Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Tunable nano-carriers from clicked glycosaminoglycan block copolymers

Ramon Novoa-Carballal[a,b], Carla Silva[a], Stephanie Möller[c], Matthias Schnabelrauch[c], Rui L. Reis[a] and Iva Pashkuleva[a]**

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Oxime click reaction is used for the synthesis of diblock copolymers of polyethylene glycol (PEG) and glycosaminoglycans (GAG) with different molecular weight (M_w) and sulfation degree. The ability of these copolymers to carry positively charged proteins is evidenced by their assembly with poly-L-lysine as a model: interpolyelectrolyte complexes with tunable size at the nanometric scale (radius of 25-90 nm) and narrow distribution are described. We demonstrate that there is a critical M_w of GAG for the formation of stable complexes and that the sulfation degree determines the size of the nano-assemblies. Highly sulfated GAGs form the smallest complexes that are stable to at least 500 mM ionic strength: properties that are not usual for GAG interpolyelectrolyte complexes. The feasibility of the synthesised block copolymers as protein carriers is further evidenced by their complexation with fibroblast growth factor (FGF-2). The described assets of GAG block copolymers together with the intrinsic GAG properties as biorecognition and biodegradability open new opportunities in the design of selective encapsulation/release nanosystems with stealth PEG corona.

1. Introduction

The attractiveness of proteins as therapeutics stems is mainly based on the exquisite specificity and selectivity by which they execute diverse biofunctions - they can catalyze specifically a certain reaction or inhibit selectively a targeted cell receptor. It is, therefore, believed that the treatment with the conventional drug therapy will be shifted towards therapeutic proteins in the future.[1] When bioactive molecules are administered, however, only a small fraction of the dose hits the relevant sites of action: most of the compound is lost either by being taken up into the wrong tissue, burst released into the target tissues or destroyed during the delivery process/route.[2] Proteins are not an exception - although protein based biopharmaceuticals have been already proposed, their efficient *in vivo* delivery continues to be a main challenge because of the instability of many proteins in serum (parenteral route) or in gastric and intestinal media (oral route). Several alternatives have been addressed in the literature to overpass this hurdle: delivery carriers such as hydrogels, liposomes, polymersomes, or inorganic carriers are among the most investigated ones, but a highly efficient delivery method has not yet been established.[3] Target controlled protein delivery, therefore, is a field seeking to address these issues in order to maximise the activity and minimise the side effects of the released molecules.[4]

The delivery systems based on block copolymers present several advantages over other polymeric release systems: sizes smaller than 200 nm, superior control of nanostructure assembly and release profile, tissue penetrating ability, reduced toxicity, and enhanced permeation and retention in tumor tissues (EPR effect).[5, 6] For example, amphiphilic diblock copolymers can be applied for the preparation of micelles that are used to encapsulate hydrophobic drugs.[7] For delivery of charged biomacromolecules, interpolyelectrolyte complexation is the easiest encapsulation method because simple mixing with a carrier bearing opposite charge leads to self-assembly. In fact, this is the most common method for gene delivery because the strong charge of the phosphate groups of RNA and DNA readily interact with polyamines (such as chitosan or polyetlylenimine) leading to the formation of complexes that are stable under physiological conditions.[8]

Some years ago, Kataoka *et al.* proposed a very elegant strategy to improve the efficiency of the delivery. They used a diblock copolymer composed of a neutral block (polyethylene glycol, PEG) and an ionic block (polyamino acid) as carriers for gene therapy.[9] The ionic block interacts with the phosphate groups of DNA or RNA and thus, ensure the formation of the complex, while the PEG block is used to confer colloidal stability of the nanocarriers and to delay the phagocytosis by enlarging blood circulation times (stealth effect).[7] Unfortunately, this approach is not applicable in the case of proteins because the complexes are disrupted at physiological ionic strength.[10] Because the stability of the complexes is closely related to the charge density of its components, Kataoka *et al.* proposed stabilisation of protein containing interpolyelectrolyte complexes (IPECs) by increasing the protein charge density through bio-conjugation.[3]

Inspired by the native environment of proteins, the extracellular matrix (ECM), and targeting higher stability of protein containing IPECs, herein we propose an alternative approach - the use of sulfated glycosaminoglycan (GAG) as ionic block in diblock PEG copolymers. GAGs are negatively charged polysaccharides that play different biological roles in ECM: they can stabilise and/or protect proteins from denaturation and enzymatic degradation; play a role in proteins storage or mediate their binding to specific receptors, acting as a local regulator of their activity.[11, 12] The main advantage of the proposed herein approach over previously described methods for preparation of nanoparticles from sulfated GAGs by chemical crosslinking [13, 14] and electrostatic interactions [15-18] is that it allows formation of complexes with neutral charge, smaller size and increased stability at physiological (and even higher) ionic strength. All these properties contribute to prolonged blood circulation times of the formed IPECs and thus, more effective delivery to the targeted site. Moreover, we have applied oxime coupling of aminooxy terminated PEG to obtain the diblock copolymers. This end modification was recently described for the preparation of polysaccharide block copolymers[19] and differs from the common grafting of polysaccharides because it preserves the native structure of the GAG (none of their lateral groups are modified) and therefore retain their bioactivity.

