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The correlation between the adsorption of adhesive proteins and cell behaviour on hydroxyl-methyl mixed self-assembled monolayers

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ABSTRACT

The objective of this study was to compare the biological effects of two key cell-adhesive proteins, fibronectin (FN) and vitronectin (VN), upon adsorption onto molecularly-designed model surfaces. Single-component and mixed self-assembled monolayers (SAMs) of alkanethiols on gold with OH and CH_3 terminal groups were prepared at 100%, 65%, 36% and 0% of OH at the surface, to generate a range of surfaces with a simple chemistry and a wettability gradient. FN and VN were adsorbed under non-competitive (single-protein solutions) and competitive (multi-protein solutions) conditions, and compared at different levels: adsorbed amount (radiolabelling), elution, functional presentation of cell-binding domains (ELISA), and role in mediating cell adhesion (antibody-based assay). The observed trends were related to mesenchymal stem cell response in terms of adhesion and overall cell morphology. Under non-competitive conditions, adsorption of both proteins increased with surface hydrophobicity. The presence of competitive proteins significantly decreased the adsorbed amounts, although both proteins were still detected in all SAMs. Adsorption of FN followed a trend similar to that of non-competitive conditions, while adsorption of VN was higher on 100%OH-SAMs. Concerning elution, retention of adsorbed VN was always higher than that of FN. For both proteins, functional presentation of cell-binding domains was more effective on the more hydrophilic 100%OH-SAMs. This fact, coupled to the ability of this type of SAMs to selectively recruit and retain VN in the presence of competitive serum proteins, seems to correlate with the better cell response observed on these surfaces, as compared with hydrophobic 0%OH(100%CH_3)-SAMs.

1. Introduction

Cell adhesion to surfaces and the subsequent biological outcome are events with high relevance to the areas of tissue engineering and nanomedicine. The rational design of surfaces with the ability of eliciting specific cell responses is important both to *in vitro* and *in vivo* applications, ranging from the development of substrates for *in vitro* cell culture to the development of implant biomaterials with improved functionalized surfaces.

The general consensus is that upon contact with physiological protein-rich media, such as plasma or serum, surfaces become instantaneously coated with layers of adsorbed proteins, and that cell response is largely mediated by the nature and bioactivity of those proteins in a highly complex and dynamic process [1]. The recognition of this fact led to the assumption that cell–surface interactions can be modulated, at least to some extent, by controlling the type, amount, conformation/orientation and surface distribution of adsorbed proteins [2,3].

Since important cell signalling pathways, including those involved in survival, proliferation and differentiation, are activated when extracellular matrix (ECM) proteins bind to cell-surface integrin receptors, the pivotal role of two key cell adhesion ECM proteins – vitronectin (VN) and fibronectin (FN) – has been the object of numerous studies [4–7]. Both proteins are present in plasma and in serum and contain the arginine–glycine–aspartic acid (RGD) cell-binding motif, although exhibiting different specificity for cell receptors [8]. The properties of FN have been extensively investigated. FN has been used as the prototypical cell adhesion protein in the majority of studies found in the literature [2,3,9–11]. However, it has long been recognised that in an *in vitro* environment, where cells are cultured in serum-containing media, VN is frequently dominant over FN in mediating cell adhesion,

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owing to its higher adsorption efficiency under competitive conditions [4–7,12–15]. Studies on the interaction of FN and VN with polymeric surfaces, namely with untreated polystyrene (PS) and tissue-culture polystyrene (TCPS), provided valuable insights into their biological effects and relative importance in modulating cell response, especially at early interaction times, when adsorbed to surfaces with different chemistries [4–7]. However, those chemistries are complex, non-homogeneous and quite undefined, rendering difficult to discriminate between the underlying effects of chemical functionality, wettability, charge and topography. The striking differences found in some of those studies between different brands of TCPS seem to corroborate these conclusions [7].

To overcome this drawback, molecularly-designed model surfaces, such as self-assembled monolayers (SAMs), appear as an alternative. SAMs of alkanethiols on gold are highly-ordered organic surfaces, formed upon immersion of gold-coated substrates in a solution of alkanethiols that spontaneously assemble at the interface through their sulphur moiety [16,18]. By varying the alkanethiol head group, different chemical functionalities can be exposed at the outer surface, generating SAMs with tailored properties [16–18].

