RESEARCH ARTICLE

REVISED Protein-mediated gelation and nano-scale assembly of unfunctionalized hyaluronic acid and chondroitin sulfate [version 2; 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, HA is covalently functionalized with moieties that can facilitate network formation through physical self-assembly, or covalent crosslinking (e.g. photocatalyzed) as the polysaccharide does not gel on its own. However, these crosslinks are not the driving force of HA self-assembly 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-crosslinked polymers as an ECM-mimetic model to study transport of hydrophilic 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. Albumin/HA mixtures formed supramolecular gels, and albumin/CS mixtures

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formed micro- and nanoparticles. These systems are formed from unfunctionalized polysaccharides, which is an attractive and simpler method of forming HA hydrogels and CS nanoparticles than functional chemistry-based approaches such as chemically modifying the polymer backbones. We also summarize the concentrations of HA and CS found in various mammalian brains, which could potentially be useful for biomimetic scaffold development in tissue engineering.

Conclusions: Simple preparation and combination of commercially available charged biomacromolecules rapidly 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 model system for ECM transport studies.

Keywords

hyaluronic acid, chondroitin sulfate, protein-polymer assembly

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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 1

The introduction now clarifies the differences between covalently tethering functional moieties that facilitate supramolecular network formation, and supramolecular networks of unfunctionalized hyaluronic acid (reviewer 1). The utility and reasons to use BSA were also clarified (reviewer 2).

The introduction and the results/discussion sections were modified to clarify the nomenclature of non-covalent interactions of HA and CS, and distinguish between the importance of these interactions for each polymer in physiology. [Figure 1](#) was updated (reviewer 1 and 2). ESI figures were moved to the main manuscript from the Extended data. We include the point made by reviewer 2 on CS/BSA interactions, as well as underscoring the point that denatured protein was key to forming nanostructures or gels.

Protein concentration was varied and the rheological properties were measured by new authors June Y. Park and Vijay K. Rana to show tunability. SEM images of the dried hydrogels were added. Further text was included to contextualize the qualitative nature of [Figure 3D](#). Electrostatically-driven crosslinking of the polymer-protein systems interfered with the voltage signal of the cantilever during AFM measurements (reviewer 1). Addition of solvents changed gel properties via diffusion of polymer/protein into solvent or diffusion of water out of the system. The [Figure 6](#) caption was edited to clarify that rhodamine under visible light is pink (reviewer 1).

The first reported methods describing the formation of HA/BSA gels resulted in gels that were sensitive to the force exerted during mixing. These methods have been expanded to be more reproducible from person-to-person. The title was modified to address reviewer comments and better represent the scope of the work. Minor mistakes in the reported concentrations as well as instrument specifications were corrected. Minor changes in the text in the abstract were made to address comments by both reviewers.

Funding from additional author JYP was added.

See referee reports

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^{1,2}. Notably, HA is the only purely supramolecular brain GAG³⁻⁷, *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^{8,9}. 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 applications¹⁰.

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](#)). While BSA is not normally found in the brain and its physiological relevance is limited, we demonstrate herein the potential for misfolded or unfolded protein to form supramolecular complex with ECM polymers to drastically change their mechanical properties. Heilshorn and colleagues previously reported on supramolecular hydrogels formed *via* protein interactions with polysaccharide backbones¹¹⁻¹³. In this work we exploit interactions between BSA and GAG polymers and induce changes in protein folding to generate structurally diverse protein-glycosaminoglycans complexes. We examine how these negatively charged biopolymers interact and self-assemble with BSA, and develop non-covalent 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 parameters¹⁴. 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 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 was added and partially denatured *via* mechanical force or heat. Various concentrations of protein were added to the viscous polysaccharide solution and mixed vigorously with a metal spatula for several minutes. The samples were sealed until they appeared transparent, typically for at least 24 h. BSA denaturing could also be accomplished with heat by incubating samples at 50°C until the solution turned opaque (typically 2 h).

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.

It was observed that BSA retaining its folded structure would not result in gel formation (see results and discussion). To form HA/CS/BSA systems, dry chondroitin sulfate (10 wt%) was added along with the dry BSA into HA solution and the same procedure as HA/BSA was used. 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).

Rheology

All rheological sweeps were conducted on an Discovery HR-2 Rheometer (TA Instruments, New Castle, DE, USA) with a 40 mm parallel plate geometry 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 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.

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.

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/ml¹⁵. 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 water¹⁶.

