Hyaluronic Acid Derivatives Prepared in Aqueous Media by Triazine-Activated Amidation

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A method is presented for the preparation of hyaluronic acid derivatives obtained through triazine-activated amidation. A number of amines were successfully reacted with hyaluronic acid carboxyl groups using 2-chloro-4,6-dimethoxy-1,3,5-triazine as an activating species in a mixture of water and acetonitrile under neutral conditions. By varying the amount of triazine reagent, it was possible to control the degree of modification. Depending on the amine chosen, degrees of modification ranging from 3 to 20% were obtained when using 0.5 equiv of the triazine to hyaluronic acid carboxyl groups. The possibility to perform the reaction in a mixture of water and acetonitrile facilitates the introduction of a wide range of both hydrophilic and hydrophobic amines. Triazine-activated amidation appears to be a highly versatile, controllable, and relatively mild technique for modification of hyaluronic acid, and we predict that it will be useful in the design of novel hyaluronic acid based biomaterials.

Introduction

The number of hyaluronic acid based products is continuously increasing; they are found in medical applications such as viscosupplementation, ophthalmic surgery aid, postsurgical adhesion prevention, tissue augmentation, and tissue engineering.[1] Since Meyer and Palmer discovered the high molecular weight polysaccharide in 1934,[2] its biological role has been investigated extensively (for a review see Laurent[3]) and has become attractive in biomaterial applications due to its unique physiochemical properties,[4] non-immunogenicity,[5] and high availability. Native hyaluronic acid has limited use due to a short turnover rate and poor mechanical stability; chemically modified derivatives are therefore developed to obtain biomaterials with tailored properties. This is generally achieved by targeting the carboxylic acid on the glucuronic acid moiety or the hydroxyl groups found on both moieties of the disaccharide repeat unit.

Prolonged in vivo residence time and improved mechanical properties can be obtained by cross-linking to form hydrogels or by adding groups that lower the aqueous solubility. The conjugation of hyaluronic acid with therapeutic agents is also a route to designing drug-delivery vehicles and biomaterials which promote specific interactions with cells. Several strategies to functionalize hyaluronic acid have been developed over the past decades; these include esterfication with alkyl or benzyl halides,[6] the use of carbodiimides for coupling of hydrazides[7] and amines,[8] and cross-linking with divinylsulfone,[9] formaldehyde,[10] and bisepoxides.[11] Although many of the modification techniques employed today have individual advantages, there is room for further improvement.

The present study describes a novel and improved method for modifying hyaluronic acid, which has a desirable combination of advantages. It is performed at neutral pH in aqueous media, and the substitution degree is highly controllable. In addition, the technique is highly versatile since it allows the regiospecific introduction of a wide range of functionalities without causing any unwanted side reactions. The described strategy involves the use of 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT, Figure 1) as an alternative reagent for coupling amines to hyaluronic acid carboxyl groups. Previous reports have shown that CDMT, which is commercially available at reasonably low cost, can be used successfully for synthesizing esters, amides, and acid anhydrides from carboxylic acids.[12]

To examine the versatility of our strategy, we have attempted to modify hyaluronic acid with a number of amines which have different functionality and would afford derivatives of hyaluronic acid with different properties. Such amines include alkyl amines that can be used to modify the solubility of hyaluronic acid, amino acids that indicate the possibility to conjugate with peptides and proteins, amines with additional reactivity that can be used for cross-linking or further coupling, and molecular probes for fluorescent labeling. The possibility to control the degree of modification was evaluated by varying the amount of added triazine reagent for each amine that was reacted with hyaluronic acid. Products were furthermore characterized by ^{1}H NMR spectroscopy, fluorescence spectroscopy, light scattering measurements, and susceptibility to hyaluronidase digestion.

