations. A functionalization of concanavalin A was selected in the previous chapter for cell capture and short-term adhesion measurements. The interaction between the cell and the cantilever was strong enough to avoid a loss of the cell from the cantilever and probably has only a limited effect in terms of the reorganization of the cell adhesion proteins. Alternatively, a functionalization of poly-L-lysine might be more efficient to interact with another cell type. In this case, the preparation of poly-L-lysine-functionalized cantilevers would be simpler and faster.

The cantilevers coated with a thin layer of zirconium (10 nm) didn’t allow the detachment of any cells. The interaction of the cantilever with the lipids of the cell membrane was probably not possible due to the presence of other molecules at the cell surface, notably sugars. However, there are literature reports of three-dimensional vesicle matrices created using multivalent zirconium
ion linkers between the liposomes\textsuperscript{186}. For this reason, the approach seems promising for the manipulation of liposomes.

### 4.3.2. Limitation of the contact time

Good results were obtained using a fibronectin functionalization and a cell contact time of thirty minutes. During these thirty minutes of contact, cell/cantilever adhesion increases. This is presumably due to a reorganization of both the focal adhesion proteins exposed at the cell surface and the cytoskeleton. This reorganization is one limitation of this functionalization because it will inevitably modify the adhesion of the cell to the substrate. Thus longer cell/cantilever contact times will result in stronger cell/cantilever interactions but will increasingly modify the cell/substrate adhesion measured. For this reason, the preparatory cell/cantilever contact time was kept to the minimum necessary to successfully detach a reasonable proportion of cells from the culture surface.

### 4.3.3. The effect of cantilever functionalization on cell adhesion

During the thirty minutes of contact between the cell and the AFM cantilever, the functionalization of the cantilever may also influence the adhesion of the cell to the substrate. For this reason, the detachment forces from glass surfaces were compared using different cantilever functionalizations to observe possible changes to cell adhesion caused by fibronectin.

The quantification of the global cell adhesion between the 3T3 cells and the glass surface was similar for all measurements independently of the cantilever functionalization (Table 4.2). The mean maximum forces of detachment were between 180 and 230 nN with no statistically significant differences for the different functionalizations. The mean works of detachment were between 2 and 3.3 pJ with no statistically significant differences. While some differences are observed in the maximum forces and works of detachment, the variations are less than a factor of two and the lowest values are not associated with the fibronectin. Any effects of the cantilever functionalization on cell-surface interaction after 30 minutes contact were too small to be detected.
### Table 4.2 Comparison of the maximum forces and works of detachment of 3T3 cells detached from glass surface using four cantilever functionalizations: concanavalin A, fibronectin, poly-L-lysine and zirconium.

<table>
<thead>
<tr>
<th>Cantilever functionalization</th>
<th>Maximum force of detachment (nN)</th>
<th>Mean</th>
<th>±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concanavalin A</td>
<td>180</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>230</td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>190</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Zirconium</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Native silicon</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Work of detachment (pJ)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>±SE</td>
<td>Mean</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>2.5</td>
<td>0.8</td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>3.3</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Poly-L-lysine</td>
<td>2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Zirconium</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>Native silicon</td>
<td>–</td>
<td>–</td>
<td></td>
</tr>
</tbody>
</table>

The acquisition of a sufficiently large data set for a statistically significant analysis of cell/surface adhesion forces was difficult under these experimental conditions. Force distance curves from twenty separate cells were used for each condition. Only three cells detached from the surface using cantilevers functionalized with concanavalin A, so only three values (15% of cell detachment) were available for analysis. The standard error was taken as characteristic of the variance in order to consider the sample size because it estimates the standard deviation of the sample mean of a population mean. The three values obtained for the two mechanical parameters of detachment using concanavalin A-functionalized cantilevers are probably not representative of a population mean. However, the results seem to indicate similar detachment forces from the glass surface for the three cells that were detached.

## 4.4. Conclusion

Force spectroscopy can be used to detach single adherent mouse fibroblasts from glass surfaces. The necessary strong adhesion between cell and cantilever can be obtained by functionalizing the cantilever with fibronectin. Fibronectin is recognized by integrins that mediate cell-surface interactions and therefore promotes cell attachment. Another advantage is that the integrins transmit the traction forces directly to the cytoskeleton. Measurements of the maximum forces and works of 3T3 cells detached from their growing substrate were used to investigate the influence of the cantilever functionalization on the adhesion of cells to surfaces. Fibroblast adhesion was shown to be similar on glass surfaces independently
of the cantilever functionalization. However, incomplete sample detachments led to less precise characterizations of cell detachment forces.

The contact time between cell and cantilever was minimized to thirty minutes to avoid a major reorganization of the focal adhesion proteins binding the cells on their growing substrate. Fibronectin-functionalized cantilevers and a contact time of thirty minutes were used to detach adherent cells for the rest of this work.
In this chapter, the detachment of living adherent cells by force spectroscopy was tested on a number of chemically functionalized surfaces in order to validate the technique. The objective is to determine if long-term adhesion measurements are useful in the development of surfaces for tissue engineering. The adhesion of living fibroblasts was studied twenty-four hours after seeding. The cantilevers were functionalized for cell detachment in long-term adhesion measurements.

The cells were grown on glass surfaces as well as on surfaces used for cell sheet engineering: glass surfaces coated with polyelectrolyte multilayers (of poly-L-lysine and hyaluronic acid) and PNIPAM films. Individual adherent cells were detached from their culture substrate using an AFM cantilever coated with fibronectin. The maximum forces of detachment of each cell were measured and taken as characteristic of the cellular adhesion.

‡Part of this chapter was included in Quantification and characterization of single cell adhesion on functionalized surfaces for cell sheet engineering. Weder G., Guillaume-Gentil O., Matthey N., Montagne F., Heinzelmann H., Vörös J. and Liley M. (submitted to Biomaterials).
5.1. Introduction

5.1.1. Tissue engineering

Tissue engineering\(^{35}\) is a multidisciplinary field involving the development of biological substitutes and/or the fostering of tissue remodelling with the purpose of repairing or enhancing tissue function\(^{192}\). Tissue engineering includes biomaterials\(^{193}\) that are designed to direct cells in the formation of functional tissue by providing both physical and chemical cues. Finally, engineering design aspects are very important for two-dimensional cell expansion and three-dimensional tissue growth.

Cells are often seeded into an artificial structure capable of supporting three-dimensional tissue formation. These structures, typically called scaffolds\(^{194}\), serve as a synthetic extracellular matrix. They often mimic the in vivo environment and allow the cells to influence their own microenvironment. Common scaffolds use natural biopolymers or synthetic polymers. The most commonly natural biopolymers include demineralised bone matrix\(^{195}\), agarose\(^{196}\), collagen\(^{197}\), hyaluronan\(^{198}\) and alginate\(^{199}\). Synthetic polymers use mainly polyglycolic acid, polylactic acid and their copolymers that are hydrolytically degradable polymers\(^{200,201,202}\). As an alternative, a variety of hydrogels, a class of highly hydrated polymer materials, are also employed as scaffold materials\(^{203,204}\).

