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Authors: Ramon Novoa-Carballal, Agatha Carretero, Raul Pacheco, Reis Rui L., and Iva Pashkuleva

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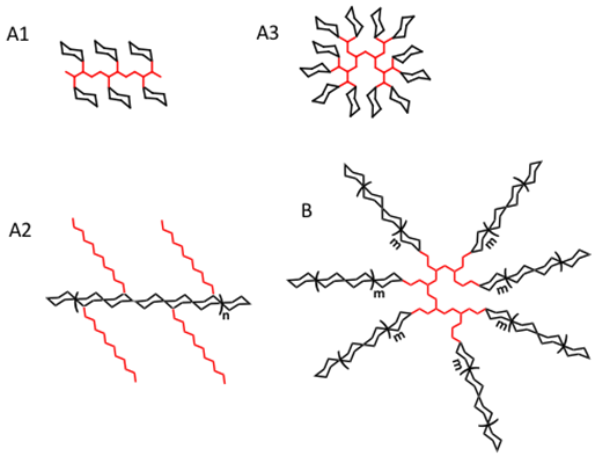
Star-like glycosaminoglycans with superior bioactivity assemble with proteins into microfibers

Ramon Novoa-Carballal,* Agatha Carretero, Raul Pacheco, Rui L. Reis and Iva Pashkuleva*

Abstract: We show that glycosaminoglycans (GAGs) with high molecular weight can be grafted via their reducing end on hyper branched synthetic cores by oxime condensation without the need of any previous functionalisation of the polysaccharide. The versatility of this reaction is demonstrated by the use of hyaluronan, chondroitin sulfate and heparin with up to 60 sugar units. The isothermal calorimetry analysis demonstrated that the generated star-like glycopolymers have superior bioactivity. Moreover, when mixed with positively charged proteins (e.g. FGF-2) they form microfiber structures instead of the spherical nanocomplexes described for linear GAGs. Our results suggest that the described star-like GAG are closer mimics of the proteoglycans at structural and functional level and therefore have a huge potential in the development of tissue engineering platforms and therapeutics by modulating the activity and presentation of various proteins such as growth factors.

Glycosaminoglycans (GAGs) are natural, negatively charged polysaccharides found in the extracellular matrix (ECM) and basement membrane of multicellular organisms where they span different biological roles.[1] Most GAGs (all but hyaluronan, HA) are secreted into the ECM as brush-like conjugates – proteoglycans (PG) – in which the polysaccharide moiety is attached by its reducing end to a core protein chain. The bioactivity of PGs is related to their crosstalk with other bioentities in the native environment: they can serve as reservoirs for proteins (growth factors, chemokines and cytokines), protect them from enzymatic degradation or act as co-factors triggering various cell signalling pathways. The main driving forces of all these processes are multivalent electrostatic interactions that occur between the proteins and GAG moieties from the PGs.[1, 2] Consequently, the sulfation pattern, the molecular weight (MW) and the nano-organisation of the GAGs when attached to the protein core (i.e. number and density of GAG chains) are key factors determining the strength of GAG/protein interactions.[1, 3]

GAGs have been proposed as therapeutics for a variety of diseases such as thrombosis, amyloid diseases, arthritis and cancer among others.[1, 4] Thus, efforts are made to mimic GAGs and their multivalent interactions by preparing glycopolymers/dendrimers (polymers/dendrimers with sugars attached to the synthetic core, scheme. 1A), and GAG modified surfaces. The surfaces are able to mimic the multivalency of PG and are relevant for protein interaction or cell adhesion studies. However, for an *in vivo* tissue engineering application 3 dimensional structures, and hence PG mimic glycoplymers/dendrimers, are required. The most common strategies for glycopolymer synthesis are (i) the polymerisation of glycan-containing monomers and (ii) the attachment of pre-functionalised glycosides to polymer/dendrimer backbones with complementary reactive groups.[5] Both approaches use mono- or short oligosaccharides (up to 4 monosaccharides) and require their chemical modification, which makes the introduction of complex glycans synthetically challenging and time-consuming.[6] The generated by these approaches glycopolymers bind different proteins, e.g. lectins, via multivalent interactions.[7] These interactions are stronger and more specific when longer oligosaccharides are used instead of monosaccharides.[8] GAGs have various binding sites of different size along their chain. As an example heparin/heparan sulfate binds different proteins via sequences whose size ranges from 5 to 18 sugar units.[2] Thus, the used oligosaccharides are, in our opinion, too short to mimic the multivalent and specific interactions governing the GAG bioactivity.[9] Moreover, PGs are biospecific as they present multivalentcy at two levels: besides the multivalency of each GAG chain, PGs have attached several GAG chains to their protein core (4-5 to hundreds depending on the PG function). This complex structure is not represented in conventional glycopolymers.[10] Herein, we describe an approach in which star- like glycopolymers are synthesised by grafting of GAGs with higher MW (up to *ca* 60 sugar units) via their reducing end to hyper branched polymers (scheme. 1B).



