Universidade do Minho
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Cells Behavior on Superhydrophobic Surfaces with Different Topographies

Outubro de 2011

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Dissertação de Mestrado
Ciclo de Estudos Integrados Conducentes ao Grau de Mestre em Engenharia Biomédica
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Trabalho realizado sob a orientação do
Professor Doutor João Filipe Colardelle da Luz Mano

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Abstract

In the study of cell-material interaction, the nature of material surface has been shown to be essential for biocompatibility. Surface wettability and topography are recognized as critical factors that influence protein adsorption and, consequently, cell behavior. So far only few works have reported cell response on surfaces exhibiting extreme wettability, therefore, the influence of topography combined with this environment is still sparse in literature.

The work presented in this thesis aimed to study the influence of superhydrophobic surfaces with different topographies on cell behavior. Bioinspired superhydrophobic rough surfaces of polystyrene (PS-R) and poly (L-acid lactic) (PLLA-R) with different micro- and nanotopographies have been obtained from smooth surfaces of the same polymers (PS-S and PLLA-S) using a simple phase-separation based methodology. Mouse osteoblastic cell line (MC3T3-E1) and a primary cell culture of bovine articular chondrocytes (bch) were used as model systems for cell response evaluation on these surfaces. Scanning electron microscopy (SEM) analysis showed that PS-R surfaces exhibited randomly distributed spheres at nanometer-scale that were agglomerated in larger micrometer structures while PLLA-R surfaces showed individual well define papilla-like structures at micrometer level with nanometer rough texture, very similar to the hierarchical architecture of lotus leaf. The water contact angle (WCA) of all surfaces was investigated over 12 weeks and showed to be stable over time. WCA measurements along with x-ray photoelectron spectroscopy (XPS) comproved that, whilst having the same surface chemistry, the superhydrophobic rough surfaces differ in wettability from the smooth ones as a consequence of the particular surface micro/nanostructures. A preliminary assay for total protein quantification was performed and demonstrated a reduction of bovine serum albumin (BSA) adsorption onto rough surfaces as compared with the correspondent smooth ones, though similar amount of protein adsorbed onto the same type of PS or PLLA surfaces. Biological assays were performed to test the ability of PS and PLLA surfaces to support cell adhesion and proliferation. Live-dead, metabolically activity assays and DNA quantification indicated that in general cells adhere and proliferate faster in the smooth surfaces as compared to the rough substrates. Nevertheless, cells were metabolically active and able to adhere and survive up to 7 days of culture on PS-R surfaces and even slightly proliferate on PLLA-R surfaces with preferential cell adhesion in specific areas as shown by SEM analysis. Both types of cells showed similar behavior when in contact with the surfaces, although MC3T3-E1 cell line showed enhanced performance. Such results indicate the relevant influence of wettability on cell behavior, which was shown to be not very influenced by the topography of the superhydrophobic surfaces or by the nature of both polymers.

Resumo

No estudo das interacções entre célula e material, a natureza da superfície do material tem-se mostrado essencial para a sua biocompatibilidade. A molhabilidade e a topografia da superfície são reconhecidas como factores críticos que influenciam a adsorção de proteínas e consequentemente o comportamento das células. Até à data apenas alguns trabalhos têm relatado a resposta celular face a superfícies exibindo extrema molhabilidade, assim sendo, a influência da topografia combinada com este ambiente ainda é escassa na literatura.

O trabalho apresentado nesta tese teve como objectivo o estudo da influência de superfícies superhidrofóbicas com diferentes topografias no comportamento celular. Superfícies superhidrofóbicas bioinspiradas de poliestireno (PS-R) e poli (L-ácido láctico) (PLLA-R) com diferentes micro- e nanotopografias foram obtidas a partir de superfícies lisas dos mesmos polímeros (PS-S e PLLA-S) utilizando uma simples metodologia baseada na separação de fases. A linha celular osteoblástica de ratos (MC3T3-E1) e a cultura primária de condrócitos articulares bovinos (bch) foram utilizadas como sistemas modelo para a avaliação da resposta celular a estas superfícies. A análise efectuada por microscopia electrónica de varrimento (SEM) revelou que as superfícies de PS-R exibiam esferas a escala nanométrica distribuídas aleatoriamente que se aglomeravam em estruturas maiores a escala micro métrica, enquanto as superfícies de PLLA-R mostraram estruturas individuais, bem definidas do género de papilas a nível micrométrico, com textura rugosa a nano-escala, muito semelhantes à arquitectura hierárquica da folha de lótus. O ângulo de contacto (WCA) de todas as superfícies foi avaliado ao longo de 12 semanas e mostrou-se estável ao longo do tempo. As medições de WCA juntamente com a técnica de espectroscopia fotoelectrónica de raios X (XPS) comprovou que, embora tendo a mesma superfície química, as superfícies rugosas superhidrofóbicas diferem em molhabilidade das superfícies lisas como consequência das micro- e nano - estruturas particulares da superfície. Um ensaio preliminar de quantificação total de proteína foi realizado e demonstrou uma redução da adsorção de albumina de soro bovino (BSA) em superfícies rugosas em comparação com as superfícies lisas correspondentes, embora quantidades similares de proteína tenham sido adsorvidas no mesmo tipo de superfícies de PS ou PLLA. Foram realizados ensaios biológicos a fim de testar a capacidade das superfícies de PS e PLLA para suportar a adesão e proliferação celular. Ensaios de viabilidade celular, actividade metabólica e quantificação de DNA indicaram que, em geral, as células aderem e proliferam mais rapidamente em superfícies lisas em comparação com as superfícies rugosas. No entanto, as células estiveram metabolicamente activas e foram capaz de aderir e sobreviver até 7 dias de cultura em superfícies de PS-R e até mesmo proliferar um pouco em superfícies de PLLA-R, com adesão celular preferencial em áreas específicas, como revelado pela análise SEM. Ambos os tipos de células mostraram comportamentos semelhantes quando em contacto com as superfícies, embora a linha celular MC3T3-E1 tenha demonstrado melhor desempenho. Estes resultados indicam a relevante influência da molhabilidade sobre o comportamento celular, o que demonstrou não ser muito influenciado pela topografia das superfícies superhidrofóbicas ou pela natureza dos dois polímeros.

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List of Abbreviations

2D	bi-dimensional
3D	three-dimensional

A

α-MEM	alpha Minimum Essential Medium Eagle

B

BCA	bicinchoninic acid
bch	bovine articular chondrocytes
BSA	bovine serum albumin

C

CPD	critical point drying

D

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide

E

ECM	extra-cellular matrix
EthD-1	ethidium homodimer
e-PTFE	expanded-polytetrafluoroethylene

F

FBS	fetal bovine serum
FPCU	poly (carbonate urethane) fluorinated
FITC	fluorescein isothiocyanate

H

HA	hysteresis angle

M

Mn	number average molecular weight
MW	weight average molecular weight

O

OTS	octadecyltrichlorosilane

P

PBS	phosphate buffered saline
PCU	poly (carbonate urethane)
PDMS	polydimethylsiloxane
PEG	poly (ethylene glycol)
Pen/Strep	Penicillin/Streptomycin
PET	poly (ethylene terephthalate)
PFDTS	perfluorodecyltriethoxysilane
PLLA	poly (L-lactic acid)
PS	polystyrene
PTFE	polytetrafluoroethylene

R

RGD	arginine-glycine-aspartic sequence

S

SA	sliding angle
SAMs	self-assembled monolayers
SD	standard deviation
SEM	scanning electron microscopy
SiNW	silicon nanowire

T

TCPS	tissue culture polystyrene
TE	tissue engineering
THF	tetrahydrofuran

W

WCA	water contact angle

X

XPS	X-ray photoelectron spectroscopy

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CHAPTER I. General Introduction

CHAPTER II. Materials and Methods

Chapter I

General Introduction

1. General Introduction

1.1. Motivation and Outline

Cell adhesion on biomedical materials is a fundamental factor for the biomaterial integration process after implantation [1]. Understanding such cell adhesion processes is critically important to the comprehension of many fundamental biological questions and for the development of biomaterials in the field of Tissue Engineering (TE) and Regenerative Medicine.

It is well documented that cell adhesion and protein adsorption onto a substrate are highly affected by wettability, topography, roughness and the chemical nature of the surface [2-4]. However, some of these surface properties are interconnected with each other, like for example, surface treatments that increase roughness typically affect wettability [5]. The close relation between some of those properties makes the individual optimization of each one far from being understood.

Few works are found in literature reporting the interaction of cells with superhydrophobic substrates, inspired by nature that combine micro and nanometer scale roughness along with a low surface energy material which leads to a water contact angle (WCA) higher than 150° [6]; the vast majority of them are limited to surfaces from hydrophilic to hydrophobic range. There is both fundamental and practical interest in combining different topographies in surfaces with extreme wettability properties in order to investigate if the influence of topography or cell type can be as important as the influence of wettability itself on cell behavior.

Polymer surface engineering is a useful tool to design biomimetic materials able to interact with the surrounding environment. The work presented in this thesis aims to prepare bioinspired superhydrophobic surfaces with different topographies on two different polymers, polystyrene (PS) and poly (L-lactic acid) (PLLA), using a phase separation based methodology in order to access their potential on the cellular response of two cell types (a cell line and a primary cell culture). The choice of these two materials was based on their actual applicability; PS is a well documented polymer, being used as control for a series of cell-material interactions concerning studies and PLLA is reported as the gold standard for biodegradable biomedical applications.

The present chapter provides an overview of the research work that has been done about the principle parameters that influence cell-material interactions. Additionally, the techniques used to produce superhydrophobic surfaces and the related cell behaviors concerning studies are also discussed.

1.2. Cell – Material Surface Interactions

In the field of biomaterials, biocompatibility is one of the most important characteristics of a biomedical material whose surface is required to interact with a biological system [7].

The concept of biocompatibility has been evolved over the years and may be redefined in the context of TE as *"the ability of a scaffold or matrix for a TE product perform as a substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signalling systems, in order to optimize tissue regeneration, without eliciting any undesirable local or systemic responses in the eventual host"* [8].

The interaction of cells with substrates is an important feature of *in vitro* biocompatibility and cytotoxicity studies. The study of cell–material interactions is primarily centered on two inextricably linked areas of investigation: the nature of the biomaterial surface and the biological response to contact with the material. Since biomaterials interact with the biological environment through their interfaces, both the kind and the strength of such interactions are largely dependent on the surface properties of the materials, as reviewed elsewhere [7, 9-11]. An accurate biophysical characterization of the surface thus becomes crucially important for understanding subsequent biological effects. Indeed, it is well established that the nature of a biomaterial surface governs the phenotypic response of interacting cells [12].

Biomaterials surface properties such as wettability [13-17], surface charge density [14, 18], free energy [19], topography [20, 21] and specific chemical groups [21-23] affect the cell adhesion, spreading and signaling, and hence regulate a wide variety of biological functions, including cell growth, migration and differentiation, synthesis of extracellular matrix and tissue morphogenesis [24-26]. The wettability, the topography and the chemistry of biomaterial surface may directly influence the cell behaviors through altering the conformation of adsorbed extracellular matrix (ECM) molecules that, consecutively, regulate cell–substrate interactions [27-29]. Besides all of these surface properties that may influence protein adsorption, cell interaction, and ultimately the host response, the type of cell, the culture condition and medium are important aspects that also influence cell behavior *in vitro* and must be taken into account.

Cell–substrate interactions can be conceptually classified into four stages: (1) adsorption of serum proteins from the physiological fluids *in vivo* or culture medium *in vitro* to the surface, (2) approach of cells to the surface, (3) cells attachment and (4) spreading of cells [3]. Therefore, understanding the interaction between proteins and material surfaces is critical and control of protein–surface interactions continues to be an important factor for consideration in the design of biocompatible surfaces.

1.2.1. Protein Adsorption

Cell response to biomaterial is not mediated by a direct contact, but rather through an interfacial layer created on material surface once it is in contact with a physiological environment as a result of non-specific adsorption of ECM proteins [30-32]. The adsorption of protein layer on the material surface is faster than eukaryotic or bacterial cell attachment [33] and influence the subsequent biological reactions including platelet adhesion and activation when in contact with physiological fluids *in vivo* [34].

It has been accepted that cell adhesion is determined by composition [35], orientation and conformation of adsorbed cell-adhesive protein from culture medium (ECM proteins), such as fibronectin and vitronectin, which contain the arginine-glycine-aspartic (RGD) amino acid sequence recognized by cell-surface integrin receptors [36-38] (Figure 1.1). Integrins are transmembranar heterodymeric glycoproteins with adhesion motifs that bind proteins and control all major cellular activities such as adhesion, cell shape changes, proliferation and migration [39, 40]. Integrin binding is followed by receptor aggregation and accumulation of actin and integrin binding proteins at cytoplasmic domains which provide focal adhesion complexes for the nucleation of actin microfilaments. These early events promote several structural changes in cells such as spreading and cytoskeletal reorganization. In addition, they initiate intracellular signaling cascades into the nucleus and cytoskeleton which regulate longer-term events such as protein production [41-43].

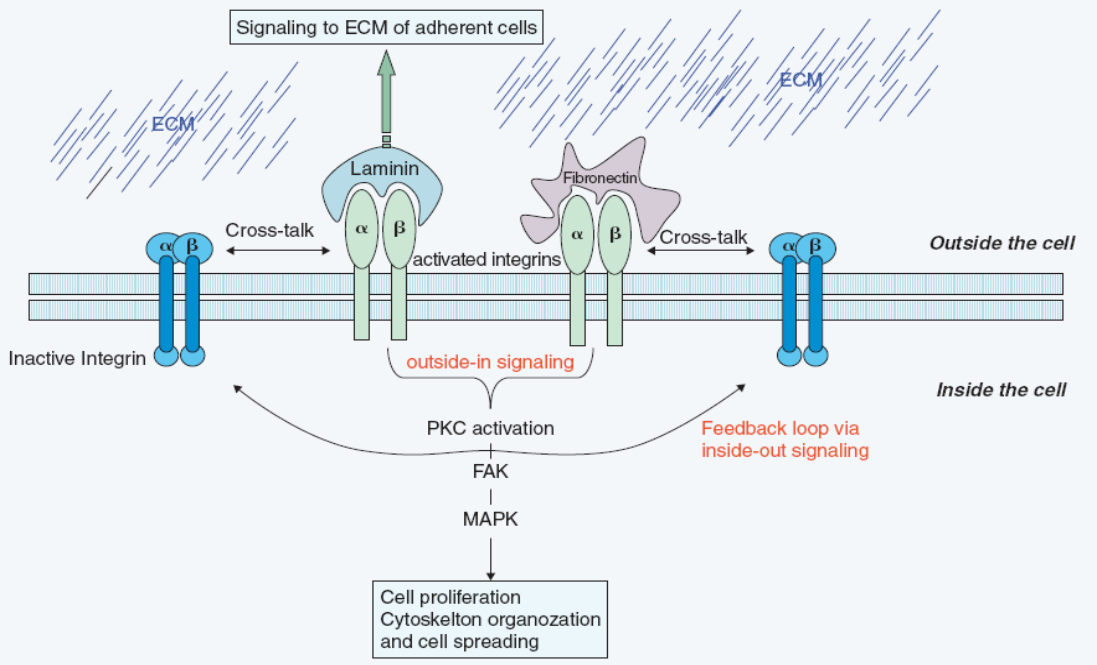

Figure 1.1 - Schematic overview of integrin-mediated activation leading to inside-out and inside-out signaling (adapted from [8]).