These artificial scaffolds have been used in various tissue engineering applications\(^{205}\) such as fractures repairing\(^{206,207}\). Ideally, they should not only promote cell adhesion, growth, and revascularization but also be biodegradable without toxic and inflammatory responses. The synchronization of the rate of new tissue formation with the rate of scaffold degradation has not been totally solved\(^{208,209,210}\). In order to avoid some of these limitations of tissue reconstruction using biodegradable scaffolds, Okano et al. developed “cell sheet engineering”\(^{211}\). This concept is based on the use of sheets of cells for tissue reconstruction instead of individual cells.
5.1.2. Cell sheet engineering

Cell sheet engineering is based on the control of cell adhesion, so that intact sheets of confluent cells may be recovered with minimal damage to cell structure and function. Clinical applications include the repair of ocular trauma by corneal epithelial cell sheet transplantation\textsuperscript{212,213}. Similarly, multilayered cell sheets have been used in cardiac tissue regeneration in rats\textsuperscript{214}.

Various polymers and copolymers of poly(N-isopropylacrylamide) (PNIPAM) have been used as coatings to control cell-surface adhesion\textsuperscript{215,216,217,211,218}. These thermally-actuated polymers show a phase transition between a collapsed state above their lower critical solution temperature (LCST) and an extended, highly hydrated state below their LCST. Above the LCST, which is typically around 32 °C, cells adhere, spread and proliferate on a PNIPAM-coated surface, while below the LCST, cells detach spontaneously from the surface. These coatings have been successfully applied to produce many types of cell sheets including oral mucosal epithelial\textsuperscript{212}, aortic endothelial\textsuperscript{219}, urothelial\textsuperscript{220} as well as periodontal ligament cells\textsuperscript{221}, cardiomyocytes\textsuperscript{222} and keratinocytes\textsuperscript{223}.

Polyelectrolyte multilayer films are also being developed, as an alternative to PNIPAM coatings, to control the adhesion of several cell types\textsuperscript{224,225,226,227} including human mesenchymal stem cells\textsuperscript{228}. Polyelectrolyte layers are built up by adsorbing polycations onto oppositely charged surfaces via electrostatic interactions\textsuperscript{229,230}. Multilayered polyelectrolyte films have been shown to desorb from conductive substrates on the application of an electrical potential\textsuperscript{231,232}. The first step in the preparation of cell sheets consist of growing cells to confluence onto polyelectrolyte thin films. The cell sheets then detach either spontaneously or under electrical control\textsuperscript{233}. Electrical desorption has been shown to be effective for several cell types. In contrast, it has been postulated that the spontaneous detachment requires weak adhesion to the substrate and high cell/cell cohesion.

An extensive literature on these two polymer-based coatings documents their properties. However, to date, there have been no quantitative investigations of their influence on cell adhesion.
5.2. Long-term adhesion to glass at 37 °C and 27 °C

Direct cell adhesion measurements were carried out by detaching single adherent fibroblasts from glass surfaces using an atomic force microscope and a temperature-controlled fluidic chamber. 3T3 cells were grown for 24 hours on a glass surface. The glass sample was placed in the fluidic chamber and a single adherent cell was selected. To ensure that the interaction between cell and surface was similar for all measurements, cells of comparable size were selected for each condition. The extremity of a fibronectin-decorated cantilever was precisely positioned above the targeted cell (Fig. 5.1A). The cantilever was gently pressed onto the single adherent cell with a predefined contact force of 1.5 nN for 30 min at constant height to allow the cell to bind to the cantilever. Finally the cantilever was withdrawn at 0.5 µm/s until the cell detached either from the surface or from the cantilever. The viability of the cell after detachment was assessed by visually observing its active re-spreading on the cantilever (Fig. 5.1B). For each condition, twenty different cells were probed. Each time a freshly prepared cantilever and new sample of cells were used.

The force exerted on the cell during this process was continuously recorded. These forces are typically displayed as a function of the distance between cantilever tip and surface in a force distance curve (Fig. 5.1C). The maximum force corresponds to the maximal deflection of the cantilever. There were two possible outcomes of the cell detachment experiments; detachment of the cell from the glass surface occurred when the adhesion between cell and cantilever was stronger than that between cell and substrate. In contrast, when the adhesion between cell and glass surface was stronger, the cell detached from the cantilever. The percentage of cell detachment gave qualitative information about the cell/surface interaction. The maximum force of detachment was evaluated separately for experiments where the cell detached from the surface and those where it did not.
Detachment experiments on glass were carried out at both 37 °C and 27 °C. Two temperatures were necessary in order to have a direct comparison with measurements on PNIPAM coatings above and below the LCST and to exclude any possible effects of a physiological response of the cells to the change in temperature.

In the detachment experiments carried out at 37 °C, seventy-five percent of the cells were detached from the glass surface whereas twenty-five percent of the cells were left on the surface. No qualitative differences were observed between force-distance curves where cells detached and those where the cells remained on the glass surface (Fig. 5.2).
Fig. 5.2. Typical force-distance curves showing the detachments of single adherent fibroblasts either from a glass surface (green and blue) or from the cantilever (red and black) at 37 °C using a fibronectin-decorated cantilever.

The mean values of the maximum forces of detachment were 230 nN for the cells detached from the surface and 150 nN for the cells detached from the cantilever (Fig. 3 and Table 1). Surprisingly, no statistically significant differences were observed between cells detaching from the glass surface or from the cantilever (two sample T-test, p-value 0.05).

The maximum forces of detachment of the cells exposed to a temperature of 27 °C for 1 h were not statistically different from those of the cells continuously incubated at 37 °C. The mean maximum force of detachment from glass at 27 °C was 200 nN (Table 1), while the mean maximum force of detachment from the cantilever was 170 nN. The maximum forces of detachment from the glass surface and from the cantilever were not statistically different. Eighty percent of the cells detached from the surface and twenty percent from the cantilever. Any possible effect of temperature was too small to be observed in this study.
Fig. 5.3. Histogram showing the distribution of the maximum forces of detachment of single adherent 3T3 cells either from the glass surface (red) or from the cantilever (blue) at 37 °C.

Table 5.1 Comparison of the maximum forces of detachment of mouse fibroblasts on glass at 37 °C and 27 °C (SE: standard error). Data were evaluated using the two sample T-test and the limit of significance was fixed at a p-value of 0.05. The values ≥0.05 indicate that the conditions are not significantly different.
5.3. Cell adhesion on polyelectrolytes at 37 °C

5.3.1. Adhesion forces

Multilayer polyelectrolyte coatings were prepared on glass by alternately spraying dilute solutions of the polycation poly(L-lysine) (PLL) and the polyanion hyaluronic acid (HA). Two coating types were tested: coatings with one layer of each polyelectrolyte (PLL/HA)\textsubscript{1} and coatings with nine layers of each polyelectrolyte (PLL/HA)\textsubscript{9}.

Detachment experiments were carried out on adherent 3T3 cells on the polyelectrolyte surfaces at 37 °C as described for glass. The results of the cell detachments from the surface were similar for (PLL/HA)\textsubscript{1} to those obtained on glass: sixty percent of the cells detached from (PLL/HA)\textsubscript{1}. The mean value of the maximum force of detachment was 200 nN (Fig. 5.4 and table 5.2). The two-sample T-test showed no statistically significant differences between cell adhesion on glass or (PLL/HA)\textsubscript{1}. Additionally, no statistically significant differences were observed between the mean values of the maximum forces of detachment from the (PLL/HA)\textsubscript{1} surface or from the cantilever.

