Supramolecular protein-mediated assembly of brain extracellular matrix glycans [version 1; referees: awaiting peer review]

Anthony Tabet\(^1\), Kamil Sokolowski\(^1\), Jarrod Shilts\(^2\), Marlous Kamp\(^1\), Nina Warner\(^1\), Dominique Hoogland\(^1\), Oren A. Scherman\(^1\)

\(^1\)Melville Laboratory for Polymer Synthesis, Department of Chemistry, University of Cambridge, Cambridge, UK
\(^2\)Wellcome Trust Sanger Institute, Hinxton, UK

Abstract

**Background:** Hyaluronic acid (HA) is the major component of the extracellular matrix in the central nervous system and the only supramolecular glycosaminoglycan. Much focus has been given to using this high molecular weight polysaccharide for tissue engineering applications. In the majority of cases, HA is covalently functionalized with moieties that can facilitate network formation through physical self-assembly, or photo-catalyzed covalent crosslinking as the polysaccharide does not gel on its own. However, these covalent crosslinks are not the driving force of HA self-assembly in biological tissues.

**Methods:** Oscillatory rheology and dynamic light scattering were used to study albumin/HA structures. Dynamic light scattering and transmission electron microscopy were used to study albumin/chondroitin sulfate (CS) structures. UV-vis spectroscopy was used to study mass transfer of a hydrophilic small molecule into the albumin/HA/CS materials.

**Results:** In this work we examine the intermolecular interactions of two major glycans found in the human brain, HA and the lower molecular weight CS, with the protein albumin. We report physiochemical properties of the resulting supramolecular micro- and nanomaterials. Albumin/HA mixtures formed supramolecular gels, and albumin/CS mixtures formed micro- and nanoparticles. We also summarize the concentrations of HA and CS found in various mammalian brains.

**Conclusions:** Simple preparation and combination of commercially available charged biomacromolecules under short time-scales can result in interesting self-assembled materials with structures at the micron and nanometer length-scales. Such materials may have utility in serving as cost-effective and simple models of nervous system electrostatic interactions and as in vitro drug release and mass transfer quantification tools.

Keywords

hyaluronic acid, chondroitin sulfate, protein-polymer assembly
Introduction
A major paradigm that has dominated the drug delivery and tissue engineering communities is the development of bio-inspired hydrogels that mimic the intermolecular interactions and mechanical properties of physiological tissue. Glycosaminoglycans (GAGs) are polysaccharides that are critical structural components of the brain extracellular matrix (ECM). One particularly abundant brain GAG, hyaluronic acid (HA) (Figure 1A), has been made into many covalently modified derivatives that have been widely explored in drug delivery and tissue engineering. However, HA is the only supramolecular brain GAG, and other ECM components including the more abundant protein-linked GAG chondroitin sulfate (CS) have been comparatively under studied. HA is often chemically functionalized because the high molecular weight polysaccharide does not gel on its own. Although functional materials based on covalently modified HA exhibit useful features for many applications, these gelation methods do not capture the physiological supramolecular interactions that are endogenously found in the brain extracellular matrix.

In this work we examine the intermolecular interactions of two major biopolymer components of the brain, HA and CS, with the model protein bovine serum albumin (BSA) (Figure 1B). We exploit known electrostatic interactions between the BSA and GAG polymers to generate structurally diverse protein-glycan complexes. We examine how these negatively charged biopolymers interact and self-assemble with BSA, and develop supramolecular systems formed from competing HA/BSA, HA/CS, and CS/BSA interactions.

Methods
Chemicals and reagents
All starting materials were purchased from Sigma Aldrich and used as received unless stated otherwise.

Electrostatic surface potential modeling
The crystal structure of bovine serum albumin (BSA) was downloaded from Protein Data Bank (ID:3V03). The second chain of the homodimer in the crystal structure was removed to display BSA in monomeric form. Using the Adaptive Poisson-Boltzmann Solver (APBS) tool available through PyMOL v2.2.2, the electrostatic surface potential was calculated under default parameters. The section of the protein surface showing the previously-described GAG binding pocket was rendered with PyMOL.

Particle formation
A 10 wt% chondroitin sulfate (C9819 Sigma) solution was prepared by mixing the polymer powder in Milli-Q H$_2$O (18 mΩ) at room temperature overnight. Bovine serum albumin (BSA; 50 mg/mL) was added to the solution and rigorously mixed for 12 hours (1000 RPM) at room temperature. Lower total concentrations of polymer and protein took more than 12 hours to form particles.

