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CHEMISTRY OF HYALURONIC ACID AND ITS SIGNIFICANCE IN DRUG DELIVERY STRATEGIES: A REVIEW

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ABSTRACT: Hyaluronic acid is an important naturally occurring polysaccharide present in extracellular matrices. The network-forming, viscoelastic and the charge characteristics of hyaluronic acid are important to many biochemical functions of living tissues. Its involvement in many diseases such as cancer, arthritis and osteoporosis, and the fact that it has specific protein receptors present on the cell surfaces, has given new impetus in drug design and synthesis of hyaluronic acid-drug conjugates. As a general arrangement, the review will focus on structure and conformation of hyaluronic acid, its chemistry and chemical methodologies that have led to a number of important hyaluronic-drug conjugates

INTRODUCTION: Hyaluronic acid (also known as hyaluronan, **1**; henceforth abbreviated as **HA**) is a naturally occurring polysaccharide of a linear repeating disaccharide unit consisting of β -(1 \rightarrow 4)-linked **D**-glucopyranuronic acid and β -(1 \rightarrow 3)-linked 2-acetamido-2-deoxy-**D**-glucopyranose, which is present in extracellular matrices, the synovial fluid of joints, and scaffolding that comprises cartilage. The unique physico-chemical characteristics, its mechanism of synthesis and its size set hyaluronic acid apart from other glycosaminoglycans. The network-forming, viscoelastic and its charge characteristics are important to many biochemical properties of living tissues.

It is an important pericellular and cell surface constituent which, through interaction with other macromolecules such as proteins, participates in regulating cell behavior during numerous morphogenic, restorative and pathological processes in the body.

The role of hyaluronic acid (HA) in diseases such as various forms of cancers, arthritis and osteoporosis has led to the development of biomaterials for surgical implants and drug conjugates for targeted delivery.

In recent years, a number of reviews on hyaluronic acid have appeared which principally deal with its biological and morphological functions, physico-chemical properties, cross linking reactions, therapeutic uses, global markets, and its industrial production¹⁻⁴.

This review will mainly focus on some of the regioselective chemical reactions of HA leading to products or intermediates of interest for

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pharmaceutical, chemical and food applications, and highlighting its importance as a vehicle for drug delivery.

Structure and Conformation: The conformation of HA in solid state by X-ray diffraction study of the oriented films revealed two forms: left-handed single helices with 2-, 3-, and 4-fold symmetries and a double helical structure are stabilised by intra-chain hydrogen bonds linking the two adjacent sugar residues, and inter-chain hydrogen bonds and cation/H₂O bridges⁵⁻¹⁰.

Evidence of conformational differences of hyaluronic acid in solid and solution state has been observed by ¹³C-NMR¹¹. In aqueous solution HA behaves like a fairly stiff coil; the stiffness has been attributed to factors such as:

- (a) The intrinsically small available conformational space at each of the two glycosidic linkages^{12, 13},
- (b) The tendency of ha chains to self-associate both intramolecularly (chain folding) and intermolecularly in neutral aqueous sodium chloride solutions¹⁴ and;
- (c) The existence of inter-residue hydrogen bonds stiffening each glycosidic linkages¹⁵.

On the basis of the low reactivity of HA (1) towards periodate oxidation and model building and molecular dynamics simulation, Scott and co-workers proposed the persistence of several inter-residue hydrogen bonds in aqueous solution^{15, 16}. These inter-residue hydrogen bonds include: one across the β-1→3 linkage, linking GlcNAc C-4 OH and GlcA O-5 and two hydrogen bonds across the β-1→4 linkage; the first one links GlcA 3-OH and GlcNAc O-5 and the second is a water-mediated hydrogen bond between the GlcNAc amide and GlcA carboxyl oxygen.

These inter-residue hydrogen bonds in HA were principally supported by ¹H- and ¹³C-NMR in DMSO¹⁷⁻²¹. NMR experiments involving a stepwise addition of water to a solution of hyaluronic acid hexasaccharide in Me₂SO-*d*₆, revealed the existence of a water-bridged hydrogen bond between the NH and the COO⁻ groups in aqueous solutions of HA²⁰.

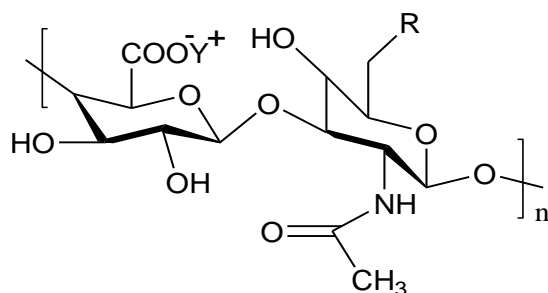
Kvam *et al*²¹ have investigated the structures of hyaluronic acid ethyl and benzyl esters and tetrabutylammonium and tetraethylammonium salts by ¹H- and ¹³C NMR spectra and have shown that the relative orientations of the monosaccharides at the (1→3) linkage in the esters and salts are different; there is only slight conformational variations around the (1→4) linkage; there are similarities in conformation between the esters in Me₂SO-*d*₆ and salts in water; and based on the chemical shifts of the ¹H resonances for NH and OH and their temperature dependence for esters and the HA and salts in Me₂SO-*d*₆, concluded that the inter-residue hydrogen bonds between the carboxyl and NH groups and between HO-4 of GlcA and O-5 of GlcNAc for salts are markedly stronger.