Experimental Section

Materials. If not stated otherwise, all chemicals were purchased from Sigma-Aldrich of reagent grade or better and used without further purification. Spectra/Por 6 RC dialysis membranes, 3.5 and 25 kDa M_w cutoff, were purchased from Spectrum Laboratories. Ion exchange was performed using Dowex H$^+$ 50W × 8-100 sulfonic resin (Aldrich)

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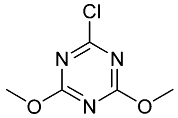


Figure 1. 2-Chloro-4,6-dimethoxy-1,3,5-triazine (CDMT).

washed thoroughly with deionized water. Hyaluronic acid sodium salt from *Streptococcus equi* (1.5×10^6 Da, Fluka) was dissolved in deionized water (5 mg/mL, pH 6.85) and mixed with Dowex H^+. The mixture was shaken at room temperature for 1 h and filtered and the solution (pH 2.85) freeze-dried to obtain hyaluronic acid in protonated form.

Instrumentation. Nuclear magnetic resonance (NMR) measurements were performed using a JEOL ECP 400 MHz spectrometer with the solvent proton signal as internal standard. Hyaluronic acid and synthesized derivatives thereof (compounds **1A**−**5A** and **1B**−**5B**) were dissolved in D_2O to concentrations of approximately 10 mg/mL and thoroughly degassed by suction before recording proton NMR spectra at 80 °C with a relaxation time of 4 s and with 128−256 scans. The reference signal from HDO was set to $\delta = 4.219$ ppm.[13] Integration of signals in spectra of hyaluronic acid modified with aminoacetaldehyde dimethyl acetal were made after performing deconvolution using JEOL Delta NMR Processing Software version 4.3.

Fluorescence measurements of 0.01 mM hyaluronic acid modified with 7-amino-4-methylcoumarin in deionized water were performed using a Perkin-Elmer LS 45 luminescence spectrometer. Excitation spectra from 200 to 370 nm were recorded at 386 nm, and emission spectra were recorded from 340 to 500 nm at an excitation wavelength of 325 nm.

The static light scattering measurements were made using a Hamamatsu photon-counting device with a 3 mW He−Ne laser, using toluene as the reference (Rayleigh ratio, $R_{tol} = 13.59 \times 10^{-6}$ cm^{-1} at 633 nm).[14] The optical constant for vertically polarized light is

$$K = 4\pi^2 n_0^{\,2} (\mathrm{d}n/\mathrm{d}c)^2 / N_A \lambda^4 \qquad (1)$$

where N_A is the Avogadro constant, λ is the wavelength, n_0 is the refractive index of the solvent, and ($\mathrm{d}n/\mathrm{d}c$) is the refractive index increment measured in a differential refractometer with Rayleigh optics at 25 °C. The Rayleigh ratio at the angle 90°, R_{90}, was determined using $[(I - I_0)/I_{tol}]R_{tol}(n_s/n_{tol})^2$. Here, n_s is the refractive index of the solvent and n_{tol} that of toluene, I is the measured intensity of the solution, I_0 the intensity of the solvent, and I_{tol} that of toluene. Molecular weight determinations were made by plotting the reduced scattering intensity, Kc/R_θ, vs $\sin^2(\theta/2)$ at a range of angles. The values of $Kc/R_{\theta=0}$ were then plotted vs the concentration, c, and the molecular weight was obtained from the intercept.[15] The radius of gyration (R_g) was estimated from the ratio of the slope to intercept of linear plots of the angular dependence of the reduced scattering intensity.

General Procedure for Triazine-Activated Amidation of Hyaluronic Acid. The following general procedure was used for the preparation of hyaluronic acid derivatives **1A**−**5A**, **1B**, and **3B**−**5B**. Hyaluronic acid (76 mg, 0.2 mmol carboxylic acid) was dissolved in 3 mL of deionized water in a 10 mL round-bottomed flask followed by the dropwise addition of 2 mL of acetonitrile while stirring. To the solution was added 22 μL (0.2 mmol) 4-methylmorpholine (NMM), causing the viscosity to increase temporarily. The solution (pH 7) was then cooled to 4 °C, and 18 mg (**1A**−**5A**) or 9 mg (**1B, 3B**−**5B**) of 2-chloro-4,6-dimethoxy-1,3,5-triazine (0.1 and 0.05 mmol, respectively) was added and stirred at room temperature for 1 h. The solution was mixed with 0.2 mmol of amine and stirred for 20 h at room temperature. Dowex H^+ (1 mL) was then added to the reaction mixture, which was stirred for 1 h and filtered. This procedure was then repeated using 1 mL of Dowex saturated with sodium. Extensive dialysis (3.5 kDa M_w cutoff) of the solution against deionized water and lyophilization afforded amide-modified hyaluronan. Compositions and specifications for synthesized hyaluronic acid derivatives (**1A**−**5A** and **1B**−**5B**) are provided in Table 1.

