MODIFICATION OF A GOLD SURFACE WITH MIXED ALKANETHIOL SELF-ASSEMBLED-MONOLAYERS AND FIBRONECTIN: DESIGN OF SURFACES FOR CONTROLLED CELL/SURFACE INTERACTIONS

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ABSTRACT

This research is based on the hypothesis that by permanently immobilizing Fibronectin (FN) on a metal substrate surface, it will be possible to enhance endothelial cells (EC)/surface interactions.

FN was covalently bound onto self assembled monolayers (SAMs) modified gold surfaces with COOH terminal groups. SAMs composed of various surface ratios of X/COOH (X=CH₃, OH, NH₂) were formed in order to control the surface conformation of FN by modulating the surface charge (potential) and wettability. Human umbilical vein endothelial cell (HUVEC) attachment and proliferation onto such surfaces was investigated.

Results have shown that SAM and SAM-protein surfaces are stable with time. An increased HUVEC attachment and proliferation was obtained on FN-free SAMs compared to a bare Au surface. However, the presence of covalently bound FN on the SAM surface further significantly enhanced EC attachment and proliferation rate. The optimum surface for the HUVEC attachment and proliferation was found to be the NH₂/COOH surface, which is positively charged and hydrophilic.
RÉSUMÉ

Cette recherche est basée sur l’hypothèse qu’en immobilisant la protéine Fibronectin (FN) sur une surface métallique, il serait possible d’améliorer les interactions entre les cellules endothéliales et la surface.

Un lien covalent a été fait entre FN et le group terminal de COOH apparentant à une couche unitaire de molécules assemblées (CUMA) sur une surface en or. Les CUMAs étaient composés de plusieurs fraction des groupes terminales X/COOH (X=CH₃, OH, NH₂) dans le but de modifier la charge et l’hydrophobicité sur la surface afin de contrôler la conformation de FN. L’attachement et la prolifération des cellules provenant d’une veine ombilicale humaine (CVOH) furent investigué sur ces surfaces.

Les résultats démontrent que les surfaces constitués de CUMA et CUMA-protéine sont stables avec le temps. Les surfaces CUMA-FN ont éprouvé plus d’activités cellulaires comparées aux surfaces de CUMA. Ces derniers étés plus avantageux que les surfaces d’or. Les surfaces composées des groups NH₂/COOH, qui étés de nature chargée et hydrophile ont obtenu le plus haut niveau d’activités cellulaires.
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1. Introduction

1.1 Problem Statement

Atherosclerosis is a coronary artery disease involving the constriction of the coronary artery due to lipid deposition on its walls, and leads to a reduced amount of oxygen supply to the heart muscle. These coronary diseases are the leading causes of death in developed countries including Canada and the USA. Currently, the most popular method used to treat such diseases is to implant a metallic stent into the coronary artery for the purpose of expanding it and improving blood circulation. However, one of the most common long-term complications of this treatment is the phenomenon of restenosis that occurs at the site of the atherosclerotic lesion following stenting \[1\]. This is often the result of damage to the endothelial (EC) lining during the stenting procedure. EC dysfunction is one of the mechanisms implicated in the subsequent development of atherosclerosis which then promotes the process of stenosis at the site by various mechanisms including inflammation and proliferation of SMCs \[2\]. Another phenomenon that can lead to restenosis is the process of thrombosis, which involves the formation of blood clots on the stent surface. This is triggered by the almost instantaneous adsorption of certain undesirable plasma proteins onto the stent surface, such as fibrinogen.

Restenosis or recurrent intimal thickening of blood vessels results in increased morbidity and health care costs. Restenosis incidence was initially believed to be significantly reduced with the development of drug-eluting stents (coated with immunosuppresant drugs) that inhibit the inflammatory response and the resulting proliferation of smooth muscle cells (SMCs) in the surrounding tissue \[3\]. However, recent large-scale clinical studies have shown that the drug-eluting stents have much higher restenosis rate than initially believed, and that their success in lowering restenosis occurrence has been shown to be at best similar to bare (non-modified) metal stents, if not worse \[4\]. In addition, the long-
term efficiency of these stents is limited because of the need to take anti-thrombotic drugs and questionable long term safety.

Although there have been a number of experimental studies that report various approaches used to modify a stent surface with (bio)molecules and cells to increase its biocompatibility and decrease the restenosis rate [5, 6, 7, 8, 9, 10, 11, 12], these approaches are far from being suitable for real applications, due to their complexity, high price, and short-term stability of the formed biomimetic surface layers.

Fibronectin (FN), a protein attached to the extra cellular matrix and found in the blood plasma, has been shown to play an important role in coordinating cellular activity such as adhesion and proliferation (explained in Section 1.2.1). Several studies have therefore investigated the influence of FN adsorbed onto metallic surfaces on subsequent cell/surface interactions. The problem with this approach is that protein adsorption onto surfaces is a reversible process thus making it possible for FN to detach and get replaced by fibrinogen or other undesirable biomolecules that may cause thrombosis. Another disadvantage is that by simply adsorbing FN, it is difficult to control its surface conformation and surface density, and thus the subsequent cell/surface interactions.

The research presented in this thesis is based on the main hypothesis that by permanently immobilizing FN on a metal surface, it would be possible to overcome negative issues related to reversible FN adsorption. The irreversible binding would eliminate the possibility of FN detachment as well as make it possible to control its surface concentration and conformation in the way to promote desirable cellular activity. Protein covalent binding onto surfaces can be tailored by using certain long or short chain alkane molecules whose head groups readily and irreversibly self assemble to specific surfaces (e.g. model surfaces such as silver, gold and silicon oxide, or commercial surfaces such as stainless steel or titanium alloys) to form a monolayer. This spontaneously formed monolayer is referred to as a self assembled monolayer (SAM), and certain types of alkane molecules that form SAMs allow FN to covalently bind to their terminal functional end groups (Section 1.2.4).
This project consisted of using 11-mercaptoundecanoic acid (MUA, HS(CH$_2$)$_{10}$COOH), an alkanethiol molecule containing a sulphuric head group (thiol) and carboxyl (COOH) terminal group, separated by an alkane -(CH$_2$)$_{10}$- spacer, to irreversibly bind FN onto model gold coated glass surfaces, to which alkanethiols readily adhere. In order to control the surface conformation of immobilized FN, and thus the subsequent cell/material interactions, single and binary alkanethiol SAMs consisting of a COOH terminal group (negatively charged and hydrophilic) combined with either a CH$_3$ (neutral and hydrophobic), OH (neutral and hydrophilic) or NH$_2$ (positively charged and hydrophilic) group were formed at various binary mixture ratios. Due to the well defined surface chemistries of these SAM surfaces, this allowed precisely controlling the charge and hydrophilicity of the surface.

The primary objective of this master’s project was to investigate the influence of physico-chemical properties (wettability and charge) of these SAM surfaces on human umbilical vein endothelial cell (HUVEC or simply EC) attachment and proliferation, and thus to determine which surface properties give the most optimum results in terms of EC/surface interactions.

This thesis is divided into 6 chapters. First, background explanations will be given to clarify the concepts that will be discussed throughout the thesis. A literature review will then describe several findings from studies that are related to this field and to this research project. A description of the project objectives will then be given, followed by the experimental methodology that was used. Finally, the results will be discussed, compared to literature, and summarized.

**1.2 Background**

This section provides brief explanations of concepts, processes and descriptions of biomolecules that are relevant to this research.

**1.2.1 Extracellular matrix**

The Extracellular matrix (ECM) consists of gradients of 3 major types of biomolecules: structural proteins (i.e. collagen and elastin), specialized proteins
(i.e. fibronectin), and proteoglycans [13]. It can be referred to as a connective tissue since it surrounds and supports cells that are within mammalian tissues. Therefore it is useful for cell migration and plays major roles in organizing, coordinating and regulating cellular activities such as attachment, proliferation and differentiation. It also plays major roles in tissue morphogenesis, homeostasis and repair. Many proteins in the ECM matrix have RGD peptides which are the central cell binding regions.

1.2.2 Fibronectin

FN is the major component of the ECM that is found in all connective tissues. It is also found in the blood plasma. It is a high molecular weight glycoprotein of 440 kDa. This protein plays primary roles in coordinating many cellular activities, which include adhesion, differentiation, proliferation and migration. It also plays a major role in many biological responses such as matrix assembly and wound healing, which is why it is of big interest in biomedical applications [14].

FN is composed of two covalently linked polypeptide subunits that are nearly identical, and are connected by a pair of disulfide linkages near their carboxy termini [15]. Each subunit is composed of the FN I (type I), FN II (type II) and FN III (type III) modules as shown in Figure 1. Figure 1 also shows the binding domains for other biomolecules such as cells. The PHSRN peptide is located in the 9th type III repeat and the RGD site, in the 10th type III repeat. These are the central cell binding sites of FN.

In general, the primary structure of a protein is the sequence of amino acids that compose it [16]. These amino acids contain a variety of chemical groups that interact together and cause the protein to fold into certain shapes in order to maintain a stable structure. Many of these shapes and patterns can be seen on different proteins and are called secondary structures. The most common shapes are the alpha-helix, beta-sheet, beta-turn and random coil, and each possesses its own characteristics and IR absorption responses. As will be further explained in Section 4.7, the proportions of each secondary structure components belonging to
a certain protein can be obtained by deconvoluting the so-called Amide I band, which is the characteristic of all proteins.

![Figure 1: Representation of FN subunit along with the binding sites for various biomolecules [14].](image1)

The tertiary structure of FN consists of the folding of the FN secondary structures to form globular domains, as seen in Figure 2 for one FN subunit (the other one is identical and symmetrical).

![Figure 2: Tertiary structure of FN showing the Cell binding sites [17].](image2)

The tertiary structure (i.e. conformation) of FN strongly depends on its environment. It has been shown that FN has a compact conformation on hydrophobic surfaces as well as in aqueous solutions, such as in the blood plasma where it is in a soluble form [14]. As seen in Figure 3, the compact form hides the RGD and PHSRN regions, which consequently makes it difficult for cell binding. However, on charged hydrophilic surfaces and when part of the ECM matrix, FN adopts more favorable conformations for cell-binding, by opening up and exposing the central cell binding domains. This is, actually, the main basis of the
surface-modification approach used in this thesis, i.e. to control the surface physico-chemical properties in order to control the FN surface conformation and subsequent cell/surface interactions.

![Figure 3: Compact conformation of FN [14].](image)

### 1.2.3 Integrins

Cells adsorb onto proteins in the extracellular matrix primarily via integrins. Integrins are cell transmembrane receptors that are specific to many cell binding sites of several ECM proteins, including FN [18]. These cell binding sites include the RGD and PHSRN motifs, as previously mentioned. Both these sites contribute to binding of two types of integrins, the $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins, which allow an increase in cell adhesion strength and stability. By mediating cell adhesion, integrins therefore support cell spreading and migration. It has been shown that the specificity of integrin binding to the central cell binding sites also trigger signals that regulate cell proliferation and differentiation [19].

The conformation of a protein strongly influences integrin binding, since a change in conformation can potentially cause an increase in the relative distance between the PHSRN and RGD sites (i.e when adopting a compact structure). This could result in an inhibition or decrease of cellular activity.
1.2.4 Cell adhesion to SAM modified surfaces

A fair amount of research has been devoted to studying the phenomenon of cell adhesion to different types of SAM modified surfaces [15, 17, 19, 20, 21, 22, 23, 24, 25]. The only purpose of using SAMs is to control the surface physico-chemical properties. Generally, the experiments involve incubating the surfaces of interest in a suspension of cells contained in a culture medium that is composed of a certain percentage of fetal bovin serum (FBS). This serum contains several types of serum proteins including FN and vitronectin, which are two proteins that enhance cell adhesion. However, bovin serum albumin (BSA), which is the main component of FBS has the opposite effect on many types of cells, including ECs. These proteins rapidly adsorb to the surface, which could influence subsequent cell adhesion. However once cells settle down on the SAM, they are quite capable of producing their own ECM matrix proteins, such as FN, which in turn can displace the initially adsorbed serum proteins and enhance cell adhesion [22]. Nevertheless, there is always competition between the ECM protein attachment (excreted by cells) and adsorption of other proteins (such as fibrinogen) that may decrease the biocompatibility of the surface, and minimize desired cell/surface interactions. Therefore, using only SAMs to modulate cell/surface interactions is not quite an efficient and cell-specific approach. Nevertheless, it represents a step up in increasing the biocompatibility of an implant, and as it will be presented in this thesis, allows to further modify the implant surface by cell-specific biomolecules (e.g. FN).

1.2.5 Self Assembled Monolayers

SAMs consist of molecules composed of an alkane chain with a certain head group and terminal functional group that self assemble on certain surfaces to form monolayers [26] (Figure 4). The head group of these molecules is capable of irreversibly adhering to certain surfaces, thus allowing monolayer formation. For this research, alkanethiols will be adhered onto on gold surfaces.
SAMs of alkanethiols have a sulfuric head group. They are represented as HS-(CH\textsubscript{2})\textsubscript{n}-X, where \(n\) represents the number of repetitive CH\textsubscript{2} groups forming an alkane chain, and \(X\) represents different terminal functional groups. The type of functional group to be used depends strongly on the application and the desired surface properties. In fact, physico-chemical properties of the whole SAM-modified surface is predominantly defined by the physico-chemical properties of the functional group.

Figure 4 shows the steps involved in SAM formation. The gold surface is first soaked in a solution of the specific alkanethiol molecules in a solvent. Alkanethiols are highly soluble in ethanol, which makes it the more commonly used solvent. The sulfuric head group has a high affinity for gold and will therefore adsorb very rapidly. However, in order to form a well-ordered monolayer (as seen in Figure 4) it is necessary to immerse the substrate in the SAM-forming solution for at least 15 hours to allow for organization of the SAM. The angle of orientation of the well-ordered SAM is typically 30° [26].

![Figure 4: Steps involved in SAM formation: (1) Soaking of gold surface in thiol/ethanol solution, (2) instantaneous adsorption of alkanethiols and (3) Organization of the SAM [26].](image)
For this project the COOH terminal SAM was used to covalently bind FN via carbodiimide coupling reactions, as illustrated in Figure 5. In this reaction, N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) are the activating agents. NHS reacts with the carboxylic acid terminal of the SAM to form an activated succinimidy ester intermediate. This allows the amine terminal of the protein (FN or another one) to easily bind to the SAM and replaces the NHS molecule.

