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Synthesis of 6-*O*-methotrexylhyaluronan as a drug delivery system

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ABSTRACT

Selective halogenation of hyaluronan and partial halogen substitution by methotrexate led to 6-chloro-6-deoxy-6-*O*-methotrexylhyaluronan, a potential antitumor drug. The remaining halogen could be further substituted by a second organic carboxylate, leading to mixed esters. 6-*O*-Acetyl-6-*O*-methotrexylhyaluronan and 6-*O*-butyryl-6-*O*-methotrexylhyaluronan were thus synthesized and characterized by NMR spectroscopy.

1. Introduction

Hyaluronan (HA, **1**, Fig. 1) is a naturally occurring polysaccharide composed of a linear repeating disaccharide unit consisting of β-(1→4)-linked D-glucopyranuronic acid and β-(1→3)-linked 2-acetamido-2-deoxy-D-glucopyranose, which is present in extracellular matrices, the synovial fluid of joints, and scaffolding that comprises cartilage.

Despite its simple structure, hyaluronan behaves quite differently from other glycosaminoglycans in its biosynthesis, its size, and its physicochemical properties.[1] Its network-forming, viscoelastic, and charge characteristics are important for many biochemical processes in living tissues. It is an important pericellular and cell-surface constituent which, through interaction with proteins, participates in regulating cell behavior during numerous morphogenic, restorative, and pathological processes in the body.

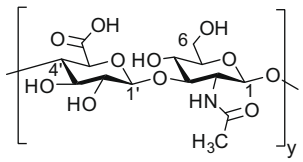

Figure 1. The repeating unit of hyaluronan.

The two most important receptors of HA are the cell-surface glycoprotein CD44 and the receptor for HA-mediated motility (RHAMM). Various tumors, for example, epithelial, ovarian, colon, stomach, and acute leukemia, overexpress the HA-binding receptors CD44[2] and RHAMM.[3] Consequently, these tumor cells show enhanced binding and internalization of HA.[4] The mechanism of hyaluronan–CD44 binding is not fully understood yet, but it has been reported that the CD44 receptor contains the specific binding domain for HA.[2,5]

The high tumor specificity of the HA–CD44 interactions and high biocompatibility of HA prompted the design and synthesis of tumor-targeting bioconjugates bearing HA and cytotoxic agents. Conjugation of low-molecular-weight HA to cytotoxic drugs such as paclitaxel, doxorubicin, mitomycin C, and butyric acid has been reported in the literature. HA–paclitaxel conjugates, exploiting a dihydrazide linker bound to the carboxylic groups of HA, were shown to be internalized into cancer cells through CD44 receptor-mediated endocytosis, followed by intracellular release of the active drug.[6,7] Two different types of doxorubicin (DOX) conjugates have been described: HA–doxorubicin, with a dihydrazide linker bound to HA carboxyl groups, and HPMA–HA–DOX conjugate, where both HA and doxorubicin are linked to the same HPMA (*N*-(2-hydroxypropyl)methacrylamide) chain through a Gly-Phe-Leu-Gly spacer.[8] The HPMA–HA–DOX conjugate clearly demonstrated better internalization and cytotoxicity as compared to the nontargeting HMPA–DOX conjugate.

HYTAD1-p20 is another paclitaxel conjugate, synthesized by carboxyl esterification of hyaluronan with paclitaxel through an alkyl spacer.[9–12] Mitomycin C has been conjugated to hyaluronan

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by way of an amide bond between the drug and the carboxyl groups of the polymer; the conjugate exhibited an excellent cancer metastasis suppression effect.[13] HA was also randomly esterified at hydroxyl groups with butyric acid;[14–16] evidence of receptor-mediated internalization was provided by fluorescein labeling. Mixed retinoic/butyric hyaluronan esters for the treatment of acute promyelocytic leukemia have been reported.[17] Carboranes have been linked to hyaluronan, via an ester linkage, for tumor targeting in boron neutron capture therapy.[18]

A different chemistry, used to conjugate lipids in view of targeting liposomes to CD44-overexpressing tumor cells, involved controlled oxidation of oligomers of hyaluronan, followed by reductive amination.[19]

Methotrexate (MTX, Fig. 2) is an antimetabolite and an analogue of folic acid used as antineoplastic drug and as a general immunosuppressant in the therapy of autoimmune diseases such as rheumatoid arthritis. The efficacy of MTX in anticancer chemotherapy is hampered by its very short plasma half-life. It is administered in relatively high dose, which often leads to drug resistance and causes nonspecific toxicities to normal proliferating cells. Adverse side effects may be minimized by targeted delivery of the drug directly to the tumor site.[20]