Propylamine Modified Hyaluronic Acid (1A and 1B). Following the general procedure, the reactions of hyaluronic acid with 16 μL of propylamine (0.2 mmol) in the presence of NMM and CDMT provided 74 mg (91%) of **1A** and 62 mg (77%) of **1B**. ^{1}H NMR (D_2O; ppm): δ 3.24 and 3.03 (m, 2H, NHCH_2), 1.94 (bs, 3H, NCO•CH_3), 1.48 (m, 2H, CH_2CH$_3$), and 0.84 (t, 3H, CH$_2$CH_3).

Aminoacetaldehyde Dimethyl Acetal Modified Hyaluronic Acid (2A and 2B). Following the general procedure, the reactions of hyaluronic acid with 22 μL of aminoacetaldehyde dimethyl acetal (0.2 mmol) in the presence of NMM and CDMT provided 74 mg (89%) of **2A.** Compound **2B** was prepared according to the general procedure in a larger scale (5×). Hyaluronic acid (380 mg, 1 mmol carboxylic acid) was dissolved in 15 mL of deionized water in a 100 mL round-bottomed flask followed by the careful addition of 10 mL of acetonitrile while stirring. To the solution was added 112 μL (1 mmol) of NMM, which raised the pH to 7 and caused the viscosity to increase temporarily. The solution was cooled to 4 °C, and 44 mg of CDMT (0.25 mmol) was added and stirred for 1 h at room temperature. The solution was mixed with 109 μL of aminoacetaldehyde dimethyl acetal (1 mmol) and stirred for 20 h at room temperature. Dowex H^+ (1 mL) was then added to the reaction mixture, which was stirred for 1 h and filtered. This procedure was then repeated using 1 mL of Dowex saturated with sodium. Extensive dialysis (3.5 kDa M_w cutoff) of the solution against deionized water and lyophilization afforded 361 mg (88%) of **2B**. ^{1}H NMR (D_2O; ppm): δ 3.38 (s, 6H, CH(OCH_3)$_2$) and 1.94 (bs, 3H, NCO•CH_3).

Glycine Ethyl Ester Modified Hyaluronic Acid (3A and 3B). A solution of 20 g of K_2CO_3 and 10 g of ethyl glycinate hydrochloride (0.07 mol) in 50 mL of deionized water was washed with diethyl ether (5×50 mL). The combined organic layers were dried with Na_2SO_4, filtered, and concentrated by evaporation, and the residue (5.73 g free amine, 78%) was used immediately. ^{1}H NMR (D_2O; ppm): δ 4.19 (q, 2H, CH_2CH$_3$), 3.40 (s, 2H, NH$_2$CH_2), and 1.25 (t, 3H, CH$_2$CH_3). Following the general procedure, the reactions of hyaluronic acid with 20 μL of glycine ethyl ester (0.2 mmol) in the presence of NMM and CDMT provided 72 mg (87%) of **3A** and 44 mg (54%) of **3B**. ^{1}H NMR (D_2O; ppm): δ 4.01 (m, 2H, OCH_2CH$_3$), 1.94 (bs, 3H, NCO•CH_3), and 1.23 (t, 3H, CH$_2$CH_3).