SAM modified surfaces are water stable and highly uniform. In order to vary the surface chemistry it is possible to combine the COOH alkanethiol with another alkanethiol possessing a different terminal functional group (CH₃, NH₂, OH) in the solution mixture. This allows modulation of surface physico-chemical properties, which in turn allows modulation of FN secondary and tertiary structures, which in turn influences cell/FN (i.e. cell/surface) interactions.

Figure 5: Carbodiimide coupling reaction: (1) Binding of COOH SAM to gold, (2) Binding of NHS to SAM, (3) Replacement of NHS by FN [15].
CHAPTER 2

2. Literature Review

This section presents literature review that is pertinent to the objectives of this project.

2.1 Effect of SAM modified surfaces on hydrophobicity

Wettability of biomedical surfaces plays an important role on cell/surface interactions, since it directly influences protein/surface (e.g. FN/surface) interactions. Thus, a number of studies on the influence of SAMs on the wettability of the modified surface have been published.

Arima et al. [22] measured the contact angle of homogenous SAM modified gold surfaces with CH$_3$, NH$_2$, OH, and COOH functional groups. The measured contact angle values were 109°, 53°, 29° and 23°, respectively. This trend also agrees with the results obtained by Keselowsky et al. [19]. Faucheux et al. [27] reached different conclusions when they formed alkyl silane SAMs with terminal functional groups CH$_3$, NH$_2$, COOH and OH on silicon wafers. The contact angle experiments indicated that the CH$_3$ functional group gave a hydrophobic surface (93.3°), NH$_2$ (58.5°) and COOH (47.7°) yielded moderately hydrophilic surfaces, and the OH (13.8) group led to the formation of a hydrophilic surface.

Arima et al. [20], in another study, produced both homogenous and binary SAMs on gold using CH$_3$, NH$_2$, OH, and COOH terminated alkanethiols. The binary SAMs were produced at several mixture ratios, the CH$_3$ group being always present in the binary mixture. The order of hydrophobicity of homogenous SAMs agreed with their previously results although the values were slightly different. SAMs composed of 100% COOH and 100% OH were observed to have the highest degree of hydrophilicity with contact angles of 21° and 22° respectively. Surfaces with 100% NH$_2$ SAMs were observed to be moderately hydrophilic with a contact angle of 51°, and the 100% CH$_3$ SAM surface was
highly hydrophobic with a contact angle of 109°. For combinations of CH$_3$/COOH SAMs, the contact angle decreased as the fraction of COOH alkanethiols in the solution increased, as can be seen in Figure A.1 in the appendix. The results clearly show that the relationship is non linear. This is an indication that the CH$_3$ alkanethiols were preferentially incorporated in the SAMs, and this was further confirmed when the actual surface fraction of the SAMs was evaluated using X-ray Photoelectron Spectroscopy (XPS, Figure A.2 in Appendix). Figure A.2 clearly demonstrates that the fraction of COOH SAMs on the surface is lower than the fraction of the COOH alkanethiol in solution. Baker et al. [28] also observed a similar trend and reached the same conclusion. The three data points that were provided in Baker et al. [28] were re-plotted and can be seen in Figure A.3 in the appendix.

Schweiss et al. [29] performed contact angle measurements on OH/COOH SAMs at different mixture ratios and concluded that all SAMs were similar in terms of hydrophilicity. Backer et al. [28], who further provided three data points for contact angles of OH/COOH SAMs also observed that the three contact angles were fairly hydrophilic, however did not seem to follow any regular trend (Figure A.4 in Appendix). A similar observation was made by Chuang et al. [30] for NH$_2$/COOH SAMs (Figure A.5 in Appendix).

All contact angle values provided by the studies mentioned above are listed in Table 1. Even though the values do not all necessarily, agree the general trend is that CH$_3$-terminated thiol forms a hydrophobic SAM, and the other three functional groups form hydrophilic SAMs, the NH$_2$ leading to the highest contact angle out of the three followed by either COOH or OH groups.
Table 1: Summary of contact angle results for homogenous SAMs of OH, COOH, NH$_2$ and CH$_3$ reported in different studies.

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
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</tr>
</thead>
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</tr>
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<td>-</td>
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<td>109</td>
<td>93.3</td>
<td>111.4</td>
<td>-</td>
</tr>
</tbody>
</table>

2.2 Influence of surface properties on FN conformation

Due to the importance of FN surface conformation on the cellular activity, a fair amount of research has been devoted to investigating the influence of surface physico-chemical properties on the conformation of adsorbed FN.

Bergkvist et al. [14] observed the change in FN conformation when adsorbed to silica (hydrophilic with a contact angle of 0-10°) and methylated silica (hydrophobic with a contact angle of 80-90°) surfaces using an atomic force microscope (AFM). It was observed, by measuring the roundness of the individual protein images, that the hydrophilic surface caused the protein conformation to be in the elongated form. For FN adsorbed onto silica, 70% of the molecules had an elongated form, 5% had a compact form and 25% had a semicompact form. The complete opposite was observed for the FN molecules adsorbed onto hydrophobic silica, suggesting that when adsorbed to hydrophobic surfaces, FN has a tendency to be in a compact form. This indicates that FN adsorbed onto hydrophilic surfaces has a more favorable structure for cell binding than when adsorbed onto hydrophobic surfaces (as explained in Section 1.2.1).

Many other studies have measured the conformation of FN adsorbed to different types of surfaces [31, 32, and 33] from which several conclusions have been drawn regarding the influence of surface properties on FN conformation and
biological activity. However, the problem with such studies is that some of the substrates had a lack of surface stability, surface homogeneity and/or complete knowledge of surface properties. As well, most of these studies only verified the effect of hydrophobicity on protein conformation, when other effects may have had an influence as well (e.g. surface charge, topography). As previously mentioned, the advantage of using SAMs is that they have well defined surface properties and are stable with time. Different types of SAMs can yield many different types of surfaces (i.e. hydrophobic, hydrophilic, positively charged, negatively charged), which allow controlling the surface properties.

Keselowsky et al. [19] investigated the effect of surface properties of homogenous SAMs on the conformation of adsorbed FN by using an antibody based assay. From these results the authors concluded that the binding affinity of HFN7.1 antibody on the different FN adsorbed surfaces decreases in the order of OH > COOH = NH₂ > CH₃, and that of 3E3 antibody, in the order of OH > COOH > NH₂ = CH₃. The OH SAM was clearly more efficient at binding the antibodies since it had the lowest FN surface density but still exhibited similar quantities of bound antibodies. From these observations, it can be concluded that the FN conformation is more optimum for cell binding on hydrophilic surfaces.

Michael et al. [17] also investigated the effect of surface chemistry on FN conformation but using the recombinant FNIII. An antibody type assay similar to Keselowsky et al. [19] was first used, except that the mAB1937 and FN1-11 antibodies were used instead of 3E3. From this experiment, it was observed that the affinity of HFN7.1 followed the pattern OH=NH₂>COOH>CH₃, which is somewhat similar to the results obtained in Keselowsky et al. [19]. Since HFN7.1 is known to bind to the flexible linker between the 9th and 10th type III repeats, the results indicate that significant structural changes must have occurred in that region, for FN adsorbed on the CH₃ SAMs. This agrees with previous studies that demonstrated that the greatest amount of denaturation and unfolding of different proteins occurred on hydrophobic surfaces [34, 35, 36, 37, 38 and 39]. The affinity of mAB1937 and FN1-11 followed the order OH=COOH=NH₂>CH₃, which led to similar conclusions concerning structural changes on the 9th (FN1-11
binding site) and 8th (mAB1937 binding site) type III repeats on hydrophobic surfaces. However from these results, since there was no significant difference in binding affinity between the OH, COOH and NH₂ SAMs, a correlation could not be made between surface hydrophilicity and structural change. This is an indication that although hydrophobicity plays an important role in protein conformation, other factors such as surface charge also have an influence.

As mentioned in Section 1.2.1, it is believed that the elongated form of FN is more favorable for cell binding, and all three studies mentioned in this section confirm this statement. Bergkvist et. al. [14] observed that hydrophilic surfaces yield a more elongated FN structure, while Keselowsky et al. [19] and Michael et al. [17] observed that the hydrophilic surfaces were more favorable for antibody binding onto the central cell binding sites, thus suggesting that the FN confirmation was more favorable for cell binding.

2.3 Influence of surface hydrophobicity on cell adhesion and proliferation to SAMs

Studies concerning cell adhesion to SAMs (in the absence of preadsorbed FN) have also been quite relevant in understanding the influence of surface properties on cell adhesion.

Arima et al. [20] tested the effect of surface hydrophobicity, charge and functional groups on HUVEC adhesion. For homogenous SAMs, there was no significant difference in cell adhesion between COOH SAMs and NH₂ SAMs both of which demonstrated the highest degree of cell attachment. The 100% OH SAM surface yielded a slightly lower degree, and the surface with the 100% CH₃ SAM exhibited very poor cell adhesion. This may lead to the conclusion that HUVEC adhesion is strongest on hydrophilic surfaces and perhaps even stronger on charged surfaces than neutral.

Figure A.6 and Figure A.7 were plotted based on data from Arima et al. [20], and illustrate the cell adhesion as a function of the solution fraction of COOH alkanethiols and contact angle, respectively. A similar trend was observed on both figures. On CH₃/COOH SAMs, cell attachment increased as the percent
of COOH alkanethiols in solution increased (Figure A.6) and contact angle decreased (increase in hydrophilicity (Figure A.7). At higher COOH alkanethiol fractions and lower contact angle values the cell density leveled off. Since at lower contact angle values, a further decrease did not necessarily result in a higher amount of cell adhesion, there was clear indication that although surface wettability was shown to strongly contribute to cell adhesion, it could not have been the only factor. Other properties belonging to the surface functional groups must have played a role as well.

Fuse et al. [21] tested cell proliferation (different cell lines) on mixed SAMs composed of OH and CH$_3$ terminal groups. The goal was to study the influence of surface hydrophobicity on cellular activity. The four curves shown in Figure A.8 in the Appendix display for each cell line, the proliferation results as a function of the surface wettability, for which the highest contact angle value pertains to the homogenous CH$_3$ SAM and the lowest to the homogenous OH SAM. Despite the fact that all cells underwent the same treatments, there was no similarity between the growth pattern of each type of cell. In fact, none of the growth patterns show a regular trend. The only common point is that mixed SAMs yielded higher cell densities than homogenous ones. For the C3H10T1/2 clone 8 and L929 cell lines, homogenous OH SAMs gave higher results than homogenous CH$_3$ SAMs, and the complete opposite occurred for the UVB6-2.1A and MC3T3E1 cell lines. These results not only agree with results of Arima et al. [20], regarding potential other roles played by the surface functional groups aside from controlling the hydrophobicity, but also demonstrate that different cell types respond in different ways to the same set of surface properties.

Arima et al. [22] tested the proliferation of HUVECs on SAMs with COOH, NH$_2$, OH and CH$_3$ terminal functional groups. Their goal was not only to examine cell adhesion but also the influence of serum proteins found in FBS (refer to Section 1.2.3). Surface plasmon resonance (SPR) and total internal reflection fluorescence microscopy (TIRFM) were used to examine the adsorption kinetics of the serum proteins and correlated them to cell adhesion. Figure A.9 in the Appendix displays the cell attachment results as a function of time. It was
observed that the initial cell attachment was strongly dependent on the surface functional group since after only 10 minutes of incubation, there were already significantly more cells on the NH$_2$ and COOH SAMs than on the OH and CH$_3$ SAMs, on which cell binding was practically insignificant. Similar trends were observed after 180 minutes. Using SPR, adsorption of serum proteins contained in the cell media was examined, and it was observed that the rate of adsorption of these serum proteins, was higher than proteins expressed by the cells, which indicates that the protein preadsorption to the surface was the first process to take place and may have influenced subsequent cell adhesion. Furthermore, in another experiment, Arima et al. [22] came to the conclusion that when ECs adhered to homogenous OH and CH$_3$ SAMs, their ability to displace BSA (the main component of FBS and cell adhesion inhibitor) was much less efficient than when they were adhered to homogenous NH$_2$ and COOH SAMs. This may explain why the OH and CH$_3$ SAMs exhibited a higher delay in cell adhesion than NH$_2$ and COOH SAMs.

Several conclusions can be drawn from the results published in papers presented in this section. The research done by Arima et al. [20] and Fuse et al. [21] suggested that surface wettability does influence cell adhesion however other factors regarding the surface functional groups may as well play a role. Furthermore, different cell lines may respond differently to certain surfaces. In their study Arima et al. [22] came to the conclusion that surface properties do indeed have an effect but mainly on initial cell adhesion. After cells adhere, they are capable of producing their own ECM proteins that enhance subsequent cell adhesion. However, as discussed in Section 2.1 the different surface properties may as well have influenced the secreted FN conformation as well as the ability to displace serum proteins, which may in turn have affected subsequent cell adhesion.
2.4 Influence of surface properties on cell adhesion to FN

Although many studies have verified the effects of surface properties on the conformation of adsorbed FN, the effects on the protein’s cell-binding activity are still poorly understood. This is crucial to understand since surface properties may affect cellular adhesion and other higher order cellular activities such as proliferation and differentiation, through the influence on the FN surface conformation.

Aside from studying the effects of surface chemistry on FN adsorption and conformation, Keselowsky et al. [19] also studied the effect of surface properties on cell adhesion. MC3T3-E1 osteoblast-like cells, that possess α5β1 integrins, were used for the cell adhesion experiments. From the results, it was observed that the α5β1 integrin binding affinity followed the pattern OH > COOH = NH2 > CH3. The results showed a similar trend as in the case of binding of HFN7.1, mentioned earlier in the text. This demonstrated that cell adhesion is mediated by the α5β1 integrin and occurs more readily when FN is adsorbed to hydrophilic surfaces which enable FN to adopt a cell-active elongated structure.

Michael et al. [17] used a centrifugation adhesion assay with fluorescently labeled cells in order to assess FN activity after adsorption to SAMs. The cell binding affinity followed the order OH=NH2>COOH>CH3. The results agree with Keselowsky et al. [19] for the CH3 and OH SAMs. Lee et al. [23], who used silicon wafers as a substrate for SAM binding, obtained different trends for the binding affinity of integrin α5β1. Erythroleukemia cells that only express the α5β1 receptor, were used. The binding affinity to FN for different types of SAMs decreased in the order of OH>NH2≈CH3≈COOH, which, aside from the OH SAM, does not agree with Keselowsky et al. [19] and Michael et al. [17]. Using two surfaces with different surface roughness (glass and silicon), Lee et al. [23] were also able to conclude that surface roughness at the nano-scale did not affect the α5β1 binding affinity to FN.
Most research groups that studied cell binding to FN adsorbed on SAM modified surfaces, only observed adhesive properties [17, 19]. Other studies have also focused on how much cell proliferation and differentiation, which are higher order cellular activities, are influenced by surface properties.