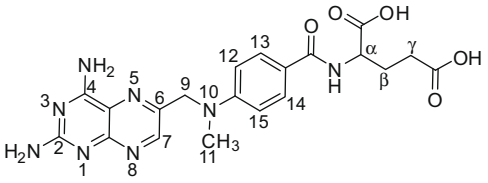
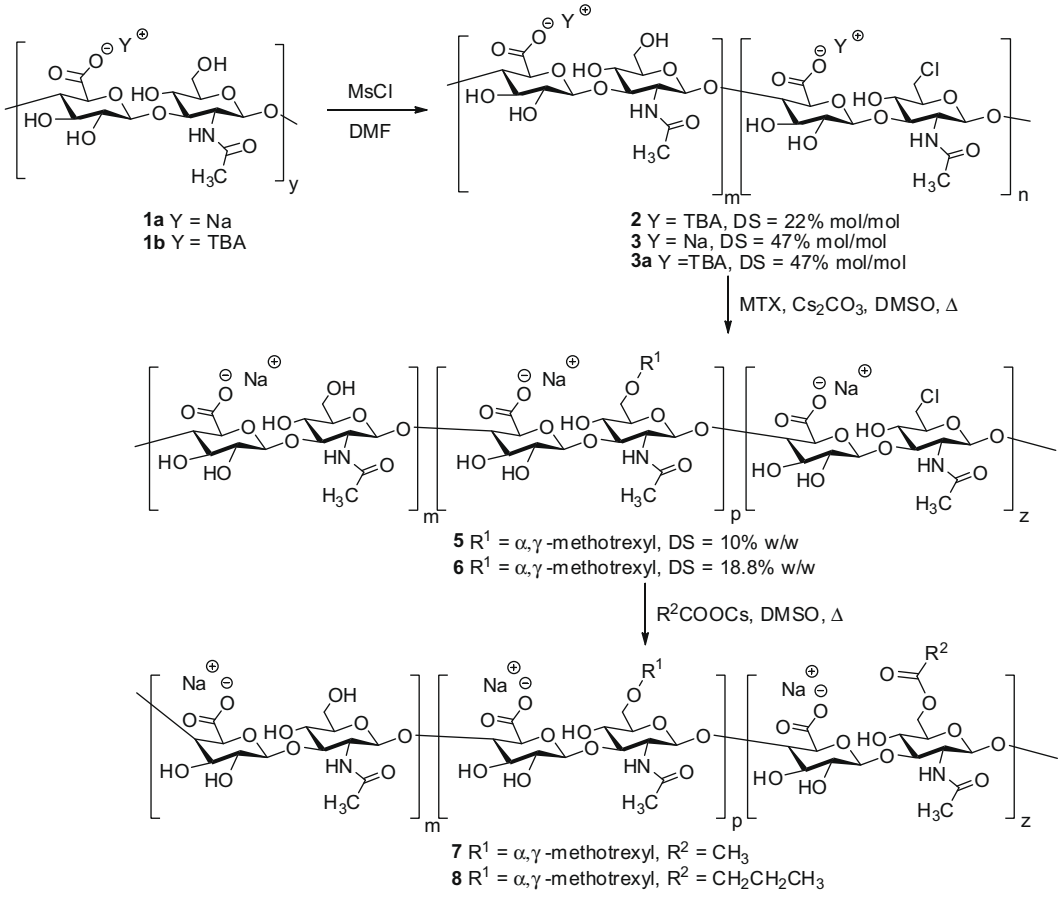


Figure 2. Methotrexate.

Most of the strategies for covalent drug conjugation described in the literature involve the carboxylic position of the D-glucuronic acid moiety of hyaluronan or consist in a random conjugation, directly or through a linker, at the hydroxyl groups of HA.[21,22] Our objective was to develop a selective methodology for covalent derivatization of HA with MTX at the primary hydroxyl group (C-6) of the 2-acetamido-2-deoxy-β-D-glucopyranose moiety of hyaluronan. The advantages of this strategy rely on leaving the carboxylates of HA unaffected, thus nonhampering the solubility in water by decreasing charges, and on specific ester formation, leading to a conjugate with a well-defined and reproducible release profile. As for MTX, no attempt was done in order to bind it through a specific carboxylate.

In the first step of the strategy, a leaving group, a chloro group, is introduced at the C-6 position and then substituted by MTX (see Scheme 1). Furthermore, conjugation conditions leave part of chloro groups unaffected, thus available for further derivatization. In this way a second acyl group could be introduced at the C-6 position, producing two mixed esters starting from **5**. In the first case an acetate group was introduced at C-6, in the latter we could produce 6-*O*-butyryl-6-*O*-methotrexylhyaluronan, which is an example of a regioselectively esterified conjugate of hyaluronan, bearing two cytotoxic drugs[11] at C-6.

As for the characterization of chemical modifications on hyaluronan, NMR spectroscopy is clearly the best tool. The NMR spectrum of the hyaluronan molecule is essentially represented by the signals of the repeating disaccharide unit. Despite the broad-



Scheme 1. Synthesis of conjugates starting from hyaluronan with a M_w of 20,000 g/mol.

ness of peaks in both the ^{1}H and ^{13}C NMR spectra, it is still possible to well characterize reaction products and to quantify the degree of chemical modification (usually referred to as the degree of substitution).

