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Cell-Protein-Material interaction in tissue engineering

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1. Introduction

The initial cellular events that take place at the biomaterials interface mimic to a certain extent the natural adhesive interaction of cells with the extracellular matrix (ECM) (Spie, 2002; Griffin & Naughton, 2002; Grinnell, 1986). In fact, the living cells cannot interact directly with foreign materials, but they readily attach to the adsorbed layer of proteins (upon contact with physiological fluids in vivo or culture medium in vitro) such as fibronectin (FN), vitronectin (VN), fibrinogen (FG), representing the so-called soluble matrix proteins in the biological fluids (Grinnell 1986). Upon longer contact with tissues many other ECM proteins, such as collagens and laminins, will also associate with the surfaces, affecting the cellular interaction. The concentration, distribution, and mobility of the adsorbed protein layer on a surface play a fundamental role in the biofunctionality of a synthetic material and are clue factors to understand the biological response of a substrate (Anselme, 2000). Cells recognize these matrix proteins via integrins - a family of cell surface receptors – that provide trans-membrane links between the ECM and the actin cytoskeleton (Hynes, 2002). When integrins are occupied they cluster and develop focal adhesions; discrete supramolecular complexes that contain structural proteins such as vinculin, talin, α-actinin, and signalling molecules, including FAK, Src and paxilin that actually anchorage the cells to the surface and trigger the subsequent cellular response (Geiger et al., 2001). Abnormalities in the cell-ECM integrin mediated interactions are associated with pathologic situations that include tumour formation (Wehrle-Haller & Imhof, 2003). Besides, integrin mediated adhesion involves not only the receptor-ligand but also post-ligation interactions with multiple binding partners (García, 2005). Thus, the initial cell-material interaction is a complex multi-step process consisting of early events, such as adsorption of proteins, followed by cell adhesion and spreading, and late events, related to cell growth, differentiation, matrix deposition and cell functioning. To measure and to quantify some of these parameters comprise the *classical* approach to characterise the cellular biocompatibility of materials (Williams, 1998).

2. Protein-material interaction

Proteins are considered to be clue factors in mediating the cell-material interaction and their status (the amount, conformation and strength of interaction) on a material surface determine the biocompatibility of the system. Even if the design of antifouling surfaces for the repulsion of proteins is an important field of activity (Chen et al, 2008), we are focused in this chapter on the effect of material properties on adsorption of matrix proteins and the influence on cell response.

Protein adsorption on material surfaces is a process driven both by the intensity of the energetic interactions between the molecular groups of the substrate's surface and of the protein (i.e., hydrogen bonding, electrostatic, van der Waals interactions) and by entropic changes as a consequence of the unfolding of the protein as bound water is released from the surface (García, 2006; Werner et al, 2006). Clearly, the amount of protein adsorbed and its conformation depend on the chemical groups of the substrate, which determine the energetic and entropic interactions with the adsorbed proteins. Protein adsorption on different substrates has been extensively investigated in the literature by different techniques. FN, albumin, laminin, collagen, lysozyme, fibrinogen, hemoglobin, and several other proteins were adsorbed on different substrates—mostly, model surfaces such as mica, glass, and self-assembled monolayers—and were investigated by different techniques that include atomic force microscopy, ellipsometry, quartz crystal microbalance, sodium dodecyl sulfate gel electrophoresis, FT-IR, spectroscopic imaging, electron microscopy, and fluorescence probe techniques (Keselowsky et al., 2003; Michael et al., 2003; Tsapikouni & Missirlis, 2007; Benesch et al., 2007; Weber et al., 2007; Steiner et al., 2007; Lord et al., 2006; Noh & Vogler, 2006; Prime & Whitesides, 2006; Sousa et al., 2007). It is convenient to describe the main adhesion proteins –and its behaviour on material surfaces- for the sake of (auto)completeness of the chapter.

fibronectin

FN is a glycoprotein found in blood, extracellular fluids, and connective tissues and attached to the cell surfaces. Both plasma FN and the cell surface forms are dimers, consisting of two subunits of 220 kDa, linked by a single disulfide bond near the carboxyl termini (Erickson & McDonagh, 1981; Erickson & Carell, 1983). Each subunit contains three types of repeating modules (types I, II, and III) that mediate interactions with other FN molecules, other extracellular matrix (ECM) proteins, and cell-surface receptors (Pankov & Yamada, 2002). The importance of FN as a mediator of cell adhesion to a substrate was recognized earlier (Pearlstein, 1980). Since then, many studies have shown the role of FN in promoting cell adhesion and regulating cell survival and phenotype expression on different surfaces (García & Boettiger, 1999; Toworfe et al., 2004; Baugh & Vogel, 2004; Lan et al., 2005; Grinnell & Feld, 1982; Altankov et al., 2000). It has been stressed that, for a fixed surface chemistry, the initial density of integrin–FN bonds is proportional to the surface density of adsorbed FN (García & Boettiger, 1999); moreover, the nature of the surface chemistry is able to modulate FN conformation (Keselowsky et al., 2003). It has been suggested that FN adsorbs preferentially on hydrophobic surfaces (Toworfe et al., 2004) and that it undergoes greater extension of its dimer arms on hydrophilic glass (Baugh & Vogel, 2004) in a conformation that favors the binding of antibodies (Grinnell & Feld, 1982) and strength the cell–material interaction (Kowalczynska et al., 2006). Even the micro/nano surface roughness has been shown to influence FN adsorption (Khang et al., 2007; Costa Martínez et al., 2008). The integrin-FN interaction, governed mainly by the $\alpha_5\beta_1$ dimer, also

leads to the formation of extracellular matrix fibrils from the newly secreted FN (Mao & Schwarzbauer, 2005) and even arrangement of those protein molecules adsorbed on the substratum (Altankov & Groth, 1996; Altankov et al., 1996). The thickness of FN matrix fibrils ranges from 10 to 1000 nm in diameter and consists of a few to hundreds of FN molecules across (Singer, 1979). FN binding to integrins induces reorganization of the actin cytoskeleton and activates intracellular signaling complexes. Cell contractility facilitates FN conformational changes, and it allows for the unfolding of the native globular FN structure, thus exposing cryptic domains that were not available in the compact form of soluble FN. Finally, fibrils are formed through FN-FN interactions, usually through binding of I1-5 to either III_{1-2} or III_{12-14} domains (Geiger et al, 2001). Cell-mediated FN reorganization, when adsorbed on a synthetic surface, seems to be also an important factor in determining the biocompatibility of a material, because poor cell adhesion and spreading has been found in cases when integrin-mediated rearrangement of FN did not occur at the material interface (Altankov & Groth, 1994; Altankov & Groth, 1996).