Several studies have shown that integrin binding to ECM proteins can regulate the switch between proliferation and differentiation [24 and 25]. For example, García et al. [24] adsorbed FN onto two types of polystyrene (highly hydrophobic (B), and negatively charged slightly hydrophobic (T)) and collagen (C), and grew C2C12 myoblast cells onto these surfaces. The levels of proliferation decreased in the order of B>T>C. Differentiation followed the trend C>T>B. Both these results indicated that differences in FN conformation lead to different levels of cell activity. García et al. [24] also observed that different levels of bound α5β1 can modulate the switch between proliferation and differentiation, and that anti-α5 antibodies inhibit differentiation while anti-αv does not.

Lan et al. [18], used BrdU culture incubation in order to analyze cell proliferation as a function of surface chemistry. The proliferation efficiency for each SAM was obtained by dividing the quantity of cells that underwent proliferation by the total number of adhered cells, and the results were represented in Figure A.10 in Appendix. It can be observed that the proliferation efficiency of the cells was significantly higher on FN adsorbed onto COOH and NH2 terminal SAMs than on OH and CH3. This led them to conclude that FN confirmation was more favorable for the binding of αvβ3 integrin, the one responsible for cell proliferation (Section 1.2.2), on hydrophilic charged surfaces.

The expression of two myogenic differentiation genes, myogenin (early stages) and troponin T (late stages), was investigated as a function of the different SAM surfaces. Both these genes exhibited similar dependence on surface chemistry. High levels of these genes were expressed with CH3 and OH terminated SAMs, intermediate levels on NH2 and low levels on COOH. This
demonstrated that the $\alpha_5\beta_1$ integrin may be responsible for differentiation, since that integrin bonded more readily on FN adsorbed to CH$_3$ and OH SAMs (as seen in Section 2.4). This is consistent with García et. al. [24] who found that differentiation was inhibited by the anti-$\alpha_5$ antibody.

Myogenic differentiation was further analyzed using immunostaining for sarcomeric myosin. Total cell adhesion decreased in the order of COOH>NH$_2$=CH$_3$>OH, whereas for the percent differentiation, the order was OH>CH$_3$>NH$_2$=COOH. The proliferation pattern was similar to the total cell adhesion whereas the differentiation pattern followed an inverse trend. This is due to differences in integrin binding. Enhanced differentiation was due to selective binding of $\alpha_5\beta_1$, and blocking of the $\beta_1$ binding site inhibited differentiation.

### 2.6 Influence of surface properties on covalently bonded proteins

Although physical adsorption of proteins (with the aim to functionalize a surface) has the advantage of being simple and in a large number of cases allows the protein to retain biological activity, the process is reversible. Other unwanted proteins can therefore replace the adsorbed ones (as explained in Section 1.1). In addition, the adsorbed protein layer can easily be removed from the surface (e.g. stent) under the blood shear. However, chemical immobilization of proteins onto surfaces results in irreversible binding. Although many studies involved immobilizing SAMs onto gold surfaces and covalently binding proteins using carbodiimide coupling [40, 41, 42, 43 and 44] for different purposes, to the best of this thesis author’s knowledge, only one study used this approach to bind fibronectin onto a gold surface [15] (further described below).

Using carbodiimide coupling, Frey et. al. [40], covalently bonded Poly(L-lysine) onto COOH SAMs and characterized each step (SAM, NHS, protein) using polarization modulation infrared reflection adsorption spectroscopy (PM-IRRAS). The purpose of that experiment was to build an immunosensor by binding an antibody onto the protein (as described below).
Other studies examined the effect of different types of SAMs as well as mixtures of SAMs on the surface concentration of covalently bound proteins.

For example Patel et. al. [45] covalently bonded and physically adsorbed the protein catalase onto homogenous SAMs of 3-mercaptopropionic acid (3-MPA) and MUA, as well as on a 10:1 (3-MPA/11-MUA) mixture of both SAMs in order to compare the protein coverage for both cases. The SAMs were immobilized onto gold coated glass slides. Before adsorbing the proteins they measured the hydrophobicity of the SAM modified surface by performing contact angle measurements. The results demonstrated that all surfaces were relatively hydrophilic, attributing the highest hydrophilicity to the 3-MPA modified surface and the lowest to the mixture. AFM imaging was used to determine the surface coverage of physically adsorbed proteins. The proteins adsorbed to mixed SAMs had the highest surface coverage of 98%. 11-MUA yielded a higher surface concentration (72%) than 3-MPA (38%). By immobilizing the protein using carbodiimide coupling, they concluded that the amount of bound protein and NHS followed the same order as adsorbed proteins, however the surface coverage was higher. SPR was used in order to obtain the amounts of EDC/NHS and catalase immobilized.

Briand et. al. [44] used mixtures of SAMs to build immunosensors. They adhered MUA mixed with either decanethiol (coded as C9CH3) or mercaptohexanol (coded as C6OH) onto gold transducers. The mixing ratios were 3:1 (other SAM/MUA). Protein A was then covalently bonded onto the SAMs using carbodiimide coupling. This protein was used as a linking agent to immobilize a rabbit antibody via bioaffinity. The goal was to study the influence of the chemical composition of SAMs as well as the resulting structure of the attached protein on sensory performance. From AFM measurements, the authors were able to conclude after binding Protein A to the SAMs that the C9CH3 mixture resulted in higher protein binding than the C6OH mixture. This agreed with Patel et. al. [45] since the more hydrophobic mixture (C9CH3/MUA) yielded higher protein binding. However, after rabbit IgG was bound via bioaffinity to Protein A, the C6OH mixture was found to be more efficient at
binding the antibody. A possible explanation is that the densely packed layer formed by Protein A on the C9CH3 mixture caused some steric hindrance. Finally the antibody’s ability to recognize and bind the corresponding antigen was tested. It was observed once again that the samples containing the C6OH/MUA mixture was more efficient as biosensors.

A number of other studies have been done with different types of mixed SAMs and different types of proteins [46, 47, 48 and 49]. Although these studies provide some useful insight about the effect of mixed SAMs on protein activity for many different application, there is practically no information on the effect of FN covalently bonded to SAMs on the cellular activity.

One particular study conducted by Plummer et. al. [15] created an ECM model surface that consisted of an FN gradient on the gold surface. The goal was to examine cell migration processes and to be able to obtain different information on cells such as their morphology and signaling. NIH-3T3 fibroblast cells were used. It was observed that the cells successfully adhered and spread onto the FN surface, while none were present on the surface free of FN (and blocked by BSA).

The authors however did not try to optimize the conformation of FN for cell proliferation and differentiation by using different terminal functional groups. No studies have been made on FN covalent binding to SAMs that involved optimizing the conformation for tailoring desired cell/surface interactions.
CHAPTER 3

3. Objectives

The major objectives of this master's project were:

- to irreversibly bind fibronectin to a model gold surface using 11-mercaptoundecanoic acid (COOH SAM) that self-assembles on the surface,

- to vary the surface physico-chemical properties of this surface by forming mixed self-assembled monolayers (SAMs) consisting of X/COOH (X=CH$_3$, OH, NH$_2$), and

- to investigate how this influences endothelial cells attachment and proliferation.

The knowledge gained in this project will serve as the initial basis for later modification of commercial biomaterial surfaces (stainless steels and Ti-based alloys) to be used as coronary stents.

The specific aims of the projects were:

- to optimize formation of mixed SAMs and chemically characterize them,

- to optimize binding of the protein and investigate the irreversibility of the protein attachment (stability of the protein layer),

- to investigate the effect of SAM surface chemistry on its wettability (contact angle),

- to investigate the effect of SAM surface chemistry on its surface charge (zeta potential),

- to investigate the effect of physico-chemical properties of the SAM on the fibronectin (FN) secondary structure,
- to investigate the effect of physico-chemical properties of the SAM and SAM-FN monolayer on the attachment and proliferation of HUVECs, and

- to select optimum surface chemistry (SAM) for HUVEC/surface interactions.
CHAPTER 4

4. Methodology and Experimental Techniques

4.1 Chemicals

The following chemicals, purchased from Sigma Aldrich, were used in this research: bovine serum albumin (BSA, cat.number: A9418), sodium chloride (NaCl, cat.number: 71381), potassium phosphate monobasic anhydrous (KH$_2$PO$_4$, cat.number: 7778770), potassium phosphate dibasic anhydrous (K$_2$HPO$_4$, cat.number: P8281), 11-mercaptopoundecanoic acid (COOH alkanethiol, cat.number: 450561), 1-undecanethiol (CH$_3$ alkanethiol, cat.number: 510467), 11-mercapto-1-undecanol (OH alkanethiol, cat.number: 447528) and N-hydroxysuccinimide (NHS, cat.number: 130672). 11-Amino-1-undecanethiol hydrochloride (NH$_2$ Alkanethiol, cat.number: 111027-004), was purchased from Assemblon, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC, cat.number: 03450) from Fluka and Fibronectin (FN, cat.number: 354008) from BD Biosciences. Quick Cell Proliferation assay Kit (WST-1) was purchased from Biovision. Table 2 shows a list the chemicals that composed the cell medium.

Table 2: List of components contained in the cell medium along with the company from which they were purchased and their catalogue number.

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<thead>
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<th>Company</th>
<th>Catalogue number</th>
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<td>Invitrogen</td>
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<td>Biomedical Technologies</td>
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<td>PBS</td>
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</table>
4.2 Formation of SAM modified gold surfaces

The gold coated glass slides were purchased from EMF. The Au was vacuum deposited using thermal evaporation. Due to the low energy of this form of physical vapor deposition, the gold films did not exhibit any long-range crystalline order and were therefore mainly amorphous.

The gold slides were first cleaned by soaking in acetone for 30 minutes. Homogenous or binary alkanethiol solutions were then prepared. The first step was to prepare a sufficient volume of each type of alkanethiol (COOH, CH₃, OH, NH₂) at a concentration of 1mM in ethanol. The binary mixtures were then prepared by varying the volume ratio of each alkanethiol solution. The COOH alkanethiol was always present and it was paired with the CH₃, OH or NH₂ alkanethiols. The different binary solutions that were made during this project were X:COOH (where X=CH₃, OH, NH₂), 0:100 (homogenous COOH SAMs), 10:90, 15:85, 30:70, 50:50, 70:30, 85:15, 90:10, 100:0 (homogenous CH₃,OH or NH₂ SAMs). SAMs were formed during a 24 hours immersion time of clean gold slides in the specific alkanethiol solution.

4.3 Contact angle measurements

The contact angle of all SAM samples was measured using a sessile drop technique with deionized water as the probing liquid. The instrument used was a Future Digital Scientific contact angle system OCA goniometer. The SCA20 software for OCA & PCA V.3.12.4 was used to calculate the contact angle and to manipulate the droplet deposition. The measurements were done on SAM modified gold slides with 1cm × 1cm dimensions. At least four measurements were made on each sample by depositing as much droplets as possible when going from one extremity of the sample to the other.
4.4 Zeta potential measurements

An Electro Kinetic Analyzer (EKA) was used for measuring the zeta potential of the different SAM surfaces. An asymmetric clamping cell was inserted into the EKA forming a channel through which an electrolyte flowed. The electrolyte used was a solution of 10mM phosphate buffer with a pH of 7.4 composed of 8mM K₂HPO₄ and 2mM KH₂PO₄. The cell consisted of two components: the sample to be tested and a poly(methyl methacrylate) (PMMA) spacer. The PMMA spacer is a small rectangular grooved spacer containing many parallel channels of rectangular shape as seen in Figure 6. The surface of interest belonging to the test sample was facing the rectangular grooves and both components were firmly pressed against each other to form the cell. Two electrodes were then plugged on each side of the cell.

![Figure 6: Plan and side view of the PMMA spacer](image)

Once the cell and electrodes were connected to the EKA and all the tubes were plugged in, the pH and conductivity meters were calibrated. All calibrations, settings and manipulations were performed using the Visio Lab for EKA version 1.03.3507 software. Before performing any measurements on gold samples, the zeta potential of a PMMA reference had to first be determined. Therefore, the EKA was rinsed twice with the electrolyte, for 16 minutes at a differential
pressure of 300 mbar and the potential of the PMMA was measured with a maximum pressure of 700 mbar. The following measurements were performed in a similar manner on SAM modified gold samples with dimensions of 2.5cm × 4.5cm, except that the rinsing time was reduced to 5 minutes.

After each operation was terminated, the software reported the average zeta potential of both the cell components (test sample and PMMA spacer). Based on that value, and the one previously obtained for the PMMA spacer it was then possible to calculate the zeta potential of the test sample.

**4.5 FN covalent binding to SAMs**

In order to covalently attach FN to the gold surface, the SAM modified gold samples were first soaked in an aqueous solution of 15mM NHS and 75mM EDC for 3 hours and then rinsed with nanopure water (Figure 5, below). In the meantime, a 2µg/ml FN solution was prepared in a pH 6 phosphate buffer solution composed of 0.056M NaOH and 50mM KH$_2$PO$_4$. The NHS modified samples were then left to soak for 4 hours in FN solution, after which they were rinsed thoroughly with nanopure water and soaked in a phosphate buffer saline (PBS) solution composed of 2M NaCl, 39.1mM NaOH and 50mM KH$_2$PO$_4$ for 2 hours in order to eliminate non-covalently bonded proteins. The schematics of the reaction are presented in Figure 5 (Section 1.2.4).

**4.6 Chemical and structural characterization of surface monolayers**

The presence of the different functional groups belonging to the molecules involved in each FN binding step was confirmed by measuring their IR spectrum using polarization modulation infrared absorption reflection spectrometry (PM-IRRAS) employing a Bruker Optics PMA50 external module in conjunction with a Tensor 27 FT-IR spectrometer. A liquid nitrogen cooled MTC detector positioned at 82° with respect to the surface normal was used. The PEM-90
photoelastic modulator was adjusted to have a wavelength setting fixed at either 1600 or 2900 cm\(^{-1}\), depending on the region of interest, with half wave retardation (\(\lambda/2\)) of 0.5. The synchronous sampling demodulator was set for an input gain of 10 and output gain of 1. Before taking measurements, the samples of interest had to first be dried with argon and then inserted into the appropriate holder in the PMA50 module. The samples were each scanned for 5 minutes with a resolution of 3 cm\(^{-1}\) and an aperture setting of 6 mm.