2. Results and discussion

For the selective halogenation of hyaluronan we resorted to a known procedure, suitable for saccharides, making use of methanesulfonyl chloride in *N*,*N*-dimethylformamide.[23] This reagent has been successfully used for the chlorination of neutral polysaccharides such as amylose,[24] pullulan,[25] laminaran,[26] cellulose,[27] and hyaluronan.[28]

The reaction of hyaluronan tetrabutylammonium (TBA) salt **1b** (obtained from HA sodium salt **1a** with a M_w of 20,000 g/mol by ion-exchange using a TBA-activated strong-ion exchanger) with a combination of methanesulfonyl chloride and *N*,*N*-dimethylformamide, initially at −10 °C and then at 50 °C for 16 h, afforded 6-chloro-6-deoxyhyaluronan (**2**), in which the primary hydroxyl group at the C-6 position of the repeating disaccharide is partially substituted by a chloro group. The reported selectivity was confirmed by NMR spectroscopy.

Chlorination can be conveniently carried out on a suspension of HA sodium salt with a M_w of 20,000 g/mol in *N*,*N*-dimethylformamide, which avoids the tetrabutylammonium exchange step and leads to more reproducible results in terms of yield and DS. Compound **3** presented a chloro degree of substitution of 47% mol/mol (Fig. 3, quantitation by ^{13}C NMR spectroscopy for compound **3**), carrying out the reaction at 60 °C for 16 h.

The ^{1}H NMR assignments in compound **3** were based on 2 D ^{1}H–^{1}H and ^{1}H–^{13}C correlation spectra (the HSQC spectrum is shown in Fig. 4, see Table 1). The resonances due to H-1 (+0.08), H-5 (+0.24), H-6a (+0.1), and H-6b (+0.07 ppm) in the modified sugar ring had slightly shifted downfield as compared to the corresponding ^{1}H resonances in the native *N*-acetyl-D-glucosamine ring (**1a**). On the basis of the integral values of the two signals at 44.2 ppm (CH_2Cl) and at 61 ppm (CH_2OH) in the ^{13}C NMR spectrum, the degree of chloro substitution in compound **3** was ascertained as 47% mol/mol, with respect to the repeating disaccharide unit.

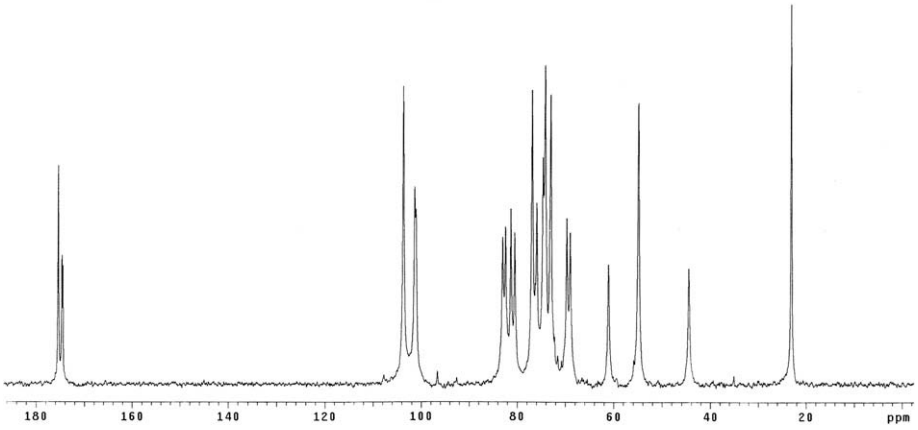


Figure 3. ^{13}C NMR spectrum of 6-chloro-6-deoxyhyaluronan **3**, DS = 47% mol/mol.

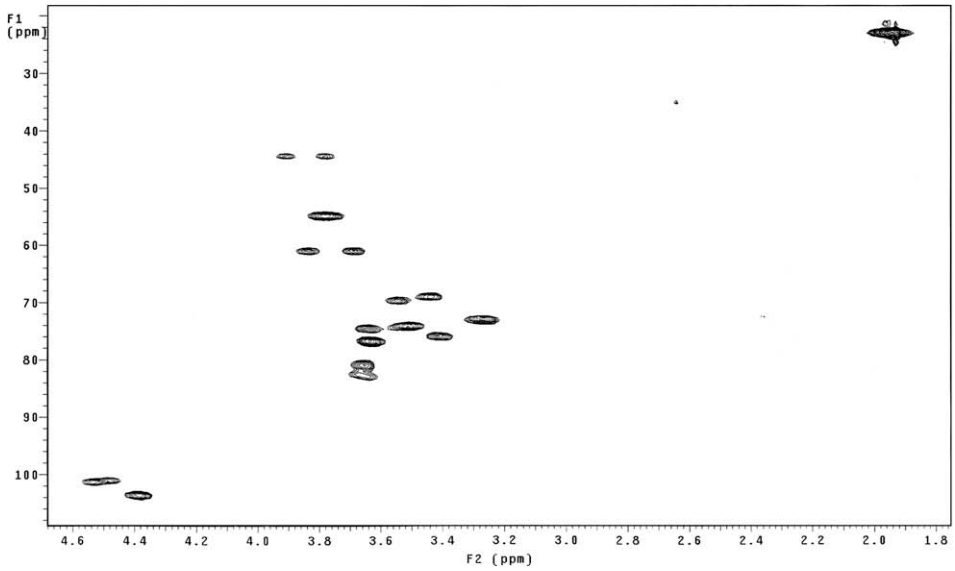


Figure 4. ^{1}H–^{13}C heterocorrelated HSQC spectrum of 6-chloro-6-deoxyhyaluronan **3**, DS = 47% mol/mol. Cross peaks between C-6 carbons and their diastereotopic protons are found at 44.5 ppm (^{13}C dimension) for –CH_2Cl and at 61 ppm (^{13}C dimension) for –CH_2OH.

