Protein Adsorption on Derivatives of Hyaluronan

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Summary: Serum protein adsorption and fibroblast cell adhesion on photo reactive hyaluronic acid (Hyal-N_3) and its sulfated derivative (HyalS-N_3) was analysed using a combination of quartz crystal microbalance (QCM) and cell adhesion assays. There was no significant differences in the amount of protein adsorbed onto the two polymers, however proteins were found to be more loosely bound to HyalS-N_3 compared with Hyal-N_3. Approximately 17% and 31% of the fibronectin interacting with Hyal-N_3 and HyalS-N_3 respectively was found to be irreversibly bound after rinsing with MilliQ water, SDS and urea. Proteins were exposed to the polymers before cell adhesion was monitored for a period of 2 hours in serum free conditions. Minimal cell adhesion was observed on albumin-coated materials as well as serum precoated Hyal-N_3. Precoating the materials with fibronectin enhanced cell adhesion, although HyalS-N_3 experienced higher levels of cell adhesion than Hyal-N_3 and similar results were found for the serum precoated materials.

Keywords: cellular response; hyaluronan; hydrogel; protein adsorption

Introduction

The modulation of biological interactions with artificial surfaces is a vital aspect of biomaterials research. Cell-surface interactions are thought to be mediated by adsorbed proteins.[1,2] The properties of an underlying substrate have been shown to influence the amount and conformation of adsorbed proteins. Surface-specific cellular responses are the result of a complex biological system that includes protein adsorption, receptor- ligand binding and signal transduction.[3] Hence an understanding of how to direct specific protein and cellular responses is critical for the development of future biomaterials.

Hyaluronan (Hyal) is a natural non-sulphated glycosaminoglycan (GAG) consisting of linear poly-anionic polymer chains of alternating N-acetyl-D-glucosamine and ß-D-glucuronic acid residues linked 1–3 and 1–4 respectively. It is highly hydrophilic due to the presence of hydroxyl and carboxyl groups and it an unusual GAG in that it does not bind to a protein core. Hyal is one of the major components of the extracellular matrix of soft tissues, such as cartilage and the epidermis, as well as the vitreous humour. It is involved in many biological functions such as joint lubrication, water regulation, connective tissue formation as well as adhesive and cellular activation processes.[4] Moreover, it has been shown to facilitate cell detachment[5] and is biodegradable.[6] Despite its hydrophilicity, Hyal is a weak polyacid with a very low charge density because only one charge is present for each repeating disaccharide unit.

Purified Hyal has been employed as a structural material due to its high molecular weight and ability to form 3D networks. Chemical modification of Hyal by the insertion of sulfate groups on the hydrophilic groups of the polysaccharide[7,8] produces a polymer with a high a high number of negative charges on the polymer chain due to the presence of sulfate groups.[9] Sulfated

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hyal (HyalS) has been found to be biocompatible, and increases cell adhesion and proliferation compared to Hyal.[10,11]

Hyal is known to bind ionically to a variety of proteins such as aggrecan and link protein for cartilage elasticity and compressibility and versican for cell adhesion, migration and proliferation.[12] Hyal is also known to covalently bind to inter-α-trypsin inhibitor to assist with extracellular matrix stabilisation in inflamed synovial fluid.[13]

In this study, the quartz crystal microbalance has been used to characterise serum protein adsorption on photo reactive hyaluronic acid (Hyal-N_3) and its sulfated derivative (HyalS-N_3). NIH3T3 murine fibroblasts have been used as a model cell line for cell adhesion assays.

Materials and Methods

Hyaluronan (Hyal, MW 240000) was supplied by Biophyl S.p.A. (Italy). Hyal was conjugated with a photo-reactive group, 4-azidoaniline hydrochloride[7] (Hyal-N_3), and sulfated as described previously (HyalS-N_3).[10] The inclusion of the sulphate group in HyalS and photo-reactive groups were verified using fourier transform infrared spectroscopy (ATR/FTIR) (data not shown).

Preparation of Surfaces

Hyal-N_3 and HyalS-N_3 were dissolved in MilliQ water (1wt%) and spin coated onto gold 5 MHz AT cut crystals (Q-Sense AB, Sweden) at 2000 rpm for 30 seconds. Polymers were crosslinked by irradiating the spin coated crystals with a UVC source (30 W) for 14 minutes at a distance of 40 cm. Complete coverage of the crystals was verified by atomic force microscopy (AFM) in tapping mode (data not shown).

Preparation of Protein Solutions

Single protein solutions of bovine serum albumin (1 mg/ml) (Sigma-Aldrich, Australia) and ovine fibronectin (50 μg/ml) were prepared in phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, Australia). The complex protein solution used was 10% fetal calf serum (FCS) (Gibco, Invitrogen, Australia) in PBS.

