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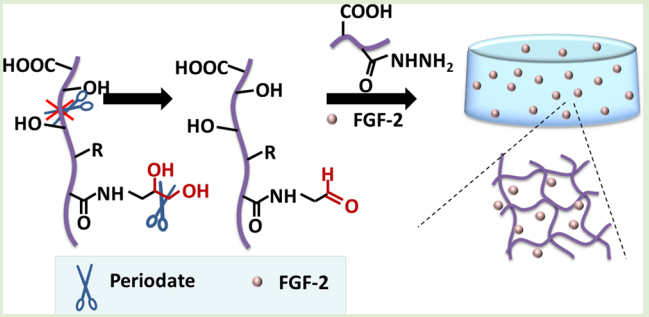
Mild and Efficient Strategy for Site-Selective Aldehyde Modification of Glycosaminoglycans: Tailoring Hydrogels with Tunable Release of Growth Factor

Shujiang Wang,‡ Oommen P. Oommen,‡ Hongji Yan, and Oommen P. Varghese*

Polymer Chemistry Division, Department of Chemistry, Ångström Laboratory, Uppsala University, Box 538, 751 21 Uppsala, Sweden

Supporting Information

ABSTRACT: Aldehydes have been used as an important bioorthogonal chemical reporter for conjugation of large polymers and bioactive substances. However, generating aldehyde functionality on carbohydrate-based biopolymers without changing its native chemical structure has always persisted as a challenging task. The common methods employed to achieve this require harsh reaction conditions, which often compromise the structural integrity and biological function of these sensitive molecules. Here we report a mild and simple method to graft aldehydes groups on glycosaminoglycans (GAGs) in a site-selective manner without compromising the structural integrity of the biopolymer. This regio-selective modification was achieved by conjugating the amino-glycerol moiety on the carboxylate residue of the polymer, which allowed selective cleavage of pendent diol groups without interfering with the C2−C3 diol groups of the native glucopyranose residue. Kinetic evaluation of this reaction demonstrated significant differences in second-order reaction rate for periodate oxidation (by four-orders of magnitude) between the two types of vicinal diols. We employed this chemistry to develop aldehyde modifications of sulfated and nonsulfated GAGs such as hyaluronic acid (HA), heparin (HP), and chondroitin sulfate (CS). We further utilized these aldehyde grafted GAGs to tailor extracellular matrix mimetic injectable hydrogels and evaluated its rheological properties. The composition of the hydrogels was also found to modulate release of therapeutic protein such as FGF-2, demonstrating controlled release (60%) for over 14 days. In short, our result clearly demonstrates a versatile strategy to graft aldehyde groups on sensitive biopolymers under mild conditions that could be applied for various bioconjugation and biomedical applications such as drug delivery and regenerative medicine.



INTRODUCTION

Aldehydes and ketones are important bioorthogonal chemical reporters, which have been extensively used for conjugation of large polymers and bioactive substances.[1,2] They are used for functionalizing several biomolecules such as nucleic acids,[3] proteins,[4] aptamers,[5] and so on. As aldehyde condensation products with hydrazide or hydroxylamine derivatives (namely, hydrazone and oxime) form pH sensitive bonds that remain stable at physiological pH, but cleavable at acidic pH, they are used to develop drug delivery systems.[6,7] Although there are several methods to develop aldehyde-functionalized biomolecules, generating aldehyde functionality on polysaccharide without changing its native chemical structure always persisted as a challenging task. Few strategies employed to acquire this functionality involve ozonolysis of enzymatic degradation product followed by reduction,[8] acidic deprotection of acetal-protected aldehydes,[9,10] as well as use of other radical based oxidants such as TEMPO (2,2,6,6-tetramethyl-1-piperidinyloxy radical).[11] All these strategies have their own limitations as they employ harsh reaction conditions, causing reduction in polymer chain length by at least 1 order of magnitude.[8,10,11] The other method employed to develop aldehyde modification on polysaccharides involves periodate oxidation of C2−C3 vicinal diols of the carbohydrate unit, which results in opening of the sugar ring structure.[12] This severely compromises the structural integrity and biological function of glycosaminoglycans (GAGs). It has been shown that opening of sugar rings by periodate results in a lack of cellular recognition of the biopolymer, which limits its use for tissue engineering application.[10] Thus we focused our efforts to develop aldehyde modifications on biopolymers under mild conditions that differ minimally from their native structure to preserve their unique physiochemical and biochemical properties.

