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Polymer chain flexibility-induced differences in fetuin A adsorption and its implications on cell attachment and proliferation

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ABSTRACT

Tissue cells are known to respond to the stiffness of the polymer substrate on which they are grown. It has been suggested that material stiffness influences the composition of the protein layer that adsorbs to the material surface, which affects subsequent cell behavior. Previously, the stiffness of a biomaterial elastomer formed from an acrylated *star*-poly(D,L lactide-*co*-ε-caprolactone) was found to influence both fibroblast proliferation as well as the adsorption of certain proteins. However, it remained unresolved as to whether material stiffness influenced protein adsorption from serum supplemented environments and which protein(s) may have been responsible for the difference in fibroblast proliferation. Using quantitative proteomics, we show that polymer stiffness influenced the composition of the protein layers that adsorb from serum supplemented media. Fetuin A was identified as a protein that influenced fibroblast proliferation and, when combined with basic fibroblast growth factor as a medium supplement, improved fibroblast proliferation over 14 days. This study is the first to correlate cell proliferation to surface adsorbed fetuin A and presents the potential new application for fetuin A as biomaterial coating or surface modifier. This work also demonstrates a novel application of quantitative proteomics for the investigation of competitive protein adsorption to biomaterial surfaces.

Statement of significance

Cells are able to respond to the stiffness of their material substrate, but the method by which they sense material stiffness is still under investigation. Previously, material stiffness was found to impact the individual adsorption of fibronectin, a protein associated with cell attachment; however, it was unclear if stiffness was able to affect protein adsorption in environments with multiple proteins. This study shows that material stiffness affects the compositions of protein layers adsorbed from supplemented media, and suggests that cells may sense material stiffness via the adsorbed protein layer. Interestingly, fetuin A was found to be affecting cell proliferation and not fibronectin. Finally, this research demonstrates the use of relative quantitation proteomics as a potentially powerful method to improve biomaterial compatibility.

1. Introduction

Mechanical properties of biomaterials, such as modulus and polymer chain flexibility, are known to affect the attachment [1–3] and proliferation [4–6] of anchorage dependent cells. However, the mechanism by which the cells sense material stiffness is not yet clearly understood and is the subject of ongoing study. Cell response to a biomaterial is believed to be mediated by the protein layer that adsorbs to the biomaterial surface [7,8], the composition and configuration of which depends on the properties of the material surface [9]. Because cells appear to respond to material stiffness, it has been suggested that substrate stiffness, itself, affects the composition and configuration of the adsorbed protein layer, which subsequently drives cellular response [10,11].

Protein adsorption is primarily an entropically driven phenomenon; the adsorption of a protein to a substrate frees water molecules bound to both the protein and the substrate's polymer chains, increasing the entropy of the system [9,12]. Material stiffness and thus polymer chain flexibility at the interface may influence both the arrangement of the water molecules bound to the polymer chain and the configurational entropy of the chains

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themselves, which may, in turn, influence the entropy increase that occurs upon adsorption, and subsequently, the quantities or conformations of the proteins that adsorb [10,11].

Few studies have investigated the means by which material stiffness influences protein adsorption. Previously, we cultured smooth muscle cells [4] and fibroblasts [10] in fetal bovine serum supplemented medium on elastomer substrates of different stiffnesses formed from crosslinking different molecular weights of an acrylated *star*-poly(D,L lactide-*co*-ϵ-caprolactone) (ASCP). For these elastomers, crosslink density impacts the material bulk stiffness and flexibility of the polymer chains without altering surface chemistry. We found that smooth muscle cell and fibroblast proliferation depended on the stiffness of the underlying elastomer and hypothesized that this cell behavior was due to differences in protein adsorption on the surfaces of these elastomers, driven by the difference in polymer chain flexibility at the interface. This hypothesis was supported by initial findings that showed differences in the average mass and viscoelastic properties of adsorbed layers of both individual proteins and fetal bovine serum.

However, it was not possible to conclude if these differences in the properties of adsorbed serum layers were due to differences in which proteins comprised the layers or conformation of the proteins therein. As such, we were unable to determine if material stiffness could affect the composition or conformation of the protein layers that adsorb in a serum supplemented cell culture environment. Furthermore, the proteins examined in the individual protein adsorption experiments (namely fibronectin and vitronectin) were selected due to their possible contribution to cell attachment and proliferation by way of acting as a source of integrin binding sites. However, it was unknown whether these proteins were actually affecting fibroblast proliferation in the serum supplemented cell culture environment.

The objective of this study was to determine if polymer chain flexibility at the material interface could affect the composition of protein layers adsorbed from serum as well as determine which protein(s) could be responsible for the observed difference in 3T3 fibroblast proliferation. Since we previously observed differences in the adsorption properties of fibronectin, we hypothesized that fibronectin, or a similar cell adhesive protein, was adsorbing from the serum supplemented media to the elastomer surfaces in different quantities or conformations, affecting the number of integrin binding sites accessible to the cells, and thus cell proliferation. However, due to the multitude of proteins in serum and plasma [13], protein layers that adsorb from these media are comprised of potentially hundreds of different proteins, and measuring the adsorption characteristics of single proteins alone does not capture the complexities of multiple protein adsorption such as protein competition and replacement [14].

