





RESEARCH ARTICLE

REVISED Protein-mediated gelation and nano-scale assembly of unfunctionalized hyaluronic acid and chondroitin sulfate [version 3; peer review: 2 approved, 1 approved with reservations]

Previously titled: Supramolecular protein-mediated assembly of brain extracellular matrix glycans

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Abstract






Background: Hyaluronic acid (HA) is a major component of the extracellular matrix (ECM) in the central nervous system and the only purely supramolecular glycosaminoglycan. Much focus has been given to using this high molecular weight polysaccharide for tissue engineering applications. In most studies, the backbone of HA is functionalized with moieties that can facilitate network formation through physical self-assembly, or covalent crosslinking (e.g. photo-catalyzed) at concentrations where the polysaccharide does not gel on its own. However, these crosslinks often utilize functional groups not found in biological tissues.



Methods: Oscillatory rheology, dynamic light scattering, and scanning electron microscopy 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 demonstrate the potential for using protein-polymer blends as an ECM-mimetic model to study transport of small molecules.

Results: We examine the intermolecular interactions of two major glycosaminoglycans found in the human brain, HA and the lower molecular weight CS, with the model protein albumin. We report the properties of the resulting micro- and nano materials. Our albumin/HA systems formed gels, and albumin/CS systems formed micro- and nanoparticles. These systems are formed from unfunctionalized polysaccharides, which is an attractive and simple method of forming HA hydrogels and CS nanoparticles. We also summarize the concentrations of HA and CS found in various mammalian brains, which could potentially be useful for biomimetic scaffold

Open Peer Review

Reviewer Status   

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development.

Conclusions: Simple preparation of commercially available charged biomacromolecules results in interesting materials with structures at the micron and nanometer length-scales. Such materials may have utility in serving as cost-effective models of nervous system electrostatic interactions and as in vitro drug release and model system for ECM transport studies.

Keywords

hyaluronic acid, chondroitin sulfate, protein-polymer assembly

Any reports and responses or comments on the article can be found at the end of the article.

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REVISED Amendments from Version 2

We thank the reviewers for their comments. The rheological data plots were reformatted per reviewer suggestions to reduce confusion. The analyses of the light scattering and rheological data are also clarified to emphasize the conclusions that can be reached within this experimental paradigm. In particular, the qualitative nature of this light scattering data was highlighted. We also expand upon the importance of mixing order in HA/BSA gel formation to highlight the difference between supramolecular and covalent systems. The illustrating figure and accompanying scanning electron microscopy and rheological data are updated. Finally, recent and relevant literature on this topic is also cited.

Any further responses from the reviewers can be found at the end of the article

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 engineering¹⁻³. Notably, HA is the only purely supramolecular brain GAG⁴⁻⁸, *i.e.* weaker and reversible non-covalent interactions dominate over covalent bond formation. Many groups have reported HA- based materials that consist of chemically functionalizing the polymer backbone with moieties that can facilitate formation of supramolecular (such as in physiology) or covalent networks^{9,10}. Networks formed

from unfunctionalized HA, however, have received considerably less attention in the literature. Furthermore, other ECM components, including the more abundant protein-linked GAG chondroitin sulfate (CS) (Figure 1A), have been comparatively under studied for biomaterial applications¹¹.

In this work we exploit interactions between two major biopolymer components of the brain, HA and CS, with the model protein bovine serum albumin (BSA) (Figure 1B). Albumin, a major component of blood, has limited permeability through the blood brain barrier (BBB) except in disease cases where the BBB is compromised^{12,13}. One study showed that microglia, the brain's resident immune cells, can synthesize albumin, potentially suggesting a more complicated role for the protein¹³. We demonstrate herein the potential for albumin subjected to thermal or mechanical force to form supramolecular complexes with unfunctionalized ECM polymers and drastically change their mechanical or topological properties. Heilshorn and colleagues previously reported on supramolecular hydrogels formed *via* protein interactions with polysaccharide backbones¹⁴⁻¹⁶. In the present work we exploit interactions between BSA and GAG polymers to generate structurally diverse protein-glycosaminoglycans complexes. We examine how these negatively charged biopolymers interact and self-assemble with BSA, and develop non-covalent and covalent systems formed from 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.