Scheme 1. (A) Schematic presentation of common glycopolymers: (A1) linear derivatives, (A2) grafted copolymers and (A3) glycodendrimers. (B) Structure of star-like glycosaminoglycans obtained by end-on grafting of polysaccharides on branched synthetic core. The synthetic moiety is presented in red and the "glyco" fragment in black.

Dr. Ramon Novoa-Carballal, MS. Agatha Carretero, Prof. Rui L. Reis and Dr. Iva Pashkuleva
3B's Research Group—Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and RegenerativeMedicine, AvePark, 4805-017 Barco Guimarães, Portugal. ICVS/3B's — PT Government Associate Laboratory, Braga/Guimarães, Portugal.
Prof. Rui L. Reis The Discoveries Centre for Regenerative and Precision Medicine, Headquarters at University of Minho, Avepark, 4805-017 Barco, Guimarães, Portugal
E-mail: ramon.novoa@dep.uminho.pt; pashkuleva@dep.uminho.pt.
Dr. Raul Pacheco
Malvern/Micrcal Products
Enigma Business Park, Grovewood Road, Malvern; WR141XZ, United Kindom

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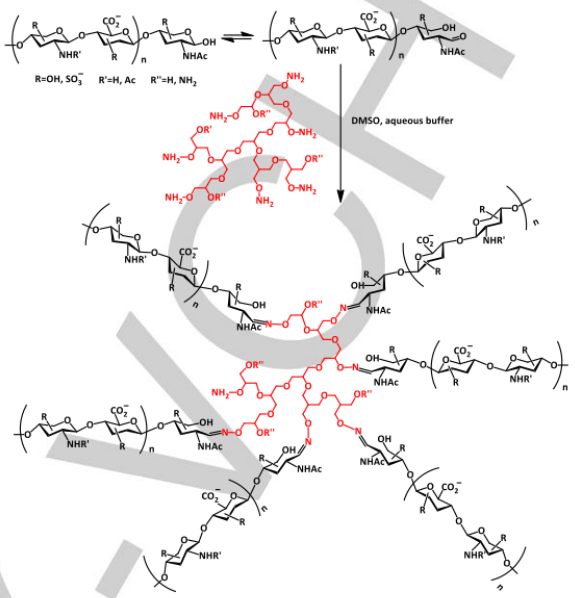
Besides the length of the branches, the obtained by us glycopolymers are distinguished from the previously described PG mimics by the chemical bond between the GAG and the core polymer.[11] Usually, the glycans are grafted to the synthetic core in a side-on manner, i.e. "oppositely" to the naturally occurring structures (Fig. 1A2).[12] This binding affects tremendously their bioactivity. Therefore, current efforts are focused on alternative synthetic methods generating glycopolymers that present GAG in a native-like manner and preserve their biofunctionality. Huisgen cycloaddition of azido functionalised polysaccharides to polypeptide core generates such brush-like glycopolymers.[13] This synthetic procedure is limited by the solvent: polysaccharides depolymerize under copper click conditions in aqueous environment due to the presence of ·OH radicals.[14] Another alternatives are hydrazone ligation with following reductive amination (that requires heating up to 85 ºC compromising the stability of the GAG) and the use of the amine group of terminal serine present in some enzymatically isolated chondroitin sulphates. [15] This last method lacks of generality and might also lead to undesired side chain reactivity in deacetylated N-acetyl groups.

Herein we present oxime coupling as an efficient and versatile method for synthesis of GAG star-like copolymers. The reaction occurs between a synthetically introduced aminooxy group and the reducing end of a native, unmodified carbohydrate. This reaction is well well-known for the linkage of oligosaccharides to lipids, peptides or into surfaces and has been recently applied to prepare GAG diblock copolymers or to introduce biotin into GAGs.[16] It is performed under mild reaction conditions and the generated bond is more stable than the hydrazone at physiological conditions.[17] In this work, we use this reaction for the synthesis of more demanding (in terms of steric hindrance) PG mimics. We obtained glycopolymers with hyaluronan (HA), chondroitin sulfate (CS) and heparin (Hep) and studied their interactions with proteins.