Chemical modifications of Hyaluronic acid: The basic repeating disaccharide units, D-glucuronic acid and N-acetyl-D- glucosamine, of hyaluronic acid provide a carboxyl group at C-5' and two free hydroxyl groups at the C-2' and C-3' positions in the β-D-GlcpA and two hydroxyl groups at C-4 and C-6 position in the β-D-GlcpNAc moiety; chemical and enzyme-catalysed reactions at some of these positions have led to a wide range of derivatives. It is of interest to note that until 1998 regioselective reactions of hydroxyl groups in hyaluronic acid were not investigated^{22, 23}.

It must be appreciated that, unlike simple carbohydrates, chemical reactions of hyaluronic acid or any other polysaccharide usually do not result in discrete compounds; a certain proportion of the molecule may be partially substituted or unsubstituted. The position and the degree of substitution in hyaluronic acid derivatives have been ascertained by High resolution NMR experiments such as 2D, ¹H-¹H and ¹H-¹³C correlation spectroscopy and ¹H-detected 2D DOSY experiments; and in some cases to study on a qualitative basis the effect of specific substitution on the conformation of the molecule.

In order to ascertain the molecular integrity of the chemical transformation products; their Molecular Weight and Molecular Weight Distribution values have been determined, using High Performance Size Exclusion Chromatography (HP-SEC).

Organic salts of Hyaluronic acid: In order to enhance the solubility of hyaluronic acid (HA) in aprotic organic solvents and to facilitate its derivatization, it has generally been first converted into its organic salt such as tetrabutyl ammonium, pyridinium or collidinium salt. This conversion of HA from inorganic to organic salt, for example, involved the treatment of a solution of the sodium salt of HA (**2**) in water with 4 N HCl (12.5 mmol) followed by neutralisation with tetrabutyl ammonium hydroxide (12.5 mmol) to afford the tetrabutylammonium salt (**3**). On a large scale hyaluronic acid tetrabutylammonium salt has been prepared using ion exchange resin in tetrabutylammonium hydroxide form²⁴. A similar process of neutralisation of HA in acid (H⁺) form with pyridine or *sym*-collidine afforded the pyridinium and the collidinium salt, respectively²³.



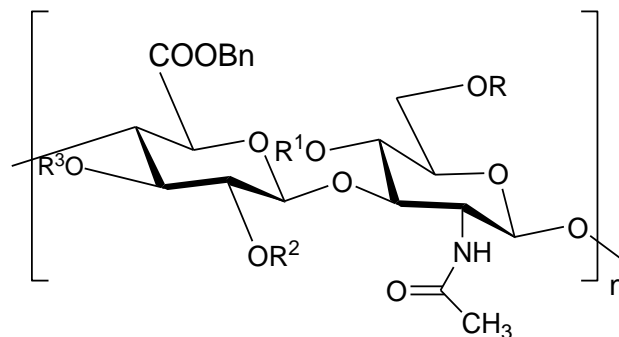
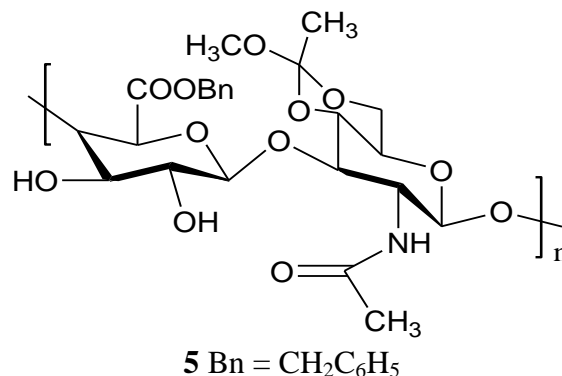
1 R = OH, Y = H; **2** R = OH, Y = Na; **3** R = OH, Y = Bu₄N

The carboxyl group of hyaluronic acid can be esterified by treatment of the HA tetrabutylammonium salt (**3**) in an aprotic solvent such as DMF or DMSO. For example, the reaction of **3** in DMSO with benzyl iodide at 30° C for 12 hours gave, after addition of small amounts of saline water and precipitation from acetone, benzyl hyaluronic acid ester²⁴ (**4**). Hyaluronic esters of ethyl-, propyl-, benzyl-, and dodecyl- alcohols have been described as medical and pharmaceutical materials such as medical sutures, films, microspheres, pellets, membranes, corneal shields and implants¹.

4, 6-cyclic-orthoester hyaluronic acid benzyl ester: The value of cyclic orthoesters as intermediates for selective acylation of carbohydrates has been demonstrated²⁵. For example, methyl α -D-glucopyranoside on treatment with trimethylorthoacetate or 1, 1-dimethoxyethene in *N,N*-dimethylformamide (DMF) in the presence

of *p*-toluenesulphonic acid is known to afford the corresponding 4,6-cyclic orthoester derivative, which on hydrolysis afforded the corresponding 6-acetate as the major and 4-acetate as the minor compound²⁶.