Gel formation
4 wt% hyaluronic acid (1.5-1.8 MDa; 53747 Sigma) solutions were prepared by mixing the polymer powder in Milli-Q H$_2$O (18 mΩ) at 40 °C for 40 hours. The solution was sealed and stored at 4 °C until further use. BSA (50 mg/mL) was added to

Figure 1. (A) Structures of chondroitin sulfate (CS) and hyaluronic acid (HA). (B) Relative surface charge densities of bovine serum albumin (BSA). Dataset 1: Dynamic light scattering and transmission electron microscopy data.
the viscous polysaccharide solution and mixed with a metal spatula for several minutes. The samples were sealed until they appeared transparent, typically for at least 24 hours. It was observed that BSA added without stirring would not result in the same behavior, and appeared to lower the solution viscosity instead. To form HA/CS/BSA systems, dry chondroitin sulfate (10 wt%) was added along with the dry BSA and allowed to homogenize with stirring (200 RPM). Poly(caprolactone) (PCL) blends were formed by mixing poly(caprolactone) diol (M₆ = 2 kDa; 5 wt%) with poly (caprolactone) diol melt (M₆ = 550 Da) and mixing at 700 RPM at 50 °C overnight. A specified amount of rhodamine B was added to the blend for Ultraviolet–visible spectroscopy (UV-Vis) studies.

Rheology
All rheological sweeps were conducted on an AR-G2 Rheometer (TA Instruments, New Castle, DE, USA) with a 40 mm parallel plate geometry at 20.0 °C. Zero gap, rotational mapping (precision bearing mapping; 2 iterations), geometrical inertia, and friction calibrations were done prior to each use of the rheometer. Samples were loaded onto the rheometer with a 600–1000 µm loading gap. A water trap was placed to prevent dehydration. Amplitude sweep were conducted to determine a strain in the linear viscoelastic region.

Dynamic light scattering
Dynamic light scattering (DLS) measurements were carried out on a Malvern Zetasizer NS90 instrument at room temperature and standard settings. Samples were analysed in a 1.5 mL PS cuvette (Fisher Brand).

Transmission electron microscopy
Transmission electron microscopy (TEM) was carried out on a FEI Philips Tecnai 20. Samples were prepared on holey carbon grids by pipetting 1 µL of desired aqueous solution and allowing it to evaporate under ambient conditions (drop-casting). Particle size distributions were calculated by counting the diameters of more than 100 particles.

UV-Vis Spectroscopy
UV-Vis spectroscopy was performed using a Mikropack DH-2000 UV-Vis-NIR Halogen light source and an OceanOptics USB2000 Fiber Optic Spectrometer. Spectra from 375 nm to 750 nm were recorded at 150 ms integration time and time intervals of 60 s.

Compiling brain glycosaminoglycan measurements
To estimate brain chondroitin sulfate (CS) levels, all articles cited as using the Blyscan assay to measure sulfated GAGs were searched against the keywords “brain” and “neural”. A total of 7 articles were found meeting the criteria of measuring sulfated GAGs in mammalian brain tissue. A separate literature search of hyaluronic acid (HA) measurements identified 2 articles. Reported concentration values were converted to molarities, representing the moles of disaccharide repeat units per volume of native brain tissue, assuming a brain density of 1.04 g/ml. In cases where brain weight was reported as dry mass instead of native tissue, masses were converted by assuming that 77% of brain weight is water.

Results and discussion
In this work we explored the electrostatically-driven self-assembly of charged proteins with negatively-charged polysaccharides endogenous to the human brain. To mimic the native interactions these polysaccharides have with surrounding proteins, we introduced BSA, which has electrostatic binding pockets complementary to anionic GAGs, analogous to the binding interfaces of ECM proteins. BSA and CS polymer solutions were mixed overnight and allowed to self-assemble into nanostructures (ESI). Two distinct populations of particles were observed to form (Figure 2, Dataset 1), and were dissimilar to the flocculation of BSA aggregates alone. The smaller particles were characterized with dynamic light scattering (DLS) and transmission electron microscopy (TEM). DLS yielded an average particle diameter (D) of 51 ± 3 nm. These structures were stable for at least 2 days in the parent suspension (Figure S1A, Extended Data). DLS autocorrelation data suggested that a large diameter species may also be present in the solution (Figure S1B, Extended Data). TEM was used to characterize these self-assembled microparticles (Figure 2D). The analysis of these micrographs indicated the presence of two distinct populations of assemblies, with the mean diameter of D = 60 ± 10 nm, that is consistent with DLS experiments, and an additional one of D = 1.5 ± 0.5 µm. In the brain, CS is covalently scaffolded onto peptide cores (e.g. aggrecan) and binds with many ECM proteins through non-covalent, supramolecular interactions. The supramolecular interactions between CS and BSA described here could provide insight into an electrostatic driving force that contributes to GAG aggregation and nanostructure formation in vivo.

We then turned our attention to HA, a linear high molecular weight polysaccharide that is the only supramolecular GAG in human physiology. HA and BSA were mixed overnight and the electrostatic interactions between mixture components led the solution to self-assemble into a supramolecular gel (Figure 3, Dataset 1'). Supramolecular gels formed via electrostatic protein-polymer interactions with polysaccharide back-bones have been previously reported. Solutions of HA alone were highly viscous but did not show gel-like properties. Oscillatory rheological measurements were used to probe the mechanics of this supramolecular gel (ESI). After introduction of BSA, electrostatic-driven network percolation resulted in a major stiffening effect and the formation of a gel with G' > 10 kPa. This material exhibited very clear shear-thinning and recovery behavior (Figure 3C).