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 1¹⁷), and were dissimilar to the flocculation of BSA

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). (E) Autocorrelation function of CS/BSA NPs. (F) Time resolved DLS of CS/BSA NPs. Dataset 1: Dynamic light scattering and transmission electron microscopy data¹⁷.

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 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 $D = 1.5 \pm 0.5$ μ m. In the brain, CS is covalently scaffolded onto protein cores (e.g. aggrecan¹⁸) and binds with many ECM proteins through non-covalent, supramolecular interactions¹⁹. 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 BSA were mixed and the electrostatic interactions between the charged moieties of the GAG and the unfolded BSA protein led the solution to self-assemble into a supramolecular gel (Figure 3, Dataset 1¹⁷). Solutions of HA alone were highly viscous but did not show gel-like properties. Oscillatory rheological measurements were used to probe the mechanical properties of this supramolecular gel. After introduction of partially denatured BSA, electrostatic-driven network percolation resulted in a major stiffening effect and the formation of a gel with $G' > 10$ kPa. This material was also self-healing and could recover its stiffness after large shear (Figure 3C). These gels' stiffness was tunable, and could span the range of brain tissue and brain cancer stiffnesses²⁰. It was observed that the same concentration of BSA with intact tertiary structure did not result in gelation (Figure 3E). This is perhaps owed to the fact that in its folded form, BSA protein exhibits singular charged binding pocket that cannot facilitate multichain interactions (Figure 1B), and thus cannot serve as an effective crosslinker. Partially denatured BSA, however, with multiple exposed charged peptide domains shows greater affinity towards multi-chain electrostatic interactions and crosslinks the system to form a physical gel. The difference in structure between these systems can be seen further in scanning electron microscopy (SEM) images (Figure 3F-H). Partially denaturing BSA results in some transition from a single binding pocket to the ability to interact with multiple chains, likely *via* electrostatic interactions. This transition may be due to changes in protein conformation making glycan binding sites more accessible, or aggregation of BSA from partial unfolding of the polypeptide²¹. This simple, electrostatic-based gelation is analogous to alginate gelation with divalent cations²². In this case, partially denatured proteins allow for bridged flocculation-type interactions which

percolates a physical, non-covalent network. This is further reinforced by the shear-recovery observed in Figure 3C. Our group recently explored the differences in effect of physical or covalent bonds in energy transfer to physical or covalent networks²³, and this shear-recovery is consistent with a physical network that can disassemble with shear and recover *in situ* over time.

Upon introduction of dry CS into a HA/BSA matrix, a sharp decrease in stiffness was observed (Figure 4A). We hypothesize this effect is due to interference from CS/BSA interactions leading to a reduction in extent of HA crosslinking. 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 HA, the BSA assemblies were more dynamic than with CS (Figure 3D, Figure 4B, Figure 5). These measurements qualitatively suggest that BSA aggregates are not static and are polydisperse, and do not suggest that there are discrete BSA particles within the HA/BSA hydrogel. When all components are combined, there appears to be an intermediate solution that is pushed towards more well defined assemblies immediately after shear but over time will revert towards the HA/BSA state (Figure 4B).

We then explored whether these HA/CS/BSA systems could be used to study transport phenomena of the model hydrophilic 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 a new ECM-mimetic model, in place of saline, to monitoring diffusion across a tissue-like gel (Figure 6). 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. 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 h. Such a system is potentially useful in modeling mass transfer of drugs between hydrophobic drug delivery materials and biological tissue such as the brain²⁴. 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 1¹⁷), we summarize the literature for CS and HA measurements of mammalian brain tissue^{25–32}. 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

Figure 3. HA/BSA gels. (A) Illustration of dynamic network formation of HA and BSA. (B) Oscillatory rheological frequency sweep of HA solution alone (40 mg/mL) and HA/BSA gels (40, 60 mg/mL). (C) Oscillatory time sweep after 100% shear of these HA/BSA gels and HA solution alone. Maroon = HA/BSA G', Gold = HA/BSA G'', Black = HA G', Grey = HA G''. (D) DLS size measurements of dynamic BSA particles alone and this HA/BSA gel. (E) Storage (G') and loss (G'') moduli of HA (40 mg/mL) with various concentrations of BSA. UD = undenatured. (F-H) SEM images of HA alone, HA/BSA (undenatured), and HA/BSA (partially denatured). Scale bar = 100 μm (top), 10 μm (bottom). Dataset 1: Rheology, dynamic light scattering, scanning electron microscopy data.

Figure 4. (A) Oscillatory frequency sweeps of HA systems loaded with BSA and with or without CSA. (B) Time-resolved dynamic light scattering experiment of CSA/BSA particles loaded with HA and sheared. Dataset 1: Rheology and dynamic light scattering data.