A similar strategy has been adopted to introduce acetate groups selectively at C-6 and C-4 of the GlcNAc unit of HA (**1**). The reaction of HA benzyl ester (**4**) in DMF with trimethylorthoacetate in the presence of *p*-toluenesulphonic acid gave the expected 4,6-orthoester (**5**), along with its hydrolysed products the 6-*O*- (**6**) and the 4-*O*- (**7**) acetate hyaluronic acid benzyl esters as minor components. Acid hydrolysis of **5** followed by *O*-4 \rightarrow *O*-6 acetyl migration using *t*-butylamine gave a mixture containing **6** as the major and **7** as the minor component²³. Conventional acetylation of the 4,6-orthoester **5** followed by hydrolysis with aqueous acetic acid afforded a mixture of 2',3',6-triacetate (**8**) and 2',3',4-triacetate (**9**).



4 R = R¹ = R² = R³ = H; **6** R = COCH₃, R¹ = R² = R³ = H; **7** R = H, R¹ = COCH₃, R² = R³ = H; **8** R = R² = R³ = COCH₃, R¹ = H; **9** R¹ = R² = R³ = COCH₃, R = H; **11** R = R¹ = H, R² = R³ = COCH₃; **12** R = R¹ = CH₃SO₂, R² = R³ = COCH₃. Bn = CH₂C₆H₅.

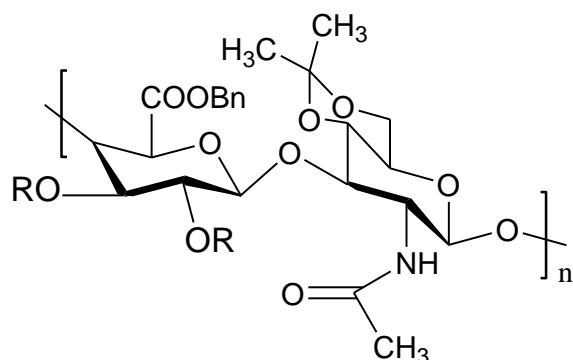
4, 6-*O*-isopropylidene hyaluronic acid:

Isopropylidene- denation of carbohydrates and their derivatives constitutes one of the most widely used modes for the protection of selected diol groups in sugar-based syntheses²⁷.

The reaction of hyaluronic acid benzyl ester (**4**) in dimethylsulphoxide (DMSO) with 2, 2-dimethoxy propane in the presence of *p*-toluenesulphonic acid at 50°C for 24 h afforded the corresponding 4,6-isopropylidene derivative (**10**), which on acetylation with acetic anhydride in DMF in the presence of *N,N*-dimethylaminopyridine (DMAP) as a catalyst gave the 4,6-di-*O*-isopropylidene-2',3'-dicaetate hyaluronic acid benzyl ester (**11**)^{22, 23}.

Hydrolysis of the 4,6-isopropylidene group in **11**, using aqueous trifluoroacetic acid in DMSO at 100°C for 3 h followed by neutralisation, dialysis and concentration gave the expected 2',3'-dicaetate HA benzyl ester (**12**). The structures of these compounds have been confirmed by ¹H and ¹³C NMR²³. As compared to the starting material **4**, the changes in the chemical shifts due to C-4' (-1.53) and to a lesser degree for C-1' (-0.1) and C-1 (-0.78) in **9** were noted; indicating substantial changes around the β-(1→4) glycosidic linkages.

These significant changes could be caused by the large 4, 6-isopropylidene group. The β-(1→3) glycosidic linkages appeared not be affected to the same extent, however, due to the absence of the OH-4...O-5' hydrogen bonds in **10**, the differences in the chemical shift value for β-(1→3) glycosidic linkages were observed. Similar conformational changes²³ were observed when HA benzyl ester (**4**) was transformed into the corresponding 4, 6-orthoester derivative **5**.



10 R = H, Bn = CH₂C₆H₅; **11** R=COCH₃, Bn = CH₂C₆H₅

Selective blocking of the 4, 6-hydroxyl groups in the GlcNAc residue of HA would allow reactions to be performed selectively at the C-2' and C-3' hydroxyl groups in the GlcA moiety.

Compound **12**, with the free hydroxyl groups at 4 and 6 positions in the GlcNAc, has been converted into the corresponding 4, 6-dimesylate **13**, by treatment with methanesulphonyl chloride in pyridine or in a combination of pyridine and catalytic amount of DMAP. The structure of **12** was supported by its ¹H and ¹³C NMR spectra²³.

6-deoxy-6-halogeno derivatives of hyaluronic acid:

The potential importance of deoxyhalogeno carbohydrates as synthetic and biological intermediates is widely recognised²⁸. Deoxyhalogeno sugars have been generally synthesised using the following methods:

- By bimolecular, nucleophilic-displacement (S_N2) reactions of sugar sulphonates using halide nucleophiles²⁹,
- By reaction with sulphuryl chloride and pyridine³⁰,
- By treatment with a combination of triphenylphosphine: carbon tetrachloride: pyridine³¹, and;
- Methanesulphonylhalide: *N,N*-dimethyl formamide complex³²⁻³⁵.

The reaction of sugars with methanesulphonyl chloride: *N,N*-dimethylformamide complex permits selective replace of primary hydroxyl groups by chlorine. However, under forcing conditions, substitution at a secondary hydroxyl group has also been observed³⁴.