It has been shown that the existence of mechanical tension is necessary for efficient integrin-mediated FN fibrillogenesis (Erickson, 2002; Smith et al., 2007). Although it is generally agreed that FN fibrillogenesis is cell-dependent process, fibrillar networks of FN have been generated also in the absence of cells by means of interactions with the underlying substrate that involves mechanical events at the molecular scale. FN fibrillogenesis upon contact with a lipid monolayer was explained through mechanical tension caused by domain separation in the lipid monolayer that pulls the protein into an extended conformation (Baneyx & Vogel, 1999). The assembly of FN into fibers was obtained also by applying forces to FN molecules via poly(dimethylsiloxane) (PDMS) micropillars at different stages of fibrillogenesis (Ulmer et al, 2008). We have recently shown that FN fibrillogenesis can take place as a consequence of the sole interaction between the protein molecules and a material surface with the appropriate surface chemistry; in concrete, a spontaneous formation of biologically active FN network was found in vitro after its adsorption on poly(ethylacrylate) (PEA) (Gugutkov et al., 2009; Rico et al., 2009).

fibrinogen

FG is a large, complex, fibrous glycoprotein normally present in human blood plasma essential for many biological functions which include haemostasis, wound healing, inflammation, and angiogenesis (Weisel, 2005). It is made up of three pairs of polypeptide chains, designated as Aα, Bβ, and γ, with molecular masses of 66, 52, and 46 kDa, respectively, which are held together by 29 disulfide bonds (Weisel, 2005). These six polypeptides are organized into independently folded units: a central E-domain, which includes the N-terminus of all six polypeptide chains, and two terminal D-domains, which include the Bß and γ chains. The carboxy-terminal of the Aα chain, the aC domain, departs from the D fragment and either associates to the E-domain to constitute a single globular domain close to it or, on the contrary, they form appendages with a certain degree of mobility. In its native form the aC association to the central domain is more common; however, there is equilibrium between these two situations (Veklich, 1993). The cleavage of the small A and B sequences from the Aα and Bβ chains by thrombin in the E-domain yields fibrin, which is able to associate and polymerize. The length of an individual FG molecule is 45–50 nm (Weisel et al., 1985; Gorman et al., 1985).

FG–surface interactions have been investigated on many substrates with different experimental techniques (Brash & Horbett, 1995). Atomic force microscopy (AFM), which is

a technique able to provide direct observation of protein conformation on different substrates has been extensively used in recent years, mainly on model surfaces, such as silica, mica, titanium graphite, and self-assembled monolayers (SAMs), flat enough so that the height magnitude is able to reveal the trinodular structure of single-adsorbed FG molecules (Toscano & Santore, 2006; Marchin & Berrie, 2003; Agnihotri & Siedlecki, 2004; Cacciafesta et al., 2000; Tunc et al., 2005; Gettens et al., 2005; Gettens & Gilbert, 2007; Ta & McDermott, 2000; Ishizaki et al, 2007; Ortega-Vinuesa et al., 1998; Mitsakakis et al., 2007; Sit & Marchant, 1999).

The effect of surface wettability, as one of the most important parameters that affects the biological response to a material, on FG adsorption has lead to different, nonconsistent conclusions. Even if there is general agreement in the decrease of FG adsorption with the increase of wettability of the substrate, (Slack & Horbett, 1992; Rodrigues et al., 2006) it is not the case concerning FG conformation. Marchin et al. observed dramatic differences in the conformation of FG adsorbed on hydrophilic mica and hydrophobic graphite: globular conformations were observed on mica, whereas on graphite the trinodular structure of the extended molecule was clearly observed (Marchin & Berrie, 2003). Sit et al. suggested that the spreading of FG increases with the hydrophobicity of the surface (Sit & Marchant, 1999). In addition, Wertz and Santore have shown through total internal reflection fluorescence that the footprint of a FG molecule is larger when adsorbed on a hydrophobic surface (graphite) than on a hydrophilic one (mica) (Wertz & Santore, 2001; Wertz & Santore, 2002). However, other authors have found the trinodular conformation both on graphite and mica (Agnihotri & Siedlecki, 2004). The adhesion force between FG and the substrate has also been investigated by AFM and it has been found to depend on the surface wettability. By functionalizing AFM tips with the protein, Kidoaki et al. found that the strength of adhesion to a hydrophobic SAM was larger than to hydrophilic ones (Kidoaki & Matsuda, 1999). Xu et al. measured adhesion forces to a series of surfaces over a broad wettability range through glow-discharge plasma modification, by using protein modified AFM tips (Xu & Siedlecki); showing a marked transition between protein adherent materials and protein nonadherent materials over the range of water contact angles of 60–65 ^{o}.

laminin

Laminins are trimeric molecules of α, β, and γ chains with molecular masses of 140–400 kDa. Several laminin isomorphs are known, with a large number of genetically distinct chains (α1 to α5, β1 to β3, and γ1 to γ3) (Burgeson et al., 2004). The laminins are important glycoprotein components of basement membranes, where they provide interaction sites for many other constituents, including cell surface receptors (Mercurio, 1995; Beck et al., 1990; Sasaki et al., 2004). Laminin plays an important role in neural cell migration, differentiation, and neurite growth (Kleiman et al., 1987; Heiduschka et al., 2001; Luckenbill-Edds, 1997; He & Bellamkonda, 2005) and it has been used as a coating for improving nerve cell adhesion and growth on different substrates (Rogers et al., 1983; Liesi et al., 1984).

Among the different material properties that influence protein adsorption (and, consequently, cell adhesion and functionality) the hydrophilicity of the system is an important one. The system based on the copolymerisation of ethyl acrylate and hydroxyethyl acrylate provides controlled hydrophilicity maintaining the same chemistry. It consists of a vinyl backbone chain with the side groups -COOCH2CH3 and -COOCH2CH2OH, respectively. Their copolymerization gives rise to a substrate in which the surface density of -OH groups can be varied without modifying any other chemical

functionality of the system. The concentration of -OH groups determines both the surface energy and the hydrophilicity of the substrate (Table 1). The interaction of the protein domains with the chemical functionalities of the substrate and with water determines the molecule's adsorbed conformation.

x_{OH}	EWC	WCA (º)
0 (pure PEA)	1.7 ±0.4	89±1
0.3	7.6±0.9	80±2
0.5	18.2±1.7	67±1
0.7	40.6±0.4	55±1
1 (pure PHEA)	134±5	45±2

Table 1. Equilibrium water content (EWC) and water contact angle (WCA) for the different substrates.