Figure 5. Time resolved dynamic light scattering of HA/BSA gels. Dataset 1: Dynamic light scattering data.

Figure 6. Measuring interfacial mass transfer kinetics between hyaluronic acid (HA)/bovine serum albumin (BSA)/chondroitin sulfate (CS) system and PCL blend. **(A)** Picture of PCL loaded with rhodamine (pink) on top of the HA/CS/BSA 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).

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.

self-assembly as well as gelation and supramolecular network formation *via* bridged flocculation. We also report on the mass transfer of a model drug from a hydrophobic phase into a glycosaminoglycans-based hydrogel, and suggest this approach may be more biologically representative of *in vivo* drug delivery compared to *in vitro* experiments that measure drug release from a matrix 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 both for its simplicity and for capturing more biologically relevant interactions compared to those derived from chemically functionalizing either polysaccharide.

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/3BXQG>¹⁷

Data is available under a CC0 1.0 License

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References

- Burdick JA, Prestwich GD: **Hyaluronic acid hydrogels for biomedical applications.** *Adv Mater.* 2011; **23**(12): H41–56.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lee KY, Mooney DJ: **Hydrogels for Tissue Engineering Applications.** *Chem Rev.* 2001; **101**(7): 1869–1879.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sasarman F, Maftai C, Campeau PM, *et al.*: **Biosynthesis of glycosaminoglycans: associated disorders and biochemical tests.** *J Inher Metab Dis.* 2016; **39**(2): 173–88.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Wiederschain GY: **Essentials of glycobiology.** *Biochemistry (Mosc).* 2009; **74**(9): 1056.
[Publisher Full Text](#)
- Papakonstantinou E, Roth M, Karakioulakis G: **Hyaluronic acid: A key molecule in skin aging.** *Dermatoendocrinol.* 2012; **4**(3): 253–8.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Conrad AH, Zhang Y, Tasheva ES, *et al.*: **Proteomic analysis of potential keratan sulfate, chondroitin sulfate A, and hyaluronic acid molecular interactions.** *Invest Ophthalmol Vis Sci.* 2010; **51**(9): 4500–15.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Toole BP: **Hyaluronan is not just a goo!** *J Clin Invest.* 2000; **106**(3): 335–6.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Hegger PS, Kupka J, Minsky BB, *et al.*: **Charge-Controlled Synthetic Hyaluronan-Based Cell Matrices.** *Molecules.* 2018; **23**(4): pii: E769.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Ranga A, Lutolf MP, Hilborn J, *et al.*: **Hyaluronic Acid Hydrogels Formed In Situ by Transglutaminase-Catalyzed Reaction.** *Biomacromolecules.* 2016; **17**(5): 1553–60.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Djeral L, Lortat-Jacob H, Kwok J: **Chondroitin sulfates and their binding molecules in the central nervous system.** *Glycoconj J.* 2017; **34**(3): 363–376.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Wong Po Foo CT, Lee JS, Mulyasmita W, *et al.*: **Two-component protein-engineered physical hydrogels for cell encapsulation.** *Proc Natl Acad Sci U S A.* 2009; **106**(52): 22067–22072.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Dubbin K, Hori Y, Lewis KK, *et al.*: **Dual-Stage Crosslinking of a Gel-Phase Bioink Improves Cell Viability and Homogeneity for 3D Bioprinting.** *Adv Healthc Mater.* 2016; **5**(19): 2488–2492.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Dubbin K, Tabet A, Heilshorn SC: **Quantitative criteria to benchmark new and existing bio-inks for cell compatibility.** *Biofabrication.* 2017; **9**(4): 44102.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Baker NA, Sept D, Joseph S, *et al.*: **Electrostatics of nanosystems: application to microtubules and the ribosome.** *Proc Natl Acad Sci U S A.* 2001; **98**(18): 10037–41.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- DiResta GR, Lee JB, Arbit E: **Measurement of brain tissue specific gravity using pycnometry.** *J Neurosci Methods.* 1991; **39**(3): 245–51.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Keep RF, Hua Y, Xi G: **Brain water content. A misunderstood measurement?** *Transl Stroke Res.* 2012; **3**(2): 263–5.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Tabet A: **Protein-mediated gelation and nano-scale assembly of unfunctionalized hyaluronic acid and chondroitin sulfate.** 2019.
<http://www.doi.org/10.17605/OSF.IO/3BXQG>
- Novak U, Kaye AH: **Extracellular matrix and the brain: components and function.** *J Clin Neurosci.* 2000; **7**(4): 280–290.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Mizumoto S, Yamada S, Sugahara K: **Molecular interactions between chondroitin-dermatan sulfate and growth factors/receptors/matrix proteins.** *Curr Opin Struct Biol.* 2015; **34**: 35–42.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Tabet A, Mommer S, Vigil JA, *et al.*: **Mechanical Characterization of Human Brain Tissue and Soft Dynamic Gels Exhibiting Electromechanical Neuro-Mimicry.** *Adv Healthc Mater.* 2019; e1900068.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Borzova VA, Markossian KA, Chebotareva NA, *et al.*: **Kinetics of Thermal Denaturation and Aggregation of Bovine Serum Albumin.** *PLoS One.* 2016; **11**(4): e0153495.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Lee KY, Mooney DJ: **Alginate: Properties and biomedical applications.** *Prog Polym Sci.* 2012; **37**(1): 106–126.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Tabet A, Forster RA, Parkins CC: **Modulating stiffness with photo-switchable supramolecular hydrogels.** *Polym Chem.* 2019; **10**: 467–472.
[Publisher Full Text](#)
- Tabet A, Wang C: **Gels Without Vapor Pressure: Soft, Nonaqueous and Solvent-Free Supramolecular Biomaterials for Prospective Parenteral Drug Delivery Applications.** *Adv Healthcare Mater.* 2019; **8**(6): e1800908.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Sood D, Chwalek K, Stuntz E, *et al.*: **Fetal brain extracellular matrix boosts neuronal network formation in 3D bioengineered model of cortical brain tissue.** *ACS Biomater Sci Eng.* 2016; **2**(1): 131–140.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- DeQuach JA, Yuan SH, Goldstein LS, *et al.*: **Decellularized porcine brain matrix for cell culture and tissue engineering scaffolds.** *Tissue Eng Part A.* 2011; **17**(21–22): 2583–2592.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)