Aldehydes generated on polymers have attracted much interest, as these could be used for a broad range of applications such as hydrogel systems for regenerative medicine,[13−15] as tissue adhesive glues,[16] and for coating biomedical devices.[17] However, using natural biopolymers that are present within the extracellular matrix (ECM) bring unique properties to these materials,[18] thereby improving its biocompatibility[19] and

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enhancing its biological recognition.[20−22] We have previously shown that hydrazone cross-linking chemistry could be utilized for designing injectable hydrogels, which provide an opportunity for minimal invasive bone regeneration applications in vivo.[13,23]

In this Article, we present a simple and versatile synthetic strategy to graft aldehyde moieties on GAGs without compromising the structural integrity of the biopolymer. This was achieved without using harsh reaction conditions or toxic reagents. We generated aldehyde units on sulfated GAGs such as heparin (HP), and chondroitin sulfate (CS,) as well as on nonsulfated GAG such as hyaluronic acid (HA). These GAGs are composed of linear polysaccharides having different functional groups such as carboxylic acids, hydroxyls, amides, and so on. Selective chemical modifications of these polymers could be utilized for designing soft hydrogels that mimic natural ECM, which is also a hydrogel composed of various protein fibrils interwoven within a hydrated network of GAG chains.[24] These ECM polymers also possess binding sites for specific growth factors, which preserves the bioactivity of these molecules by noncovalent interaction.[25] Thus utilizing these biopolymers for developing 3D scaffold for delivering therapeutic protein would be an ideal choice for tissue engineering applications. We therefore utilized our synthetic strategy to design hydrazone cross-linked ECM mimetic hydrogels with tunable release of growth factor by regulating the composition of hydrogel.

EXPERIMENTAL SECTION

Synthesis of Aldehyde Derivative of GAG. HA, HP, or CS (400 mg HA; 600 mg HP; and 500 mg CS; 1 mmol of disaccharide repeating units) was dissolved in 100 mL of deionized water. To this reaction mixture, *N*-hydroxybenzotriazole (HOBt; 153 mg, 1 mmol) and 3-amino-1,2-propanediol (91 mg, 1 mmol) were added and stirred until it was completely dissolved. Thereafter, the pH of the resultant solution was adjusted to 6.0 (using 1 M HCl) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC; 57.5 mg, 0.3 mmol) was added and stirred overnight. The solution was loaded into a dialysis bag (Spectra Por-3, MWCO 3500 for HA and CS and Spectra Por-7, MWCO 1000 for HP) and dialyzed against dilute HCl (pH = 3.5) containing 0.1 M NaCl (2 × 2L, 48 h), followed by dialysis in dilute HCl (pH 3.5) (2 × 2L, 24 h). The solution was lyophilized to obtain amino-glycerol-modified HA (**3a**), HP (**3b**), and CS (**3c**).

The lyophilized GAG derivatives **3a−c** were selectively oxidized by $NaIO_4$ following the general method. Briefly, GAGs **3a−c** (0.5 mmol of disaccharide repeating units) were dissolved in 45 mL of deionized water. Predissolved $NaIO_4$ solution (107 mg, 0.5 mmol dissolved in 0.5 mL water) was added to the reaction mixture and stirred for 5−15 min (Table 1). The unreacted $NaIO_4$ was quenched by addition of 5-fold excess ethylene glycol (160 μL, 2.5 mmol) and stirred for additional 1 h. The resultant solution was loaded into a dialysis bag (Spectra Por-3, MWCO 3500 for HA and CS and Spectra Por-7, MWCO 1000 for HP) and dialyzed against deionized water (2 × 2L, 48 h). The obtained solution was lyophilized to obtain aldehyde modified HA (**4a**), HP (**4b**), and CS (**4c**) and stored at −20 °C.

The percentage of aldehyde modification for HA and CS was determined by ^{1}H NMR by treating aldehyde-modified HA and CS with *tert*-butyl carbazate followed by $NaCNBH_3$ reduction. The ratio of *tert*-butyl signal at 1.4 ppm and *N*-acetyl signal at 2.0 ppm of GAG was used to ascertain the percentage of aldehyde functionalization (Table 1). For HP, the ^{1}H NMR could not be used due to the absence of reference peak because of the random sulfation pattern. The percentage aldehyde functionalization was equivocally quantified by UV spectrophotometric analysis for HA, HP, and CS as indicated below.