The reaction has been rationalised: the initial step, slow and presumably rate limiting, is the formation of imminium ion (Me₂N⁺=CHOSO₂Me) Cl⁻, which then reacts with an alcohol (ROH) to give an intermediate (Me₂N⁺=CHOR) Cl⁻. Bimolecular, nucleophilic, substitution at the alkyl group by chloride ion affords the chlorodeoxy product. The last step of the S_N2 reaction was found not to be rate-limiting³².

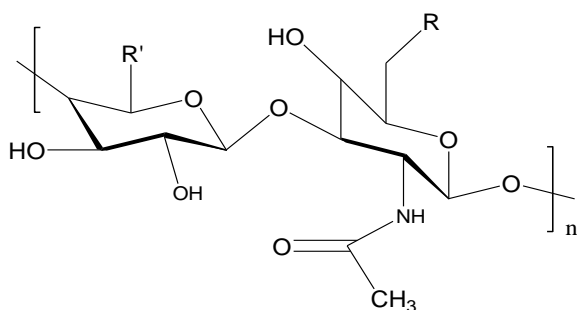
The halogenation of HA - sodium salt, - *sym*-collidinium salt or - tetrabutylammonium salt in *N,N*-dimethylformamide has been described using methanesulphonylhalide: *N,N*-dimethylformamide complex^{35, 36}. As a general methodology, a suspension of HA sodium salt (**2**) in dry DMF at 20°C was treated with 1.25 mol of methanesulphonyl chloride at -10°C for ~ 1 hour and then heated 60°C for 18.5 h. The reaction mixture was then poured in portions into a mixture of ice and 1 M sodium carbonate with vigorous mixing, maintaining the pH at 9.5. The resulting brownish suspension was stirred at pH 9.5 at room temperature for about 48 h, affording a clear solution, which was filtered and subjected to tangential flow filtration. The solution was concentrated and freeze-dried to give 6-chloro-6-deoxy-hyaluronic acid sodium salt (**14**).

The presence of chlorine group in **14** was confirmed by ¹³C NMR. The C-6 resonance of the GlcNAc residue shifted from δ 61.18 (due to CH₂-OH) to δ 44.55; indicating that the 6-OH group was replaced by chlorine. The degree of chlorine substitution in 6-chloro-6-deoxy-hyaluronic acid sodium salt (**14**) has been calculated from their ¹³C NMR spectra, from the integral of CH₂Cl signal and from the sum of CH₂Cl and CH₂OH integrals:

$$DS_{Cl} = 100 \times I_{CH_2Cl} / (I_{CH_2OH} + I_{CH_2Cl}).$$

A standard ¹³C NMR sequence was used, assuming similar relaxation times for CH₂OH and CH₂Cl signals³⁵.

Similarly, treatment of hyaluronic acid *sym*-collidinium salt with a combination of methanesulphonyl bromide and DMF afforded the 6-bromo-6-deoxy-hyaluronic acid sodium salt (**15**). The presence of bromine in **15** at the C-6 position (δ 34.5) of the GlcNAc residue has been confirmed by ¹³C NMR³⁶.



14 R = Cl, R' = COO⁻ Na⁺; **15** R = Br, R' = COO⁻ Na⁺; **16** R = N = N⁺ = N⁻, R' = COO⁻ Na⁺; **17** R = NH₂, R' = COO⁻ Na⁺; **18** R = NH₂, R' = COO⁻ Bu₄N⁺

6-amino-6-deoxyhyaluronic acid: The amino deoxy derivatives of carbohydrates are of interest because they are components of biological materials such as glycoprotein and bacterial polysaccharides. They are usually synthesised by catalytic reduction of the corresponding azido derivatives, which in turn can be prepared from the corresponding halodeoxy compounds. A direct, high yielding, synthesis of 6-amino-6-deoxyhyaluronic acid has been achieved by selective amination of the C6-chlorinated hyaluronic acid in aqueous media.

Treatment of 6-chloro-6-deoxyhyaluronic acid (sodium or *sym*-collidinium salt) with sodium azide in DMF or DMSO at 100°C for 40 hours gave, after dialysis against distilled water and freeze drying, the expected 6-azido-6-deoxyhyaluronic acid²³ (**16**). The presence of the azido group at C-6 position in **16** has been confirmed by ¹³C NMR spectroscopy, which revealed the shift of C6-Cl signal at δ 44.55 to δ 51.34 due to azido group. The IR (KBr) spectrum showed a strong peak at ν 2110.7 due to the azido group. The reduction of the 6-azido compound **16** (sodium salt) with SnCl₂.2H₂O in methanol at room temperature, for 96 hour, afforded 6-amino-6-deoxyhyaluronic acid²³ (**17**). The ¹³C NMR spectrum revealed a peak at δ 42.09. The IR (KBr) spectrum showed a minor peak at ν 2110.7 due to the azido group, indicating the presence of some unreacted azido group.

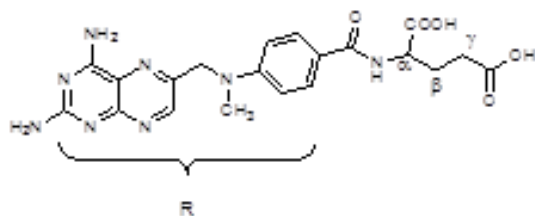
A simpler and one-step conversion of 6-chloro-6-deoxyhyaluronan (**14**) to the corresponding 6-aminodeoxy derivative **17** was subsequently achieved by heating compound **14** in excess aqueous ammonium hydroxide; with the further advantage of using water as the solvent for the reaction.

