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UMinho | 2011

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Dissertação de Mestrado
Mestrado Integrado em Engenharia Biomédica
Ramo de Engenharia Clínica

Trabalho realizado sob a orientação da
Doutora Lígia Rodrigues
e do
Doutor Theo van Kooten

Outubro de 2011

Acknowledgements

I would like to thank Dr. Lígia Rodrigues for being always present. I am very grateful for all the patience, comprehension and help; without it I am sure I wouldn't be able to do the best work I could. Every word, every advice, every incentive were more than I would ever expect from a supervisor.

From Dr. Theo van Kooten I got the best orientation during my research work development. I am thankful for the patience and the teaching, for having someone that gave me orientation, that wanted me to learn how to solve problems, that was there to listen to my ideas and that always had time to help me.

To Dr. Miguel Gama I would like to thank the wise words that made me move on and not giving up the opportunity of working abroad and learn new working methods, cultures and traditions.

I would like to thank the Biomedical Engineering Department colleagues, which made me feel home and always made me smile. To Joram, Thomas, Joana and Das I express special thanks, because they were more than colleagues, they were friends.

Finally, a really heartfelt gratitude to my family, which supported me during the last 5 years and never gave up on me, to Diana, the best friend and roommate, and to my fiancé Steffen, the most patient and sweet partner one can have.

Abstract

Poly(dimethylsiloxane) or PDMS is one of the materials of choice to be used in implants due to some of its properties, such as low-cost, versatility, elasticity, chemical inertness, biocompatibility, non-toxicity, among others. However, PDMS also presents some drawbacks. The major limitation of PDMS is its hydrophobic nature, which makes the transferring and spreading of aqueous solutions difficult and may lead to complications in cell culture, as well as its low adhesiveness for cell attachment.

The PDMS used in the current work was modified by atom transfer radical polymerization (ATRP), which is a technique used to carry out a controlled/living radical polymerization that is easy to apply and makes possible the use of different monomers and reaction media. The surface was coated with poly(ethylene glycol) methyl methacrylate (PEGMA), a polymer known for its ability to reduce biomolecules adsorption. With this coating, anti-fouling properties are expected, as well as a more hydrophilic surface. Contact angles were measured showing that the PEGMA coating turned the surface more hydrophilic. Moreover, by atomic force microscopy the surface topography was assessed. It was possible to observe that the coating possessed a high roughness, thus suggesting that ATRP is a suitable technique to create brushes in the PDMS surface. However, the contact angle variability that was found in the modified PDMS samples suggests that the technique is not reproducible.

The interaction of the modified PDMS with human skin fibroblasts (HSkF) and human umbilical vein endothelial cells (HUVECs) was assessed in order to determine the biocompatibility of the surface. To observe these interactions, an immunocytochemistry assay was used to stain the cell nuclei and the vinculin and fibronectin complexes. The stained structures were visualized with confocal microscopy. The staining of the cell nuclei made possible the estimate of the number of cells in the surface, and the formed fibronectin was also quantified. Some colorimetric assays were also performed (MTT and CV) to quantify the metabolic activity per cell, giving some insight about cell viability and adhesion.

It was possible to conclude that the modified surfaces decrease cell adhesion, which is expected due to the anti-fouling properties of PEGMA. Then, if an increase in cell adhesion is desired, PDMS coated with PEGMA is not the most suitable material for vascular implants. However, due to the anti-fouling properties, a better hemocompatibility can be achieved.

Resumo

O poli(dimetilsiloxano) ou PDMS é um dos materiais de eleição no desenvolvimento de implantes devido a algumas das suas propriedades, tais como baixo-custo, versatilidade, elasticidade, inércia química, biocompatibilidade, não toxicidade, entre outras. No entanto, o PDMS apresenta algumas desvantagens. A maior limitação do PDMS é a sua natureza hidrofóbica, a qual pode levar a complicações na cultura de células, bem como a sua reduzida aderência para a adesão celular.

O PDMS usado neste trabalho foi previamente modificado por polimerização radicalar controlada por transferência de átomo (ATRP), que consiste numa técnica usada para levar a cabo uma polymerização radicalar controlada fácil de aplicar e que torna possível o uso de diferentes monómeros e meios de reacção. A superfície foi revestida com poli(etileno glicol) metil metacrilato (PEGMA), um polímero conhecido pela sua capacidade de reduzir a adsorção de biomoléculas. Com este revestimento, esperava-se a obtenção de uma superfície anti-adesiva e mais hidrofílica. Os ângulos de contacto foram medidos confirmando que o revestimento tornou a superfície mais hidrofílica. Adicionalmente, a topografia da superfície foi avaliada por microscopia de força atómica (AFM). Observou-se que o revestimento apresenta uma elevada rogusidade, mostrando que o ATRP é uma técnica apropriada para criar “escovas” na superfície do PDMS. No entanto, as grande variabilidade dos ângulos de contacto medidos no PDMS modificado indicam que a técnica não é reprodutível.

A interacção do PDMS modificado com fibroblastos da pele humana (HSkF) e células endoteliais da veia do cordão umbilical humano (HUVECs) foi avaliada de modo a determinar a biocompatibiltidade da superfície do material em estudo. De modo a observar as interacções, utilizou-se técnicas de imunocitoquímica que permitiram marcar o núcleo celular e complexos de vinculina e fibronectina com fluorescência. As estruturas marcadas foram visualizadas com micorscopia confocal. A marcação do núcleo celular permitiu estimar o número de células na superfície, e a fibronectina formada foi também quantificada. Alguns estudos colorimétricos foram também utilizados (MTT e CV) com o intuíto de quantificar a actividade metabólica por células, fornecendo informação acerca da viabilidade e da adesão celular.

Foi possível concluir que a superfície modificada diminui a adesão celular, o que era expectável devido às propriedades anti-adesivas do PEGMA. Assim sendo, se um aumento na adesão celular for desejado, o PDMS revestido com PEGMA não é o

material mais apropriado para implantes vasculares. No entanto, devido às propriedades anti-adesivas, uma melhor hemocompatibilidade pode ser alcançada.

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Abbreviations

GABGs- Coronary Artery Bypass Grafts

VAGs- Vascular Access Graft

PET- Polyethylene Terephthalate

ePTFE- Expanded Polytetrafluoroethylene

PU- Polyurethane

PEtUs- Poly(ether)urethanes

PDMS- Poly(dimethyl siloxane)

UV- Ultraviolet

ATRP- Atom Transfer Radical Polymerization

PEGMA- Polyethylene Glycol Methyl Methacrylate

PHEMA- Poly(2-hydroxyethyl methacrylate)

PEO- Poly(ethylene oxide)

PEG- Poly(ethylene glycol)

ECM- Extracellular Matrix

GAGs- Glycosaminoglycans

RGD- Arg-Gly-Asp

VEGF- Vascular Endothelial Cell Growth Factor

FGF- Fibroblast Growth Factor

EGF- Epithelial Cell Growth Factor

FAK- Focal Adhesion Kinase

HSkF- Human Skin Fibroblasts

HUVECs- Human Umbilical Veins Endothelial Cells

RPMI- Roswell Park Memorial Institute

PBS- Phosphate Buffer Saline

EDTA- Ethylenediamine Tetraacetic Acid)

PFA- Paraformaldehyde

BSA- Bovine Serum Albumin

DAPI- 4',6-diamidino-2-phenylindole

MTT- “3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide)

CV- Crystal Violet

Framework

The cardiovascular diseases are becoming more and more common mostly due to the increasing use of blood-contacting devices. One example of cardiovascular interventions is the replacement of obstructed parts of arteries, which involves the use of vascular grafts. The most common materials used in the synthetic vascular grafts are the polyethylene terephthalate (PET), expanded polytetrafluoroethylene (ePTFE), but they present a thrombogenic nature that can cause serious complications. Thus, some alternatives have been studied, like the poly(dimethyl siloxanes) (PDMSs) that has been used for vascular vessels and as a coating, presenting some promising results. However, this material is hydrophobic, which will repel proteins from serum and consequently will decrease cell adhesion to the surface. Hence, some coating techniques like the ATRP can be used in order to modify the surface by covering it with hydrophilic brushes.

The aim of this work is to evaluate the biocompatibility of modified silicone rubber for vascular applications. Specifically, this work aims to assess if the PDMS surfaces modified with polyethylene glycol methacrylate (PEGMA) brushes by atom transfer radical polymerization (ATRP) are suitable for vascular grafts applications. These modified surfaces were kindly provided by the Department of Biological Engineering, University of Minho, Portugal. The hydrophobicity of the modified surfaces will be determined, since the goal of the modification was to turn it more hydrophilic thus increasing the interaction of the biomaterial with endothelium cells. Cell-biomaterial interactions will be studied and to enhance cell adhesion the surfaces will also be coated with fibronectin and gelatin.

Chapter 1. Introduction

Biomaterials, that were unknown 50 years ago, are now widely used in medicine, dentistry and biotechnology. Back on that time, medical devices manufacturers, regulatory approval processes and the biocompatibility concepts did not exist. (1) The development of biomaterials was not based in any scientific criteria. On the contrary, when biomaterials were used to create new devices, these were tested on a trial-and-error basis using animals and even humans.

However, this scenario has changed dramatically due to the needs of the continuous and ever-growing practice of medicine, and currently there are thousands of biomedical devices. Besides the biomedical devices, diagnostic products, pharmaceutical preparations and disposable devices, now the list of biomaterials includes intelligent drug delivery, tissue culture, tissue engineering and hybrid organs. (2)

The biomaterials can be divided into four major classes: polymers, metals, ceramics and natural materials. The polymers represent the major class of biomaterials and are used in several biomedical applications in the orthopedic, dental, soft tissue and cardiovascular areas. (1)

Implants made of biomaterials can range from temporary implants, as venous catheters, to long-term implants, as artificial joints. It is highly probable that most individuals in developed countries will use biomaterials and medical devices at some point in their lives. (3) However, some implants and extracorporeal devices can have adverse interactions with the patient, which constitute a device failure, and thus can cause injury or death of the patient. These complications arise largely from the tissue-biomaterial interaction and infection. (1)

Vascular implants (or grafts) are one type of implants that have been widely used in order to enhance the insufficient functioning of blood vessels. There are several types of vascular implants, which can be grouped in three main classes: coronary artery bypass grafts (CABGs); small-diameter grafts; and large-diameter grafts. The CABGs are used in aortocoronary bypass procedures and have a diameter between 1–4 mm. The small-diameter grafts (4 –6 mm) are used peripherally to relieve lower-extremity ischemia and as vascular access graft (VAG) to treat endstage renal

diseases. Regarding the large-diameter grafts (> 7 mm) are used in the thoracic and abdominal cavities, typically for the replacement of aortic sections. (3)

1. Vascular implants

The medicine practice has always involved the use of blood-contacting devices, such as the simple blood bags and catheters. Nowadays, these devices are even more present in our reality and in the so-called modern medicine, being more complex and intimately connected to cardiovascular diseases. Cardiovascular diseases, which include any disorder that affects both the heart and the blood vessels, are one of the main causes of death in the World. (4) According to the World Health Organization (WHO), in 2002 there were 393.000 deaths caused by cardiovascular diseases in Portugal, and around 167 million deaths worldwide. The use of the blood-contacting devices is deeply connected to the cardiovascular diseases, thus being its major application in the biomedical field.

The vascular grafts are included in the group of blood-contacting devices, being widely used to restore the blood flow in patients with different cardiovascular problems. The replacement of obstructed parts of arteries is becoming a very common medical procedure, so the use of vascular grafts is increasing.

The ideal features of a vascular graft include an easy handling, mimicking the native blood vessels, being non thrombogenic, immunologically inert, resistant to infection and puncture trauma, being able to retain tensile strength, and being manufactured at reasonable costs. There are two main groups of vascular substitutes, the biological grafts and synthetic grafts. (5)

The biological grafts or autografts consist in taking tissue from one site on a patient and transplant it to another site on the same patient. Usually it consists in an autogenous vessel, like the saphenous or the arm veins. Currently, the autografts are the vascular grafts with the best performance. However, due to the unavailability, poor quality or the failure (thrombosis, emboli production, intimal hyperplasia) of autologous conduits, the use of prosthetic grafts is often required. (2; 6)

The prosthetic grafts are manufactured from synthetic materials, like polyester and expanded polytetrafluoroethylene. (6) Regardless of being extremely useful and necessary these devices are not perfect, presenting some biocompatibility issues and some undesirable side effects, thus requiring the search for more suitable materials and alternative coating techniques. Though a large variety of polymeric materials are

used (e.g. polyethylene terephthalate (PET), expanded polytetrafluoroethylene (ePTFE) (7)), they present a thrombogenic nature that can cause serious complications, and consequently the failure of the devices. It is recognized that these materials have excellent mechanical and physical properties. Nevertheless, they are also characterized by some hemo-incompatibility, thus activating the humoral and cellular defense mechanisms of the body when contacting with blood. (4; 8) To overcome this biocompatibility problem, the patients are usually exposed to systemic anticoagulation regimens, which in a long-term situation can increase the probability of hemorrhage, even if the levels of anticoagulants are controlled. Indeed thrombosis is, for example, the cause of 80% of vascular graft dysfunction. In addition to this problem, there is a risk of damaging the endothelium lining of blood vessels during implantation of some devices like vascular grafts, which can lead to proliferation of smooth muscle cells or vascular stenosis (decrease in vessel diameter). (9)

For that reason, searching for more suitable materials and coating techniques is highly recommended. Alternative polymers that have been studied are the polyurethane (PU) elastomers, which are thermoplastic polymers with outstanding physical properties. A group of the PU, the aromatic poly(ether)urethanes (PEtUs), due to their flex life, tensile strength, elongation and good blood compatibility, have been used as chorinc implants (e.g. ventricular assist devices, intra-aortic balloons). However, these polymers have been presenting variable clinical results, showing some thrombogenicity and tendency to degrade uncontrollably. (7) Furthermore, the poly(dimethyl siloxanes) (PDMSs) constitute another example of materials that have been used for vascular vessels and as a coatings, presenting some promising results (8). The PDMS presents better blood contact properties when compared to the PU. (7)

2. Silicone Biomaterials

Silicones, or poly(diorganosiloxanes), are a class of synthetic polymeric biomaterials with high chemical stability that were not explored before 1940. Six years later this polymer was referred for the first time as suitable for biomedical applications, and nowadays is one of the materials that found several applications into the biomedical field being widely used as an implant or any other invasive device. (1; 3; 10) The key milestones in the development of silicone are represented in the Table 1.

Table 1. Key milestones in the development of silicone. [Adapted from (1)]

Year	Milestone
1824	Silicon is discovered by Berzelius. It is obtained through the reduction of potassium fluorosilicate with potassium: $4K + K_2SiF_6 \longrightarrow Si + 6KF$.
1863	Tetraethylsilane: the first silicon organic compound is synthesized by Friedel and Craft.
1940	After Hide of Dow Corning demonstrates the thermal stability and the high electrical resistance of silicone resins, and Rochow of General Electric finds a method to separate silicone form silicon and methylchloride, silicones become commercial materials.