Table 1. Optimization of Periodate Oxidation Reaction

	time (min)	modification (%)[a]	modification (%)[b]
HA−CHO	5	9	8
	10	9	8.5
HP−CHO	5	nd	5
	10	nd	8.4
	15	nd	11.1
CS−CHO	5	10	9.8
	10	12	12.5
	15	13	13

The amino-glycerol modifications were performed with 0.3 mol equivalent EDC at pH 6. [a]Percentage of aldehyde modification as obtained from NMR. [b]Percentage of aldehyde modification as obtained from UV measurements; nd = not determined.

UV Spectrophotometric Analysis. The degree of aldehyde modification was determined spectrophotometrically by measuring the rate of periodate consumption before quenching the reaction with ethylene glycol (Table 1). Briefly, 5 μmol of GAGs **3a−c** (with respect to disaccharide repeating units) and native GAGs **1a−c** (2 mg of HA; 3 mg of HP; and 2.5 mg of CS) was dissolved in 1 mL of distilled water to reach a concentration of 5 mM. To this solution $NaIO_4$ (1 mg, 5 μmol) was added, and UV absorbance at 290 nm was monitored at different time intervals. Each experiment was performed in triplicate.

Determination of the Rate of Oxidation. For reactions of type $A + B \xrightarrow{k_2} C$, the second-order rate constant can be obtained from equation (eq 1) when $c_A^0 \neq c_B^0$.[26]

$$\ln \frac{c_A}{c_B} = (c_A^0 - c_B^0)k_2 t + \ln \frac{c_A^0}{c_B^0} \quad (1)$$

Here t is time (s), k_2 is the second-order rate constant, c_A^0 and c_B^0 are the concentrations (mol·L^{-1}) of A and B at time $t = 0$, and c_A and c_B are the concentrations (mol·L^{-1}) of A and B at time t. The oxidation rate k_2 for native and amino glycerol-modified GAGs were determined by plotting $\ln(c_A/c_B)$ against 't' (Table 2). A representative kinetic plot is shown in Figure S1b in the Supporting Information (SI).

Table 2. Second-Order Rate Constants for Periodate Oxidation of Native and Amino Glycerol-Modified GAGs

polymer	k_2 (L·mol^{-1}·s^{-1})
HA	$1.1 \times 10^{-4} \pm 2.2 \times 10^{-5}$
HA−10% CHO	0.72 ± 0.042
HA−20% CHO	0.66 ± 0.041
HP	$7.7 \times 10^{-4} \pm 4.72 \times 10^{-5}$
HP−20% CHO	0.21 ± 0.042
HP−50% CHO	0.22 ± 0.02
CS	$1.2 \times 10^{-4} \pm 1.54 \times 10^{-5}$
CS−10% CHO	0.24 ± 0.021
CS−30% CHO	0.23 ± 0.05

Protein Release Experiment. *Release of Bovine Serum Albumin (BSA) from Hydrogel.* BSA release experiment was performed on all gels. Briefly, HA-aldehyde (HA−CHO), HP-aldehyde (HP−CHO), CS-aldehyde (CS−CHO), and HA-hydrazide (HA−CDH) were separately dissolved in phosphate buffered saline (PBS; pH 7.4) to reach a concentration of 20 mg/mL. BSA was dissolved in Milli-Q water to reach a concentration of 5 mg/mL. For HA−HA gel, 50 μL BSA solution was mixed with 100 μL HA-CDH solution first, followed by addition of 100 μL HA-CHO solution to prepare gel containing 0.2% (w/v) BSA. Gel was incubated 24 h at room temperature before performing the release experiment. HA−HP and HA−CS gels (where 50 μL of HA−CHO was replaced by 50 μL of HP−CHO or CS−CHO) containing 0.2% (w/v) BSA were prepared the same way. Each gel was incubated in 1.25 mL of PBS (pH 7.4) at room temperature,

and 1 mL of release medium was taken out and measured at 489 nm at different time points. The release medium was placed back to the vial after each measurement. Concentration of released BSA was determined from the standard plot with known concentration of BSA.