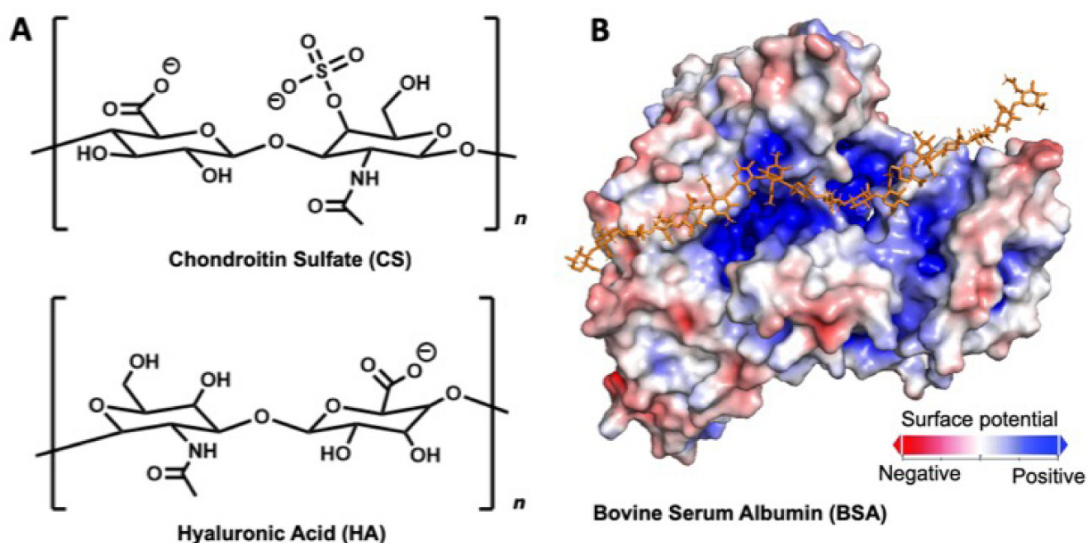


Figure 1. (A) Structures of chondroitin sulfate (CS) and hyaluronic acid (HA). (B) Relative surface charge densities of bovine serum albumin (BSA). BSA's binding pocket is indicated with the example HA chain.

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 parameters¹⁷. 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; A3294 Sigma) was added to the solution and rigorously mixed for 12 h (1000 RPM) at room temperature. Lower total concentrations of polymer and protein took more than 12 h 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 h. The solution was sealed and stored at 4 °C until further use. To electrostatically crosslink HA, BSA (A3294 Sigma) solutions were first heated to 80 °C for 2 h. The resulting solutions were mixed with HA solutions at room temperature and mixed rigorously at 1000 RPM until homogeneous, typically 20 min. The BSA concentration was varied but the HA concentration for HA/BSA systems was at 1 wt%. HA/BSA samples with heat treated albumin were observed to be opaque, whereas blends with unheated protein were transparent. It was observed that unheated BSA would not result in gel formation (see results and discussion). To form HA/CS/BSA blends, dry chondroitin sulfate (10 wt%) and dry BSA were added to HA solutions and mixed vigorously with a metal spatula. Poly(caprolactone) (PCL) blends were formed by mixing poly(caprolactone) diol ($M_n = 2$ kDa; Sigma 189421; 5 wt%) with poly(caprolactone) diol melt ($M_n = 550$ Da; Sigma 189405) 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). The covalent HA/BSA system used for SEM studies was formed by heating all components together to 80 °C for 2 h.

Rheology

Rheological sweeps were conducted on an Discovery HR-2 Rheometer (TA Instruments, New Castle, DE, USA) with 8 mm, 20 mm, or 40 mm geometries all at 20 °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 sweeps were conducted, usually at 10 rad/s, to determine the strains within 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.

Scanning electron microscopy

Scanning electron microscopy (SEM) was carried out on a Tescan MIRA3 SEM. Samples were freeze dried in small volumes and thin flakes were carefully mounted onto sample stubs covered in carbon conductive adhesive tapes. Each sample was then placed into a platinum sputtering system and sputter-coated to 10 nm thickness. To load the sample, the SEM chamber was vented and the sample stubs were loaded and tightened into places. After closing the sample compartment and allowing the system to reach vacuum, images were captured using operating voltage ranging from 10–30 kV.

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/mL¹⁸. 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 water¹⁹.