ECM contains proteins like fibronectin, vitronectin, collagen type I, laminin, among others, but contrarily to fibronectin and vitronectin, serum albumin lacks cell specific domains, and thus does not support the receptor-mediated binding of cells to biomaterials. Nevertheless in the absence of ECM proteins, cells may bind to surfaces by non-receptor chemical bounding, by means of hydrogen bounds, electrostatic, polar or ionic interactions. This chemical bounding occurs between various molecules existent on the cellular membrane and surface functional groups [44].

Proteins adsorption to any surface is affected not only by protein properties but also by surface properties such as wettability [3], charge [45], roughness [46] and surface chemistry [47, 48]. Beyond surface and protein properties, medium conditions such as pH [49] and salt concentration are also important factors for this complex process.

Protein interaction with surface requires that proteins adhere with the right conformation and amount in order to obtain the most favorable cell adhesion and proliferation. The proper folding of protein structures is of vital importance to their biological functions. The well-defined three-dimensional (3D) conformation of protein is coupled essentially with many biological processes, ranging from molecular recognition and transportation to cell growth and regulation [50-54].

Cell behavior might be controlled by playing with surface modification which is critical to elicit specific cellular responses and direct new tissue formation. Peptides, polysaccharides and other bioactive ligands may be covalently grafted or adsorbed onto the surface or even included in the bulk to promote specific cell adhesion. The biomolecular recognition of the material by the cells is usually achieved by incorporating cell-binding peptides in the form of native long chain of ECM proteins [55], or, more frequently, in the form of short peptide sequences derived from ECM proteins, such as RGD peptides [56]. Although the incorporation of RGD peptides is the most advantageous thanks to their stability, straightforward synthesis and absence of random folding of long chain proteins during adsorption onto the biomaterial surface. Such short peptides are often covalently attached to the polymer, e.g., via hydroxyl-, amino-, or carboxyl- groups [56].

1.2.2. Surface Wettability

Surface properties of biomaterials, such as wettability and topography, are critical for cell adhesion. Wettability is one of the most important factors that affect the cytocompatibility of biomaterials. The adhesion and growth of cells on a surface are considered to be strongly influenced by the balance of hydrophilicity/hydrophobicity, frequently described as wettability. Such property interferes in cell

attachment and protein adsorption [57-59], platelet adhesion/activation [60], blood coagulation [61], eukaryotic cells [62] and bacterial adhesion [63].

Although it has been reported that there is no obvious correlation between wettability and cell behavior, many studies have demonstrated that cells tend to attach better on hydrophilic surfaces than on hydrophobic surfaces presenting moderate wettability with WCA of 40-70° [13-15, 19, 35, 64-69]. This broad range of WCA results may be due to the use of different materials, different surface topographies and different surface chemistries applied to alter wetting behavior.

The introduction of hydrophobic, hydrophilic groups or biochemical cues covalently bound or adsorbed on surfaces has an effect on wettability; molecules or groups rich in oxygen should lead to an increase in the wetting behavior. Then, the control of the surface wettability can be performed by manipulating the type of exposed chemical groups and the ratio of oxygen on the surface. As higher the oxygen ratio is, higher the wettability of the surfaces becomes. In the same way, surface treatments that increase roughness also affect wettability; it is well known that the roughness of a hydrophobic solid enhances its hydrophobicity [70]. Typically, hydrophobic surfaces present better affinity for proteins [71-74]. However, up to now only few investigations on protein adsorption and cell behavior cover the entire range of surface wettability [75, 76], but some comparisons can be found in literature between superhydrophilic and superhydrophobic surfaces [77, 78].

1.2.3. Surface Topography

The topography of a surface substantially affects the macroscopic behavior of a material. Nowadays, the influence of surface topography in biological response is a matter of investigation [79].

Topography affects not only cell adhesion but also differentiation, proliferation, ECM production, gene expression, cell morphology and orientation by interfering in the assembly of the focal adhesion points [80-82]. Cells cultured on smooth surfaces tend to generate more organized ECM, including more homogeneous distribution of focal adhesions. However, on rougher surfaces, focal adhesions are located at cell edges, where the contact with the substrate takes place [83].

Micro- and nanopatterned surfaces have been studied in the literature for a better understanding of cell response to topographic features, mainly concerning cell adhesion. The interaction of cells with native topographical substratum is done in several ways, often through a known phenomenon called contact guidance [84]. Contact guidance is an important example of a naturally occurrence that is characterized by the response of cells to structures on the micro- and nanometer scale [85].

Cells can sense the topography and align using filopodia which is driven by cytoskeletal actin bundles. As the filopodia encounter a favorable guidance cue, they become stabilized following the recruitment of microtubules and accumulation of actin. There is further evidence that nascent focal adhesions then form at the filopodial tips. Once cells locate a suitable feature, through the filopodia present on the leading edge of cells, lamellipodium are formed to move the cell to the desired site [86]. However, when cells cannot find a suitable site for adhesion, they adopt shapes which correspond to certain cytoskeleton organizations that intracellularly might have some transduction significance or enhance some signaling pathways, which would not occur with other shapes.

The response of cells to bi-dimensional (2D) synthetic topographic substrates depends on many factors, including cell type [75, 87], feature geometry and size and the physical properties of the bulk material such as substrate stiffness [88].

Cells respond to topographical cues in many ways and it has been reported for decades. A wide variety of cell types including BHK cells, neuronal cells, fibroblasts, macrophages, epithelial cells, endothelial cells and smooth muscle cells have been studied in several substratum features such as grooves, ridges, steps, pores, wells and nodes in micro- and nano scale [87, 89, 90] to understand the interactions between cells and different topographies. Campbell and von Recum found that the influence of pore sizes on cell adhesion was greater than that of surface hydrophilicity/hydrophobicity [91]. Nonetheless, some types of cells such as human-derived leucocytes, keratinocytes and monocytes have shown a lower response to microtopography (1 μm of pitch and depth) [92].

In addition to the type of pattern, surface roughness is a parameter that can also influence cell behavior. Random surface topographies are more easily fabricated and less expensive. Furthermore, smooth and rough surfaces have been shown to have different effects on cell adhesion and proliferation [3]. PLLA substrates with different surface roughnesses (but without a pattern) were studied and cell phenotype, adhesion and proliferation were shown to be significantly improved on smooth surfaces. In addition, cell morphology was completely different on rough substrates [11].

Cell morphology and proliferation on silicon wafers of various roughnesses and topographies created by chemical etching in caustic solution and by corundum sandblasting were analyzed by Schweikl et al. The cell number of MG-63 osteoblasts was significantly lower on sandblasted surfaces compared with other rough surfaces but no differences were detected with 3T3 mouse fibroblasts. The different surface roughnesses and topographies were recognized by osteoblasts. The cells spread well on smooth surfaces but appeared smaller on a rough and unique pyramid-shaped surface and on a rough sandblasted surface [21].

Martínez et al. showed that the micro-roughness of PLLA surfaces could induce chondrocyte orientation and promoted changes in their shape [93].

Wan et al. demonstrated that OCT-1 osteoblast-like cells adhesion was enhanced on PLLA and PS surfaces with nano- and micro-scale roughness when compared to the smooth surfaces [94]. It was also shown that introducing roughness on titanium surfaces increased attachment of osteoblastic-like cells [59]. However, some authors found that proliferation was not improved by roughness but it was greater on smooth surfaces [94-96].

1.2.4. Surface Chemistry

Surface chemistry is an important surface property as well as wettability and topography. As a matter of fact, the chemical groups exposed onto the surface dictate the wettability and further the absorption of proteins onto the surface. Accordingly, the amount and the conformation of the proteins will vary; different integrins of the cell will bind to the surface according to the functional groups exposed on it.

Ratner and co-works demonstrated a systematic approach to the characterization of a surface with the attempt to understand the role of chemical functional groups on a surface interacting with proteins and cells. A range of oxygen contents and types and concentrations of functional groups (hydroxyl, carbonyl and carboxyl) were introduced on PS and poly (ethylene terephthalate) (PET) surfaces by plasma modification. The surfaces were exposed to bovine aortic endothelial cells and cell growth and spreading were measured. The cell growth was linearly correlated with the oxygen content of the PS-treated surfaces. The methanol treatments resulted in more oxygen incorporation and more cell growth than the acetone treatment for bovine aortic endothelial cells. However, this same correlation did not hold for unmodified PET films which were high in surface oxygen content, yet exhibited poor growth. From such observations the authors concluded that specific functional groups rather than simple oxygen content may be responsible for cell growth [97].

The effect of surface chemistry on adhesion and proliferation of tissue cells was also investigated using self-assembled monolayers (SAMs) of various alkanethiols and alkylsilanes as model surfaces independent of the particular biomaterial. Hydrophilic and hydrophobic properties were created and controlled on these model surfaces by termination with groups like COOH and NH_2 or CH_3, CF_3, OH and poly (ethylene glycol) (PEG). WCA data indicated that oxidized wafer surfaces displayed high hydrophilicity, modification with PEG created a hydrophilic surface, and an amino group (NH_2) led to a moderately wettable surface. A hydrophobic surface was formed by hydrocarbon chains terminated by CH_3, but this hydrophobicity was even further increased by a fluorocarbon (CF_3) group. Cell proliferation

of MG-63 osteoblasts and 3T3 mouse fibroblasts on these surfaces was different depending primarily on the chemistry of the terminating groups rather than on wettability. Cell proliferation on CH_3 was as high as on NH_2 and hydrophilic oxidized surfaces, but significantly lower on CF_3 [21]. In another study human umbilical vein endothelial cell adhesion revealed to be higher on –COOH and -NH_2 functionalized surfaces than on –OH and –CH_3 functionalized ones [3]. Therefore, the nature of the surface chemical groups can always be a surface property as important as wettability and topography that must be taken in consideration for the development of biological surfaces.

1.3. Superhydrophobic surfaces

Superhydrophobicity has recently drawn a great deal of attention from both fundamental and practical application points of view. Although superhydrophobicity has been studied since the mid 1930s, interest in this phenomenon has grown substantially in the past few years due to recent recognition of its potential applications in various areas.

Superhydrophobic surfaces are characterized by a WCA higher than 150° and a hysteresis angle (HA) lower than 10° [98]. The interest in superhydrophobic surfaces is being driven by the desire to produce these surfaces for functional applications including anti-biofouling, non wettable textiles, transparent and antireflective self-cleaning coatings, humidity-proof coatings for electronic devices, fluidic drag reduction, enhancing water supporting forces, controlled transportation of fluids, superhydrophobic valves, prevention of water corrosion, oil-water separation and microcondensation [99]. However, in the biomedical field the use of such surfaces has been poorly studied. The non-wettable character has been proposed for particles production [100-103], open microfluidic devices [104], substrates for high-throughput analysis [78] and anti-bioadhesion applications seeking to prevent protein adsorption and cell adhesion, mostly in blood compatible materials [105, 106]. Nonetheless, in the literature only a few authors have used superhydrophobic surface as supports for cell response studies [75-77, 107-109].

1.3.1. Natural Superhydrophobic Surfaces

Many superhydrophobic examples can be found in nature. One of the most well known example is the lotus leaves (*Nelumbo nucifera*) investigated by Barthlott and Neinhuis [110]. High resolution scanning electron microscopy (SEM) study of the surface of lotus leaf shows papillae structures spaced out 20–40 μm, each covered with epicuticular wax crystalloids with a diameter of 100–200 nm (Figure 1.2 (a)) which provides the low surface free energy. These complex surface textures consisting of micro-

and nano-scale hierarchical structures amplify the hydrophobicity of the leaf surface to attain a WCA of 150–160◦ and a critical sliding angle (SA) lower than ∼2◦. Other plants with superhydrophobic leaves include rose, tulipa, iris, asphodelus, drosera, eucalyptus, euphorbia and gingko biloba [110].

Superhydrophobic surfaces can also be found in insects and birds. The water striders legs, the wings of butterflies and cicadae (Figure 1.2 (b)), the Nambibian beetles and the feathers of many birds living on water (Figure 1.2 (c)) are examples that exhibit superhydrophobicity [111, 112].

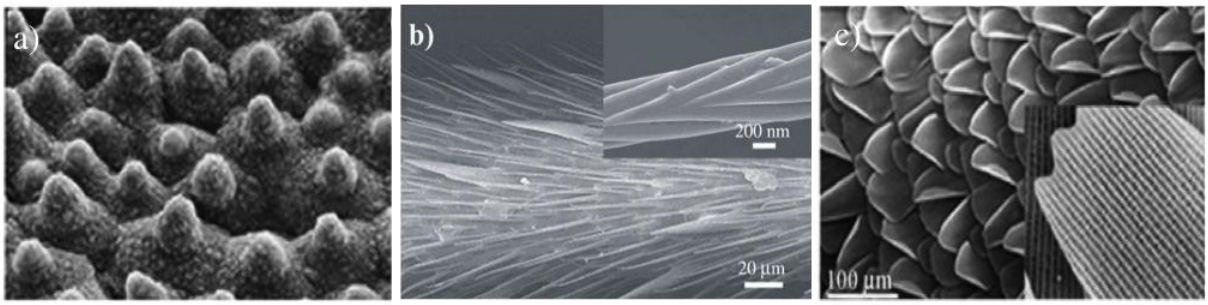

Figure 1.2 - Microstructures of biological surfaces: (a) Lotus leave, (b) water strider's leg, (c) butterfly's wing (adapted from [113]).

1.3.2. Synthetic Superhydrophobic Surfaces

Nature always gives inspiration for the fabrication of functional materials by mimicking the structural design or stimuli-responsive capability of biomaterials.

Several studies have proved that the combination of micro and nanometer-scale roughness found in nature, along with a low surface energy material leads to a WCA higher than 150°, a HA lower than 10°, a low SA and a self-cleaning effect [98, 114].

For a liquid droplet on a flat film, the wettability is determined by the surface free energy of a solid substrate, which is commonly given by the Young's equation (Figure 1.3 (a)) where θ_Y is the static WCA for a smooth surface and γ_{lv}, γ_{sv} and γ_{sl} are the different surface tensions (liquid/vapor, solid/vapor, and solid/liquid) involved in the system [115]. In actuality, few solid surfaces are truly flat; therefore, the surface roughness factor should also be considered during the evaluation of the surface wettability.

The principles of superhydrophobicity were first outlined by Wenzel in 1936 [116] and then by Cassie and Baxter in 1944 [117]. These authors proposed two distinct models to explain the wetting behavior of rough surfaces. In the Wenzel model roughness increases a solid surface area; a geometric feature that enhances solids hydrophobicity. In the Cassie–Baxter model the surface roughness leads to a superhydrophobic behavior as a consequence of the fact that the liquid does not intrude into the lower

regions of the topographic features and a fraction of the surface of the drop in contact with the substrate is suspended by air pockets.