7-Amino-4-methylcoumarin Modified Hyaluronic Acid (4A and 4B). Following the general procedure, the reactions of hyaluronic acid with 35 mg of 7-amino-4-methylcoumarin (0.2 mmol) in the presence of NMM and CDMT provided 72 mg (89%) of **4A** and 48 mg (59%) of **4B**. ^{1}H NMR (D_2O; ppm): δ 7.78 (d, 1H, Ar-H), 7.73 (s, 1H, Ar-H), 7.50 (d, 1H, Ar-H), 6.32 (s, 1H, Ar-H), 2.44 (s, 3H, CH_3), and 1.94 (bs, 3H, NCO•CH_3).

Furfurylamine Modified Hyaluronic Acid (5A and 5B). Following the general procedure, the reactions of hyaluronic acid with 18 μL of furfurylamine (0.2 mmol) in the presence of NMM and CDMT afforded 68 mg (83%) of **5A** and 48 mg (60%) of **5B**. ^{1}H NMR (D_2O; ppm): δ 7.46 (s, 1H, Ar-H), 6.41 (s, 1H, Ar-H), 6.34 (s, 1H, Ar-H), and 1.94 (bs, 3H, NCO•CH_3).

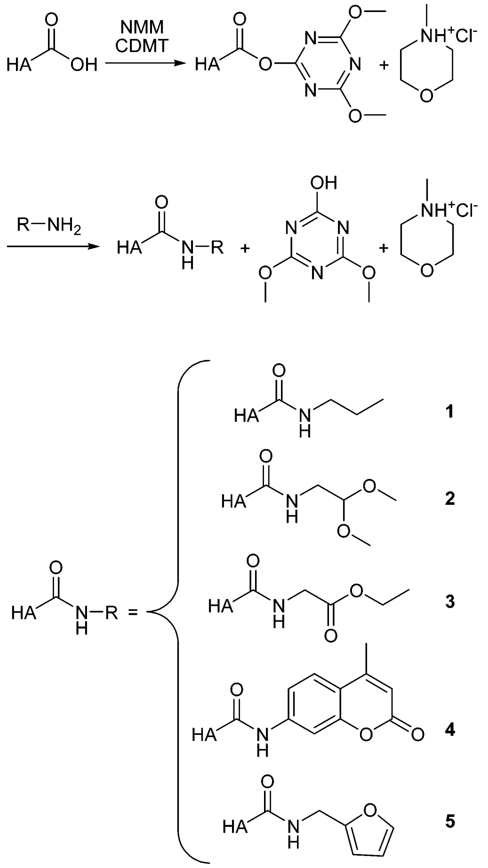
Biostability Analysis of Aminoacetaldehyde Dimethyl Acetal Modified Hyaluronic Acid. Hyaluronic acid modified with 17% aminoacetaldehyde dimethyl acetal, according to the procedure for **2A**, was subjected to hyaluronidase (HAase) digestion to investigate if the modification process had influenced the biological nature of the native polymer. A stock solution of HAase (1000 U/mL) was prepared by dissolving 10 mg of bovine testicular hyaluronidase (1013 U/mg, Sigma), 213 mg of sodium phosphate, 87 mg of sodium chloride, and 58 mg of citric acid in 10 mL of deionized water. A 75 mg amount of the modified substance and 75 mg of sodium hyaluronate were each dissolved in 13.5 mL of Dulbeccos phosphate-buffered saline (DPBS, Biochrom AG) and 1.5 mL of the HAase solution. Control solutions with 75 mg of both hyaluronan substances were prepared in 15 mL of DPBS in the absence of HAase. All four solutions were incubated at

Table 1. Specifications for Synthesized Hyaluronic Acid Derivatives

		A[a]		**B**[b]	
derivative no.	amine	yield (%)	SD[c] (%)	yield (%)	SD[c] (%)
1	propylamine	91	20	77	9
2	aminoacetaldehyde dimethyl acetal	89	20	88	11
3	glycine ethyl ester	87	20	54	7
4	7-amino-4-methylcoumarin	89	3	59	2
5	furfurylamine	83	12	60	2

[a] 0.5 equiv of CDMT to hyaluronic acid carboxyl groups. [b] 0.25 equiv of CDMT to hyaluronic acid carboxyl groups. [c] Degree of substitution as determined by ^{1}H NMR spectroscopy.