The structure of compound **17** was supported by their ¹³C NMR, DEPT, and 2D heterocorrelated NMR spectra, which showed in the ¹³C NMR spectrum a decrease of the peak at 44.5 ppm due to CH₂-Cl and the appearance of a peak at 41 ppm due to CH₂-NH₂ (**Fig. 3**).

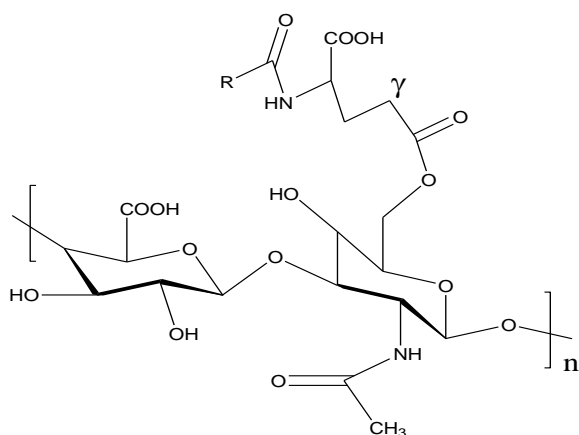
A positive Kaiser test (ninhydrin test) confirmed the presence of primary amino groups³⁷. In order to enhance the solubility of Compound **17** in organic solvents, such as DMF and DMSO, it was converted into the corresponding tetrabutyl ammonium salt **18**.

Hyaluronic acid-methotrexate conjugates: Small molecule drugs such as antitumor compounds have recently been conjugated to synthetic and natural polymers. The advantages envisaged in this strategy are reduced toxicity, increased solubility and stability, localisation and controlled release of the drug³⁸⁻⁴².

Methotrexate (**19**) is an antimetabolite and an analogue of folic acid used in the treatment of diseases such as inflammatory pathologies, autoimmune or neoplastic diseases. For example, it is used for the treatment of Crohn's disease, inflammation of the colon, ulcer colitis⁴³, rheumatoid arthritis, and osteoarthritis⁴⁴. However, its therapeutic use is limited because of its high systemic toxicity and short plasma half-life. In cancer treatment 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 tumour site^{43, 44}.



19 Methotrexate



20 Hyaluronic Acid – Methotrexate Conjugate

Regioselective conjugation of methotrexate with HA has been investigated^{35, 36}. Treatment of a solution of 6-bromo-6-deoxy-hyaluronic acid sodium salt (**15**) in DMSO with a solution of methotrexate (**19**) in DMSO and cesium carbonate under nitrogen at 80° C for 24 hour gave the desired product, hyaluronic acid-6-methotrexate conjugate. The product was analysed by ¹H and ¹³C NMR for the presence of methotrexate covalently linked and integrity of the polymer³⁶.

The S_N2 reaction of 6-chloro-6-deoxy-hyaluronic acid sodium salt (**14**, MW 20,000) has been performed with methotrexate in the presence of cesium carbonate in DMSO at 80° C for 40 hour; as is the case with biopolymer reactions in general, partial displacement, of the C-6 chloro group in **14** occurred to afford a mixture of 6-chloro-6-deoxy-6-*O*- α,γ -methotrexylhyaluronic acid. Its structure was supported by ¹H and ¹³C NMR³⁵. For simplistic reason and the economy of space, only the structure of compound 6-*O*- γ -methotrexylhyaluronic acid has been depicted (**20**). The chlorine group in 6-chloro-6-deoxy-6-*O*- α,γ -methotrexylhyaluronic acid has been subsequently replaced by acetate and butyrate group by treatment with the corresponding cesium salt in DMSO; structures of the resulting 6-*O*-acetyl-6-*O*- α,γ -methotrexylhyaluronic acid and 6-*O*-butryl-6-*O*- α,γ -methotrexylhyaluronic acid were determined using NMR spectroscopy³⁵.

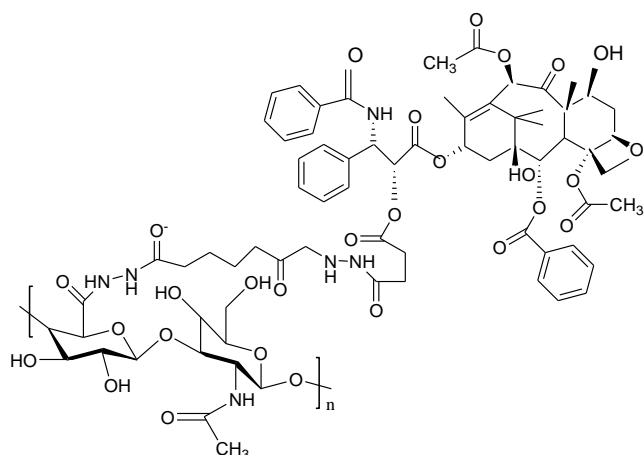
Treatment of a solution of 6-bromo-6-deoxy-hyaluronic acid sodium salt (**15**) in DMSO with a solution of methotrexate (**19**) in DMSO and cesium carbonate under nitrogen at 80° C for 24 hour has been described to afford HA-6-methotrexate (**20**). The product has been analysed by ¹H and ¹³C NMR to establish the presence of covalently linked methotrexate and integrity of the polymer³⁷.