The amount of protein adsorbed on the different surfaces was quantified by image analysis of the Western blot bands obtained by analysing the supernatant after adsorption on the material surface (Rodríguez Hernandez et al., 2009; Rico et al., 2009). Two different curves have been observed (included in Figure 1) corresponding to FN and FG adsorption from a solution of concentration 20 μg/mL. The adsorbed FN depends non-monotonically on the -OH density of the substrate, and FN surface density shows a minimum at approximately x_{OH}=0.5. Both higher and lower concentrations of hydroxyl groups in the substrate result in higher amounts of the adsorbed protein. For all concentrations of the original solution, the highest protein adsorption occurs on the most hydrophilic substrate. The situation is completely different when FG is adsorbed on the same family of substrates. The amount of adsorbed protein diminishes monotonically as the OH density increases. The difference of adsorbed FG between pure PEA (OH_0) and OH_{30} is approx. 0.9 μg/cm², a huge fall for such a small OH increment, whereas these differences tend to diminish as substrata become more hydrophilic (<0.1 μg/cm).

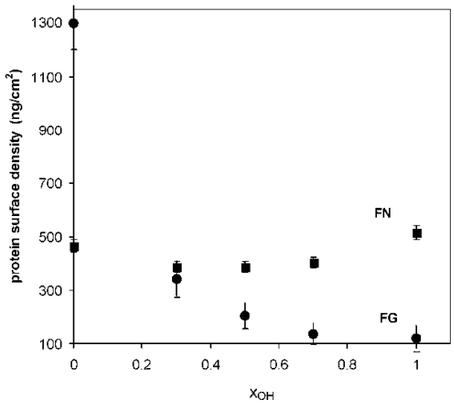


Fig. 1. Surface density of fibronectin (FN, squares) and fibrinogen (FG, circles) adsorbed on substrates with controlled OH density.

The conformation of the protein adsorbed on the substrate can be directly observed by Atomic Force Microscopy. AFM studies of the adsorption of protein on non-model surfaces in cases where the roughness of the substrate is of a size of the order of the protein height

cannot be conclusive if monitored with the height signal since the protein features are usually blurred; one would have to polish the neat material to get a surface flat enough (Veklich et al., 1993) Instead of the height, the phase signal of AFM, which is a magnitude sensitive to the different viscoelastic behaviors, (Tamayo & García, 1997; Cleveland et al., 1998; Tamayo & García, 1998; García et al., 1998) can be used to distinctly reveal protein conformation under conditions of usual, non-model polymer surfaces (Holland & Marchant, 2000; Rodríguez-Hernández, 2007). Figure 2 shows protein conformation and distribution after adsorption on the different substrates from a 20 μg/mL protein solution, which is the concentration typically employed when coating a substrate with the protein for cell culture purposes (Keselowsky et al., 2003; Erickson & Carell, 1983; Altankov et al., 2000; Altankov & Groth, 1994; Altankov & Groth, 1996). The more hydrophobic surfaces induce the formation of protein networks, whose density decreases as the fraction of -OH groups increases. FN network is well developed on the PEA (-OH_0) and -OH_{10}. Protein aggregates with elongated shape are still formed on the -OH_{30} surface, but only weakly connected protein filaments are identified. A higher amount of hydroxyl groups (from x_{OH}=0.5 on) prevents the formation of a protein network on the materials surface, and only disperse (micro) aggregates of the protein are observed on the -OH_{50}, -OH_{70}, and PHEA (-OH_{100}) substrates. The surface density of these globular FN aggregates seems to increase with the fraction of hydroxyl groups from x_{OH}=0.5 to 1.

FG distribution after adsorption on the different substrata at different magnifications reveals rather than single FG molecules, AFM images show protein patterns with different topologies. Nevertheless, some differences between the conformations of FG on the different substrates are worth mentioning. The formation of a FG network takes place on pure PEA (OH_0), but the co-continuity of the protein network is lost when small amounts of OH are introduced in the system (OH_{10} and OH_{20}). However, from this hydroxy content on, FG–FG interactions are somehow enhanced and variable fibril network topologies show up again.

FN conformation on the substrate is not related to the total amount of protein adsorbed on it. This nonmonotonic dependence of adsorbed protein on -OH fraction can be understood if protein adsorption on a substrate's surface is analyzed in terms of the number of available sites on the surface; it is clear that not only the energetic interactions between the substrate and the protein play a role in the adsorption process, but also the conformation of the protein—the configurational entropy—must trigger the amount of molecules directly adsorbed on the substrate: globular conformations of FN on the more hydrophilic substrates must lead to a higher amount of the protein adsorbed. The other way around, minimum adsorption at x_{OH}=0.5 must be a consequence of two opposite processes: energetic and entropic interactions that lead to less efficient FN packing for this substrate composition. In a similar way, FG conformation on the substrate is not directly related to the total amount of protein adsorbed on it. It has been found that the footprint of a FG molecule is larger when adsorbed on a hydrophobic surface than on a hydrophilic one (Wertz & Santore, 2001; Wertz & Santore, 2002), and higher amounts of adsorbed proteins on the most hydrophobic surface result in an ordered FG–FG adsorption, which leads to the formation of a network on the substrate. As hydrophilicity increases, the amount of FG directly in contact with the substrate decreases, as well as the footprint of the molecule, which results in the formation of isolated FG aggregates.

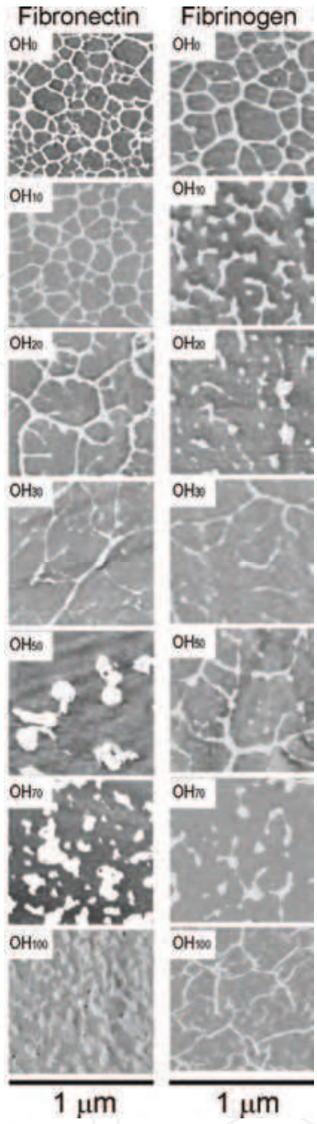


Fig. 2. AFM phase images of fibronectin and fibrinogen adsorbed on substrates with controlled hydroxyl density. Both proteins tend to form networks on the most hydrophobic surface that disaggregates as the hydrophilicity of the substrate increases. (these results are extracted –with permission- from refs Rico et al., 2009 and Rodríguez Hernández et al., 2009).

