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Protein covalent immobilization via its scarce thiol versus abundant amine groups: Effect on orientation, cell binding domain exposure and conformational lability

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ABSTRACT

Quantity, orientation, conformation and covalent linkage of naturally cell adhesive proteins adsorbed or covalently linked to a surface, are known to influence the preservation of their subsequent long term cell adhesion properties and bioactivity. In the present work, we explore two different strategies for the covalent linking of plasma fibronectin (pFN) – used as a cell adhesive model protein, onto a polystyrene (PS) surface. One is aimed at tethering the protein to the surface in a semi-oriented fashion (via one of the 4 free thiol reactive groups on the protein) with a heterofunctional coupling agent (SSMPB method). The other aims to immobilize the protein in a more random fashion by reaction between the abundant pendant primary amine bearing amino acids of the pFN and activated carboxylic surface functions obtained after glutaric anhydride surface treatment (GA method). The overall goal will be to verify the hypothesis of a correlation between covalent immobilization of a model cell adhesive protein to a PS surface in a semi-oriented configuration (versus randomly oriented) with promotion of enhanced exposure of the protein's cell binding domain. This in turn would lead to enhanced cell adhesion. Ideally the goal is to elaborate substrates exhibiting a long term stable protein monolayer with preserved cell adhesive properties and bioactivity for biomaterial and/or cell adhesion commercial plate applications. However, the initial restrictive objective of this paper is to first quantitatively and qualitatively investigate the reversibly (merely adsorbed) versus covalently irreversibly bound protein to the surface after the immobilization procedure.

Although immobilized surface amounts were similar (close to the monolayer range) for all immobilization approaches, covalent grafting showed improved retention and stronger "tethering" of the pFN protein to the surface (roughly 40%) after SDS rinsing compared to that for mere adsorption (0%) suggesting an added value to the covalent grafting immobilization methods. However no differences in exposure of the cell binding domains were observed (ELISA results) before SDS rinsing, suggesting that pFN protein grafting to the surface is initially kinetically driven be a stochastic random adsorption phenomenon. Covalent grafting acts in the final stage as a process that simply tethers and stabilizes (or freezes) the initial conformation/orientation of the adsorbed protein on the surface. In addition covalent linkage via the SSMPB approach is likely favored by surface-induce exposure of one of the normally hidden free thiol group pair, thus optimizing covalent linkage to the surface.

However after SDS rinsing, this "tethering"/"freezing" effect was significantly more prominent for the GA grafting approach (due to greater number of potential covalent links between the protein and the surface) compared to that for the SSMPB approach. This hypothesis was buttressed by the improved resistance to denaturation (smaller conformational lability) for the GA compared to the SMPB approach and improved exposure of the cell binding domain for the former (>50%) even after SDS rinsing.

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These results are promising in that they suggest covalent tethering of fibronectin to PS substrate in a monolayer range, with significantly improved irreversible protein surface bonding via both approaches (compared to that for mere adsorption). The latter are likely applicable to a wide range of proteins.

1. Introduction

Surface immobilization of peptides or proteins for biomedical applications is an important issue – in particular when cell-material interactions are of concern [1–3]. Initial physico-chemical surface modification may significantly improve substrate-biological media interactions, while maintaining desired material bulk mechanical properties. Much work on the subject of surface modification for subsequent immobilization of adhesive and/or bioactive proteins or biomolecules has been done [4–14].

Processes adding new chemical functions on the surface of a material include radiative (UV, gamma), chemical or plasma treatments [15,16]. An advantage of cold plasma treatment over all other physical treatments, is that it allows the incorporation of a large possible range of desired chemical functions on the surface of even relatively chemically inert surfaces. In addition, the type and density of deposited chemical functions as well as the final surface energy may be controlled by modulating the physico-chemical features during plasma treatment (plasma energy, nature of the gas used and treatment time).

Cold nitrogen plasma treatment, in particular, allows for the incorporation of reactive primary amines on the surface of polystyrene and many other polymers [17–19] for subsequent covalent grafting of organic bioactive molecules [20] such as amino acids, small peptides or proteins. This latter approach was chosen in this paper for subsequent grafting of a plasma fibronectin protein (pFN).