Although numerous studies have examined FN adsorption on SAMs [2,3,9], only a few studies have used VN [19,20]. Moreover, the great majority of studies have used proteins adsorbed from pure solutions [2,3,9], while only a few used more complex protein-rich solutions [19]. Thus, comparing the behaviour of the two proteins, especially under competitive conditions and using well-defined surfaces, remains to be done.

In this study, single-component and mixed SAMs of OH- and CH_3-terminated long-chain alkanethiols ($HS(CH_2)_nX$, $n \geq 10$) were used. FN and VN were adsorbed under non-competitive (single-protein solutions) and competitive (multi-protein solutions) conditions, and their behaviour was compared at different levels: adsorbed amount (radiolabelling), displacement (elution tests), functional presentation of cell-binding domains using monoclonal antibodies (ELISA) and selective mediation of cell adhesion (antibody-based assay). The trends observed for FN and VN adsorption were then related to cell response in the short-term, evaluated in terms of adhesion, spreading, cytoskeleton organization and focal adhesions formation. Human adult mesenchymal stem cells (hMSC) were selected for this study, since they currently represent one of the most promising stem cell populations for regenerative medicine applications. Given the potentially high impact of these cells, new methodologies are required to expand them *in vitro*, under strictly controlled conditions.

2. Materials and methods

2.1. Preparation and characterisation of SAMs

2.1.1. Gold substrates

Gold on silicon substrates was prepared as already described [21,22]. Briefly, a 5 nm underlayer of Cr and a 25 nm layer of Au were deposited by ion beam sputtering from the respective targets (99.9% purity) onto Si wafers, which were subsequently coated with 1.5 μm of photoresist (PFR7790EG, JSR Electronics) to protect the film surface, and diced into square samples (1×1 and 0.5×0.5 cm^2) using a DISCO DAD 321 automated saw.

2.1.2. Monolayer formation

Pure thiol solutions of 11-mercapto-1-undecanol ($SH\text{-}(CH_2)_{11}OH$; 97%, Aldrich) and 1-hexadecanethiol ($SH\text{-}(CH_2)_{15}CH_3$; 92%, Aldrich) were prepared in ethanol (Merck, 99.8%) at 1 mM, under a dry N_2 atmosphere. Binary solutions were prepared by mixing pure solutions at $OH:CH_3$ v/v ratios of 9:1 and 8:1, in order to obtain 65% and 36% of OH groups at the surface, respectively [21,22]. Just before being used, Au substrates were washed twice with acetone, rinsed with ethanol and immersed 5 min in *piranha* solution (7:3 v/v H_2SO_4:30% H_2O_2). Substrates were sequentially washed with ethanol/distilled–deionised water/ethanol for 2 min in an ultrasonic bath. The clean Au substrates were dried with Ar and incubated in alkanethiol solutions for 24 h (at room temperature and under N_2). SAMs were finally washed three times with ethanol, dried and maintained under Ar until used.

2.1.3. Contact angle measurements

The water contact angles (θ_w) of SAMs were determined using the sessile drop method. Measurements were performed in a Data Physics (model OCA 15) system with video camera (CCD) and SCA 20 software. SAMs were placed in a thermostatic chamber (25 °C) saturated with water to prevent evaporation. Water drops (distilled–deionised with conductivity ≤1 μS/cm, 4 μL) were applied using a gas-tight syringe (Hamilton) mounted on an electronic unit. Images were acquired every 2 s over 600 s. To calculate θ_w, droplet profiles were fitted using the Young–Laplace ($\theta_w \geq 90°$), the ellipse ($30° < \theta_w < 90°$) or the tangent ($\theta_w < 30°$) methods, and values were obtained by extrapolating the time dependent curve to zero.

2.2. Cell culture studies

2.2.1. Isolation and culture of hMSCs

Heparinized human bone marrow was obtained from healthy donors after informed consent. Low-density bone marrow MNC were separated on a Ficoll–Histopaque density-gradient centrifugation (1.077 g/mL; Sigma). For each donor, MSCs were isolated by magnetic cell sorting for Stro-1 positivity (R&D Systems Inc.) [23], and CD45 and Gly-A negativity (Miltenyi Biotec). Cells were expanded in gelatin-coated flasks with mesenchymal stem cell basal medium (Poietics; Lonza) supplemented with MSCGM SingleQuot Kit. Cells from the 5th passage were used in this study.