FN and FG stands for a situation in which similar distribution and conformation of the protein is observed on the substrates (Figure 2) but very different tends in the amount of adsorbed protein are found (Figure 1). When laminin is adsorbed on this set of substrates, protein molecules show globular like morphology on the hydrophilic PHEA and gradually extend as the amount of –OH groups on the surface diminishes, up to a point in which the protein conformation tends again to a more compact, less extended conformation. Additionally, the formation of a laminin network takes place on the 50:50 copolymer in which the N-terminal domain of all three chains of the protein are linked. This polymerized

supramolecular aggregation is the typical form of laminin in the basement membrane and its formation depends on time, temperature, and concentration (Luckenbill-Edds, 1997). The formation of protein networks on our surfaces must be conditioned not only by the different conformation of the molecule on the substrate, but also by the surface density of adsorbed protein.

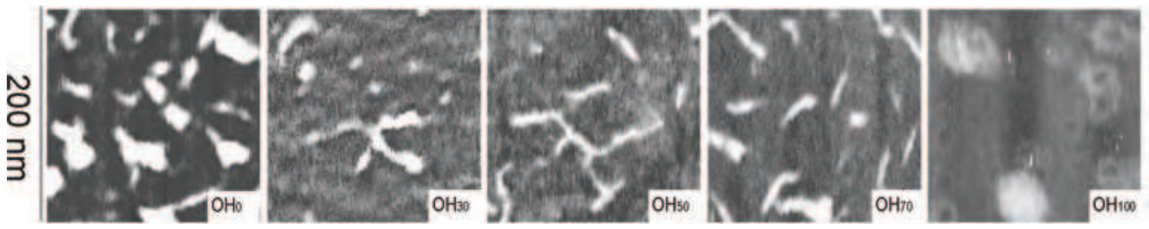


Fig. 3. AFM phase image of laminin molecues adsorbed on substrates with controlled hydroxyl density. Globular protein molecules are observed both on the hydrophilic and hydrophobic polymers while the protein tends to extend their arms for intermediate hydroxyl densities (extracted –with permission- from ref. Rodríguez Hernández et al., 2007).

3. Development of provisional extracellular matrix on biomaterials interface

Cell-matrix interaction in vivo is a complex bi-directional and dynamic process. Cells in the tissues are constantly accepting information on their environment from cues in the ECM (Altankov & Groth, 1994) and, at the same time, cells are producing and frequently remodelling their matrix (Grinnel, 1986; Hynes, 2002; Avnur & Geiger, 1981). Therefore, it is not surprising that many cells cannot adapt and poorly survive in vitro and, conversely, when a foreign material is implanted in the body, the adjacent tissue cells do not interact properly because of lack of their ECM.

A large and growing body of evidence shows that the cells in vivo need to accept distinct physico-chemical signals from the surrounding ECM and because a tight connection between the cytoskeleton and ECM the cells also respond to these properties. Mechanical properties are also important. For instance, as the stiffness of the surrounding ECM is in the same order of magnitude as cells, they are able to reorganize this matrix (Rhees & Grinnel, 2007; Kolakna et al., 2007). However, on stiffer materials cells may fail to do so, and it is an obstacle for their biocompatibility. A line of previous investigations has shown that fibroblasts and endothelial cells tend to rearrange adsorbed matrix proteins, such as FN and FG (Altankov et al., 1997; Tzoneva et al., 2002), as well as collagen (Maneva-Radicheva et al., 2008) in a fibril-like pattern. Using model surfaces –mostly self-assembled monolayers (SAMs) - it has been shown that this cellular activity is abundantly dependent on the surface properties of materials, such as wettability (Altankov et al., 1996), surface chemistry and charge (Pompe et al., 2005; Altankov et al., 2000). It has been shown that for this kind of substrates (SAMs) fibroblast on hydrophilic surfaces may reorganise FN in ECM-like structures whereas on hydrophobic surfaces almost no rearrangement of FN occurs (Fig. 4).

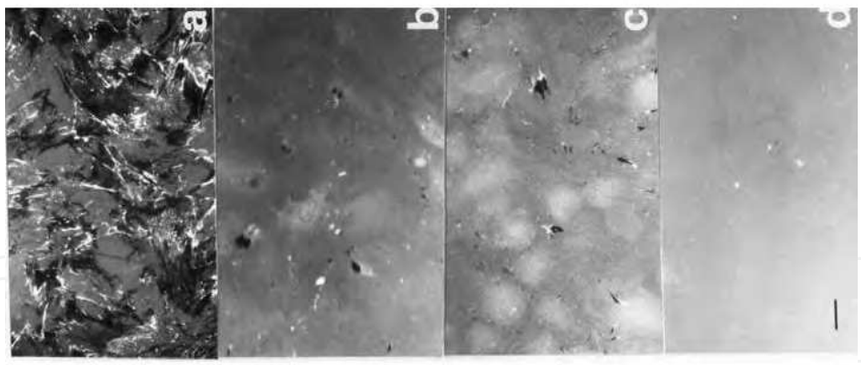


Fig. 4. Reorganization of adsorbed FITC-fibronectin on different wettable model substrates: a) glass (WCA=23^0); b) aminopropyl silane (WCA=67^0); c) octadecyl silane (WCA=87^0); d) silicon (WCA=107^0).

These experiments suggests that loosely adsorbed FN on hydrophilic surfaces provide a better substrate for cell growth presumably due to the fact that cells need to modify adsorbed FN for their normal function. Therefore, they remove and organise FN from the substrate into specific fibrillar structures, similar to FN matrix fibrils. Early events of integrin receptor β_1 functioning is also different on hydrophilic and hydrophobic materials. It is known that $\alpha_5\beta_1$ binds FN to the cell surface and induced conformational changes required for FN polymerisation (Smith et al., 2007). It has been shown that clusters of β_1 integrin might organise in a specific linear pattern on the dorsal cell surface of adhering fibroblasts on hydrophilic glass, presumably matching the initial positional organisation of FN matrix. However, on hydrophobic glass, even if the cells formed normal focal adhesions –similar to those on FN-coated glass- they did not develop a linear organisation of the FN receptor (Altankov et al., 1997). This evidence raises the possibility that tissue compatibility of such materials may be connected with the allowance of cells to remodel surface associated proteins presumably as an attempt to form their own matrix, e.g. materials that bind proteins loosely will support the organization of a provisional ECM. This view however does not consider the real molecular architecture of the adsorbed proteins layer and also causes limitations to the materials selection.