2. Results and discussion

2.1. Synthesis of sulfated GAG diblock copolymers (GAG-*b*-PEG)

Oxime click reaction was performed as previously described for chitosan, dextran and hyaluronan (Figure 1).[19] We have synthesised five new sulfated GAG-*b*-PEG block copolymers that are listed in Table 1.

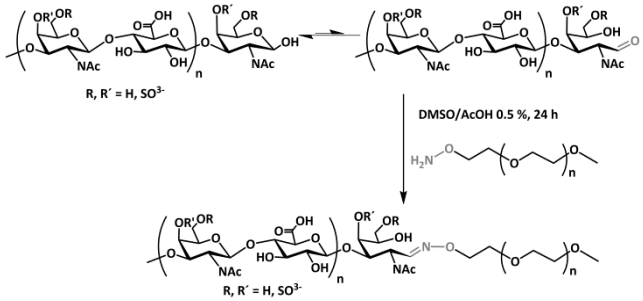

Figure 1. Oxime click reaction used for the synthesis of sulfated GAG-*b*-PEG copolymers.

Table 1. Properties of used GAGs and prepared from them GAG-*b*-PEG copolymers

GAG	10^3 Mn, [g/mol] (M_w/M_n)[a)]	DS[b)]	Intrinsic viscosity [units][a)]	GAG-*b*-PEG	10^3Mn[c)], g/mol (M_w/M_n)[a)]	Intrinsic viscosity [units][a)]
CS	2.7 (1.20)	0.7	0.03	CS_{3k}-*b*-PEG	7.9 (1.10)	0.15
	14 (1.39)	0.9	0.30	CS_{14k}-*b*-PEG	19(1.80)	0.32
	24(1.33)	0.9	0.32	CS_{24k}-*b*-PEG	29 (1.70)	0.34
HAS	12 (1.80)	1.7	0.14	HAS_{12k}-*b*-PEG	17 (1.40)	0.22
	36 (1.50)	3.0	0.17	HAS_{36k}-*b*-PEG	41 (1.40)	0.21

[a)] Determined by GPC-MALS with triple detection; [b)]determined by elemental analysis; [c)] determined by NMR. Abbreviations: CS chondroitin sulfate; HAS hyaluronan sulfate; PEG polyethylene glycol; DS degree of sulfation per disaccharide unit

Diblock copolymers were characterised by ^{1}H NMR (Figures 2 and S1-S5) and gel permeation chromatography (GPC, Figures S6-S11). Two new signals are observed in the ^{1}H NMR of the low molecular weight CS-*b*-PEG (CS_{3k}-*b*-PEG) (Figure 2, inset) corresponding to the formed oxime bond. Moreover, the signal of H1 in terminal GalNAc in the ^{1}H NMR spectrum of the precursor GAG (δ = 5.21 ppm) is not visible in the spectrum of the obtained block copolymers confirming the successful reaction between the GAGs and PEG-ONH_2 (Suppl Figure S1).

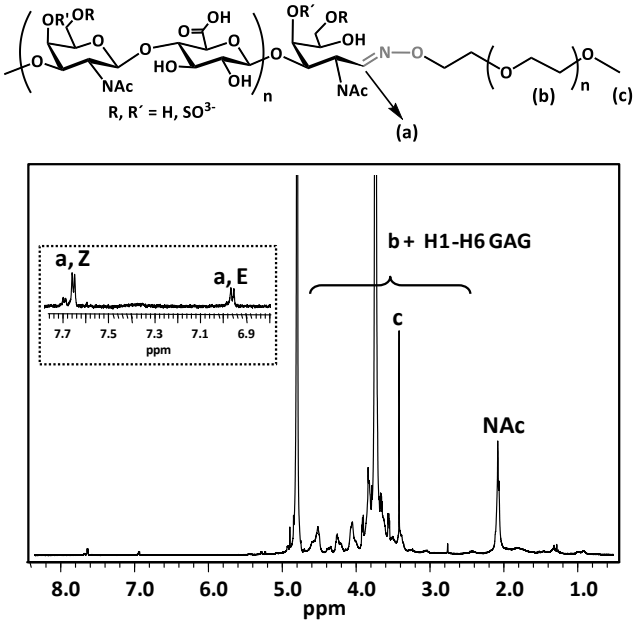

Figure 2. ^{1}H NMR (300 MHz, D_2O, 298K) of CS_{3k}-*b*-PEG.