Hyaluronic acid-paclitaxel conjugate: Paclitaxel an antileukemic and antitumor agent was first isolated from the bark of the Pacific yew tree, *Taxus brevifolia*. Paclitaxel is a poorly soluble antimitotic chemotherapeutic agent which causes tumor cell death by disrupting mitosis. Its solubility was greatly increased by conjugation to HA. Hyaluronic acid is over expressed at sites of tumour and provides a matrix to facilitate invasion. Hence, to overcome the solubility problem and to target the tumour cells, a hyaluronic acid conjugate of Taxol was prepared⁴⁵.

The conjugation synthetic strategy involved:

- Synthesis of Taxol-2'-hemisuccinate by treatment of Taxol with succinic anhydride in a mixture of dichloromethane and pyridine;
- The hemisuccinate derivative was treated with *N*-hydroxysuccinimide diphenyl phosphate in acetonitrile in the presence of triethylamine to give Taxol-*N*-hydroxysuccinimide;
- Hyaluronic acid was functionalised by treatment with adipic dihydrazide in aqueous system at pH 4.75 to give hyaluronic acid-adipic dihydrazido derivative; and finally;
- The two intermediates, Taxol-*N*-hydroxysuccinimide and HA-dihydrazide, were stirred together in aqueous DMF in the presence of 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide at room temperature for 24 hour to afford the desired HA-Taxol conjugate (**21**).

Compound **21** exhibited selective toxicity toward the human cancer cell lines (breast, colon and ovarian) that are known to express hyaluronic acid receptors; no toxicity was noted against a mouse fibroblast cell line at the same concentrations.

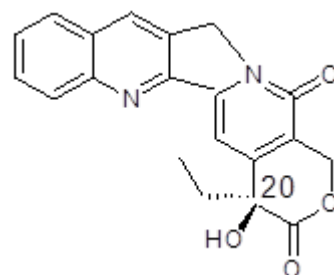


21 Hyaluronic Acid – Taxol Conjugate

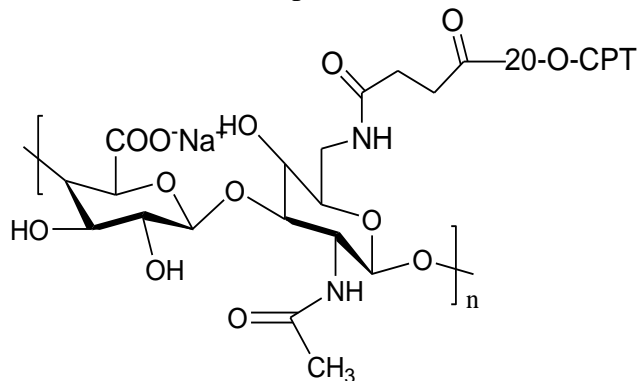
The balance between hyaluronic acid and conjugated drug is critical in obtaining the optimal cytotoxic effect; as the carboxyl group of the polymer is responsible for its targeting properties, high loading of the drug may affect the CD44 recognition ability of the polymer-drug conjugate⁴⁶.

In 2009, a new approach to address limited drug substitution in hyaluronic acid-drug conjugates has been proposed by Norbedo *et al*³⁷; since C-6 hydroxyl groups of HA are not involved in CD44 recognition, higher degrees of substitution may not significantly affect the targeting properties of the resulted conjugates⁴⁷.

Hyaluronic acid-camptothecin conjugate: Camptothecin 20-(S) (CPT, **22**) is a naturally occurring alkaloid isolated from *Camptotheca acuminata* with a significant antitumor activity against a variety of human solid tumours⁴⁸. However, because of its limited solubility in water and severe toxicity its trial as an anticancer drug was stopped⁴⁹. It is also important to note that the intact lactone ring is critical for the CPT antitumor activity. In order to overcome these drawbacks, water soluble analogues of camptothecin, Hycamtin and Camtosar, were synthesised, which are approved for the treatment of ovarian and colon cancer, respectively⁵⁰⁻⁵².



22 Camptothecin 20-(S)



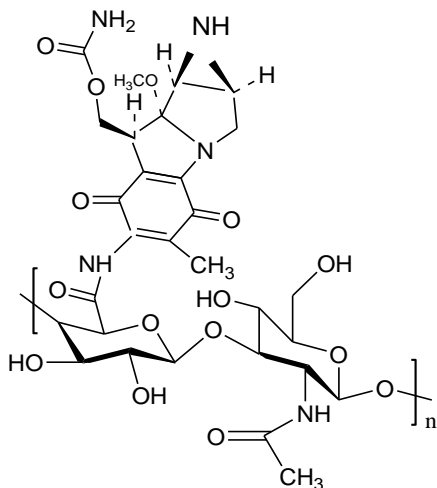
23 Hyaluronic Acid – Camptothecin 20-(S) Conjugate

To impart targeting ability and to enhance the water solubility, camptothecin has been conjugated with the hyaluronic acid at the C-6 position of the *N*-acetyl-D-glucosamine moiety. The conjugation strategy adopted was similar to the hyaluronic acid-Taxol conjugate:

- The C-20 hydroxyl group of camptothecin was converted to the corresponding hemisuccinate
- The CPT-20-*O*-hemisuccinate was then treated with 6-amino-6-deoxyhyaluronan TBA salt in the presence of *N*-hydroxysuccinimide/*N,N*-diisopropylcarbodiimide in dimethyl sulphoxide; and;
- The resulting HA-20-*O*-CPT conjugate TBA salt was converted to the corresponding Na salt³⁷ (**23**).