2.1. Properties

The silicone backbone consists in repeating silicon atoms bonded to oxygen atoms, with R groups (organic groups) attached to the silicon atom (Figure 1). (1; 11) Usually the organic groups are methyl groups, which gave origin to the name silicone. This happened because Kipping (1904) believed that there was a structural similarity with ketones, which was proved to be incorrect. However the name was kept. (1; 12)

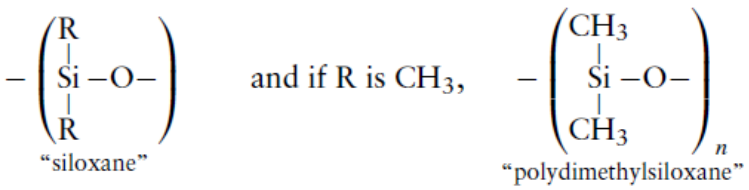

Figure 1. Repeat unit of siloxane and of PDMS. [Taken from (1)]

There are several features that make this material suitable for biomedical applications, such as its good chemical and physical properties, its blood compatibility, low toxicity, good thermal and oxidative stability, low modulus and anti-adhesive properties. (1; 13; 14) It is the presence of organic groups attached to an inorganic backbone that provides excellent properties to the silicone, enabling this material to be used as a fluid, an emulsion, a compound, a resin or an elastomer. (1) Silicone finds many applications due to its hydrophobic nature, which makes it suitable to water

repellent applications. On the other hand, its low surface energy makes it suitable to applications where a material with non-stick characteristics is required. Furthermore, there are also many applications in the electronic field, due to the excellent dielectric properties exhibited by silicone. (12)

However, silicone also presents some biocompatibility issues, due to the presence of siloxane oligomers or some catalyst residues that can diffuse out from the material and cause inflammation, and presents a surface that is difficult to modify due to its hydrophobic character. Also, silicone favors protein adsorption, which can cause problems like blood coagulation and biofilm formation. (10; 14)

2.2. Poly(dimethylsiloxane) (PDMS)

The most common poly(diorganosiloxane) structure is the poly(dimethylsiloxane) or PDMS (-Me_2SiO-) (Figure 1), which was first used for external prostheses in 1960, and from then on has been one of the materials of choice to be used in implants. (3; 12; 15) PDMS is a linear silicone that is in the fluid state at room temperature and is soluble in organic solvents. Its physical properties depend widely from its molar mass. A low molar mass it's in the origin of materials with low viscosity, and high molar mass materials have a viscoelastic behavior and are gum-like. (12) PDMS has a low surface tension (20.4 mN/m) and can wet most surfaces. Because of its methyl groups that point to the outside, PDMS gives rise to very hydrophobic films and presents a surface with good releasing properties. This material is widely used in many applications due to the characteristics mentioned above and due to its lack of toxicity. (1)

The introduction of chemical crosslinks in this material enables the formation of silicone elastomers or rubbers. These materials present a high reversible extensibility, which is called rubberlike elasticity. Then, due to the resulting three-dimensional network, the silicone rubber only swells in organic solvents, thus it doesn't dissolve anymore. The silicone resins, which present low extensibility and low degrees of swelling in the presence of organic solvents, are formed when there is a high crosslinking degree. Both the silicone fluids, elastomers and resins are highly stable. Their thermal stability is so high that they can support temperatures up to 250 °C in air and up to 350 °C in vacuum. (12)

PDMS shows several characteristics that makes it attractive for biomedical applications. It has a low-cost and presents versatility, elasticity, chemical inertness,

biocompatibility, non-toxicity, excellent optical properties, gas permeability, lack of autofluorescence, transparence down to 280 nm, reversible deformation. Moreover, PDMS makes it possible to reproduce features in the micron scale with high fidelity and is easy to process. (16; 17; 18; 19; 20; 21; 22; 23; 24)

Despite all the interesting properties, PDMS presents also some drawbacks. The major limitations of PDMS are its hydrophobic nature, which makes the transferring and spreading of aqueous solutions difficult and may lead to complications in cell culture, as well as its low adhesiveness for cell attachment. (19; 20; 24) Moreover, PDMS may present large residual deformations (18), which can difficult its application in the micromachining field. Although being widely used as an implant material, PDMS leads to intermittent local and systemic adverse immunological effects, which includes the formation of a fibrotic capsule around the implant that can result in contraction causing severe pain and local tissue damage. (21)

2.3. Applications

Silicone biomaterials are used in several areas, due to its attractive properties. Some of the applications (Table 2) where silicone can be used are vascular grafts, urinary and intravenous catheters, heart valves, artificial joints, breast implants, contact lenses, voice prostheses, oxygenators, finger joints, artificial skin, among others. (3; 13; 10)

Table 2. Applications of silicone biomaterials in the biomedical field. [Adapted from (3)]

Application	Examples
Implants	• Retinal tamponade; • Gel swelling agents.
Lubricants	• Silicone valves; • Syringes; • Needles; • Condoms.
Soft tissue implant fillers	• Brest implants; • Testicular implants.
Membranes	• Blood oxygenators; • Dialysis.
Encapsulants	• Cochlear implants; • Pacemakers/Defibrillators.
Plastic surgery	• Soft tissue implant envelopes; • Tissue expanders; • Maxillofacial implants.
Ophthalmology	• Punctum plugs; • Intraocular lenses.
Urology	• Penile implants; • Incontinence devices.
Cardiology	• Artificial heart valves.

PDMS itself found its way in several applications, like numerous active and passive implantation devices, microfluidic devices (separations systems, micromixers, micropumps), microreactors, hydrophobic vent valves, microdevices for cell-culture, cell-based assays, DNA hybridization assays, among others. (16; 17; 18; 19; 20; 21; 25; 26)

3. Surface Modification

Polymer surfaces are the boundaries between the bulk polymer and the surrounding environment. It is the surface that is responsible for the interaction of the polymer with the outer environment. Polymer surfaces are usually hydrophobic and chemically inert, which can lead to undesirable protein adsorption and cell adhesion. In order to overcome this problem, a great amount of research has been carried out in order to develop proper surface modifications of polymeric materials. (27)

As previously mentioned, PDMS has been widely used in biomedical applications although this polymer also presents some drawbacks. (28) Hydrophobicity is usually the main drawback, which can compromise its use in several medical devices (catheters, vascular grafts). The hydrophobic character of PDMS surface enhances the adsorption of numerous proteins, which can lead to microbial adhesion and, lately, to biofilm formation, and causes a lack of interaction between the device and the tissues. (28; 29)

To overcome the problems caused by the hydrophobic character of PDMS surface, the surface energy has to be increased in order to increase the hydrophilicity, and consequently PDMS surface functionality. Increasing the hydrophilic character of the surface, with no influence in the bulk properties of PDMS, can be achieved with surface modification techniques. (28) For the PDMS surface modification different treatments may be used, namely chemical, physical and a combination of both. (27)

3.1. Physical Treatments

The physical modification of a polymer surface can be achieved in two different ways, one that involves the chemical modification of the surface layer and the other that involves the deposition of an external layer on top of the material surface. In the first case the physical techniques require the generation of high-energy species on the surface, like radicals, ions or molecules in the excited electronic state. In the second case it requires the deposition of atoms or atomic clusters coatings on the polymer surface. (28)

There are several physical techniques, including sputtering and flame, corona, cold and hot plasma, ultraviolet (UV), laser, electron-beam and ion-beam treatments. Some of these techniques were already used to modify the PDMS surface, but the most used ones are corona, plasma and laser treatments. (28)

Plasma Treatment

Plasma treatment is the most common technique used to modify the hydrophobic character of a surface. This simple treatment leads to modifications that depend on the characteristics of the created plasma and on the system used. (29)

When a surface is modified with a plasma technique, depending on the gas that is used, the treatment can introduce groups in the surface or can activate the surface, which will make possible the linkage of water soluble polymers afterwards. (29)

There are two types of gases that can be used in the plasma treatment, reactive gases and inert gases. When a reactive gas is used, the reaction that occurs between the polymer surface and the activated gas will lead to the surface modification. On the other hand, if an inert gas is used, free radicals are formed in the polymer surface. (30)

Owen *et al*, in 1994, modified the PDMS surface with a plasma treatment. This group reported an increase in the wettability of PDMS surface. Several gases like argon, helium, oxygen and nitrogen were used, and a thin silica like layer was produced in the PDMS surface with all the gases. However, the surface modification is reversible. The surface rapidly recovers its hydrophobic character, which can be explained with the reorientation of surface silanol groups into the bulk polymer making possible the movement of free PDMS chains to the surface. (23; 29; 31)

3.2. Chemical Treatments

The chemical modification of a polymeric surface can be done in two different ways, a modification achieved by the direct chemical reaction with a solution (wet treatment) or a modification by covalent binding of macromolecular chains to the surface (grafting). Among different techniques the one most widely used is the surface grafting.

Besides the surface grafting, other techniques can be used to achieve a chemical modification of the polymer surface. Some of these techniques include surface oxidation, etching, hydrolysis and functionalization. (28)

Surface Grafting

Depending on the polymer, different grafting techniques can be chosen to modify its surface (Figure 2). If the polymer surface presents suitable functional groups, an option is to attach large macromolecules to the appropriate surface (*grafting on*). The other option is to exploit these functional groups as possible sites to start the polymerization of a monomer (*grafting from*). (28; 32)

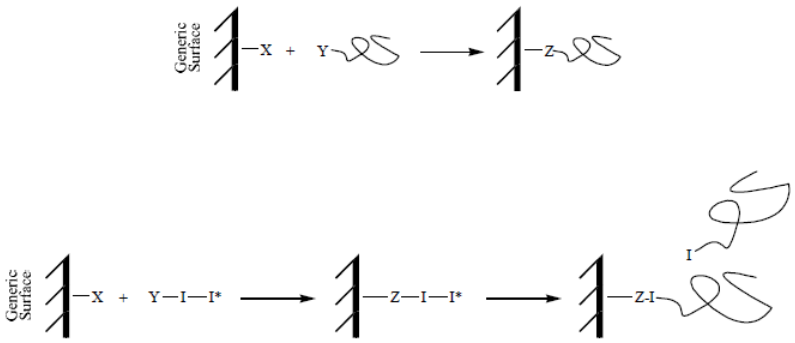

Figure 2. Surface grafting of a generic surface. On the top figure it is possible to observe a representation of the grafting on approach. In the bottom the grafting from approach is represented. [Taken from (33)**]**

The *grafting from* approach (Figure 2: bottom) presents several advantages when compared to the *grafting on* (Figure 2: top). Although the *grafting on* approach offers better define and characterized structures in the surface (the structures can be isolated and purified before being grafted), the *grafting from* approach has reduced preparative steps (the macromolecular material to be grafted doesn't need to be prepared and isolated) and the surface density is not influenced by the dimension and mutual sterical constraints of the grafting material (the surface density depends on the density of the initiation groups). (32) Nevertheless, the formation of islands or mushrooms on the *grafted on* surface is often observed. (33)

Additionally, if the polymer has no chemically-reactive functional groups in its surface that allows the grafting initiation, it is possible to create radicals in the surface by chemical treatments followed by an initiation reaction or by irradiation. The irradiation can be carried out in the presence of a monomer (simultaneous irradiation) or the monomer can be added after the irradiation (pre-irradiation). (28)

Grafting modification that is induced by radiation is an extremely promising method for surface modification and can be used in most of the polymers that are

known. This technique enables the development of materials with new properties, since it allows to change the nature, morphology and structure of the modifying polymer and to control the thickness of the grafted layer. (28)

Several radiation sources have been used, with corona, plasma or glow-discharge being the most common. When the radiation source is chosen, there are some factors to be considered, as the availability, the impact on the modification process and the penetration depth. (28)

Among the *grafting from* techniques, atom transfer radical polymerization (ATRP) has been widely used and discussed, being a promising technique. It can be performed in mild conditions (room temperature in aqueous solution) and can be used in a wide range of polymers. In addition, this technique has negligible transfer reactions, due to the presence of monomers only in the end of the growing chains. (32)

Atom Transfer Radical Polymerization (ATRP)

Atom transfer radical polymerization (ATRP), which consists in a catalyzed reversible redox process, is one of the techniques used to carry out a controlled/living radical polymerization. (34; 35) This technique is easy to apply and makes possible the use of different monomers and reaction media (aqueous or organic) (36; 37) The monomers that may be used are styrenes, methacrylates (e.g. polyethylene glycol methyl methacrylate (PEGMA) and poly(2-hydroxyethyl methacrylate) (PHEMA)), methacrilamides, methacrylic acids and 4-vinylpyridine. (35) Furthermore, this technique allows to obtain polymer chains with controlled molecular weights and low poly-dispersity. (34) ATRP is also a suitable technique for growing polymeric brushes on surfaces. (37)

The name of the technique is based in the fact that both the activation and the deactivation of the radicals involve an atom transfer reaction. Being a radical polymerization process, this technique has four main steps: initiation, propagation, transfer and termination.

The initiation step consists in the formation of a reactive site, thus initiating the polymerization. After the initiation, the propagation step begins, with the monomers being added one by one to the active chain end. During the reaction, the active site can be transferred to the monomer, the initiator, the polymer or the solvent itself, in the transfer step. This step may result in the terminated molecule or in some new active site where the propagation may occur. The final step is the termination, which results in

inert macromolecules. This may occur by coupling reactions of two active centers or active transfer between active chains. (33)

All of these steps may be found in the ATRP process, as represented in Figure 3. First the initiator covers the polymer surface. Many commercially available initiators (e.g. alkyl halides) can be used, as long as they present a weak halogen-heteroatom bond. The initiator will provide the polymer a surface with simple halogen as end groups, which is easily converted into useful functionalities. The transition metal complexes used (Ru, Cu, Fe, Ni, among others) are responsible for the conversation into useful functionalities, removing the halides from the polymer surface. The surface is then ready to a polymerization. (35)

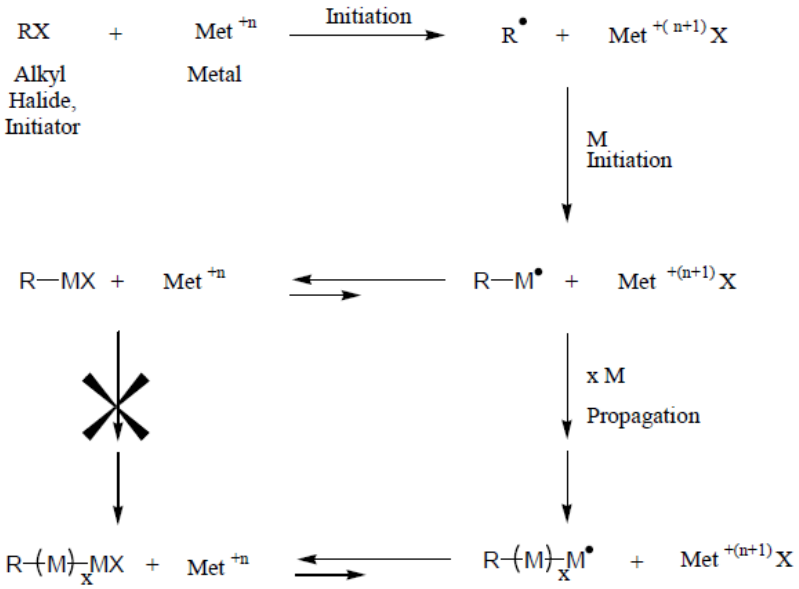

Figure 3. Schematic representation of the ATRP process. After the initiator (X) covers the polymer surface (R), the transition metal complex (Met) will remove the halides from the polymer surface. The monomer (M) will then interact with the polymer surface and the propagation of the monomers begins, culminating with the formation of polymeric chains in the polymer surface. [Taken from (33)]

In order to achieve a controlled polymerization of the monomers, the initiation process has to be fast enough to provide a constant concentration of growing polymer chains, and there has to be a dynamic equilibrium between the inactive chains and the growing radicals. (38)

Xiao *et al* (2002) used ATRP to modify the PDMS surface. They used polyacrylamide chains to increase the hydrophilicity of PDMS. The amount of polyacrylamide reached a maximum after 15 min. However, after 30 min of exposure

visual inspection revealed PDMS damage. It was also observed that the treatment could be performed optimally before the occurrence of bulk damage. The surface became hydrophilic and, unlike the plasma treatment, the hydrophilic character of the modified PDMS lasted for at least one month. (39)

3.3. Coating Materials

Several materials have been used in order to increase PDMS surface hydrophilicity. Among these materials it is possible to find polymers and biomolecules. (28; 29; 37; 39; 40) Some polymers that are widely used in order to increase the hydrophilicity are the poly(ethylene oxide) (PEO), the poly(ethylene glycol) (PEG), the polyacrylamides (PAAm), among others.