Results and discussion

In this work we explored the self-assembly of charged proteins with negatively-charged polysaccharides endogenous to the human brain. To facilitate supramolecular crosslinking of these polysaccharides, 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 rigorously overnight and allowed to self-assemble into nanostructures. Two distinct populations of particles were observed to form (Figure 2B–D, Dataset 1²⁰), 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

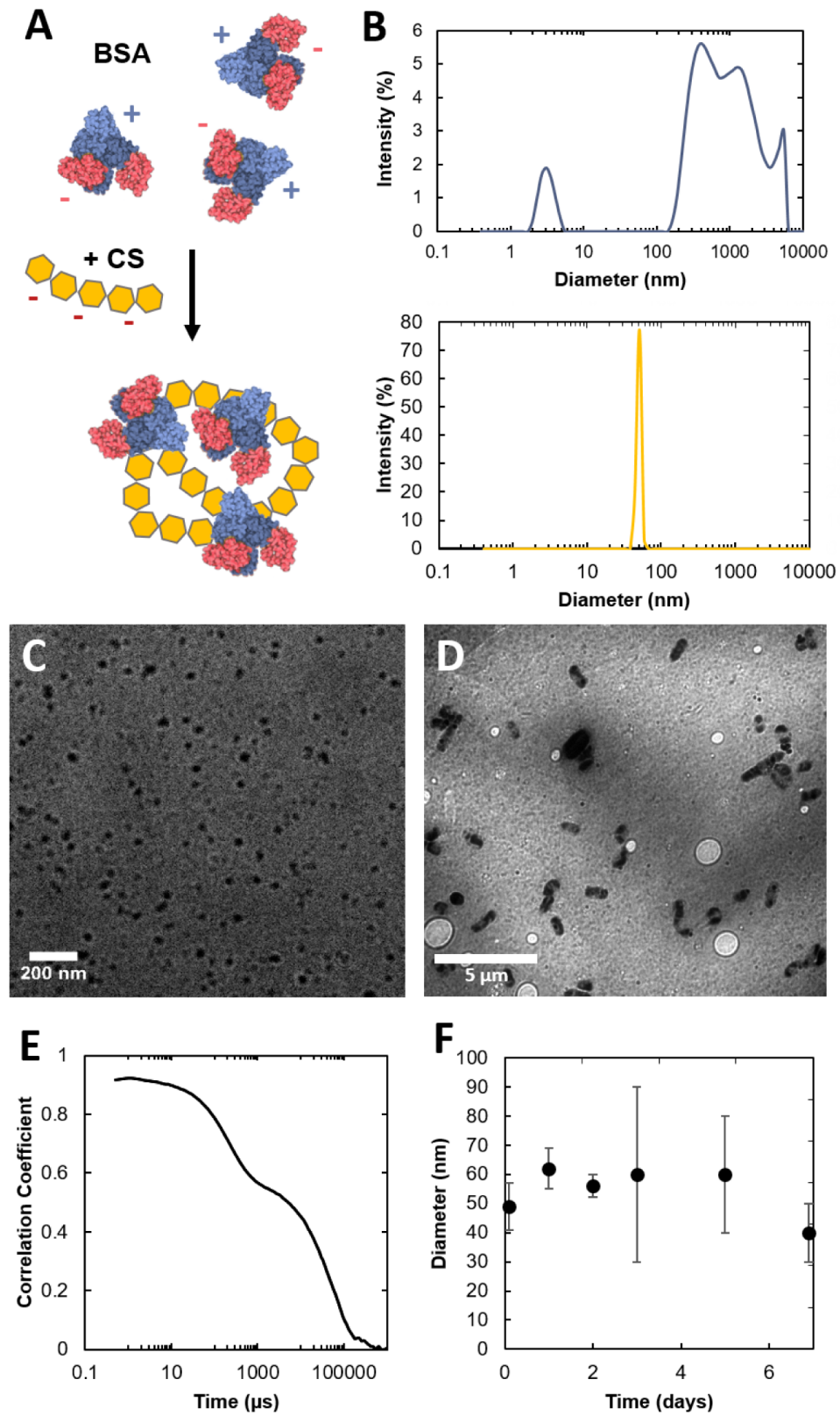


Figure 2. Self-assembly of chondroitin sulfate (CS) and bovine serum albumin (BSA) particles. (A) Schematic of the formation of dense CS-BSA particles. (B) DLS size plot of dynamic BSA aggregates (top) and CSBSA particles (bottom). (C–D) TEM image of CS-BSA nanoparticles (C) and microparticles (D). (E) Autocorrelation function of CS/BSA NPs. (F) Time resolved DLS of CS/BSA NPs. Dataset 1: Dynamic light scattering and transmission electron microscopy data²⁰.