Table 1
NMR data of 6-chloro-6-deoxyhyaluronan (**3**)

Position	^{1}H (ppm)	^{13}C (ppm)	C-6
$NCOCH_3$	1.96	22.6	OH, Cl
C-1	4.49	100.7	OH
C-1	4.54	101	Cl
C-1′	3.49	103.3	OH, Cl
C2	3.79	54.7	OH, Cl
C-2′	3.28	72.7	OH, Cl
C-3	3.68	82.4	OH, Cl
C-3′	3.52	73.8	OH, Cl
C-4	3.45	68.7	OH
C-4	3.55	69.3	Cl
C-4′	3.67	80.7	OH, Cl
C-5	3.42	75.7	OH
C-5	3.65	74.3	Cl
C-5′	3.64	76.5	OH, Cl
C-6	3.7	60.7	OH
C-6	3.84	60.7	OH
C-6	3.79	44.2	Cl
C-6	3.92	44.2	Cl

Table 2
NMR data for 6-chloro-6-deoxy-6-*O*-methotrexylhyaluronan (**5**)

Position	^{1}H (ppm)	^{13}C (ppm)	C-6
$NCOCH_3$	1.96	23	OH, Cl, MTX
C-1	4.5	101.1	OH, Cl, MTX
C-1′	4.4	103.8	OH, Cl, MTX
C-2	3.78	54.9	OH, Cl, MTX
C-2′	3.29	73	OH, Cl, MTX
C-3	3.65	83.16	OH, Cl, MTX
C-3′	3.52	74.2	OH, Cl, MTX
C-4	3.45	69	OH, Cl, MTX
C-4′	3.67	80.6	OH, Cl, MTX
C-5	3.43	76	OH, Cl, MTX
C-5′	3.65	77	OH, Cl, MTX
C-6	3.70, 3.85	61.2	OH
C-6	3.93, 3.78	44.5	Cl
C-6	3.70, 4.18	65.7	MTX
MTX C-5	8.56	150	MTX
MTX C-7	2.3	34.13	MTX
MTX C-8	3.14	39.3	MTX
MTX C-10, C-14	6.83, 6.59	112.3, 112.7	MTX
MTX C-11, C-13	7.63, 7.54	129.66	MTX
MTX C-17	4.78	55.7	MTX
MTX C-18	2.21, 2.04	27.5	MTX
MTX C-19	2.47	31.3	MTX

The chlorination reaction was also attempted on a hyaluronan of a different molecular size, starting from hyaluronan sodium salt with a M_w of 200,000 g/mol. Under the same conditions used to make **3**, compound **4** was obtained, presenting a chloro degree of substitution of 50% mol/mol. The slightly higher DS with respect to compound **3** can be an effect of both the error in the NMR measurements and the use of a suspension in this reaction, which can affect the outcome depending on the characteristics of the starting material. Anyway, this trial confirms a more general applicability of these conditions.

Treatment of 6-chloro-6-deoxyhyaluronan tetrabutylammonium salts (**2** and **3a**) in dimethyl sulfoxide with MTX in the presence of cesium carbonate (80 °C for 40 h) afforded the methotrexate derivatives **5** and **6**, respectively (see Fig. 5, Table 2), through substitution of chloride by the cesium salt of MTX. The use of tetrabutylammonium salts is in this case necessary to ensure solubility in dimethyl sulfoxide; in fact, carrying out the reaction on the sodium salt **3** prevented the conversion to the desired conjugate.

According to the NMR spectra, the repeating disaccharide units in compounds **5** and **6** were partially esterified by MTX at the C-6 position (CH_2OMTX), leaving a fraction of chlorodeoxy (CH_2Cl) groups unaffected. The ^{13}C signal due to esterified C-6 appeared at 65 ppm as a broad peak, and the ^{1}H resonances due to H-6a and H-6b shifted downfield by 0.02 and 0.33 ppm, as compared to the corresponding proton resonances in compound **1a**.

The ester linkage between MTX and the C-6 position of the *N*-acetyl-D-glucosamine residue in HA–MTX conjugates could occur through α- or γ-carboxylic groups of the L-glutamic acid moiety; no attempt was made to distinguish the ester linkage.