Once the scans were completed, the obtained interferogram was deconvoluted to give the infrared spectrum of the sample, using the Bruker OPUS Spectroscopy v.5.0 software. The spectrum contained all the absorption peaks for the specified range, and depending on the peak position, the type of functional group was determined (explained in more detail in Section 5.1). Using the OPUS software it was possible to save the spectrum and perform several manipulations such as cutting it so that only the range of interest is exposed, performing a baseline correction thus leveling all the peaks to zero, smoothing the curves, performing second derivative analysis, as well as integrating and curve fitting it. The integration function allowed obtaining the area under a specific peak which is generally proportional to the surface concentration of the compound represented by that peak. The curve fit and second derivative functions were used on the Amide I bands for obtaining the secondary structure of surface bound FN, as described in detail in the following section.

4.7 Determination of FN secondary structure

As described in the previous section, surface bound proteins can be conveniently and accurately characterized using PM-IRRAS. A protein IR spectrum has two distinct peaks in the 1600-1700 cm\(^{-1}\) and 1500-1600 cm\(^{-1}\) regions of the IR spectrum, termed as Amide I and Amide II bands, respectively. These bands, which are characteristic to all proteins, arise from the different vibrational modes of the polypeptide backbone. The Amide I band represents mainly 80% of the C=O stretching vibration of the Amide group coupled to
approximately 10% C-N stretching and 10% in-plane N-H bending modes [51]. The Amide II band represents mainly 60% N-H bending coupled to some C-N stretching (40%) of the protein’s Amide groups.

The secondary structure of surface bound FN was determined by deconvolution of the Amide I band. In order to perform deconvolution, it was first necessary to estimate the number of secondary structure component bands within the Amide I region, along with their respective peak positions. This was done by performing a second derivative analysis on the Amide I band using the derivative function of the OPUS software, to yield a spectrum similar to the one shown in Figure 7. As can be seen, the second derivative peaks helped identify the different absorption band positions pertaining to the Amide 1 spectrum.

Figure 7: Second derivative spectrum of an FN Amide I band where FN was covalently bonded to a homogenous COOH SAM, displaying the peaks of the different secondary structure components.

The determined peaks were then inputted in the OPUS curve fit function and the fitting calculations were initiated. The same peak positions were used for
curve fitting the Amide I bands of all samples in order to maintain consistency. The peak positions as well as the type of curve (Lorentz) were fixed and the software used an iterative least square approach to adjust the widths and the heights of each component band so as to obtain the best fit between the simulated and experimental Amide I peak. Figure 8 shows an example of a deconvoluted spectrum obtained from a SAM-FN sample along with all the underlying peaks. The output of the analysis was the height, width, intensity and integral (area) of each underlying peak. By summing up all the integrals and calculating for each individual peak the proportion of its integral with respect to the total area, it was possible to obtain the relative contribution of that particular secondary structure to the overall response of the protein in the Amide I region. The next step was to associate the different absorption bands to the appropriate secondary structure components. This process was not straightforward since these peaks vary with different types of proteins and measurement techniques, and therefore the literature was not quite consistent. With the help of several literature sources [52, 53 and 54], and thorough analysis, the associations seen in Table 3 were established. The purpose of this curve fitting method is not necessarily to determine the exact quantification of secondary structure components but more to verify and follow the changes in conformation [52]. For components possessing multiple Amide I absorption bands such as beta sheets (Table 3), their relative quantity was determined by adding all the relative peak areas pertaining to that structure.
Figure 8: Deconvoluted spectrum of a SAM-FN sample along with all the underlying peaks each pertaining to a specific secondary structure component.
Table 3: Chosen peak position association to secondary structure component.

<table>
<thead>
<tr>
<th>Peak Position (cm⁻¹)</th>
<th>Secondary Structure Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1618</td>
<td>Beta Sheet</td>
</tr>
<tr>
<td>1625</td>
<td>Beta Sheet</td>
</tr>
<tr>
<td>1630</td>
<td>Beta Sheet</td>
</tr>
<tr>
<td>1638</td>
<td>Beta Sheet</td>
</tr>
<tr>
<td>1648</td>
<td>Random Coil</td>
</tr>
<tr>
<td>1655</td>
<td>Alpha Helix</td>
</tr>
<tr>
<td>1662</td>
<td>Beta Turn</td>
</tr>
<tr>
<td>1670</td>
<td>Beta Turn</td>
</tr>
<tr>
<td>1677</td>
<td>Beta Sheet</td>
</tr>
<tr>
<td>1685</td>
<td>Beta Turn</td>
</tr>
<tr>
<td>1690</td>
<td>Beta Turn</td>
</tr>
</tbody>
</table>

4.8 Cell Attachment and Proliferation

SAM and SAM-FN samples were first prepared as explained above and stored overnight at room temperature in a 7.4 PBS solution similar to the one described above but with a NaCl concentration of 0.1M. Each sample was produced in 18 replicates, half of which was used for cell attachment and the other half for cell proliferation. Three incubation times were used for each cellular activity (attachment/proliferation) for which three replicates were made, giving a total of nine samples per activity. After all samples were soaked in PBS overnight, they were placed in plates containing 24 wells, each of which was filled with cell medium containing approximately 40,000 HUVECs. The samples were then incubated at 37°C for 1, 2 and 4 hours (cell attachment) and 2, 5 and 8 days (cell proliferation). After the samples were incubated for the required amount of time, they were transferred into new welled plates and rinsed with PBS.
to remove any non-specifically adhered cells. The wells were then filled with a media/WST-1 proliferation kit solution at a volume ratio of 10:1 and incubated at 37°C for a sufficient amount of time to allow the kit and the cells to properly react. The occurrence of a reaction was identified when the incubated solution became colored, the intensity of which was related to the amount of adhered cells. A small quantity of the newly formed colored solution from each well was removed and placed in a smaller well on a different plate. The plates were then placed in a microfilter plate reader and the absorbance was measured. A calibration curve relating the absorbance to the amount of cells was used in order to determine the actual cell density for a specific absorption. The spectrometer used was a Universal Microplate Reader, EL800 from Bio-TEK instrument, and the reference wavelength was ~650nm.
CHAPTER 5

5. Results and Discussion

5.1 SAM characterization

The chemical and structural characterization of SAMs formed on the gold surface was investigated by PM-IRRAS since it was important to ensure their presence on the gold surface. Figure 9 shows the absorption peaks of the CH\textsubscript{2} groups of the alkyl chain forming the COOH SAM. The peaks at 2927 cm\textsuperscript{-1} and 2855 cm\textsuperscript{-1} represent the asymmetric and symmetric C-H stretching vibrations of the CH\textsubscript{2} groups, respectively. Thus the results in Figure 9 clearly demonstrate the presence of the COOH SAM on the gold surface. A shift in the peak from 2918 to 2927 indicates that the SAMs have a disordered structure. Namely, the magnitude of the shift corresponds to a change from a condensed, almost “all-trans” conformational phase to a liquid-like, gauche-disordered phase. This is most likely due to the surface non-homogeneity (surface roughness and charge, crystal orientation) of the polycrystalline gold used in these experiments [55].

The stability of the homogenous COOH SAM was tested by immersing it for two days in a 0.1M sodium hydroxide (NaOH) solution. This solution is highly aggressive and easily removes chemical layers that are not tightly bound to a surface. As shown in Figure 10, there was an insignificant change in peak height which indicates that the formed SAM is a highly stable structure.
Figure 9: PM-IRRAS spectrum of COOH SAMs in the 2840-2980 cm\(^{-1}\) region, showing the asymmetric (2927 cm\(^{-1}\)) and symmetric (2855 cm\(^{-1}\)) CH\(_2\) stretching peaks.

Figure 10: Comparison of COOH SAM peaks in the 2840-2980 cm\(^{-1}\) region after SAM formation (a) and after immersing the sample in NaOH for two days (b).
Figure 11 shows the spectrum for the same SAM but in the 1750-1200 cm\(^{-1}\) region. The peaks at 1716 cm\(^{-1}\) and 1567 cm\(^{-1}\) correspond to the asymmetric and symmetric stretching of the carbonyl group belonging to the carboxyl terminal of the SAM, respectively. The occurrence of C-H bending of the CH\(_2\) group and C-O stretching of the carbonyl group is also evidenced by the peaks at 1462 cm\(^{-1}\) and 1269 cm\(^{-1}\), respectively.

![PM-IRRAS spectrum of COOH SAMs in the 1200-1750 cm\(^{-1}\) region showing the asymmetric (1716 cm\(^{-1}\)) and symmetric (1567 cm\(^{-1}\)) carbonyl stretching, the C-H bending (1462 cm\(^{-1}\)) and C-O stretching (1269 cm\(^{-1}\)) peaks.](image)

The PM-IRRAS spectrum of the OH SAM recorded in the 2840-2980 cm\(^{-1}\) region is similar to the one shown in Figure 9, except that the CH\(_2\) asymmetric stretching peak was shifted to a lower wavenumber 2926 cm\(^{-1}\) rather than 2927cm\(^{-1}\) (see Figure A.11 in appendix). Figure 12 shows the OH SAM spectrum in the 1200-1750 cm\(^{-1}\) region. The peaks pertaining to the C-O stretching of the carbon atom attached to the oxygen of the OH group and the C-H bending of the CH\(_2\) groups can once again be observed. The lack of carbonyl groups on that molecule can be seen by the absence of the carbonyl peaks in the 1500-1700 cm\(^{-1}\) region.
Figure 12: PM-IRRAS spectrum of OH SAMs in the 1200-1750 cm\(^{-1}\) region, showing the C-H bending (1463 cm\(^{-1}\)) and C-O stretching (1264 cm\(^{-1}\)) peaks.

The NH\(_2\) SAM also contains an alkyl chain of CH\(_2\) groups and therefore shares a similar spectrum in the 2840-2980 cm\(^{-1}\) region as the OH and COOH SAMs (see Figure A.12 A.12 in appendix).

Figure 13 represents the spectrum of the CH\(_3\) SAMs in the 2840-2980 cm\(^{-1}\) region. The two CH\(_2\) stretching peaks can once again be observed and the order of these SAMs seems to be slightly better than the other SAMs, since the asymmetric C-H stretching is slightly shifted to a lower wavenumber, 2924 cm\(^{-1}\). Due to the presence of the CH\(_3\) terminal group, the corresponding C-H asymmetric and symmetric stretching vibrations can also be observed at 2965 cm\(^{-1}\) and 2879 cm\(^{-1}\), respectively [56]. These peaks were absent in all the other SAM spectra in that region.
Figure 13: PM-IRRAS spectrum of CH₃ SAMs in the 2840-2980 cm⁻¹ region, showing the asymmetric (2927 cm⁻¹) and symmetric (2855 cm⁻¹) CH₂ stretching as well as the asymmetric (2965 cm⁻¹) and symmetric (2879 cm⁻¹) CH₃ stretching peaks.

Figure 14 shows the absorption peaks of the CH₃ SAM in the 1200-1750 cm⁻¹ region. The peak at 1463 cm⁻¹ can once again be observed due to the C-H bending of the CH₂ group. The other two peaks are due to C-H bending of the CH₃ terminal group [56].
Figure 14: PM-IRRAS spectrum of CH$_3$ SAMs in the 1200-1750 cm$^{-1}$ region, showing the CH$_2$ bending (1264 cm$^{-1}$) of and CH$_3$ bending (1463, 1372 cm$^{-1}$).

The results presented in this section clearly confirmed the presence of all the SAMs that were used in this research, on the gold surfaces. The results also confirmed that the formed SAMs are not well-ordered, but rather of an amorphous structure. The homogenous COOH SAM was shown to be rather stable and it was assumed that the other SAMs were stable as well since they have the same sulfuric headgroup and size of the alkyl chain.

5.2 NHS characterization and SAM surface composition

This section will describe the NHS spectrum and what the main peaks represent. It will also discuss how the NHS peaks can potentially be used to obtain the SAM surface composition of COOH SAMs.

Figure 15 shows a spectrum that was obtained after NHS was reacted with a COOH SAM modified surface (reaction shown in Figure 5). The peaks at 1746 and 1786 cm$^{-1}$ correspond to the asymmetric and symmetric stretching of the
carbonyl groups belonging to NHS, respectively. The small peak at 1818 cm\(^{-1}\) demonstrates that the NHS has indeed covalently bonded to the SAMs since it belongs to the carbonyl stretching of the ester formed by the NHS/MUA bond [57].

![Figure 15: Spectrum of NHS covalently bonded to COOH SAMs showing the asymmetric (1746 cm\(^{-1}\)), symmetric (1786 cm\(^{-1}\)) and the ester (1818 cm\(^{-1}\)) C-O stretching peaks.](image)

The area under 1746 cm\(^{-1}\) peak is proportional to the amount of covalently bonded NHS. This was used to evaluate the NHS binding kinetics, which is presented in Figure 16. The graph shows that after 30 minutes the NHS binding to the 100% COOH SAM stops under the experimental conditions applied. Similar measurements were performed on samples prepared with different surface ratios of CH\(_3\)/COOH alkanethiols, and the optimum NHS binding time for all ratios was calculated to be 3 hours. Namely, with a decrease in the relative amount of COOH on the surface, the NHS binding time increases.
Figure 16: Relative NHS peak area as a function of reaction time. The area under 1746 cm$^{-1}$ peak was normalized with respect to the area obtained after 2.5 hours of binding. The solid line is plotted only as a visual aid and does not represent efforts to model/fit the experimental data.

It was also demonstrated (explained below) that the relative amount of covalently bonded NHS depends on the relative amount of COOH groups on the surface. Figure 17 shows the relative areas under the 1746 cm$^{-1}$ peak as a function of the molar fraction of COOH terminal alkanethiols in the binary thiol mixtures for all three pairs of mixed SAMs. It is important to note that in Figure 17, as well as in most of the other figures presented later, the 0% solution fraction of COOH on the x-axis represents samples composed of homogenous SAMs of alkanethiols with CH$_3$, OH and NH$_2$ terminal functional groups. No NHS binding was performed on these samples due to the absence of the COOH group.

When looking at Figure 17, it can be seen that for OH/COOH mixed SAMs the relative amount of covalently bonded NHS is practically equal to the percentage of COOH SAMs in the solution. This could indicate that the fraction of surface bound COOH SAM is equal to its fraction in the binary solution and therefore both molecules with polar head groups have an equal affinity to the gold surface. Schweiss et al. [29] came to the same conclusion after performing XPS measurements of the surface compositions of their samples. This is considered to be a good indication that NHS binding is representative of the amount of COOH SAMs.
Figure 17: Relative areas of NHS peaks as a function of percent COOH alkanethiols in the binary solution for all three pairs of mixed SAMS. The area under 1746 cm\(^{-1}\) peak was normalized with respect to the area obtained with a SAM containing 100% of COOH on the gold surface. The error bars represent the standard deviation based on two replicates.