27. Medberry CJ, Crapo PM, Siu BF, *et al.*: **Hydrogels derived from central nervous system extracellular matrix.** *Biomaterials*. 2013; **34**(4): 1033–1040.
[PubMed Abstract](#) | [Free Full Text](#)
28. Kočí Z, Výborný K, Dubišová J, *et al.*: **Extracellular Matrix Hydrogel Derived from Human Umbilical Cord as a Scaffold for Neural Tissue Repair and Its Comparison with Extracellular Matrix from Porcine Tissues.** *Tissue Eng Part C Methods*. 2017; **23**(6): 333–345.
[PubMed Abstract](#) | [Publisher Full Text](#)
29. Baiguera S, Del Gaudio C, Lucatelli E, *et al.*: **Electrospun gelatin scaffolds incorporating rat decellularized brain extracellular matrix for neural tissue engineering.** *Biomaterials*. 2014; **35**(4): 1205–1214.
[PubMed Abstract](#)
30. Tubert AR, López FB, Matías ÉA, *et al.*: **Vectors and sequences for the treatment of diseases.** 2016.
31. Laurent UB, Tengblad A: **Determination of hyaluronate in biological samples by a specific radioassay technique.** *Anal Biochem*. 1980; **109**(2): 386–394.
[PubMed Abstract](#) | [Publisher Full Text](#)
32. Delpech B, Maingonnat C, Girard N, *et al.*: **Hyaluronan and hyaluronectin in the extracellular matrix of human brain tumour stroma.** *Eur J Cancer*. 1993; **29A**(7): 1012–1017.
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Reviewer Report 08 August 2019

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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.

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

Yes

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Yes

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Yes

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

Yes

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

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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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?

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Partly

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Yes

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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

<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.

References

1. Hegger PS, Kupka J, Minsky BB, Laschat S, et al.: Charge-Controlled Synthetic Hyaluronan-Based Cell Matrices. *Molecules*. 2018; **23** (4). [PubMed Abstract](#) | [Publisher Full Text](#)
2. Broguiere N, Isenmann L, Zenobi-Wong M: Novel enzymatically cross-linked hyaluronan hydrogels support the formation of 3D neuronal networks. *Biomaterials*. **99**: 47-55 [PubMed Abstract](#) | [Publisher Full Text](#)
3. Ranga A, Lutolf M, Hilborn J, Ossipov D: Hyaluronic Acid Hydrogels Formed in Situ by Transglutaminase-Catalyzed Reaction. *Biomacromolecules*. 2016; **17** (5): 1553-1560 [Publisher Full Text](#)

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Reviewer Expertise: hydrogels, tissue engineering

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