The purity of the block copolymers (absence of free PEG added in excess) was confirmed by GPC (Figure S6). Surprisingly, we have observed similar elution volumes for the block copolymers and for the respective GAG precursors that are probably due to interactions occurring between the columns and the PEG. Nevertheless, the higher molecular weight of GAG-*b*-PEG was confirmed by the increase of the intrinsic viscosity compared to the glycan precursors (Table 1). Once the absence of free PEG was demonstrated, we determined the molecular weight (M_w) of the block copolymers from the ^{1}H NMR spectra[19] (S2 in Suppl. Info). The success of the synthetic approach for hyaluronan sulfate (HAS) and chondroitin sulfates (CS) of different M_w together with the results described previously for dextran, chitosan and hyaluronic acid proves the viability of oxime click reaction as a general tool for the preparation of the PEG diblock copolymer of polysaccharides containing a reducing end.

2.2 Nano-complex formation

Recently, we have described the formation of interpolyelectrolyte nano-complexes of hyaluronan-block-polyethylene glycol (HA-*b*-PEG) and poly-L-lysine (PLL) used as a model for a positively charged protein. We have demonstrated that the formed nano-assemblies have hydrodynamic radii ranging from 45 to 150 nm with low dispersity indices. Unfortunately, these assemblies are not stable at physiological conditions - disruption of the complexes is observed at physiological ionic strength.[20] In fact, hyaluronan is the only non-sulfated GAG and thus, among GAGs

it has the smallest negative charge. On the other hand, it is well documented that sulfated polymers (for example polystyrene sulfonate) are strong polyelectrolytes because they are completely dissociated in solution independently of the pH. [21-23] We therefore hypothesised that the substitution of HA-*b*-PEG by CS-*b*-PEG may be enough to obtain GAG-based complexes that are stable at physiological (or higher) ionic strength (Figure 3). We have also prepared diblock copolymers with a charge density higher than the one of CS, by using semisynthetic hyaluronan sulfate because of the recently reported strong interactions of HAS with growth factors.[24]

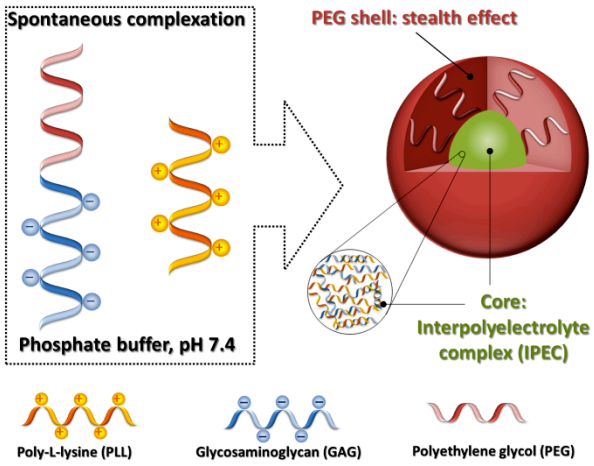

Figure 3. Schematic presentation of the complexation process between GAG-b-PEG and Poly-L-Lysine (PLL).

For comparison purposes the complexes described here were formed with poly-L-lysine (PLL) as previously reported for HA-*b*-PEG. PLL is commonly used as a model for positively charged protein because 80% of its amino groups are charged at pH 7.4. Moreover, the interpolyelectrolyte interaction between PLL and CS has been confirmed previously.[25]

IPECs were prepared by simple mixing of PLL of *ca*. 9 kDa with the respective GAG-*b*-PEG (Table 1) in phosphate buffer with low ionic strength (10 mM $NaHPO_4$, 2.8 mM HCl, ionic strength I = 20 mM) at pH 7.4. We have tested different ratios of GAG-*b*-PEG to PLL and screened the zeta potential (ζ) in order to optimise the assembly process. The mixtures for which ζ *ca.* 0 was determined were further optimised and the IPECs sizes and dispersity (PDI) were obtained by dynamic light scattering (DLS, Table S1).

Table 2. Characteristics of the GAG-*b*-PEG/PLL complexes (10 mM $NaHPO_4$, 2.8 mM HCl, pH 7.4, ionic strength, I = 20 mM)

GAG	10^{-3} Mn[a], [g/mol] (Mw/Mn)	DS	R_h [nm]	PDI	ζ potential [26]
CS	2.7 (1.20)	0.7	208	0.49[b]	0.4
	14 (1.39)	0.9	40±1	0.05±0.04	-1.2
	24 (1.33)	0.9	94±8	0.08±0.02	0.3
HAS	12 (1.80)	1.7	22±1	0.09±0.02	-0.1
	36 (1.50)	3.0	25±1	0.16±0.05	0.4

[a] Determined by GPC-MALS with triple detection [b] not optimised due to the high PDI. Abbreviations: CS chondroitin sulfate; HAS hyaluronan sulfate; DS degree of sulfation per disaccharide unit; R_h Apparent hydrodynamic radius; PDI polydispersive index

Three different batches were prepared for each selected ratio in order to evaluate the reproducibility of the IPECs preparation (presented as standard deviation in Table 2). Typical size distribution of CS-*b*-PEG and HAS-*b*-PEG complexes is presented in Figure 4.