Hyaluronic acid - mitomycin c conjugate:

Mitomycin C has been linked to the HA molecule by way of an amide bond between the drug and the carboxyl group of the D-glucuronic acid moiety. An amide linkage formation is a dehydration reaction and requires anhydrous systems. However, as hyaluronic acid is difficult to dissolve in an anhydrous organic solvent, reaction conditions were developed to use a water based system⁵⁹. The general scheme of the conjugation reaction followed: (a) activation of the carboxyl groups of the D-glucuronic acid moiety of the HA-Na salt (**1**) using *N*-hydroxysuccinimide in the presence of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) in pyridine and water under acidic conditions (pH ~ 4.7); (b) the reaction mixture was treated with sodium acetate buffer to decompose the excess carbodiimide, and (c) the resulting *N*-hydroxysuccinimide-HA intermediate was then treated with an aqueous solution of mitomycin C in a phosphoric acid buffer to afford HA-Mitomycin C conjugate (**24**).



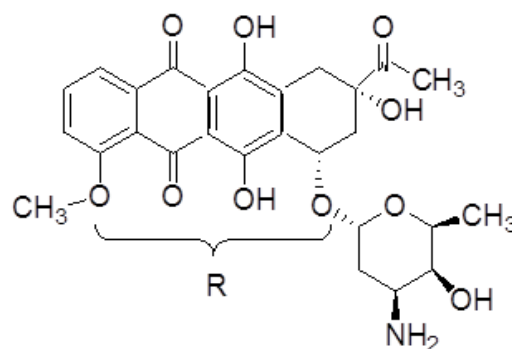
24 Hyaluronic Acid – Mitomycin - Conjugate

The cancer metastasis suppressing test performed with the HA-Mitomycin C conjugate **24** in mouse with Lewis lung carcinoma cells, as compared to mitomycin alone, exhibited excellent cancer metastasis suppression effect both in single administration and consecutive administrations. Especially, the conjugate produced a striking effect in the three consecutive administrations.

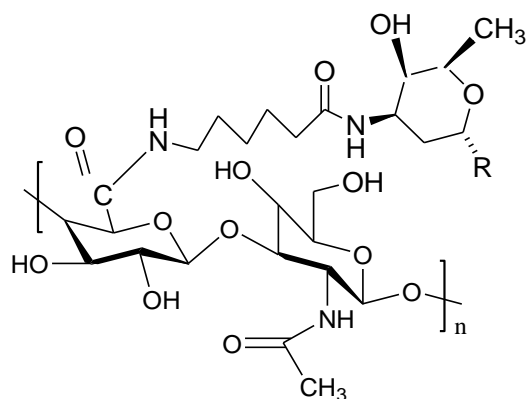
The conjugate also exhibited strong suppressing effect against cancer metastasis through lymph nodes and the MethA tumour growth⁵³.

Hyaluronic Acid-Daunomycin Conjugate:

Daunomycin (**25**) has been conjugated with HA, using ϵ -aminocaproic acid spacer arm. The carboxyl group of the hyaluronic acid was activated using *N*-hydroxysuccinimide and EDC, the excess was decomposed by dipotassium phosphate buffer and then treated with ϵ -aminocaproic acid to afford the intermediate HA-5'- ϵ -aminocaproic acid amide, which was then treated with Daunomycin C in water, pyridine and *N,N*-dimethylformamide (DMF) in the presence of EDC. At the end of the reaction the excess EDC was removed using sodium acetate buffer and the HA-Daunomycin conjugate (**26**) was precipitated from acetone⁵³.



25 Daunomycin



26 Hyaluronic Acid – Daunomycin Conjugate

Daunomycin has also been conjugate to HA directly. However, in order to overcome the solubility problem of hyaluronic acid-Na salt in dry organic solvent the HA-Na salt, some or all of the hydroxyl groups were acetylated prior to the conjugation reaction.

For example, the reaction of the carboxylic group of the acetylated HA is performed in the following sequence of reactions:

- A solution of the acetylated derivative of HA in dry DMF was treated with isobutyl chloroformate to activate the C-5' carboxyl group of the HA,
- The activated HA was then treated with a solution of Daunomycin in DMF in the presence of triethylamine,
- The acetyl groups were then removed by treatment with sodium hydroxide at pH 12.5, and (d) the solution was neutralised with acetic acid and the HA-drug conjugate was precipitated from acetone.

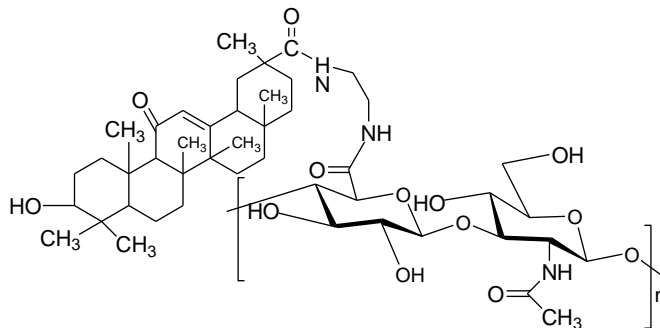
Synthesis of hyaluronic acid conjugates of 5-Fluorouracyl, Cytosine and Epirubicin have also been described⁵³.

