Exploring the Efficiency of a Surface-Tethered Hyaluronan Model for Testing Endothelial Cell Responses

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Statement of Purpose: Crosslinked gels (hylans) containing long-chain (MW>1x10^6 Da) hyaluronan (HA), a connective tissue GAG, show exceptional biocompatibility for vascular implantation but poorly interact with vascular endothelial cells (ECs). To overcome this, it may be necessary to incorporate smaller, more biologically responsive, HA fragments into hylan gels. Since fragmented HA can be pro-inflammatory, it is important to determine an optimal size distribution of HA fragments on the hylan surface that will recruit and support a functional EC monolayer while limiting an inflammation response. To assess these responses we seek to create HA-tethered cell culture surfaces.

Methods: HA with molecular weights of 1000 KDa, 200 KDa and 22.48 KDa (HA 1000, HA 200, HA 22.48) were separately immobilized onto each chamber of a 4-well glass chamber slide using an adaptation of a previously described method.[1] To bind HA, the chamber slides were rendered reactive by amination with an amino-silane.[2] A 750 µl volume of 3% (v/v) 3-aminopropyl-trimethoxysilane/ ethanol was applied to the wells for 20 minutes, briefly washed with 100% ethanol and air dried. The chamber slides were then oven heated (115°C, 1 h), rinsed with 95% ethanol, and air-dried. Successful immobilization of amines was confirmed with an amine reactive-fluorescent probe, SEM and XPS. The surface density of amine groups was quantified by spectrophotometric detection (λ= 498 nm) of the 4, 4'-dimethoxytrityl cation upon its release when the amine groups are reacted with sulfo-succinimidyl-4-O-(4,4'-dimethoxytrityl) butyrate (sulfo-SDTB).[3] Using a carbodiimide/N-hydroxysulfosuccinimide (NHS) reaction chemistry, the bound amines were then reacted with carboxyl groups present on the HA chains, then soaked in DI water for 24 hours and air dried.[4] Surface-bound HA was visualized through immunofluorescence, using a biotinylated HA binding protein (HABP) and rhodamine-conjugated streptavidin along with SEM and XPS. Dissolution of HA from the surface during incubation in culture medium over 21 days was assessed by (i) detection of bound HA using toluidine blue and (ii) digestion of bound HA with *hyaluronidase SD*. Analysis of the digest containing HA disaccharides is in progress and will be visualized using FACE. The band content will be quantified by comparison with the intensity of standard bands containing known disaccharide amounts. The pH of the incubation medium was also monitored over 21 days to ensure lack of acid-induced degradation of HA. In ongoing cell culture experiments, rat aortic endothelial cells (RAEC) have been seeded onto glass, aminated, and HA-coated surfaces and are being cultured in MCDB-131 medium supplemented with VEGF (10 ng/ml), fetal bovine serum (10%), ECGS (50 µg/ml), glutamine (4 mmol/L), penicillin/streptomyosin (1%) and heparin (30 UI/ml) for 21 days. At 1, 4, 7, 14, and 21 days of culture, the cell are subject to a DNA assay to assess cell proliferation on the respective substrates. The density of live cells is also being assessed using a fluorescent cell label, Calcein AM

Results / Discussion: Fluorescence microscopy indicated successful amine attachment to the glass slide. The amine reactive dye was applied to an unmodified glass slide to confirm no nonspecific binding occurred. A bright green coloration showed the successful reaction of the fluoraphor with the APTMS primary amine. The sulpho-SDTB assay revealed a surface density of 9 ± 3 amine groups /nm^2 HA was covalently bound to APTMS and imaged using confocal microscopy. The rhodamine fluorescence revealed a HA surface comprised of flat sheets and fibrous networks. HA of smaller fragment sizes contained a higher percentage of fibrous networks. For a control, HA slides were labeled with neutravidin. FITC labeled fluorescent HA (90 KDa) was also bound to the glass slide. Amine slides were imaged under FITC fluorescence as a control. The glass, APTMS and HA surfaces were examined with SEM. The glass surface was plasma treated to promote cell attachment and appears smooth with speckled marks due to the plasma treatment. Upon the introduction of APTMS groups, small rod shaped structures appear on the surface indicating successful incorporation of APTMS onto the glass. HA was then bound to APTMS. HA 1000 appeared as a flat homogeneous sheet while HA 200 and HA 22.48 contained both flat sheets and fibrous networks. The XPS results showed that unmodified glass slides contained large quantities of silicone and oxygen as predicted by the chemical structure of glass. After the incorporation of APTMS, the surface contained a significant amount of nitrogen indicating the presence of amine groups. An increase in carbon and decreases in silicone and oxygen was attributed to the chemical structure of APTMS. HA surfaces were accurately measured to be comprised primarily of carbon and oxygen with a small amount of nitrogen. TBO bound to the carboxyl groups of HA attached to the glass surface removing TBO molecules from an aqueous solution. TBO has a high absorbance at 630 nm and the depletion of TBO in solution was quantified using a spectrophotometer. The reaction between HA and the amine groups on APTMS involves activating the carboxyl groups on HA with NHS. If these carboxyl-NHS complexes near primary amines, the carboxyl binds to it. Otherwise, they remain intact. TBO also binds to the carboxyl groups of HA and is affected by the number of NHS molecules blocking their attachment. In addition, the chance of a free carboxyl group is higher on a long strand of HA. Therefore, this assay was only able to compare surface bound HA within a specific molecular weight and a direct measurement of HA on the surface cannot be reported with this method because of the NHS inhibition of the carboxyl groups. Overall, the amount of HA on the surface did not change during a 21 day period within each MW group. No change in pH of the medium was observed during this period. Cell culture studies indicate viable cells on glass, amineated, and HA-coated surfaces. Cells on the glass and HA slides were spread, the latter tended to form clumps, while those on the aminated slides were rounded. Proliferation experiments are currently in progress.

Conclusions: The results show that fairly homogeneous HA surfaces were created on a glass slide by amine activation and a carbodiimide reaction. A minimal loss of bound HA was observed when incubated in culture media for 21 days. FACE and cell culture experiments will follow. These results are useful towards determining the size- and dose-specific responses of surface-tethered HA on endothelial cell response and tailoring the design of HA scaffolds suited to different tissue engineering applications.

References:

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