Fig. 5.4. Histogram showing the distribution of the maximum forces of detachment of single adherent 3T3 cells on polyelectrolytes films at 37 °C: (PLL/HA)\textsubscript{1} in red and (PLL/HA)\textsubscript{9} in blue. The cells detach either from the surface (dark color) or from the cantilever (light color). All the cells detach from the surface on (PLL/HA)\textsubscript{9}.
In contrast, the results obtained on (PLL/HA)$_9$ were very different: All the adherent cells were detached from the surface. The mean maximum force of detachment was 100 nN – approximately half of the value observed on glass or on (PLL/HA)$_1$ (Fig. 5.4 and table 5.2).

<table>
<thead>
<tr>
<th></th>
<th>Mean (nN)</th>
<th>±SE</th>
<th>Two sample T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass 37°C</td>
<td>From surface</td>
<td>200</td>
<td>22</td>
</tr>
<tr>
<td>(PLL/HA)$_1$</td>
<td>From surface</td>
<td>220</td>
<td>53</td>
</tr>
<tr>
<td>(PLL/HA)$_9$</td>
<td>From surface</td>
<td>100</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 5.2 Comparison of the maximum forces of detachment of mouse fibroblasts on glass at 37 °C and 27 °C (SE: standard error). Data were evaluated using the two sample T-test and the limit of significance was fixed at a p-value of 0.05. Values ≥0.05 indicate that the conditions are not significantly different.

5.3.2. Characterization of PLL/HA substrates*

The buildup of (PLL/HA)$_n$ multilayers is characterized by the initial formation of isolated polyelectrolyte complexes that grow by addition of new polyelectrolyte layers and by mutual coalescence of the complexes$^{234}$. The growth and coalescence of these complexes finally leads to the formation of continuous films, and the number of layer pair depositions required to reach a continuous coverage of the substrate depends highly on the working conditions. Figure 5 shows the morphologies of the (PLL/HA) coatings used in this study, which both consisted of randomly distributed polyelectrolyte complexes. The deposition of (PLL/HA)$_1$ yielded nano-droplets covering less than 1% of the substrate, with heights ranging from 5 to 20 nm. (Fig. 5.5B) The surface modification with (PLL/HA)$_9$ resulted in polyelectrolyte complexes covering more than 50% of the substrate, with droplet heights around 100 nm in average. (Fig. 5.5C)

*AFM characterization of the PLL/HA layers was carried out by O. Guillaume-Gentil at ETH Zurich (Switzerland).
Fig. 5.5. Characterization of PLL/HA modified substrates by atomic force microscopy. (A) Unmodified glass substrate. (B) (PLL/HA)$_1$ on glass. (C) (PLL/HA)$_9$ on glass. The graphs show one representative profile for each AFM image.

5.3.3. Discussion of polyelectrolyte coated surfaces

Cell adhesion on (PLL/HA) multilayers has been shown to be relatively low$^{233,235}$. In general, polyelectrolyte multilayers based on biopolymers or polypeptides like PLL/HA are considered soft, highly hydrated materials, and it has been shown that their cell adhesive properties relate mainly to their hydration degree and viscoelastic properties, regardless of serum protein adsorption$^{236}$. As such, one can consider that (PLL/HA)$_1$ coatings do not significantly affect the hydration and stiffness of the glass substrate due to the low amount of adsorbed polyelectrolytes and the low surface coverage. However, (PLL/HA)$_9$ coatings yielded bigger complexes and higher surface coverage, significantly affecting the substrate properties, and consequently its cell adhesive properties.
5.4. Cell adhesion on PNIPAM at 37 °C & 27 °C

5.4.1. Principle

The second surface chemistry investigated was a reactive PNIPAM layer. This polymer exhibits a lower critical solution temperature (LCST) in aqueous solution at approximately 32 °C. Above the LCST, the PNIPAM layer is present in a compact relatively hydrophobic form, while below the LCST the layer changes to an expanded, swollen and hydrophilic state.

The PNIPAM layers were produced by grafting PNIPAM chains to a thin layer of gold on glass substrates using a self-assembled monolayer (SAM-COOH) of thiols (HS(CH₂)₁₁CO₂H) as an intermediate coupling agent. The amine-terminated PNIPAM was spin-coated onto the reactive SAM of thiols and the substrate was then annealed above the glass transition temperature to enable the amine groups to react with the SAM-COOH.

5.4.2. Adhesion forces

Cell detachment experiments were carried out on adherent 3T3 cells incubated for 24 hours at 37 °C on the PNIPAM-functionalized surfaces as described for the glass and polyelectrolyte substrates. The results obtained for PNIPAM at 37 °C were similar to those obtained on glass and on (PLL/HA)₁. Sixty percent of the cells detached from PNIPAM at 37 °C. The mean value of the maximum force of detachment was 270 nN (Fig. 5.6 and table 5.3). No statistically significant differences were observed in the maximum forces of detachment for the cells detached from the PNIPAM surface or from the cantilever.

Cell detachment experiments were also carried out at 27 °C. At the start of the experiment the temperature was reduced to 27 °C for 20 minutes during which time the PNIPAM layer changed from a compact to an extended configuration. After twenty minutes, the cells showed a retracted morphology but were still firmly attached to the surface (Fig. 5.7). The temperature was maintained at 27 °C for the rest of the detachment experiment, which was carried out as described for glass and polyelectrolytes. Cell adhesion was dramatically reduced at 27 °C. All the cells were released from the surface. The mean maximum force of detachment was 10 nN (Table 5.3).
Fig. 5.6. Histogram showing the distribution of the maximum forces of detachment of single adherent 3T3 cells on PNIPAM at 37 °C in red and at 27 °C in blue. The cells detach either from the surface (dark color) or from the cantilever (light color). All the cells detach from the PNIPAM surface at 27 °C.

Fig. 5.7. Schematic and optical images showing the morphological response of single adherent 3T3 cells on thermo-responsive 100%-PNIPAM layers (A) at 37 °C and (B) after cooling the sample at 27 °C for 20 min. 100%-PNIPAM induces a retraction of the cell at 27 °C.
### Table 5.3 Comparison of the maximum forces of detachment of mouse fibroblasts on 100%-PNIPAM and glass at 37 °C and 27 °C (SE: standard error). Data were evaluated using the two sample T-test and the limit of significance was fixed at a p-value of 0.05. Values ≥0.05 indicate that the conditions are not significantly different whereas values <0.05 indicate that the conditions are significantly different.

<table>
<thead>
<tr>
<th></th>
<th>PNIPAM 100% 37°C</th>
<th>Glass 37°C</th>
<th>Glass 27°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>From surface</td>
<td>270 ± 50</td>
<td>0.3829</td>
<td>0.2368</td>
</tr>
<tr>
<td>From cantilever</td>
<td>350 ± 83</td>
<td>0.4302</td>
<td></td>
</tr>
<tr>
<td>PNIPAM 100% 27°C</td>
<td>10 ± 3</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

#### 5.4.3. Discussion of PNIPAM functionalized surfaces

The study of PNIPAM layers showed a remarkable switching behavior between the adhesive state at 37 °C and the non-adhesive state at 27 °C. While this behavior has been extensively studied and investigated in past years, this is the first study to quantify these changes in adhesion. The mean maximum force of detachment at 27 °C was around 10 nN indicating a reduction in cell adhesion of more than an order of magnitude between the adhesive and non-adhesive states.