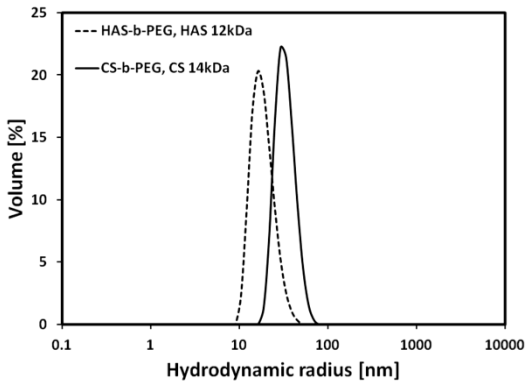

Figure 4. Typical size distribution for complexes between GAG-*b*-PEG and PLL formed in PBS with low ionic strength (10 mM $NaHPO_4$, 2.8 mM HCl, I = 20 mM) at pH 7.4.

It is well known that high ζ potential (30 ≤ ζ ≤ -30) confer stability of IPECs in solution while flocculation occurs when the ζ potential is close to zero. In fact, this dependence has been described in detail for CS/PLL complexes.[25] Here, we demonstrate the formation of stable complexes between CS-*b*-PEG and PLL with ζ potentials close to zero. The presence of PEG shell around the complexes (Figure 3) confers them stability that is not ζ dependent. Similar results have been observed for polypeptide-*b*-PEG/PLL complexes and for the recently reported HA-*b*-PEG/PLL complexes.[20] For comparison purposes, we prepared complexes of CS (24 kDa, DS 0.9) and PLL (i.e. without PEG shell). As expected, the formed IPECs have much bigger hydrodynamic radius (R_h = 2600 nm) and higher dispersity (PDI = 0.6).

The analysis of the sizes and PDI (Table 2) of the formed IPECs reveals that the formation of narrow and nanosized complexes is possible using either CS or HAS. These complexes are very stable – they did not change for at least one month. We have also evaluated the influence of the GAG molecular weight and sulfation degree on the properties of the obtained IPECs. Varying these two parameters we obtained assemblies with R_h in the range 20 - 90 nm. As can be seen from the data summarised in Table 2, a minimal size of the GAG is needed for formation of stable IPECs - CS with molecular weight of 2.7 kDa forms only polydisperse complexes. Additionally, we observed that GAG with higher sulfation degree (sulfated HA) lead to formation of smaller nano-assemblies. It is noteworthy that the complexes with sulfated GAGs are smaller than the HA-*b*-PEG/PLL complexes (R_h of *ca*. 45-150 nm)[20] in spite of the higher hydration expected for the sulfated GAGs. This can be explained with the stronger electrostatic interaction between sulfate and amino groups in comparison of the carboxylic/amino interaction leading to exclusion of water molecules from the hydrated polysaccharide chain.

2.3 Microscopy studies

The morphology and the size of the nano-complexes were further

investigated by scanning transmission electron microscopy (STEM). We have experienced some difficulties in the direct STEM analysis because the obtained IPECs are quite soft similarly to the previously reported HA-*b*-PEG/PLL complexes.[20] We have therefore crosslinked the IPECs with glutaraldehyde and evaluated the effect of this treatment on the size of the complexes (Table 3). The DLS data confirm that the crosslinking has no significant effect over the size. Figure 5 shows the STEM and TEM images of IPECs obtained between PLL and GAG-*b*-PEG with different properties: as expected, spherical objects with different sizes are observed.

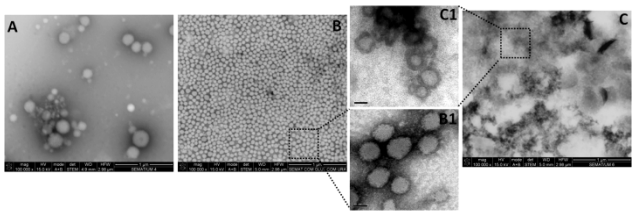

Figure 5. Representative STEM (A, B and C) and TEM (B1 and C1) images of the IPECs obtained from PLL and CS_{24k}-*b*-PEG (A), CS_{12k}-*b*-PEG (B and B1) and HAS_{12k}-*b*-PEG (C and C1). Scale bars for the STEM micrographs are equal to 1μm and for TEM to 50nm.