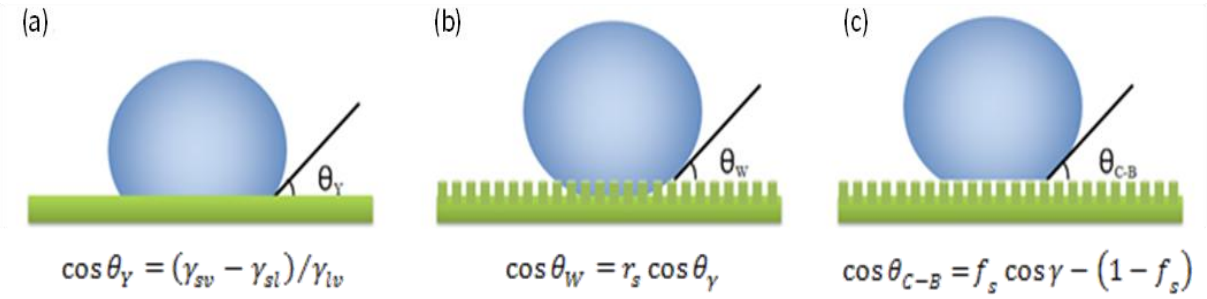

Figure 1.3 - Effect of surface structure on the wetting behavior of solid substrates. (a) A liquid drop on a flat substrate (Young model). (b) Wetted contact between the liquid and the rough substrate (Wenzel model). (c) Non-wetted contact between the liquid and the rough substrate (Cassie-Baxter model) (adapted from [118]).

The Wenzel model describes homogeneous wetting by the equation in Figure 1.3 (b) where θ_W is the static WCA for a rough surface. The surface roughness r_s is the ratio of the actual area over the apparent surface area of the rough substrate.

The Cassie–Baxter model describes heterogeneous wetting by the equation in Figure 1.3 (c) where f_s is the solid fraction that is in contact with the liquid under the droplet.

Nowadays, transitional states between the Wenzel and Cassie–Baxter states have been discovered [70]. Feng et al. proposed a superhydrophobic state, called the "Cassie impregnating wetting state" or "petal effect". Both describe superhydrophobic surfaces with high adhesive forces to water, and this means that the wetted surface area is smaller than in the Wenzel model but larger than in the Cassie–Baxter model [119].

Since the Kao Corporation [120] demonstrated for the first time the fabrication of synthetic superhydrophobic surfaces in 1996, a plethora of techniques have been developed to produce a variety of shaped surface features on different length scales, using various materials from polymers to metals. Many routes to prepare superhydrophobic surfaces require complicated, sometimes expensive equipment, although some surfaces can be fabricated fairly easily.

1.3.2.1. Techniques

Many techniques have been used to prepare superhydrophobic surfaces, as reviewed by several authors [98, 118, 119, 121, 122]. These processing methodologies can be basically divided into two

approaches: produce a rough surface from a hydrophobic material (low surface energy material) or modify a rough surface with a material of low surface energy.

The methodologies used to produce superhydrophobic surfaces by roughening low surface energy materials are frequently one-step processes and have the simplicity as an advantage. However these methods are limited to a restricted set of materials, including fluorocarbons [123-127], silicones, in particular polydimethylsiloxane (PDMS) [128-131], organic materials such as polyethylene [132], PS [133, 134], polyamide [135], polycarbonate [136] and alkylketene dimer [137] and certain inorganic materials such as zinc oxide (ZnO) [138, 139] and titanium dioxide (TiO_2) [140].

The production of superhydrophobic surfaces by a completely different approach, i.e., first preparing a rough substrate and then modifying it with a low surface energy material, separates the surface wettability from the bulk properties of the material and enlarges potential applications of superhydrophobic surfaces.

A wide range of techniques to produce rough surfaces have been investigated over the past years. Some of these techniques are summarized in Table 1.1.

Table 1.1 - Techniques for superhydrophobic surfaces production.

Processing Techniques			
Anodic oxidation	[141-143]	Sol-gel processing	[138, 140, 144-148]
Laser etching	[128, 129, 149-151]	Layer-by-layer	[152-156]
Plasma etching	[124, 157-159]	Colloidal assembly	[160, 161]
Chemical etching	[162-164]	Self-assembly	[165-167]
Lithography	[168-172]	Template method	[173-175]
Electrical/chemical reaction and deposition	[147, 152, 153, 176]	Phase separation	[75, 76, 78, 100-104, 107, 176, 177]
Electrospinning	[125, 131, 133, 178, 179]		
Chemical vapor deposition	[180-184]		

There are several methods commonly used to modify the chemistry of a surface, including covalent bonds that can be formed between gold and alkyl thiols, silanes that are often used to decrease the surface energy and physical binding, adsorption and coating that can also change the surface chemistry [121].

The surface tension of well known substituent end-groups decreases in the following order: CH_2 > CH_3 > CF_2 > CF_3. This is the reason why, among the numerous molecular structures, fluorinated polymers/fluoroalkylsilanes with CF_3 endgroups are widely used as unwettable coating [185].

In many reports, 1H, 1H, 2H, 2H-perfluorodecyltriethoxysilane (PFDTS) is employed to modify the chemistry of the surfaces and reach the superhydrophobicity, as reviewed by Raza et al [185]. The hydrophobic perfluorinated alkyl chain has been associated with low surface energy and higher thermal stability [186].

Lima et al. implemented bioinspired superhydrophobic surfaces including PS, copper and aluminum with a surface fluorination with PFDTS in order to increase the superhydrophobicity, suitable for producing smart hydrogel beads [100].

In the biological field, organosilane has been widely used in the application of DNA microarray and protein microarray for DNA/protein immobilization or for surface passivation to prevent non-specific binding, particularly in the spatial control of cell adhesion [187].

CELL-SUPERHYDROPHOBIC SURFACES INTERACTION STUDIES

In literature it is suggested that protein adsorption onto superhydrophobic surfaces occurs being dependent on the rough feature sizes [188] and that cell adhesion is also possible [189].

Shiu et al. reported a study that superhydrophobic spots were generated onto Teflon® by localized oxygen plasma treatment to produce roughness [190]. NIH 3T3 fibroblasts adhered more onto the superhydrophobic Teflon® spots than on the smooth areas. The same surfaces were studied in another work and have exhibited short-term protein resistance, meaning that proteins can actually adsorb to superhydrophobic surface [191].

Expanded-polytetrafluoroethylene (e-PTFE) superhydrophobic artery prostheses were prepared by ion etching and oxygen glow-discharge and implanted in pig and rabbit models. After four weeks of implantation, *in vitro* experiments have shown significantly more platelets adhesion on superhydrophobic e-PTFE than on polytetrafluoroethylene (PTFE) [192].

In another work, PTFE was modified by low pulse high frequency oxygen plasma immersion ion implantation in order to increase the roughness and modify the surface oxygen content which permitted to obtain superhydrophobic PTFE. Rat calvaria osteoblasts adhesion, proliferation and alkaline phosphatase activity was significantly higher on the superhydrophobic PTFE than on the unmodified PTFE [189].

Zhou et al. investigated nanostructured PDMS superhydrophobic surfaces prepared by a template method associated with chemical coating of perfluorooctyl-triethoxysilane. Adhesion of human platelets decreased about 80% on the superhydrophobic surfaces comparing with the smooth PDMS [193].

Poly (carbonate urethane) (PCU) with different ratios of fluorinated alkyl side chains were nanostrutured with aligned carbon nanotubes to obtain superhydrophobic surfaces (FPCU). Smooth samples have shown human platelets adhesion, spreading and activativation after 30 minutes of incubation. On the other hand, on superhydrophobic samples just few platelets adhered and these were rounded and much less activated when compared to the smooth samples. Therefore, superhydrophobic PCU fluorinated nanostructured with carbon nanotubes surfaces improved blood biocompatibility comparing with smooth PCU fluorinated surfaces [109].

Self-organized layers of vertically orientated TiO_2 nanotubes providing defined diameters were grown on titanium by anodic oxidation. After coating TiO_2 nanotube layers with SAMs of octadecylphosphonic acid they showed a diameter-dependent wetting behavior ranging from hydrophobic up to superhydrophobic. Cell adhesion, spreading and growth of mesenchymal stem cells on the unmodified and modified nanotube layers were investigated and compared. Cell adhesion was found to be independent of nanotube diameter in superhydrophobic modification. Cell attachment was considerably enhanced after 24 h on superhydrophobic surfaces. This effect was, however, of a temporary nature and was essentially lost after 3 days. Adsorption experiments with ECM proteins showed that specific adsorption of those proteins on methyl-terminated SAMs could not account for this temporary effect [194].

Vertically aligned silicon nanowire (SiNW) arrays prepared by the stain etching technique were investigated by Piret et al. After preparation, a chemical modification with octadecyltrichlorosilane (OTS) led to the formation of superhydrophobic SiNW surface. A micropatterned superhydrophilic/ superhydrophobic SiNW surface was fabricated using standard optical lithography techniques. Chinese Hamster Ovary K1 cells were cultured on patterned superhydrophilic/superhydrophobic silicon nanowire surfaces. It was found that the cells adhered selectively to the superhydrophilic regions while cell adhesion was almost completely suppressed on the superhydrophobic surface. Cell adhesion in the superhydrophilic regions was also accompanied by SiNW dissolution in the culture medium whereas the superhydrophobic surface remained unaffected [195].

PHASE-SEPARATION BASED METHODOLOGY

Among all techniques employed to produce artificial superhydrophobic surfaces, phase-inversion based methodologies have gained great interest because of their simplicity, economy and one-step procedure.

Through phase-separation technology, a thermodynamical instability is established in a homogeneous multicomponent polymer solution that, under certain conditions, tends to separate into more than one phase in order to lower the total free energy. Polymer solution separates into two phases, a polymer-rich and a polymer-poor phase. The polymer-rich phase solidifies and the polymer poor phase is removed by extraction, evaporation or freeze drying leaving a highly porous polymer network. The selection of proper solvents is an essential step in the phase separation method. The polymers are usually dissolved in solvents with a low melting point and that is easy to sublime, such as naphthalene, phenol or 1, 4 dioxane [196].

Erbil et al. reported a simple method for forming superhydrophobic surfaces in polypropylene by combining solvents and adequate drying temperatures. Non-solvents act as a polymer precipitator, increasing the extend of polymer phase separation between the liquid and polymer rich phases, and this increases crystallization [197]. The first study of biodegradable superhydrophobic surfaces produced by phase-inversion based technology involving the use of a solvent and non-solvent to produce fragile superhydrophobic PLLA films was reported by Shi et al. in 2008 [107]. When the polymer solution contacts the non-solvent and if the two liquids are miscible, there is a strong tendency for the last one to diffuse into the polymer solution. During the phase separation of the solution, crystallization of the polymer takes place, resulting in solid-liquid demixing accompanying the liquid-liquid demixing. This induces the creation of particular structure on the surfaces, which exhibits a hierarchical micro/nano organization.

1.3.2.2. Synthetic Polymers Employed for Superhydrophobic Surfaces Production

Synthetic polymers are more controllable and predictable than naturally derived polymers, whereas chemical and physical properties of the polymer can be tailored to match specific mechanical and degradation characteristics. The wide variety of copolymers, polymer blends and composites with other materials, such as bioceramics, bioactive natural materials or different synthetic polymers, broaden the range of properties of this class of materials. Moreover, risks like toxicity, immunogenicity and infections are much lower for pure synthetic polymers [198].

POLYSTYRENE

PS has been a widely used polymer to produce tissue culture plates and flasks since about 1965. Figure 1.4 shows the chemical structure of PS. Many cell types adhere to and move on the surfaces of such materials and present a morphology that is very similar to that seen when the cells are grown on glass. However, it has long been known that PS must be subjected to a surface treatment to render the dishes suitable for cell attachment [199]. Nonetheless, PS has been used as control for several fundamental studies concerning cell-material interaction.

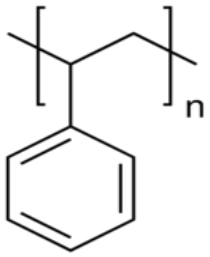

Figure 1.4 - Chemical structure of polystyrene

Surperhydrophobic surfaces of PS have been employed in several applications such as particles production [100-103], open microfluidic devices [104] and substrates for high-throughput analysis [78].

Oliveira et al. studied cell adhesion and proliferation of three different cell lines (SaOs-2, L929 and ATDC5) on rough superhydrophobic PS surfaces (WCA 151°) compared to smooth unmodified PS (WCA 80°). L929 fibroblasts, ATDC5 chondrocyte-like and SaOs-2 osteoblast-like adhered more on rough surfaces than on smooth ones meaning that the introduction of surface roughness and the consequent increase of surface area are important features to enhance cell attachment, which was even higher on rough surfaces than on tissue culture polystyrene (TCPS) with exception of the case of L929. L929 fibroblasts were able to proliferate on the rough surfaces whereas ATDC5 chondrocyte-like and SaOs-2 osteoblasts-like cells were not. For all cell types the proliferation was significantly higher on the TCPS [75].

In a recent study developed by Ballester-Beltrán et al. fibronectin adsorption and adhesion of MC3T3-E1 cells were investigated on standard and superhydrophobic PS surfaces. Fibronectin was adsorbed on superhydrophobic surfaces in lower density and altered conformation as compared with the corresponding standard PS. As a consequence cell adhesion occurred without formation of mature focal adhesion plaques and scarce phosphorylation of FAKs However, cells were able to proliferate up to 21 days on the superhydrophobic substrate, although in a significantly lower density when compared with standard PS [200].

POLY (L – LACTIC ACID)

Many biomaterials have been used for TE applications, among them polyesters, such as (PLLA), have been well recognized for their excellent biodegradability, biocompatibility, nontoxicity and their biocompatible degradation products [201]. Therefore, PLLA is one of the few synthetic degradable polymers approved by the US Food and Drug Administration for a variety of human clinical and environmental applications [201-203]. Figure 1.5 shows PLLA chemical structure. Like other poly (α – hydroxy acids), PLLA has been used as a gold standard for a series of biodegradable biomedical applications.

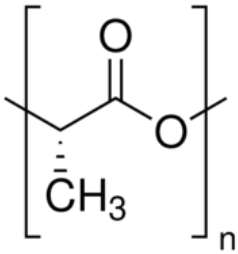

Figure 1.5 - Chemical structure of poly (L-lactic acid).

Song et al. studied the adhesion of a mouse lung fibroblast cell line (L929) on superhydrophobic PLLA surfaces (WCA 153.6°). The adhesion of L929 decreased significantly when compared with the results of smooth PLLA surfaces (WCA 71.3°) and the cell morphology was much more round [76].

In another study, Alves et al. examined that superhydrophobic PLLA surfaces prevent rat bone marrow derived cells adhesion and proliferation, when compared with smoother surfaces prepared by simple solvent casting (WCA 78.2°). Cell density decreased from day 1 to day 7 of culture and virtually no cells could be found in 21 days of culture [108].

1.4. Conclusions and Future Aspects

Surface properties play a key role on the biocompatibility of a biomaterial. Cell behavior on any surface is mainly influenced by surface properties including wettability, topography, roughness, charge density and surface chemistry (functional groups), which influence the quantity and conformation of ECM proteins that adhere to the surface and dictate the protein–surface interaction that is an important determinant of the fate of a biomedical device, once it is introduced in a biological environment. The type of cell is an important factor that should be taken in consideration on cell-material interaction studies.