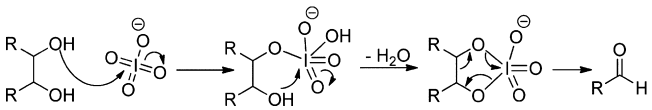
Release of Human Basic Fibroblast Growth Factor (FGF-2) from Hydrogel. Gels for FGF-2 release were prepared in the same way as for BSA release experiment. Briefly, 250 μL hydrogels containing 75 ng of FGF-2 were incubated at room temperature in 1.25 mL of PBS (pH 7.4) containing 0.5% (w/v) BSA. For different time points, separate gels were prepared to minimize standard deviation. The release mediums from gels prepared on different time intervals were collected in reverse order to obtain different time points (1, 2, 7, and 14 days) on day 14. All the experiments were performed in triplicate.

FGF-2 concentrations in release medium were measured using a FGF-2 ELISA kit from the Invitrogen, according to manufacturer's instructions. Briefly, 50 μL/well of dilute buffer followed by 50 μL/well of sample was added to each well in a coated 96-well plate and incubated at room temperature for 2 h. A dilution series of known concentrations of human FGF-2 in release medium was used to generate a fresh calibration curve for the assays. Each well was emptied and washed three times with wash buffer; thereafter, 100 μL/well of biotin-anti-FGF-2 was added and incubated at room temperature for another 1 h. The medium was removed from each well and washed three times with wash buffer, and 100 μL/well of a 1:1000 dilution of streptavidin–horseradish peroxidase was added and incubated again at room temperature for 30 min. The medium was removed again from each well and washed three times with wash buffer, followed by addition of 100 μL/well of 3,3,5,5-tetramethylbenzidine. The plate was incubated for 30 min in the dark, followed by quenching of the reaction using 100 μL/well of stop solution. The plate was read at 450 nm after 10 min to determine the FGF-2 level in the released medium.

RESULTS AND DISCUSSION

In search for a mild and reproducible synthetic strategy to develop aldehyde functionality on GAGs, we looked at the periodate oxidation chemistry, previously studied by several groups.[12] The advantage of this chemistry is that it can be performed under aqueous conditions and in neutral pH. The rate-limiting step for this reaction involves the formation of cyclic iodate ester intermediate followed by concerted bond cleavage to generate aldehyde residues (Scheme 1).[27] This implies that the stereochemistry of the vicinal diols is crucial for successful oxidation step. Since all GAGs have glucopyranose structure with C2–C3 vicinal diols in *trans* geometry, we envisioned that installation of glycol residue on the GAG backbone would permit generation of flexible vicinal diols. This would facilitate selective and efficient oxidation of the side chains without any backbone oxidation, as the two types of diols will have different reaction kinetics.

Scheme 1. Mechanism of $NaIO_4$-Mediated Oxidation of Vicinal Diols



With this rational, we synthesized amino-glycerol derivatives of HA, HP, and CS **3a–c** using carbodiimide chemistry with HOBt as the activating agent (Scheme 1; Table 1).[28] ^{1}H NMR analysis of this product, however, did not give much information as the glycerol signals overlapped with the carbohydrate signals. Further, unlike HA and CS, HP possesses a random sulfation pattern, which also complicated the NMR analysis. Thus we attempted to find an alternative route to verify the synthesis and oxidation step. Since $NaIO_4$ has a unique UV absorbance at 220 and 290 nm, we decided to determine the consumption of this reagent by measuring the absorbance at 290 nm.[29] The weaker 290 nm was chosen over 220 nm in order to prevent interference of absorbance from GAGs. First we checked the detection limit of $NaIO_4$ at 290 nm to see whether the $NaIO_4$ concentration could be detected at the concentration range used for the oxidation reaction. The standard curve showed a linear plot for $NaIO_4$ concentrations from 0.2 mM to 7 mM (Figure S1a in the SI). This allowed direct determination of rate of oxidation for different GAGs with minimal standard deviation. Gratifyingly, we observed an instant aldehyde formation **4a–c** with a distinct oxidation pattern for amino-glycerol tagged GAG as compared to their native counterpart (Figure 1). The percentage of aldehyde as quantified by spectrophotometric method was in close agreement with the ^{1}H NMR analyses (as confirmed by integrating the *t*-butyl signal at 1.4 ppm of Boc-hydrazide derived hydrazone with respect to the *N*-acetyl signal at 2.0 ppm from HA and CS).