#### 5.5. Controlling cell adhesion using a patterned PNIPAM coating

##### 5.5.1. Principle

A patterned PNIPAM coating was used to control cell adhesion by locally detaching the cell from the surface on lowering the temperature at 27 °C. The patterned film was obtained using the grafting method previously described except that the uniform layer of gold was replaced by a patterned gold layer. The gold layer was composed of squares of 3 x 3 µm, and covered approximately 30% of the glass substrate (Fig. 5.8).
5.5.2. Adhesion forces

Cell adhesion measurements on PNIPAM-patterned glass surfaces were performed using the experimental procedure described previously. The results obtained for PNIPAM-patterned glass at 37 °C were similar to those obtained on plain glass. Eighty-five percent of cells detached from PNIPAM-patterned glass at 37 °C. The mean value of the maximum force of detachment was 240 nN (Fig. 5.9 and table 5.4).

The results obtained at 27 °C were different from those obtained at 37 °C. Cooling the sample to 27 °C for 1 h did not alter the morphology of the cells (Fig. 5.10). Ninety percent of cells detached from PNIPAM-patterned glass at 27 °C. The mean value of the maximum force of detachment was 110 nN (Table 5.4). A statistical comparison of the maximum forces of detachment showed a clear difference (p-value 0.0007).
Fig. 5.9. Histogram showing the distribution of the maximum forces of detachment of single adherent 3T3 cells on PNIPAM-patterned glass at 37 °C in red and at 27 °C in blue. The cells detach either from the surface (dark color) or from the cantilever (light color).

Fig. 5.10. Schematic and optical images showing the morphological response of single adherent 3T3 cells on thermo-responsive 33%-PNIPAM layers (A) at 37 °C and, (B) after cooling the sample at 27 °C for 20 min. 33%-PNIPAM does not induce any major morphological modifications of the cell at 27 °C.
<table>
<thead>
<tr>
<th></th>
<th>Mean (nN) ±SE</th>
<th>Two sample T-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PNIPAM 33% 37°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From surface</td>
<td>240* 35</td>
<td>0.6419 0.4546</td>
</tr>
<tr>
<td><strong>PNIPAM 33% 27°C</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>From surface</td>
<td>110** 19</td>
<td>0.0007 0.0035</td>
</tr>
</tbody>
</table>

Table 5.4 Comparison of the maximum forces of detachment of mouse fibroblasts on 33%-PNIPAM and glass at 37 °C and 27 °C (SE: standard error). Data were evaluated using the two sample T-test and the limit of significance was fixed at a p-value of 0.05. Values ≥0.05 indicate that the conditions are not significantly different whereas values <0.05 indicate that the conditions are significantly different.

*This value was calculated for the 17 cells that detached from the surface (85% of cell detachment)

**This value was calculated for the 18 cells that detached from the surface (90% of cell detachment)

5.5.3. Discussion of PNIPAM patterned surfaces

A patterned glass/PNIPAM surface has been also studied. The glass adhesive substrate was covered with an array of small gold squares functionalized with PNIPAM. The array was chosen so that each adherent cell covered on average 29 squares and one third of the glass surface was covered with the gold-PNIPAM coating. A reduction in temperature to 27 °C should therefore result in a local de-adhesion of the cell at each of the 3 x 3 micron squares of PNIPAM under the cell, while leaving the gross morphology of the cell and, to a large extent, the cytoskeleton unchanged. Ideally this would reduce the maximum detachment force of the cell by one third, so that highly adherent cells could be detached using fibronectin-coated AFM cantilevers.

The results reported here show that the 3T3 cells used in this study proliferate well on the patterned PNIPAM/glass substrates and have similar morphologies to those grown on plain glass surfaces. In addition, at 37 °C, cellular adhesion to the PNIPAM/glass surfaces is indistinguishable from that on plain glass. When the temperature is reduced to 27 °C, there is no observable change in cellular morphology, but there is a clear and significant reduction in mean detachment forces from 240 nN to 110 nN.
5.6. Discussion

5.6.1. Detachment of adherent cells

The maximum force needed to detach a cell from the culture surface was measured separately for each individual cell and was taken as characteristic of the adhesion of the cell. On most surfaces, a very broad distribution of results was obtained, indicating a large heterogeneity in the attachment of the cells. Despite the wide distribution of individual values, statistically significant results were obtained. One notable exception was the (PLL/HA)$_9$ surface which had a strikingly narrow distribution of cell adhesions.

The results for cells detaching from the culture surface or from the cantilever were evaluated independently. Surprisingly, similar distributions of adhesion strengths were observed between cell/culture surface and the cell/cantilever for most of the culture surfaces. This can clearly be seen from the percentage detachments of the cells from the culture surfaces (60-85% of the cells detached). Only on the two substrates with much lower maximum detachment forces – the (PLL/HA)$_9$ and the PNIPAM 100% (27 °C) surfaces – was it possible to detach all the cells from the culture surfaces. These results suggest that the adhesion between cell and cantilever also varies widely. Thus the exact percentage of cells detaching from the culture surface and the mean maximum force obtained for a population of cells depends, for most of the surfaces studied here, on the interplay of the two distributions: cell/surface adhesion and cell/cantilever adhesion. Moreover, small changes in experimental parameters such as the cell/cantilever contact force or the retraction speed, may have a strong influence on the percentage of cells that detach from the quartz surface.

It was possible to quantify cell adhesion on glass, PNIPAM and (PLL/HA) polyelectrolyte surfaces as well as on mixed glass/PNIPAM surfaces at 37 °C and 27 °C. Very similar mean maximum detachment forces (200 – 270 nN) were observed on glass (at both 27 °C and 37 °C), PNIPAM (100% and 33%) at 37 °C and on (PLL/HA)$_1$ surfaces: all substrates on which the 3T3 cells studied proliferate well. On (PLL/HA)$_9$ a very different cellular adhesion was observed, with a much lower maximum detachment force of 100nN. This reduction in cellular adhesion associated with an increase in the number of PLL/HA layers has been documented qualitatively in the literature$^{237,235}$. It should therefore be possible to correlate cell detachment forces with the
number of (PLL/HA) layers as well as with initial cellular proliferation and the later detachment of confluent cell sheets.

5.6.2. Limitation of the sample size

The acquisition of a sufficiently large data set for a statistically significant analysis of cell behavior is one of the major limitations of the technique. Single cell force spectroscopy is currently a laborious technique because each cell must be individually bound to a clean freshly-mounted AFM cantilever. Similarly each cell/substrate sample must be tested at the same time after cell seeding (24 hours in this case). Force distance curves from twenty separate cells were used for each experimental condition. In our hands, data from two separate cells could be acquired per day per atomic force microscope under ideal circumstances.