The size of ca. 20 particles was measured from the images obtained for the samples CS_{14k}-*b*-PEG, CS_{24k}-*b*-PEG and HAS_{12k}-*b*-PEG (Table 3). The values determined by TEM are in good agreement with DLS data and follow the same tendency as observed by DLS.

Table 3. Comparison of the GAG-*b*-PEG/PLL complexes sizes determined by different methods

Sample	R_h (nm)	R_h (nm) after crosslinking	Radius (nm) STEM
CS_{24k}-*b*-PEG	94±8	107	104±20
CS_{14k}-*b*-PEG	40±1	48	30±4
HAS_{12k}-*b*-PEG	22±1	27	22±2

Abbreviations: CS chondroitin sulfate; HAS hyaluronan sulfate; DS degree of sulfation per disaccharide unit; R_h Apparent hydrodynamic radius; PDI polydispersive index; PEG polyethylene glycol; STEM scanning transmission electron microscope

Previous studies of IPECs between sulfated polysaccharide and chitosan or trimethyl chitosan (TMC)[15-18, 27-29] reported relatively narrow distributions and sizes between 150-300 nm but only when excess of one of the polyelectrolyte is used (otherwise flocculation occurs). However, the low charge of chitosan at pH 7.4 minimises the electrostatic interaction and thus, the chitosan based particles are not stable at physiological conditions. TMC-CS has higher charge at physiological pH but the particles obtained in PBS swell to around 1-2 μm due to the screening effect. The presence of PEG attached to the GAG moiety brings important benefits to the reported herein IPECs as compared to these previous results: neutral particles with colloidal stability. Moreover, PEG also reduces the aggregation of the complexes leading to formulations with smaller sizes. As a result, the obtained IPECs are well tailored to penetrate into tissues and cells and with an optimal size to be accumulated into e.g. tumor tissues due to the enhanced permeation and retention effect.[5, 6]

2.4 Resistance to high ionic strength

The primary objective of the preparation of sulfated GAG-*b*-PEG copolymers is their use in the formation of neutral IPECs that are stable at physiological ionic strength. Thus, the complexes prepared at low ionic strength (*ca*. 20 mM) were analysed in terms of stability by stepwise addition of concentrated NaCl solution. The R_h and count rate for the GAG-*b*-PEG/PLL complexes was followed 30 min after each step (Figure 6).

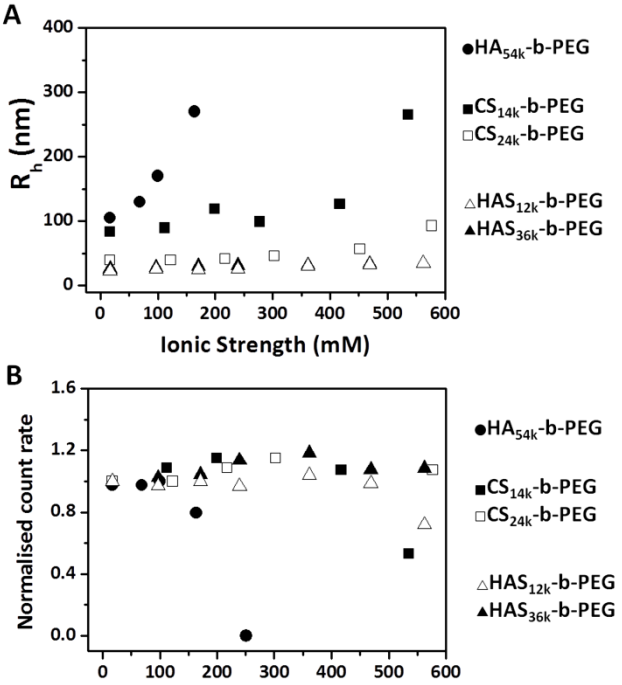

Figure 6. Hydrodynamic radius (A) and count rate (B) for GAG-*b*-PEG/PLL complexes as a function of the ionic strength. The results are compared to the previously reported results for HA-*b*-PEG/ PLL complexes[20]

It is clear that the count rate starts to decrease at 100 mM for the HA based complexes (Figure 6B). These complexes are disrupted between 150 and 250 mM ionic strength.[20] At the same ionic strengths, the sulfated GAGs-*b*-PEG/PLL complexes remain stable. Moreover, no change in the count rate was detected up to 500 mM ionic strength for all tested sulfated GAG-*b*-PEG. These results confirm our starting hypothesis that the stronger negative charge of sulfate groups results in formation of stable complexes because of the higher number of electrostatic contacts between the amino groups from the PLL and GAG sulfate groups in spite of the possible screening effect due to the presence of salt in the solution. Moreover, the strong swelling observed in HA-*b*-PEG between 20 and 100 mM ionic strength does not occur in the IPECs formed from sulfated GAGs-*b*-PEG (Figure 6A).