As anticipated, the oxidation of native GAGs with 1 mol equiv of periodate (with respect to the disaccharide units) did not yield any reasonable oxidation for nearly 2 h under our reaction condition (<3% oxidation, Figure 1). However, with amino-glycerol-functionalized GAGs, complete oxidation of the glycerol unit could be observed in shorter time. The oxidation time for HA was ~5 min, for HP ~40 min, and for CS ~10 min. This bimolecular reaction showed second-order kinetics with rate constants k_2 = ~0.7 L·mol^{-1}·s^{-1} for HA; ~0.2 L·mol^{-1}·s^{-1} for HP and CS modifications, respectively (Table 2). The native GAGs demonstrated k_2 = ~1.1 × 10^{-4} L·mol^{-1}·s^{-1} for HA; ~7.7 × 10^{-4} L·mol^{-1}·s^{-1} for HP and ~1.2 × 10^{-4} L·mol^{-1}·s^{-1} for CS, respectively. The representative second-order kinetic plot is given in Figure S1b in the SI. This clearly suggests that amino-glycerol modification furnished

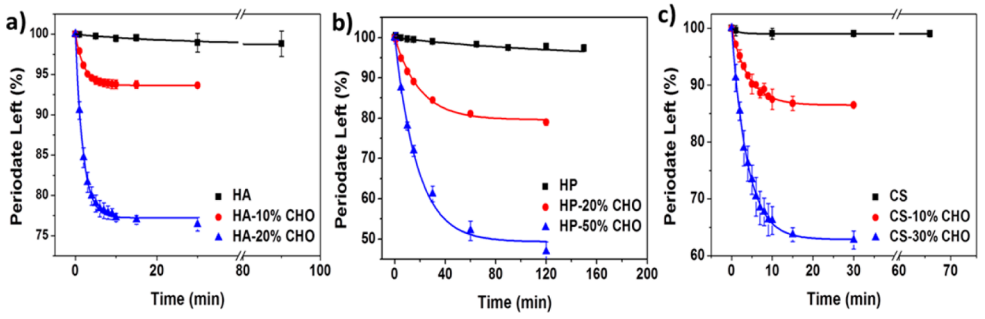


Figure 1. Kinetic plots showing the percentage of periodate left at different time points. Rate of oxidation for (a) HA (b) HP, and (c) CS.

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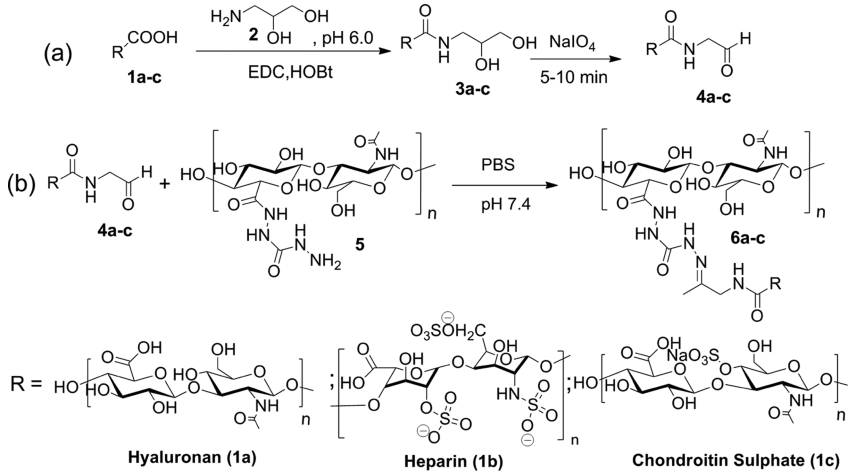
Scheme 2. (a) Synthetic Strategy for Designing Aldehyde Modified GAGs; (b) Hydrazone Crosslinking Strategy to Obtain Injectable Hydrogels



extremely faster oxidation kinetics of nearly 4 orders of magnitude as compared to the native GAGs providing a regioselective control for generating aldehydes on sensitive biopolymers.