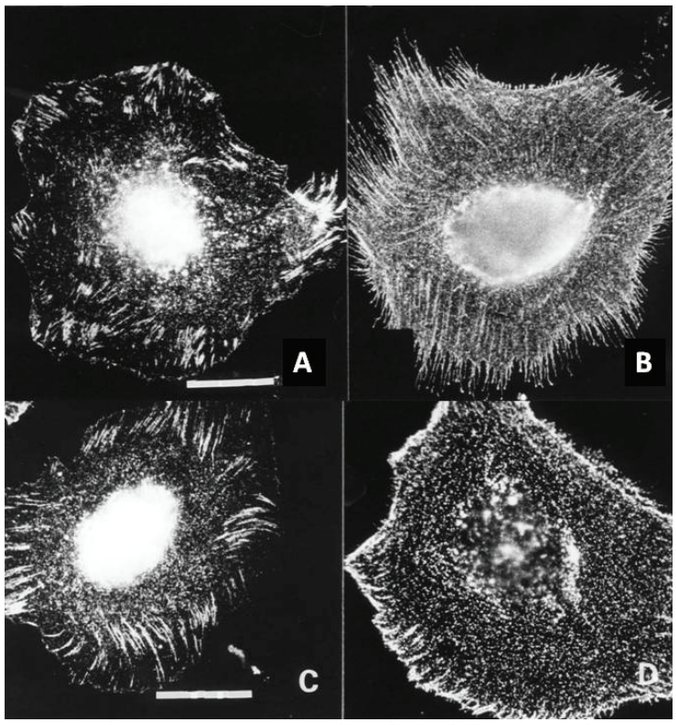


Fig. 5. Organization of β_1 integrins on the dorsal (B,D) and ventral (A,C) cell surface of fibroblasts adhering on hydrophilic glass (A,B) and hydrophobic ODS (C,D) surfaces. Note: Focal adhesions are well developed on both hydrophilic and hydrophobic substrata (A and C) while linear arrangement of ß1 integrins are visible on hydrophilic glass only (B) and absent on hydrophobic ODS (D).

The arrangement of natural FN matrix is also dependent on the ability of cells to reorganize the adsorbed FN layer on the material substrate. When hydrophilic and hydrophobic glass are culture with human fibroblast for longer time (72 h), significant amounts of FN are deposited by cells on the hydrophilic substrate, organised in fibrils and clusters, oriented in the direction of the cell polarization. However, on the hydrophobic glass, less FN fibril formation was observed although cell spreading was almost in the same extent as on the hydrophilic glass (Altankov & Groth, 1996). Figure 6 shows organization of extracellular fibronectin matrix on different wettable model substrates of increasing hydrophobicity as measured by the water contact angle (WCA): glass (WCA=23^o) aminopropyl silane (WCA=67^o), octadecylsilane (WCA=87^o), and silicon (WCA=107^o). A clear trend for less FN matrix formation with increasing the hydrophobicity of substratum is demonstrated on Figure 6 below.

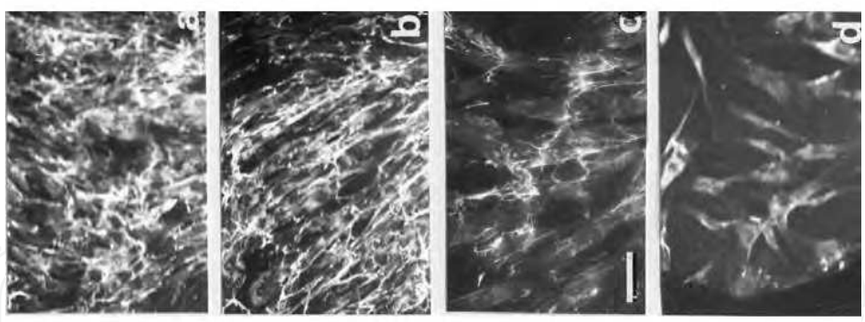


Fig. 6. Organization of extracellular fibronectin matrix on different wettable model substrates: a) glass (WCA=23^0); b) aminopropyl silane (WCA=67^0); c) octadecyl silane (WCA=87^0); d) silicon (WCA=107^0).

Recent investigations however, have shown that even if cells need the adequate environment to synthesize their own matrix at the cell material interface, it is not mandatory that this happens only on hydrophilic substrata. The system based on the copolymerisation of ethyl acrylate and hydroxyethyl acrylate, that we have already commented concerning protein adsorption in the previous section, is an example of how things can happen in a different way. There, it was found that the cells are able to synthesize and deposit FN matrix fibrils on some of the material surfaces. The formation of FN fibrils, the so-called fibrillogenesis, is a process either mediated by integrins or, as it is accounted for previously, induced by the substrate. However, FN fibrils could not be found on the more hydrophilic samples (-OH_{100} and -OH_{70}) while on the sample with intermediate composition, -OH_{50} the fibroblasts deposit only small fibrils, located mostly beneath the cells. As the hydroxyl fraction decreases (and the surface becomes more hydrophobic) the FN deposition increases, which moreover is organized into a typical matrix-like structure similar to those on the hydrophilic glass (Altankov et al. 1996). Nevertheless, FN reorganisation does not happen as expected anymore: no reorganization of FN takes place whatever the hydroxyl fraction of groups in the sample, that is, FN reorganization does not depend on the hydrophilicity for this family of substrates. It is noteworthy, however, that the values for the wettability of the PHEA samples (WCA 45^o) correspond to values that are optimal for the cellular interaction in other systems. Conversely, surfaces with about 90^o WCA, characteristic for pure PEA, and where the best cellular interaction was found, usually abrogate cellular interaction (Garcia, 2006; Grinnell & Feld, 1982; Arima & Iwata, 2007). Collectively this suggests that even unable to organize the preadsorbed FN on the substrate, the fibroblasts respond on the FN network previously formed during protein adsorption on the substrate (Figure 2), presumably because the conformation of the protein provides the adequate signals which stimulate their normal matrix-forming activity.