Natural chondroitin sulfates have a degree of sulfation per disaccharide unit (DS) around 1 (Table 2) and this DS is enough to prepare complexes that are stable at ionic strengths ranging from 20 to 500 mM. In this range we did not observe significant difference in the stability of the studied complexes and dependence of DS (studied DS = 1, 1.7 and 3). Thus, we can state that at physiological ionic strength sulfation is required for stabilisation of complexes and DS is crucial in the size control as demonstrated above.

Another property that can influence the stability of the IPECs is the molecular weight of the GAG (since we have always used the same M_w of the PEG block). Generally, the interactions between the polyelectrolytes increase with the molecular weight until a threshold over which no important differences are observed.[30] Such a tendency was described for the IPECs obtained from HA-*b*-PEG: higher M_w leads to formation of more stable complexes with a less pronounced swelling when salt is added.[20] In the case of sulfated GAG-*b*-PEG, the stronger interaction between the sulfate and the amino groups leads to lower M_w threshold and no differences are observed as a function of the M_w.

2.5 Complexation of CS-*b*-PEG with basic fibroblast growth factor (FGF-2)

Proteins, including growth factors, are less flexible and have lower charge density than PLL making the interpolyelectrolyte interaction weaker. The preparation of GAG-*b*-PEG/protein complexes should be optimised for each protein and various GAGs should be screened to find optimal encapsulation efficiency, size etc. As a proof of concept and aiming to demonstrate the feasibility of GAG-*b*-PEG for proteins encapsulation, we have investigated the complexation of CS_{24k}-*b*-PEG with basic fibroblast growth factor (FGF-2). FGF-2 is a globular protein (R_h = 1.45 nm) that contains a number of basic residues and has an isoelectric point of pI = 9.6[11, 31-33] (Figure 7). Thus, we expected that it will make stable IPECs with the sulfated GAG-*b*-PEG.

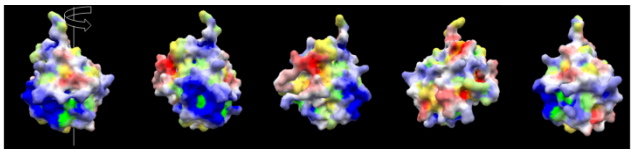

Figure 7. Charge distribution on the surface of FGF-2 (Protein Databank identifier PDB ID: 1bld, http://ef-site.hgc.jp/): blue and red indicate positively and negatively charged domains, respectively. The hydrophobic domains are visualized in yellow.

Indeed, mixing of FGF-2 with CS_{24k}-*b*-PEG (phosphate buffer, pH 7.4) results in the formation of a milky solution immediately indicating an ongoing complexation. We have tested different ratios between FGF-2 and CS_{24k}-*b*-PEG (Table S2) as for the GAG-*b*-PEG/PLL complexes. Complexes with low PDI and sizes below 100 nm (DLS) were generated at mass ratio CS_{24k}-*b*-PEG:FGF-2 ca 0.3. The IPEC obtained at this ratio (98±7 nm and PDI 0.22±0.02) was further analysed by nanoparticle tracking analysis (NTA). One of the advantages of this method is the use of very small amount of sample. It allows visualisation of the particles and gives information about the particles size and their concentration in the solution; the trajectory of hundreds of particles is tracked by the software and the size is determined from the rate of Brownian motion. Initially, we have analysed solutions of each component separately (Suppl video 1 and 2 for FGF-2 and CS_{24k}-*b*-PEG respectively). We did not observe any particle formation in these solutions. Upon mixing both components at ratio 0.3, formation of particles is observed (Suppl video 3) and their concentration ($4.8x10^8$ particles/mL) demonstrates a high efficiency of the complexation process. The particles size obtained by NTA (Figure 8, Suppl Figure S16) agrees well with the DLS values. It must be noticed that the particles are stable against the high dilution needed for the NTA analysis which is an indication of strong electrostatic complexation. However, we believe that the sulfation pattern (regioselectivity) is another important parameter that must be investigated because is expected to influence the properties of complexes formed between sulfated GAG-*b*-PEG and specific proteins such as growth factors.[14, 15]

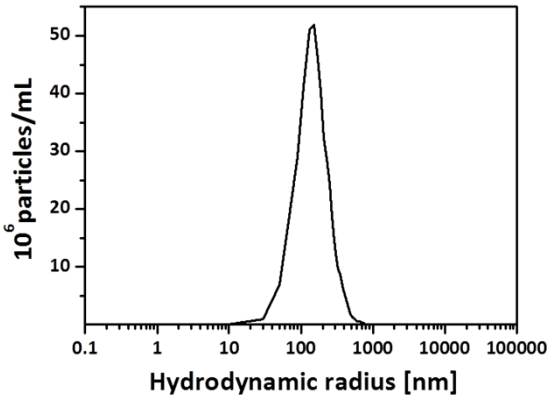

Figure 8. Size distribution obtained by nanoparticle tracking analysis of the CS_{34k}-*b*-PEG /FGF-2 complexes (mass ratio 0.3)