stable for at least 2 days in the parent suspension (Figure 2E). DLS autocorrelation data suggested that a large diameter species may also be present in the solution (Figure 2F). 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 $D = 1.5 \pm 0.5$ μm . In the brain, CS is covalently scaffolded onto protein cores (e.g. aggrecan²¹) and binds with many ECM proteins through non-covalent, supramolecular interactions²². The non-covalent interactions

between CS and BSA described here, potentially owed to electrostatic interactions or phase separation, could provide insight into driving forces 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 heat treated BSA were mixed and the interactions between the charged moieties of the GAG and the albumin solution led to the self-assembly of a supramolecular gel (Figure 3–Figure 4, Dataset 1²⁰). Solutions of HA alone

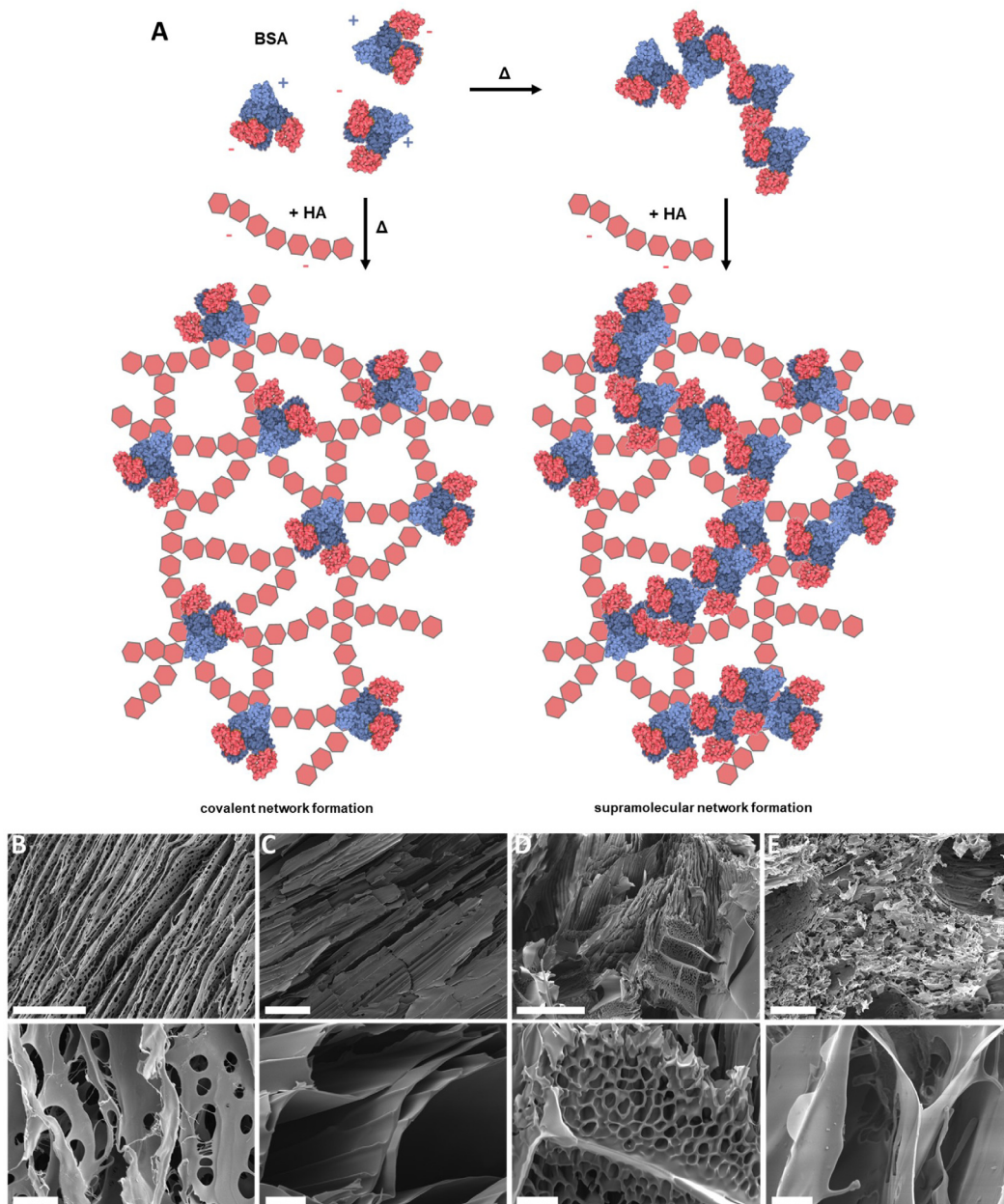