A significant reduction in the standard errors associated with the means of the different force distributions would require the number of measurements to be increased by a factor of at least four. In future studies, an increased cell/cantilever adhesion – giving detachment of all cells from all surfaces - might allow a more precise characterization of cell detachment forces: a slightly larger sample size would be available and any skew in the distributions due to incomplete sample detachment would be eliminated.

5.7. Conclusion

Cell adhesion was quantified on a number of different chemically functionalized surfaces: plain glass, glass coated with polyelectrolyte layers (PLL and HA), homogeneous PNIPAM films and patterned surfaces with areas of PNIPAM and others of plain glass. On several surfaces, measurements of cell adhesion were performed at both 37 °C and 27 °C. For most of the cell culture surfaces very similar values were obtained for the maximum forces of detachment; for glass at both 37 °C and 27 °C, 100% or patterned PNIPAM surfaces at 37 °C and for (PLL/HA)₁ surfaces the mean maximum detachment forces were between 200 and 270 nN. The measurements of cell adhesion on glass showed that a reduction of the temperature from 37 °C to 27 °C did not induce significant changes in cell adhesion due to physiological changes in the cell.

Very different results were observed on (PLL/HA)₉ layers, where mean maximum detachment forces were 100 nN. This reduced level of cell adhesion is clearly sufficient to allow good initial
cell proliferation followed, according to literature reports, by detachment of confluent cell sheets. Reducing the temperature of the homogeneous PNIPAM layers to below the LCST reduced the cell adhesion by a factor of more than one order of magnitude. This effect has been extensively studied and reported in the literature. However, this is the first time, to our knowledge, that the effect has been measured quantitatively.

Finally, preliminary results on patterned glass/PNIPAM surfaces, showed that these substrates can be used to reduce cell surface adhesion – in this case by a factor of two - while keeping cell morphology unchanged. It is hoped that these substrates may be used to investigate the adhesion of highly adherent cells to surfaces in a semi-quantitative way.

Single cell force spectroscopy was used to study the effects of surface modifications on cell adhesion. These effects of modified surfaces on cell adhesion were quantified. Force spectroscopy allows unrivalled spatial and temporal control of cells, as well as highly quantitative force actuation and measurement under physiological condition. Force spectroscopy is a suitable tool for the development of surfaces in cell sheet engineering.
In this chapter, the potential and limitations of force spectroscopy to detach adherent cells were explored by altering the cell type and the topography of the surface. The adhesion of living immortalized and primary fibroblasts to their culture substrate was studied twenty-four hours after seeding.

The cantilevers were functionalized for cell detachment in long-term adhesion measurements.

Both primary fibroblasts and a continuous cell line were studied on flat and topographically structured quartz surfaces. Individual cells were detached using an AFM cantilever, pressed onto the cell for thirty minutes and then withdrawn. The force-distance curves obtained allowed the maximum force, work and distance of detachment to be determined.

6.1. Introduction

6.1.1. Topography and cell adhesion

Adherence-dependent cells are profoundly influenced by the topography of the substrate to which they adhere. Cellular responses such as adhesion, morphology, alignment, guidance, gene expression and proliferation are all influenced by topography\textsuperscript{238,239,240,241,242,243}. The effects may differ widely depending on the dimensions and form of the topography\textsuperscript{244}.

Brunette \textit{et al.} divided cells into “rugophobic” cells, such as fibroblasts, that prefer smooth surfaces over rough ones, and “rugophilic” cells, such as osteoblasts\textsuperscript{245,246}. In the last ten years, it has been shown that the rugophobia/rugophilia classification depends both on the dimensions of the topography and on the cell type. A large and sometimes contradictory literature on this topic seems to indicate a number of other poorly-controlled factors that influence cell response to topography.

Taking the example of fibroblasts, many studies have shown that surface nanotopography affects fibroblast response, with both increased\textsuperscript{247,244} and decreased\textsuperscript{248} adhesion observed. Nanometric islands have been reported both to speed up initial cell adhesion\textsuperscript{249} and to have no influence at all when compared with planar samples\textsuperscript{250}. Numerous studies have shown a reduction in cellular adhesion and a decrease in spreading when fibroblasts are grown on sub-micron topographies\textsuperscript{251} such as islands\textsuperscript{252}, columns\textsuperscript{253} or pits\textsuperscript{254}. In contrast, microtopography has been used to transform non-adhesive into adhesive substrates for fibroblasts\textsuperscript{255} by increasing cellular adhesion.

While the effects of nano-sized topography on fibroblast adhesion remain unclear, most authors report that sub-micron topographies inhibit fibroblast adhesion and result in cell rounding.

6.1.2. Topography and cell type

There are certainly several reasons why studies of cell/surface adhesion are so confused. In many studies, surface chemistry is poorly controlled, and experimental parameters vary widely between different research groups. However, the effects of topography on cell adhesion may also differ widely depending on the cell type\textsuperscript{256}. Immortalized and primary cells from the same tissue origin have been shown both to exhibit distinct cell morphologies and mechanical properties\textsuperscript{179}. The effects of surface topography on cell adhesion can be very different for different continuous cell
lines and different from those of primary cells\textsuperscript{257}. Although the effects of surface topography on cell adhesion were demonstrated to depend on the cell type, there is a lack of suitable methods to quantify cell/surface interactions.

Here, force spectroscopy is used to directly measure the adhesion of two cell types to their culture substrates. The adhesion of immortalized and primary fibroblasts to polylysine-treated quartz surfaces after 24 hours of growth \textit{in situ} was first quantified. It was then compared with the adhesion on identical quartz surfaces that have been structured with a sub-micron pillar motif.

### 6.1.3. Description of the sub-micron topography

Two different sub-micron topographies on quartz surfaces were tested. Each consisted of quartz pillars with an average diameter of 400 nm and an approximate surface coverage of 30 \%. In one case the pillars were 270 nm high and in the other 520 nm high. Both topographies are shown in figure 6.1. The wide distribution of pillar diameters is due to the use of a polymer blend mask in the fabrication process. Differences in the shape of the pillars are due to differences in the fabrication process and particularly the lift-off step. After fabrication the samples were thoroughly cleaned to ensure a controlled and reproducible surface chemistry. All traces of chromium and amorphous silicon used as etch masks were removed using a chrome etchant and potassium hydroxide solution. Possible fluorine traces from the dry etching step were removed by an oxygen plasma.

![Fig. 6.1. Scanning electron microscopy pictures of sub-micron pillars in quartz with a height of 270 nm (A), 520 nm (B) and a flat control surface (C).](image)
6.2. **Long-term adhesion to flat quartz**

Cell adhesion was measured directly by detaching single adherent 3T3 fibroblasts from quartz surfaces coated with poly-L-lysine. The forces exerted on the cell during this process were continuously recorded and the values were represented in a force-distance curve. Each force-distance curve consisted of one peak independently of whether or not the cell detached from the quartz surface. Three mechanical parameters were extracted from each curve i.e. the maximum force, the work and distance of detachment.

The percentage of cells detached from the surface gives the first qualitative information about the relative adhesion forces. Seventy percent of cells detached from the flat quartz. The maximum force of detachment was evaluated separately for experiments where the cell was detached from the surface and those where it did not. No significant statistical differences were observed between cells detaching from the quartz surface and from the cantilever (two sample T-test, p-value 0.05) (Fig. 6.2). The mean values of the maximum forces of detachment were 420 nN for the cells detached from the surface and 310 nN for the cells detached from the cantilever (Table 6.1B).