Synthesis of star-like GAGs

We have chosen hyperbranched polyglycerol (hPG, scheme 2) as a template. It has several advantages such as single step synthesis, easy large scale production (in contrast to the synthesis of dendrimers) and safe use as demonstrated by numerous biomedical applications.[18] hPG (number average molecular weight, Mn, of 8.1 kDa) was synthesised as previously described.[19] Functionalisation with aminooxy group (-ONH_2) was performed by Mitsunobu reaction (details are presented in the supporting Information (SI). We achieved 40% functionalisation (as shown by NMR), corresponding to ca. 30 –ONH_2 groups per molecule.

Next, we assayed the coupling of the prepared hPG-OH with hyaluronic acid (HA, Mn 4 kDa), chondroitin sulfate (CS, Mn 5 kDa) and heparin (Hep, Mn 14.5 kDa) at the conditions previously optimised for diblock copolymers.[16a]



Scheme 2. Synthetic pathway used to obtain star-like GAG copolymers. The synthetic template, hPG, is presented in red and the GAG in black.

Although we observed by gel permeation chromatography (GPC) formation of glycopolymers for all studied GAGs at these conditions (Tables S1 and S2), we have detected considerable amount of unreacted GAGs. Thus, further optimisation of the reaction conditions (excess of GAG, temperature, reaction time and use of aniline as catalyst) was carried out (detailed discussion is included in the SI). The use of aniline shortened significantly the reaction times (Table S1 and Fig. S2) due to the formation of an iminium ion intermediate that stabilises the aldehyde at the GAGs reducing end.[20] The use of large GAG excess did not lead to higher molecular weight products, i.e. a limited number of chains can be attached to the hPG core. The number of the grafted GAG chains was estimated from the NMR and GPC and correspond to 4-6 GAG chains per synthetic core (Table 1 details in the SI).

Table 1. Molecular weight and number of GAG chains for the star-like GAG copolymers determined by GPC and NMR.

GAG	GAG Apparent Mn [kDa] (PDI)	GAG Absolute Mn [kDa] (PDI)	Apparent Mn [kDa] (PDI)	Number of GAG chains GPC/NMR
HA	9.9 (1.3)	4.0 (1.2)	69 (3.4)	6/5.1
	16.2 (1.2)	7.6 (1.2)	117 (1.5)	6/4.1
CS	12 (1.2)	5.0 (1.1)	59 (2.8)	4/3.8
Hep	36 (1.2)	14.6 (1.2)	159 (3.0)	4/4.5

Interaction of star-like GAGs with proteins

Branched structures with 4-5 GAG chains resemble PGs with discrete number of GAG chains such as syndecans and perlecan. These PGs interact with different proteins (e.g. lectins, growth factors, cytokines) in the pericellular space thus, activating different pathways.[21],[22] Initially, we used poly-L-lysine (PLL, Mn *ca*. 9kDa) as a model positively charged protein because it is well documented that GAGs interact with proteins via Cardin–Weintraub sequences.[23] Our results showed no interactions between PLL and HA or CS star-like glycopolymers at physiological ionic strength. Similar behavior has been previously reported for diblocks of polyethyleneglycol (PEG) with HA and CS of comparable molecular weights.[16f, 16h]

Star-like Hep behaved differently: micrometric fibers with length of 0.5-2 mm and width of *ca*. 0.02 mm were formed in the presence of PLL (Fig. 1A). Closer observation of these microstructures showed that they are congregation of numerous smaller fibers (Fig. 1B, C). The fibrillar structures are only formed when an excess of the glycopolymer is used (mass ratio PLL/hPG-Hep of 0.2 or lower, scheme. S11). When PLL is in excess (mass ratio PLL/hPG-Hep of 2), we observed flocculation (Fig. S11). Intermediate ratios (mass ratio PLL/hPG-Hep of 0.4-0.6) resulted in formation of mixtures of fibers and flocculated particles (Figs. S11). We compared these results with the data from the interactions between unmodified Hep and PLL. Previous studies report formation of spherical nanocomplexes between these two components.[24] We confirmed these data by reproducing the experiments with the Hep used by us for the synthesis of hPG-Hep: nanocomplexes were detected by dynamic light scattering when PLL in solution was stepwise added over a Hep in solution (DLS, Figs. S9, S10), until flocculation was observed (Fig. S12).