Scheme 1. Proposed General Synthetic Route for Triazine-Activated Amidation of Hyaluronic Acid (HA)



37 °C; after 6, 24, and 48 h, 5 mL of each solution was withdrawn and dialyzed (25 kDa M_w cutoff) against 2 L of deionized water for 20 h and finally lyophilized. The remaining products were weighed and the cumulative weight loss plotted as a function of time (graphic presentation is provided in the Supporting Information).

Results and Discussion

Triazine-Activated Amidation of Hyaluronic Acid. The reaction of CDMT with carboxylic acids and primary amines in the presence of 4-methylmorpholine (NMM) proceeds according to the proposed synthetic route presented in Scheme 1 and involves the formation of an intermediate triazine-activated ester.[16] The reaction conditions can be considered relatively mild since the reactions are performed at room temperature and at pH 7. Each amidation reaction was performed in a mixture of water and acetonitrile (3:2) since this was found capable of dissolving both hyaluronic acid and CDMT. By using a combination of a polar and nonpolar solvent, the introduction of both hydrophilic and hydrophobic amines is made possible. Previously published methods relating to the reactions of hyaluronic acid with hydrophobic substances involve the use of organic solvents, such as dimethyl sulfoxide or dimethyl formamide, in which hyaluronic acid is made soluble by changing the counterion on the carboxylic acid groups to tetrabutylammonium.[6,17,18] To obtain products with different degrees of modification, each amidation reaction was performed twice using ratios of 1:2 and 1:4 of CDMT to hyaluronic acid repeat units (**1A**−**5A** and **1B**−**5B**, respectively). Treatment with Dowex before dialysis was found necessary not only to remove morpholinium but also the excess amine which would otherwise act as counter ions in their protonated form toward nonmodified carboxylic acid groups. This was observed with ^{1}H NMR spectroscopy for products not treated with Dowex through the appearance of an extra set of signals with chemical shifts different from those for each of the amines (data not shown).

Characterization. All products were characterized using ^{1}H NMR spectroscopy according to the procedure described in the Experimental Section. Typical spectra for products **1A**−**5A** are presented in Figure 2. Evidence of amide bond formation is seen in the ^{1}H NMR spectrum for propylamine-modified hyaluronic acid (Figure 2, **1A**) by the appearance of two multiplet signals (δ 3.24 and δ 3.03) from the protons adjacent to the amide bond due to their diastereotopic nature.

Degrees of modification (SD) were determined by comparing integrated signals from the substituent groups with the corresponding methyl signal on the *N*-acetylglucosamine moiety (δ = 1.94 ppm); these are presented in Figure 3. Deconvolution was performed to increase the accuracy of integral values in spectra where signals from the substituent groups were present in the same range as other signals (**2A**,**B**). The correlation of SD to the amount of added triazine reagent suggests a high degree of control, a feature that is necessary for designing hyaluronic acid derivatives with tuned properties and high reproducibility. Repeated experiments using the triazine-activated amidation of hyaluronic acid with aminoacetaldehyde dimethyl acetal, in larger scales with degrees of modification ranging from 5 to 25%, indicate that the degree of substitution can be controlled with an accuracy of ±2%. Functionalization of hyaluronic acid with aminoacetaldehyde dimethyl acetal is a route to the introduction of cross-linking sites, since hydrolysis of the acetals yields aldehydes, which in turn can react with cross-linking agents containing hydrazides or amines.[8] The furfurylamine-modified hyaluronic acid also provides possible further functionalization via Diels−Alder reactions.

As shown in Figure 3, the degree of modification varies between the different amines, which can be explained by their different nucleophilicities and/or solubilities as well as by hydrolysis of the intermediate active ester due to the presence of water. The low SD for 7-amino-4-methylcoumarin-modified hyaluronic acid (**4A**,**B**) was probably caused by the poor solubility of the amine in both water and acetonitrile. It may, however, also be a result of low reactivity.