Figure 3. HA/BSA gels. (A) Illustration of strategies to form gels from BSA and HA. Changing the order of addition and protein concentration allows facile tuning of the mechanical and topological properties of the network. (B–E) SEM images of HA alone, HA/BSA (untreated and undenatured; 1:5), HA/BSA covalent gels²³ (1:5) which are not otherwise considered in this study, and supramolecular HA/BSA gels (1:5; see Figure 4). Scale bar = 100 μm (top), 10 μm (bottom). Dataset 1: Scanning electron microscopy data.

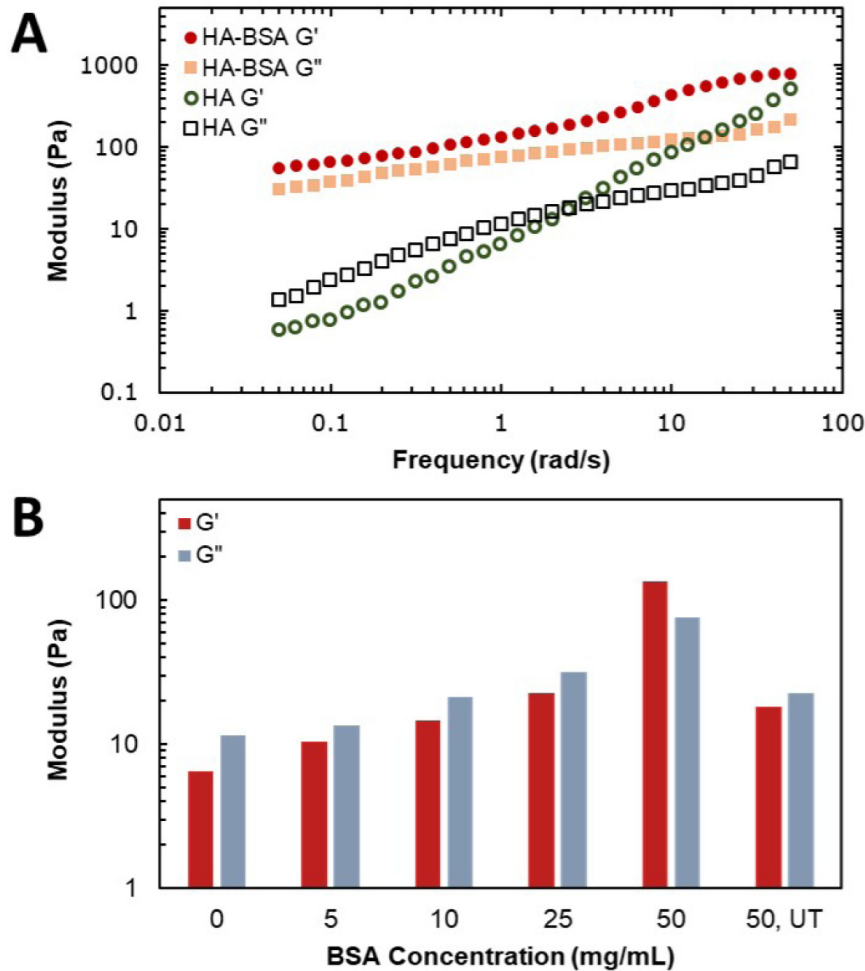


Figure 4. Rheological characterization of HA/BSA systems. (A) Oscillatory rheological frequency sweep of HA solution alone (10 mg/mL) and HA/BSA gel (BSA: 50 mg/mL). **(B)** Storage (G') and loss (G'') moduli of HA (10 mg/mL) with various concentrations of BSA at 1 rad/s. UT = untreated BSA. Dataset 1: Rheology data.