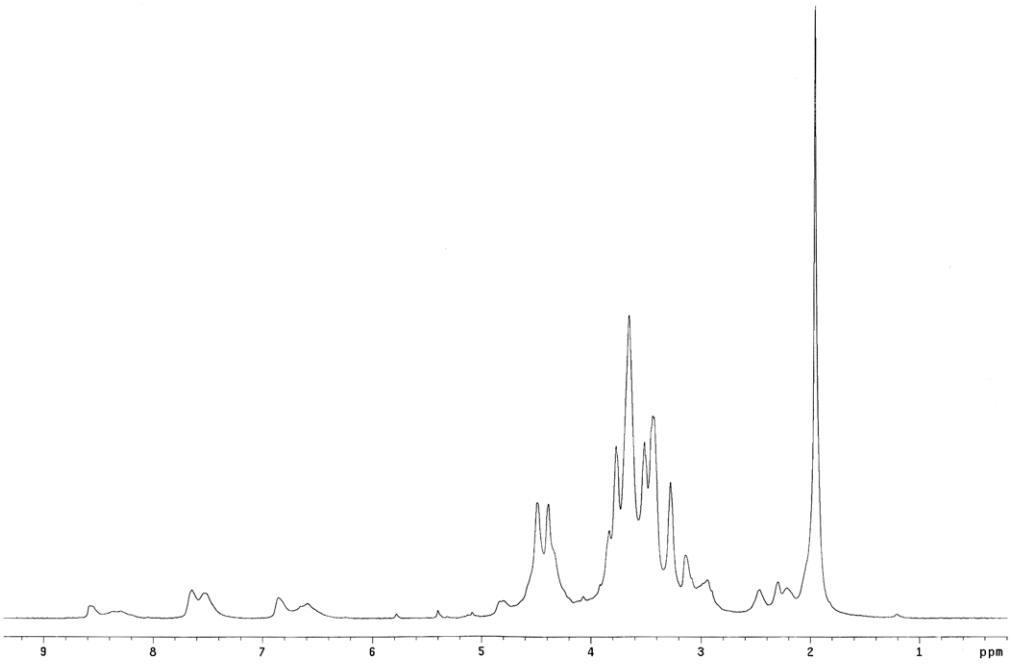


Figure 5. ^{1}H DOSY NMR spectrum of HA–MTX conjugate **6**. Only signals of polymeric species are visible: the aromatic (6.6–8.8 ppm) and glutamic chain protons (2.2–2.7 ppm) of methotrexate clearly confirm conjugation.

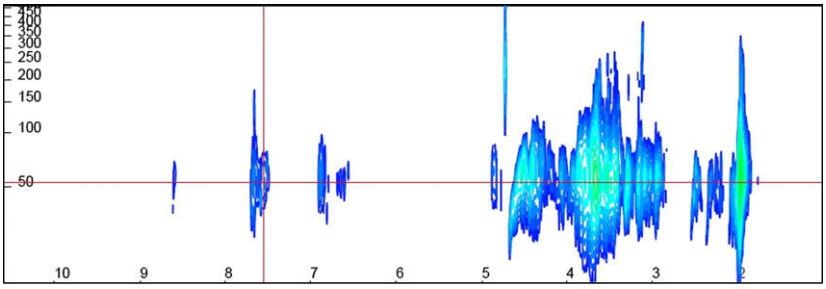


Figure 6. ^{1}H-detected 2D-DOSY map of 6-chloro-6-deoxy-6-*O*-methotrexyl hyaluronan (**5**) in D_2O solution at 25 °C.

To quantify the amount of loaded MTX as well as of bound and not-bound impurities in the conjugates, an HPLC method was developed, based on standard methods available for MTX. Under these conditions, small-molecule impurities, comprising free MTX, can be determined in a sample of conjugate. The only significant impurity was found to be free MTX, with values under 0.2% w/w. To quantify bound MTX, the samples were subject to hydrolysis in 0.1 N NaOH and then analyzed under the same conditions. The HPLC traces showed the peak of total MTX, and hence, from difference, the quantity of conjugated MTX. According to this method, the conjugated MTX content in **5** was 10.0% w/w (10% mol/mol), while in conjugate **6** it was 18.8% w/w (20.5% mol/mol), corresponding to substitution of around 42–45% of the initial chloro group.

The covalent conjugation of MTX at the C-6 position was confirmed by a 2D DOSY (Diffusion Ordered SpectroscopY) NMR spectrum in D_2O at 25 °C. NMR diffusion experiments can discriminate between large and small molecules, as a consequence of their different diffusion coefficients. These coefficients are a function of several parameters, such as temperature, solvent, ionic strength, viscosity, but especially size and shape of the molecule. The 2D DOSY map of HA–MTX (Fig. 6), with the chemical shift on the horizontal axis and the diffusion values on the vertical axis, expressed in $\mu m^2/s$, unequivocally demonstrated that all of the resonances of HA as well as methotrexate possess the same diffusion coefficient, indicating conjugation.

Treatment of 6-chloro-6-deoxy-6-*O*-methotrexylhyaluronan sodium salt (**5**, 10% w/w of MTX) with an excess of cesium acetate afforded compound **7**. The structure of **7** was supported by its NMR spectra, which revealed the absence of the ^{13}C peak at 44.5 ppm, the presence of a ^{13}C peak at 21.1 ppm and the ^{1}H signal at 2.1 ppm due to the 6-acetate (Table 3), showing efficient substitution of the chloro group left after the first conjugation with MTX. Similarly, treatment of **5** with excess cesium butyrate afforded compound **8**. The disappearance of the signal at 44.5 ppm confirmed the complete substitution of the chloro group by butyrate, whose signals were also present in NMR spectra (Table 4). DOSY NMR spectra confirmed that in compounds **7** and **8** the second acyl group, introduced after MTX, is linked to the polymer.