PEG and its derivates have been used to change the hydrophilicity and the biomolecule-repelling properties of PDMS surfaces. This polymer is frequently used due to its low toxicity, low immunogenicity, and its ability to prevent protein, cell, and bacteria adhesion. However, PEG has only two hydroxyl groups available per chain, because of its linear structure. This can lead to some limitations in the surface modification. (37; 41)

PEO is structurally similar to PEG, but has a higher molecular weight. The surface coating with this polymer usually results in the reduction of biomolecules adsorption (e.g. proteins and bacteria). PEGMA, which is also used as a coating material, has a similar structure and similar properties. (37)

Other solution may be the use of the hydrophobic nature of PDMS and immobilize blood proteins on its surface. Because of the hydrophobic interaction, the proteins would bond and stay on the surface and remain part of the fluid that is washed off. (42) Some extracellular matrix (ECM) proteins (collagen, laminin, fibronectin) may also be used as a coating, promoting cell adhesion and migration. (40)

3.4. Vascular Grafts Modification

Vascular grafts have drawn high interest in the medical society, with the increased need of regeneration of vascular tissues. One of the most common materials utilized in vascular grafts is ePTFE. (43) Although this material presents interesting properties, some graft failure has been observed, due to thrombosis and intimal occlusion of the vessel. In order to avoid these limitations, an increased effort to seed the vascular grafts has been performed by several researchers. (44)

Cezeaux and co-workers (1998) modified the surface of ePTFE using vacuum ultraviolet, with the purpose of increasing the endothelial cell adhesion. The modified surfaces could not increase endothelial cell adhesion, however the surfaces yielded an increased cell proliferation. These results suggest that the vacuum ultraviolet surface modification can be used to obtain more suitable surfaces for endothelial cell colonization of ePTFE vascular grafts. (44)

Adali *et al* (2010) reported a new approach for the cell seeding of grafts, namely the *in situ* endothelialization of implanted grafts inside the body. For that purpose, the endothelial progenitor cells (EPCs), which consist in a small population of CD34+ circulating mononuclear cells capable of attaining endothelial cell characteristics *in vitro*, are the best candidates. The limitation of using such cells for *in situ* endothelialization of grafts is due to the fact that these cells are mainly located in the bone marrow and only small amounts of EPCs circulate in the blood. Thus, these cells need to be mobilized to the implant site and seed their surface. The proposed strategy is coating the graft with capture molecules that attract the circulating EPCs and increase their adhesion to the surface. (4)

Furthermore, Larsen *et al* (2006) synthesized a novel peptide fluoro-surfactant polymer modification that facilitates the adhesion and growth of endothelial cells on ePTFE vascular grafts. The peptide fluoro-surfactant polymer consists of a poly(vinyl amine) backbone with RGD sequences and perfluorocarbon pendant branches. Endothelial cells showed a specific adhesion to the RGD sequences and retained an hemostatic function. (45)

4. Body-Biomaterial Interactions

Replacing injured tissues with biomedical devices is currently the main approach in biomaterials science, because substitutes of biological origin are recognized by the immune system. This happens due to the presence of biological motifs in this substitutes that are immunologically recognizable. (2)

Once the biomaterials are intended to contact directly with living tissues and biological fluids, they are targets for the protective mechanisms within the body (protein adsorption, hemostasis, inflammation, foreign body response). During the past decade it was recognized that all implantable biomaterials invoke an almost identical inflammatory and foreign body response, despite the biomaterial nature. Currently

obstacles related to the design of biomaterials involve the interaction of biomaterials with the body and the reaction of the body to biomaterials. (46)

Therefore, understanding and predicting the interaction between tissues or body fluids with biomaterials is crucial to all kinds of medical technologies. The reconstructive medical implants require a perfect integration of the biomaterial with the surrounding tissues to restore adequate function, with no release of harmful products. (2)

4.1. Cell-Biomaterial Interaction

Cell adhesion to biomaterials surface is a critical step in the integration of implants. Therefore, the interaction of cells with biomaterials is an important feature of *in vitro* biocompatibility and cytotoxicity studies. This adhesion is mediated by adsorbed proteins, like immunoglobulins, vitronectin, fibrinogen and fibronectin. (47; 48)

The main parameters of cell-biomaterial interactions are the cell adhesion and the cell spreading. Cell adhesion and spreading are the consequence of a series of molecular events (Figure 4) that occur in and around the cells, which are mediated by the trans-membrane receptors present in the extracellular matrix (ECM). (49)

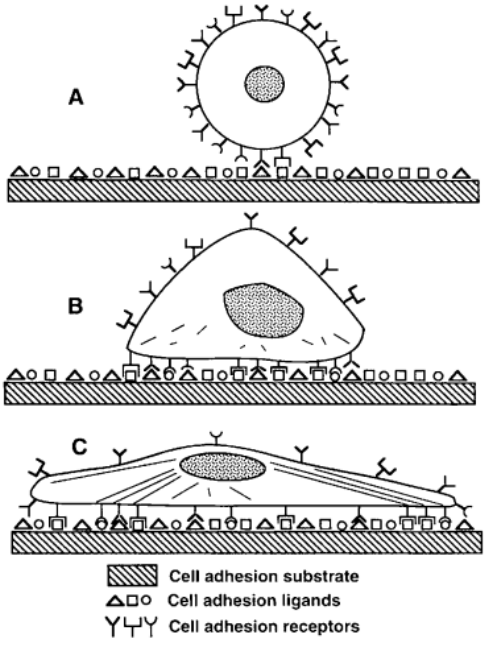

Figure 4. Progression of the mammalian cell adhesion. (A) Initial contact of the cell with the material covered by proteins. (B) Formation of bonds between the cell surface receptors and the cell adhesion ligands in the proteins. (C) Cytoskeletal reorganization with a progressive spreading of the cell in the surface of the implant, increasing the attachment strength. [Taken from (1)**]**

4.1.1. Extracellular Matrix (ECM)

The ECM is secreted by the cells that populate a tissue or organ. Its composition will be determined by factors like the mechanical forces, the oxygen requirements, the gene expression patterns. This matrix plays a crucial role in the mammalian development and physiology, and both the amino acid sequence and the quaternary components of the ECM are greatly preserved across species lines. (49)

The composition of the ECM is a mixture of functional and structural proteins (collagen, fibronectin), glycosaminoglycans (GAGs), glycoproteins and small molecules (figure 5). All this components are arranged in a unique three-dimensional architecture. (49)

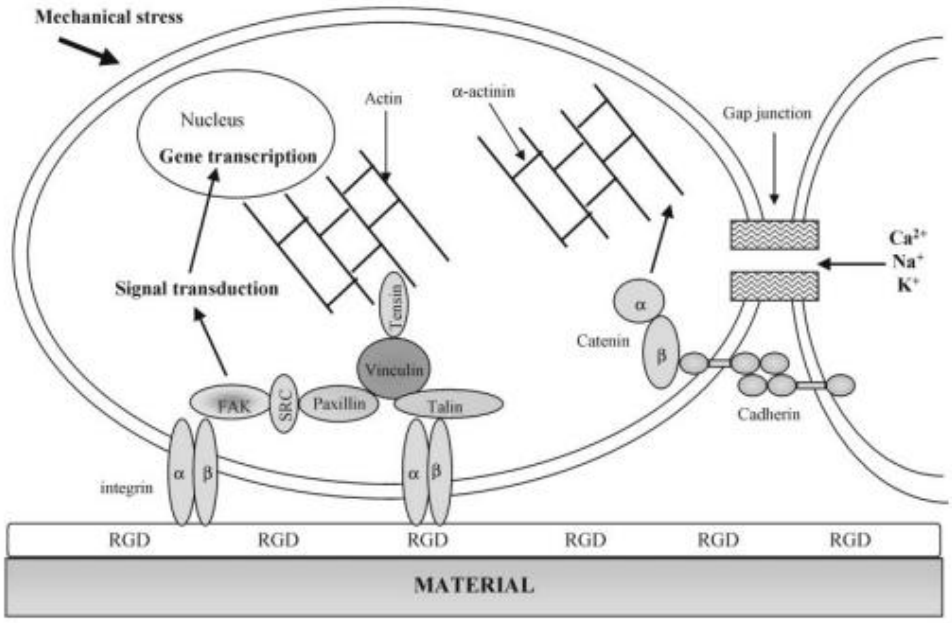

Figure 5. Cell proteins involved in cell adhesion on a biomaterial. [Taken from (50)**]**

Collagen

Collagens represent more than 90% of the dry weight of the ECM, being the most abundant proteins in the mammalian ECM. More than 20 different types of collagen where identified and the type I collagen is the major structural protein in tissues. Type I collagen is abundant in tendinous and ligamentous structures, providing the necessary strength that these tissues require. Other types of collagens can be found in the ECM in much lower amounts than the type I collagen, although providing different mechanical and physical properties to the ECM. One example is the type IV

collagen, which is present within the basement membrane of most vascular structures and tissues with an epithelial cell component. (49)

Fibronectin

Fibronectin is the second most abundant protein in the ECM. It is a large dimeric glycoprotein that exists either in the soluble state or as a tissue isoform. This protein is a mediator of mammalian cells adhesion, because it possesses ligands for adhesion of several cell types. Fibronectin is rich in the tripeptide Arg-Gly-Asp (RGD), which is recognized by the cell surface receptors, the integrins, thus being extremely important in cell adhesion. When bound via integrins, these proteins trigger a number of signal transduction pathways that activate events like cell spreading, proliferation, differentiation and migration. This protein is critical for normal biologic development, especially the development of vascular structures. (49; 51)

Laminin

Laminin is an adhesion protein present in ECM that exists in numerous forms, depending in the specific mixture of the several peptide chains. It is determinant in the formation and maintenance of vascular structures. This protein is one of the most critical ECM factors in the process of cell and tissue differentiation. (49)

Glycosaminoglycans

It is possible to find several mixtures of GAGs in the ECM, depending on the tissue location, the age of the host and the microenvironment. The GAGs have various functions, like binding growth factors and cytokines, promoting water retention and contributing to gel properties of the matrix. Heparin and hyaluronic acid are two GAGs present in the ECM. (49)

Growth Factors

Growth factors and cytokines, although in small amounts, are also present in the ECM. However, they act as potent modulators of cell behavior. There is an extensive list of growth factors present in the ECM, including the vascular endothelial cell growth factor (VEGF), the fibroblast growth factor (FGF) family, and the epithelial cell growth factor (EGF), among others. These factors can be found in different isoforms, each with a specific biologic activity. (49)

4.1.2. Adhesion Molecules - Integrins

The adhesion molecules include four main classes – selectins, immunoglobulin super family, adhesins and integrins- and are capable of interacting with specific ligands situated on the membrane of neighbor cells or on the ECM. (50)

Among the four classes of adhesion molecules, the integrins are the main cell surface receptors for proteins within the ECM. (52) The integrin family is composed of 22 heterodimers with two types of subunits, α and β, that are non-covalently associated. There have been discovered 16 α subunits and 8 β subunits, that combined in different ways can origin a diversity of structures with various ligand-binding possibilities. In the integrin structure, each subunit has a large extracellular domain, a transmembrane domain and a short cytoplasmic domain. (48; 50; 52; 53; 54; 55)

Integrins can bind to specific amino acid sequences, such as the RGD motif that is present in many ECM proteins. (49; 53; 54) Besides the ECM proteins, integrins can interact with components of the cytoskeleton and signaling molecules through their intracellular domain. Being an interface between the extracellular and the intracellular environment, integrins can translate the attachment of external ligands to internal information, inducing adhesion, spreading, cell migration and cell growth and differentiation. (50)

After binding specifically to a ligand, the integrins cluster together into focal adhesions. Focal adhesions consist of additional cytoskeletal proteins, adapter molecules and kinases, being an area of close contact between the cell and the ECM. The integrins are present in these areas in higher amount than their normal membrane distribution. Focal adhesions are barely formed on hydrophobic surfaces, and well developed in surfaces that sustain cell adhesion. (53; 54)

When clustered, the recruitment of tensin and focal adhesion kinase (FAK), as well as their phophorylation start, resulting in the recruitment of talin, vinculin and α-actinin, which are responsible to link the F-actin fibers to the plasma membrane. The rearrangement of F-actin fibers induces changes in the organization of the cytoskeleton (and consequently in the cell shape), affecting cell adhesion and mobility. (53; 54)

4.2. Adhesion Proteins

When a biomaterial is implanted, in a question of seconds to minutes, a layer of proteins rapidly adsorbs and covers its surface. (47; 56) Therefore, instead of the original surface of the implanted material, the cells will recognize this protein layer. It is possible to say that the adhesion proteins convert the biomaterials into a biologically recognizable material. The adsorption of these adhesion proteins is the basis for all the reactions that may occur in the body. (1)

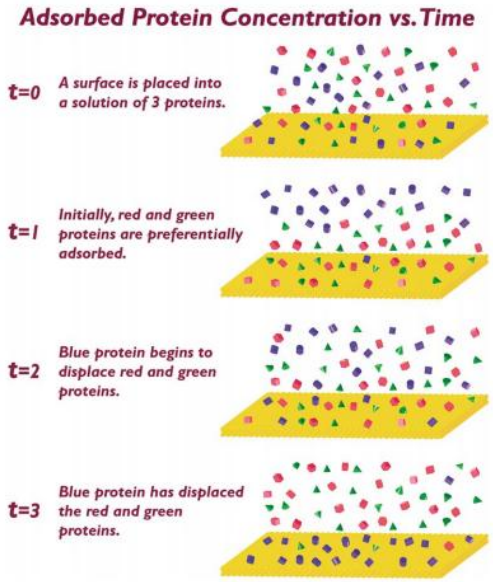

Figure 6. Surface placed in a protein mixture. In a matter of seconds the surface is covered by a layer of adsorbed proteins. Initially, the "red" and the "green" proteins adsorb in a higher concentration. With time, these proteins are displaced by the "blue" protein. This can happen because the concentration of proteins in the adsorbed layer is usually different from the concentration in the solution, being able to change in time. [Taken from (47)]

The surface properties of the biomaterials (chemistry and hydrophobicity) determine the type, amount and conformation of the adsorbed proteins. The composition of this protein layer can be different, depending of the fluid composition and adsorption time. In figure 6 it is possible to observe that initially the "red" and the "green" proteins are present in a higher concentration, but with time these proteins are displaced by the "blue" proteins. Besides the composition of the protein layer, the conformation and the orientation of the protein can also change with time, as represented in figure 7 (44,54).

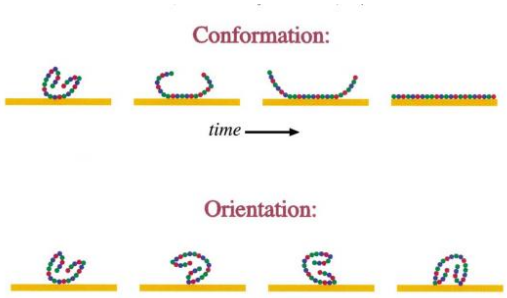

Figure 7. The top scheme shows a protein denaturing with increasing adsorption time. The bottom scheme shows a proteins adsorbing to the surface in different orientations. This is possible because the conformation and the orientation of adsorbed proteins depend on adsorption conditions and surface properties. [Taken from (47)**]**

The layer of proteins that is formed will increase the cell adhesiveness, since the cells have receptors in the cell membrane, which bind specifically to the adhesion proteins. This layer of adhesion proteins also increases the cell spreading in the surface.

4.3. Host Immune Response

Nowadays it is known that there are no inert biomaterials. The medical implants, being foreign to the host body, will trigger tissue responses during the healing process that are dependent on the nature of the biomaterial and the implant site. (57) Indeed, the host-biomaterial interaction is a very complex process that will control the biological performance of the medical implants. (56)

When the biomaterial is implanted in the body a wound is created and a series of events, initially similar to the ones in normal wound healing, will occur. The process of implantation disturbs the homeostatic mechanism in the body, thus activating the healing process. (58)

The way the implant is accepted by the host and how well the host will heal depends largely on the way the complex wound healing around the device will occur. The wound healing process includes four phases, namely the inflammation, the foreign body reaction, the fibrous encapsulation and the matrix formation and remodeling. (57; 58)

The host body reacts similarly to nearly all the biomaterials, as represented in figure 8. After one month of implantation, all the biomaterials are found to heal basically in the same way.