Quantification of Adsorbed Protein

Quantification of protein adsorption was carried out using a quartz crystal microbalance with dissipation monitoring (QCM-D) (Q-Sense AB, Sweden) at $37 \pm 0.1\,^{\circ}C$. The QCM-D, described in detail elsewhere,[15] measures frequency (f) and dissipation (D) at the fundamental frequency (5 MHz) and three successive overtones (15, 25 and 35 MHz). Polymer coated QCM-D crystals were exposed to PBS and stable f and D measurements established before the addition of the test protein solution for 1 hour and then rinsed with PBS until consistent f and D measurements were obtained (usually two rinses). The degree of reversible binding of fibronectin on each of the polymers was also assessed by QCM-D by incubating the polymers with 50 μg/ml fibronectin for 1 hour before rinsing with MilliQ water (18 $M\Omega \cdot cm$) five times (10 min each) followed by two rinses with 2% sodium dodecyl sulfate (SDS) and two rinses with 3 M urea. Adsorbed mass estimates were obtained using the Voigt model which assumes that the adsorbed layer is of uniform thickness, conserves its shape and does not flow.[16]

Cell Adhesion Assay

Murine fibroblast cell line NIH3T3 (EGFP)[17] was grown in DMEM supplemented with 10% newborn calf serum (Gibco, Invitrogen Corporation), 100 U/ml penicillin and 100 μg/ml of streptomycin. Cells were maintained in a humidified incubator (5% CO_2/95% air atmosphere at $37\,^{\circ}C$).

Hyal-N_3 and HyalS-N_3 coated glass coverslips were placed in 24-well tissue culture polystyrene (TCPS) plates, rinsed with PBS and incubated with either albumin, fibronectin or 10% FCS in DMEM for 1 hour at $37\,^{\circ}C$ followed by two rinses with PBS. NIH3T3 (EGFP) cells were seeded onto the protein coated surfaces in serum free DMEM at a cell density of 1.5×10^5 cells/ml for a period of 2 hours. Non-adherent cells were removed by rinsing

with PBS. Fluorescence microscope images of adhered cells were taken at 2 hours post cell seeding at 100× magnification from which the number of adhered cells could be determined. Each experiment was performed in triplicate. TCPS wells were used as control materials.

Results and Discussion

Dry polymer thicknesses for Hyal-N_3 and HyalS-N_3 were approximately 14 ± 2 nm and 15 ± 4 nm respectively, as determined by QCM-D. Hydrated polymer thickness was determined by measuring hydrogel coating thickness before and after the addition of PBS to hydrate the polymers in the QCM-D. The hydrated thicknesses for Hyal-N_3 and HyalS-N_3 were found to be 19 ± 4 and 21 ± 5 nm respectively, yielding water contents of approximately 40% for the two photo-immobilised polymers. In synovial fluid and cartilage Hyal functions as a lubricant through repulsion between charges and contributes to the maintenance of osmotic pressure due to its ability to absorb and hold large amounts of water.[18] HyalS-N_3 is more hydrophilic than Hyal-N_3 due to the presence of sulphate groups,[19] although this did not result in a significant difference ($P < 0.05$) in water uptake between the two materials in this study. Hyal-N_3 and HyalS-N_3 were found to have similar viscoelastic properties as indicated by their comparable D values of $154 \pm 5 \times 10^{-6}$ and $154 \pm 3 \times 10^{-6}$, respectively, as measured by QCM-D in PBS before the addition of protein. Hence, the measured changes in f and D, or changes in the mass and viscoelasticity, with protein exposure are due to adsorption and absorption of adhesion proteins,[20–22] not the underlying surface.

The mass of irreversibly bound protein (Figure 1A) indicates that there is no significant difference ($P < 0.01$) between the same protein bound to each of the surfaces as monitored by the QCM-D. There can, however, be differences in the speciation and conformation of adsorbed proteins.[23] Hydrated polymers may allow the indiffusion of proteins into their matrix,[24] which can be detected by the QCM-D through changes in D. Protein adsorption on both Hyal-N_3 and HyalS-N_3 was observed to be predominantly surface adsorption due to the positive shift in D with protein adsorption. Both Hyal-N_3 and HyalS-N_3 adsorbed approximately monolayer levels of albumin at 270 ± 30 and 350 ± 80 ng/cm^2 respectively. Hyal-N_3 has previously been reported to experience very low albumin adsorption, although quantitative values were not provided.[25] Photo-immobilised polymers also experienced similar levels of fibronectin adsorption with Hyal-N_3 experience 500 ± 100 ng/cm^2 and HyalS-N_3 experience 590 ± 80 ng/cm^2 (Figure 1A). Fibronectin has previously been shown to adsorb in higher amounts onto HyalS-N_3 in comparison to Hyal-N_3[19] due to the higher hydrophilicity of HyalS-N_3,[26,27] although this is not observed with the QCM-D