The investigation of each parameter that influences individually cell response is extremely difficult due to the close relation between some of them, e.g. wettability depends both on the surface roughness and on surface chemistry. Therefore, further studies are required to seek new ways to understand how such properties are individually crucial to cell behavior.

The influence of extreme environments on protein adsorption and cell behavior such as extreme wettability is still sparse in literature. In general, it is reported that proteins can actually adsorb to superhydrophobic surfaces but with altered conformation, even so few cells can adhere to such surfaces. However, some results are contradictory and it is still not very clear, depending on the cell type. Accordingly to the Cassie-Baxter model, in cell culture conditions, air should be entrapped between the surface and cells/medium during the culture time. In these cases cells could only contact with the surface in some points, especially on the top of the asperities. However, some studies showed that the cells could proliferate better on superhydrophobic surfaces as compared with the smooth material. There is clearly the need of finding new ways of investigating the effect of wettability in superhydrophobic surfaces on cell attachment by changing as less as possible the number of variables in order to understand which parameters contribute the most. Further studies of protein-cell interaction, cell adhesion, proliferation and gene expression are required to a better understanding of cell behavior to these environments.

2. References

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CHAPTER II

MATERIALS AND METHODS

1. Materials and Methods

1.1. Materials

The smooth PS films used in this work were purchased from GoodFellow Cambridge Limited, England, with a thickness of 0.25 mm.

A high stereoregular PLLA with a Mn of 69.000 and a Mw/Mn of 1.734 was obtained from Cargill Dow LLC, USA. The glass transition temperature and melting temperature of this polymer were 60 and 162 °C, respectively [1].

Tetrahydrofuran (THF) and sterile agarose were purchased from Sigma-Aldrich. 1, 4-Dioxane (p.a.≥ 99.5%) and absolute ethanol were obtained by Fluka and Panreac, respectively.

1.2. Methods

1.2.1. Superhydrophobic Surfaces Production

One of the main purposes of this work was to study the biological influence that surface properties such as extreme wettability and different topographies have on protein adsorption and cells behavior.

Rough PS and PLLA superhydrophobic surfaces were obtained by a phase-separation based methodology proposed by Song et al. [2].

PREPARATION OF PS SUPERHYDROPHOBIC SURFACES

PS substrates were obtained cutting the smooth PS films into small squares of 5 x 5 cm^2 and cleaned by immersion in 70% ethanol (v/v) in an ultrasonic bath for 10 minutes.

Figure 2.1 illustrates the process used to obtain the superhydrophobic surfaces. A 70 mg ml^{-1} solution of PS (injection molding grade) in THF was prepared and then mixed with absolute ethanol in a ratio of 2: 1.3 (v/v). The mixture was uniformly dispensed onto PS substrates, resulting in the formation of an opaque or semi-transparent layer. After 10s on air, the substrates were then immersed in absolute ethanol for 1min. Afterwards the surfaces were dried at room temperature, resulting in rough superhydrophobic PS.

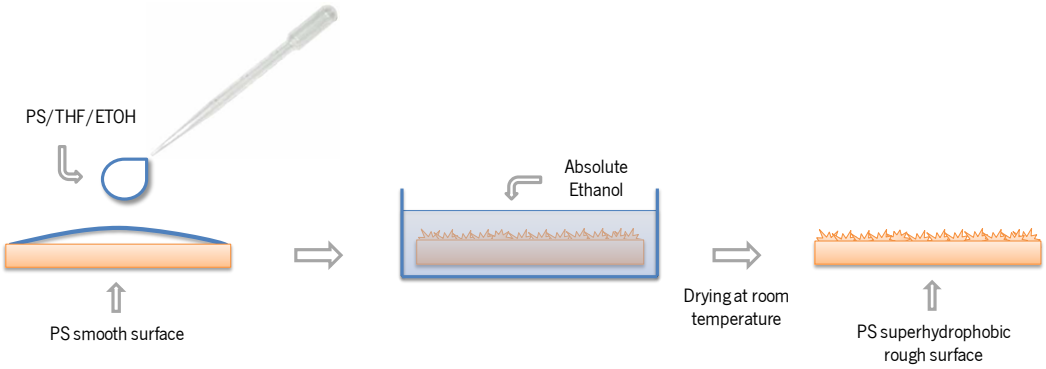

Figure 2.1 – Schematic representation of the methodology used to produce PS superhydrophobic surfaces.

PREPARATION OF PLLA SUPERHYDROPHOBIC SURFACES

Flat smooth PLLA substrates were produced by melting PLLA powder between two glass slides subjected to compression at 200°C followed by cooling in water as reported by Song et al. [2].

A 13% (wt/v) PLLA solution in 1, 4-dioxane was casted on the substrates. After an evaporation period of a few seconds the substrates were immersed in absolute ethanol for 1h to induce the phase separation of the casted solution. The samples were first dried under nitrogen flow and then in the vacuum oven at 30°C for 24 hours to eliminate all solvent residues. When the samples were completely dried, the upper part was removed giving rise to rough superhydrophobic. Figure 2.2 illustrates the methodology used to produce smooth and rough surfaces of PLLA.

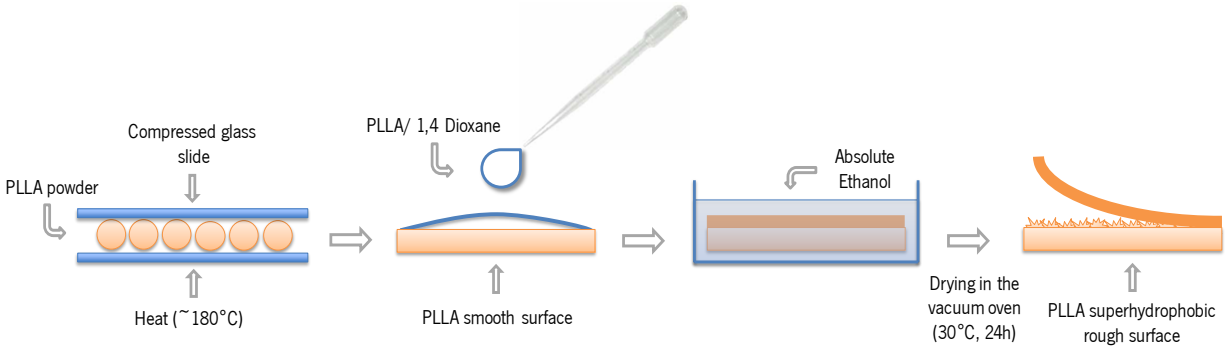

Figure 2.2 - Schematic representation of the methodology used to produce PLLA superhydrophobic surfaces

All surfaces were punched into circular samples with a diameter of 8 mm and the samples' nomenclatures used along this thesis are summarized in Table 2.1.

Table 2.1 – Sample nomenclatures

POLYMER	TOPOGRAPHY	
	SMOOTH	ROUGH
Polystyrene	PS-S	PS-R
Poly (L-acid lactic)	PLLA-S	PLLA-R

1.2.2. Characterization

SCANNING ELECTRON MICROSCOPY (SEM)

SEM allows the observation and characterization of heterogeneous organic and inorganic materials on a nanometer (nm) to a micrometer (μm) scale. Its popularity relies on its capability of obtaining 3D-like images of the surfaces of a very wide range of materials [3].

Surface topography was analyzed, before and after phase-inversion method, by a Leica Cambridge S-360 scanning electron microscope (Leica Cambridge, UK) to evaluate the hierarchical micro/nano organization of surface structures. All samples were precoated with a conductive layer of sputtered gold. The analyses were performed at an accelerating voltage of 15kV at different magnifications.

CONTACT ANGLE MEASUREMENT

The wettability of different surfaces was characterized by WCA measurements. Static WCA measurements were performed using the sessile drop method on an OCA15+ goniometer (DataPhysics, Germany) under ambient conditions at room temperature. Milli-Q water (6 μL) was dropped on the surfaces and pictures were taken after water drop stabilization. Three samples of each surface type were measured five times.

The WCA measurements of all samples were carried out in the same week of their preparation and 12 weeks later.

X-RAY PHOTOELECTRON SPECTROSCOPY (XPS)

X-ray photoelectron spectroscopy (XPS) is a non destructive, Ultra High Vacuum requiring technique to study the chemical nature of a material surface within the probing depth of approximately 10 nm. All elements with a concentration higher than 0.5 atomic % can be detected, except for H and He [4].

The surface chemical composition of smooth and rough surfaces was investigated by XPS using a Physical Electronics Quantera SXM (scanning XPS microprobe) system with monochromatic Al Kα radiation (h_υ= 1486.6 eV/15kV) shot at an angle of 45° toward the surfaces. Survey spectra were obtained with a pass energy of 224 eV and a step size of 0.8 eV. The software used was a Compass for XPS control, Multipak v.8.0 for data reduction. The measurements were carried out in triplicate 12 weeks after samples' preparation.

1.2.3. Protein Adsorption Assay

BCA ASSAY

Protein absorption on smooth and rough surfaces was analyzed by a colorimetric detection and quantification of total protein using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co, USA). This system utilizes BCA as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. The purple coloured reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}) [5]. This water-soluble complex exhibits a strong absorbance at 562nm that is linear with increasing protein concentration.

Surfaces were fixed on the bottom of an ultra low attachment plate and immersed in 500 μg/ mL of bovine serum albumin (BSA, Sigma-Aldrich) in phosphate buffered saline (PBS, Gibco). The same plate was used as control and after 24 hours of incubation in a humidified atmosphere with 5% CO_2 and at 37°C, the remaining protein in solution was assayed for total protein quantification. The assay was performed according to manufacturer's instructions. Accordingly, protein concentrations were determined with reference to standards of BSA. A series of dilutions of known concentration were prepared from the protein and assayed alongside the unknowns before the concentration of each unknown was determined based on the standard curve. The absorbance was read on a microplate spectrophotometer (Tecan) at 562 nm (n=3).

The total protein adsorbed in each surface is expressed subtracting the absorbed protein area on the well that was not covered by the sample from the total absorbed protein of the control.

1.2.4. Cells Culture

MOUSE OSTEOBLASTIC CELL LINE CULTURE

The murine osteoblastic cell line MC3T3-E1 were suspended in alpha Minimum Essential Medium Eagle (α-MEM, Invitrogen) supplemented with 10% of heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich), 2 mM α-glutamine (Invitrogen), 100U/100 μg/ml penicillin/streptomycin (Pens/Strep, Invitrogen) and 1mM sodium pyruvate (Invitrogen), cultured in plastic tissue culture flasks and incubated in a humidified atmosphere with 5% CO_2 and at 37°C. Adherent MC3T3-E1 cells were expanded and the medium was changed every third day, until the cells achieved 80% of confluence.

BOVINE ARTICULAR CHONDROCYTE CULTURE

Bovine articular chondrocytes (bch) were isolated from harvested bovine cartilage from the patellar-femoral groove of calf legs through enzymatic digestion. Cartilage tissue was cut in small pieces and chondrocytes were isolated by incubation in Duldbecco's modified Eagle's medium (DMEM, Invitrogen) containing 0.2% collagenase type II overnight at 37°C. The isolated chondrocytes were washed in PBS, centrifuged and re-suspended in chondrocyte proliferation medium containing DMEM high glucose (Gibco) with 10% FBS (Sigma-Aldrich), 0.1mM non essential amino acids (Sigma-Aldrich), 100U/100 μg/ml Pen/Strep (Invitrogen), 0.4 mM proline (Sigma-Aldrich) and 0.2mM Ascorbic-acid-2-Phosphate (Invitrogen), culture expanded in plastic tissue culture flasks and incubated in a humidified atmosphere with 5% CO_2 and at 37°C. The medium was changed every third day until the cells achieved 80% of confluence.

Prior to cell seeding of both cell types separately, the surfaces were sterilized by immersion in 70% (v/v) of ethanol for 2 hours, rinsed three times with PBS and then fixed to the bottom of 48-well plates with a gelseal (GE Healthcare Bio-Science Corp.). Each well containing the samples was filled with a heated agarose solution (agarose/PBS) 3% (wt/v). The plates were left 1 hour in the fridge at 4°C to solidify and create an agarose mould. TCPS wells were used as control and agarose moulds were also made inside of these wells. After solidification the agarose moulds were punched with the same size and direction of the samples and the remaining holes were filled with culture medium leaving the samples preincubated in medium overnight.

The agarose moulds were used in this work in order to prevent the contact of cell suspension with the bottom and walls of the wells and to ensure that cell attachment was not decreased due to medium culture repellence and sample floating.

At confluence cells were detached using 0.25% trypsin/EDTA solution (Sigma-Aldrich) after medium removal and washed with PBS. Cells were re-suspended in each culture medium and seeded on surfaces from each group as well as in TCPS with $1x10^4$ cells in 300 µL of medium (per sample). The mediums were changed every two days and before each assay the agarose moulds were removed carefully.

1.2.5. Cell Viability, Adhesion and Proliferation Studies

Cell viability and metabolic activity of MC3T3-E1 and bch were studied using a MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-dimethyl tetrazolium bromide] and live/dead assay. The MTT assay was performed at day 1 and live/dead assay at day 1 and 7, according to manufacturer's specifications.

Cell adhesion on smooth and rough surfaces was studied by a DNA quantification assay carried out after 3 days of culture using both cell types. In order to study the influence of these surfaces on cell proliferation, the alamar blue assay was performed with MC3T3-E1 cell line at 1, 3 and 7 days.

The surfaces were further observed by SEM to investigate MC3T3-E1 and bch cells morphology at days 1, 3 and 7.

MTT QUANTIFICATION

3-(4, 5- dimethyl-2-thiazolyl)-2, 5-dimethyl tetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt that may be used in measurement of metabolic activity of viable cells. Dissolved MTT can be converted to a water insoluble purple formazan by mitochondrial dehydrogenase enzymes of living cells that catalyze the cleavage of the tetrazolium ring in MTT [6-8]. The formazan can be solubilized using solvents and the absorbance of dissolved material, measured spectrophotometerally, comes as a function of concentration of converted dye.

In brief, the culture medium of each cell culture was removed from the wells and rinsed twice with 400 µL of PBS. Then, 400 µL of complete culture medium and 40 µL of MTT solution (5 mg/mL) were added to each well. After homogenization, the plate returned to cell culture incubator (humidified atmosphere with 5% CO_2 at 37°C) for 4 h. Subsequently, the supernatant was carefully discarded and the remaining MTT-formazan crystals dissolved by adding 400 µL of dimethyl sulfoxide (DMSO). The plate was under agitation for 5 min in an orbital shaker (200 rpm). The content of each well was transferred to an identified microtube and centrifuged at 1300 rpm for 2 min. From the supernatant 200 µL aliquots were transferred into a 96-well plate and a control with 200 µL of DMSO was also prepared. Afterwards the absorbance was read on a microplate spectrophotometer (Tecan) at 540 nm

with background subtraction at 690 nm. The results are expressed in percentage relative to the untreated cells control (n=3).

LIVE/DEAD ASSAY

The viability/cytotoxicity of cultured cells can be visualized with a Live/dead staining. Live cells are distinguished by the presence of intracellular esterase activity, determined by the enzymatic conversion of the non-fluorescent cell-permeant calcein-AM to the intensely fluorescent calcein. Ethidium homodimer-1 (EthD-1) is only able to enter cells with damaged membranes and after binding to nucleic acids producing a bright red fluorescence in dead cells [9].