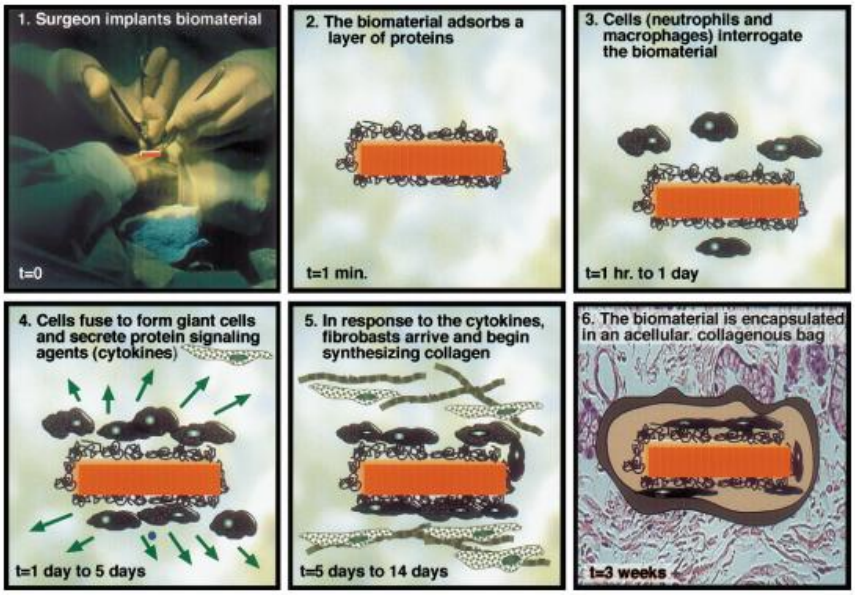

Figure 8. Reaction of the host to the implanted biomaterial. (1) The biomaterial is implanted in the surgical site by the surgeon. (2) A layer of proteins quickly adsorbs to the implant surface. (3) The neutrophils and the macrophages examine and attack the biomaterial. However the implant is too large to be ingested. (4) The macrophages find that they cannot digest the implant, so they fuse into giant cells to engulf the implant. The giant cells send out cytokines to attract other cells. (5) The fibroblasts arrive and start synthesizing collagen. (6) The implant is completely entrapped in an acellular, avascular collagen bag. [Taken from (47)**]**

The inflammation process can be trigged by surgery trauma or the presence of a foreign body in the host. In the 24 hours after the surgery cellular and non cellular responses will take place. The first event is a non cellular response, the vasodilatation of the local vessels, which culminates with the increased permeability of the vascular endothelium and the edema formation. In parallel the complement cascade is activated by the membrane damage. Then the cellular response also takes place with cell recruitment. (59) Several molecular signals will act as chemoattractants at the implant site, recruiting the inflammatory cells. (58)

The first cells migrating to the injury site are the neutrophils, which are responsible for the phagocytosis, the engulfing and the degradation of the foreign body. Once neutrophils start their function, the monocytes circulating in the blood enter the tissue and become macrophages, which are also responsible for the phagocytosis and release several biochemical factors that can activate other cells. (59) The activated macrophages adhere to the material and spread on its surface, trying to phagocyte it. Because they cannot digest or engulf the implant, they fuse together and origin a foreign body giant cell, which can phagocytize larger particles. It is the presence of the

foreign body giant cells long after the implantation that indicates the existence of a chronic inflammation. (47)

The foreign body giant cells are still not capable of engulfing the implant, so they signal the host body for the presence of this large mass that needs to be walled off. Then the fibroblasts arrive and generate the collagen capsule. (47)

In the end of the inflammatory response, the tissue remodeling starts. First, the dead cells are phagocytized and removed, then granulation and neovascularization take place. The provisional matrix is dissolved and it´s replaced for a mature and functional matrix, which provides a biomechanical scaffold for cell attachment and anchorage of macromolecules. It is when the provisional matrix starts to disappear that the deposition and remodeling of collagens determine the healing capacity of the wound. (57; 59)

There are two types of factors that can influence the wound healing process, the intrinsic factors and the extrinsic factors. The bulk nature of the material, its porosity, roughness and changes in the surface chemistry are intrinsic factors of the implant. The extrinsic factors are, for example, the surgical procedure, the condition of the patient (diabetic, immunocompromised) and the anatomical location of the implant. (57)

The end of the inflammation process is characterized by the scar maturation, with the type III collagen being replaced by type I collagen. The type I collagen is stronger and less elastic, allowing the wound to gain tensile strength, which results in a dense and fibrous tissue. (59)

Besides this universal immune response that is only slightly affected by the structure and chemical composition of the foreign material, there can be a more specific immune response. This response is determined by a humoral response, characterized by the production of freely circulating antibodies mediated by B lymphocytes, and a cell mediated response, carried out by the T lymphocytes. The immune system is responsible for triggering the inflammatory response to foreign tissues that can culminate with rejection, but this system can adapt and develop a specific memory to a specific foreign material, resulting in a hypersensibility or allergy to that material. (59)

Chapter 2. Materials & Methods

In the current work, modified silicone rubber surfaces have been evaluated regarding their interaction with mammalian cells under culture conditions (*in vitro*). Two cell lines were used as model cell lines to assess the effect of the PDMS surface modification on cell adhesion, namely human skin fibroblasts (HSkF) and human umbilical veins endothelial cells (HUVECs). Silicone surfaces modified by atom transfer radical polymerization (ATRP) (Figure 9) under the scope of the BIOSURFA project were kindly provided by the Department of Biological Engineering, University of Minho, Portugal.

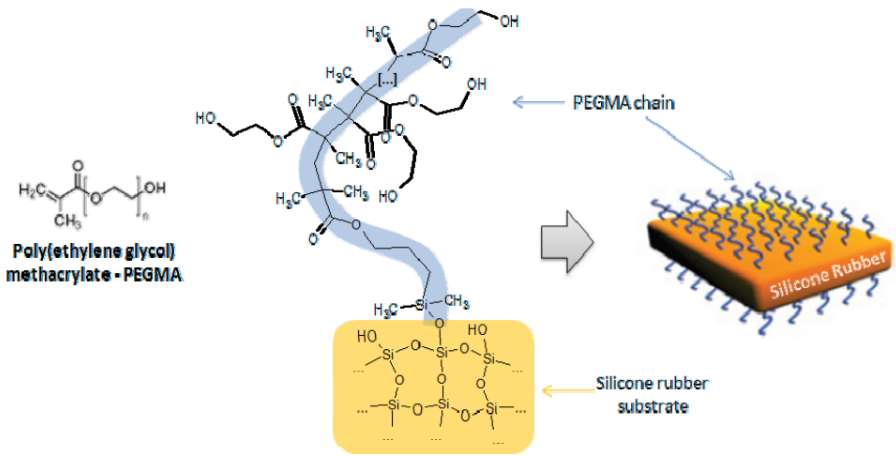

Figure 9. Representation of poly(ethylene glycol) methyl methacrylate (PEGMA) polymer brushes formed in the PDMS surface due to the ATRP modification. [Taken from (60)**]**

The surface modification process starts with the surface activation. In this step, both PDMS (Stockwell Elastomerics, Inc.) surfaces (top and bottom) were exposed during 10 min to UV radiation and for 30 min to Ozone. Then, a 94% solution of the initiator (1-Trichlorosilyl-2-(P,M-Chloromethylphenyl)Ethane(94%)) was evaporated under vacuum, in order to have a chemical vapor deposition of the initiator onto the PDMS surfaces. After washing and drying the PDMS samples, they were polymerized with PEGMA using the following monomer and catalysts: [PEGMA]:[CuII]:[CuI]:[bpy] (PEGMA: Copper II: Copper I: 2,2'-Bipyridine) in the molar proportions of 22:1:0,2:2. The samples were washed and dried again in order to obtain the final modified PDMS surfaces.

1. Surface Modification

The PDMS surfaces, which were modified by ATRP at University of Minho, were coated with a layer of fibronectin and a layer of gelatin before cell culturing, to achieve a higher cell adhesion.

1.1. Fibronectin Coating

Fibronectin (Sigma F2006) was stored in vials at -20°C in a concentration of 1 mg per ml of PBS. For the coating, a 5 μg/ml concentration was used. Next, 750 μl of fibronectin solution was added to each well and the plate was incubated for 30 min at room temperature. After that, the solution was removed and the cells were added according to the procedure described in section 4.1.

1.2. Gelatin Layer

For the HUVEC cells experiments, after placing the PDMS sample, 1 ml of gelatin was added per well. The plate was incubated for 30 min at 37°C, in the CO_2 incubator. After the 30 min, the gelatin was discarded and the cells were added according to the procedure described in section 4.1.

2. Surface Characterization

2.1. Contact Angles

The material used in the current work was the modified silicone rubber surfaces abovementioned, in which it is expected to encounter PEGMA brushes. One way to evaluate the presence of these brushes at the surface is through the determination of its hydropobicity/hydrophilicity. Therefore, contact angles of the modified and unmodified PDMS were measured using the Spindler&Hoyer contact angle meter and the Novell software ContactAngle.

A contact angle can be measured by adding a drop of liquid to a solid surface. The contact angle is an indication of the surface hydrophilicity/hydrophobicity since it measures how much a droplet spreads on a given surface. Using a water droplet (Figure 10), the more hydrophilic the surface is the lower the contact angle will be. On the other hand, higher contact angle values represent more hydrophobic surfaces.

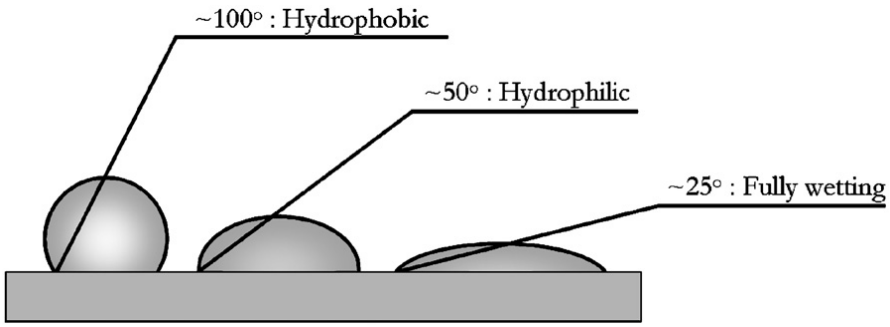

Figure 10. Water drops formed in the surface. Contact angle between the water drop and the surface depends on the surface hydrophobic/hydrophilic character. [Taken from (61)**].**

The contact angle was measured at room temperature, using the sessile-drop method with water. For the modified PDMS, the contact angle was measured in 5 different surfaces (5 replicates). On the contrary, for the unmodified PDMS the contact angles were measured in 2 replicates. Three droplets were measured per sample.

2.2. Atomic Force Microscopy (AFM)

Surface topography can be studied by AFM (atomic force microscopy). (62) Using this technique it is possible to probe surface roughness and assess if the PEGMA coating was uniform. AFM was used to determine if the fibronectin coating was successfully accomplished.

The image acquisition by AFM results from a tip attached to a flexible cantilever moving across the sample surface. This will make possible to assess the surface morphology on an atomic scale, by detecting changes in the surface height from measuring the location of the reflected laser beam in the quadrant photodetector. The result is a surface topographical map from which surface roughness values can be determined. (61)

The AFM (Dimizion 3100, Bruker) was used in the contact mode, at room temperature in air with a Bruker np tip, to characterize the modified PDMS surfaces. The software used was the NanoScope IVa Controller. Measurements (3 measurements per sample) were conducted on two modified PDMS surfaces. After fibronectin was adsorbed to these surfaces, they were again analyzed in AFM. The surfaces were then seeded in a ratio of $6.7x10^4$ cells per well and incubated (5% CO_2, 37ºC) for 24 h. Then, the medium was removed and the cells were incubated with

distilled water until all the cells detached from the surface. Afterwards the surfaces were observed by AFM.

3. Surface Cytotoxicity

To evaluate if some toxic substances could be released from the modified PDMS samples a cytotoxicity assay was conducted (Figure 11). Two modified PDMS samples were cut into 4 squares of 1 cm^2 and one of the samples (4 squares) was subjected to a washing procedure.

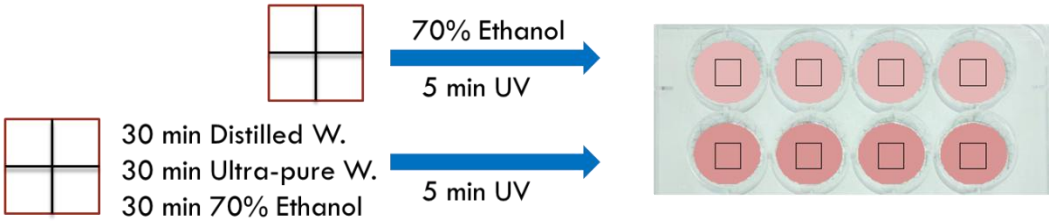

Figure 11. Scheme of the cytotoxicity assessment experiment. First, samples were cut into 4 small squares (1 cm^2). One of the samples was washed and the other was only sterilized. The 4 squares of each sample were placed in a 12 well-plate and the cells were seeded.

In the washing procedure, the 4 squares of one sample were placed in a 50 ml falcon with distilled water for 30 min under agitation. Next, the squares were moved into another 50 ml falcon with ultra-pure water for 30 min under agitation. Finally, the samples were placed in a new 50 ml falcon with 70% ethanol for 30 min under agitation. The non-washed sample was sterilized with 70% ethanol. Then, the squares of both samples were sterilized by 5 min exposure to UV radiation (both sides of the sample) and placed in the wells of a 12 well-plate. Finally, 2 ml of culture medium were added to each well and the plates were incubated for 24 h (37°C, 5% CO2).

After 24 h the samples were placed in a new 12 well-plate, with 2 ml of fresh medium in each well, and were incubated for another 24 h (37°C, 5% CO2). The previous 12 well-plate was kept in the fridge. This step was repeated again in order to dilute the possible leachables twice.

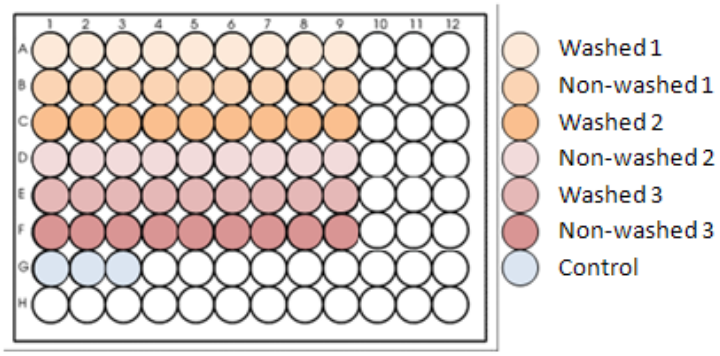

Figure 12. Schematic representation of the 96 well-plate used to seed the cells. Cells were seeded in a concentration of 5 x 10^4 cells/ml. After 24 h the medium was changed. Row A: medium incubated with the washed modified PDMS in the first 12 well-plate. Row B: medium incubated with the non-washed modified PDMS in the first 12 well-plate. Row C: medium incubated with the washed modified PDMS in the second 12 well-plate. Row D: medium incubated with the non-washed modified PDMS in the second 12 well-plate. Row E: medium incubated with the washed modified PDMS in the third 12 well-plate. Row F: medium incubated with the non-washed modified PDMS in the third 12 well-plate. Row G: control with fresh medium.

The cells were seeded in a 96 well-plate (Figure 12) in a concentration of 5 x 10^4 cells/ml (for details on cell culture see section 4.1). Each well contained 100 µl of medium with cells, i.e. 5000 cells per well. The plates were incubated for 24 h (37°C, 5% CO2). Next, the medium was removed and 100 µl of the medium with possible leachables were added to each well. The 96 well-plate was incubated for 24 h (37°C, 5% CO2).

In order to improve the cytotoxicity assessment and to obtain more conclusive results, some modifications have been introduced to the procedure described before. Modified PDMS samples were cut, washed and sterilized prior to the experiment. Afterwards, PDMS samples were placed into a 12 well-plate with 1 mL of culture medium and incubated for 72 h (37°C, 5% CO_2). After the referred incubation time, the culture medium was diluted according to the following ratios: 1:2, 1:4 and 1:8. Cells were seeded in a 96-well tissue plate and incubated for 24 h (37ºC, 5% CO_2). Next, the medium was replaced by the one that has previously been in contact with the samples, both diluted and undiluted, and cells were left to incubate for 72 h.

To measure the percentage of metabolically active cells and the percentage of viable cells in each well, after the incubation period the MTT and crystal violet (CV) assays were performed (for details see sections 2.4.1 and 2.4.2). The percentage of metabolically active viable cell was calculated dividing the two values.

3.1. MTT Assay

MTT ("3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) is a tetrazolium salt that has been widely used in order to screen the biochemical activity of cell cultures. Cells are capable of reducing this salt in their mitochondria, resulting in a formazan precipitate that can be dissolved with n-propanol and quantified spectrophotometrically. (48)

First, the medium of the 96 well-plates was discarded, the wells were washed with 1 ml of sterile PBS (37ºC) and 100 µl of MTT (Sigma) in a concentration of 0.5 mg/ml (diluted in culture medium) were added to the wells. The plate was incubated (37°C, 5% CO_2) during 2 h. After checking for cellular changes in coverage and morphology, the medium with MTT was gently removed and the wells were washed with 1 ml PBS (37ºC). Once the wells were again washed with PBS (37ºC), 120 µl of 2-propanol were added to each well and the plates were put on the orbital shaker at high speed (1100 rpm) until the formazan was completely dissolved. The absorbance was then measured at 570 nm in an automated plate reader (FluoStar Optima).

3.2. Crystal Violet (CV) Assay

Following the MTT determination, the same test plate with cells was washed 3 times with a PBS plus 0.05% Tween 20 solution. Then, 50 µl of 0.1% crystal violet (Sigma) solution were added to each well and the plate was incubated for 20 min at room temperature while shaking. After that, the plate was washed 4 times with running tap water. The cell-bound stain was extracted with 33% acetic acid (100 µl per well) while shaking for 15 min. The supernatant was transferred to a new 96 well-plate and the optical density was measured at 560 nm.