Upon introduction of dry CS into a HA/BSA matrix, a sharp decrease in stiffness was observed (Figure S3, Extended Data'). We hypothesize this effect is due to both interference from CS/BSA interactions and the reduction of entanglement of HA. DLS data showed that when combined with either HA or CS, BSA exhibited hierarchical assemblies that were not observed for BSA alone. Furthermore, when combined with
Figure 2. Self-assembly of chondroitin sulfate (CS) and bovine serum albumin (BSA) particles. (A) Schematic of the formation of dense CS-BSA particles. (B) DLS size plot of dynamic BSA aggregates (top) and CS-BSA particles (bottom). (C–D) TEM image of CS-BSA nanoparticles (C) and microparticles (D).

HA, the BSA assemblies were more dynamic and polydispersed than with CS (Figure 3D, S2).

We then explored whether these HA/CS/CS systems could be used to explore mass transfer of the model hydrophilic drug rhodamine B (rhodB). Many parenteral drug-delivery studies will monitor *in vitro* release kinetics into saline as a model for *in vivo* release. Here we explore the feasibility of monitoring release kinetics into a gel instead of saline (Figure 4, Extended Data). A hydrophobic blend of poly(caprolactone) (PCL) chains at different molecular weights (ESI) loaded with rhodB was carefully added on top of the hydrogel phase (Figure 4, Extended Data). We found that it was possible to monitor the interfacial concentration of rhodB in the hydrogel phase with UV-Vis spectroscopy. Interestingly, we observed a large bolus release of the hydrophilic drug from the hydrophobic phase to the hydrophilic phase at the interface until the same concentration was reached, subsequently an equilibrium or pseudo-steady-state concentration was reached after 14 hours. Such a system is potentially useful in modeling mass transfer of drugs between hydrophobic drug delivery materials and biological tissue such as the brain.

Prior tissue engineering studies have largely neglected the question of how the exact composition of the brain ECM might inform efforts to create biologically-mimetic hydrogels. To estimate the physiological concentrations of CS and HA that occur *in vivo* (Figure 5, Dataset 1), we summarize the literature for CS and HA measurements of mammalian brain tissue. These estimates clustered in the millimolar range for CS disaccharides, and 100 micromolar range for HA.
Figure 3. Oscillatory rheological sweeps of 40 mg/mL hyaluronic acid (HA) and 50 mg/mL bovine serum albumin (BSA) gels. (A) Schematic illustration dynamic network formation of HA and BSA. (B) Oscillatory rheological frequency sweep of HA solution alone and HA-BSA gels. (C) Oscillatory time sweep after 100% shear of HA-BSA gels and HA solution alone. (D) DLS size measurements of dynamic BSA particles alone and HA-BSA gel. Dataset 1: Rheology and dynamic light scattering data.

Conclusions

In this work we characterize supramolecular interactions between HA, CS, and BSA. We report nano- and microparticle self-assembly, gelation, and network interference behaviors. We also report on the mass transfer of a model drug from a hydrophobic phase into a glycan-based hydrogel. Finally, we summarize reported concentrations of HA and CS in different animal models. This report may inform the development of biomaterials for tissue engineering that capture or exploit supramolecular interactions between brain extracellular matrix glycans.
**Figure 4.** Measuring interfacial mass transfer kinetics between hyaluronic acid (HA)/bovine serum albumin (BSA)/chondroitin sulfate (CS) system and PCL blend. (A) Picture of initial two phases measured via UV-Vis spectroscopy. (B) Concentration at interface after large bolus release over time. Hydrophobic phase drug concentration = 10 µg/mL. Average hydrophilic phase equilibrium concentration = 14.5 µg/mL. The periodic fluctuations in concentration were attributed to trapped air interfering with the interface. (Dataset 1: UV-Vis data.)

**Figure 5.** Summary of concentrations of hyaluronic acid (HA) and chondroitin sulfate (CS) in the brain of various species. The concentration of HA in healthy tissue, glioblastoma, and astrocytoma is also plotted.
Data availability
Underlying data for this study is available from Open Science Framework

OSF: Dataset 1: Supramolecular Protein-Mediated Assembly of Brain Extracellular Matrix Glycans, https://doi.org/10.17605/OSF.IO/3BXQG

Data is available under a CC0 1.0 License

Extended data
Electronic supplementary information (ESI) document is available from Open Science Framework.

OSF: Extended Data. Supramolecular Protein-Mediated Assembly of Brain Extracellular Matrix Glycans ESI https://doi.org/10.17605/OSF.IO/NG6H7

The ESI contains the following Supplementary Figures -

Figure S1: Autocorrelation function of chondroitin sulfate (CS)/bovine serum albumin (BSA) NPs. (B) Time resolved DLS of CS/BSA particles in Milli-Q water.

Figure S2: Time resolved dynamic light scattering of hyaluronic acid (HA)/bovine serum albumin (BSA) gels.

Figure S3: (A) Oscillatory frequency sweeps of hyaluronic acid (HA) systems loaded with bovine serum albumin (BSA) and with or without chondroitin sulfate (CS). (B) Time-resolved dynamic light scattering experiment of CS/BSA particles loaded with HA and sheared.

Extended data is available under a CC0 1.0 License

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References

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