![Fig. 6.2. Histogram showing the mean values of the maximum forces of detachment obtained for the detachments from the surface (grey) and from the cantilever (black). Vertical lines indicate the means and the error bar is the standard deviation.](image)
The mean values for the work of detachment were 7.9 pJ for the cells detached from the surface and 6.9 pJ for the cells detached from the cantilever (Table 6.1C). Again no significant statistical differences were observed between the two populations.

The distance of retraction from the surface required to totally detach the cell was also analyzed. The mean values of the distance of detachment were 33 µm for the detachments from the substrate and 38 µm for the detachments from the cantilever (Table 6.1D). The two-sample T-test showed no significant difference between the two populations.

6.3. Effect of sub-micron topography

The percentage of cells detached from the quartz surfaces was greatly increased on both pillar topographies. The percentage of cell detachment was 95 % on the 270 nm pillars and 100 % on the 520 nm pillars. The maximum forces of detachment were dramatically reduced on the microstructures, with mean values of 210 nN on the 270 nm pillars and 180 nN on the 520 nm pillars (Table 6.1B). Wide dispersions in the force values were observed on both flat and structured surfaces reflecting the heterogeneity of the cell adhesion properties within the same population (Fig. 6.3).

Similarly, the work of detachment was reduced on the pillars. The mean values on the 270 nm and 520 nm pillars were 2.6 pJ and 2.7 pJ (Fig. 6.4 and Table 6.1C). The analysis of the distance of detachment showed that shorter distances were required to totally detach the cells from the pillars. The values were 22 µm on both 270 nm and 520 nm pillars (Table 6.1D). No statistically significant differences were observed between the 270nm and 520nm pillars.
Chapter 6 Effect of sub-micron topography on cell adhesion

Fig. 6.3. Histogram showing the distribution of the maximum forces of detachment of single adherent 3T3 cells grown for 24 h on quartz surfaces: Flat (grey), 270 nm pillars (white) and 520 nm pillars (black).

Fig. 6.4. Histogram showing the distribution of the works of detachment of single adherent 3T3 cells grown for 24 h on quartz surfaces: Flat (grey), 270 nm pillars (white) and 520 nm pillars (black).
Table 6.1 Comparison of the percentages of cell detachment and the mean values of the maximum force of detachment, the work of detachment, and the distance of detachment of 3T3 cells on three quartz surfaces: flat, 270 nm pillars and 520 nm pillars (SE: standard error). *These values were calculated for the 19 cells that detached from the quartz surface (95 % of cell detachment).

### A. Percentage of cell detachment (%)

<table>
<thead>
<tr>
<th>Surface</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz flat</td>
<td>70</td>
</tr>
<tr>
<td>270 nm pillars</td>
<td>95</td>
</tr>
<tr>
<td>520 nm pillars</td>
<td>100</td>
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</tbody>
</table>

### B. Maximum force of detachment (nN)

<table>
<thead>
<tr>
<th>Surface</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz flat From the surface</td>
<td>420 ±37</td>
</tr>
<tr>
<td>From the cantilever</td>
<td>310 ±50</td>
</tr>
<tr>
<td>270 nm pillars</td>
<td>210 ±36</td>
</tr>
<tr>
<td>520 nm pillars</td>
<td>180 ±29</td>
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</table>

### C. Work of detachment (pJ)

<table>
<thead>
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<th>Surface</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
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</tr>
<tr>
<td>From the cantilever</td>
<td>6.9 ±1.7</td>
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<tr>
<td>270 nm pillars</td>
<td>2.6 ±0.7</td>
</tr>
<tr>
<td>520 nm pillars</td>
<td>2.7 ±0.7</td>
</tr>
</tbody>
</table>

### D. Distance of detachment (µm)

<table>
<thead>
<tr>
<th>Surface</th>
<th>Mean ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartz flat From the surface</td>
<td>33 ±3</td>
</tr>
<tr>
<td>From the cantilever</td>
<td>38 ±8</td>
</tr>
<tr>
<td>270 nm pillars</td>
<td>22 ±2</td>
</tr>
<tr>
<td>520 nm pillars</td>
<td>22 ±2</td>
</tr>
</tbody>
</table>

6.4. **Comparison with primary mouse fibroblasts**

A key point in the study of cell/surface interactions and the role of topography is the choice of cell type. Both immortalized cell lines and primary cells have been studied and very different results have been found for different cell lines and primary cells. For this reason, the study was extended to the more biologically-relevant, but harder to study, primary fibroblasts. The primary fibroblasts tested were obtained from mouse embryos. As 3T3 cells showed no differences in adhesion when the height of the submicrometer pillars was changed from 270 nm to 520 nm, the primary fibroblasts were detached only from the 520 nm pillars.
6.4.1. Cell adhesion

It was found that – with the experimental parameters used for 3T3 cells – very few primary cells detached from the flat quartz surface: 90 % of the cells were left on the surface. On the 520 nm pillars the percentage of detachment increased to 30 %.

Since so few cells detached, the analysis of the force distance curves essentially characterizes the interaction between cell and cantilever. The mean values of the maximum force of detachment were 190 nN on the flat quartz and 210 nN on the 520 nm pillars (Table 6.2B). The mean values obtained for the work of detachment were 1.7 pJ on the flat surface and 3.8 pJ on the 520 nm pillars (Table 6.2C). The differences between the values obtained on flat quartz and on the pillars were not statistically significant (two-sample T-test, p-value 0.05) as can be expected from the fact that they mostly reflect the cell/cantilever interactions. Finally, the mean values for the distance of detachment were between 13 and 16 µm showing that the primary cells were less stretched than the 3T3 cells during detachment (Table 6.2D).

<table>
<thead>
<tr>
<th></th>
<th>A. Percentage of cell detachment (%)</th>
<th>B. Maximum force of detachment (nN)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quartz flat: 10</td>
<td>Quartz flat: 190 ± 41</td>
</tr>
<tr>
<td></td>
<td>520 nm pillars: 30</td>
<td>520 nm pillars: 210 ± 58</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. Work of detachment (pJ)</td>
<td>Mean ±SE</td>
<td>D. Distance of detachment (µm)</td>
</tr>
<tr>
<td>Quartz flat: 1.7 ± 0.5</td>
<td></td>
<td>Quartz flat: 13 ± 2</td>
</tr>
<tr>
<td>520 nm pillars: 3.8 ± 1.4</td>
<td></td>
<td>520 nm pillars: 16 ± 3</td>
</tr>
</tbody>
</table>

Table 6.2 Comparison of the percentages of cell detachment and the mean values of the maximum force of detachment, the work of detachment, and the distance of detachment of PMEF cells on two quartz surfaces: flat and 520 nm pillars (SE: standard error).

6.4.2. Cell proliferation

Cell proliferation on the test substrates was investigated to confirm the viability of the cells on the flat and structured surfaces. The Alamar Blue assay incorporating a non-toxic fluorometric growth indicator based on detection of metabolic activity was used. The quartz surfaces were particularly attractive for the 3T3 cells showing an optimal proliferation activity that was not
modified by the topography (Fig. 6.5A). In contrast, the primary cells were less viable than the 3T3 cells, with a slow and constant proliferation that was reduced on the pillars (Fig. 6.5B).