4. Cell-Biomaterial Interaction

4.1. Cell Culture

The human skin fibroblasts (HSkF) used in the experiment belong to the ATCC (American type culture collection) collection CRL-2429 (cell line CCD 1112SK). The human umbilical veins endothelial cells (HUVECs) were provided from the Department of Medical Biology of the University Medical Centre of Groningen. The medium used for the HSkF was the RPMI (Roswell Park Memorial Institute) complete (Gibco, UK), composed of 90% of RPMI medium (Gibco), 10% of fetal bovine serum (FBS, Gibco

10099-141, UK), 1% of penicillin-streptomycin (Gibco 15140-122, UK) and 1 % of L-alanyl-LGlutamine (GlutaMAX-I, Gibco 35050-038, UK). Regarding the HUVECs, the medium was supplied for the same department that kindly provided the cells.

The HSkF were stored in vials that were immersed in liquid nitrogen. To thaw the HSkF, a vial was removed from the liquid nitrogen and transferred to a water bath (37°C) until an ice clump was floating in the vial. Then, the vial was immersed in a 70% ethanol solution (room temperature) for decontamination, cleaned with a Kleenex tissue and transferred to the laminar flow cabinet[1] (Clean Air Techniek B.V., Woerden, Netherlands).

Then, 20 ml of the culture medium previously warmed (37ºC) were transferred to a centrifuge tube. After opening very carefully the vial, its content was transferred into the warm medium and the solution was homogenized. The solution was then centrifuged for 5 min at 1200 rpm (Heraeus Labofuge 400R centrifuge), the supernatant was discarded and the pellet was resuspended in 5 mL of fresh medium. This solution was transferred to a 25 cm^2 tissue culture polystyrene flask (Greiner) and incubated in the CO_2 incubator (Steri-Cycle, Thermo Forma, Marietta, Ohio, USA) at 37°C, 5% carbon dioxide and humidity saturated atmosphere.

The cells were kept in T-flasks until confluence was reached. Whenever confluence was reached, the cells were sub-cultured. The medium was removed from the flask and few milliliters of 1 % PBS (phosphate buffer saline; 137 mM NaCl, 1.47 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 2.68 mM KCl) solution were added to wash the flask and remove any remaining serum[2]. Afterwards, the PBS solution was discarded and trypsin/EDTA (ethylenediamine tetraacetic acid) 0.05% (Gibco, UK) was added. For a 25 cm^2 flask the recommended volume of trypsin is 1 ml and for a 75 cm^2 flask is 3 ml. The cells were then incubated in the CO_2 incubator during 2-15 min to allow cell detachment from the flask. The detachment time depends on several variables like the cell type, the enzyme added and the passage number. After cell detachment, 4 or 5 mL of culture medium were added to the cell suspension in the case of 25 cm^2 or 75 cm^2 flask, respectively. The cell suspension was then transferred to a 50 ml falcon. Furthermore, to wash any remaining cells in the flasks, 2.5 or 5 ml culture medium were added to the 25 cm^2 or the 75 cm^2 flask, respectively, and transferred to the 50 ml

[1] From this step forward, all the procedures are carried out in the laminar flow cabinet.

[2] Trypsin is inactivated by serum, so the flask has to be washed in order to remove any serum that may remain in the flask.

falcon. After determining the cell density in a Burker-Turk counting chamber (0.0025mm^2, Labor Optik) and dividing the cellular suspension into new culture flasks according to the optimal passage ratio, fresh culture medium was added to each flask so the total volume on the 25 cm^2 or the 75 cm^2 was 6.5 ml or 15 ml, respectively. The flasks were then incubated in the CO_2 incubator at 37ºC in a 5% carbon dioxide and humidity saturated atmosphere.

4.1.1. Specific conditions

The HSkF were sub-cultured (when they reached 80% confluence), usually after 3 to 4 days, in 1:2 splitting ratios.

The HUVECs require a thin layer of gelatin (Sigma) to adhere, grow and reach confluence. The gelatin-coated culture flasks were pre-incubated in the CO_2 incubator for 30 min, and afterwards the gelatin that was not solid was removed immediately before adding the cell suspension to the flask. During the cell sub-culturing some extra care was needed, like pipetting to the lateral side of the flasks, so that the gelatin layer was not disturbed. These cells were subcultured after 3 to 4 days in 1:3 splitting ratios.

4.2. Cell Interaction with Modified and Unmodified PDMS

In order to evaluate the influence of the PEGMA coating in cell adhesion, both unmodified and modified PDMS surfaces were incubated with the cell lines above referred.

The modified PDMS samples were cut in 1 cm^2 squares, and then sterilized with 70% ethanol and 5 min of UV radiation in both sides. Next, each square was placed in a well of a 12 well-plate. For the experiments conducted with fibroblasts, two 12 well-plates were used since two different incubation times were tested. The first four wells contained the unmodified PDMS samples, the next four the modified PDMS samples, and the last four the modified PDMS samples with a fibronectin coating (Figure 13). The same setup was used for the HUVEC cells, but in this case an extra plate was used for each time, with four modified PDMS samples covered with a gelatin layer, in a total of four plates (Figure 14).

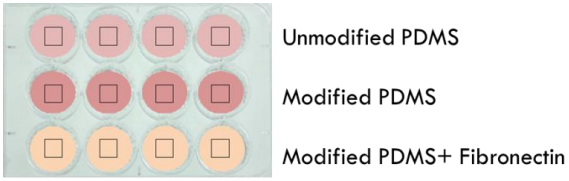

Figure 13. Distribution of PDMS samples in the 12 well-plate, for the experiments with HSkF. The first 4 wells contain unmodified PDMS surfaces, the next 4 contain modified PDMS surfaces and the last 4 wells contain modified PDMS with a fibronectin coating. The 12 well-plate was prepared in duplicate, one to be incubated for 48 h and the other for 120 h.

Figure 14. Distribution of PDMS samples in the 12 well-plate, for the experiment with HUVECs. In the left plate, the first 4 wells contain unmodified PDMS surfaces, the next 4 contain modified PDMS surfaces and the last 4 wells contain modified PDMS with a fibronectin coating. In the second plate, the first 4 wells contain modified PDMS with a gelatin coating. Both the 12 well-plate were prepared in duplicate, one to be incubated for 48 h and the other for 120 h.

The cells were seeded in the wells containing the PDMS samples in a ratio of $6.7x10^4$ cells per well. Afterwards, the plates were incubated (5% CO_2, 37ºC) at two different time points, namely 48 and 120 h. After the 48 h, the cells of one plate were fixed using 0.9 ml of PFA (paraformaldehyde; 3.7% paraformaldehyde in CS buffer: 0.1 M PIPES, 1 mM EGTA, 4% (w/v) polyethylene glycol 8000 (all Sigma), pH 6.9) during 5 min. Then, extra 0.9 ml of PFA was added, this time for 10 min, and after removing the PFA, 1 ml of PBS was added. The plate was then wrapped up with aluminum foil and stored at 4ºC. The same procedure was carried out in the second plate, after the 120 h of incubation. Finally, cells in both plates were stained with fluorescent markers according to the procedure described in the “Immunocytochemistry Assay” section. After staining the samples were observed and pictures were taken with Confocal Microscopy.

Based on the results obtained, the previously described experiment was repeated for the HUVECs, but using a different distribution of the samples (Figure 15). With this new sample distribution, it was possible to conclude about the surface influence in cell adhesion and proliferation since the only variable of the experiment

was the surfaces being used. The incubation times changed from 48 and 120 h (2 and 5 days) to 3, 4 and 5 days.

Figure 15. Distribution of PDMS samples in the 12 well-plate, for the second experiment with HUVECs. In the left plate, the first 4 wells contain unmodified PDMS surfaces with adsorbed fibronectin, the next 4 contain modified PDMS surfaces with adsorbed fibronectin and the last 4 wells only contain a fibronectin coating. In the second plate, the first 4 wells have no additional surface or coating. Both the 12 well-plate were prepared in triplicate, one to be incubated for 3 days, the other for 4 days and the last for 5 days.

4.2.1. Immunocytochemistry Assay

The immunocytochemistry assay was performed in 2 (out of 4) wells for each surface. After cells fixation, 1 mL of 5% BSA (bovine serum albumin; Sigma) in PBS was added to each well for 10-20 min to block non-specific background. Then, 750 µL of the primary antibody diluted in PBSA (PBS + 1% BSA) were added to each well for 1 hour, at 20°C. Before incubating the secondary antibody each well was washed three times with 1 mL of PBSA. The secondary antibody, previously diluted in PBSA was incubated for 1-2 h at 20°C. Because of the fluorescent label the plates were covered up with aluminum foil during the incubation times. Afterwards, the wells were washed 4 times for 5 min with 1 mL of PBSA and 2 times for 5 min with PBS. Then, the plates were again covered up with aluminum foil and were ready to be observed under Confocal Microscopy.

Two different first antibodies were used, a polyclonal rabbit-α-human fibronectin and a monoclonal mouse-α-human vinculin, in order to stain the fibronectin and the vinculin networks, respectively. Therefore, two second antibodies were used as well, a donkey-α-rabbit IgG (immunoglobulin) with a redX (rhodamine) fluorescent label and a goat-α-mouse IgG with a FTIC (fluorescein isothiocyanate) fluorescent label. In order to use these antibodies, adequate dilutions had to be performed. For the rabbit-α-human fibronectin a 1:400 dilution was used. For all the other antibodies, mouse-α-human

vinculin, donkey-α-rabbit IgG and goat-α-mouse IgG, the dilution ratio was 1:100. Finally, DAPI (4',6-diamidino-2-phenylindole) was also added to stain the nuclei of the cells (4 µg/mL), as was TRITC-labelled phalloidin (Sigma, 2 µg/mL).

4.2.2. Confocal Microscopy

Cells were observed using a LEICA TCS SP2 (LEICA, Germany) confocal microscope. To observe the cells conformation and quantify the cell number and the expression of fibronectin and actin, the Leica Confocal Software (version 2.61) and ImageJ program (version 1.41) were used. Pictures were taken in three different places in each sample. Using the Leica Software, several confocal images stacks that correspond to several optical slices were taken. The thickness of each image stack was chosen with the purpose of including all the relevant structures. After that, it was possible to create a two dimensional projection, that is an overlay of all images of each sample. The scale and resolution were the same for all the pictures; therefore the results could be compared. The area of the pictures was of 375µm x 375µm and the number of pixels was of 1024 per 1024.

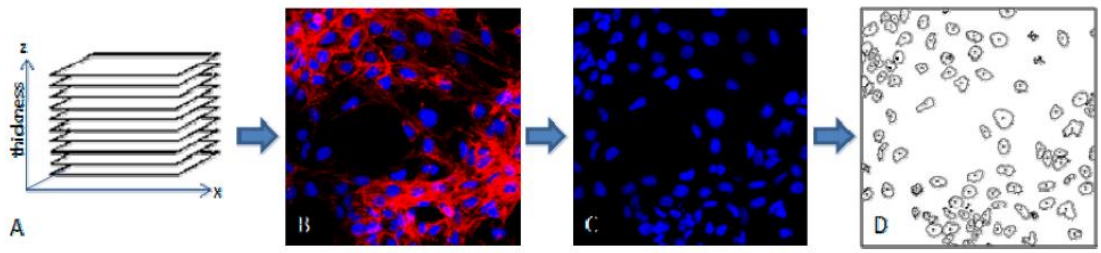

Figure 16. Scheme representing the image processing for cell number quantification. (A) image stack (B) overlay image (C) selection of DAPI staining fluorescence (D) particle analysis image. [Taken from (60)**]**

After the overlay image was obtained (Figure 16B), the DAPI staining fluorescent was selected (Figure 16C). Then, it was possible to count the number of nucleus and consequently quantify the cell density. For this purpose, Scion Image Software (version β 4.0.2) was used. After setting some parameters in the software it was possible to count the intact nuclei (Figure 16D). The same software was used to determine the percentage of fibronectin in each sample.

5. Statistics

The statistical analysis of the experiments was conducted using two methods, the *t*-test and the ANOVA. The *t*-test was used to compare the results obtained for 2 different groups, considering a significance level (α) = 0.05. Whenever 3 or more different groups were compared, the ANOVA was used, also with an α = 0.05. Regarding the ANOVA statistical analysis, when 3 or more independent samples with only one variable (e.g. concentration) were compared the one-way ANOVA was performed. However, when two variables were present (e.g. time and concentration) the two-way ANOVA was required.

Moreover, the ANOVA model only allows to state that 3 or more independent samples are statistically different. Using ANOVA it is not possible to determine which groups are different. Therefore, the *t*-test was used to compare the independent samples in groups of 2 (N=3, sample A was compared with sample B, after was compared with sample C, and finally sample B was compared with sample C).

The two statistical methods used give a *p*-value (probability that the variance is not significant) from the *F* statistic. When the *p*-value is higher than α it can be concluded that the samples do not present significant statistical differences. On the other hand, if $p < 0.05$ the samples present significant statistical differences.

Chapter 3. Results & Discussion

This chapter gathers the results obtained in the present work, namely on the contact angle measurements and the assessment of cell-biomaterials interactions, as well as the discussion in view of the current state-of-the-art.

1. Surface Characterization

Biomaterials performance depends largely on the protein adsorption which will influence the future responses of the host to the biomaterial. Controlling these interfacial phenomena by surface modification has become crucial in the development of biomaterials for implantation purposes. (63)

As mentioned in Chapter 1, PDMS is a hydrophobic material, which is prone to adsorb proteins or small hydrophobic molecules. Due to this limitation, PDMS was modified by ATRP in order to obtain a more hydrophilic surface. Surface contact angles were measured, and the surfaces were observed by AFM to confirm the occurrence of a successful surface modification. AFM was also used to visualize the same surfaces after fibronectin coating and after cell adhesion.

1.1. Contact Angles

The water contact angles were measured in 5 independent modified PDMS surfaces, and 3 measurements were carried out for each independent sample. Regarding the unmodified PDMS, 2 independent surfaces were tested, also with 3 measurements for each sample. The results obtained for the contact angles measurement in both unmodified and modified PDMS are gathered in Table 3.

Table 3. Contact angles measured for the modified and unmodified PDMS. Results represent the average of 5 (modified PDMS) and 2 (unmodified PDMS) independent measures ± standard deviation.

	Contact Angle
Modified PDMS	68.1 ± 6.3
Unmodified PDMS	88.3 ± 2.7

For the unmodified PDMS, an 88.3 ± 2.7 water contact angle is observed, thus demonstrating its hydrophobic character. According to the literature, the contact angle formed between deionized water and the PDMS is usually around 105°, which means that the contact angle represented in Table 3 is low. (19; 64; 65) However, Armani and coworkers referred that a contact angle between 90 and 120° can be measured. (66) Usually, the 90° contact angle corresponds to the receding contact angle, which is the smallest possible angle. This angle is measured when the minimum volume necessary to form a water droplet in the surface is used. (67) Thus, the measured contact angle may be low due to the use of a small water volume. Considering the standard deviation, the contact angle measured in the unmodified PDMS samples is in accordance to the literature. For example, Chen and co-workers (2004) reported a contact angle of 89° for the PDMS. (67)

On the other hand, the modified PDMS presented a 68.1 ± 6.3 water contact angle value, which is lower than the one obtained for the unmodified sample. The statistical analysis of the data demonstrates that there is a significant statistical difference between both unmodified and modified samples ($p < 0.05$). This lower contact angle value indicates that the surface is more hydrophilic, thus confirming the existence of PEGMA brushes at the surface, and therefore a successful modification procedure. Although this contact angle is close to the optimal contact angle for the best cell adhesion (60°), the value is still higher, which can influence cell adhesion. (68)

Furthermore, a significant statistical difference was observed when comparing the different modified samples ($p < 0.05$). This result indicates that the coating varies from surface to surface, which suggests that the coating technique used was not reproducible. The non-reproducibility can be explained by the difficulty in controlling the homogeneity of the polymer brushes formed in the surface, due to chain breaking that may occur during the propagation step. (69)

1.2. AFM

The topography of a modified surface is an extremely important parameter to be assessed, because it affects directly protein adsorption and cell adhesion and proliferation on the surface. Atomic force microscopy (AFM) is an extremely useful tool that allows studying the modified surfaces. This type of microscopy provides a real image of the surface morphology and nanostructure and topographical information about surface roughness.

In order to observe the surface topography of the modified PDMS and to assess if the fibronectin coating is present, pictures from the modified PDMS were taken before and after adding fibronectin to the coating. The pictures are illustrated in Figure 17.