Live/dead assay (Invitrogen) was performed by initially removing the culture mediums from the wells and rinsing the seeded samples with sterile PBS. The samples were stained with calcein-AM (2 μm) and EthD-1 (4 μM) in PBS and incubated for 30 minutes in dark at 37°C in a 5% CO_2 humidified atmosphere. The samples were immediately examined in an inverted fluorescent microscope (Nikon Eclipse E600) using a fluorescein isothiocyanate (FITC)/Texas Red filter.

DNA QUANTIFICATION ASSAY

Quantification of total DNA was performed with Quant-iT PicoGreen dsDNA assay kit (Molecular Probes/Invitrogen, USA) that allows the measurement of the fluorescence produced when PicoGreen dye is excited by UV light while bound to dsDNA.

Before DNA quantification, a standard curve was created according to the manufactures description. After 3 days of culture, samples were rinsed twice with sterile PBS and transferred to eppendorfs, where 200 μL of distillated water was added and then sonicated for 5 seconds for 4 times. Samples were vortexed and 10 μL of each plus 90 μL of PicoGreen solution were added to an opaque white flat bottom 96 well plate. After 2-5 minutes of incubation at room temperature (protected from light) the plate was read using a microplate reader (Victor^3, Perkin-Elmer, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm (n=3).

ALAMAR BLUE ASSAY

Alamar Blue is a proven cell viability and cell proliferation indicator that uses the natural reducing power of living cells to convert resazurin to the red fluorescent molecule, resorufin. Viable cells continuously convert resazurin to resorufin, thereby generating a quantitative measure of viability—and cytotoxicity [10]. The amount of fluorescence produced is proportional to the number of living cells.

Alamar Blue was investigated as a measure of metabolic activity by the Alamar Blue reduction assay (Biosource, DAL 1100). Proliferation measurements were made fluorometrically. Briefly, cell culture medium was replaced with 10% (v/v) (total culture medium) of Alamar Blue solution in each well. After 4 h of incubation in a humidified atmosphere with 5% CO_2 and at 37°C, the fluorescence of triplicates samples (200 µL) was measured in a 96-well plate, using a microplate reader (Victor^3, Perkin Elmer, U.S.A.) at an excitation wavelength of 545 nm and an emission wavelength of 590 nm (n=3).

SCANNING ELECTRON MICROSCOPY (SEM) OBSERVATION

MC3T3-E1 and bch morphology was evaluated by SEM after the samples being fixed with 10% (v/v) formalin for 30 minutes, dehydrated using graded ethanol solutions (70%, 80%, 90% and 100% (v/v), 30 minutes in each) and critical point dried (Balzers CPD 030). All samples were coated with gold using a sputter coater (Cressington) for 60 seconds at a current of 40 mA. The analysis was performed on a Philips XL 30 ESEM-FEG microscope at an accelerating voltage of 10kV.

1.2.6. Statistical Analysis

All the results on this study are reported as mean ± standard deviation (SD). Experimental data were analyzed using the one-way ANOVA test to assess statistical significance of the results, with $p<0.05$ (*) and (#) or $p<0.01$ (**) considered as being statistically significant.

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Chapter III

Cells Behavior on Superhydrophobic Surfaces with Different Topographies

CELLS BEHAVIOR ON SUPERHYDROPHOBIC SURFACES WITH DIFFERENT TOPOGRAPHIES

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Abstract

Surface wettability and topography are recognized as critical factors in biomaterials that influence cell behavior. So far only few works have reported cell response on surfaces exhibiting extreme wettability, especially reporting the influence of topography combined with this environment.

In the present study, bioinspired superhydrophobic rough surfaces of polystyrene and poly (L-lactic acid) with different micro/nanotopographies have been obtained from smooth surfaces using a simple phase-separation based methodology. In order to evaluate the cell behavior on such surfaces, a mouse osteoblastic MC3T3-E1 cell line and a primary cell culture of bovine articular chondrocytes (bch) were used as model systems. Smooth and rough surfaces have been characterized by scanning electron microscopy, contact angle measurements and X-ray photoelectron spectroscopy. A preliminary assay for total protein quantification was performed and showed a reduction of BSA protein adsorption onto rough surfaces as compared with the correspondent smooth ones, though similar amount of protein adsorbed onto the same type of PS or PLLA surfaces. Biological assays were performed to test the ability of PS and PLLA surfaces to support cell adhesion and proliferation. Results indicated that both types of cells showed similar behavior when in contact with the surfaces, although the cell line showed enhanced performance. In general cells adhere and proliferate faster in the smooth surfaces as compared to the rough substrates, indicating the relevant influence of wettability on cell behavior, which seems not very influenced by both polymer nature and topography of the superhydrophobic surfaces.

Keywords: cell behavior, biomimetic, polystyrene, poly (L-lactic acid), superhydrophobic surfaces, topography.

1. Introduction

Biomaterials interact with the biological environment through their interfaces. Both the kind and the strength of such interactions are largely dependent on the surface properties of the materials, such as wettability, topography/roughness, surface charge and chemical functionalities [1]. Understanding such interactions process is critically important to the comprehension of many fundamental biological questions and for the development of biomaterials in the field of Tissue Engineering (TE) and Regenerative Medicine.

Surface properties including wettability and topography are recognized as critical factors that directly influence cell behavior through altering the conformation of absorbed extracellular matrix (ECM) proteins that, consecutively, regulate cell-substrate interactions [2-5]. Cells respond to topographical cues in many ways and a wide variety of cell types have been studied in several substratum features such as grooves, ridges, steps, pores, wells and nodes in micro- and nano scales to understand the interactions between cells and different topographies [6-8]. Nevertheless, surface treatments that increase roughness at that scale level typically affect wettability [9].

Superhydrophobic surfaces combine micro and nanometer scale roughness along with a low surface energy material which leads to a water contact angle (WCA) higher than 150° [10]. Many examples of superhydrophobic surfaces can be found in nature, but synthetic ones have been produced using a large number of techniques [11, 12]. Superhydrophobic surfaces have been proposed to be used in several functional applications including anti-biofouling, non wettable textiles, transparent and antireflective self-cleaning coatings or humidity-proof coatings for electronic devices [13, 14]. We have shown that such surfaces could be useful in several biomedical-related areas including as substrates for particles production [15-18], open microfluidic devices [19] and the production of arrays for high-throughput analysis [20]. Anti-bioadhesion applications, seeking to prevent protein adsorption and cell adhesion have been mostly studied in blood compatible materials [21, 22]. Nonetheless, few works are found in literature reporting the use of superhydrophobic surfaces as supports for cell response studies [23-28].

Superhydrophobic surfaces may display different topographies, but to our knowledge the influence of such different textures on cell behavior were never reported. Cell behavior is also extremely dependent not only on surface properties but may be affected by cell type [23, 29]. There is both fundamental and practical interest in combining different topographies in surfaces with extreme wettability properties in order to investigate if the influence of topography or cell type can be as important as the influence of wettability itself on cell behavior.

In this work, biomimetic superhydrophobic surfaces with distinct topographies were obtained from two smooth polymeric surfaces, polystyrene (PS) and poly (L-lactic acid) (PLLA), by a simple and economical phase separation methodology. Our aim was to investigate the influence of surface topography and the chemical nature in superhydrophobic surfaces on the cellular response using two cell types (a cell line and a primary cell culture) and compare with the correspondent smooth surfaces. PS is a well documented amorphous aromatic polymer, being used as control for a series of cell-material interactions concerning studies and PLLA is a biodegradable, semi-crystalline polyester proposed to be used in several biomedical applications. The different thermal behavior of these two materials allows to generate different surface textures when substrates are processed by a phase separation methodology. This fact will be used in this work to produce superhydrophobic surfaces with distinct topographies and different chemistries.

2. Materials and Methods

2.1. Materials

The smooth PS films used in this work were purchased from GoodFellow Cambridge Limited, England, with a thickness of 0.25 mm.

A high stereoregular PLLA with a Mn of 69.000 and a Mw/Mn of 1.734 was obtained from Cargill Dow LLC, USA. The glass transition temperature and melting temperature of this polymer were 60 and 162 °C, respectively [30].

Tetrahydrofuran (THF) and sterile agarose were purchased from Sigma-Aldrich. 1, 4-Dioxane (p.a.≥ 99.5%) and absolute ethanol were obtained by Fluka and Panreac, respectively.

2.2. Methods

2.2.1. Superhydrophobic Surfaces Production

PREPARATION OF PS SUPERHYDROPHOBIC SURFACES

PS substrates were obtained cutting the smooth PS films into small squares of 5 x 5 cm^2 and cleaned by immersion in 70% ethanol (v/v) in an ultrasonic bath for 10 minutes.

A 70 mg ml^{-1} solution of PS (injection molding grade) in THF was prepared and then mixed with absolute ethanol in a ratio of 2: 1.3 (v/v). The mixture was uniformly dispensed onto PS substrates, resulting in the formation of an opaque or semi-transparent layer. After 10s on air, the substrates were then immersed in absolute ethanol for 1min. Afterwards the surfaces were dried at room temperature, resulting in rough superhydrophobic PS.

PREPARATION OF PLLA SUPERHYDROPHOBIC SURFACES

Flat smooth PLLA substrates were produced by melting PLLA powder between two glass slides subjected to compression at 200°C followed by cooling in water [25].

A 13% (wt/v) PLLA solution in 1, 4-dioxane was casted on the substrates. After an evaporation period of a few seconds the substrates were immersed in absolute ethanol to induce the phase separation of the casted solution. The samples were first dried under nitrogen flow and then in the vacuum oven at 30°C for 24 hours to eliminate all solvent residues. When the samples were completely dry, the upper part was removed giving rise to rough superhydrophobic surfaces of PLLA.

All PS and PLLA surfaces were punched into circular samples with a diameter of 8 mm and their nomenclatures were the following: PS-S and PLLA-S for the smooth PS and PLLA substrates, respectively, and PS-R and PLLA-R for the corresponding rough surfaces.

2.2.2. Characterization

SCANNING ELECTRON MICROSCOPY (SEM)

Surface topography was analyzed, before and after being processed using the phase-inversion method, by a Leica Cambridge S-360 scanning electron microscope (Leica Cambridge, UK). All samples were pre-coated with a conductive layer of sputtered gold. The analyses were performed at an accelerating voltage of 15kV at different magnifications.

CONTACT ANGLE MEASUREMENT

The wettability of different surfaces was characterized by CA measurements. Static CA measurements were performed using the sessile drop method on an OCA15+ goniometer (DataPhysics, Germany) under ambient conditions at room temperature. Milli-Q water (6 µL) was dropped on the surfaces and pictures were taken after water drop stabilization. Three samples of each surface type were measured five times. The CA measurements of all samples were carried out in the same week of their preparation and 12 weeks later.

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The surface chemical composition of smooth and rough surfaces was investigated by XPS, using a Physical Electronics Quantera SXM (scanning XPS microprobe) system with monochromatic Al Kα radiation (h_υ= 1486.6 eV/15kV) shot at an angle of 45° toward the surfaces. Survey spectra were obtained with pass energy of 224 eV and a step size of 0.8 eV. The software used was a Compass for XPS control, Multipak v.8.0 for data reduction. The measurements were carried out in triplicate 12 weeks after samples' preparation.

2.2.3. Adsorbed Protein Quantification

BCA ASSAY

Protein absorption on samples was analyzed by a colorimetric detection and quantification of total protein using a bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co, USA). This system utilizes BCA as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. The purple coloured reaction product exhibits a strong absorbance at 562nm that is linear with increasing protein concentration.

Surfaces were fixed on the bottom of an ultra low attachment plate and immersed in 500 μg/ mL of bovine serum albumin (BSA, Sigma-Aldrich) in phosphate buffered saline (PBS, Gibco). The same plate was used as control and after 24 hours of incubation in a humidified atmosphere with 5% CO_2 and at 37°C, the remaining protein in solution was assayed for total protein quantification. The assay was performed according to manufacturer's instructions. Accordingly, protein concentrations were determined with reference to standards of BSA. A series of dilutions of known concentration were prepared from the protein and assayed alongside the unknowns before the concentration of each unknown was determined based on the standard curve. The absorbance was read on a microplate spectrophotometer (Tecan) at 562 nm (n=3).

The total protein adsorbed in each surface is expressed subtracting the absorbed protein area on the well that was not covered by the sample from the total absorbed protein of the control.

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Prior to cell seeding, the surfaces were sterilized by immersion in 70% (v/v) of ethanol for 2 hours, rinsed three times with PBS and then fixed to the bottom of 48-well plates with a gelseal (GE Healthcare Bio-Science Corp.). Each well containing the samples was filled with a heated agarose solution (agarose/PBS) 3% (wt/v). The plates were left 1 hour in the fridge at 4°C to solidify and create an agarose mould. Tissue culture polystyrene (TCPS) wells were used as control and agarose moulds were also made inside of these wells. After solidification the agarose moulds were punched with the same size and direction of the samples and the remaining holes were filled with culture medium leaving the samples preincubated in medium overnight.

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The surfaces were further observed by SEM to investigate MC3T3-E1 cell line and bch morphology at days 1, 3 and 7.

MTT QUANTIFICATION

MTT assay measures the metabolic activity of viable cells, once the dissolved MTT can be converted to a water insoluble purple formazan by mitochondrial dehydrogenase enzymes of living cells that catalyze the cleavage of the tetrazolium ring in MTT. In brief, the culture medium of each cell culture was removed from the wells and rinsed twice with 400 µL of PBS. Then, 400 µL of complete culture medium and 40 µL of MTT solution (5 mg/mL) were added to each well. After homogenization, the plate returned to cell culture incubator (humidified atmosphere with 5% CO_2 at 37°C) for 4 h. Subsequently, the supernatant was carefully discarded and the remaining MTT-formazan crystals dissolved by adding 400 µL of dimethyl sulfoxide (DMSO). The plate was under agitation for 5 min in an orbital shaker (200 rpm). The content of each well was transferred to an identified microtube and centrifuged at 1300 rpm for 2 min. From the supernatant 200 µL aliquots were transferred into a 96-well plate and a control with 200 µL of DMSO was also prepared. Afterwards the absorbance was read on a microplate spectrophotometer (Tecan) at 540 nm with background subtraction at 690 nm. The results are expressed in percentage relative to the untreated cells control (n=3).

LIVE/DEAD ASSAY

Live/dead assay (Invitrogen) was performed by initially removing the culture mediums from the wells and rinsing the seeded samples with sterile PBS. The samples were stained with calcein-AM (2 µM) and ethidium homodimer-1 (4 µM) in PBS and incubated for 30 minutes in dark at 37°C in a 5% CO_2 humidified atmosphere. The samples were immediately examined in an inverted fluorescent microscope (Nikon Eclipse E600) using a fluorescein isothiocyanate (FITC) and Texas Red filters. Calcein- AM is cleaved by intracellular esterases and produces green fluorescence because it is capable of permeating

the plasma membrane of viable cells. Ethidium homodimer-1 is only able to enter cells with damaged membranes and after binding to nuclei acids produces red fluorescence in dead cells.