The antitumor activities of these new hyaluronan derivatives are at present under evaluation.

Table 3
NMR data for 6-*O*-acetyl-6-*O*-methotrexylhyaluronan (**7**)

Position	^{1}H (ppm)	^{13}C (ppm)	C-6 position
$COCH_3$	2.1	21	AcO
$NCOCH_3$	1.96	23	OH, MTX, AcO
C-1	4.5	101	OH, MTX, AcO
C-1′	4.4	103.7	OH, MTX, AcO
C-2	3.78	54.8	OH, MTX, AcO
C-2′	3.28	73	OH, MTX, AcO
C-3	3.65	83.1	OH, MTX, AcO
C-3′	3.52	74.1	OH, MTX, AcO
C-4	3.9	68.9	OH, MTX, AcO
C-4′	3.67	80.4	OH, MTX, AcO
C-5	3.43	75.9	OH, MTX, AcO
C-5′	3.66	76.8	OH, MTX, AcO
C-6	3.70, 3.85	61.	OH
C-6	3.71, 4.19	65.9, 65.77	MTX, AcO
MTX C-5	8.5	150	MTX
MTX C-7	2.3	33.9	MTX
MTX C-8	3.11	39.6	MTX
MTX C-10, C-14	6.83, 6.59	112.3, 112.7	MTX
MTX C-11, C-13	7.6	129.7	MTX
MTX C-17	4.35	55.4	MTX
MTX C-18	2.21, 2.04	27.5	MTX
MTX C-19	2.49	31.5	MTX

Table 4
NMR data for 6-*O*-butyryl-6-*O*-methotrexylhyaluronan (**8**)

Position	^{1}H (ppm)	^{13}C (ppm)	C-6
$COCH_2CH_2CH_3$	2.19	23.8	But
$N\text{-}COCH_3$	1.96	23	OH, MTX, But
C-1	4.5	101.05	OH, MTX, But
C-1′	4.4	103.53	OH, MTX, But
C-2	3.77	54.86	OH, MTX, But
C-2′	3.28	73.1	OH, MTX, But
C-3	3.65	82.9	OH, MTX, But
C-3′	3.51	74.1	OH
C-3′	3.42	75.8	MTX, But
C-4	3.9	69	OH, MTX, But
C-4′	3.67	80.5	OH, MTX, But
C-5	4.58	76.16	OH, MTX, But
C-5′	3.65	76.8	OH, MTX, But
C-6	3.70, 3.84	61	OH
C-6	4.25, 4.41	63.6	But
C-6	3.7, 4.2	65.42	MTX
	3.43	72.15	MTX, But
MTX C-5	8.5	150	MTX
MTX C-7	2.35	33.9	MTX
MTX C-8	3.1	39.4	MTX
MTX C-10, C-14	6.65, 6.70	112.4, 112.8	MTX
MTX C-11, C-13	7.56	129.5	MTX
MTX C-17	4.33	55.5	MTX
MTX C-18	2.21, 2.04	27.5	MTX
MTX C-19	2.43	31.5	MTX

3. Experimental

3.1. General methods

Hyaluronan sodium salt **1a** (20,000 and 200,000 g/mol) was purchased from Bioiberica (Spain). MTX (Amethopterin) was bought from Fermion (Finland). An isocratic reversed phase HPLC method with UV detection at 302 nm was used to measure free and bound MTX in HA–6-MTX; total MTX content was measured after complete hydrolysis of the conjugate in 0.1 N NaOH for 30 min. The amount of conjugated MTX was obtained by difference between total and free MTX amount.

NMR spectra were recorded on a Varian Mercury 200 and on a Varian Inova 500 NMR spectrometer, in D_2O solutions. Gradient (g) ^1H–^1H correlation spectroscopy was performed with the pulse sequence gCOSY. Heteronuclear (^1H–^{13}C) 2D chemical shift correlation spectroscopy was performed using gHSQCAD (^1H–^{13}C) and gHMBCAD (^1H–^{13}C long-range) pulse sequences. Assignments are presented in tabular form in Tables 1–4. The HA–MTX solutions for DOSY NMR spectra were prepared in deuterated water at a concentration of 0.5 mM. The gradient strength was calibrated by water ($D = 2.229 \times 10^{-9}$ m^2 s^{-1} at 298.2 K), and the maximum gradient strength was 53 G cm^{-1}. The Varian Doneshot pulse sequence (a modification of the Dbppste sequence) was used for the measurement of diffusion coefficients. The samples were loaded into a 4-mm diameter NMR tube, and the experiments were performed by keeping the *z*-gradient pulse length constant and gradually increasing the gradient strength in 40 steps. The gradient pulse length was 40 ms in order to measure the diffusion coefficient of all components. The diffusion coefficient (D) was obtained by fitting the following equation:

$$\ln(I_g/I_0) = -[\gamma^2\delta^2G^2(\Delta - \delta/3)]D$$

where I_g and I_0 are the intensities of the NMR signal in the presence and absence of field gradient pulses; γ is the gyromagnetic constant for ^1H; δ is the duration of the *z*-gradient pulse; G is the gradient strength; and Δ is the time interval between the gradient pulses (200 ms). Experimental data were processed using the software GIFA 5.2 with the Inverse Laplace Transformation (ILT) method, using the Maximum Entropy approach.[29]

The TBA (tetrabutylammonium) content in compounds **1b**, **2**, and **3a** was measured by ^1H NMR spectroscopy, by integration of the TBA methyl peak at 0.9 ppm versus the HA methyl peak at 2.0 ppm.