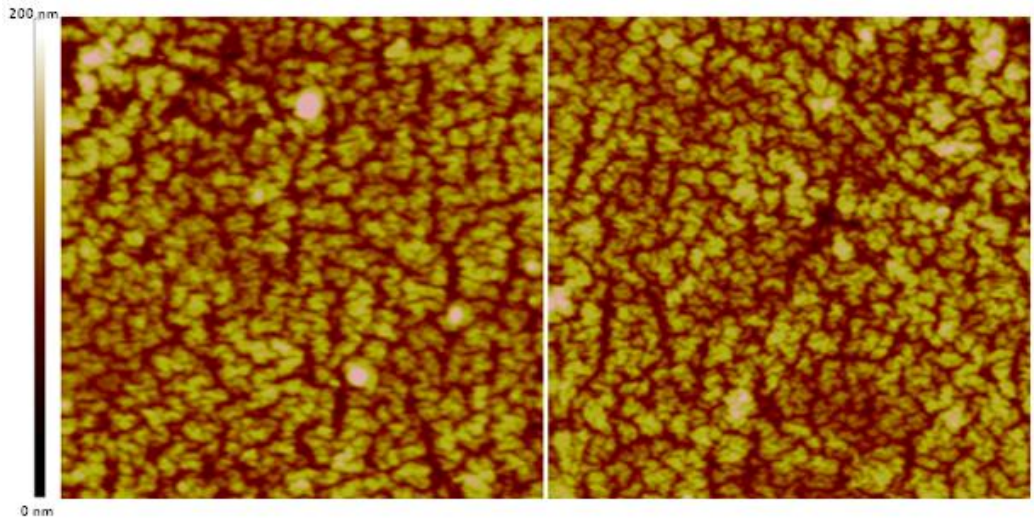

Figure 17. Images obtained by atomic force microscopy (AFM) from the modified PDMS without (left) and with (right) a fibronectin coating. Both the pictures correspond to an area of 5 x 5 µm. Surface roughness is represented between 0 and 200 µm.

From Figure 17, it is possible to observe that the PEGMA coating resulted in a surface with a high roughness, with coating heights that can reach more than 100 nm. No control (unmodified PDMS) was observed, once the main goal of this experiment was to assess if the fibronectin was adsorbing to the PEGMA coating. However, from literature it is known that the common roughness of bare PDMS is around 0.52 nm. (29; 67) Since a roughness higher than 100 nm is observed in the modified PDMS, it is possible to conclude that the ATRP procedure resulted in the coating of the PDMS surface with dense and long PEGMA brushes. From the high roughness observed, it is possible to conclude that ATRP is a suitable technique to coat PDMS surface. However, a roughness between 100 nm and 1 µm (submicron roughness) can have a dual effect on cell adhesion, growth, viability and maturation. Zhao *et al* (2006) concluded that a submicron roughness in titanium surfaces lead to a lower cell adhesion and spreading comparing to the flatter surface. Though, the cells adhered to the submicron roughness surface produced more factors that are responsible for promoting cell differentiation. (70) Thus, the surface roughness that was observed can be responsible for a lower cell density.

After incubating the surfaces with fibronectin, the surfaces were again observed with AFM (Figure 17, right side). No considerable differences in the surface roughness are observed. However, Hull and co-workers (2008) determined the roughness of fibronectin adsorbed to a surface (1 cm x 1 cm). The researchers tested different concentrations of fibronectin and reached a range of roughness from 7 to 11 nm (concentrations of fibronectin from 1 to 100 µg/ml). (71)

In the current experiment a fibronectin concentration of 5 µg/ml was used, so an increase in the surface roughness should be less than 11 nm. The surface roughness before the fibronectin adsorption was higher than 100 nm, which would mean an increase in the roughness of around 10%. In this case, it is not possible to confirm if the fibronectin is or not adsorbed to the PEGMA, thus hampers to draw any conclusion regarding the fibronectin coating.

After coating the surfaces with fibronectin, they were seeded with cells and incubated. After the incubation period, the surfaces were incubated with ultra-pure water in order to detach the cells from the surface. The surfaces were then analyzed with AFM to observe if there were any changes in the topography, indicating that some coating degradation could be occurring during its incubation with cells. The results are illustrated in Figure 18.

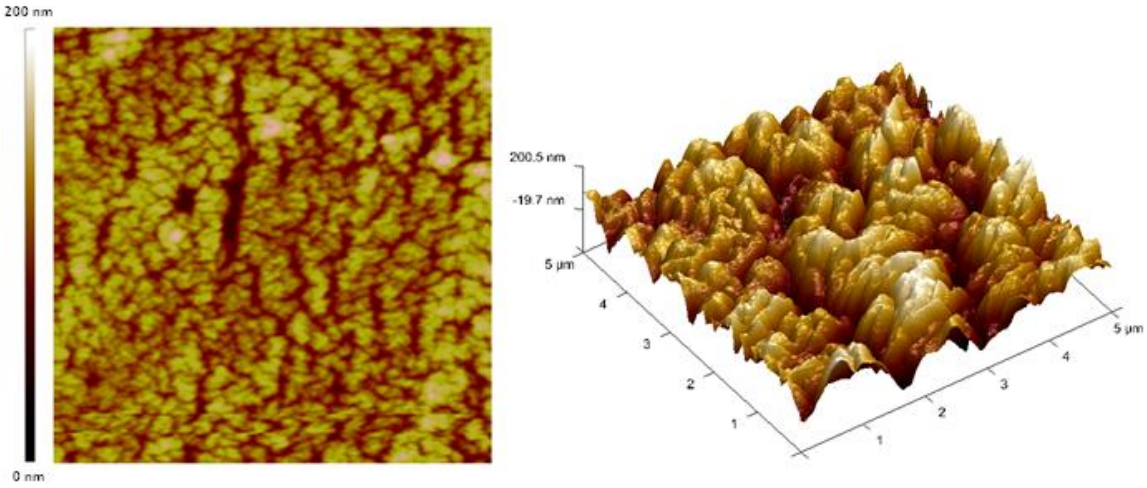

Figure 18. Images obtained by atomic force microscopy (AFM) from the modified PDMS with a fibronectin coating after cell seeding. The picture on the left has a normal resolution and corresponds to an area of 5 x 5 µm. The picture on the right is a 3D picture from a high resolution picture taken from the surface.

From Figure 18, no visible changes in the surface topography could be observed. These results suggest that there was no degradation of the coating during its incubation with cells. Nevertheless, the fact that the PDMS surface is coated with PEGMA brushes, which makes the surface coating non homogeneous, difficult the confirmation of such surface degradation

2. Surface Cytotoxicity

In order to evaluate if leachable compounds were being released from the surfaces or if there were some compounds that were not completely washed from the surfaces after the modification procedure, thus interfering with the cell-biomaterials interactions, the surfaces cytotoxicity was evaluated (for details see section 3). Two complementary assays were used, namely MTT (colorimetric assay to evaluate cell metabolism) and CV (colorimetric assay to evaluate viable cells). The ratio between these values was used to determine the metabolism per cell (Figures 19 and 20). These results allowed monitoring the biochemical activity of viable cells. (48) The first assessment was conducted with the fibroblasts.

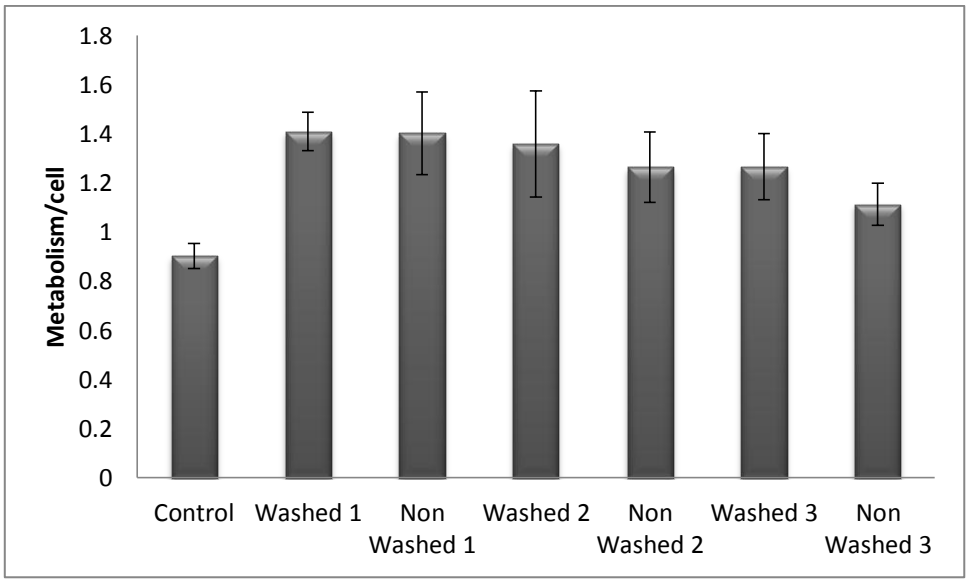

Figure 19. Ratio between the MTT values and CV values for each dilution of the medium in contact with washed and non-washed modified PDMS surfaces. Control consists of the fibroblasts seeded in fresh medium. Results correspond to the average of 3 independent assays ± standard deviation.

From Figure 19 it is possible to observe the different metabolism per cell present in the control and the testing samples. Through statistical analysis (ANOVA), it

is possible to determine that there is a significant statistical difference between the control and the wells with medium incubated with both the washed and non-washed surfaces ($p < 0.05$). This increase in the metabolic activity per cell is due to an increase in the MTT. The CV values are similar in the different samples, which indicate that there is a higher cell metabolism in the different testing samples than in the control. These results point to the presence of some compound in the medium that is stimulating the fibroblasts and increasing their metabolic activity.

Huang *et al* (2011) also observed an increase in the metabolic activity when they seeded hepatocytes with modified polyester (PES) surfaces. In their experiment, this increase in the metabolic activity indicated a stronger adhesion of the cells to the modified PES which resulted in higher numbers of cellular adhesion and, consequently, a higher metabolism. (72) However, in the current work the fibroblasts were not seeded in contact with the modified PDMS, and therefore the results are not comparable. Since in the current study, the only variable condition in the experiment was the medium with which the cells were incubated, this means that only the presence of some compound (leachable) on the medium can be the cause of an increase in the cellular metabolism.

Comparing the results from the wells with medium incubated with the washed surfaces and the results from the wells with medium incubated with the non-washed surfaces, there is a statistically significant difference ($p < 0.05$). This indicates that a washing procedure prior to cell seeding introduces differences in the metabolism per cell. Observing Figure 19, a higher metabolism per cell is present in the wells with medium incubated with surfaces submitted to a washing procedure. This result indicates that the differences observed may not result from an increase in cell metabolism due to some leachable compound. If some compound released from the surfaces was stimulating the cells, the washed surfaces should present a lower metabolism per cell. However, the metabolism is higher. Thus, the higher metabolism may result from some interference of some medium chemicals in the MTT measurement, which results in a higher activity of the enzyme responsible for MTT reduction. (73)

Also, there is a significant statistical difference ($p < 0.05$) between the results obtained for the wells with different dilutions of the medium incubated with the non-washed surfaces. The metabolic activity per cell decreases with increasing dilutions, which indicates once more that the non-washed surfaces release some compound that stimulates the fibroblasts. Regarding the washed surfaces, no significant statistical difference ($p > 0.05$) between the different dilutions of the medium are observed. This

discrepancy in the results suggests some arbitrary interference of a medium compound in the MTT assay.

Similar results were obtained in the same experiment conducted with HUVECs (Figure 20).

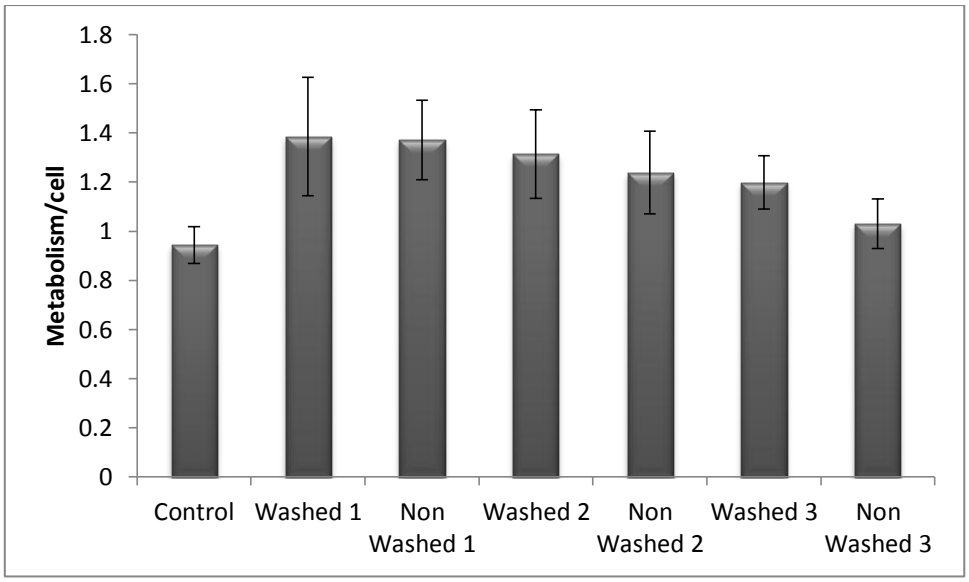

Figure 20. Ratio between the MTT values and CV values for each dilution of the medium in contact with washed and non-washed modified PDMS surfaces. Control consists of the HUVECs seeded in fresh medium. Results correspond to the average of 3 independent assays ± standard deviation.

The results illustrated in Figure 20 show a similar tendency to the results obtained for the fibroblasts (Figure 19). However, for the HUVECs, the MTT and the CV show a similar variation, which indicates that any increase in the metabolic activity per cell is the result of both the increase in the MTT measurement and the CV measurement. For the HUVECs, there is a significant statistical difference ($p < 0.05$) between the wells with the different dilutions of the medium incubated with the washed surfaces. On the other hand, no significant statistical difference ($p > 0.05$) is observed between the wells with the dilutions of the medium incubated with the non-washed surfaces. This trend suggests that an inefficient homogenization of the cells suspension before distribution through the wells may be on the basis of the observed differences.

In order to evaluate if the incubation time could result in different values of metabolism per cell and decrease the interferences observed, some changes were introduced in the experimental protocol. These experiments were conducted with both fibroblasts and HUVECs cells. Briefly, the medium was incubated with the modified PDMS (washed and non-washed) and afterwards it was diluted at different ratios (1:2, 1:4, 1:8). Moreover, the incubation time of the cells with the different medium was

increased to 72 h. The results obtained with this new experimental setup are illustrated in Figures 21 and 22.

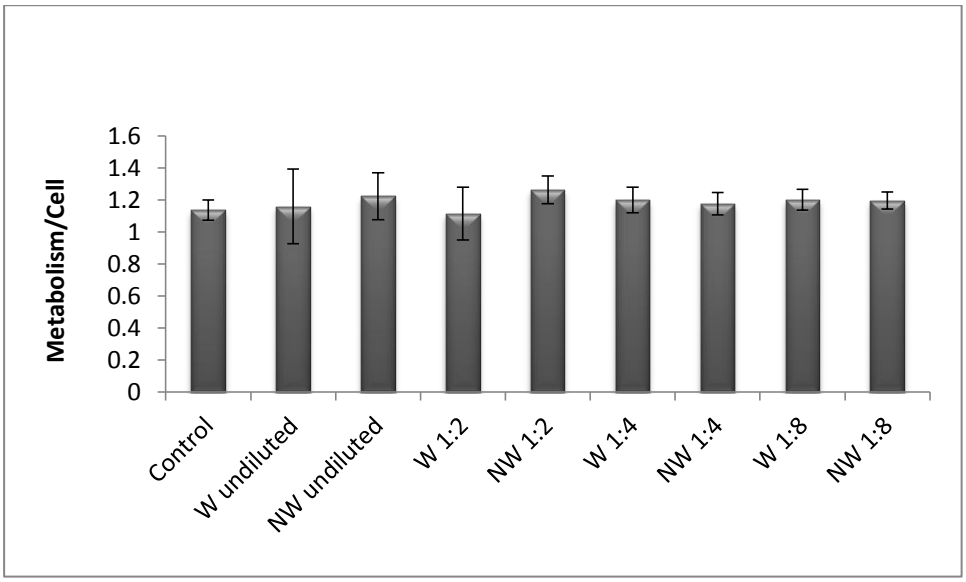

Figure 21. Ratio between the MTT values and CV values for each dilution of the medium in contact with washed and non-washed modified PDMS surfaces. Fibroblasts were seeded with medium that was previously incubated with modified PDMS and diluted afterwards. Control consists of the fibroblasts seeded in fresh medium. Results correspond to the average of 3 independent assays ± standard deviation.