For the case of CH\(_3\)/COOH SAMs, the curve slope (triangles) is lower than that of the OH/COOH SAM (circles). This indicates that the non polar CH\(_3\) molecules may have a higher affinity to the gold surface than the COOH terminal molecules. Zhuk et al. [58] obtained the surface fraction of COOH SAMs on gold for a large diversity of mixture ratios using XPS and also came to the same conclusion. Figure A.13 in the appendix clearly demonstrates that at low COOH alkanethiol fractions, there is a preferential formation of CH\(_3\) SAMs. All these results reinforce the assumption that the relative COOH SAM surface fraction can be estimated using NHS peaks.

Arima et al. [20] (Figure A.2), who used another approach to calculate the surface fraction of the CH\(_3\) functional group for CH\(_3\)/COOH SAMs, came to the same conclusion regarding the preferential adsorption of the CH\(_3\) alkanethiol. This involved using the C-H asymmetric stretching peak of that group (at 2964 cm\(^{-1}\)). The area under that peak, the authors postulated, reflects the relative quantity of CH\(_3\) on the surface. A similar experiment was performed during this
research and the results are displayed in Figure 18. It can be seen that the slope of the line is closer to unity than the one in Figure 17 (triangles) which, indicates that there was no significant preferential adsorption of the CH₃ group. The intensity of the CH₃ stretching mode is influenced by the orientation of the functional group with respect to the surface (the surface selection rule), and the results in Figure 18 cannot be taken to represent the true surface CH₃/COOH ratio with high confidence.

![Graph](image)

Figure 18: Surface fraction of COOH SAMs based on the relative areas of the C-H asymmetric stretching of the CH₃ group peak as a function of percent COOH alkanethiols in the binary solution. The area under 1964 cm⁻¹ peak was normalized with respect to the area obtained with a SAM containing 100% of CH₃ on the gold surface. The error bars represent the standard deviation based on two replicates.

The data for the NH₂/COOH SAM in Figure 17 (squares) clearly indicate that the NH₂ terminal molecules bind more readily on the gold surface than the COOH molecules, as indicated by a small slope of the line (0.27). Chuang et al. [30] also concluded that the gold surfaces were “amine-rich” after soaking them in binary solutions of COOH and NH₂ alkanethiols at different mixture ratios (Figure A.14 in the appendix). A possible origin of such behaviour could be related to a difference in the relative solubilities of the alkanethiols in the ethanol solution [30].

The observations drawn from the results presented in this section provided a simple and promising insight on developing a method to evaluate the surface
fraction of COOH SAMs. However due to the sensitivity of the signal to the orientation of the molecules, there is some uncertainty regarding the true surface composition. Therefore, the results presented in the remaining portion of the thesis will refer to the solution composition of the SAM-forming compounds, rather than the surface composition. The same approach has also been commonly adopted in literature.

5.3 Protein binding, characterization and quantification

As mentioned before, surface bound proteins can be conveniently and accurately characterized using the PM-IRRAS (Section 4.7). An example of a FN spectrum covalently bonded to COOH SAMs is shown in Figure 19. The presence of the Amide I and Amide II bands is reflected by the peaks at 1650 and 1546 cm\(^{-1}\), respectively.

![Figure 19: IR Spectrum of FN covalently bonded onto the homogenous COOH SAM modified gold surface showing the NHS (1741 cm\(^{-1}\)), Amide I (1650 cm\(^{-1}\)) and Amide II (1546 cm\(^{-1}\)) peaks.](image)
Since NHS had to be used prior to covalently binding the protein, its presence is apparent from the peak at 1741 cm\(^{-1}\). If the protein was simply adsorbed and therefore the use of NHS was not required, that peak would have been absent in the protein spectrum. For all covalently bonded protein spectra, the size of the NHS peak decreased significantly from its original size, since the NHS molecule was replaced by FN after binding (reaction scheme in Figure 5). However, the peak always remained present, and was still quite prominent on samples with a high fraction of COOH groups, which indicates that the protein does not necessarily bind to all carboxyl groups. This is quite logical considering the difference in size between the protein and NHS molecule.

In order to optimize binding of FN to the surface-bound NHS, BSA was used instead of FN since it is less costly. However, it should be emphasized that the reaction chemistry remains the same.

Figure 20 shows a set of IR spectra of BSA bonded to a 100% COOH SAM recorded at different times. With an increase in time, the two Amide peaks also increase, while the NHS peak decreases. The corresponding relative amount of BSA surface concentration was obtained by evaluating the area under the Amide I band, which is proportional to the protein surface concentration. Figure 21 demonstrates the reaction kinetics between BSA and NHS. With time, the amount of surface-bound BSA increases and gradually approaches a plateau (not attained during the time interval presented), while the surface concentration of the free (non-reacted) NHS decreases. The opposite trend of the BSA and NHS surface concentration clearly demonstrates the occurrence of the reaction between the protein and surface-bound NHS.

The stability of BSA was also tested at high shear and temperature conditions. After BSA has been covalently bonded to a homogenous COOH SAM, the sample was suspended in a beaker containing a solution of PBS (pH 7.4) and a magnetic stirrer. The solution was heated to 60°C and the stirred vigorously for 24 hours. The SAM-BSA sample was scanned using the PM-IRRAS at several time intervals and the Amide I band of each scan was recorded. Figure 22 shows selected scan results, and despite the rather long experiment
time, high temperature, and shear the result clearly indicates that the sample (i.e. BSA) was relatively stable (covalently attached to the surface) since the decrease in the integrated peak area is insignificant. This confirms that the employed protein immobilization method (covalent attachment) is appropriate.

Figure 20: IR spectra showing the decrease in NHS peak and the increase of the corresponding BSA peak with respect to time. (a) NHS peak before BSA binding, (b) NHS and BSA peaks after 15 minutes of BSA soaking, (c) after 30 minutes of BSA soaking, (d) after 60 minutes of BSA soaking.
Figure 21: Relative peak areas of NHS and BSA as a function of reaction time for a 100% COOH SAM. The values were obtained from the data presented in Figure 20.

Figure 22: Amide I band of BSA covalently bonded onto homogenous COOH SAMs at different times in high temperature/shear setup: initially (a), after 30 minutes (b), after 3 hours (c) and after 24 hours (d).

The next step in this research was to investigate the relationship between the surface composition of the SAM (X/COOH, where X=CH₃, OH and NH₂) and
FN surface amount. The results are presented in Figure 23 as the dependence of the Amide I peak area of the surface-bound FN on the bulk solution fraction of COOH in the mixed X/COOH (one plot for each SAM pair). As can be observed, the amount of surface-bound protein on NH$_2$/COOH SAMs (Figure 23a) does not seem to be significantly dependent on the fraction of COOH ($p=0.34$, $\alpha=0.05$). For CH$_3$/COOH ($p=0.004$) and OH/COOH ($p=0.01$) SAMs there is a statistically significant variation although the fraction of COOH groups does not seem to directly influence the quantity of immobilized protein. Therefore, these results indicate that there is no direct relationship between the relative amount of COOH on the X/COOH surface and the quantity of covalently bonded FN. These results are logical knowing that the difference in size between the protein and NHS molecule is rather large. Namely, only a small number of NHS molecules (which are attached only to COOH-SAM molecules) immobilized on the Au surface is needed to bind a layer of FN molecules, and it appears that a 15% fractional surface coverage by NHS is as equally sufficient in binding a layer of BSA as a 100% fractional coverage.
Figure 23: Amide I peak areas of surface bound FN for all ratios of CH$_3$/COOH (a), OH/COOH (b) and NH$_2$/COOH SAMs (c) obtained from PM-IRRAS spectra. The error bars represent the standard deviation based on three replicates.
5.4 Influence of physico-chemical properties of mixed SAMs on surface hydrophobicity

Contact angle measurements were performed on all the prepared samples in order to test the influence of different SAM types and ratios on surface hydrophobicity. The contact angle values obtained for bare gold and homogenous SAM surfaces can be seen in Table 4. It is evident that the CH$_3$ SAM is a hydrophobic surface while all other SAMs are almost equally hydrophilic (p-value of 0.1). The trend observed is consistent with the literature (Table 1).

Table 4: Contact angle of gold and Homogenous SAM surfaces.

<table>
<thead>
<tr>
<th>Homogenous SAM terminal groups</th>
<th>Contact Angle ($^\circ$)</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_3$</td>
<td>94.7</td>
<td>1.59</td>
</tr>
<tr>
<td>NH$_2$</td>
<td>56.5</td>
<td>3.64</td>
</tr>
<tr>
<td>COOH</td>
<td>51.2</td>
<td>4.03</td>
</tr>
<tr>
<td>OH</td>
<td>49.5</td>
<td>2.70</td>
</tr>
<tr>
<td>Bare gold</td>
<td>81.7</td>
<td>3.80</td>
</tr>
</tbody>
</table>

Figures 24, 25 and 26 show the results that were obtained for CH$_3$/COOH, OH/COOH and NH$_2$/COOH SAMs at different solution compositions, respectively. From Figure 24 it can be seen that the contact angle of CH$_3$/COOH SAM mixtures is not linearly related to the solution fraction of COOH terminal alkanethiols. Similar trends were also observed by Arima et al. [20] (Figure A.1) and Baker et al. [28] (Figure A.3) as mentioned in Section 2.1. Furthermore based on their contact angle results, Baker et al. [28] concluded that there was preferential binding of the CH$_3$ alkanethiols onto the gold surface.
Figure 24: Contact angle of CH$_3$/COOH SAMs with respect to the solution fraction of COOH alkanethiols where 0% represents the homogenous CH$_3$ SAM. The error bars represent the standard deviation based on three replicates.

The contact angle of different surface compositions of the OH/COOH SAM (Figure 25) does not follow any regular trend, even though the contact angle of the homogeneous OH and COOH SAMs was practically equal (Table 4). For certain mixtures, the surfaces actually exhibited a significantly higher contact angle. Nevertheless, all surfaces are relatively hydrophilic, as illustrated in Figure 25. This irregularity agrees with the results reported by Schweiss et al. [29] and Baker et al. [28] (Figure A.4), as previously explained in Section 2.1. According to Baker et al. [28], the irregularity in trend is due to the relatively high-energy surfaces exhibited by mixtures of OH and COOH groups. In order to reduce these energies, such surfaces have the tendency to adsorb air-born organic contaminants from the laboratory air, to which they are susceptible after they are dried and prepared for contact angle measurements. Since homogenous OH and COOH SAMs are almost equally hydrophilic, small inconsistencies can be quite apparent when they are mixed and may therefore lead to irregular trends.
Figure 25: Contact angle of OH/COOH SAMs with respect to the solution fraction of COOH alkanethiols where 0% represents the homogenous OH SAM. The error bars represent the standard deviation based on three replicates.

SAMs composed of NH$_2$ and COOH terminal alkanethiols also do not show any regular trend in terms of contact angle vs. solution fraction of the COOH groups, as can be seen from Figure 26, which is due to the same reasons as for OH/COOH SAMs [30], and is in accordance with the results published by Chuang et al. [30]. As mentioned in Section 2.1, a similar curve was obtained by plotting data points from Chuang et al. [30] (Figure A.5).

Figure 26: Contact angle of NH$_2$/COOH SAMs with respect to the solution fraction of COOH alkanethiols where 0% represents the homogenous NH$_2$ SAM. The error bars represent the standard deviation based on three replicates.
5.5 Influence of physico-chemical properties of mixed SAMs on zeta potential

Zeta potential measurements were also performed on all the prepared samples in order to later investigate the influence of surface charge on the cell/surface interactions. The results for CH$_3$/COOH, OH/COOH and NH$_2$/COOH samples are plotted in Figures 27, 28 and 29, respectively.

Figure 27 illustrates that with the increase in surface fraction of negatively charged COOH end groups, the corresponding zeta potential of CH$_3$/COOH SAMs also increases in the negative direction, which was quite an expected trend.

![Figure 27: Zeta potential of CH$_3$/COOH SAMs with respect to the fraction of COOH alkanethiols in solution where 0% represents the homogenous CH$_3$ SAM. The error bars represent the standard deviation based on three replicates.](image)

The plot in Figure 28 shows the zeta potential results for OH/COOH SAMs. Both the OH and COOH groups are hydrophilic, but the OH SAM does not have any surface charge (zero), while the COOH SAM is negatively charged, as already discussed. Thus, one would expect an increase in zeta potential in the positive direction with an increase in the OH surface fraction, which is what Figure 25 indeed demonstrates, although not in a linear manner as expected. Namely, at low OH surface fractions (<30%), the surface charge is still dominated by the negative COOH charge, which is due to the ‘charge shielding effect’ of the
COOH groups. However, with an increase in OH surface fraction, this effect starts decreasing and the negative surface charge decreases. In conclusion, the result clearly shows that the 100% OH SAM is less negatively charged than the 100% COOH SAM, as expected.

![Graph](image)

Figure 28: Zeta potential of OH/COOH SAMs respect to the fraction of COOH alkanethiols in solution where 0% represents the homogenous OH SAM. The error bars represent the standard deviation based on three replicates.

When considering NH$_2$/COOH mixed SAMs, since the NH$_2$ group is hydrophilic and positively charged, one could expect to see an increase in zeta potential in the positive direction with an increase in the NH$_2$ fraction on the surface. This is, indeed, clearly demonstrated in Figure 29 at low NH$_2$ alkanethiol solution fractions (up to 50%). However, at NH$_2$ fractions above 50%, the zeta potential of the surface remains constant, indicating the ‘surface charge saturation’. Nevertheless, the result in Figure 29 clearly demonstrate that the NH$_2$ covered surface is more positive then the COOH covered surface, which is in agreement with literature, and also expected considering individual charge properties of the two head groups.
Figure 29: Zeta potential of NH$_2$/COOH SAMs respect to the fraction of COOH alkanethiols in solution where 0% represents the homogenous NH$_2$ SAM. The error bars represent the standard deviation based on three replicates.

5.6 Influence of physico-chemical properties of mixed SAMs on the secondary structure of covalently bound fibronectin

As already mentioned, cell/surface interactions are greatly modulated by FN/surface and cell/FN interactions. Cell/FN interactions depend on the conformation (secondary and tertiary) of the surface-bound (or adsorbed) FN, which in turn depend on the physico-chemical properties of the surface. Thus, it is very important to investigate the conformation of covalently attached FN on the SAM-modified gold surface. In this work, the dependence of the FN secondary structure on the physico-chemical properties of mixed SAMs on the gold surface was investigated.