In this study, we used relative quantitation proteomics to determine the composition of protein layers adsorbed from serum supplemented media to two different stiffnesses of ASCP elastomer and attempted to identify which adsorbed protein(s) could be driving the previously observed stiffness dependent fibroblast proliferation. Relative quantitation proteomics is a powerful emergent technique for comparing the quantities of individual proteins on multiple surfaces [15]. Upon identifying a protein, alpha-2-HS-glycoprotein (fetuin A), which adsorbed asymmetrically to the elastomer, fetuin A layers on each elastomer were characterized using surface plasmon resonance (SPR) and quartz crystal microbalance with dissipation (QCM-D). Finally, fibroblasts were cultured on the elastomer surfaces in medium supplemented with fetuin A and with or without basic fibroblast growth factor (FGF-2) to determine the protein's effect on fibroblast proliferation.

2. Materials and methods

2.1. Prepolymer Fabrication

Elastomers were prepared from 2000 g/mol and 5000 g/mol acrylated *star*-poly(D,L lactide-*co*-ϵ-caprolactone) (ASCP) according to the procedure described in Amsden et al. [16]. Elastomers fabricated from 2000 g/mol and 5000 g/mol pre-polymer are henceforth referred to as E2 and E5, respectively. These molecular weights were chosen because they have been previously shown to induce different degrees of smooth muscle cell [4] and fibroblast proliferation as well as adsorb different amounts of fibronectin, IgG, and serum proteins [10]. To prepare the ASCP, a 1:1 M ratio of D,L-lactide (Purac, The Netherlands) and ε-caprolactone (Fluka, Switzerland) were added to a flame dried glass ampoule with glycerol (Fisher Scientific, Canada) as the initiator, and tin(II) 2-ethylhexanoate (Sigma, Canada) as a catalyst. The ampoules were sealed under vacuum and reacted for 24 h at 130 °C. For these polymers, the molecular weight is controlled by the ratio of moles of glycerol: moles of monomer added to the polymerization. The resulting polymer was dissolved in dry dichloromethane (Fisher Scientific, Canada) and acrylated at their termini by the dropwise addition of acryloyl chloride (Sigma, Canada), in the presence of triethylamine (Sigma, Canada) and dimethylaminopyridine (DMAP) (Sigma, Canada) as a catalyst. The resulting ASCP prepolymer was purified by overnight precipitation in cold (−20 °C) isopropanol (Fisher Scientific, Canada). Number average molecular weights (2100 g/mol and 5200 g/mol for E2 and E5, respectively) with degrees of acrylation (above 99% for both pre-polymers) were assessed using ^{1}H NMR (500 MHz, Bruker).

2.2. Microsphere preparation

To increase the available surface area for protein adsorption, E2 and E5 elastomers were fabricated as microspheres. ASCP prepolymers were dissolved in toluene in a 1:1 w/v ratio with 2,2 dimethoxy-2-phenyl-acetophenone (DMPA) (Sigma, Canada) as a photoinitiator. 3 mg photoinitiator was added per gram of prepolymer. 4 mL de-ionized (DI) water and a small stir bar were added to a 5 dram glass vial. 1 mL of pre-polymer was pipetted into the stirred vial (1200 rpm) in a thin stream. A UV light (Lightningcure LC8, Hamamatsu) was then placed over the stirring vial to crosslink the pre-polymer droplets (300–400 nm wavelength, 30 mW/cm^2, 5 min). Crosslinked microspheres were removed from the water by sieving over a 22 μm sieve and were dried in an oven at 120 °C for 10 min, then overnight at room temperature to remove excess water and solvent before use. Microsphere diameter was measured by image analysis (Image J) of SEM images (Hitachi S-2300 SEM, 15 kV) (Fig. 1). Average microsphere diameters for the E2 and E5 microspheres were 184 ± 57 μm and 370 ± 132 μm respectively.

2.3. Protein adsorption

To determine which proteins adsorbed to E2 and E5 in a cell culture environment, Dulbecco's modified Eagle's medium (DMEM, D6429, Sigma, Canada) supplemented with 10% fetal bovine serum (FBS, Fisher Scientific, Canada) and DMEM supplemented with 10% adult bovine plasma (with 10 U/mL sodium heparin, Cedarlane, Canada) were incubated with E2 and E5 microspheres at 37 °C for 24 h. 2 mL microcentrifuge tubes were filled with either 0.5 g of E2 or 1 g of E5 microspheres, equalizing the available adsorption surface area. The microspheres were incubated in unsupplemented DMEM for 12 h prior to protein adsorption. The unsupplemented media was then removed and replaced with 1 mL of FBS or plasma

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