From Figure 21, no statistically significant differences ($p > 0.05$) are observed between either the washed and non-wahsed surfaces, the several dilutions in both the situations, and both the medium incubated with washed and non-wased surfaces and the control. This indicates that the metabolism of fibroblasts in contact with the medium incubated with the washed and non-washed modified PDMS after 72 h is similar to the results obtained for the control experiment. Therefore, the results suggest that the surfaces do not release any cytotoxic compound, either they are previously washed or not. Thus, these modified surfaces can be used to assess cell adhesion without previous washing and no cytotoxic effect from the surfaces is expected.

Regarding the HUVECs, the results obtained with the same experiment are represented in Figure 22. Contrarily to what happened in the previous experiment, where the results obtained for the fibroblasts and the HUVECs are similar, in this experiment there are differences in the results obtained for the different cell lines.

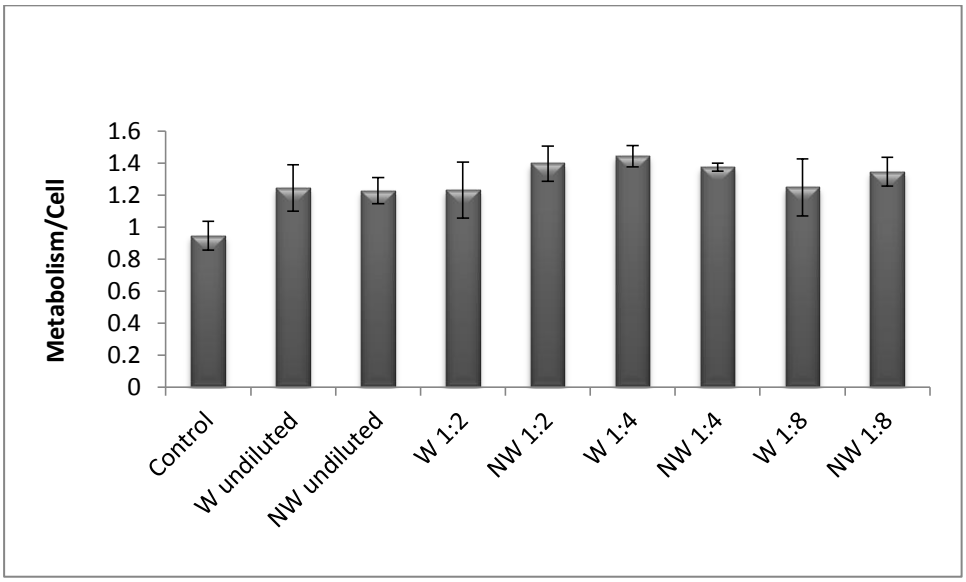

Figure 22. Ratio between the MTT values and CV values for each dilution of the medium in contact with washed and non-washed modified PDMS surfaces. HUVECs were seeded with medium that was previously incubated with modified PDMS and diluted afterwards. Control consists of the fibroblasts seeded in fresh medium. Results correspond to the average of 3 independent assays ± standard deviation.

From Figure 22, it is possible to conclude that as observed previously, there is no significant statistical difference ($p > 0.05$) between the wells with medium incubated with a washed surface and with a non-washed surface. No statistically significant differences are either observed between the different medium dilutions, for both the washed and non-washed surfaces. These results are in accordance with the results obtained for fibroblasts using the same experimental protocol.

However, a significant statistical difference ($p < 0.05$) is observed between the wells with the medium incubated with the washed and non-washed modified PDMS and the control wells. The differences result both from an increase in the metabolic activity and an increase in the cell viability. Although this indicates that the medium incubated with the surfaces stimulates the HUVECs, which become more metabolically active and present higher cell viability, the lack of differences between dilutions indicates the opposite. Thus, the differences may also result from a defficient homogenization of the cell suspension.

3. Cell-Biomaterial Interaction

Assessing the cell morphology and its distribution on the biomaterial surface is an important step in the evaluation of the interactions between cells and biomaterials. Imaging these interactions may provide significant information about cell phenotype and function. (74)

In order to evaluate the interactions between cells and the modified PDMS surfaces, an immunocytochemistry assay was performed as described in the Materials & Methods section. The confocal microscopy images shown in Figure 23 were obtained in that assay conducted after seeding fibroblasts on wells containing the modified PDMS (with and without an additional fibronectin coating) and unmodified PDMS, at two distinct time points (48 and 120 h).

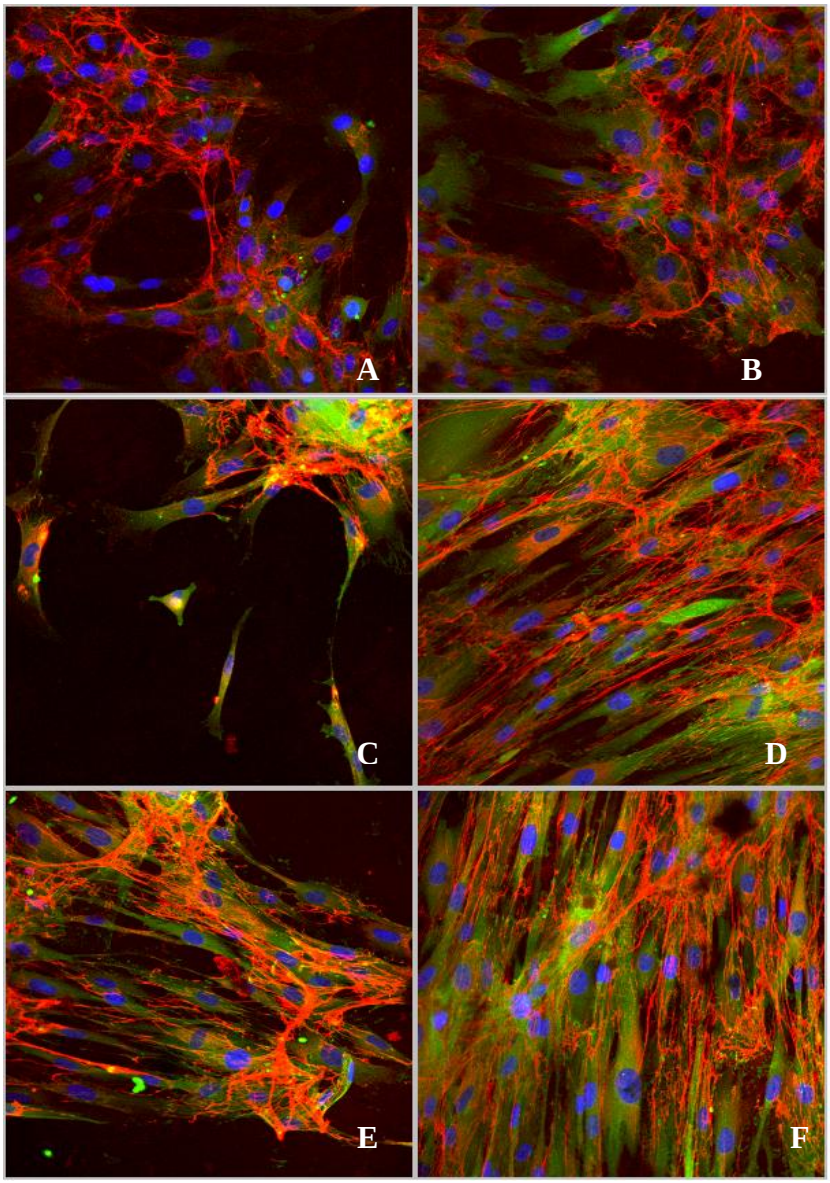

Figure 23. Images obtained by confocal microscopy of the immunochemistry assay conducted after exposing fibroblasts to the modified PDMS for 48 and 120 h. A and B - fibroblasts adhered to unmodified PDMS, after 48 and 120 h, respectively. C and D - fibroblasts adhered to modified PDMS, after 48 and 120 h, respectively. E and F - fibroblasts adhered to modified PDMS with a fibronectin coating, after 48 and 120 h, respectively.

Figure 23 clearly demonstrates the differences on the fibroblasts adhesion to the different surfaces and at different time points. The green staining represents vinculin, a structural protein present in the cytoplasmic side of focal adhesions. In red it is possible to observe the fibronectin, and in blue the cell nuclei. It is important to notice that the cells present a good and stretched conformation, thus indicating that these materials are non-cytotoxic. (75) Regarding the different surfaces, it can be observed that there are more cells adhered to the unmodified PDMS (Figure 23A and 23B) as compared to the ATRP modified PDMS (Figure 23C and 23D). This result was

expected since the PEGMA brushes introduced at the PDMS surface turned it more hydrophilic (see section on contact angles measurements) and should decrease the protein adsorption, thus decreasing cell adhesion. As referred by Wu and co-workers (2008), PDMS hydrophobic nature makes it prone to the adhesion of proteins, which is enhanced by the use of serum in the culture medium, thus inducing cell adhesion. On the other hand, hydrophilic surfaces have lower levels of protein adsorption, which leads to lower cell adhesion levels. (63)

The same trend is observed comparing modified PDMS with and without a fibronectin coating. Fibronectin is a glycoprotein that binds to the cell integrins, thus promoting adhesion. This glycoprotein contains the sequence RGD, which is necessary for focal adhesion formation. Focal adhesions are known to be critical for cell survival. (76) So, as expected, more cells are adhered to the modified PDMS surface with a fibronectin coating (Figure 23E and 23F).

Finally, comparing the unmodified PDMS with the modified PDMS with a fibronectin coating, the differences found are not clear although it is possible to visualize a better conformation (more stretched) of the fibroblasts in the second case. (75) As discussed above, this can be explained by the presence of the fibronectin that is well-known to promote cell adhesion.

Regarding the stained structures, in all the pictures it is possible to observe assembled fibronectin fibers, which got organized in networks with increasing density over time. This indicates a good cell attachment to the surfaces. (48) Vinculin can also be observed, but no focal adhesions (patches containing vinculin) are present. It is possible to observe that both the proteins seem to be co-localized after 120 h and, as reported by Singer (1982), fibronectin-vinculin complexes might correspond to focal adhesions. This indicates that even though vinculin patches are not visible, the focal adhesions may be present. (77)

Cell proliferation is present because, comparing the results obtained for the different time points, cell density increases for all the surfaces. This also indicates the presence of focal adhesions, which are necessary for cell spreading, although these ones are not visible.

After obtaining confocal images of the cells adhered to the surfaces, the nuclei in each image were counted and it was possible to compare the average number of cells adhered in the different time points and surfaces. The results are illustrated in Figure 24.

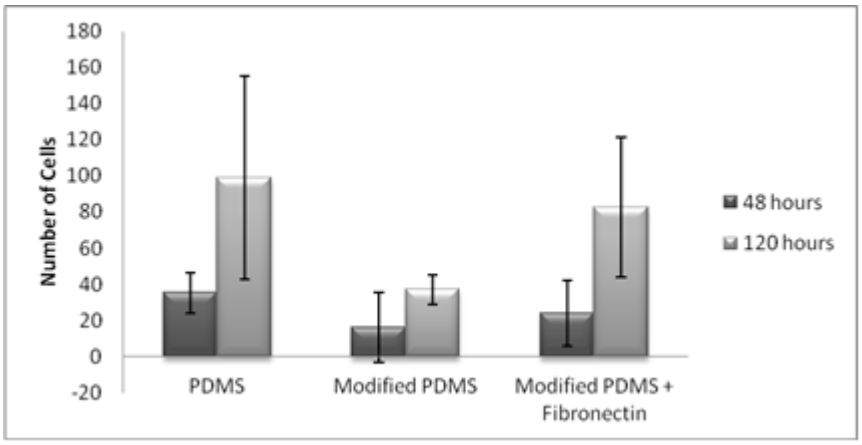

Figure 24. Average number of nuclei counted after 48 and 120 h of cells exposure to unmodified PDMS, modified PDMS and modified PDMS with a fibronectin coating. Results correspond to the average of 6 counts ± standard deviation.

Results gathered in Figure 24 show high standard deviations mainly due to the experimental method. In this method, three random pictures were taken to each sample (2 samples for each surface). Since cell distribution in the surfaces is not homogeneous, these random pictures take into account large variations of cell numbers.

Furthermore, after 48 h, and due to the considerable standard deviation observed, the cell adhesion between the different surfaces presents no significant statistical difference ($p > 0.05$).

After 120 h, significant statistical differences ($p < 0.5$) between the number of cells in the unmodified and modified PDMS, and between the modified PDMS and modified PDMS with a fibronectin coating, can be observed. However, no statistically significant difference is found between the number of cells adhered to the unmodified PDMS and the modified PDMS with a fibronectin coating ($p > 0.05$). The fact that a higher cell adhesion is present in surfaces coated with fibronectin, comparing to the PEGMA modified PDMS surfaces, is in accordance with the literature, since the fibronectin being a protein that establishes cell adhesion, is known to activate cell spreading, proliferation and differentiation. (51) However, no differences are observed between the unmodified PDMS and the modified PDMS with adsorbed fibronectin, which may be caused by the high standard deviations that were obtained.

The fact that no significant differences are observed in the first 48 h may be related to the explanation suggested by Tziampazis and co-workers (2000). These authors suggest that when cells attach to ligands adsorbed in a surface they will be

able to rearrange the membrane receptors and the surface ligands, which in the future will ease the cell binding to the surface. Therefore, in the beginning few binding sites will be available in all the surfaces, but over time, due to the rearrangement of the surface ligands, more cells will be able to adhere to the surface and to spread. (78)

Using suitable software (Scion Image, Scion Corporation, US) it was possible to estimate the percentage of fibronectin present at the surfaces in the different time points (Figure 25). Quantifying the fibronectin is important because, as referred before, this protein contains a necessary peptidic sequence (RGD) that is necessary for focal adhesion formation, being an indicator of a higher cell adhesion. (76)

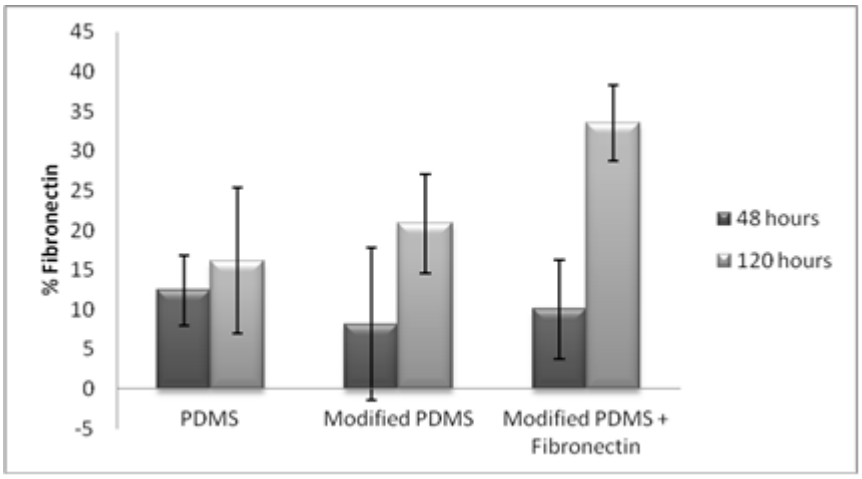

Figure 25. Estimate of the fibronectin percentage present after 48 and 120 h using unmodified PDMS, modified PDMS and modified PDMS coated with fibronectin. Results correspond to the average of 6 experiments ± standard deviation.

A higher percentage of fibronectin is an indication of a stronger cell adhesion. From Figure 25 it is possible to infer that after 48 h no significant statistical difference between the fibronectin percentages in the unmodified PDMS and the modified PDMS with a fibronectin coating is observed ($p > 0.05$). However, significant statistical differences ($p < 0.05$) are present between either the unmodified PDMS and the modified PDMS, and the modified PDMS and the modified PDMS with a fibronectin coating. Higher percentages of fibronectin are detected in both the unmodified PDMS and the modified PDMS with a fibronectin coating, which indicates a stronger cell adhesion to these surfaces.

After 120 h there is a statistically significant difference between the modified PDMS with a fibronectin coating and the other samples ($p < 0.05$). This result is in accordance with the results reported by Webb *et al* (2000), which concluded that the attachment of fibroblasts on a surface is a function of the amount of fibronectin

immobilized in the surface. (79) Furthermore, no significant statistical difference ($p > 0.05$) is found between the unmodified and the modified PDMS.

It is possible to conclude that the modified PDMS produces a weaker cell adhesion, but when a fibronectin coating is added the strength of the cell adhesion increases and a higher fibronectin network is produced over time.