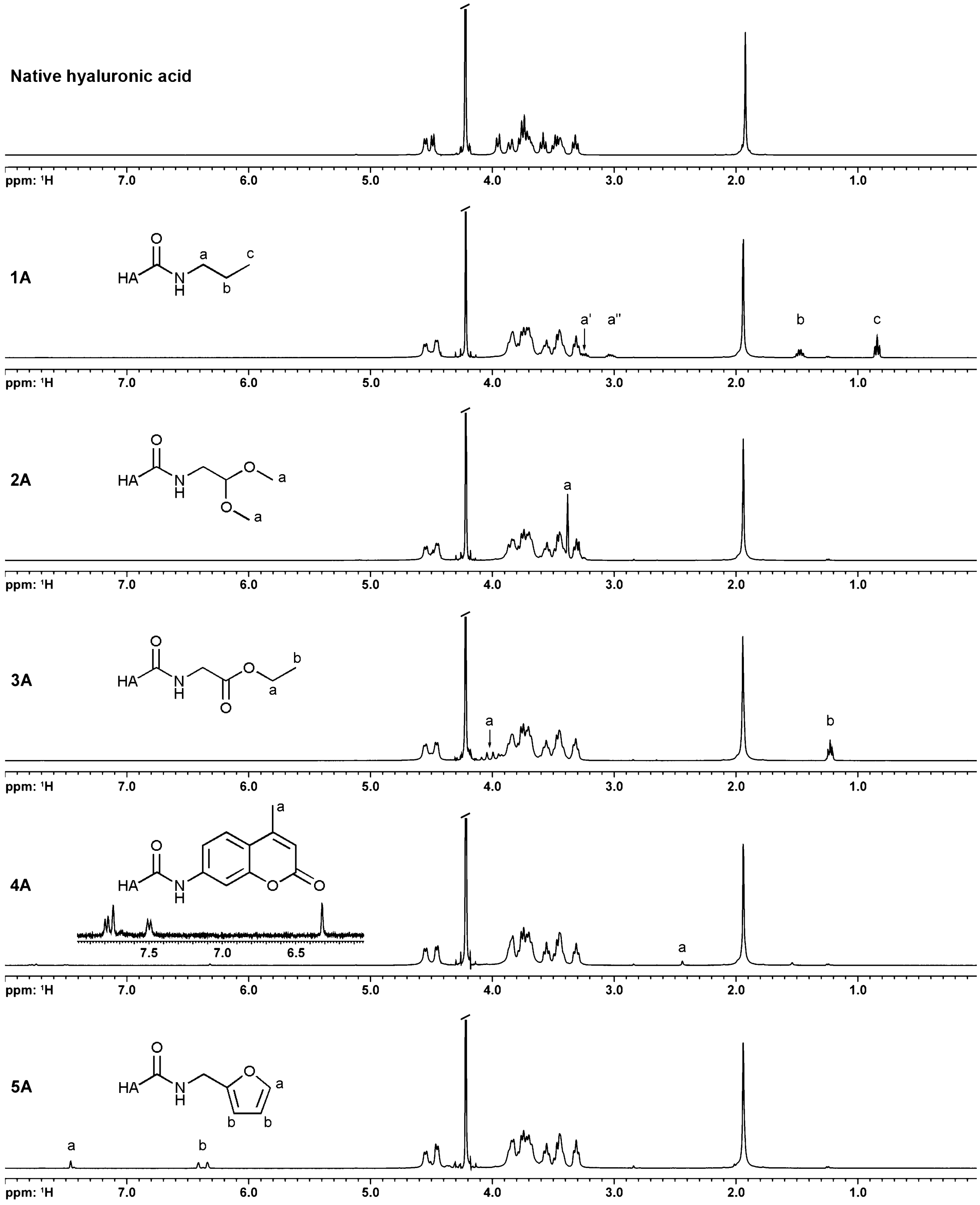


Figure 2. ^{1}H NMR spectra of native hyaluronic acid in protonated form (top) and hyaluronic acid derivatives (compounds **1A**−**5A**) recorded in D_2O at 80 °C.