were viscous but did not show gel-like properties. Oscillatory rheological measurements were used to probe the mechanical properties of these materials. Introduction of BSA resulted in the formation of a gel with stiffness comparable to central nervous system tissue²⁴. It was observed that the same concentration of BSA without the heat treatment did not result in gelation (Figure 4B). This is perhaps owed to the fact that in its folded form, the BSA protein has a singular charged binding pocket that cannot facilitate multi-chain interactions (Figure 1B), and thus cannot serve as an effective crosslinker. Heat-treated BSA, however, shows greater affinity towards multi-chain interactions and crosslinks the system to form a physical gel. The differences in topology between these systems can be seen further in scanning electron microscopy (SEM) images (Figure 3B–E). The covalent and supramolecular systems differ from HA with non-treated BSA. The underlying structural

changes that occurs when this solution of BSA is heated remains to be elucidated and is exciting future work.

We then studied a HA/CS/BSA blend generated *via* mechanical force, similar to the CS/BSA particle system. DLS data qualitatively showed that when combined with these charged polysaccharides, BSA exhibited different behavior than for the protein alone or the protein with CS alone (Figure 2, Figure 5). These studies suggest that the presence and composition of polymers in a protein-polymer mixture change albumin's behavior. We explored whether these HA/CS/BSA systems could be used to study transport phenomena of the model drug rhodamine B (rhodB). Many parenteral drug-delivery studies monitor *in vitro* release kinetics into saline as a model for *in vivo* release. Here we explore the potential for this blend to replace saline and to monitor diffusion across an interface

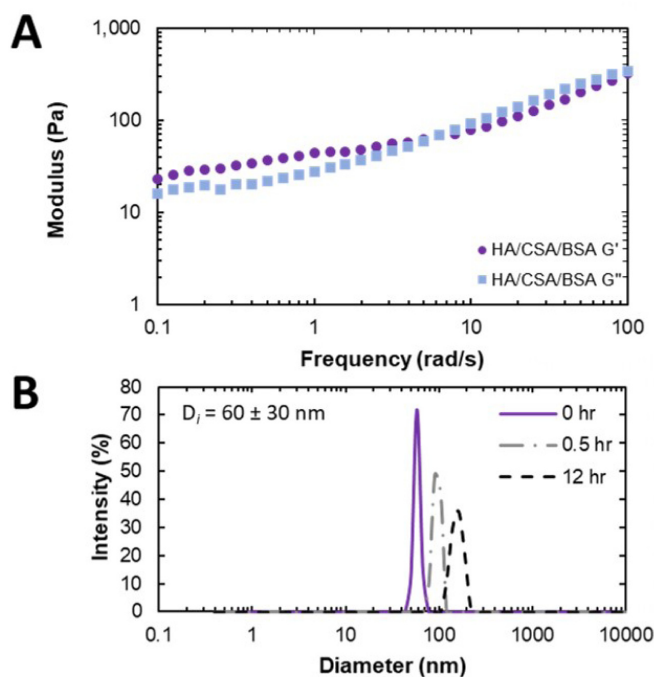


Figure 5. Blend characterization. (A) Oscillatory frequency sweeps of protein-polysaccharide blend. (B) Qualitative time-resolved dynamic light scattering experiment of the sheared blend. Dataset 1: Rheology and dynamic light scattering data.

(Figure 6). A hydrophobic blend of poly(caprolactone) (PCL) chains at different molecular weights loaded with rhodB was carefully added on top of the hydrophilic blend. We found that it was possible to monitor the interfacial concentration of rhodB with UV-Vis spectroscopy. Interestingly, we observed a large bolus release of the water-soluble drug from the hydrophobic phase to the hydrophilic phase at the interface until the same concentration was reached, subsequently an equilibrium or pseudosteady-state concentration was reached after 14 h. Such a system is potentially useful in modeling mass transfer of drugs between hydrophobic drug delivery materials into hydrophilic physiological conditions²⁵.