DNA QUANTIFICATION ASSAY

Quantification of total DNA was per formed with Quant-iT PicoGreen dsDNA assay kit (Molecular Probes/Invitrogen, USA) that allows the measurement of the fluorescence produced when PicoGreen dye is excited by UV light while bound to dsDNA.

Before DNA quantification, a standard curve for DNA analyses was generated with λ DNA according to the manufactures description. After 3 days of culture, samples were rinsed twice with sterile PBS and transferred to eppendorfs, where 200 µL of distillated water was added and then sonicated for 5 seconds for 4 times. Samples were vortexed and 10 µL of each plus 90 µL of PicoGreen solution were added to an opaque white flat bottom 96 well plate. After 2-5 minutes of incubation at room temperature (protected from light) the plate was read using a microplate reader (Victor^3, Perkin-Elmer, USA) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm (n=3).

ALAMAR BLUE ASSAY

Alamar Blue is a proven cell viability and cell proliferation indicator that uses the natural reducing power of living cells to convert continuously resazurin to the red fluorescent molecule, resorufin. The amount of fluorescence produced is proportional to the number of living cells.

Alamar Blue was investigated as a measure of metabolic activity by the Alamar Blue reduction assay (Biosource, DAL 1100). Proliferation measurements were made fluorometrically. Briefly, cell culture medium was replaced with 10% (v/v) (total culture medium) of Alamar Blue solution in each well. After 4 h of incubation in a humidified atmosphere with 5% CO_2 and at 37°C, the fluorescence of triplicates samples (200 µL) was measured in a 96-well plate, using a microplate reader (Victor^3, Perkin Elmer, USA) at an excitation wavelength of 545 nm and an emission wavelength of 590 nm (n=3).

SCANNING ELECTRON MICROSCOPY (SEM) OBSERVATION

MC3T3-E1 and bch cells morphology was evaluated by SEM, after the samples being fixed with 10% (v/v) formalin for 30 minutes, dehydrated using graded ethanol solutions (70%, 80%, 90% and 100% (v/v), 30 minutes in each) and critical point dried (Balzers CPD 030). All samples were coated with gold using a sputter coater (Cressington) for 60 seconds at a current of 40 mA. The analysis was performed on a Philips XL 30 ESEM-FEG microscope at an accelerating voltage of 10kV.

2.2.6. Statistical Analysis

All the results on this study are reported as mean ± standard deviation (SD). Experimental data were analyzed using the one-way ANOVA test to assess statistical significance of the results, with $p<0.05$ (* and #) or $p<0.01$ (**) considered as being statistically significant.

3. Results and Discussion

3.1. Surfaces Physical-Chemical Characterization

SEM pictures evidencing the morphology of the PS and PLLA rough surfaces are shown in Figure 3.1. Such surfaces exhibit a hierarchical micro and nano-structured roughness induced when the polymers were dissolved in the respective solvents and mixture with the non-solvent (ethanol) forcing polymers precipitation. The mass transfer of the non-solvent and solvent across the interface leads the homogeneous solution to become thermodynamically unstable and induces phase separation. PS and PLLA form both poor and rich polymers phase. In the poor phase, polymer nuclei are formed by precipitation. The rich polymer phase aggregates around these nuclei in order to decrease surface energy (tension). During polymer precipitation within the rich PS and PLLA phase, a continuous deposition of spheres on the surface takes place in order to decrease even more surface tension [31].

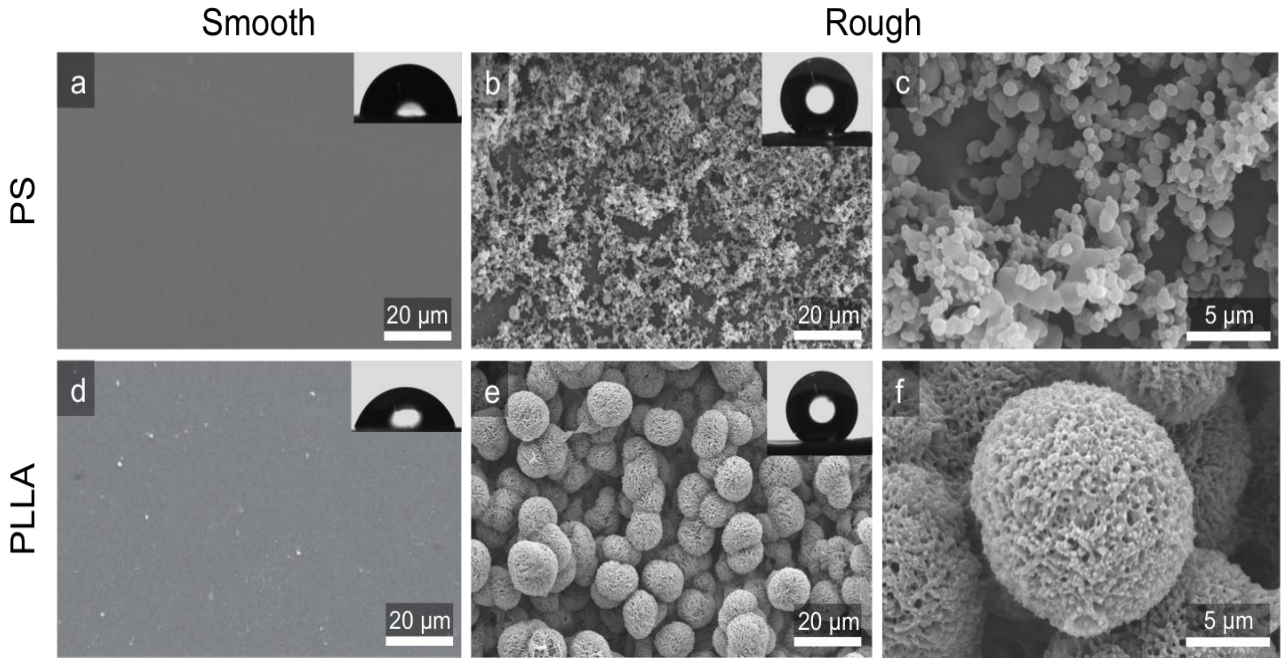

Figure 3.1 - SEM microphotographs of PS (a, b, c) and PLLA (d, e, f) surfaces before (a, d) and after (b, c, d, e, f) phase inversion based methodology. The insets show photographs of a water droplet deposited on the corresponding surfaces.

Despite the methodology used to prepare both PS and PLLA rough surfaces be the same, such surfaces have different topographies mainly due to the different structural nature of the polymers: PS is amorphous while PLLA is a semi-crystalline polymer. PS-R exhibits randomly distributed spheres with sizes from 50 nm to 200 nm that are agglomerated in larger micrometer structures (Figure 3.1 c), while PLLA-R shows well define individual papilla-like structures with sizes of 10 µm exhibiting clear

rough texture at the nanometer level (Figure 3.1 f), similar to the papillae nanostructures of the lotus leaf [32].

The wetting behavior and surface chemistry of smooth and rough surfaces were investigated by water contact angle (WCA) measurements and XPS technique, respectively. The phase separation method transforms the hydrophobic smooth surfaces into rough superhydrophobic surfaces (WCA>150°), which wettability is stable over time, as confirmed by the water contact angle measurements (Figure 3.2).

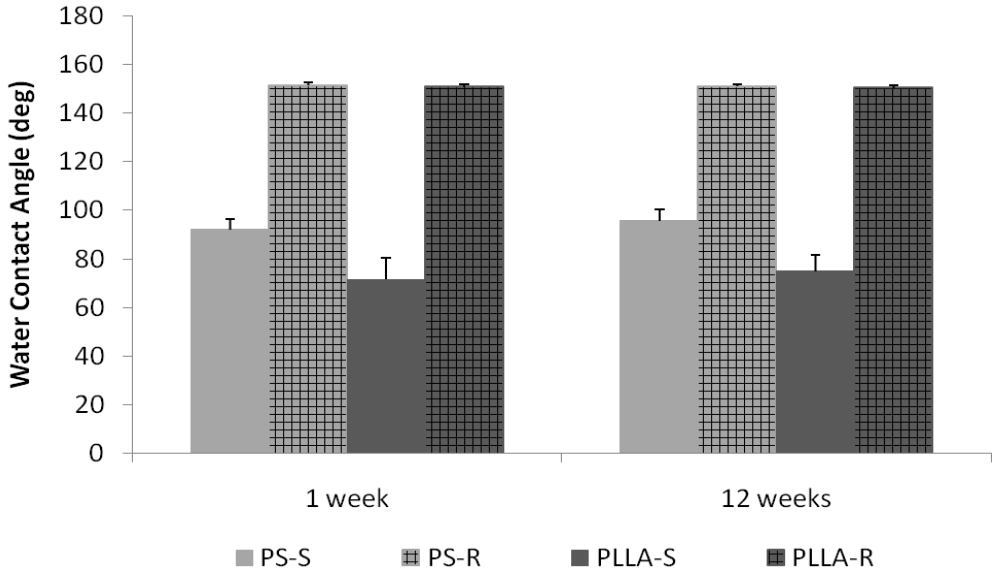

Figure 3.2 – Water contact angle on the different surfaces measured at week 1 and week 12 using the sessile drop method.

The high-resolution C1s spectra of the PS and PLLA samples are shown in Figure 3.3 and revealed three peaks, which were decomposed into Gaussian peaks using a nonlinear fitting algorithm. For PS-S and PS-R surfaces, the high-resolution spectrum consists of a hydrocarbon peak at 285.0 eV, an aromatic carbon peak at 284.7 eV and a broad aromatic shake up peak at about 291.5 eV. In the case of PLLA-S and PLLA-R surfaces, the C1s 285.0 eV peak was assigned to the main backbone carbon peak, 287.33 eV to C-O group and 289.53 eV to O=C-O group. As expected, O1s spectra did not show any significant differences between PS-S and PS-R and between PLLA-S and PLLA-R. These results are consistent with the molecular structure of PS and PLLA, respectively.

XPS analysis suggested that the chemistry of rough and smooth surfaces were similar for each polymer, which means that the chemistry is maintained on the rough surfaces that only differ from original smooth surfaces from topographic features [19]. The superhydrophobicity is an exclusively consequence of surface roughness, as a combination of micro and nanometer scale roughness, along with a low surface energy material [33].

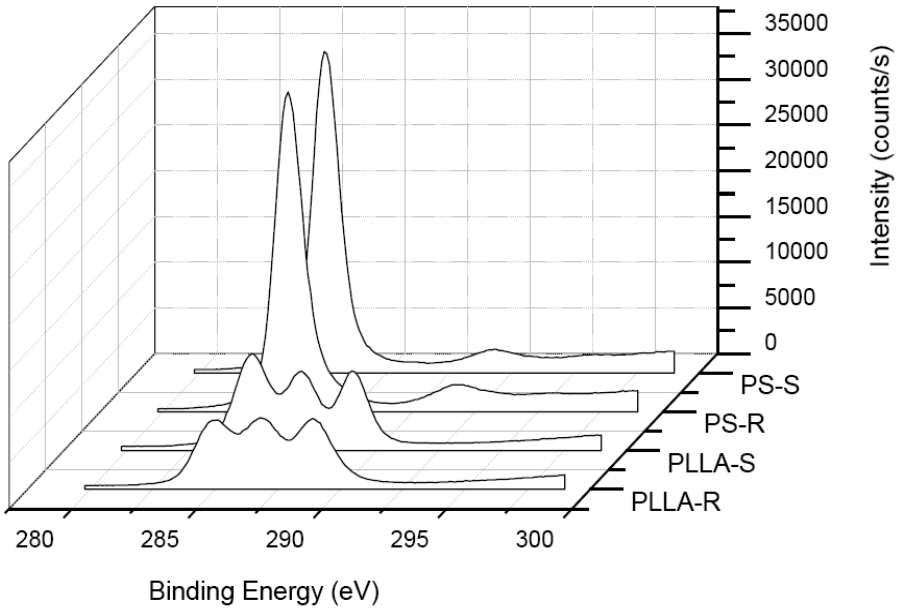

Figure 3.3 - X-ray photoelectron spectroscopy high resolution C1s spectrum of smooth and rough surfaces of PS (PS-S, PS-R) and PLLA (PLLA-S, PLLA-R).

3.2. Protein absorption on surfaces

It is widely accepted that cell response to biomaterial is not mainly mediated by a direct contact, but rather through an interfacial layer created on material surface once it is in contact with a physiological environment as a result of a competitive adsorption of proteins from the milleu [34]. The composition, conformation and orientation of this protein layer critically determines cell responses, such as cell adhesion [35].

It is well known that surface topography at both micro and nanometer levels influences protein adsorption and distribution between topographic cues [36-38]. However, just a few studies reported protein adsorption studies on surfaces exhibiting extreme conditions of wettability [20, 39, 40].

For the proof-of-concept bovine serum albumin (BSA) adsorption was analyzed by BCA protein assay in order to investigate the effect of the nature of the substrate. The quantitative adsorption results onto smooth and rough surfaces after 24 h are shown in Figure 3.4.

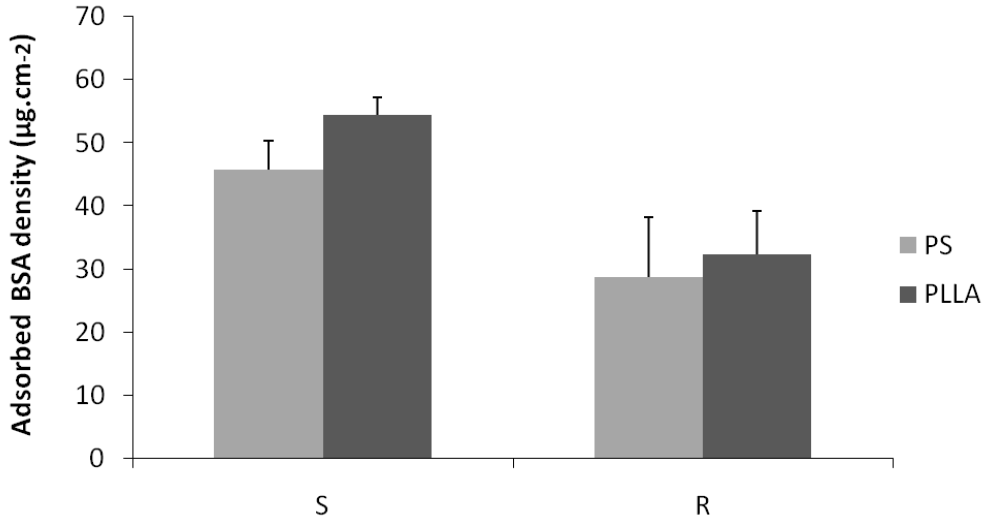

Figure 3.4 - BCA assay showing albumin adsorption on PS and PLLA surfaces after 24 hours of immersion in 500 µg/mL of BSA.