After successfully optimizing the synthesis of aldehyde-modified GAGs, we set forward to develop injectable hydrogels using hydrazone chemistry (Scheme 2b). We adopted carbodihydrazide (CDH) derived hydrazone for this purpose, exploiting its unique intrinsic stabilization property reported earlier by our group.[30] With this chemistry we developed three types of hydrogels containing either HA alone or HA conjugated with HP or CS GAGs designated as HA−HA, HA−HP, and HA−CS hydrogel, respectively. The hydrazide and aldehyde content in these GAGs were maintained ∼10% with respect to the disaccharide units. The HA−HA hydrogel **6a** was derived by mixing HA-aldehyde **4a** and HA-hydrazide **5** in 1:1 ratio. However, to develop HA−HP and HA−CS hydrogels (**6b** and **6c**, respectively), roughly 50 wt % of HA-aldehyde **4a** was substituted with HP-aldehyde **4b** or CS-aldehyde **4c** component. The total solid content (16 mg/mL or 1.6% w/v) was maintained for good comparison between the groups. The gelling time for all the three types of hydrogels was found to be around 30 s. The efficiency of the hydrazone cross-linking was confirmed by attenuated Fourier transform infrared (FTIR) spectroscopic analysis of lyophilized hydrogels. FTIR spectrum of lyophilized gel samples displayed a disappearance of the aldehyde peak at 1729 cm^{-1} in all the three gel samples, indicative of efficient cross-linking (Figure S2). The appearance of peak at 1618 cm^{-1} is attributed to the formation of the desired hydrazone (characteristic C═N stretching) cross-linking. The sulfate residue in the HP and CS containing gels could be confirmed by S−O stretching frequency at 1233 cm^{-1}. The signal observed at 1553 cm^{-1} and 1028 cm^{-1} corresponded to carboxylate (C═O) and alcohol (C−O) residues.

We further evaluated the mechanical stability of these hydrogels before and after swelling (24 h) in PBS (pH 7.4). Rheological evaluation using frequency sweep of 0.1−10 Hz showed that HA−HA gels possessed higher rigidity with storage modulus G′ ∼ 2000 Pa, which was reduced to ∼1700 Pa after swelling (Figure 2 and Table 3). The HA−HP and HA−CS hydrogels were softer and exhibited a larger variation upon swelling with G′ reducing from ∼1500 to ∼800 Pa and ∼1100 to ∼600 Pa, respectively (Figure 2 and Table 3). This

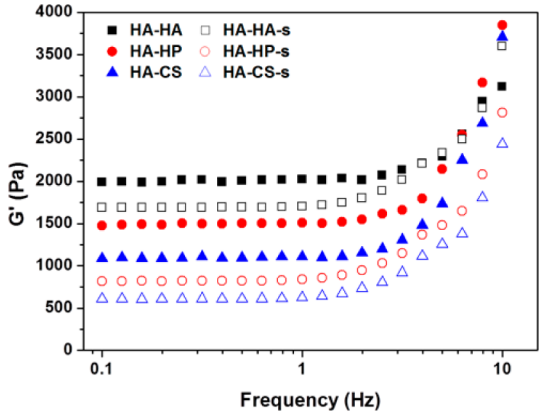


Figure 2. Rheological analysis of hydrogels before and after swelling. Frequency sweep data showing storage modulus (G′) as a function of frequency.

Table 3. Rheology Data of Hydrogels

sample	G′ (Pa)	G″ (Pa)	M_c (kg/mol)	ξ (nm)	sw%[a]
HA−HA	1978 ± 48	3.16 ± 0.6	19.98	12.8	13 ± 2.8
HA−HP	1474 ± 58	4.7 ± 0.8	26.8	14.1	23 ± 0.7
HA−CS	1085 ± 31	2.9 ± 0.5	36.42	15.6	43 ± 1.6
HA−HA[b]	1686 ± 179	7.6 ± 2.6	23.44	13.5	
HA−HP[b]	811 ± 13	7.1 ± 1.1	48.73	17.2	
HA−CS[b]	611 ± 53	3.9 ± 1.1	64.68	18.9	

[a]Percentage swelling ratio of gels after 24 h of swelling. [b]Gel after 24 h of swelling in PBS (pH 7.4).

probably could be due to differences in polymer structure and chain length. This difference in hydrogel properties was also reflected in molecular weight between cross-links (M_c)[31] and average pore size (ξ)[32] between HA−HA and other hydrogels (Table 3). HA−HA hydrogel formed a compact network with smaller M_c and ξ. The M_c, ξ, and swelling % (sw%) were calculated according to the equation given in the SI. This also resulted in slower hyaluronidase-mediated enzymatic degradation of the HA−HA gel compared to the other hydrogels (Figure S3 in the SI). This is in agreement with our previous results, which showed that reduction in pore size reduces degradation rate.[30] It is worth mentioning that increasing the