Finally, 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 7, Dataset 1²⁰), we summarize the literature for CS and HA measurements of mammalian brain tissue^{26–33}. These estimates clustered in the millimolar range for CS disaccharides, and 100 micromolar range for HA.

Conclusions

In this work we characterize non-covalent interactions between HA, CS, and BSA. We report nano- and microparticle self-assembly as well as gelation and supramolecular network formation.

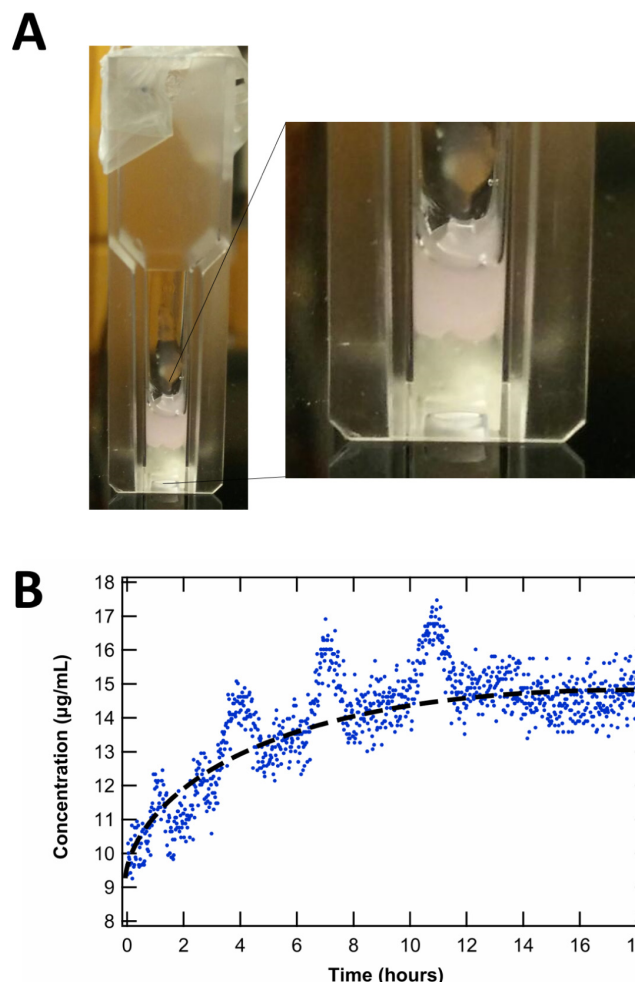


Figure 6. Measuring interfacial mass transfer of a small molecule between hydrophilic and hydrophobic phases. (A) Picture of PCL loaded with rhodamine (pink) on top of the HA/CS/BSA blend system, representing the hydrophobic and hydrophilic interface for potential modeling of mass transfer in drug delivery *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.

We also report on the mass transfer of a model drug from a hydrophobic phase into a protein-polysaccharide hydrophilic phase, and suggest this approach may be more useful *in vitro* approach than measuring drug release into saline. Finally, we summarize reported concentrations of CNS HA and CS in different animal models and humans, which may be important in the design of matrices for CNS tissue engineering. This work offers a robust method of forming hydrogels from unfunctionalized HA, and is attractive for its simplicity compared to those derived from chemically functionalizing the polysaccharide backbone.