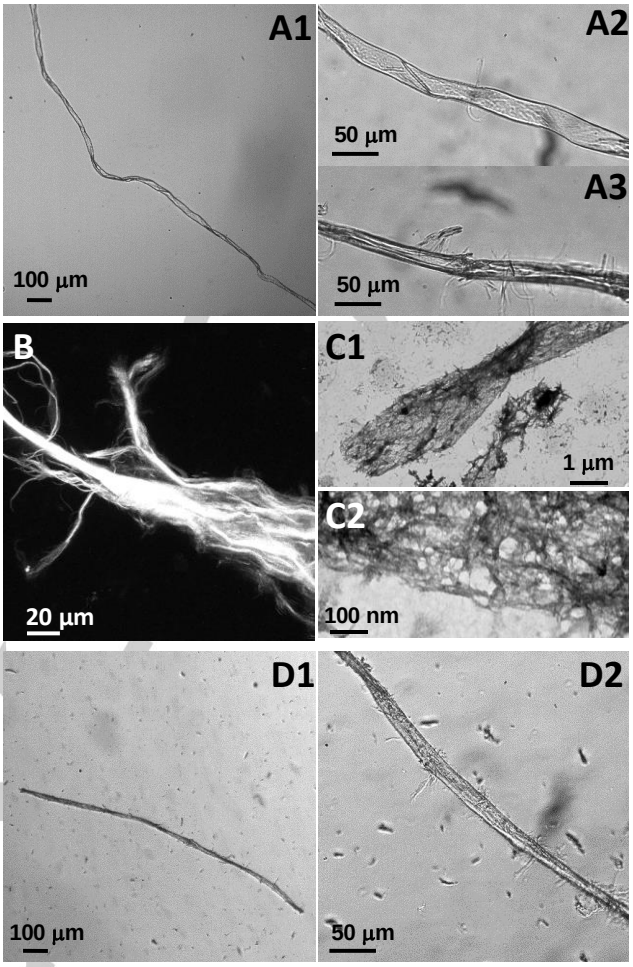
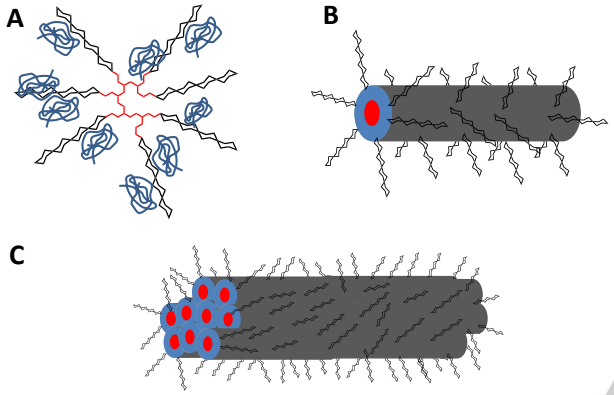


Figure 1. Optical (A, D), confocal (B) and transmission electron microscopy (C) images of fibers generated from PLL and star-like heparin (A, B, C; PLL/hPG-Hep mass ratio of 0.2) or FGF-2 and star-like heparin (D, FGF-2/hPG-Hep mass ratio 1.8) in buffer (150 mM NaCl, 10 mM Na2HPO4, 2.8 mM HCl, pH 7.4).

We obtained very similar results when PLL was replaced by basic fibroblast growth factor (FGF-2): microfibers with similar structure and dimension were generated upon the addition of FGF-2 to the hPG-Hep (Fig. 3C). As for PLL/hPG-Hep, formation of fibers was only observed at low FGF-2/hPG-Hep mass ratios (excess of hPG-Hep). Of note, the viscosity of the solution did not change upon the addition of either PLL or FGF-2, suggesting no formation of continuous network of crosslinked molecules or gel formation. The assembled structures are phase separated from the solution and remain as colloids. In fact, the formation of aggregated micelles ("supermicelles") by hPG derivatives has been previously described.[25] Our results are in agreement with the described tendency of such hyperbranched polymers for supramolecular association although the generated assemblies have different shape (spherical vs fibrillary structures described herein). The transition of spherical to cylindrical and finally vesicular

structures can be tailored by changing the hydrophilic-hydrophobic ratio in copolymers and it also applies for star-block copolymers,[26]. Our results (DLS and microscopy) suggest initial formation of cylindrical micelles/nanofibers upon the addition of PLL or FGF-2 to hPG-Hep, followed by a rapid growth and aggregation of these structures (Scheme 3). Similar assembly into fibrillar superstructures has been observed by the addition of positively charged poly(4-vinylpyridine) to a structural analogue of hPG-Hep: a linear acrylate with branches made of a copolymer of polystyrene sulfonate and poly(ethylene oxide).[27]



Scheme 3. Representation of the formation of microfibers: (A) complexation of hPG-Hep with FGF-2 or PLL that leads to (B) formation of cylindrical micelles. (C) Further assembly of these nanostructures into microfibers. The synthetic hPG core is presented in red, the PLL or FGF-2/Hep complex in blue and free Hep in black.

