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RESEARCH ARTICLE

Supramolecular protein-mediated assembly of brain extracellular matrix glycans [version 1; peer review: 2 approved with reservations]

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V1 First published: 20 Nov 2018, 7:1827
<https://doi.org/10.12688/f1000research.16929.1>
Second version: 12 Apr 2019, 7:1827
<https://doi.org/10.12688/f1000research.16929.2>
Latest published: 08 Nov 2019, 7:1827
<https://doi.org/10.12688/f1000research.16929.3>

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

Open Peer Review

Approval Status

	1	2	3
version 3 (revision) 08 Nov 2019			
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Any reports and responses or comments on the article can be found at the end of the article.

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

This article is included in the **Nanoscience & Nanotechnology** gateway.

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Author roles: **Tabet A:** Conceptualization, Investigation, Methodology, Writing – Original Draft Preparation, Writing – Review & Editing; **Sokolowski K:** Investigation, Writing – Review & Editing; **Shilts J:** Investigation, Writing – Review & Editing; **Kamp M:** Investigation, Writing – Review & Editing; **Warner N:** Investigation, Writing – Review & Editing; **Hoogland D:** Investigation, Writing – Review & Editing; **Scherman OA:** Conceptualization, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: AT and JS thank The Winston Churchill Scholarship Foundation of the United States for funding support. *The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

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How to cite this article: Tabet A, Sokolowski K, Shilts J *et al.* **Supramolecular protein-mediated assembly of brain extracellular matrix glycans [version 1; peer review: 2 approved with reservations]** F1000Research 2018, **7**:1827 <https://doi.org/10.12688/f1000research.16929.1>

First published: 20 Nov 2018, **7**:1827 <https://doi.org/10.12688/f1000research.16929.1>

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 engineering¹. However, HA is the only supramolecular brain GAG², and other ECM components including the more abundant protein-linked GAG chondroitin sulfate (CS) have been comparatively under studied³. 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 matrix⁴.

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^{5,6} to generate structurally diverse proteoglycan 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 H₂O (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 H₂O (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 data⁷.

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 ($M_n = 2$ kDa; 5 wt%) with poly(caprolactone) diol melt ($M_n = 550$ 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 μ m 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 μ L 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 1⁷), 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 Data⁸). DLS autocorrelation data suggested that a large diameter species may also be present in the solution (Figure S1B, Extended Data⁸). 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 $D = 60 \pm 10$ nm, that is consistent with DLS experiments, and an additional one of $D = 1.5 \pm 0.5$ μ m. In the brain, CS is covalently scaffolded onto peptide cores (e.g. aggrecan⁴) and binds with many ECM proteins through non-covalent, supramolecular interactions⁹. 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 1⁷). Supramolecular gels formed *via* electrostatic protein-polymer interactions with polysaccharide back-bones have been previously reported¹⁰. 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 Data⁸). 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, S2⁸).

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 Data⁸). 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 Data⁸). 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 brain¹¹.

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 1⁷), we summarize the literature for CS and HA measurements of mammalian brain tissue^{12–19}. 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/3BXQG7>

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/NG6H78>

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 NPs 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

Grant information

AT and JS thank The Winston Churchill Scholarship Foundation of the United States for funding support.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Version 1

Reviewer Report 12 February 2019

<https://doi.org/10.5256/f1000research.18510.r42740>

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Jessica CF. Kwok

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The article 'Supramolecular protein-mediated assembly of brain extracellular matrix glycans' by Tabet et al., describes the self-assembly of glycosaminoglycans hyaluronan (HA) and chondroitin sulfate (CS) in the presence of bovine serum albumin (BSA). The authors report that HA/BSA forms hydrogel upon mixing while CS/BSA forms nano- and micro-particles.

The recognition of supramolecular assembly with biological materials has increased our understanding of how biological systems function in the last years. Here, the authors aim to address how HA and CS self-assemble for their function, emphasising on their role in the central nervous system. However, the study appears partial and the hypothesis is not clear.

Hypothesis is not clear: Why do you use serum albumin? Serum albumin is not normally present in the brain tissue but confines inside the blood vessels in the brain. The penetration of serum albumin to the brain is protected by the blood-brain barrier. Extravasation of albumin to the brain will only happen in pathological conditions. This means that serum albumin is not the normal contributor to brain extracellular matrix (ECM) glycan assembly. This needs to be stated clearly in the article. Resulting from this, the title is also misleading, because the model described is thus not related to brain ECM.