The low SD for **4A**,**B** is difficult to determine by ^{1}H NMR spectroscopy due to the large difference in magnitude of the compared peak integrals. However, fluorescence spectroscopy revealed a similar relative difference in SD for the two products (Figure 4). It is reasonable that a higher SD could be obtained by increasing the amount of CDMT, although the current products contain sufficient amounts of the probe to record fluorescence spectra at concentrations as low as 10 μM (based on the total number of hyaluronic acid repeat units). The possibility to bind fluorescent probes such as 7-amino-4-methylcoumarin covalently to hyaluronic acid can be of great value in studying interactions with biological environments.

Hyaluronic acid is susceptible to degradation through a number of mechanisms due to the labile nature of the glycosidic bond.[1] To determine the effect of modification on the molecular weight, we also performed light scattering measurements, both on an unmodified sample as well as on sample **2A**, both in water and in 0.10 M NaCl. This method was used in favor of, e.g., viscosity or chromatographic techniques, since one obtains the molecular weight and size directly. We first measured d*n*/d*c*

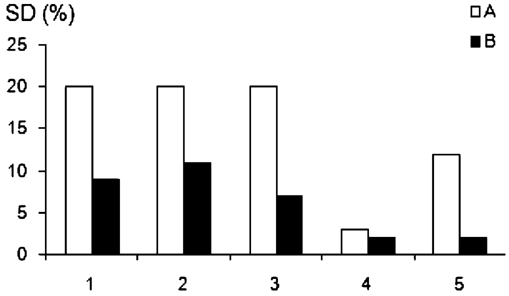


Figure 3. Degrees of substitution (SD) for hyaluronic acid modified with propylamine (1), aminoacetaldehyde dimethyl acetal (2), glycine ethyl ester (3), 7-amino-4-methylcoumarin (4), and furfurylamine (5). Data were obtained using ^{1}H NMR spectroscopy by comparing signals from substituent groups with corresponding methyl signals from the *N*-acetyl group on the *N*-acetylglucosamine moiety of hyaluronic acid; see the spectra presented in Figure 2.

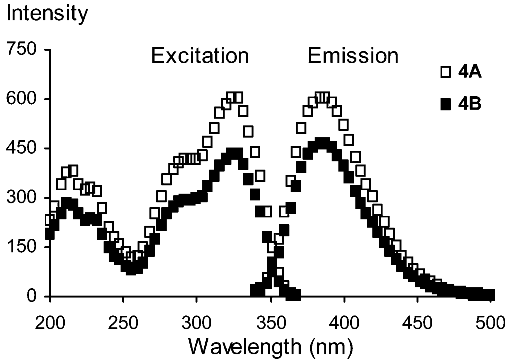


Figure 4. Fluorescence excitation and emission spectra for 10 μM water solutions of hyaluronic acid modified with 7-amino-4-methylcoumarin.

which was equal to 0.143 mL/g for both types of samples, which is within the range earlier reported for hyaluronic acid in water.[19] Preparing the samples in water for the light scattering measurements, we observed that the modified molecules **2A** resulted in a rather viscous solution but still possible to filter into the light scattering cell using a 0.8 μm Sartorius Minisart N filter. The original unmodified (sodium) form of the hyaluronic acid, however, gave a very viscous solution which was not possible to filter. By instead using 0.10 M NaCl as solvent, the original solution could now be filtered and the molecular weight and radius of gyration were determined. For **2A** M_w and R_g were determined both in water and 0.10 M NaCl, and in Figure 5 the angular dependence of the reduced scattering function is shown in NaCl together with the concentration dependence of the reduced scattering function extrapolated to $\theta = 0°$.