Fig. 6.5. Alamar Blue assay results from 3T3 mouse fibroblasts (A), and primary mouse embryonic fibroblasts (B). They were cultured on structured and flat quartz for 4 days and each value represents the mean of 6 samples.

6.4.3. Cell morphology

Differences in morphology were observed between the immortalized and primary fibroblasts and on the different quartz surfaces. F-actin staining (phalloidin) and scanning electron microscopy were used to compare cell morphologies.

3T3 cells grown on flat quartz had a spread morphology with a mean diameter of 50 µm (Fig. 6.6). In contrast, a spherical morphology was observed on both pillar structures with a mean diameter of 30 µm. The fixing and dehydration of the cells considerably limited the extent to which the images may be interpreted, particularly the position of the cell membrane on the pillar structures.

The primary cells had a highly spread and flattened morphology on flat quartz and were significantly larger at around 150 µm in diameter (Fig. 6.7). The 520 nm pillars dramatically modified their morphology resulting in a spindle-shaped, flattened morphology with a considerable reduction in spreading.
Fig. 6.6. Comparison of 3T3 cells by confocal microscopy (ACE) showing the actin fibres (labeled with AlexaFluor 488 Phalloidin) and scanning electron microscopy (BDF) on different quartz surfaces: flat (AB), 270 nm pillars (CD) and 520 nm pillars (EF).

Fig. 6.7. Comparison of PMEF cells by confocal microscopy (AC) showing the actin fibres (labeled with AlexaFluor 488 Phalloidin) and scanning electron microscopy (BD) on two quartz surfaces: flat (AB) and 520 nm pillars (CD).
6.5. Discussion

6.5.1. Detachment of adherent cells
The force distance curves obtained during cell detachment were analyzed to obtain three characteristic mechanical parameters: the maximum force applied during the detachment; the work necessary for cell detachment; the distance of retraction at which the cell detached from the surface. Of the three, the maximum force was the most intuitively accessible and seemed most characteristic of the different experimental conditions used in this study. The rest of the discussion is focussed on this parameter.

The maximum forces applied in the detachment experiments on 3T3 cells on quartz (Fig. 6.2) reveal, firstly, that the adhesion between cell and substrate varies widely. In order to investigate the sensitivity of this technique to changes in cell/surface adhesion, two important experimental parameters were altered: the topography of the quartz surface and the cell type. Changing the quartz substrate from a flat surface to one covered with sub-micron pillars resulted in a significant reduction in cell adhesion. The fraction of cells detached from the substrate went from 70% to 95-100% while the mean maximum force applied to the cell during detachment was reduced by a factor of approximately two. These results are in accordance with most literature reports on the effect of topography on fibroblast adhesion\textsuperscript{252,253,251,254,258,259,260}.

6.5.2. Cell adhesion to quartz and glass
Adhesion strength for 3T3 cells detaching from quartz surfaces coated with poly-L-lysine or from glass surfaces were different. While the percentages of cell detachment were similar on both surfaces (70%-75%), the maximum forces of detachment were different. The mean maximum forces of detachment were 420 nN from flat quartz surfaces coated with poly-L-lysine and 230 nN from plain glass surfaces. The difference of the maximum forces of detachment is probably due to the presence of poly-L-lysine on quartz. In contrast, the explanation of the similar percentages of detachment remains unclear. The fibronectin-based functionalization of the AFM cantilevers is capable of supporting at least a mean maximum force of detachment of 420 nN. The cantilever functionalization was also capable of detaching all the cells from the quartz surfaces with sub-micron pillars. Therefore, there is an explanation that is independent of the efficiency of the cantilever functionalization. The cells might have exhibited a different
morphology modifying their area of contact with the AFM cantilever but in this case, the percentages of cell detachment should have been different. Alternatively, poly-L-lysine may active cell adhesion not only on the surface but also on the cantilever, resulting in higher maximum forces of detachment and similar percentages of cell detachment than from glass.

### 6.6. Limitation of the cell type

When the 3T3 cells were replaced by PEMF cells, only 10% of the cells detached from flat quartz, while 30% detached from the sub-micron pillars on quartz. The absolute numbers of cells detached from the surfaces are so low that no meaningful quantitative analysis of cell/substrate adhesion is possible. The force distance curves obtained with PEMF cells do, however, give information about the cell/cantilever interactions. The mean maximum forces obtained on PEMF cells were approximately 200 nN on both flat and structured quartz. In contrast the mean maximum forces obtained for the detachment of the 3T3 cells from the cantilever were around 300 nN. A statistical analysis finds the probability that these distributions are the same to be 5% (p=0.05), the limit of statistical significance chosen for this study. It is likely, that a reduction in adhesion to the cantilever contributes to the failure to detach PEMF from the quartz surface.

The differences in cell/cantilever adhesion may be linked to inherent differences in the adhesive properties of 3T3 and PEMF cell surfaces, or to the speed of reaction of the different cell types. It is likely, however, that the very different morphologies of 3T3 and PEMF cells also play a role. 3T3 cells on quartz are relatively thick – at least at the cell nucleus – when compared to the extremely flat, spread form of the PEMFs. The raised cell nucleus allows a relatively large area of contact between cell and AFM cantilever (which is tilted at 10° to the horizontal). In contrast, it is very difficult to obtain a large contact area between PEMF and cantilever.

Despite the failure to detach the PEMF, it is possible to draw some qualitative conclusions about their adhesion to the quartz surfaces. Firstly, the PEMF adhere more strongly to the flat quartz surfaces than the structured quartz surfaces. Secondly, a comparison between the detachment of 3T3s from the 520 nm pillars (100% detachment with a mean maximum force of 180 nN) and PEMF from the same structure (30% detachment with a mean maximum force of 210 nN) shows clearly that the PEMF adhere more strongly to the structured quartz surfaces than the 3T3s.
The failure to detach a significant number of PEMF cells highlights a key issue with this approach: if the number of cells detached from the substrate is close to 100% then the values obtained for the detachment parameters (force, work and distance) may be considered representative of the cell’s adhesion, irrespective of the forces between cell and cantilever. However, as the percentage of cells detached from the substrate decreases, the accuracy of the values obtained also decreases: a bias is introduced into the distribution because only the least adherent cells can be measured.

**6.7. Conclusion**

Force spectroscopy can be used to detach single adherent fibroblasts from flat and structured quartz surfaces by traction normal to the surface. Measurements of the percentage of cells detached from the substrate surface and the maximum force necessary for detachment were used to investigate different factors influencing the adhesion of fibroblasts to surfaces. 3T3 mouse fibroblast adhesion was shown to be higher on flat quartz surfaces and lower on quartz surfaces with a structure of sub-micron pillars. The rugophobia of fibroblasts on sub-micron topography has been reported by other authors \[^{252,253,251,254,258,259,260}\] but in this work the adhesion forces were quantified. No differences in adhesion were observed when the height of the sub-micron pillars was changed from 270nm to 520nm.