Protein adsorption on superhydrophobic surfaces is suggested to be more resistant than on more wettable surfaces, even if there is no precise quantification of the total amount adsorbed in the literature [14, 40]. For each smooth (S) or rough (R) class of surfaces, no statistical differences were found between the protein adsorbed between PS or PLLA substrates. The amount of protein absorbed onto rough surfaces was found to be tendencially lower when compared with smooth surfaces, indicating the relevant influence of wettability on protein adsorption that seems not very influenced by the topography of the rough surfaces. These results is in agreement with previous works [20, 39, 41] and may be explained by the principles of the model proposed by Cassie and Baxter [42] to address the superhydrophobic behavior. This model postulated that the surface roughness leads to a superhydrophobic behavior as a consequence of the fact that the liquid does not intrude into the lower regions of the topographic features and a fraction of the surface of the drop in contact with the substrate is suspended by air pockets. So it is predictable by this model that the protein solution may be prevented from being in contact with the entire surface. However, nowadays transitional states between the Cassie–Baxter and the so-called Wenzel states have been proposed, which describe superhydrophobic surfaces with high adhesive forces to liquids [33, 43]. In this case, when the protein solution droplet fills the grooves of the rough substrates the wetting behavior changes from the Cassie-Baxter state to the Wenzel state (the liquid interface fully conforms to the roughness of the surface) [44], which means that the wetted surface area is smaller than in the Wenzel state but larger than in the Cassie–Baxter state, explaining why the water repellency of PS-R and PLLA-R were not sufficient to avoid protein absorption.

3.3. Cell Viability, Adhesion and Proliferation

The influence of surface topography on cell behavior has been widely investigated. It is well documented that surface microtopography promotes changes in cell adhesion patterns, cell orientation and cell shape on the substrate [45]. Cells cultured on smooth surfaces tend to generate more organized ECM, including more homogeneous distribution of focal adhesions. However, on rougher surfaces, focal adhesions are located at cell edges, where the contact with the substrate takes place [46]. The biological response of superhydrophobic rough surfaces has lead to contradictory results when compared to smoother surfaces. Some authors reported better cell performance on rough surfaces [23, 47-49] and others the opposite [25, 26, 39, 49]. In general, only few cells cell can adhere on superhydrophobic surfaces, therefore they do not proliferate [26, 39]. On the contrary, some studies have shown cell proliferation or survival [47, 49] and even differentiation [48]. Nevertheless, it has been reported that cell behavior can be extremely dependent on the cell type [23, 29].

Attachment, adhesion and spreading belong to the first phase of cell-material interaction and the quality of this stage influences the capacity of cells to proliferate on contact with the material. In order to investigate the biological performance of PS and PLLA surfaces and evaluate the influence of surface wettability, topography and chemistry a cell line and a cell primary culture were used as models for *in vitro* evaluation. Osteoblastic MC3T3-E1 cell line and bovine articular chondrocytes (bch) were seeded on the different surfaces and viability, adhesion and proliferation studies were investigated.

Cell viability/cytotoxicity was assessed using the MTT quantification (Figure 3.5) and live-dead assays (Figure 3.6). For the same smooth or rough surfaces, the viability of bch cells was tendentially lower that of the MC3T3-E1 cells. The cell viability in the rough surfaces is generally lower than in the smooth surfaces, especially for the case of the bch cells. As seen before for the case of the protein adsorption, cell viability seems to be not dependent on the chemical nature of the two polymers and also not sensitive to the topography of the superhydrophobic surfaces. The strongest changes are seen between the smooth surfaces and the equivalent rough ones. As expected the cell line (MC3T3-E1) tends to have a better biological performance than the primary cell culture (bch).

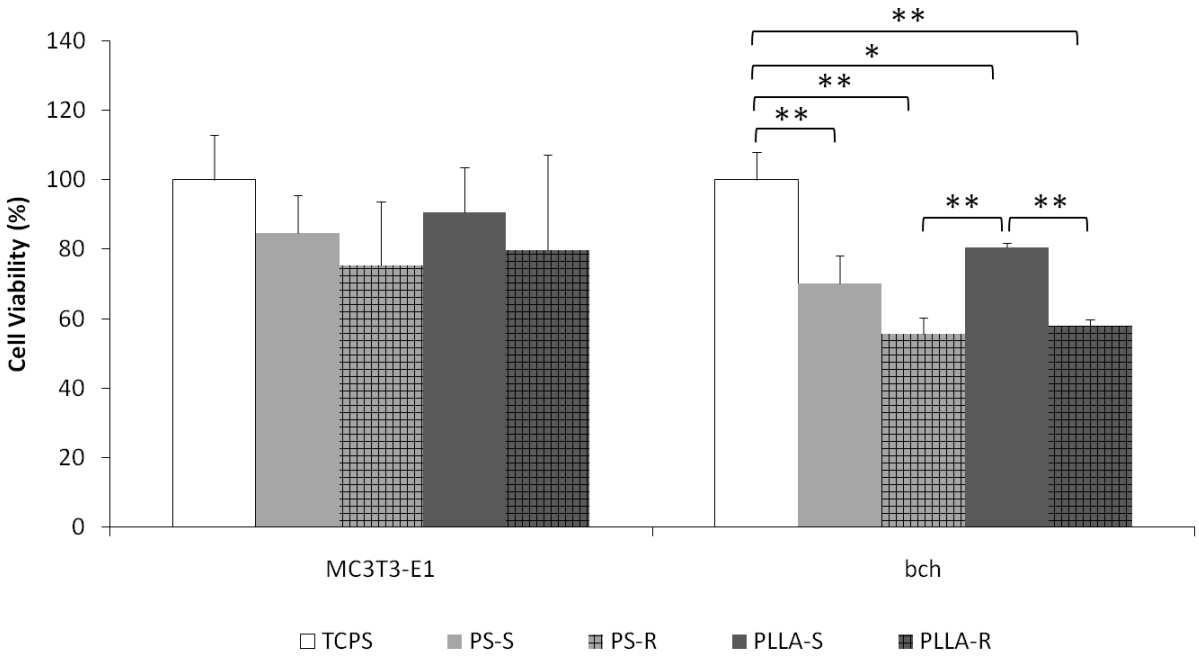

Figure 3.5 - MTT quantification of MC3T3-E1 cell line and bovine articular chondrocytes (bch) on the different surfaces and on TCPS control after 1 day in culture. Significant differences between different surface types on the same culture day were found for $p<0.05$ (*) and $p<0.01$ (**).

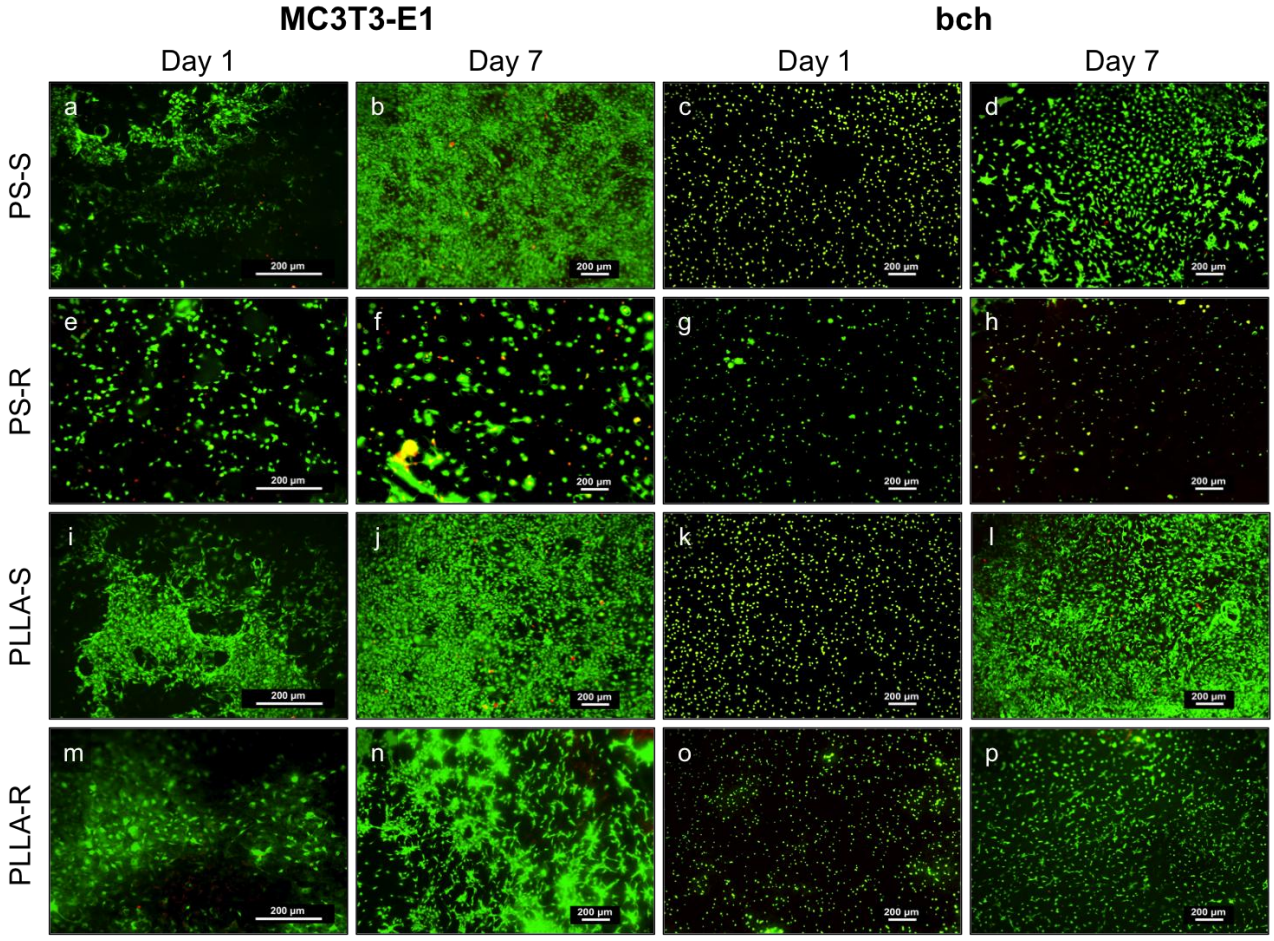

Figure 3.6 - Live-dead assay showing MC3T3-E1 and bch cells at the PS surfaces (e -h) and at the PLLA surfaces (i-p) at day 1 and 7 of culture. Cells were stained with calcein-AM/ethidium homodimer (dead cells stain red and living cells green) and visualized using fluorescence microscopy. Cell density: $1x10^4$ cells/300 µL.

Live-dead assay displayed distinct cell attachment and cell proliferation performance – see Figure 3.6. In general more live cells (green) are seen on smooth surfaces as compared to the rough counterpart after 1 day of culture, being consistent with the MTT results. After 7 days in culture, cells start to be confluent on smooth surfaces, but the same it is not visible on the rough surfaces. One could then conclude that both types of cells prefer to adhere and proliferate on smooth surfaces than on rough ones.

The DNA quantification assay allowed to have a clean picture of the activity of MC3T3-E1 and bch cells on the developed surfaces - see Figure 3.7. As seen before in the qualitative live-dead assay, the adhesion of both types of cells was shown to be greater on smoother surfaces. MC3T3-E1 cell line showed to adhere more than bch cells to the same kind of surface, revealing again the more attachment tendency of the cell line. The results also strengthen the low influence of both polymer nature and topography of the superhydrophobic surfaces on cell adhesion.

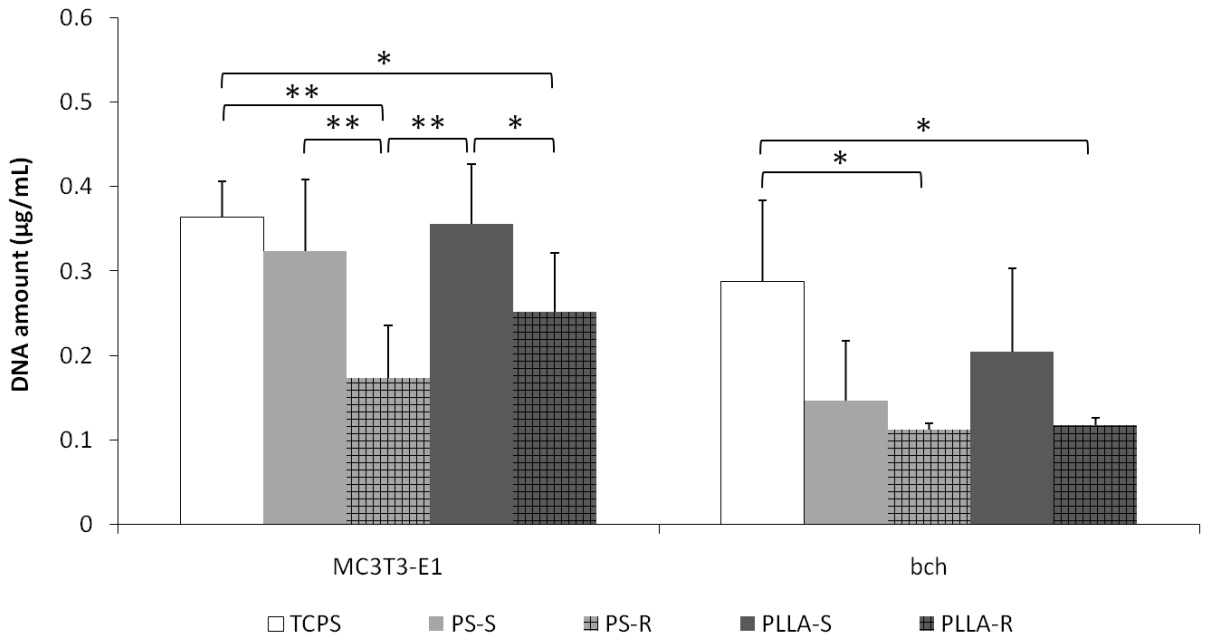

Figure 3.7 – DNA quantification of MC3T3-E1 cell line and bch cells on the produced surfaces and the control after 3 days of culture. Significant differences between different surface types on the same culture day were found for $p<0.05$ (*) and $p<0.01$ (**).

In order to evaluate the performance of smooth and rough surfaces on cell proliferation, alamar blue assay was performed and MC3T3-E1 cell line was used to study the effect of these surfaces on the metabolic activity of cultured cells up to 7 days of culture - see Figure 3.8.

At day 1, the rough surfaces presented slight lower values as compared with the correspondent smooth surfaces. From day 1 to day 3, cell activity increased for the smooth surfaces while small

differences were observed for the rough surfaces. The data observed for day 3 is quite consistent with the DNA quantification results (Figure 3.7). From day 3 to day 7, cell activity increased significantly for the smooth surfaces and again no proliferation of cells could be seen in the rough surfaces. At day 7, strong differences were observed between smooth and rough surfaces that contrasted with the small differences found between the polymers: no differences could be detected between PS-S and PLLA-S and just a slightly higher value of fluorescence intensity was found in PLLA-R when compared to the PS-R. Resuming this set of data: (i) Cell activity significantly increased with increasing of culture time on PS-S and PLLA-S that exhibit similar values for each time points; (ii) the values between PS-R and PLLA-R are similar except for day 7; PLLA-R demonstrates a significant increase between day 1 and day 7, but even in this case the difference is much lower that the smooth counterpart. (iii) The cells almost no proliferate in the rough surfaces.