The secondary structure of FN was evaluated using the OPUS curve fit function by fitting the Amide I band, according to the procedure described in Section 4.7. FN was covalently bonded on all three types of mixed SAMs, but a set of measurements in which the protein was also physically adsorbed to homogenous SAMs with 100% CH$_3$, OH and NH$_2$ terminal functional groups was also performed for comparison. After protein attachment or adsorption, all samples were thoroughly rinsed, left immersed in PBS overnight and scanned the
following morning. A small volume of a more concentrated protein solution (1mg/ml) was also deposited onto bare gold samples and left to dry. Since the protein was quite concentrated, this produced several layers of FN on the surface. This sample was then used to evaluate the secondary structure of native FN, which is here used as a control sample.

Figures 30, 31 and 32 show the dependence of the secondary structure of FN adsorbed to homogenous CH₃, OH and NH₂ SAMs (0% data point), and FN covalently bound to binary mixtures of CH₃/COOH, OH/COOH and NH₂/COOH SAMs (and also the homogenous COOH SAM). The results for the native protein are included as a control. The figures present four curves, each of which represents the relative content of the different types of secondary structure components (beta-sheet, random coil, alpha-helix, beta-turn).

![Graph](image)

Figure 30: Secondary structure components for CH₃/COOH SAMs where 0% represents the homogenous CH₃ SAM. The protein is chemically bound to the binary SAMs and 100% COOH SAM, while it is adsorbed on the homogenous CH₃ SAM. The error bars represent the standard deviation based on three replicates.
Figure 31: Secondary structure components for OH/COOH SAMs where 0% represents the homogenous OH SAM. The protein is chemically bound to the binary SAMs and 100% COOH SAM, while it is adsorbed on the homogenous OH SAM. The error bars represent the standard deviation based on three replicates.

Figure 32: Secondary structure components for NH$_2$/COOH SAMs where 0% represents the homogenous NH$_2$ SAM. The protein is chemically bound to the binary SAMs and 100% COOH SAM, while it is adsorbed on the homogenous NH$_2$ SAM. The error bars represent the standard deviation based on three replicates.
Three statistical analysis of the results was performed in order to:

- evaluate the influence of SAM composition on the covalently bonded FN secondary structure (Table 5, 3\textsuperscript{rd} column),
- evaluate whether chemical binding of FN to SAMs induces a change in its secondary structure with respect to the native structure (Table 5, 4\textsuperscript{th} column).
- compare the differences in secondary structure of covalently bonded FN to adsorbed FN (Table 5, 5\textsuperscript{th} column),

From the third column in Table 5 it is clear that for each set of SAMs, the secondary structure of covalently bound FN is not statistically significantly influenced by the surface ratio of the SAMs. For each secondary structure component, the $p$-value is higher than 0.05. When observing the forth column (middle $p$-value column), which compares the secondary structure of covalently bonded protein to its secondary structure in native state, it is quite evident that for the beta-sheet, random coil and beta-turn components of all samples, the $p$-values obtained are below 0.05. This clearly indicates that for these components, there is indeed a statistically significant difference between the structure of native FN and FN covalently bound to the SAMs. For the case of the alpha-helix component, there was no statistically significant difference in the secondary structure content between the native FN and FN covalently bonded to all three sets of SAMs ($p > 0.05$).

The 5\textsuperscript{th} column in Table 5 compares the secondary structure components of FN covalently bonded to SAMs with the structures of FN physically adsorbed to the same SAMs. The $p$-values of certain secondary structure components were found to be below 0.05 (beta sheet of CH$_3$/COOH SAM-FN and alpha helix NH$_2$/COOH of SAM-FN samples). This indicates that for these components, there is a statistically significant difference in secondary structure between adsorbed and covalently bonded FN.
Thus, the major conclusions that can be drawn from the secondary structure results presented in Table 5 are that the change in physico-chemical properties of the SAMs (within the same SAM type) does not induce secondary structure changes of FN covalently bonded to them. However, the chemical binding, in turn, does induce secondary structure changes relative to the structure of native FN. The results presented in this section clearly indicate that although there is no significant dependence of FN secondary structure on the SAM composition of a specific SAM pair, there is a significant difference when the comparison is made between the structure of FN chemically or physically bound to the SAM, and native FN. Nevertheless, the results in Figures 30, 31 and 32 show that these relative changes are not significant. Furthermore, cell/surface interactions presented later in the thesis will show that the change in secondary structure of FN from the native form does not influence endothelial cell attachment and proliferation on the SAM-FN modified surface. In fact, it will be shown that the presence of FN on the SAM-covered surface is greatly beneficial to cell/surface interactions.
Table 5: $p$-values comparing secondary structure components of all SAM-FN samples for each specific SAM pair.

- third column: $p$-values for covalently bound FN;
- forth column: $p$-values calculated on the basis of comparison of samples with covalently bound FN to the secondary structure of native FN;
- fifth column: $p$-values calculated on the basis of comparison of samples with covalently bound FN to the secondary structure of FN adsorbed on the same SAMs.

<table>
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<th>SAMs</th>
<th>Secondary structure component</th>
<th>Samples with only covalently bonded FN</th>
<th>Samples with covalently bonded FN and native FN</th>
<th>Samples with covalently bonded and adsorbed FN</th>
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5.7 Influence of surface properties on cell attachment and proliferation

5.7.1 Overview

Endothelial cell (EC) testing experiments were performed on both samples modified only with selected SAMs, and samples modified with the same SAMs but to which FN was covalently bound. This was done in order to evaluate the effect of FN on EC/surface interactions and to separate the effect of the underlaying SAM. The ratios of mixed SAMs of X:COOH, where X=CH₃, OH, NH₂, that were used were 0:100, 15:85, 50:50, 70:30, 100:0. Obviously, FN could only be covalently bonded on samples that contained the COOH functional group. The EC attachment was tested for 1, 2 and 4 hours, and EC proliferation for 2, 5 and 8 days. For each type of measurement, 3 replicates were made.

5.7.2 Results for CH₃/COOH-based SAMs

Figure 33 and Figure 34 show the EC attachment and proliferation results for (a) CH₃/COOH and (b) CH₃/COOH-FN samples. Table 6 shows the order in which EC attachment and proliferation decreases as a function of the fraction of COOH in the solution. These results are also compared to bare gold surfaces (Au). In this table, if a $p$-value of the cell density between two sample averages was determined to be above 0.05, it was assumed that there was no significant difference between the samples, and the symbol (~) was used. The effect of binding FN to the SAM on the EC attachment and proliferation is summarized in Table 7, as a percentage in the increase in EC surface density (the EC density on the surface covered only with the bare SAM is taken as control). It is important to note that throughout the text, the percentages used to refer to the different SAM compositions will be the percent of COOH alkanethiols in solution.
Figure 33: Cell attachment on (a) CH$_3$/COOH SAM, and (b) CH$_3$/COOH SAM with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Figure 34: Cell proliferation on (a) CH$_3$/COOH SAM, and (b) CH$_3$/COOH SAM with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Table 6: Order of decreasing EC attachment (a) and proliferation (b) as a function of the SAM composition expressed as a percentage of COOH groups in the CH$_3$/COOH alkanethiol solution mixture. The table shows the data for both the gold surface covered with only a SAM, and the gold surface covered with a SAM to which FN was covalently attached.

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<th>sample type</th>
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<td>SAMs 85-100-50-30-Au-0</td>
<td>85-100-30-50-0-Au</td>
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<tr>
<td>SAM-FN 85-50-30&gt;100&gt;Au</td>
<td>85-100&gt;50&gt;30&gt;Au</td>
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Table 7: Influence of FN bonded on the CH$_3$/COOH SAM on the EC attachment (a) and proliferation (b), expressed in terms of the percentage of the cell surface density increase with respect to the density on the corresponding SAM surface measured in the absence of FN.

<table>
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<table>
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<th>% COOH alkane thiol in solution</th>
<th>proliferation time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>30</td>
<td>37</td>
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<td>85</td>
<td>20</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
</tr>
</tbody>
</table>
5.7.2.1 EC attachment on CH₃/COOH-based SAMs

Figure 33a and Table 6a demonstrate that at the attachment time of one and two hours, the SAM presence on the Au surface and its composition does not significantly influence EC attachment, except when the surface is covered with a 85% COOH SAM. However, at the attachment time of four hours, the presence of the SAM on the gold surface does influence the EC attachment, as demonstrated by the increase in EC surface density between the bare gold and 0% COOH (i.e. 100% CH₃) surface. By further increasing the ratio of COOH on the surface, the EC surface density only slightly, but not significantly increases \((p>0.05)\), reaching the maximum value on the 100% COOH surface. The trends from Figure 39a are more clearly summarized in Table 6a.

Figure 33b shows that the presence of covalently bound FN on the SAM-covered gold surface does influence the EC attachment. For the attachment time of one hour, the EC cell density for the 30, 50 and 85% COOH SAMs is not significantly different. However, the cell density on these surfaces is significantly higher than on the 0% (no FN on this surface), 100% COOH SAM surface and bare gold surface (see Table 6). After two hours of attachment time, the presence of FN starts to show a significant influence on the EC attachment. Namely, the two FN-free surfaces (bare gold and 0% COOH) show a significantly lower EC surface density then the remaining four surfaces on which FN is present (30, 50, 85 and 100% COOH). The 85% and 100% COOH surfaces seem to yield the highest EC surface density. A similar trend is observed after four hours of attachment, but in this case, in addition, the presence of pure CH₃ SAM (0% COOH SAM) does influence the EC cell attachment in comparison to the bare gold surface. The statistical analysis showed that no significant difference in EC surface density exists on the 50, 85 and 100% COOH surfaces.

The effect of FN attached to the SAM on the EC attachment is summarized in Table 7a, as a percentage of EC surface density increase with respect to the density in the absence of FN. There is no any particular trend in the behaviour with respect to the solution fraction of COOH alkanethiols. Thus, while for the attachment time of one hour, the highest increase was obtained for the surface
covered by 50%CH$_3$-50%COOH-FN (50% SAM-FN), the 100% SAM-FN gave the highest increase (of 50%) after the second hour of EC attachment. After four hours of attachment the increase in EC surface density decreased for all samples when compared to the second hour which indicates that FN had the highest effect on EC attachment after 2 hours. This confirms that the rate of cell attachment was higher on SAM-FN surfaces since CH$_3$/COOH SAM surfaces with no FN exhibited a delay in EC adhesion. As explained in Section 1.2.4 this is due to the fact that EC cells have to start producing their own ECM proteins in order to displace preadsorbed serum proteins such as BSA that was present in the FBS solution containing HUVECs, and enhance subsequent cell attachment. On SAM-FN samples the presence of FN allowed drastically reducing that delay in cell adhesion since FN was already ‘available’ on the surface for cell binding, and it also prevented BSA adsorption and the need for its replacement by cell-expressed FN. In conclusion, the results in Table 7 clearly demonstrate that the presence of FN on the SAM-covered surface significantly increases the attachment of EC at all times.

Due to the absence of any visible regular trend presented in Figure 33 and Table 6a, it is not possible to directly draw any conclusions from these figures on the influence of surface physico-chemical properties on the EC attachment. However, this is clearly visible in Figure 35a and Figure 36a, which illustrate the dependence of the surface cell density on the contact angle and zeta potential (surface charge), respectively, of the SAM and SAM-FN samples. The cell attachment data points were taken from the 4 hour attachment results. It is important to note that for the SAM-FN curves seen in the plots, the data does not include the 0% COOH SAM since this samples contained no covalently bound FN. In order to statistically compare the data presented in Figure 36 and 37, the corresponding $p$-values were calculated.

Figure 35a shows that for the case of SAM surfaces with no covalently bound FN, EC attachment decreased as the contact angle increased, thus indicating that EC attachment occurs more readily on hydrophilic surfaces. The most hydrophilic surface (lowest contact angle) had a statistically significantly
higher cell density than all the rest of the samples. However, there was no significant statistical difference between the remaining samples. For the case of surfaces with covalently attached FN, there was no statistically significant difference in cell density between the first two data points, and there was a statistically significant decrease at the third data points. Cell attachment on the most hydrophobic sample (highest contact angle) was statistically significantly lower than on all other samples.

Figure 36a shows that for SAM samples without any surface bound FN, the sample with the most negative zeta potential (100% COOH SAM) exhibited the highest amount of cell attachment. The cell density decreased as the negative charge decreased, and reached a minimum at the surface with the lowest negative charge (100% CH₃ SAM). Although there was a statistically significant difference in cell density between the most negatively and least negatively charged samples, there was no statistical significant difference between each neighboring data point. Similarly to the results above, for SAM-FN samples the cell density also increased with increasing negative charge of the SAM surface, however there was no significant difference in cell adhesion between the two most negatively charged samples (85 and 100% COOH SAM-FN) as well as between the second and third data points (85 and 50% COOH SAMs-FN). However, all SAM-FN samples yielded a significantly higher cell density than the least negatively charged sample (30% COOH SAM-FN). From all the data presented in Figure 35a and Figure 36a, it can be concluded that the surface wettability and charge influence the cell attachment (the more hydrophilic and negatively charged samples exhibited the highest degree of cell attachment). However, this dependence is not straightforward, indicating that the surface chemistry, i.e. variation in surface functional groups (and their surface ratio in mixed SAMs) also play a role in the cell attachment process. This agrees with Arima et al. [20] who stated that no direct relationship was observed between cell adhesion and contact angle.
Figure 35: Cell attachment (4 hours) (a) and proliferation (8 days) (b) as a function of the contact angle of CH$_3$/COOH SAM and SAM-FN surfaces of varying CH$_3$/COOH ratio. The error bars represent the standard deviation based on three replicates.
Figure 36: Cell attachment (4 hours) (a) and proliferation (8 days) (b) as a function of zeta potential of the contact angle of CH$_3$/COOH SAM and SAM-FN surfaces of varying CH$_3$/COOH ratio. The error bars represent the standard deviation based on three replicates.

5.7.2.2 EC proliferation on CH$_3$/COOH-based SAMs

The cell proliferation results are presented in Figures 35, 35b and 36b, as well as in Table 6b and Table 7b, in a similar manner and order as the cell attachment results.