These results suggest that the distinction between hydrophilic and hydrophobic features of a substrate is insufficient to explain the general trends underlying the cell-material interaction, and more factors must be taken into account. For instance, the conclusion that the ability of fibroblasts to secrete ECM proteins is greatly reduced on hydrophobic substrates (Altankov et al., 1996) even if cell adhesion takes place clearly differs from the results in this recent work. Rather, fibroblast functional behaviour on a synthetic substrate depends in a subtle way on the particular substrate chemistry that triggers the process of

protein adsorption. Both protein conformation on the substrate and the intensity of the protein-material interaction play a fundamental role on cell behavior: the adequate protein conformation on the substrate—leading to a substrate induced FN fibrillogenenis—results in excellent cell adhesion and matrix formation (for low -OH contents), even if preadsorbed FN cannot be removed by cells. Alternatively, if protein conformation is good enough so as to support initial cell adhesion, cells will be able to remove the initial FN layer and secrete their own extracellular matrix (as it happens in the control glass). Higher -OH fractions in the substrate lead to inadequate protein conformation on the substrate, which does not support good cell adhesion and consequently leads to diminished functionality.

Despite our knowledge on ECM organization, relatively little is known about the fate of these already arranged matrix proteins. Recent data indicate that polymerized forms of the matrix proteins have properties distinct from protomeric, non-polymerized ones. For example, the state of collagen polymerization has been shown to alter its growth regulatory properties (Schofield, 1978). Emerging evidence also indicates that the ECM form of FN (matrix fibrils) is functionally distinct from the soluble FN (Hynes, 1990; Wagenaar-Mller et al., 2007), having implications on the long-term behaviour of this protein. Thus, depending on the allowance of materials surface to support the development of fibrillar matrix, the biological properties of a material may be altered. Much is known about the interactions between different ECM proteins, but surprisingly less is our knowledge about the ECM composition, organization, and stability at the biomaterials interface. Can it be regulated? A distinct hierarchy of matrix remodelling is already evident from the in vitro studies (Velling et al., 2002). Integrin-mediated assembly of FN into fibrils is well documented (Mosher et al., 1992; Christopher et al., 1997; Sottile & Hocking, 2002), but the fact that FN may tether other matrix proteins to the cell surface, and therefore is required for their organization, is new in the field (Maneva-Radicheva et al., 2008; Dzamba, 2008). It provides also new insights on the mechanisms for other fibrillar matrix proteins assembly, such as collagen and trombospondin (Velling et al., 2002; Sottile & Hocking, 2002). It has been also shown that FN fibrillogenesis is also required for rearrangement of substratum associated fibrinogen (Tzoneva et al., 2002). That is to say, it has been observed that endothelial cells were able to reorganise both adsorbed (on the substrate) and soluble (added to solution after cell adhesion to the substrate) FG in specific fibrillar structures only on hydrophilic glass, while this phenomenon is inhibited on hydrophobic substrata. Thus, endothelial cells mediated fibrinogen fibrillogenesis is altered on hydrophobic substrata, in the same way that FN fibrillogenesis is. Additionally, it was reported that endothelial cell spreading on FG was affected by cell synthesized FN (Dejana et al, 1990). This finding was supported with the observation of a different pattern of integrin organisation during the interaction with substratum-bound soluble FNG (Tzoneva et al., 2002).

It is known that the adhesion on endothelial cells to adsorbed FG is mediated by $\alpha_v\beta_3$ integrin (Cheresh et al, 1989). Indeed, the β_3 integrin clusterizes in structures resembling focal adhesion contacts when endothelial cells adhere to FG-coated substrata. Conversely, on the dorsal cell surface FG fibrils were not co-localized with β_3 integrin, representing a punctuate distribution, in contrast to β_1 integrin, which showed a well-pronounced linear pattern of organisation. The absence of β_1 integrin from the focal adhesion plaques is an indication that the FN receptor does not participate in endothelial cell adhesion to FG. Integrin β_1, however, has clearly shown to be involved in FN fibril formation (Dejana et al, 1990). The co-localisation of FN and FG fibrils was found on the dorsal cell surface of

endothelial cells (Tzoneva et al., 2002). Also, the incorporation of FN fibrils into matrix fibrils starts from the distinct place at the cell periphery, near to the focal adhesions, suggesting the leading role of FN in this process. The existence of this joint fibrillogenesis, i.e. coassembly of FG and FN, found for endothelial cells have been reported also for epithelial cells (Guadiz et al., 1989) and fibroblasts (Pereira et al., 2002). Even collagen IV, which is a non-fibrillar protein could undergo fibril-like linear rearrangement along with FN as we recently show (Maneva-Radicheva et al., 2008), a fact that needs to be further elucidated.

In the last decade many studies have been focused on the modifications of biomaterials surfaces with synthetic or natural ECM fragments to provoke an adequate recruitment of cells for in vivo tissue regeneration (Massia & Hubel, 1990; Werner et al., 2006; Ma et al., 2007). However, when a foreign material is implanted in the body, it hampers the local organization of ECM and alters the biocompatibility of the implant, a process further complicated from the non-specific inflammatory response. A possible reason for the lack of adequate tissue response is attributed by many authors to the different dimensionality of the implant. Nowadays tissue engineering strives to replace the damaged tissues with natural or synthetic scaffolds designed to mimic the 3D organization and mechanical properties of ECM. Nevertheless, many of the bioengineered devices such as stents, prosthesis, membranes, metal implants, etc, simply can not avoid the 2D contact with cells. Therefore we argue, if this 3D architecture is always obligatory. When epithelial or endothelial cells reside on the basement membrane, they meet a rather flat environment, which they assess more as topography and as source of positional signals that guide their functionality. The basement membrane is actually a two dimensional structure, common to many types of tissues, providing underlinement for baso lateral cell attachment and functional polarization (Campbell & Teranova, 1988). Therefore, a future prospective for the development of biohybrid organs may require the construction of modules based on 2D permeable membranes colonized with cells that will mimic to a maximal extent the functional arrangement of basal membrane. On the other hand however, dimensionality of cell-material interaction raises a number of obstacles that need to be solved. For example, the blood contacting devices such as small diameter vascular grafts, stents, synthetic heart valves and assist systems, have suffered from a common problem, the lack of significant endothelial cells in-growth and function. While endothelial cells procurement technologies for seeding implants have improved, adhering endothelial cells usually dedifferentiate and act in a counterproductive manner, very often accelerating device failure (Ludwig et al., 2007). Thus, it seems that endothelial cells do not meet an adequate environment, as on the natural basal membrane, but what they are actually missing remains unclear.