2.2.2. Cell adhesion and spreading

SAMs were placed in TCPS plates for suspension cells (Sarstedt), to minimise cell adhesion to the bottom of the wells, and sterilised in 70% v/v ethanol for 30 min. After washing twice with sterile PBS, SAMs ($n = 4$ for each condition) were pre-conditioned in culture medium with 10% v/v foetal bovine serum (FBS) for 30 min at 37 °C, seeded with hMSCs at 3000 cells/cm^2 and incubated for 90 min at 37 °C under a humidified atmosphere of 5% v/v CO_2 in air. TCPS coverslips and SAMs without cells were processed like the other samples and used as controls. To estimate cell adhesion, samples were washed twice with pre-warmed PBS, transferred to a new plate and incubated in MTS solution (Cell Titer Proliferation Assay, Promega) for 3 h. The supernatants were recovered and optical density read at 490/620 nm in a microplate spectrophotometer (PowerWave XS, BioTek). A calibration curve showed the absorbance readings to be proportional to the cell number.

For scanning electron microscopy (SEM), cells were fixed with 1.5% v/v glutaraldehyde in 0.14 M sodium cacodylate (pH 7.4), for 30 min at room temperature. Dehydration was carried out by sequential immersion in serial diluted solutions of 50, 60, 70, 80, and 90% v/v ethanol in water, followed by immersion in 99.8% v/v ethanol. Samples were then transferred to hexamethyldisilazane (HMDS), air-dried under laminar flow at room temperature overnight and sputter-coated with gold using a JEOL JFC-100 Fine Coat Ion Sputter device. Analyses were made using a JEOL JSM-6301F microscope operating at 10 kV.

2.2.3. Cytoskeleton organization and focal adhesions formation

Cell adhesion on SAMs pre-conditioned in culture medium with 10% FBS was promoted as described above. After 24 h of incubation, samples were washed twice with pre-warmed PBS, fixed in 4% v/v paraformaldehyde for 15 min, incubated in permeabilising buffer for 5 min, and blocked with 1% w/v bovine serum albumin (BSA) in PBS at room temperature. To evaluate focal adhesions formation and cytoskeleton organization, vinculin and tubulin were immunostained with primary mouse anti-human monoclonal antibodies (hVIN-1 and TUB 2.1, 1:100 in 1% w/v BSA/PBS, Sigma), followed by rabbit anti-mouse Alexafluor-488 conjugated Fab fragments (Molecular Probes, 1:200 in 1% w/v BSA/PBS). Actin filaments were stained with Alexafluor-conjugated phalloidin (Molecular Probes, 1:40 in 1% w/v BSA/PBS). Nuclei were counterstained with 1 μg/mL 4,6-Diamidina-2-phenylin (DAPI, Sigma) for 10 min. Samples were washed with PBS, mounted with Vectashield® (Vector) in glass slides and photographed with an inverted fluorescence microscope Axiovert M100 (Carl Zeiss).

2.3. Protein adsorption studies

2.3.1. Protein radiolabelling and adsorption to SAMs

Bovine FN and VN (F1141 and V9881, Sigma) were ^{125}I-radiolabelled using the Iodogen method [24]. Labelled proteins (^{125}I-bFN, ^{125}I-bVN) were purified by size exclusion chromatography using a Sephadex G-25M column (PD-10, Amersham Pharmacia Biotech). The iodination yield was ≥92%, as estimated by trichloroacetic acid precipitation (10% v/v). The effect of labelling on the adsorption behaviour of both proteins was first investigated. Control adsorption experiments using a range of solutions at the same total protein concentration but varying ratios of labelled-to-unlabelled proteins (5–50%) were run, showing that the adsorbed amounts remained constant, independently of the labelled-to-unlabelled ratio (data not shown), which suggests that there is no preferential adsorption of one of the forms [25].

Adsorption studies were then performed using different solutions to which freshly prepared ^{125}I-bFN or ^{125}I-bVN was added as tracers. Single-protein