Once the aim of the present work is to evaluate if the modified PDMS is suitable to be used as a vascular graft, the same experiments were conducted using HUVECs (human umbilical vein endothelium cells). Figure 26 illustrates the images obtained by confocal microscopy in the immunocytochemistry assay.

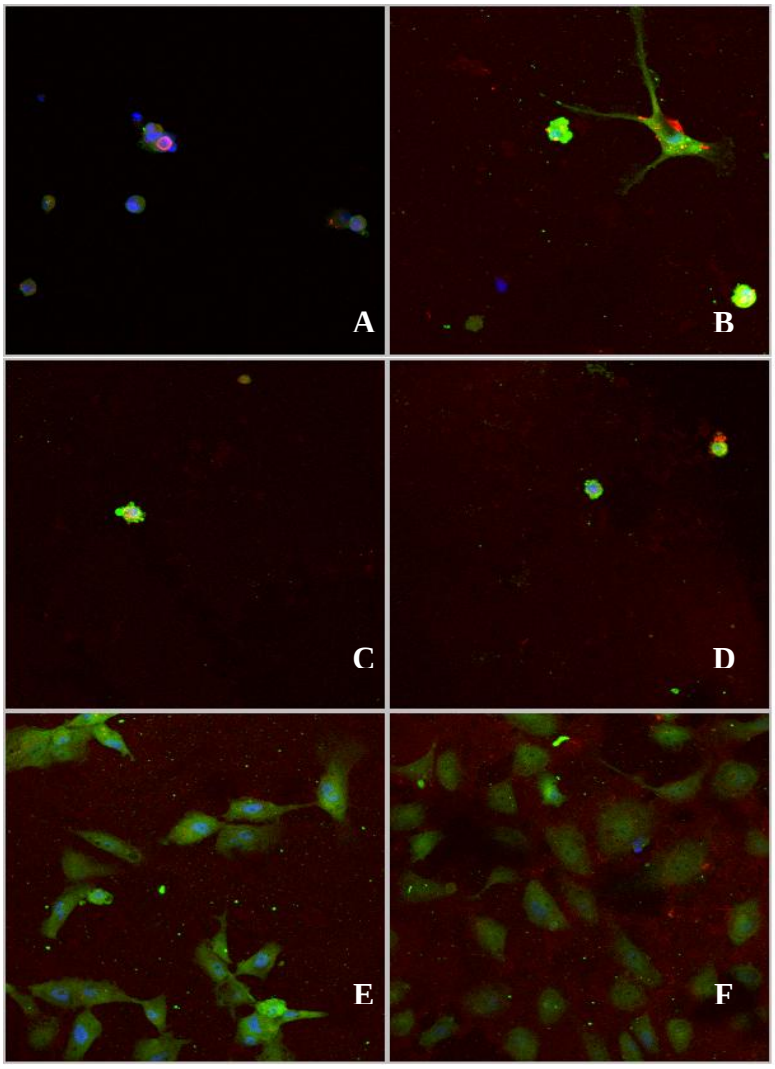

Figure 26. Images obtained by confocal microscopy of the immunochemistry assay conducted after exposing HUVECs to the modified PDMS for 48 and 120 h. A and B - HUVECs adhered to unmodified PDMS, after 48 and 120 h, respectively. C - HUVECs adhered to modified PDMS, after 48 h. D - HUVECs adhered to modified PDMS coated with fibronectin after 48 h. E and F - HUVECs adhered to modified PDMS coated with gelatin, after 48 and 120 h, respectively.

Results from Figure 26 reveal that HUVECs behavior when in contact with the different surfaces under study is completely different from the fibroblasts. Clearly, few cells adhered to the unmodified PDMS (Figure 26A and 26B) even after 120 h. Regarding the modified PDMS without (Figure 26C) and with (Figure 26D) a fibronectin coating, very few cells adhered after 48 h. In all the previously referred cases, no cells could be found after 120 h, consequently no images were taken at that time points.

The lack of cell adhesion on the unmodified PDMS is expectable, because as referred by Kooten and co-workers (2004) endothelial cells don't adhere to hydrophobic surfaces. This lack of cell adhesion is explained by the inaccessibility of adhesion sites in the surface. However, with the increase in the hydrophilicity (modified PDMS) a higher cell adhesion is expected, because endothelial cells readily form focal adhesions on hydrophilic polymers, which is not observed. In a previous work from Kooten *et al* (2004), limitations in the endothelial cell adhesion to hydrophilic polymers were also reported. The authors coated a polymeric surface in order to make it more hydrophilic and endothelial cells were seeded, but after 24 h these cells began to detach. The explanation may be the fact that the anti-fouling properties of PEGMA decrease protein adsorption or that there is some decrease in the quality of the coating elements associated to the surface aging. (48)

According to Sanborn *et al* (2002), the expression of cytoskeletal proteins and, in consequence, cell adhesion should be enhanced when a fibronectin coating is present (64). However, in the current work cell adhesion was not enhanced by the presence of a fibronectin coating. It is unclear what could have caused this lack of cell adhesion. In the same work from Kooten *et al* (2004) above mentioned, when they assessed the endothelial cell adhesion on surfaces coated with fibronectin, the presence of fibronectin was not enough to avoid cell loss. A possible explanation is some malfunction of the gap junction intracellular communication, which is a process mediated by the gap junctions that contributes to an optimal rate of cell growth and death. (48; 80)

Only for the modified PDMS coated with gelatin it is found a considerable number of cells adhered (Figure 26E and 26F). However, after 120 h the cells show morphological signs of apoptosis, which include chromatin condensation and apoptotic body formation (Figure 26F). (81) These results clearly demonstrate that throughout the days something is causing the occurrence of cell apoptosis.

Copper and 2,2'-bipyridine are the catalysts used in the ATRP process, and according to the literature 2,2'-bipirydine (chelating ligand) metal complexes (namely with gold (III), copper (I/II)) have anti-proliferative effects. (82) Since the modified samples, although sterilized, were used without further washing, there is the possibility that some trace of these compounds is present in the medium. Thus, it was necessary to assess if the surfaces somehow were releasing this kind of compounds to the culture medium or if some leachable compounds could be released from the surface, thus interfering with adhesion and cell viability.

To confirm if the HUVEC apoptosis previously observed in the immunocytochemistry assay is not an isolated result this assay was repeated. Interestingly, the new results do not confirm the occurrence of cell apoptosis.

These cells were also seeded in the 12 well-plate wells coated with gelatin to determine if any cell death could be due to the surface presence. Because cell apoptosis occurred between 48 and 120 h, cells were now incubated for 3, 4 and 5 days, in order to determine in which day they start the apoptotic process. Results are illustrated in Figure 27.

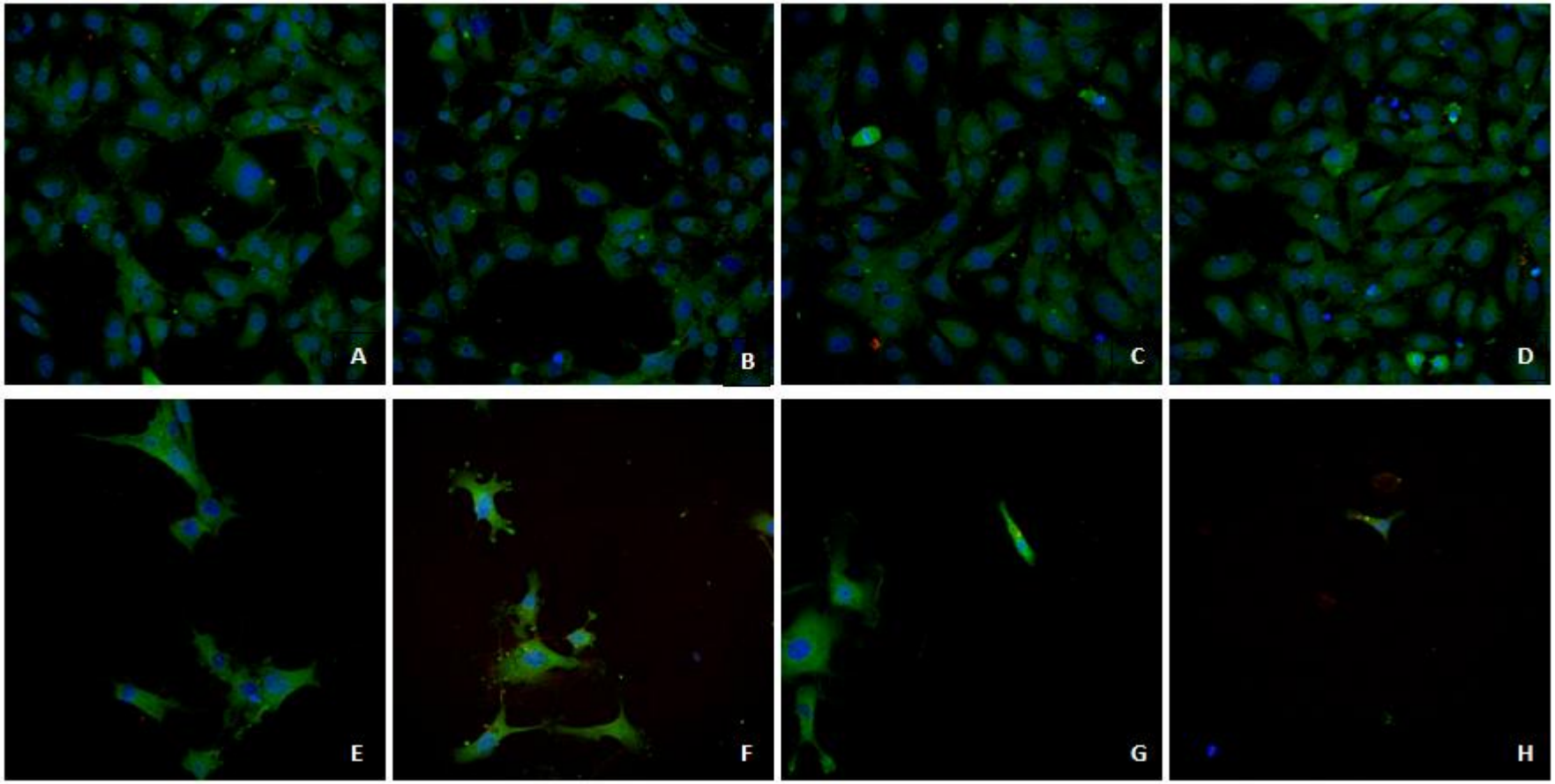

Figure 27. Images obtained by confocal microscopy of the immunochemistry assay conducted after seeding the HUVECs in different surfaces for 3, 4 and 5 days. A and B - HUVECs adhered to the 12 well-plate wells with no gelatin, after 3 and 5 days, respectively. C and D - HUVECs adhered to the 12 well-plate wells with gelatin, after 3 and 5 days, respectively. E and F - HUVECs adhered to modified PDMS coated with gelatin after 3 and 5 days, respectively. G and H - HUVECs adhered to unmodified PDMS coated with gelatin, after 3 and 5 days, respectively. Pictures from day 4 are not represented, because they didn't represent any additional information.

Results from Figure 27 reveal that HUVECs behavior changed relatively to the last immunocytochemistry assay performed with these cells (Figure 26). In the current experiment no cell apoptosis is observed. However, opposite to the previous results (Figure 26) where a good cellular density was achieved, few cells adhered to the modified PDMS with a gelatin coating (Figure 27E and 27F). Unger and co-workers (2005) also coated PES fibers to increase the adhesion of endothelial cells. Cell adhesion in the PES fibers with no coating was weak, and after 36 to 72 h no cells were visible. Nevertheless, when the PES fibers were coated with gelatin the cell adhesion was enhanced and a higher number of HUVEC could be observed in the surface. (83) Therefore, based on these researchers findings, in the present work a higher cell adhesion would be expected. Nevertheless, the difficulty in distributing the gelatin homogeneously over the surface may lead to significant discrepancies in cell adhesion. (84)

Regarding the lack of cell apoptosis, it can indicate that the previous results were isolated cases (outliers). The presence of cell apoptosis in the previous results occurred due to unknown and unexpected causes.

Observing the results of the wells coated with gelatin (Figure 27C and 27D) and the wells with no gelatin coating (Figure 27C and 27D), there is a high density of cells adhered. There are no differences between the coated and non-coated wells, both with a high cell adhesion. This is understandable, because the 12 well-plates used are special tissue culture plates composed of treated PS. Thus, a good cell adhesion is expected in this material, contrary to the untreated PS.

Since the cells adhere and proliferate in the wells of the culture plate and not in the modified PDMS, it is possible to conclude that the surfaces under study in the present work decrease cell adhesion, which is in accordance with the expectations due to the coating with non-adhesive PEGMA brushes. (85) The modified PDMS is then not suitable to be used in vascular implants, once it decreases HUVEC adhesion, which may lead to an inefficient integration of the vascular graft on the implant site. However, it is possible that the anti-fouling properties may lead to a decreased activation of different cascade reactions, resulting in a better hemocompatibility. (4)

Chapter 4. Conclusions

In the current work the biocompatibility of modified silicone rubber for vascular applications was assessed. Atom transfer radical polymerization (ATRP) was used to coat silicone rubber surfaces with polyethylene glycol methacrylate (PEGMA) brushes, with the aim of reducing protein adsorption and overcome the PDMS limitations caused by its hydrophobicity.

Regarding the surface modification, it is possible to conclude that the PEGMA coating decreased the contact angle, turning the surface more hydrophilic. However, differences were found between the modified PDMS samples, indicating that the coating is perhaps not uniform. Moreover, by AFM, it was possible to observe that the coating presented a roughness higher than 100 nm, therefore ATRP is a suitable technique to create brushes on the PDMS surface. However, a high roughness can lead to some limitations in cell adhesion. The following experiments showed that the modified surfaces could be used to evaluate cell adhesion without previous washing since no cytotoxic effect from the surfaces should be expected.

From the immunocytochemistry assay, performed to evaluate the interaction of HSkF and HUVECs with the modified PDMS, it is possible to conclude that the modified PDMS decreases cell adhesion, due to the anti-fouling properties of PEGMA. However, when human fibronectin is adsorbed to the modified PDMS surface, HSkF adhesion increases On the other hand, the unmodified PDMS shows a higher HSkF adhesion than the modified PDMS, which is explainable by the fact that PDMS is known to adsorb serum proteins easily.

In the case of HUVECs, neither the unmodified, modified or modified PDMS with adsorbed fibronectin yield any cell adhesion. The lack of cell adhesion on the unmodified PDMS is expected, because endothelial cells don’t adhere to hydrophobic surfaces.

The results gathered in the present work show that the modified surfaces decrease cell adhesion, which is expected due to the anti-fouling properties of PEGMA. For vascular applications, this decrease in HUVEC adhesion may lead to an inefficient integration of the vascular graft on the implant site, which makes the modified PDMS not suitable to be used in vascular implants. However, it is possible that the anti-fouling properties may lead to a decreased activation of different cascade reactions, resulting in a better hemocompatibility.

Chapter 5. Recommendations and Future work

The results obtained in the present work showed that ATRP is an appropriate technique to create polymer brushes in a polymeric surface. The fact that the PEGMA coating gave to the material some anti-fouling properties, may be interesting to some applications were no interaction between the material and biomolecules is desirable, such as for catheters and dialysis systems.

The surfaces should be further characterized regarding its homogeneity and coating distribution. This characterization could be made by AFM or Scanning electron microscopy (SEM) and X-ray Photoelectron Spectroscopy (XPS).

If cell adhesion is desired, the interaction between PEGMA and some proteins involved in cell adhesion should be studied. The interaction between PEGMA and, for example, fibronectin should be clarified. In this work it was not possible to assess if fibronectin adsorbs correctly to the PEGMA brushes. A higher resolution in the AFM could be used, or alternatively some enzyme assays (as Enzyme-linked immunosorbent assay or ELISA).

With a vascular application as the aim of the PDMS modification, a coating with biomolecules in order to increase the adhesion of endothelial cells could be the solution. In this case, the interaction of the material with other cell type, as macrophages, could be interesting. The blood coating and platelets formation in the presence of the biomaterial could also be crucial to determine its applicability as a vascular graft. A possible hemocompatibility could be measuring fluorescent labelled platelets adhered to the surface.

Finally, the techniques used to determine the cell adhesion to the surface could be complemented. Some proliferation testing, as the measurement of Ki67 gene by the cells using ELISA, could be performed. The presence of some proteins (such as cadherins and focal adhesion kinase) in the cells could be determined using Western Blotting. Furthermore, based on the high metabolism and cell apoptosis that were observed, some genotoxicity tests should also be performed, in order to evaluate the biosafety of the surfaces under study.

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