Hyaluronic Acid Glycyrrhetic Conjugate:

Hyaluronic acid-glycyrrhetic acid-graft conjugate (HA-GA, **27**) has been synthesized as a carrier for intravenous administration of paclitaxel, which combined hyaluronic acid (HA) and glycyrrhetic acid (GA) as the active targeting ligands to liver tumor⁵⁴.

Paclitaxel has been entrapped in HA-GA nanoparticles with high efficiency up to 31.16 weight % and entrapment up to 92.02%; it exhibited significant cytotoxicity to HepG2 cells than B16F10 cells due to simultaneously over expressing HA and GA receptors.

The study also includes: physicochemical characteristics, cellular uptake efficiency, and *in vivo* fates of HA-GA conjugates (**27**).



27 Hyaluronic Acid – Glycyrrhetic acid – Graft Conjugate

The reaction sequence for the synthesis of HA- GA conjugate **24** as follow:

- First GA was aminated, using ethylene diamine in dichloromethane in the presence of N-hydroxysuccinimide (NHS) and N,N-dicyclohexyl carbodiimide (DCC) to afford the corresponding succinimido glycyrrhetic acid [G-CO-NH-(CH₂)₂-NH₂]; and;
- The succinimido GA was reacted with hyaluronic acid in DMF in the presence of N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide to afford the HA-GA conjugate, which was isolated after precipitation from acetone, dialysis against deionized water, and freeze drying.

Biopolymer Bioscaffolds as Bioreactors:

Biopolymer scaffolds have been generated in recent years to construct bio-artificial tissues or organs for treatment of patients⁵⁵, and for enzyme catalyzed acylation of carbohydrates⁵⁶. Methodologies available to generate biopolymer scaffolds are known like, for example, fiber bonding, gas foaming, phase separation/emulsification, solvent casting and particulate leaching, interpenetrating polymer network, chemical cross-linking, and photo cross-linking. This Section will cover the recently patented work on the synthesis of biopolymer scaffold and their application to produce regio-selectively sugar alkyl esters, in particular sugar fatty acid esters⁵⁷. The commercial importance of sugar fatty acid esters as surfactants has been well recognized⁵⁸; sucrose 6-acetates is one of the key intermediates in the production of Sucralose (Splenda®), a commercially important high intensity sweetener^{59, 60}.

The invention relates to the process for the production of biopolymer scaffolds as bioreactors for the synthesis of sugar 6-*O*-acylates, in particular sugar fatty acid esters. The process comprises the following steps:

- (a) Producing a stable biopolymer scaffold of appropriate pore size and pore distribution, by using a combination of polysaccharide and polyethylene glycol dimethacrylate;
- (b) Immobilizing or encapsulating both the enzyme (a lipase) and the substrate (sucrose), preferably in solid form, into the polysaccharide-PEG scaffold, preferably during the preparation step of the scaffold;
- (c) Reacting the sucrose immobilized or encapsulated together with the enzyme into the scaffold, with an acylating reagent in *t*-amyl alcohol to selectively afford sucrose 6-*O*-esters.

As a general procedure, to a solution of the polysaccharide, e.g., hyaluronic acid or chitosan, and the PEG dimethacrylate in water at pH 5 was added IRGACURE [bis(2, 4, 6-trimethylbenzoyl)-phenylphosphine oxide - CAS Registry Number: 162881-26-7] (2959, 0.5%) from CIBA and 36 μ l *N*-vinyl-pyrrolidinone; the solution was stirred in dark at ambient temperature for few minutes and then added the enzyme (e.g., *T. lanuginosa*) and the solid substrate (sucrose) and then irradiated with UV light (365nm) till the gelation. It was then freeze dried to give the desired biopolymer scaffold.

The hyaluronic acid-PEG-Enzyme-Sucrose scaffold in *t*-amyl alcohol was magnetically stirred (~200 rpm) in the presence of molecular sieves for 1 h at room temperature and then vinyl laurate (5 equiv.) was added. The temperature was then raised to 30-40°C and the reaction was carried out for 24 h to give after removing the solvent sucrose 6-laurate as the major and sucrose di-laurate as the minor product. The scaffolds were recovered, dried to remove residual solvents then regenerated with sucrose by treatment with aqueous sucrose solution and the reaction with vinyl laurate was repeated.

The major strengths of the biopolymer scaffolds are:

- (i) The three-dimensional structure of the scaffold is robust to withstand repeated use in organic solvents at the required temperature,
- (ii) They have appropriate porosity to retain the enzyme and the substrate within the structure,
- (iii) They allow the influx of the acylating reagents and the efflux of the final products during the enzymatic reaction;
- (iv) It does not use toxic organic solvents such as dmf, dmsO or pyridine,
- (v) The enzyme is stable and retains its activity at 40°C for several weeks, allowing its repeated recycling and to develop a continuous process, and
- (vi) The process uses sustainable, regenerable, nanomaterials.

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