Primary embryonic mouse fibroblasts were also studied and were found to be rugophobic. However, only very few PEMFs could be detached from the surface using the experimental parameters optimized for 3T3s. For this reason it was not possible to quantify the PEMF adhesion to the surface. Qualitatively, it is clear that PEMF adhesion is lower on structured quartz than on flat quartz. The PEMF also adhere more strongly at least one of the quartz surfaces than the 3T3s. Cell/cantilever adhesion seems lower for PEMF than for 3T3. A less favourable cell morphology resulting in smaller contact area between cell and cantilever may contribute to this effect. This reduced cell/cantilever adhesion for the PEMFs seems to be a major factor in the different percentages of cell detachment. The different cell/cantilever adhesions obtained for different cell types is a significant limitation of the technique because it renders comparisons between cell types difficult. The experimental parameters of cell detachment have to be similar and optimized for both cell types when adhesion forces have to be compared.
Chapter 6 Effect of sub-micron topography on cell adhesion
The main objective of this work was to use force spectroscopy to quantify the long-term global adhesion between cells and surfaces and their response to modified surfaces. This objective was achieved. Force spectroscopy was used to quantify the global adhesion forces of individual adherent cells. Adherent cells were detached from glass surfaces modified with polyelectrolytes and a thermo-responsive polymer, as well as from quartz surfaces with a structure of sub-micron pillars. The cells were detached after twenty-four hours in culture using fibronectin-decorated AFM cantilevers that were pressed onto the adherent cells for thirty minutes. The mechanical parameters acquired during detachment were used to quantify the cell adhesion forces.

The first step was to verify if cell adhesion has to be studied independently of cell cycle or not. Short-term adhesion was used to demonstrate that binding proteins at the cell membrane remain similar both in number and in their binding properties during interphase and mitosis. The rounding up and greatly reduced adhesion of cells during mitosis was associated with an increased stiffness of the cell caused by a cytoskeletal reorganization. Mitosis was not paired with large differences in the binding proteins at the cell membrane but with cytoskeletal changes. In this way, it was demonstrated that cell adhesion can be studied independently of cell cycle as
long as mitotic cells are not chosen due to their different morphology. However, the demonstration was performed using short-term adhesion with osteoblasts captured on the AFM cantilever. It is hoped that results are similar for the adherent fibroblasts studied in the rest of the thesis. A more powerful demonstration, verifying this, would use adherent fibroblasts, synchronized in the different phases of the cell cycle, and detached from their culture substrate.

Long-term cell adhesion was quantified on glass surfaces coated with polyelectrolyte bilayers. Large differences in cellular adhesion were observed depending on the number of polyelectrolyte bilayers. The reduced level of cell adhesion associated with an increased number of bilayers was, however, sufficient to allow good initial cell attachment and proliferation. Wide dispersions in the force values were observed within the same condition reflecting the heterogeneity of the cell population. However, the statistical analysis of the values indicated highly significant differences between the conditions.

The measurements of cell adhesion on glass showed that a reduction of the temperature from 37 °C to 27 °C did not induce significant changes in cell adhesion due to physiological changes in the cell. This result allows global cell adhesion measurements, not exceeding one hour, to be performed without an accurate control of the temperature (between 27 °C and 37 °C).

Long-term cell adhesion was quantified on thermally-responsive PNIPAM brushes. Cell adhesion was dramatically reduced by lowering the temperature to below the LCST. The observation of cells between the adhesive and non-adhesive states indicates only if cells are detached or not. In contrast, the quantification of the adhesion forces details the PNIPAM’s effect on cell adhesion in both states. Glass surfaces patterned with periodic PNIPAM micro-domains were used to reduce cell surface adhesion while keeping cell morphology unchanged. This approach to reduce cell adhesion and to keep cell morphology could be interesting in the stimulation of single adherent cells that are detachable. The stimulation could be performed at 37 °C and the detachment at 27 °C for further single cell analysis.

Long-term cell adhesion was shown to be higher on flat quartz surfaces than on quartz surfaces with a structure of sub-micron pillars. This was already reported by other authors but here the adhesion forces were quantified. In contrast to cell adhesion, cell proliferation was not affected by the sub-micrometer pillars. No differences in adhesion were observed when the height of the sub-micrometer pillars was changed. Three-dimensional positioning of cells was not clearly
determined by microscopic analysis due to artifacts coming from the cell fixation. The microfabrication of higher quartz pillars in order to eliminate any contact of the cells with the surface between the pillars was not possible.

Finally, an immortalized cell line was compared with primary cells in long-term adhesion experiments. The failure to detach the primary cells revealed the importance of the cell type. This allowed the potential and limitations of the technique in measuring cell/surface interactions to be discussed in more detail. The differences of detachment between the two cell types may be linked to several independent reasons including the contact area with the AFM cantilever and how quickly the cells adhere to the cantilever. The cell surface-to-volume ratio is important because in cases where the cell adhesion forces are greater than the cell cohesion forces, detachment of such cells is not possible. Primary cells may break in case of strong connection with the AFM cantilever.

As with most new techniques, single cell force spectroscopy needs to mature. The modification of experimental parameters during this study highlighted some biological and physical limitations. A key issue with the technique is the adhesion of the cells to the AFM cantilever used to detach them. The cantilever functionalization is crucial to promote cell attachment. The contact time cell/cantilever has to be minimized to limit the inevitable reorganization of the adhesion cell/substrate. The connection could be addressed by a mechanical approach instead of functionalizing the cantilever. A mechanical connection would suppress the reorganization of the adhesion of the cell to the substrate and would reduce the time of the measurements. Hollow force-controlled AFM cantilevers were used for local dispensing of a solution into individual cells\textsuperscript{261}. The adaptation of these micro-channeled cantilevers for aspiration instead of injection could be used to capture single cells. The concept should be first validated for cell capture of non-adherent cells.

Another significant limitation was the acquisition of a sufficiently large data set for a statistically significant analysis of cell behavior. Adhesion measurements that use single cells are time consuming because only one cell can be characterized at a time. Therefore, it would be useful to adjust the size of the representative sample depending on the magnitude of the effect which is sought. The detection of the influence of one polyelectrolyte bilayer on cell adhesion may require fifty or hundred measurements whereas ten measurements could have been sufficient for one and
nine bilayers. Since the cell dynamics limits the speed of individual experiments, the throughput has to be increased by parallelizing the AFM experiments. Performing experiments in parallel also offers the possibility of testing various parameters under the same experimental conditions. Parallelizing the AFM experiments requires the use of a cantilever array. One of the major challenges is the development of a readout system for cantilever arrays.

A large proportion of cells were detached from glass and quartz surfaces. Surface modifications with polyelectrolytes, PNIPAM and sub-micron topography allowed the detachment of all the adherent cells. In this work, cell adhesion measurements were focused on surfaces dedicated for cell sheet engineering and bone implant applications. Many effects on cell adhesion were already reported but this was the first time that global cell adhesion was measured quantitatively.

Single cell force spectroscopy is a powerful tool for the study of long-term adhesion. Under suitable experimental conditions, force spectroscopy allows quantification of adhesion forces between adherent cells and their culture substrates. Quantitative data of this nature should open the way for more rigorous investigation and a comparison of the influence of different parameters on cell/substrate adhesion. Force spectroscopy is highly complementary to molecular biology approaches and to existing qualitative techniques already used in cell adhesion studies.
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**Prize**


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