3. Experimental Section

3.1 Materials

High molecular weight chondroitin sulfate (CS, bovine trachea, 50 kDa as determined by GPC) and hyaluronidase (type IV) were purchased from Sigma Aldrich. Hyaluronan was purchased from Kraeber & Co. GmbH, Germany. Recombinant Human FGF-2 was purchased from Peprotech (UK). MeO-PEG-ONH_2 (5.2 kDa by MALDI-TOF) was prepared by a two-step procedure from commercial MeO-PEG-OH as previously described.[19] All other reagents were purchased from Sigma-Aldrich. Dialysis membranes with molecular weight cut off 100 kDa (cellulose ester) were purchased from Spectrum Laboratories. Ultrafiltration membranes (regenerated cellulose) were purchased from Millipore.

3.2 Methods

3.2.1 Digestion of chondroitin sulfate and hyaluronan combined with fractionation by ultrafiltration

CS (2 g) was dissolved in phosphate buffer (0.1 M, pH 5.3) and digested with hyaluronidase (15.4 mg IV, 22976 units) at 37 ºC during 48 h. Subsequently the reaction was stopped by heating the solution at 70 ºC during 25 minutes and centrifuged at 10.000 rpm for 30 min. The digested CS was then fractionated by ultrafiltration using 10, 5 and 1 kDa cut off membranes. The fraction retained by the 1 kDa membrane was analysed by GPC, NMR and elemental analysis.

3.2.2 Sulfation of hyaluronan

The HAS derivatives with a degree of sulfation per disaccharide unit (DS) of 1.7 and 2.0, respectively, were synthesised as recently described. [34] Briefly, in a first step the sodium salt of hyaluronan was transformed into tetrabutylammonium salt (TBA-HA) using Dowex WX8 ion exchanger. The following sulfation reactions of the TBA-HA

were performed under argon in DMF at room temperature. For the HAS with DS = 1.7 a SO_3-pyridine complex (molar polymer/SO_3 ratio 1 : 7) was used as sulfation agent whereas a SO_3-DMF complex (molar polymer/SO_3 ratio 1 : 20) was added to prepare the highly sulfated hyaluronan (DS = 3.0). The sulfated products were isolated from the reaction mixture by precipitation into acetone and neutralised with ethanolic NaOH solution. The formed sodium salts of the HAS were washed with acetone and purified by dialysis against distilled water followed by lyophilisation and drying of the resulting polymers under vacuum. For analytical data see Table 1.

3.2.3 Synthesis of the polysaccharide block copolymers

CS or HAS was dissolved in AcOH (0.5%) buffer solution at pH 3 (50 mg/mL). MeO-PEG-ONH_2 (5.2kDa, 5 Eq.) was dissolved in DMSO (50 mg/mL). Both solutions were mixed and stirred at 45ºC for 24h. The mixture was then added into excess of dioxane and freeze dried. The obtained white foam was dissolved in water and excess of ethanol was added. The resulted opalescent solution was dialysed against ethanol (cut off 100 kDa) until the excess of MeO-PEG-ONH_2 was eliminated as observed by GPC. After dialysis the product was suspended in ethanol and dissolved by addition of water. The ethanol was concentrated under reduced pressure. Finally, the water solution of the block copolymer was freeze dried to obtain white foam. The products were characterised by GPC and ^{1}H NMR. The detailed characterisation including the spectra and eluograms is included in the supplementary information.

3.2.4 Interpolyelectrolyte complexation

Solutions of sulfated GAG-*b*-PEG and PLL or FGF-2 were prepared in a phosphate buffer with low ionic strength (10 mM $NaHPO_4$, 2.8 mM HCl, pH 7.4, ionic strength I = 20 mM). IPECs were formed by adding a solution of PLL or FGF-2 to the solution of GAG-*b*-PEG under vigorous stirring. The respective concentrations are listed in Table S1 for PLL and in Table S2 for FGF-2.

3.2.5 Gel permeation Chromatography (GPC)

GPC measurements were performed on two different sets of three columns connected inside a Malvern Viscotek TDA 305 with refractometer, right angle light scattering and viscometer detectors. All samples were measured with a Malvern A-Guard Precolum (10 µm, 6×50 mm), A2000 (6 µm, 7.8×300 mm) and A2500 (6 µm, 7.8×300 mm) or with Malvern A-Guard Precolum (10 µm, 6×50 mm), A2500 (6 µm, 7.8×300 mm) and PLaquagel-OH-Mixed (8 µm, 7.5×300 mm). The system was kept at 30ºC. We have used 0.1M NaN_3, 0.01M NaH_2PO_4, pH=7.5 as eluent at rate of 1 mL/min. PEG 20kDa (Malvern) and Pullulan 200kDa (PSS standard services) were used to calibrate the detectors.