4. Remodelling of the extracellular matrix

Except organization, the ECM undergoes proteolytic degradation, which is a mechanism for the removal of the excess ECM usually approximated with remodelling. Remodelling of ECM occurs in various physiological and pathological processes, such as normal development, wound healing and angiogenesis, but also in atherosclerosis, fibrosis, ischemic injury and cancer. Thus, matrix remodelling is a subject of an extensive biomedical research, but how it relates to the biocompatibility of materials remains unclear. Upon

implantation, foreign materials often trigger an uncontrolled deposition of fibrous matrix that, difficult to be predicted, hampers the biocompatibility of the implant.

In fact, ECM remodelling is a dynamic process which consists of two opposite events: assembly and degradation. These processes are mostly active during development and regeneration of tissues but, when miss-regulated, can contribute to diseases. Perturbing matrix remodelling, for example by preventing the turnover of collagen type I or altering the level of matrix-degrading proteases, has been shown to result in fibrosis, arthritis, reduced angiogenesis, and developmental abnormalities (Schofield, 1978; Wagenaar-Mller et al., 2007; Heyman et al., 2006; Holmbeck et al., 1999). The invasive behaviour of cancer cells is also due to up-regulation of matrix remodelling (Reisenawer et al., 2007; Carino et al., 2005). ECM organization in vivo is regulated by the 3D environment and the cellular tension that is transmitted through integrins (Hynes, 2002). It is difficult, however, to create such an environment on the biomaterials surface. Thus, identifying factors that control matrix deposition on the materials interface is an essential step for understanding the mechanisms involved in the pathological host response.

The proteolytic remodelling of matrix proteins such as FN, VN and FG, as well as, collagens and laminins at the biomaterials interface has only recently received attention, although the pericellular proteolysis is extensively studied in various pathological conditions. The proteolytic cleavage of ECM components represents a main mechanism for ECM degradation and removal (Koblinski et al., 2000; Mohamed & Sloane 2006). Several families of proteases operate at the ECM level, including matrix metalloproteinases (MMPs), cysteine proteases and serine proteases. Proteolysis may also regulate the ECM assembly, editing the excess ECM components. During enzymatic remodelling of ECM structures, bioactive fragments and growth factors can be released that will affect cell growth, morphogenesis, tissue repair, and also various pathological processes. The major enzymes that degrade ECM and cell surface associated proteins are MMPs, a family of secreted and membrane bound proteinases. The role of MMPs in both development and diseases has been recently extensively studied and reviewed because is tightly linked with the mechanisms for tumour invasion and metastasis (Page-McCaw et al., 2007). MMPs are family (20 members) of zinc dependent endopeptidases, which together with adamalysin-related membrane proteinases that contain disintegrin and metalloproteinase domains (ADAMs or MDCs), such as thrombin, tissue plasminogen activator (tPA), urokinase (uPA) and plasmin are involved in the degradation of ECM proteins. MMPs are either secreted or anchored to the cell membrane by a transmembrane domain or by their ability to bind directly uPAR and integrin $\alpha_v\beta_3$ (Buck et al., 1982).

In fact, the basement membrane is the most altered structure during remodelling and it depends particularly on the degradation of type IV collagen that is a primary structural component of basement membrane, integrating laminin and nidogen into a microscopically visible two-dimensional network. Assembly of the basement membrane, a strongly specialized form of ECM that underlies epithelial and endothelial cells, but is also in contact with many other cell types, is initiated by laminin. It self-assembles into heterotrimers that bind to the cell surface integrin receptors. The network-forming collagen type IV is the next main component of the basement membrane, however the mechanism of its assembly remains unclear. It is widely accepted that collagen IV also self-assembles into a meshwork by antiparallel interactions and extensive disulfide bounding of four molecules to form 7S domains. The lateral interactions between C-terminal globular domains create an irregular

two-dimensional meshwork that is actually the main constructive element of the basement membrane. Observations have been made however, that collagen IV may be linearly organized during early basement membrane assembly (Fleischmajer et al., 1998) suggesting another type of cell-dependent arrangement. While the molecular mechanisms which endow the spatial distribution and organization of collagen IV in basement membrane are still debatable, our recent results (Maneva-Radicheva et al., 2008) surprisingly suggest that material surface-associated collagen IV also undergo cell dependent rearrangement through reversible association with FN fibrils. It is obvious that ECM remodelling is poorly understood at the biomaterials level.

5. Substrate engineering

Different model substrates have been prepared in the recent years aiming to learn more about cell-material interaction, especially in what cell adhesion is concerned. These works are mainly focused on the effect of material properties on the biological performance of the substrate and, only a few of them, investigate this effect by addressing first protein adsorption and conformation on the material surface and then by correlating this phenomenon with cell behavior. Despite the belief that the issue of cell-protein-material interaction is critical to the engineering of new biomaterials, clear links between the material, the adsorbed protein layer and their influence on the cell remain far from being understood; in particular the behaviour of surface associated matrix proteins is generally missing. Even if the cell material interaction is not a direct one, but it is mediated by ECM proteins previously adsorbed on the substrate's surface, it is said that cells response to three different kinds of surface parameters: chemical, topographical and mechanical. The influence of surface chemistry on protein adsorption and cell adhesion has been addressed mostly on surfaces with well controlled chemistry, in order to investigate the role of concrete chemical groups in self-assembly monolayers SAM (e.g. OH, COOH, NH_2, CH_3 and their mixtures) (Keselowsky et al., 2003; Keselowsky et al., 2004; Faucheux et al.; 2004; Lee et al., 2006; Barrias et al., 2009). Studies on fibronectin (FN) and vitronectin (VN) adsorption under non-competitive and competitive (multi-protein solutions, including FBS) conditions suggested the major role of VN in cell-materials interaction. Different chemistries in SAM substrates were shown to modulate the structure and composition of focal adhesion complexes and fibrillar adhesions, closely linked to the capacity of cells to polymerise FN into fibrils (Keselowsky et al., 2004; Faucheux et al.; 2004). Other attempts to correlate surface chemistry with protein adsorption and cell adhesion were done by preparing substrates based on copolymers, that allow to modulate the material properties (wettability, hydrophilicity, substratum charge, topography, etc.) within the same chemical family (Allen et al., 2006). The response of cells to different material chemistries is a complex process and even minute changes in composition of the substrate produce amplified differences in cell responses (Bae et al., 2006). Plasma surface modifications have allowed to introduce different chemical groups in order to improve cell adhesion and study the effect on the ECM proteins conformation as well as focal adhesion formation (Silva et al., 2008; van Kooten et al., 2004; Pompe et al., 2007) and it is a versatile technique that allows one to produce chemical gradients in the same substrate so that the response to a large range of different chemistries on a single sample can be investigated as well as the effect not only of surface properties but their variations (Zelzer et al., 2008). It has been argued that sequence of

events –contact, attachment, spreading and proliferation- is similar among different surfaces but with very different dynamics, leading in the case of poorly compatible surfaces to long induction periods in which cells are in a life-or-death struggle to improve the pericellular environment by excretion of matrix proteins (Liu et al., 2007).