The result of the light scattering measurements showed that the molecular weight for the original molecule was 1.2×10^6 g/mol, which is close to the value stated by the manufacturer. For the modified molecules **2A** the molecular weight was 5.4×10^5 g/mol in water and 5.2×10^5 g/mol in NaCl, thus giving the same value within experimental limits. These results show that during the modification reaction, some hydrolysis of the original hyaluronic acid takes place, which approximately reduces the molecular weight by a factor of 2. This corresponds to scissions most likely occurring at the glycosidic linkages, although a uronic acid β-elimination is also a possible cause. There are several possible steps involved in the modification process which may cause scission, such as lyophilization, the temporary exposure to acidic environment, or reactive oxygen species.

We also calculated the radius of gyration, and this explained why it was not possible to perform any measurements on the original form of the hyaluronic acid in water. For **2A**, R_g was equal to 78 nm in 0.10 M NaCl but increased to 186 nm in water. For the unmodified sample, R_g in 0.10 M NaCl was 132 nm and would thus increase to a very large value in water, which was also seen, as the corresponding solution was very viscous and could not be filtered.

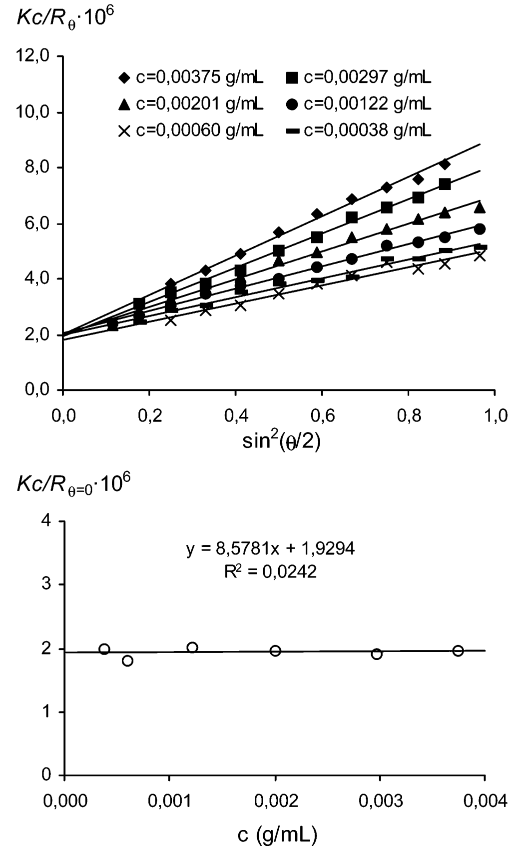


Figure 5. Angular dependence of the reduced scattering function, Kc/R_θ, for polymer **2A** in NaCl at different concentrations (top), and the corresponding extrapolated value at $\theta = 0°$ as a function of concentration (bottom).

Biomedical applications of hyaluronic acid often require chemical modifications. It is, however, important that the biological properties of the native polymer are retained in the product. An investigation was therefore conducted to determine if a hyaluronic acid derivative obtained by the triazine-activated amidation was susceptible toward degradation by hyaluronidase digestion, as described in the Experimental Section. The cumulative weight loss over 48 h was 18% for hyaluronic acid modified with aminoacetaldehyde dimethyl acetal and 14% for native sodium hyaluronate, when subjected to HAase of 100 U/mL. The results indicate that the biological nature of the modified polymer is preserved.

Conclusions

We present here a new technique for the chemical modification of hyaluronic acid, which enables the coupling of a wide range of hydrophilic and hydrophobic amines to hyaluronic acid carboxyl groups by a reaction performed in aqueous media under relatively mild conditions. The technique involves the use of 2-chloro-4,6-dimethoxy-1,3,5-triazine as an activating species, the number of hyaluronic acid repeat units that are modified is determined by how much of the triazine that is added, and the decrease in molecular weight is limited. The triazine-activated amidation is a convenient route for introducing new functionalities to hyaluronic acid and can be applied for the design of hyaluronic acid based biomaterials.

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Supporting Information Available. ^{1}H NMR spectra of compounds **1B−5B** and graphic presentation of results from biostability analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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