Figure 34a and Table 6b demonstrate that for SAMs without covalently attached FN, at the proliferation time of two days, the presence of the CH$_3$ group (0% COOH SAM) on the Au surface does not significantly influence EC
proliferation. However, there is a significant increase in the surface cell density when going from the 0% to the 30% COOH SAM, the latter yielding almost the same surface cell density as the 50% COOH SAM. With a further increase in COOH ration in the SAM to 85%, the surface cell density also increases. However, there is no significant difference in cell proliferation between the 85% and 100% COOH surfaces. At the proliferation time of five days, the bare Au surface, and the 0% and 50% COOH SAM surfaces show the lowest degree of cell proliferation, and there is no statistical difference in the cell density among these three surfaces. However, the presence of a SAM on the Au surface seems to have a statistically significant influence on the 30%, 85% and 100% COOH SAM surfaces, with the 85% COOH surface yielding the highest cell density. After eight days of proliferation, the 0% COOH SAM surface yielded statistically the lowest surface cell density, while there was no significant statistical difference among the 50%, 30% COOH SAM and bare gold samples. The 85% COOH SAM surface yielded the highest cell surface density followed by the 100% COOH SAM surface.

Figure 34b when compared to Figure 34a shows that the presence of covalently bound FN on the SAM-covered gold surface does influence the EC proliferation and, as seen in Table 6b, all SAM-FN samples at all three proliferation times gave a significantly higher cell proliferation rate than the 0% COOH SAM (control surface), on which covalently-bound FN was not present. For the proliferation time of two days, the difference in EC density between the 50, 30, 100 and 85% COOH SAMs-FN samples was significant, and cell proliferation reached a maximum on the 85% COOH SAMs-FN. All these samples exhibited a significantly higher cell density than the bare gold surface. After five days of proliferation, there was no significant increase in cell density among the 30, 50 and 100% COOH SAM-FN surface, however the 85% COOH SAM-FN surface yielded a significantly higher amount of cells. Once again all samples, except the 0% COOH SAM had a higher cell density than bare gold. After eight days of proliferation, the cell density increased in the same order as the one after two days of proliferation but that the difference between the 100 and
50% COOH SAM-FN as well as the one between the 50 and 30% SAM-FN was significant. The general observation from the presented results is that the 85% COOH SAM-FN surface seems to yield the highest cell density at all proliferation times.

Refering to Table 7b it can once again be observed that binding of FN to the SAM did indeed significantly enhance EC proliferation on all SAM surfaces. It is quite clear that for all three times of EC proliferation, the FN enabled a higher percent increase in cell proliferation on 30 and 50% SAMs than on 85 and 100% SAMs. As shown in the results above (Table 6b and Figure 34), the 30 and 50% COOH SAM ratios seem to yield a significantly lower cell proliferation than the 85 and 100% COOH SAMs, for which cell proliferation was quite high even without the covalently bound FN. Therefore, since the 30 and 50% COOH SAM surfaces exhibited poor cell proliferation the addition of FN had a greater enhancement effect than on 85 and 100% COOH SAM surfaces.

In order to verify if there is a regular trend relating EC proliferation with surface charge and wettability, Figure 35b and Figure 36b were plotted. Similarly to Figure 35a and Figure 36a, these figures demonstrate the EC proliferation as a function of contact angle and zeta potential, respectively. The cell proliferation data points were based on the eight day proliferation results.

Figure 35b (triangles) shows for cell binding to SAM surfaces, that with an increase in surface hydrophobicity, the EC surface density first increases and then decreases, yielding a maxima at a contact angle of 63° (85% COOH SAM), while the lowest EC surface density was obtained on the most hydrophobic surface (0% COOH SAM). However, there is no statistical difference in the EC surface density on the three most hydrophobic surfaces (contact angle from 83° to 95°). Further, the results in Figure 35b also show that binding of FN to the SAM does not change the shape of the curve (squares), indicating that the SAM, not FN, seems to have the predominant influence on the surface wettability and thus cell proliferation. However, at the same time the bound FN significantly increases the surface cell density, as clearly seen from the difference between the two curves.
The influence of surface charge (zeta potential) on the EC proliferation is shown in Figure 36b, and a similar trend is recorded for samples with and without covalently attached FN. After eight days of cell proliferation, the two most negatively charged SAM samples (85 and 100% COOH) showed a significantly higher cell proliferation than all the remaining SAM and SAM-FN surfaces. The difference in cell density between the two most negatively charged samples and the remaining ones is higher for SAM (triangles) than SAM-FN (squares) samples, which can be clearly seen from the length of the verticle line on both curves in Figure 36b. As mentioned above and observed in Table 7, this is due to the low cell binding ability of surfaces containing a low amount of COOH groups (50 and 30% SAMs).

The same conclusion as for cell attachment can be drawn for cell proliferation, after analyzing the data in Figure 35b and Figure 36b. Although the data show that the surface wettability and charge significantly influence EC proliferation, no regular trend in the behaviour, i.e. straightforward relationship between these three parameters, can be established. This indicating that the surface chemistry, i.e. variation in surface functional groups (and their surface ratio in mixed SAMs) also play a role in the cell proliferation process.

5.7.3 Results for OH/COOH-based SAMs

This section will present the cell results obtained for OH/COOH SAM and SAM-FN samples. Unlike the detailed analysis that was made in the Section 5.7.2, only major observations will be discussed in this section.
Figure 37: Cell attachment on (a) OH/COOH SAMs and (b) OH/COOH SAMs with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Figure 38: Cell proliferation on (a) OH/COOH SAMs and (b) OH/COOH SAMs with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Table 8: Order of decreasing EC attachment (a) and proliferation (b) as a function of the SAM composition expressed as a percentage of COOH groups in the OH/COOH alkanethiol solution mixture. The table shows the data for both the gold surface covered with only a SAM, and the gold surface covered with a SAM to which FN was covalently attached.

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<th>Sample type</th>
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<th>4</th>
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</thead>
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<td>SAM-FN</td>
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<td>85<del>100&gt;50</del>30~Au</td>
<td>100<del>85&gt;50&gt;30</del>Au</td>
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<td>SAMs</td>
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</tr>
<tr>
<td>85~100&gt;50&gt;30&gt;0</td>
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<tr>
<td>85<del>100&gt;50</del>30~Au</td>
</tr>
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</table>

Table 9: Influence of FN bonded on the OH/COOH SAM on the EC attachment (a) and proliferation (b), expressed in terms of the percentage of the cell surface density increase with respect to the density on the corresponding SAM surface measured in the absence of FN.

<table>
<thead>
<tr>
<th>% COOH alkane thiol in solution</th>
<th>Attachment Time (Hrs)</th>
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<td>88</td>
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<tr>
<td>100</td>
</tr>
<tr>
<td>17</td>
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</table>
5.7.3.1 EC attachment on OH/COOH-based SAMs

In general, the trends observed in Figure 37a and Table 8a demonstrate that cell attachment increases with increasing solution fraction of COOH SAMs. However there was no significant statistical difference between the 100 and 85% COOH SAM samples. When comparing the results after the first and second hour of attachment, the presence of SAM on the Au surface did not significantly enhance the cell attachment on 85 and 100% COOH SAMs, and actually did the opposite for the remaining samples (50, 30 and 0% COOH SAMs). However, after 4 hours of attachment, the 85% and 100% COOH SAM surfaces showed the enhanced cell attachment when compared to the Au surface.

Figure 37b shows that the presence of covalently bound FN on the SAM covered gold surface does influence the EC attachment when compared to the bare gold surface and the results in Figure 37a. Once again, at all attachment times, the EC cell density increased significantly as the percent COOH alkanethiol increased, however there was no significant difference between the 100 and 85% COOH SAM-FN after 2 and 4 hours. Unlike the cell attachment results on the SAM-covered Au surfaces (no FN) described above, there was a statistically significantly higher cell density on all the SAM-FN surfaces when compared to the Au surface after 4 hours. As can be observed in Table 8a, the 85 and 100% samples always yielded the highest cell density at all attachment times.

The effect of FN attached to the SAM on the EC attachment is summarized in Table 9a, as a percentage of EC surface density increase with respect to the density of EC on the same surface in the absence of covalently attached FN. After two hours of attachment, the enhancement effect of FN seems to be higher for all sample ratios than after 1 hour of attachment. As explained in Section 5.7.2, this can be attributed to the delay in cell secretion of ECM proteins (fibronectin) by ECs on the SAM samples without covalently bound FN. For the four hour attachment time, the percent enhancement increased for the 30 and 50% COOH SAM-FN samples when compared to the value after two hours but decreased on 85 and 100% COOH SAM-FN samples. This could imply that there
was a longer delay in ECM protein expression on the 30% and 50% COOH FN-free SAMs than on 85 and 100%.

In order to investigate the influence of wettability and surface charge (zeta potential) on EC attachment, Figure 39a and Figure 40a were plotted. Similarly to what was stated in Section 5.7.2, the attachment times were based on the results after 4 hours.

Figure 39a shows that for SAM and SAM-FN surfaces, the trend relating cell attachment to contact angle is similar. The samples that gave higher contact-angle values (less hydrophilic samples) exhibited significantly higher cell densities. However, it must be noted that the contact angle data, shown in Figure 25 is also irregular and, as explained in Section 5.4, this was due to the high energy surfaces exhibited by mixtures of OH and COOH SAMs.

Figure 40a depicts the EC attachment as a function of the zeta potential of SAM and SAM-FN surfaces, respectively. For both SAM and SAM-FN surfaces, it can be seen, going from the more positive to the more negative potential, that the cell adhesion increases significantly as the zeta potential increases in negativity until it reaches a maximum at the two most negatively charged surfaces for which there was no statistically significant difference in cell density. Since, despite that increasing trend, the most negatively charged sample did not necessarily yield the highest EC density, this leads to a similar conclusion as the results in Section 5.7.2 regarding the role played by the variation in surface functional groups.
Figure 39: Cell attachment (4 days) (a) and proliferation (8 days) (b) as a function of contact angle of OH/COOH SAM and SAM-FN surfaces of varying OH/COOH ratio. The error bars represent the standard deviation based on three replicates.
Figure 40: Cell attachment (4 days) (a) and proliferation (8 days) (b) as a function of zeta potential of OH/COOH SAM and SAM-FN surfaces of varying OH/COOH ratio. The error bars represent the standard deviation based on three replicates.

5.7.3.2 EC proliferation on OH/COOH-based SAMs

Figures 38, 39b and 40b as well as Table 8b and Table 9 illustrate the cell proliferation results for OH/COOH SAMs in a similar order and manner as the cell attachment results.

Figure 38a and Table 8b clearly show that for all three proliferation times, all SAM and SAM-FN samples enabled a significantly higher EC proliferation rate than the 0% COOH SAM surface (there is no FN chemically bound to this SAM). EC proliferation on SAM and SAM-FN surfaces increased statistically
significantly as the ratio of COOH alkanethiols in solution increased. However, it seems that for the case of OH/COOH SAMs without covalently bound FN, only the SAMs containing a COOH solution fraction of 85 and 100% had a significant effect on EC proliferation when compared to the naked Au surface. For the case of SAMs with covalently bound FN, all samples yielded a significantly higher cell density than the naked Au surface. Generally, it can once again be observed that the highest degree of cell proliferation occurred on 85 and 100% COOH SAMs, however there was generally no significant difference in EC density between these two surfaces.

The results presented in Table 9b, clearly show the positive effect of covalently bound FN on the EC proliferation rate. The greatest enhancement effect was obtained on the 30% and 50% COOH surfaces for all three proliferation times.

The influence of contact angle and zeta potential on EC proliferation can be seen in Figure 39b and Figure 40b, respectively.

Figure 39b shows the influence of contact angle on cell proliferation. For both SAM and SAM-FN surfaces, the trends are quite similar to the ones seen in Figure 39a for EC attachment. It can once again be concluded that no significant trend can be drawn relating the surface wettability of OH/COOH to cell proliferation. Similarly, Figure 40b demonstrates that the dependance of EC proliferation rate on the zeta potential for the OH/COOH SAM and SAM-FN surfaces yields a similar trend as the EC attachment trends in Figure 40a. Consequently, similar conclusions could also be drawn from these results.
5.7.4 Results for NH$_2$/COOH-based SAMs

Figure 41: Cell attachment on (a) NH$_2$/COOH SAMs and (b) NH$_2$/COOH SAMs with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Figure 42: Cell proliferation on (a) NH2/COOH SAMs and (b) NH2/COOH SAMs with covalently bonded FN. The error bars represent the standard deviation based on three replicates.
Table 10: Order of decreasing EC attachment (a) and proliferation (b) as a function of the SAM composition expressed as a percentage of COOH groups in the NH₂/COOH alkanethiol solution mixture. The table shows the data for both the gold surface covered with only a SAM, and the gold surface covered with a SAM to which FN was covalently attached.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Attachment Time (Hrs)</th>
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<td>1</td>
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<td></td>
</tr>
<tr>
<td>SAMs</td>
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<tr>
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<td>85→50→30→100→Au</td>
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<tr>
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<td>85→50→30→100→Au</td>
<td>85→50→30→100→Au</td>
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</table>

Table 11: Influence of the presence of FN bonded on the NH₂/COOH SAM on the EC attachment (a) and proliferation (b), expressed in terms of the percentage of the cell surface density increase with respect to the density on the corresponding SAM surface measured in the absence of FN.

<table>
<thead>
<tr>
<th>% COOH alkane thiol in solution</th>
<th>Attachment Time (Hrs)</th>
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</table>

<table>
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<th>% COOH alkane thiol in solution</th>
<th>Proliferation Time (Days)</th>
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<th>5</th>
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<tr>
<td>100</td>
<td>32</td>
<td>23</td>
<td>17</td>
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</table>
5.7.4.1 EC attachment on NH\textsubscript{2}/COOH-based SAMs

From Figure 41a and Table 10a it is quite clear that cell attachment does not seem to depend on the composition of the NH\textsubscript{2}/COOH SAM. However, the results demonstrate that all the SAM-covered surfaces enhanced EC attachment in comparison to the Au surface.

Figure 41b and Table 10a show the results for EC attachment on SAM surfaces to which FN was chemically bound (SAM-FN surfaces). All the surfaces exhibited a significantly higher degree of EC attachment when compared to the naked Au surface. Although after two and four hours, these samples also yielded a statistically significantly higher cell density than the 0% COOH SAM (on which there was no FN since the whole surface is covered with only the NH\textsubscript{2} – terminated SAM), it was quite surprising to observe that after one hour, this surface yielded a statistically higher cell density than the 100% SAM-FN sample. This is an indication that the initial rate of cell adhesion to the free NH\textsubscript{2} groups on the NH\textsubscript{2}-terminated SAM surface (0% COOH SAM) is faster then the adhesion to the 100% COOH on which FN was already available. The author of this thesis currently does not have any explanation for this behavior.