The degree of chloro substitution (DS%, mol/mol) in compounds **2**, **3**, and **4** was calculated from their ^{13}C NMR spectra, from the integral (I) of CH_2Cl signal and the sum of CH_2Cl and CH_2OH integrals: $DS_{Cl} = 100 \times I_{CH_2Cl}/(I_{CH_2OH} + I_{CH_2Cl})$. A standard ^{13}C NMR sequence was used, assuming similar relaxation times for CH_2OH and CH_2Cl signals. Using a quantitative ^{13}C NMR sequence led to very small differences; however, within the usual error of this measurement.

The DS of MTX in HA–6-MTX was calculated from its ^1H NMR spectrum, by integration of the five aromatic protons of MTX (6.6–8.8 ppm), the four protons of the glutamic acid residue of MTX (2.2–2.7 ppm), and the three methyl protons of HA at 2.0 ppm. The DS values of MTX in compounds **5** and **6** by NMR and HPLC are in good agreement.

Water content of the conjugates was measured by thermogravimetric analysis (TGA). The molecular weight (M_w) and molecular weight distribution of the hyaluronan conjugates were measured by a HP–SEC (column: TSK PWxl from TosoBioscience, G6000 + G5000 + G3000 6, 10, 13 μm particle size) at 40 °C (mobile phase: NaCl 0.15 M + 0.01% NaN_3) coupled with a MALS detector (WYATT DAWN EOS—WYATT, USA, λ = 690 nm, d*n*/dc = 0.167 mL/g), a UV detector (λ = 305 nm) and an Interferometric Refractive Index detector (λ = 690 nm). The analysis allows the measurement of M_w (weight average molecular weight), M_n (number average molecular weight), and P.I. (polydispersity index).

Tangential flow filtrations were performed using a Centramate system from Pall, with 10,000 g/mol MWCO suspended screen membranes. The quantities in moles for hyaluronan and its derivatives in the examples are referred to the repeating disaccharide unit.

3.2. Hyaluronan, TBA salt (1b)

A solution of hyaluronan sodium salt **1a** (10 g, M_w 20,000 g/mol) in Milli-Q water (300 mL) was circulated in a column of cation-exchange resin (Amberlite IR-120 in TBA form) for 24 h at a flow rate of 4 mL/min; the solution was then concentrated on a rotary evaporator and freeze dried to afford **1b** (15.4 g). TBA content: 123% mol/mol.

3.3. 6-Chloro-6-deoxyhyaluronan, TBA salt (2)

To a solution of **1b** (4.00 g, 6.46 mmol) in 80 mL of DMF, methanesulfonyl chloride (5.0 mL, 65 mmol) was added dropwise, with stirring at −10 °C under nitrogen over 30 min. After an additional 30 min, the mixture was heated to 50 °C and then stirred for 16 h. After cooling, the mixture was quenched by the addition of 10% TBA hydroxide to pH 10. After stirring at this pH for 4 days, the suspension became a brown solution. It was filtered and purified by tangential flow filtration using a membrane with a molecular weight cut off of 10,000 g/mol. The aqueous solution was then freeze dried to afford **2** (2.80 g; 69%) as a white solid. Degree of chloro substitution: 22% mol/mol. TBA content: 126% mol/mol.

3.4. 6-Chloro-6-deoxyhyaluronan, Na salt (3)

To a suspension of hyaluronan sodium salt (4.00 g, 9.98 mmol, M_w 20,000 g/mol) in 70 mL of DMF, methanesulfonyl chloride (7.75 mL, 99.8 mmol) was added dropwise, with stirring at −10 °C under nitrogen over 30 min. After an additional 30 min, the mixture was heated to 60 °C and then stirred for 16 h. After cooling, the mixture was quenched by pouring into cold 1 M Na_2CO_3 (300 mL). The pH was adjusted to 9.5 and maintained, while stirring, for 48 h. The resulting brownish solution was filtered and purified by tangential flow filtration using a membrane with a molecular weight cut off of 10,000 g/mol. The aqueous solution was then freeze dried to afford **3** (2.72 g; 66%) as a white solid. Degree of chloro substitution: 47% mol/mol.

3.5. 6-Chloro-6-deoxyhyaluronan, TBA salt (3a)

A solution of **3** (2.5 g) in Milli-Q water (100 mL) was circulated in a column of cation-exchange resin (Amberlite IR-120 in TBA form) for 24 h at a flow rate of 4 mL/min; the solution was then concentrated on a rotary evaporator and freeze dried to afford **3a** (3.8 g). TBA content: 95% mol/mol.