These results are very encouraging from the tissue engineering/regenerative medicine perspective since the obtained fibers can be used as PG mimics for example as systems for protection and release of growth factors. Thus, we further studied the interactions between hPG-Hep and FGF-2 by isothermal titration calorimetry (ITC). ITC allows to precisely measure the thermodynamic parameters (enthalpy, entropy, free energy, binding constant), and stoichiometry of the interaction of any two biomolecules in a single experiment and in solution. Previously, it has been used to determine the affinity of low molecular weight Hep (Mw 3 kDa) to FGF-2. The results demonstrated that two FGF-2 molecules bind to this oligosaccharide (with 8 to 10 sugar units) with a K_D of 0.45 μM and a ΔG of -36 KJ and an interaction with a significant electrostatic percentage but with more specific non-ionic interactions playing an important role.[28] The stoichiometry was in accordance with the crystallographic data, showing a tetra to penta-saccharide interacting with one FGF-2 molecule,[2b] and static light scattering data showing among 6[29] to 10[30] FGF-2 molecules binding to heparin of 16 kDa (similar to the Hep Mw used in this investigation).

Our initial experiments were performed to determine the binding affinity and stoichiometry of the Hep used for the synthesis of the hPG-Hep. Experiments were performed under similar conditions described before, *i.e.* Hep was added to FGF-2 (Table 2, Figs. S16, S17). The stoichiometry obtained shows eight FGF-2 molecules per Hep, in very good agreement with light scattering data and previous ITC data.[28],[29],[30] The results obtained for K_D (0.3 μM) and ΔG (-37 KJ), are also in very good agreement with those described for low molecular weight Hep.

Table 2. Stoichiometry and thermodynamic parameters of the interaction of Hep and hPG-Hep obtained by isothermal titration calorimetry: FGF-2 (23.2 μM; 0.4 g/L) in the cell; and 40 injections of Hep (165 μM; 2.4 g/L) or hPG-Hep (39 μM; 2.8 g/L) in the syringe (0.25 and 0.30 μL injections respectively). The thermodynamic and binding parameters were derived from the nonlinear least squares fit to the binding isotherm.

Sample	Number of sites	K_Dx10^{-6} (M)	ΔH (KJ/mol)	ΔG (KJ/mol)
Hep	8.2±0.1	0.30±0.06	-22.3±0.50	-37.3
hPG-Hep	35±1.0	0.98±0.30	-21±1.22	-34.0

ΔG and ΔH for the system hPG-Hep/FGF-2 were similar to those obtained for Hep/FGF-2 showing that the nature of the interaction(s) between the Hep and FGF-2 is preserved in the hPG-Hep and the binding mechanism is similar. On the other hand, the stoichiometry changed significantly: 4.5-fold increase of the FGF-2 bound to hPG-Hep was determined as compared to Hep/FGF-2. This result demonstrates that: 1) the used chemistry does not affect the integrity of the Hep binding sites and 2) Hep chains in the copolymer are separated enough to bind FGF-2 in a highly dense complex.

In conclusion, we demonstrate that the described herein star-like GAGs are functional mimics of PG. Because Hep PGs bind specifically numerous growth factors, cytokines and chemokines, the hPG-Hep can find application is various regenerative and therapeutic approaches. Moreover, the described synthetic procedure opens new opportunities to address the challenging multivalency and the associated biospecificity.

Acknowledgements

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Keywords: Glycosaminoglycan • Glycopolymer • Oxime • FGF-2 • Heparin

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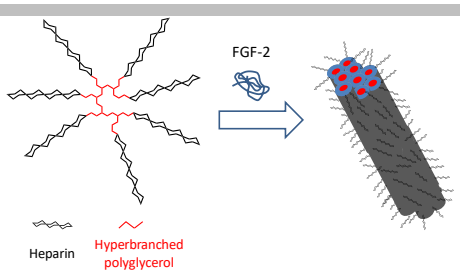

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