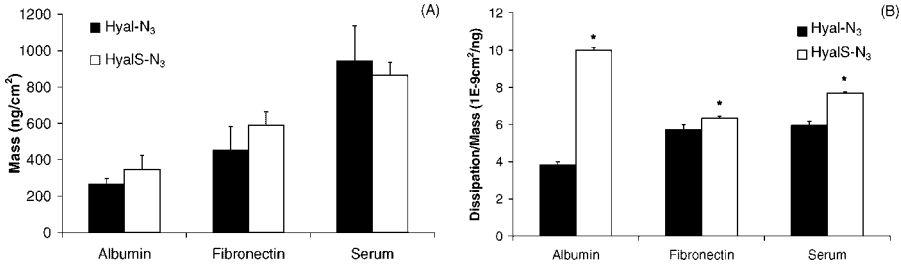

Figure 1.
Comparison of serum protein mass attached to Hyal-N_3 and HyalS-N_3 polymers after 1 hour incubation at 37 °C and PBS rinses as determined by QCM-D, displayed as (A) mass and (B) dissipation per unit mass. Error bars: SD (n = 6), * = t test, $P < 0.01$ with respect to Hyal-N_3. Adsorbed mass estimates derived using the Voigt model.

measurements. Analysis of the results obtained by Barbucci et al.[19] show that the total amount of fibronectin extracted from much thicker Hyal-N_3 and HyalS-N_3 polymers was approximately 4.1 ± 0.1 μg/cm^2 and 5.2 ± 0.2 μg/cm^2 respectively after elution with double distilled water, SDS and urea, while the amount of fibronectin remaining on the materials after double distilled water rinses was approximately 2.2 μg/cm^2 for each. The QCM-D results (Figure 1A) show the amount of fibronectin remaining on the polymers after PBS rinses is approximately the same for both Hyal-N_3 and HyalS-N_3, which is in agreement with Barbucci et al.[19] Differences in the concentration of fibronectin exposed to the polymers, along with thickness of the gels, could account for disparity in the magnitude of the results. Although a lower concentration of fibronectin was used in this study, only 3% of the available fibronectin was adsorbed onto the polymer surfaces. Higher levels of serum adsorption were observed on both Hyal-N_3 and HyalS-N_3 compared to fibronectin and albumin. The adsorption of proteins from a complex solution, such as serum, involves a series of adsorption and displacement steps where smaller biomolecules in high concentration will adsorb faster, but are then replaced by molecules with a higher surface affinity.[28]

Changes in D measured by the QCM-D relate to changes in the viscoelasticity of the adsorbed layer. D measured for protein binding to Hyal-N_3 and HyalS-N_3 was normalised against the mass of the adsorbed layer (Figure 1B). Proteins adsorbed onto HyalS-N_3 were significantly ($P < 0.01$) more dissipative than proteins adsorbed onto Hyal-N_3 suggesting that there are differences in the type of binding to the different photo-immobilised polymers. The results indicate that proteins were more rigidly adsorbed on Hyal-N_3 than HyalS-N_3.

Analysis of the reversibility of fibronectin adsorption onto the photo-reactive polymers was also analysed by QCM-D. Polymers were rinsed with MilliQ five times followed by two rinses with 2% SDS and finally two rinses with 3 M urea (Figure 2). MilliQ water was able to remove approximately 30% and 62% of the fibronectin interacting with HyalS-N_3 and Hyal-N_3 respectively. After treatment with both 2% SDS and 3 M urea approximately 31% and 17% of the initially interacting fibronectin remained on HyalS-N_3 and Hyal-N_3 respectively. This result is in contrast to the report by Barbucci et al.[19] who