3.6. 6-Chloro-6-deoxyhyaluronan, Na salt (4)

To a suspension of hyaluronan sodium salt (4.00 g, 9.98 mmol, M_w 200,000 g/mol) in 70 mL of DMF, methanesulfonyl chloride (7.75 mL, 99.8 mmol) was added dropwise, with stirring at −10 °C under nitrogen over 30 min. After an additional 30 min, the mixture was heated to 60 °C and then stirred for 16 h. After cooling, the mixture was quenched by pouring into cold 1 M Na_2CO_3 (300 mL). The pH was adjusted to 9.5 and maintained, while stirring, for 48 h. The resulting brownish solution was filtered and purified by tangential flow filtration using a membrane with a molecular weight cut off of 10,000 g/mol. The aqueous solution was then freeze dried to afford **4** (2.93 g; 72%) as a white solid. Degree of chloro substitution: 50% mol/mol.

3.7. 6-Chloro-6-deoxy-6-*O*-methotrexylhyaluronan, Na salt (5)

A solution of **2** (1.20 g, 1.93 mmol) in Me_2SO (100 mL) was treated with a solution of methotrexate (2.20 g, 4.84 mmol) in Me_2SO (25 mL) and Cs_2CO_3 (1.58 g, 4.85 mmol) with stirring under nitrogen. The resulting mixture was heated and stirred at 80 °C for 40 h, then cooled to room temperature and poured into ice-water. The pH was adjusted to 7 with dilute HCl; the mixture was stirred at room temperature for 2 h and then dialyzed against 1 M NaCl

(2 × 2.5 L). The insoluble material was filtered off through a sintered glass filter (class IV), and the solution was purified by tangential flow filtration using a 10,000 g/mol membrane, and then filtered through 1.2 μm, 0.45 μm and 0.22 μm pore size filters. The yellow solution was concentrated on a rotary evaporator and freeze dried to obtain a yellow fluffy solid (0.39 g; 45%). Conjugated methotrexate content: 10.0% w/w (HPLC), 10% mol/mol (^{1}H NMR). Water content: 10.5% w/w; M_w: 33,000, P.I. 2.8.

3.8. 6-Chloro-6-deoxy-6-*O*-methotrexylhyaluronan, Na salt (6)

A solution of **3a** (3.00 g, 4.83 mmol) in Me_2SO (250 mL) was treated with a solution of 5.50 g of methotrexate (12.1 mmol) in Me_2SO (60 mL) and Cs_2CO_3 (3.94 g, 12.1 mmol) with stirring under nitrogen. The resulting mixture was heated and stirred at 80 °C for 40 h, then cooled to room temperature and poured into ice-water. The pH was adjusted to 7 with dilute HCl solution; the mixture was stirred at room temperature for 2 h and then dialyzed against 1 M NaCl (4 × 2.5 L). The insoluble material was filtered off through a sintered glass filter (class IV), and the solution was purified by tangential flow filtration against a 10,000 g/mol membrane and then filtered through 1.2 μm, 0.45 μm and 0.22 μm pore size filters. The yellow solution was concentrated on a rotary evaporator and freeze dried to obtain a yellow fluffy solid (0.95 g; 40%). Conjugated methotrexate content: 18.8% w/w (HPLC), 20% mol/mol (NMR). Water content: 9.8% w/w; M_w: 18,000, P.I. 2.5.

3.9. 6-*O*-Acetyl-6-*O*-methotrexylhyaluronan, Na salt (7)

To Me_2SO (15 mL), **5** (150 mg, 0.30 mmol) was added and stirred under nitrogen at 60 °C for 1 h. The resulting homogeneous suspension was treated with solid CsOAc (576 mg, 3.0 mmol) at 80 °C for 24 h. The mixture, after cooling to room temperature, was purified by dialysis against water, filtered, and then freeze dried to give 110 mg (84%) of **7**. Conjugated methotrexate content: 7.2% w/w (HPLC), 6.9% mol/mol (NMR); free methotrexate: 0.07% w/w (HPLC). Acetyl content: 10% mol/mol (NMR). Water content: 11% w/w; M_w: 27,000, P.I. 2.2.

3.10. 6-*O*-Butyryl-6-*O*-methotrexylhyaluronan, Na salt (8)

To Me_2SO (15 mL), **5** (150 mg, 0.30 mmol) was added and stirred under nitrogen at 60 °C for 1 h. The resulting homogeneous suspension was treated with 660 mg (3.0 mmol) of solid cesium butyrate (prepared from butyric acid and cesium hydroxide) at 80 °C for 20 h. The mixture, after cooling to room temperature, was purified by dialysis against water and then freeze dried to give 122 mg (92%) of **8**. Conjugated methotrexate content: 7.8% w/w (HPLC), 7.5% mol/mol (NMR). Butyryl content: 10% mol/mol (NMR). Water content: 10.5% w/w; M_w: 29,000, P.I. 2.3.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.09.021.

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