3.2.6 NMR spectroscopy

NMR spectra were recorded at Bruker Avance 300 MHz, in D_2O. Chemical shifts are reported in ppm (δ units) downfield from 3-(trimethylsilyl)-propionic acid-d_4 (D_2O).

3.2.7 Electron microscopy

Scanning transmission electron microscopy (STEM) was performed with a NOVA Nano SEM 200 FEI microscope. Transmission electron microscopy (TEM) was carried out with a JEOL- JEM-1010. Samples with and without crosslinking were studied. Crosslinking was performed by addition of glutaraldehyde to the complex suspension under vigorous stirring (0.625 equivalents with respect to amino groups of PLL). After 24 h the samples were analysed by DLS. The carbon coated copper grids (400 meshes, 3 mm diameter) were immersed into the suspension and excess solvent was instantly absorbed by a filter paper. Then, 0.2% uranyl acetate was dropped on the sample and left for 5 min. Excess of the liquid was removed as described for the previous step.

3.2.8 Dynamic light scattering (DLS)

DLS measurements were performed in 1 cm polystyrene cells at an angle of 173° on a Malvern NanoZS with a He–Ne laser with the wavelength of 633 nm. The Zeta potential was determined in folded capillary cells (Malvern). Selected samples were measured after filtration (Millipore syringe filters with pore size of 0.2 µm) showing no significant differences in the size, PDI and count rate to non-filtrated samples. The CONTIN algorithm (intensity weighted) was applied to obtain the size distribution. The average hydrodynamic radius (R_h) and dispersity index (PDI) were determined by fitting the correlation function with the cumulant method. Small amounts (50-100 µL) of 2M NaCl were added to the IPEC solutions stepwise and the changes in the R_h, PDI, and count rate were recorded.

3.2.9. Nanoparticle tracking analysis (NTA)

NTA experiments were performed using a NanoSight NS500 instrument (Salisbury, UK). This system includes a charge-coupled device (CCD) camera that allows visualisation and tracking Brownian motion of laser-illuminated particles in suspension. The sample (CS_{24k}-*b*-PEG/FGF-2 at mass ratio of 0.3) and the control solutions of FGF-2 and CS_{24k}-*b*-PEG were filtered (Millipore filters with pore size of 0.2 µm) and then injected into the system. Initially, we have tested solutions at concentrations used for DLS analysis (Table S2 in bold). However, the particles concentration for the IPEC was very high for NTA and we needed to dilute the solutions 50 times and then to analyse them. Video images were analysed by the NTA analytical software version 2.3. The measurements were made at room temperature, and each video sequence was captured over 60 s (Supplementary Info).

4. Conclusions

We have demonstrated that the stabilisation of protein containing IPECs can be achieved by increasing the charge density of the ionic block discarding the need of protein modification (as proposed by Kataoka *et al.)*. Moreover, the native extracellular partners of the proteins – glycosaminoglycans – were explored as a counter polyions required for the self-assembly. The used herein oxime click reaction allows preservation of all structural features of GAGs (functional groups, molecular weight, regio- and sterio- chemistry) and thus, their bioactivity. The introduced PEG moiety confers stability of the obtained neutral IPECs at physiological ionic strength. These properties together with the possibility for tunable size emphasise the enormous potential of sulfated GAG-*b*-PEG copolymers for the engineering of delivery systems for positively charged proteins.

Acknowledgements

The research leading to these results has received funding from the European Union's Seventh Framework Programme under

Find and Bind (NMP4-SL-2009-229292) and POLARIS (REGPOT-2012-2013-1-316331) projects. RNC thanks Xunta de Galicia for his Postdoctoral grant. SM and MS acknowledge financial support from the Deutsche Forschungsgemeinschaft (TR 67, A2). The authors would like to acknowledge Dimitry V. Pergushov for the helpful discussion on interpolyelectrolyte complexation.

Notes and references

[a] *3B's Research Group – Biomaterials, Biodegradables and Biomimetics, University of Minho, Headquarters of the European Institute of Excellence on Tissue Engineering and Regenerative Medicine, AvePark, 4806-909 Taipas, Guimarães, Portugal; ICVS/3B's – PT Government Associate Laboratory, Braga/Guimarães, Portugal; E-mail: pashkuleva@dep.uminho.pt*

[b] *Department of Organic Chemistry and Center for Research in Biological Chemistry and Molecular Materials (CIQUS), University of Santiago de Compostela, Jenaro de la Fuente s/n, 15782 Santiago de Compostela, Spain.; E-mail:* rnovoausc@yahoo.es

[c] *Biomaterials Department, INNOVENT e.V., Pruessingstrasse 27 B, 07745 Jena, Germany.*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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