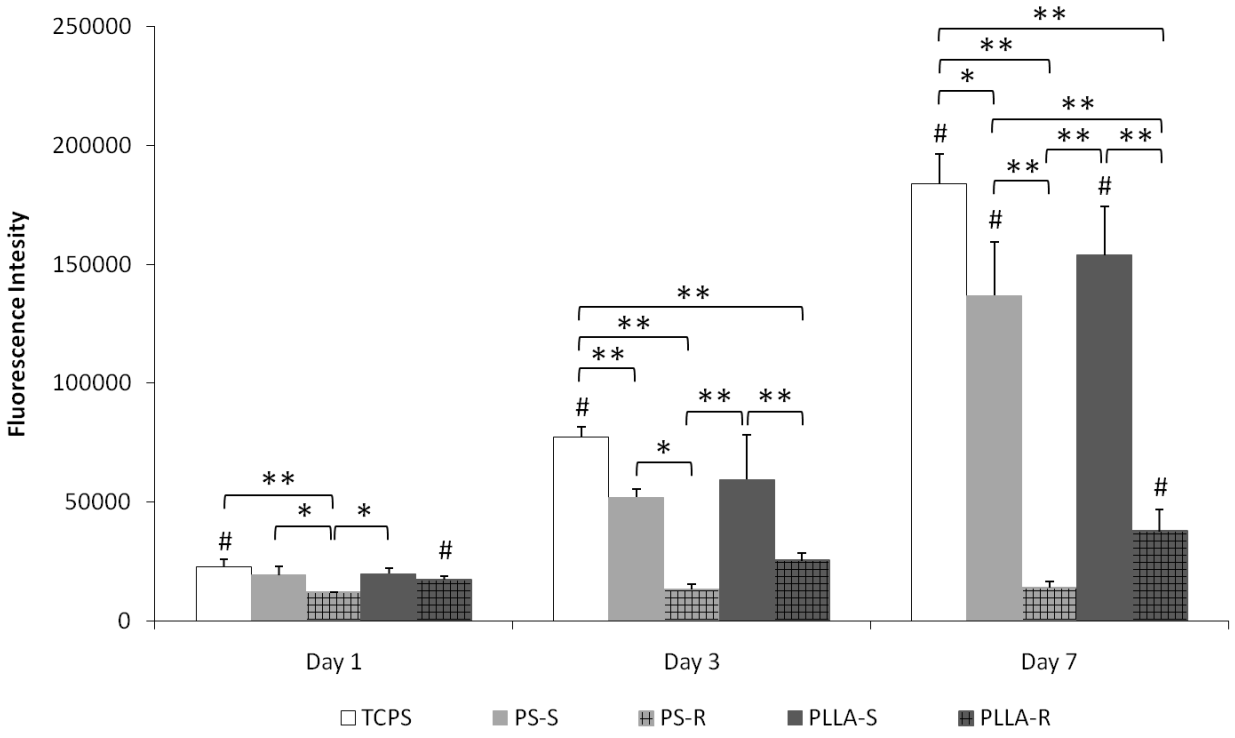

Figure 3.8 – Alamar blue assay of MC3T3-E1 cell line on the produced surfaces and the control, after 1, 3 and 7 days of culture. Significant differences between different surface types on the same culture day were found for $p<0.05$ (*) and $p<0.01$ (**). (#) represents significant differences between the same surface type on different culture days ($p<0.05$).

These results can be explained based on the distribution, conformation, and strength of adhesion between proteins and the substrate that modulate the interaction between cell and surfaces. It has been reported that there is a favored cell attachment on surfaces with moderate wettability, which permits the adsorption of serum proteins with labile and reversible bonds. The moderate degree of wettability of the substrates allows cells to deposit their own adhesion proteins, exchanging with the

more rapidly adsorbed serum proteins [28, 37, 50]. This mechanism was proposed to be slower on extremely hydrophobic or hydrophilic surfaces and adsorbed proteins showed altered conformation of the domains involved in cell adhesion, which led to lack of mature focal adhesion formation [39], justifying the fact that cells do not adhere and proliferate so well on those surfaces. For superhydrophobic surfaces one should also consider the scenario in which protein adsorption and cell attachment may be prevented by the fact that there is a significant fraction of the surface area that do not contact with the liquid medium. The Cassie-Baxter hypothesis limits the mass transfer of protein to the surface and may reduce the amount of fixing point for cell adhesion.

After contact to surfaces, cells alter their cell membrane and morphology to stabilize the cell-material interface. In this study, the morphology of MC3T3-E1 and bch cells onto smooth and rough surfaces was further analyzed by SEM after 1, 3 and 7 days of culture (Figure 3.9).

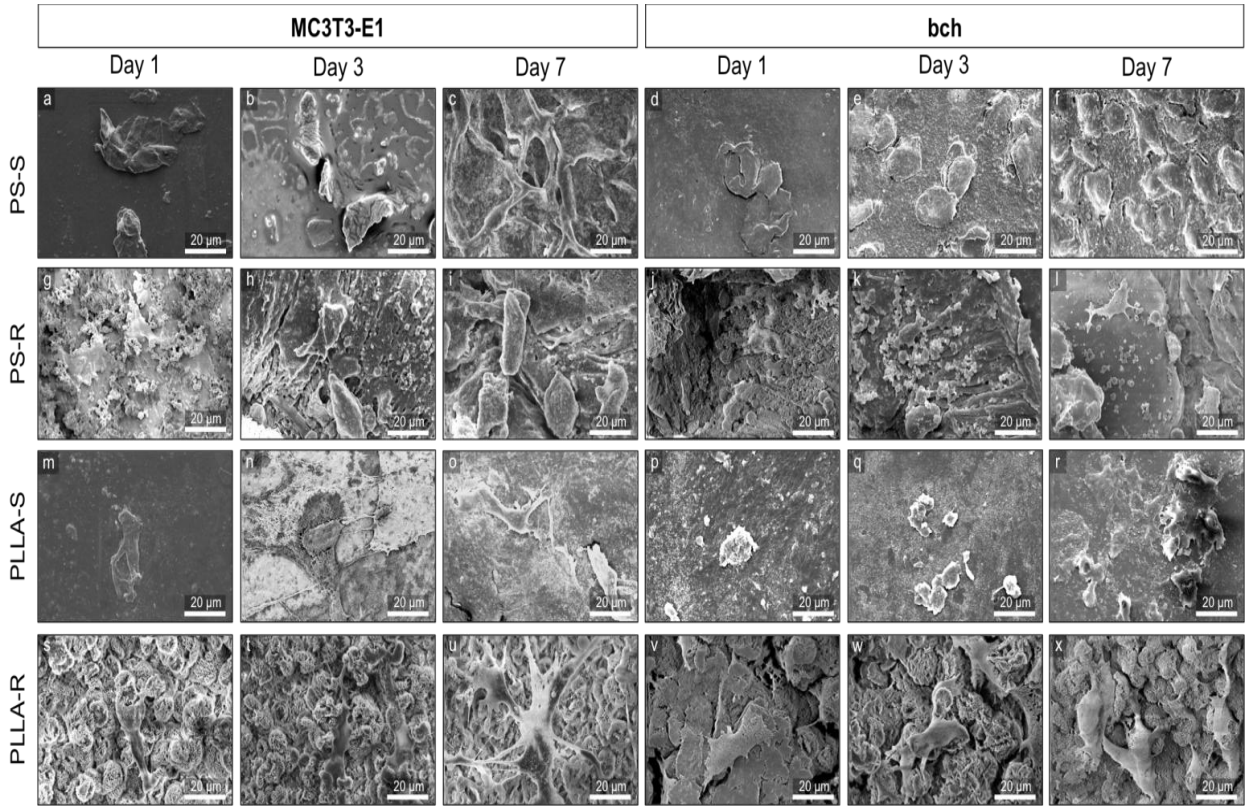

Figure 3.9 – SEM micrographs showing the morphology of MC3T3-E1 and bch cells over the PS (a-l) and PLLA surfaces (m-x) after 1, 3 and 7 days in culture. Cell density: $1x10^4$ cells/300 µL.

Due to the considerable difference between the surfaces properties, such as wettability and topography, the cell adhesion behavior is expected to be different, as shown by the previous assays. Different topographies may promote changes in cell adhesion, cell orientation and cell shape on the substrate: on rough surfaces, focal adhesions are typically situated at the extremities of the cells, where

the contact with the substrate takes place, whereas on smooth surfaces cells tend to generate more organized ECM, including more homogeneous distribution of focal adhesions [45, 46].

MC3T3-E1 cells on PS-S and PS-R surfaces were more spread on day 1, but after 3 and 7 days in culture cells started to become confluent on PS-S and adopted an elongated shape on this surface. Regarding the cell PS-R interactions and the cell morphology we found that cells located on the surface showed an elongated morphology with high cell-cell interactions but with a low cell-material attachment. On the contrary, bch cells kept their round phenotype on PS-S surfaces during the 7 days of culture. However, on PS-R cells only adhered to the asperities of the surfaces, where is possible to observe not only round morphologies but also spread ones.

The shape of MC3T3-E1 cell line presented some differences among PLLA surfaces. On PLLA-S surfaces, cells exhibited an elongated morphology, but cells preferred to stretch on rough surfaces. The morphology of the bch cells was maintained for 3 days in culture on PLLA-S surfaces; however, at day 7 cells were spread. On PLLA-R surfaces, both types of cells were well spread and connected mainly to some points of asperities at the surfaces for the whole culture time. Bch cells adopted a characteristic spread stellate-like morphology when in contact with PLLA-R surfaces that is, at least partially, modulated by the nano roughness of papillae-like structures of these surfaces. Consistent with a Cassie-Baxter scenario, this behavior should be a consequence of the non complete contact between the medium suspension and the surface, due to the existence of air trapped in the micro and nano-cavities, giving rise to areas uncovered by the proteins [26]. Also for the PS surface, it could be observed that bch cells exhibit a more round morphology on PS-S and that the adhesion of such cells on PS-R takes place in just some points of the cells' body.

The round shape of chondrocytes is an indicator of phenotype retention and is essential for matrix formation. The dedifferentiation of chondrocytes in culture is usually associated with changes in cell morphology, from a rounded to a spread one, as shown preferentially in PLLA-R or PS-R surfaces.

4. Conclusions

Bioinspired superhydrophobic rough surfaces of PS and PLLA with different micro/nanotopographies have been obtained from smooth surfaces using a simple phase-separation based method. PS-R surfaces exhibited randomly distributed structures while PLLA-R surfaces showed individual well define papilla-like structures with rough texture. Regarding the total protein quantification, results showed that similar amounts of BSA protein were adsorbed in the same type of PS or PLLA surfaces, although a protein adsorption was found to be reduced in rough surfaces as compared with the correspondent smooth ones. The viability, adhesion and proliferation studies indicated that rough surfaces reduced the performance of MC3T3-E1 cell line and primary bch cells when compared to smooth surfaces. Nevertheless, cells were metabolically active and able to adhere and survive up to 7 days on PS-R surfaces and even slightly proliferate on PLLA-R. Both types of cells showed similar behavior when in contact with the surfaces, although MC3T3-E1 cell line has demonstrated enhanced performance. Such results strengthen the low influence of both polymer nature and topography of the superhydrophobic surfaces on cell behavior and show the relevant influence of wettability in the main differences found between smooth and rough surfaces.

5. References

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Chapter IV

General Conclusions and Future Research

General Conclusions and Future Research

The potential of surfaces with extreme wettability differing from topography on cell behavior was evaluated in this work. A mouse osteoblastic cell line (MC3T3-E1) and a primary cell culture of bovine articular chondrocytes (bch) were used as model systems.

Inspired by Nature, superhydrophobic rough surfaces of PS (PS-R) and PLLA (PLLA-R) with different micro/nanotopographies have been obtained from smooth surfaces (PS-S and PLLA-S) using a simple and economical phase-separation based methodology. Despite the method used be the same, SEM analysis showed that PS-R exhibited randomly distributed structures at nanometer-scale that were agglomerated in larger micrometer ones while PLLA-R surfaces showed individual well define structures at micrometer level with nanometer rough texture, very similar to the lotus leaf architecture. WCA measurements along with XPS comproved that whilst having the same surface chemistry, the superhydrophobic rough surfaces differ in wettability from the smooth ones as a consequence of the particular surface micro/nanostructures and that extreme wettability is maintain over time. Similar amounts of BSA protein were adsorbed onto the same type of PS or PLLA surfaces, although protein adsorption was found to be reduced in rough surfaces as compared with the correspondent smooth ones.. So we hypothesize that, due to the transitional states between the Cassie - Baxter and Wenzel states, water repellency of PS-R and PLLA-R were not sufficient to avoid protein absorption. Biological assays were performed to test the ability of PS and PLLA surfaces to support cell adhesion and proliferation. MTT, live-dead, DNA quantification and Alamar Blue assays indicated that rough surfaces reduced the performance of both types of cells when compared to smooth ones. Nevertheless, cells were metabolically active and able to adhere and survive up to 7 days on PS-R surfaces and even slightly proliferate on PLLA-R with preferential cell adhesion in specific areas as shown by SEM analysis. Both types of cells showed similar behavior when in contact with the surfaces, although MC3T3-E1 cell line has demonstrated enhanced performance. PLLA-R surfaces present a better surface for cells to adhere compared to PS-R and also a relevant topography with preferential cell adhesion in specific areas as shown by SEM analysis. Such results strengthen the low influence of both polymers nature and topography of the superhydrophobic surfaces on cell behavior and show the relevant influence of wettability in the main differences found between smooth and rough surfaces.

Further studies concerning the influence of transitional states on protein-material interactions are required to a better understanding of cell behavior on these extreme environments as well as the investigation of gene expression on different topographies. Additionally to surfaces properties, differences are found whether the materials are 2D or 3D. It is well established that a 3D environment

mimics more closely the living organism than a 2D environment. Nevertheless, fundamental studies are usually performed in 2D structures. Therefore, there is both practical and fundamental interest in expanding such studies towards 3D structures in order to investigate only cellular response to surface properties.

Superhydrophobic surfaces where cells cannot adhere and proliferate can have several applications, such as in membranes used for periodontal guided tissue regeneration, where cells from distinct origins contact different sides of the membrane. In this case, a cell-adhesive surface could be placed on one side of the membrane facing a bone or cartilage defect to guide osteoblasts or chondrocytes, respectively, to the defect area, whereas superhydrophobic surfaces could be produced on the other side of the membrane that contacts epithelium in order to inhibit epithetial cells migration and proliferation to the host.

Spots can be easily patterned on superhydrophobic surfaces where cells can adhere and proliferate similarly as they are on TCPS, as already reported by our group. Such spots can be coated with proteins or any other modification can be made One application of these surfaces could be the production of high-throughput systems for analyzing of cell-material, cell–cell or cell–protein interactions. Such surfaces can also be used to localize cell proliferation just on some areas or even be used to create patterned co-cultures when researchers are looking for highly organized and vascularised organ tissues, which is still one of the biggest challenges in tissue engineering. Patterned surfaces can be the used as chips for combinatorial analyses of liquid drops or 3D constructs.

One interesting and challenging approach is to build microfluidics based on biodegradable superhydrophobic surfaces and integrate them with biodegradable scaffolds. The microfluidic channels could be the vasculature and be integrated with the existing one. Microfluidic cell chips, with the ability to associate microfluidics with surface patterning, control of the microenvironment properties and further control of receptor-mediated interaction, open the doors to the creation of highly organized tissues.