Surface topography is also a key parameter that is able to modify cell response independently of the chemical composition of the substrate. Even though sometimes topography is only a manifestation of material chemistry, it can be modulated in an independent way. The effect of topography on cell adhesion has been widely studied. Different microtopograhies can promote changes in cell adhesion pattern, cell orientation and cell shape on the substrate (Anselme et al., 2000). Cells cultured on smooth surfaces tend to generate a more organized ECM, with a 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 (Brunette, 1986). Micro and nano patterned surfaces have been prepared for a better understanding of the cell response to topographic features, mainly in what cell adhesion is concerned. Anisotropic surfaces prepared by lithographic and microfabrication techniques can induce cell reorientation following microgrooves, the so-called contact guidance phenomenon (Ohara & Buck, 1979; Lim, 2007); and the scale of anisotropic topography plays an important role in deciding cell alignment (Affrossman et al., 2000). Different techniques have been used to produce controlled isotropic topographies at different scales which include photolithography, electron beam lithography, colloidal lithography, polymer solvent demixing techniques during a high speed spin-casting process (Denis et al., 2002; View et al., 2000; Kriparamanan et al., 2006; Dalby et al., 2004). Polymer solvent demixing techniques make use of phase separation during a high speed spin-casting process. This technique allows obtaining nanotopographic motifs in a broad range (from 9 to 100 nm); however the effect of nanotopography on cell response remains an open question. It seems that the interval 10-30 nm gives rise to better adhesion and higher stimulation of intracellular signaling than going up to 100 nm (Lim et al., 2008; Dalby et al., 2008; Zinger et al., 2005). Cell differentiation and gene expression have also been described to be influenced by surface topography (Dalby et al., 2007; Dalby et al., 2006; Pirouz-Dolatshahi et al., 2008). The effect of surface nanotopography on cell behavior should be a consequence of different protein adsorption patterns. Scarce experimental data exist on the effect of surface nanotopography on protein adsorption but it seems that nanotopography is able to enhance protein adsorption as compared to the same plane chemistry (Khor et al., 2007). Nevertheless, the effect of surface nanotopography on matrix remodelling has not been investigated in the literature and only some qualitative effect has been recently shown (Costa Martínez et al., 2008; Pegueroles et al., 2009). We have developed the technology to prepare different microtopographies with tailored surface chemistry which lead to different cell function as a consequence of the extracellular matrix organisation at the cell-material interface (González-García et al., 2009; Pelham & Wang, 1997).

Mechanical properties of the substrate play also an important role in cell response regardless surface chemistry and topography but it is not completely understood. Cell behaviour depends sensitively on the rigidity of the extracellular matrix. When cells are cultured on classic (rigid) polystyrene dishes, they develop micron-sized focal adhesions connected by actin fibers. However, these structures are gradually lost as cells are cultured on softer substrates, as prepared for example by changing the crosslinking density of gels

and, more recently, polyelectrolyte multilayers (Ryan et al., 2001). Cell spreading and motility are higher in stiff substrates than soft ones, what favours cell-cell interaction compared to the cell-material one and leads to more organised cell aggregates (Wong et al., 2003). Cell proliferation increases on stiff surfaces and, in the case of a rigidity gradient on the substrate, cells migrate to stiffer regions (Lo et al., 2000; Bischofs et al., 2003; Pegueroles et al., 2009). This kind of cell behaviour has been found for different cells types (fibroblasts, muscular VSMC cells, chondrocytes and neurons) independently of the protein coating of the substrate (fibronectin, collagen, etc). It is thought that cells are able to react to substratum rigidity by means of a real tactile exploration, by contractile forces and interpreting the substrate deformation. Because the stiffness of the environment is a passive quantity, it has to be actively sensed by the cell by contracting it and measuring some kind of mechanical response (Schwarz, 2007; Shin, 2008). The relationship between the mechanical properties of the matrix and the activity of cells must lead to the maintenance of a functional mechanical state. The effect of substrate stiffness on the dynamic behaviour of surface associated matrix proteins is generally missing, i.e. is protein conformation determined by substrate stiffness? What is the role of the strength of interaction between the protein layer and the substrate on matrix remodelling as a function of the stiffness of the underlying substrate? Recent studies have indicated that the cells not only detect the roughness, chemistry, or stiffness of the substrates, but can also detect dimensionality (Hollister, 2005). For example, pore sizes of engineered trabecular bone depend on the initial scaffold geometry. Because it is clear that the ECM can affect cell fate and differentiation, tissue engineering is looking toward cells and the developmental biology for guidance in the design of scaffolds (Wnek, 2003).

6. Conclusions and future trends

Understanding the cell-protein-material interaction is fundamental for developing more powerful tools in tissue engineering and regenerative medicine strategies. The design of model substrates including the presence of well defined properties (chemistry, topography, stiffness) and even the gradient of these properties in three dimensional environments must lead in the near future to learn more about the specific roles of protein adsorption and the very dynamic process related to the cell fate of synthetic substrates: cell adhesion, matrix reorganisation, deposition and degradation at the cell-material interface.

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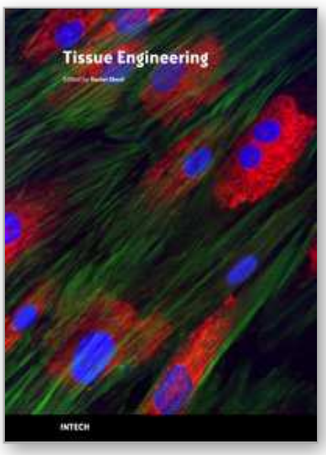
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The Tissue Engineering approach has major advantages over traditional organ transplantation and circumvents the problem of organ shortage. Tissues that closely match the patient's needs can be reconstructed from readily available biopsies and subsequently be implanted with minimal or no immunogenicity. This eventually conquers several limitations encountered in tissue transplantation approaches. This book serves as a good starting point for anyone interested in the application of Tissue Engineering. It offers a colorful mix of topics, which explain the obstacles and possible solutions for TE applications.

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