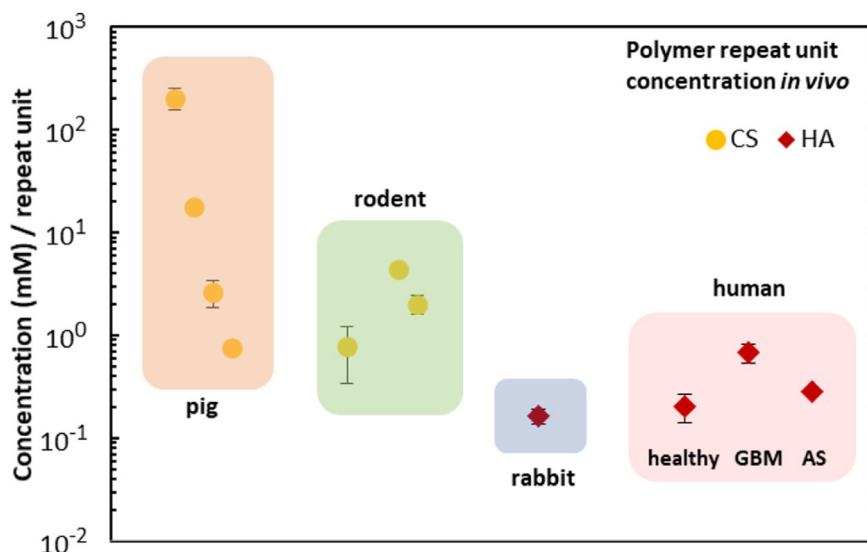


Figure 7. Summary of concentrations of hyaluronic acid (HA) and chondroitin sulfate (CS) in the brain of various species. The concentration of HA in healthy tissue, glioblastoma, and astrocytoma is also plotted.

Data availability

Underlying data for this study is available from Open Science Framework

OSF: Dataset 1: Protein-mediated gelation and nanoscale assembly of unfunctionalized hyaluronic acid and chondroitin sulfate, <https://doi.org/10.17605/OSF.IO/3BXQG²⁰>

Data is available under a CC0 1.0 License

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Michelle A. Calabrese 

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The paper by Tabet and co-workers examines the interactions between a model protein, BSA, and hyaluronic acid (HA) and/or chondroitin sulfate (CS) in forming self-assembled networks using rheology, DLS, imaging, and other techniques. The work is of a generally high standard and is suitable for indexing, but several points (below) should be addressed to improve the quality of the paper.

Rheology measurements:

Why does the gap range between 600 μm and 1 mm? The text says “amplitude sweep” was conducted – at what frequencies were they done or was just one done?

DLS measurements:

What is the solution concentration used here? Interactions between particles, especially electrostatic interactions, can cause two modes to appear in the DLS data; these do not necessarily correspond to sizes. As such, it is important to specify the concentration in the materials and methods section, and what interactions are expected. One way to check if the additional peaks are from interactions or not is to further dilute the samples. Additionally, what model used to produce the radii listed? Especially in cases where electrostatic interactions may be significant, the extrapolation of the data to “radius” may not be accurate; this should be considered by the authors.

The analysis of the DLS data between Fig. 2-5 is also a bit confusing. How are we to interpret DLS data on a gel like this? Again, the radius/modeling assumptions seem like a stretch here so that should be stated... Also in Figure 5, could the larger sizes be coming out of aggregation that's occurring/changes to structure that are occurring due to any interfaces in the cuvette? Because the effect is so slow/fairly minor, and it's hard to really understand the DLS from a gel like this to start with, I think this interpretation should be re-examined.

What makes something “more dynamic” (page 6 second to last paragraph). Based on the labelling, it seems as though both the gels (Fig. 5) and the particles (Fig. 4b) change over a 12h time scale so I'm not sure what more dynamic means. The authors state that the width of the distribution in the HA/BSA is why

you assume there are not discrete particles because there is just one peak in the time zero data.

Figure 3:

1. c) At what frequency is this conducted? More detail is needed. Do you know that the change in modulus with the time sweep is real vs. can be attributed to drying? A water trap will not necessarily stop this over the 5 hour experiment course. What happens if you put this in a small Couette? Do you get a visual change if you leave these in a vial over time in terms of the flowability/pourability? Have you tried the measurements with a light oil or similar around the sample edges (not in the trap)?
2. The experiment that part c) is done after is not clear. Was the solution simply sheared to 100% (in which case, include the rate at which this was done)? Or was it oscillatory shear to 100% at a particular frequency? These details should be specified.
3. e) at what frequency is this comparison done at?

Minor points:

With respect to the rheology methods section, “to determine a strain in...” should be “to determine the strains within” since multiple strains are within the LVE window.

The legend in Fig. 3b,c is hard to differentiate between in black and white. While most rheologists will know which moduli is which, just based on symbol/coloration, it is difficult to tell which is G' and G'' in the key. I think it would help if G' was open circles and G'' was open squares for HA and have the same with closed symbols for HA+BSA.

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

Yes

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?

Partly

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?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: rheology, self-assembly, hydrogels

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.

Reviewer Report 15 April 2019

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Laura De Laporte

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

No further comments.

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Yes

Are the conclusions drawn adequately supported by the results?

Yes

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.

Version 1

Reviewer Report 12 February 2019

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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?

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Partly

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Not applicable

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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

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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.

References

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