Fibronectin (FN) is an adhesive protein with a molecular mass of 450 kDa, found in blood plasma or in the extracellular matrix (ECM) [21,22] and formed by two monomeric peptide chains linked together by two disulfide bonds. Also present in the extra cellular matrix (ECM), it interacts with cells to control adhesion, cytoskeletal organization and cell signaling [23–25]. It is widely used in vitro for cell-surface interactions, and in vivo for improved host tissue integration and improved biocompatibility of artificial implants as well as for tissue engineering applications [26]. Another main widespread use of FN is in the context of cell culture dishes and biochip functionalization [27,28]. In this domain, the amounts, conformation and/or orientation of the surface immobilized protein plays a critical role in the preservation of its final cell adhesive bioactivity properties or biosensor sensitivity [29]. In particular, bioadhesive properties are expressed when the protein is immobilized in an orientation and/or conformation enabling it to express or "expose" its cell binding domain (e.g. RGD sequence) to the oncoming cell. Grafting methods using a heterobifunctional arm to attach the protein to the surface in order to achieve preserved binding capacity or bioactivity of the immobilized protein appears to be a logical and viable approach. In addition, protein grafting to the surface yields higher protein layer "stability" (resistance against elution and removal) toward long term immersion in cell culture medium (and resistance to surface removal) compared to simple adsorption [30,31]. Although, this former approach has been abundantly described in the literature for surface immobilization of cell adhesive RGD amino acid sequences, peptides or FN fragments, it has been more scarcely for surface immobilization of the whole pFN molecule. This may be due to the purification cost of human plasma FN (pFN) [32,8] and other associated problematic issues such as virus or prion contamination risks.

In this work, we propose to covalently immobilize the whole pFN protein on an aminated polystyrene surface (PS) via two synthetic routes involving different chemical groups on the protein and to compare resistance to removal of the immobilized protein layer from the surface by an eluting agent (sodium dodecylsulfate, SDS) compared to that for simply adsorbed. The first grafting route involved reaction of one of the abundant primary protein amines (available on lysine residues) with a glutaric anhydride (GA) treated surface (GA method) resulting in a covalent linkage of the protein in a non-oriented configuration (random or statistical grafting).

It is worth noting here that this latter approach has considerable advantage over a more conventional glutaraldehyde protein surface binding approach. Since one problem encountered with the use of glutaraldehyde is the intra- and inter-molecular protein cross-linking via available lateral amino groups (on lysine for instance) reducing reaction yield efficiency and cell binding domain accessibility. Our approach circumvents this impediment since all reacting amino groups are involved exclusively in the linkage reaction with the surface (no side-reactions).

The second approach involved reaction between one of the two free thiol groups on each of the two homologous peptide chains of the pFN molecule, with the maleimide function of the sulfo-succinimidyl-4-(p-maleimidophenyl) butyrate heterofunctional linker (SSMPB method) [33]. The latter approach yields a protein in a *semi-oriented* configuration (oriented grafting) since theoretically only two free thiol linkage positions are possible on each of the two homologous chains of the pFN molecule. The weakly non specifically bound proteins were eliminated by phosphate buffer (0.15 M, pH 7.4) and 1% SDS w/v (sodium dodecyl sulfate) rinsing according to a procedure described elsewhere [28].

2. Materials and methods

2.1. Nitrogen cold plasma treatment of polystyrene

In order to deposit primary amine groups, the polystyrene surfaces received the following cold nitrogen plasma treatment. Polystyrene (plastic plate form 12 or 24 wells NUNC, from thermofisher, Denmark) were treated in sterile conditions for the incorporation of amine functions. The large volume radiofrequency (RF) cold plasma reactor (Manufacturer: ISYTECH, Lannion, France) is located in the CTTM laboratory in an ISO 7 clean room. This reactor is composed of three main parts: a processing chamber (60 l), a pumping system and a 600 W radiofrequency generator (13.56 MHz – INTEGRO^{TM} 136 RF GENERATOR). A 5 min plasma treatment was performed in the following conditions: nitrogen was introduced at a gas flow of 10 standard cubic centimeters and at a pressure of 30×10^{-3} mbar. The power was set at 200 W.

2.2. Fibronectin preparation and purification

Plasma fibronectin (pFN) (98% w/w pure, 450–470 kDa) obtained from ERRMECe laboratory at the University of Cergy Pontoise was purified from human cryoprecipitated plasma according to a well-established protocol [34]. The purified pFN solution was then filtered through a 0.2 μm filter and stored at 8 °C in a 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4)