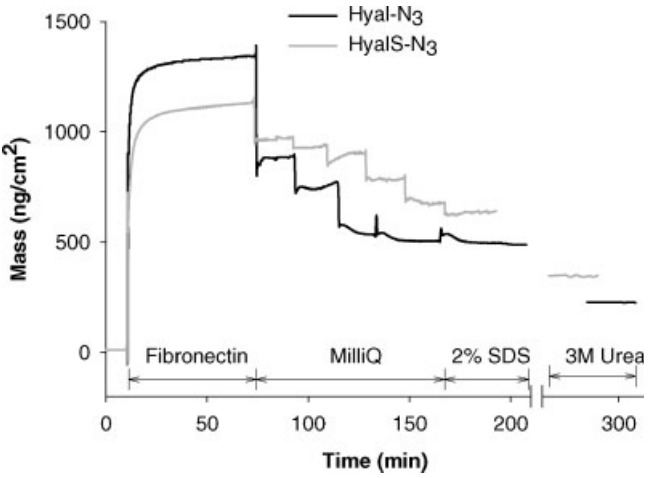

Figure 2.
Mass of fibronectin adsorbed to Hyal-N_3 and HyalS-N_3 after consecutive rinses with MilliQ water, 2% SDS and 3 M urea.

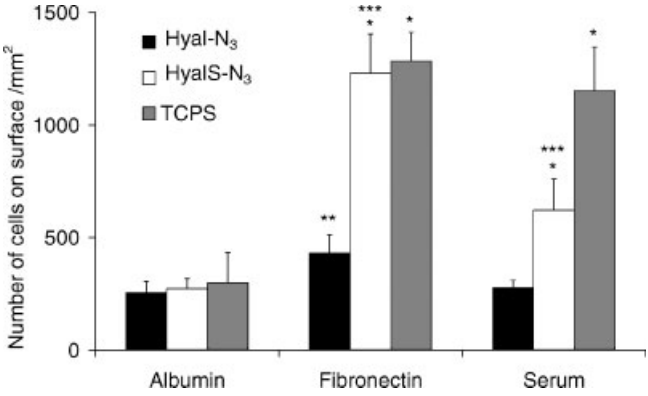

Figure 3.
Number of cells resting on or adhered to protein precoated Hyal-N_3, HyalS-N_3 and oxidised polystyrene (TCPS) after 2 hours incubation in the 24-well plates and a PBS rinse. Error bars: SD (n = 6). * = t test, $P < 0.01$ with respect to Hyal-N_3 with the same protein coating. ** = t test, $P < 0.01$ with respect to albumin coated Hyal-N_3 and *** = t test, $P < 0.01$ with respect to albumin coated HyalS-N_3.

reported that fibronectin could not be detected on these polymers after the similar rinsing treatment as determined by ATR/FTIR. The difference in result may be due to differences in the thickness of the polymers.

Cell adhesion on protein precoated Hyal-N_3 and HyalS-N_3 was also observed after 2 hours incubation in TCPS plates. Cells in contact with the test materials were rinsed with PBS before imaging (Figure 3). Protein coated (albumin, fibronectin or serum) TCPS plates were used as control surfaces and showed low levels of cell adhesion on the albumin coating, while maximal levels of cell adhesion were shown on the fibronectin and serum coatings. Low levels of cell adhesion were observed on each of the protein precoated Hyal-N_3 polymers although fibronectin precoated Hyal-N_3 was found to support significantly ($P < 0.01$) more cell adhesion than albumin and serum precoated Hyal-N_3. Low levels of cell adhesion were also observed on albumin precoated HyalS-N_3 after PBS rinses. In contrast high levels of cell adhesion were observed on fibronectin, with levels comparable to fibronectin and serum precoated TCPS. The level of cell adhesion on serum precoated HyalS-N_3 was found to be significantly ($P < 0.01$) higher than on albumin precoated HyalS-N_3 but significantly ($P < 0.01$) less than the level of cell adhesion obtained on fibronectin precoated HyalS-N_3. These results are in agreement with previous studies which show that HyalS-N_3 is able to promote cell adhesion and proliferation while Hyal-N_3 has been shown to have an inhibitory effect on cell adhesion.[19]

Conclusions

Similar amounts of protein adsorption were measured on both Hyal-N_3 and HyalS-N_3 after rinses with PBS, while rinsing with MilliQ, SDS and urea revealed a higher level of irreversible fibronectin binding on HyalS-N_3 compared with Hyal-N_3. Proteins adsorbed onto HyalS-N_3 were found to be more loosely bound than those adsorbed onto Hyal-N_3. Cell adhesion to Hyal-N_3 was minimal when precoated with either albumin or serum, whilst fibronectin precoating enhanced cell adhesion on this material, but not to the level found with tissue culture polystyrene. Cell adhesion was enhanced on fibronectin-precoated HyalS-N_3 in comparison with fibronectin-precoated Hyal-N_3, as well as albumin and serum precoated HyalS-N_3. Fibronectin

coated HyalS-N_3 was the only material to reach a level of cell adhesion comparable to TCPS. Hence it appears that protein speciation as well as the underlying substrate plays a role in the modulation of biological activity including cell adhesion.

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