Similarly to the previously presented results on the influence of surface-bound FN on the EC/surface interactions, Table 11a clearly demonstrates a positive influence of FN on EC attachment, ranging from 17% on the 100% COOH surface after one hour of attachment, to 44% on the 85% COOH surface after two hours of attachment. However, no particular trend relating the influence of SAM composition on the EC attachment is visible from the presented data.

In order to investigate the influence of surface wettability and charge on the EC adhesion (after 4 hours) for the NH\textsubscript{2}/COOH and NH\textsubscript{2}/COOH-FN SAMs, Figure 43a and Figure 44a and were plotted.

Both figures show that there is not any statistical difference among the results, thus clearly demonstrating that the NH\textsubscript{2}/COOH surface wettability and charge does not at all influence EC attachment. This a surprising result considering the influence (at least partial) of these two surface parameters on the
EC adhesion on the other two types of SAMs (CH$_3$/COOH and OH/COOH, Figures 33, 34 37 and 38). This, in turn, indicates that some other phenomena seem to predominantly govern the EC attachment to the NH$_2$/COOH-based SAMs (e.g. conformation of the SAMs, van der Waals forces and hydrogen bonding, etc.). Namely, since EC/surface interactions are predominantly governed by the conformation of either the cell-expressed FN (FN-free SAM surfaces) or chemically-bound FN (SAM-FN surfaces), it appears that the surface wettability and charge do not influence the FN surface conformation, and thus the EC attachment and proliferation (Figure 43).

Figure 43: Cell attachment (4 days) (a) and proliferation (8 days) (b) as a function of contact angle of NH$_2$/COOH SAM and SAM-FN surfaces of varying NH$_2$/COOH ratio. The error bars represent the standard deviation based on three replicates.
Figure 44: Cell attachment (4 days) (a) and proliferation (8 days) (b) as a function of zeta potential of NH$_2$/COOH SAM and SAM-FN surfaces of varying NH$_2$/COOH ratio. The error bars represent the standard deviation based on three replicates.

5.7.4.2 EC proliferation on NH$_2$/COOH-based SAMs

The proliferation results for NH$_2$/COOH SAMs and SAMs-FN are illustrated in Figures 42, 43b and 44b as well as in Table 10b and Table 11b.

Figure 42a and Table 10b show that for all three proliferation times the SAM samples yielded a statistically higher surface cell density than the Au sample. However, similarly to the EC attachment results, no regular trend was observed relating cell proliferation to the solution fraction of COOH SAMs.
Figure 42b and Table 10b show that for all three proliferation days, all FN-modified SAM samples (SAM-FN) exhibited a statistically higher degree of cell proliferation than the 0% COOH SAM (covalently bonded FN on this SAM) which in turn was statistically higher than the Au surface. In general, all SAM-FN surfaces seem to yield a similar affinity for cell proliferation. This agrees with the observations made for cell attachment to the same SAM-FN surfaces Table 10a.

When looking at Table 11b, it can be seen once again that FN did indeed have an enhancing effect on cell proliferation when compared to SAMs without FN. However, there is no particular trend relating the enhancement effect to the ratio of the COOH group. The enhancement effect ranged from 17% for the 100% COOH SAM surface after 8 days of EC proliferation to 32% on the same surface after 2 days of EC proliferation. However, when comparing these values to the values in Table 7b and Table 9b, for the CH₃/COOH and OH/COOH SAMs, respectively, it can be seen that the FN enhancement effect is the weakest on the NH₂/COOH SAMs, since the former two surfaces gave the maximum FN enhancement effect for EC proliferation of 47% and 72%, respectively.

Figure 43b shows show the dependence of EC surface density after 8 days of proliferation on the contact angle (surface wettability) and zeta potential (surface charge), respectively, for NH₂/COOH SAMs and SAMs-FN surfaces. These trends are very similar to those related to the cell attachment (Figure 43a and Figure 44a), i.e. they show that there is not any statistical difference among the results, thus clearly demonstrating that the NH₂/COOH surface wettability and charge does not influence EC proliferation.

Thus, these results indicate that some other phenomena seem to predominantly govern EC proliferation on the NH₂/COOH-based SAMs, in a similar way as the cell attachment, as already discussed in the previous section (e.g. conformation of the SAMs, van der Waals forces and hydrogen bonding, etc.).

It can be concluded from the results in this section that positively and negatively charged surfaces have a similar effect on EC adhesion and proliferation. Finally, since the NH₂/COOH SAM surfaces were found to be
hydrophilic at all ratios and did not follow any regular trend (Figure 26), no relationship could be established between the surface wettability and EC activity.

5.7.5 Comparison and selection of surface properties promoting highest EC activity

In this section, relative comparison of the samples that gave the most optimum cell density results are analyzed, based on the separate set of measurements performed with only these selected samples. Namely, as established previously, for all SAM pairs, the samples with chemically bound FN gave higher cell density results than the corresponding ones with bare SAMs. Furthermore, for the case of CH₃/COOH and OH/COOH SAMs-FN, the most hydrophilic and negatively charged samples (100 and 85% COOH SAMs) had a better affinity for cell adhesion and proliferation than the other ratios. On the other hand, mainly the 85%-COOH and occasionally the 50%-COOH samples of NH₂/COOH SAMs-FN were shown to provide a higher cell density, both of which were hydrophilic and charged. Therefore all these types of SAM-FN samples were retested for cell proliferation for 2 and 8 days of incubation. The homogenous SAMs of CH₃, NH₂ and OH alkanethiols were in addition used as control surfaces. The results of this new set of measurements are presented in Figure 45 and Table12.
Table 12: Order of decreasing cell attachment (a) and proliferation (b) based on the percent of COOH SAM in solution.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Time (Days)</th>
<th>2</th>
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<tbody>
<tr>
<td>Homogenous SAMs</td>
<td>NH$_2$&gt;OH&gt;Au&gt;CH$_3$</td>
<td></td>
</tr>
<tr>
<td>SAMs + FN</td>
<td>15%NH$_2$&gt;50%NH$_2$&gt;15%OH~100%COOH&gt;15% CH$_3$&gt;Au</td>
<td></td>
</tr>
<tr>
<td>b)</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>Homogenous SAMs</td>
<td>NH$_2$&gt;OH&gt;Au&gt;CH$_3$</td>
<td></td>
</tr>
<tr>
<td>SAMs + FN</td>
<td>15%NH$_2$&gt;50%NH$_2$&gt;100%COOH&gt;15%OH~15% CH$_3$&gt;Au</td>
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5.7.5.1 EC adhesion to homogenous SAMs

For homogenous SAMs, the trend was the same for both incubation days (Figure 45 and Table 12a). After analyzing results for homogenous COOH SAMs discussed in the previous sections of the thesis, it was concluded that the surface
cell density for EC proliferation decreased in the order of \( \text{NH}_2\text{--COOH}>\text{OH}>(\text{Au})>\text{CH}_3 \), which may signify that the cell proliferation is enhanced on both the positively (\( \text{NH}_2 \)) and negatively (\( \text{COOH} \)) charged hydrophilic surfaces, while it is decreased on the neutral hydrophilic (\( \text{OH} \)) and hydrophobic surface (\( \text{CH}_3 \)). Arima et al. [20] came to the same conclusions regarding EC adhesion to homogenous SAMs.

Further, it was explained in Section 1.2.4, that the surface functional groups have a major effect on initial cell attachment since they not only have a direct influence on the cell adhesion/proliferation but also on the almost instantaneous adsorption of serum proteins contained in the media. Once the cells express their own ECM proteins on a SAM surface, the different physico-chemical surface properties further influence the displacement of (unwanted) FBS proteins (e.g. BSA in our case), the expressed protein surface conformation, and therefore the subsequent cell/surface interactions.

Arima et al. [22] observed that the delay in cell adhesion to homogenous SAMs was higher on OH and CH\textsubscript{3} groups than on NH\textsubscript{2} and COOH, most likely due to difference in efficiency at displacing BSA. Furthermore it was demonstrated in Section 2.2 that the FN conformation was favorable for cell adhesion on hydrophilic samples and unfavorable on hydrophobic ones [14, 17, 19].

### 5.7.5.2 EC adhesion to optimized SAM-FN surfaces

As it can clearly be seen in Figure 45, the SAM samples with covalently bonded FN yielded a significantly higher cell density than the homogenous SAM samples without FN. The comparison of cell density on the mixed SAM-FN surfaces is presented in Table 12. For a proliferation time of two days, the sample containing 15% NH\textsubscript{2} groups (85% COOH groups) yielded a statistically higher EC surface density than the sample containing 50% NH\textsubscript{2} groups, both of which, in turn, yielded a statistically higher EC surface density than the remaining SAM-FN samples. There were some minor differences between the trend observed after two and eight proliferation days, but it could generally be concluded that the 15%
NH$_2$ SAM is the most optimum surfaces for HUVEC proliferation, followed by the 50% NH$_2$.

There were no studies performed involving EC interaction with mixed SAMs with covalently bonded FN, and therefore no comparisons could be made with literature. However, it was clearly demonstrated in Chapter 2 that surface functional groups influence the conformation of adsorbed FN, and it is therefore quite likely that they may also influence the conformation of covalently bound FN. Since the best SAM-FN samples examined in this thesis were relatively negatively charged and hydrophilic, it is quite likely that the open (elongated) conformation of covalently bound FN was the major factor that positively influenced EC adhesion and proliferation, which is in agreement with literature. Namely, as presented in Section 2.2, Bergkvist et. al. [14] observed that FN adsorbed on a hydrophilic silica surface had an elongated form, favourable for cell binding, while when adsorbed to hydrophobic methylated silica, FN had a tendency to be in a compact form not favourable for cell binding.
CHAPTER 6

6. Conclusions

This research project involved modifying a gold surface with mixed SAM monolayers of different physico-chemical properties, and irreversibly binding extracellular matrix protein fibronectin (FN) to these SAMs, with the purpose of studying the effect of surface physico-chemical properties on the FN secondary structure, and HUVEC attachment and proliferation.

The following conclusions could be made:

- NHS IR peak analysis showed that the relationship between the X:COOH (X=CH₃, OH, NH₂) surface SAM ratio and the corresponding ratio in the SAM-forming precursor solution depends on the type of X-precursor used to form the mixed SAM.

- The COOH SAM surface was found to have the highest negative charge (zeta potential), while the CH₃ SAM surface was found to be the most hydrophobic surface. There was no significant difference in the degree of wettability between homogenous COOH, NH₂ and OH SAMs.

- In terms of the HUVEC/surface interactions, most naked SAM-modified surfaces (with no FN bound to the SAM) yielded higher cell attachment and proliferation surface densities than the bare gold surface (control surface).

- The presence of chemically bound FN to the SAM was found to significantly enhance HUVEC attachment and proliferation.

- HUVEC attachment and proliferation to CH₃/COOH SAM and CH₃/COOH FN SAM surfaces was highest on the most hydrophilic and negatively charged surfaces. However the dependence was not found to be directly proportional to the surface wettability or charge.
- For OH/COOH SAM and OH/COOH-FN SAM surfaces, cellular activity increased as the surface negativity increased and reached a plateau at the two most negatively charged samples.

- The surface charge and wettability was found not to influence HUVEC adhesion and proliferation to mixed and homogenous NH$_2$/COOH SAM and NH$_2$/COOH-FN SAM surfaces.

- The 15% NH$_2$ / 85% COOH-FN SAM surface was found to be the most optimum surface for HUVEC proliferation.

**Recommendations for future work:**

- Observe the protein tertiary structure conformation (surface conformation) as a function of SAM composition, surface charge and wettability in order to elucidate how these affect the exposure of FN cell-binding sites to HUVECs.

- Study the interaction of these surfaces with smooth muscle cells and compare the results to HUVECs.

- Translate the findings to a commercial stent material – stainless steel 316L.
Appendix A

Figure A.1: Water contact angles plotted against compositions of reaction mixture. SAMs were formed on gold thin layers in reaction mixtures of alkanethiols carrying terminal CH₃, and COOH. Re-plotted from Arima et al. [20].

Figure A.2: Fraction of COOH SAMs on the gold surface vs fraction of COOH alkanethiols in the binary solution [20].

Figure A.3: Contact angle of CH₃/COOH SAMs vs fraction of COOH alkanethiols in the binary solution. Re-plotted from Baker et al [28].
Figure A.4: Contact angle of OH/COOH SAMs vs fraction of COOH alkanethiols in the binary solution. Re-plotted from Baker et al. [28].

Figure A.5: Contact angle of NH$_2$/COOH SAMs vs fraction of COOH alkanethiols in the binary solution. Re-plotted from Chuang et al. [30].

Figure A.6: Relationship between compositions of reaction mixture of mixed CH$_3$/COOH SAMs and the number of adherent HUVECs. Re-plotted from Arima et al. [20].
Figure A.7: Relationship between water contact angles of mixed CH$_3$/COOH SAMs and the number of adherent HUVEC. Re-plotted from Arima et al. [20].

Figure A.8: Cell growth patterns on mixed SAMs for 3 or 6 days: a) C3H10T1/2 clone 8, b) L929; c) UVB6-2.1A and d) MC3T3E1 [21].
Figure A.9: Fraction of adherent cell on CH$_3$– (open circles), OH– (filled circles), COOH– (open triangles) and NH$_2$–SAMs (filled triangles) determined from TIRFM images acquired during the cell-adhesion process of HUVECs [22].

Figure A.10: Surface chemistry modulates cell proliferation after 1 day in culture. Cultures were incubated in BrdU for 12 h and analyzed for BrdU incorporation by immunostaining [18].
Figure A.11: PM-IRRAS spectrum of OH SAMs in the 2840-2980 cm\(^{-1}\) region, showing the asymmetric (2927 cm\(^{-1}\)) and symmetric (2855 cm\(^{-1}\)) CH\(_2\) stretching peaks.

Figure A.12: PM-IRRAS spectrum of NH\(_2\) SAMs in the 2840-2980 cm\(^{-1}\) region, showing the asymmetric (2927 cm\(^{-1}\)) and symmetric (2855 cm\(^{-1}\)) CH\(_2\) stretching peaks.
Figure A.13: XPS determination of the COOH/CH₃ ratio in monolayers compared with the ratio introduced into the original solution [58].

Figure A.14: Surface fraction of COOH SAMs as a function of their solution fraction. Re-plotted from Chuang et al. [30].
References

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