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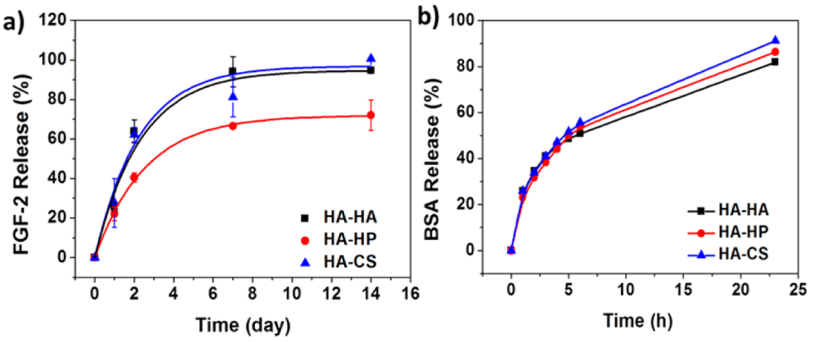

Figure 3. Release profile of proteins in different gels: (a) FGF-2; (b) BSA.

total solid content in material preparation could enhance the mechanical properties of the hydrogel.

Finally we utilized these hydrogel systems to evaluate their efficacy to deliver two different types of therapeutic proteins, namely, FGF-2 and BSA (Figure 3). FGFs are an important class of growth factor, which act as a crucial extrinsic cue for stem cell maintenance and differentiation[33] and possesses angiogenic[34] and neuroprotective[35] properties. It has been reported that a sustained release of FGF-2 for over 5 days is required to achieve regenerative benefit in rat spinal cord injury model.[36] Similarly, sustained release of FGF-2 is essential for the embryonic neural stem cell to remain proliferative and undifferentiated.[37] Since HP and heparan sulfate (HS) possess specific binding sites for FGF-2, which stabilizes this protein within the ECM,[38] we anticipated that HA−HP gel could attenuate the release profile of this growth factor. The HA−HA and HA−CS hydrogel, on the other hand, may not show this effect. BSA, on the other hand, represents a biocompatible protein that is extensively used for drug delivery applications.[39] This protein however, does not have any specific affinity for GAGs and therefore is not anticipated to discriminate between the three hydrogel systems. Interestingly, we observed a sustained release of FGF-2 for over 14 days (60%) in HA−HP gel and a faster release in HA−HA and HA−CS gels (90%) during the same period. BSA, on the other hand, did not show any substantial difference in the release profile from the three hydrogel systems (released within 24 h). The FGF-2 release was determined using ELISA, while the BSA release was estimated by the spectrophotometric method. The release of two proteins from the hydrogel system followed simple diffusion. As the pore size ξ of the hydrogel ranged from 12.8 to 15.6 nm, no substantial difference in the release rate for BSA was observed. However, binding of FGF-2 to heparin modulated its release from the heparin containing hydrogel. Although, such release kinetics do not mimic in vivo scenario, where enzymatic degradation of hydrogel is anticipated, the differences in release profile of different proteins could be very useful for tissue engineering applications that require different release rates of therapeutic proteins within the therapeutic window.

CONCLUSION

In conclusion, we demonstrate a simple and versatile method for site selective incorporation of aldehyde functionality in a polysaccharide-based biopolymer. This was achieved by taking advantage of differences in reaction kinetics of periodate oxidation (four-orders of magnitude) for vicinal diols within glucopyranose and conjugated glycol system. Although we demonstrated this fast and regioselective reaction for HA and sulfated GAGs, namely, HP and CS, it could be easily adapted for other polysaccharides. We utilized these GAG-derived aldehydes to develop injectable hydrogels with tunable release kinetics of therapeutic proteins. We show that the composition of the hydrogel influenced the release kinetics of HP binding protein FGF-2, but not for BSA. Our result clearly demonstrates a versatile strategy to graft aldehyde groups on sensitive biopolymers under mild conditions that could be applied for various bioconjugation and biomedical applications.

ASSOCIATED CONTENT

Supporting Information

Materials and methods; periodate oxidation kinetics; synthetic procedure; IR analysis; hydrogel preparation; swelling and degradation studies; rheological analysis; protein release. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*E-mail: oommen.varghese@kemi.uu.se; Fax: +46 18 471 3477.

Author Contributions

‡The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. These authors contributed equally.

Notes

The authors declare no competing financial interest.

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