Please define clearly the meaning of 'supramolecular' in the article. In both the Abstract and the Introduction, the authors claimed that HA is the 'only supramolecular glycosaminoglycan'. What do you mean by this? However, in the main text, the authors described CS/albumin interaction as 'supramolecular interactions'. Please define clearly the meaning of the term.

Regarding the CS/albumin interaction, the authors attribute particle formation from mixing CS with serum albumin is due to supramolecular interactions between CS and BSA. How can you rule out that this is not due to phase separation of your materials instead? Are the nano- and micro-particles stable over time?

Regarding the model of drug release, the authors used rhodamine by laying it on top of the hydrogel. This is showing diffusion of molecules through the gel, but not the release of rhodamine from the gel. Please correct the terminology accordingly.

Regarding the title, please change glycans to glycosaminoglycans.

Minor changes:

In the abstract, the first sentence: HA is 'a' major component of ECM in the CNS, but not "the" major component. CS is present in higher concentration than HA in the CNS, which is also indicated by the analysis reported in Figure 5 in the article. Please correct the sentence.

Figure 1: The CS shown in Figure 1A is chondroitin 6-sulfate, while the CS used in the experiment (Sigma C9819) is a chondroitin 4-sulfate. Could you please amend the figure to represent the structure of the CS used?

Figure 1: The HA structure is wrong. HA is formed by disaccharide repeats of [GlcNAc- β 1,4-GlcA- β 1,3]. The figure shows a [GlcNAc- β 1,4-GlcA- β 1,4] linkage. Please correct this accordingly.

Page 4, first paragraph in the result section: 'CS is covalently scaffolded onto peptide cores (e.g. aggrecan)'. Please change peptide into protein. Aggrecan is a big protein with molecular weight larger than 250 kDa even without the attachment of glycosaminoglycan chains.

Figure 3C: what are the three lines in Figure C? Please state clearly, either in the figure or in the legend.

Page 5, I assume 'HA/CS/CS' systems actually mean 'HA/CS/BSA' systems?

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Not applicable

Are all the source data underlying the results available to ensure full reproducibility?

No source data required

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Extracellular matrix, glycosaminoglycan, central nervous system, neuroplasticity, neuroregeneration

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 03 December 2018

<https://doi.org/10.5256/f1000research.18510.r40915>

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Laura De Laporte

DWI — Leibniz-Institute for Interactive Materials, Aachen, Germany

The paper looks at the interaction of natural GAG, such as HA and CS, with BSA as model protein. Overall the data is clear but some improvements can be made.

Are the particles formed with CS/BSA hard particles or nano, microgels? You can do AFM to check this, by measuring the swelling behavior in media and determining the Young's modulus.

For the HA/BSA interaction, gels are formed. Here more analysis can be done. The rheometer data only shows one data point. The G' for multiple gels can be measured to do statistics. Ideally these measurements should also be compared with media as the salts will affect the mechanical properties of the gel. Also, the gels seem to be very stiff for these natural materials. You decrease the concentrations to check whether you can vary the stiffness. Nervous tissue is usually in the range below 1 kPa. The authors mention the gel is shear thinning but this is not proven with rheology. Even a video of the gel would be helpful to have an idea about its properties, like transparency, shear thinning properties via pipetting, and gelation. In addition, even though SEM may cause some artifacts, it will still give you an idea about the internal structure of these gels compared to other gels reported in the literature. In Figure 3C, there is not legend and unclear what the different conditions are. For Figure 3D, as you have a gel, how can you also have individual particles for DLS measurements? Please make a note about this in the text.

Figure 4: As Rhodamine is fluorescent, you can show fluorescent images to demonstrate the diffusion of the molecule into the gel.

Some minor comments to improve the text:

Abstract:

- Self-assembly
- HA has been crosslinked covalently via other mechanisms than photo-catalyzed ^{1,2,3}.

Introduction:

- The authors mention that HA has been made into many covalently modified derivatives. Do they mean that functional groups were covalently bound to the HA backbone to make derivatives, or that functionalized HA can covalently bind to other molecules to form drug delivery and TE materials. This is repetitive with a sentence later in the intro: 'HA is often chemically modified.....'

Methods:

- The GAG binding pocket of BSA is not shown in Figure 1B.
- BSA should only be written full once, after that just use the abbreviation.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: hydrogels, tissue engineering

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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