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Synthesis of Sulfonated Hyaluronan Derivatives Containing Nucleic Acid Bases

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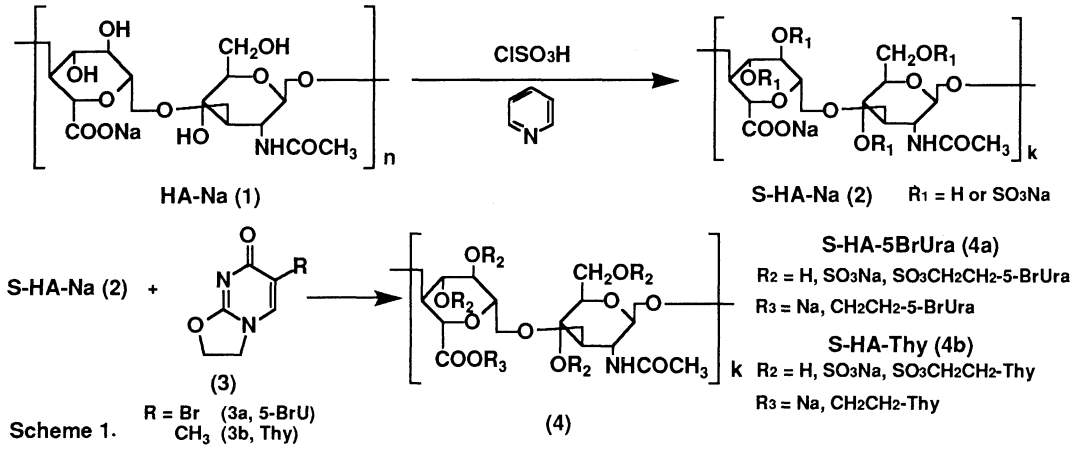
The conjugation of nucleic acid base with sulfonated hyaluronan was achieved by the ring opening reaction of 1, 2-O-ethano derivatives of nucleic acid bases. The conditions of sulfonation of sodium hyaluronate were studied. Thymine and 5-bromouracil base were quantitatively conjugated to sulfonated hyaluronan in 15% and 24%, respectively.

As hyaluronic acid (HA) is of a great interest in the pharmaceutical and clinical practice, it received much attention for specific properties and other applicabilities. However, a few studies on chemical modification of hyaluronan have been reported.[1] We imply that one of interesting modifications of HA is hybridization with nucleic acid moiety. Our group has reported a number of syntheses and applications of stable nucleic acid analogs as synthetic polymer containing nucleic acid bases or nucleosides in side chain.[2] It is expected that the conjugation of nucleic acid base with HA derivatives is a good strategy to obtain a new type of biocompatible nucleic acid analogs. In this case, the nucleic acid moieties could play an important role with respect to specific interactions with DNA, while the HA backbone should provide a series of biocompatibility.

Modification of HA could be achieved at either acetamide group or carboxylate group. Deacetylation of acetamide group was proved to be an effective way for the conjugation of nucleic acid bases with water solubility and specific interaction properties.[3] On the other hand, the modification of carboxylate group gave the water insoluble analogs, because carboxylate group of HA made for water solubility. Thus the modification of carboxylate group with water solubility could be obtained such as pathways of carboxymethylation, sulfonation and organic solvent soluble tetra-*n*-butylammonium salt.



Scheme 1.

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A series of sulfonated polysaccharide has received much attention for its bioactivity and antibacterial action. In the case of sulfonated hyaluronan, R.E.Drzymala et al. have reported that sulfonation of deacetyl HA derivative showed arteriosclerosis property.[4] Through these attractive viewpoints, this report is focused on the study of the synthesis of sulfonated HA derivative and the introduction of nucleic acid bases.

Table 1. Sulfonation of Sodium Hyaluronate [a]

Run	Time / h	Temp / °C	Yield / % (g)	DS [b]	$[\eta]$ [c] / dL/g
1	1.0	60	70 (4.4)	1.1	7.57
2	1.0	90	79 (5.9)	2.0	5.82
3	4.0	90	43 (3.0)	1.6	2.50
4	2.0	Reflux	27 (1.8)	1.3	0.85

a) Exptl. condition: HA-Na = 5 g, $ClSO_3H$ = 5 mL (6.3eqv. against sodium hyaluronate unit), Pyridine = 80 mL
b) Degree of sulfornation per unit was caculated from the results of elementary analysis.
c) Limiting viscosity was measured in H_2O at 25 °C.

The synthesis of HA containing nucleic acid base began with the preparation of sulfonated HA (S-HA-Na, **2**) which was derived from the reaction of sodium HA (**1**) with chlorosulfonic acid (Scheme 1). The procedure is as follows : to a suspension of sodium HA (**1**, 12.5 mmol) in dried pyridine, chlorosulfonic acid (6.3 mmol) was added dropwise and stirred at 90 °C for 1 h. After cooling to room temperature, the precipitate was collected and dissolved in 300 mL of distilled water. The pH was adjusted to 8 by 1.0 mol dm^{-3} NaOH and the solution was concentrated under reduced pressure. The deposited salt was excluded by filtration and the filtrate was poured into 1 L of acetone. The obtained precipitate was dissolved in 300 mL of distilled water and dialyzed through cellulose tube against 2 L of distilled water for 10 days. Then the solution was concentrated under reduced pressure and poured into an excess of acetone. Resulting precipitate was collected and well dried to give light yellow product (**2**) in 78% yield.

Sulfonation was confirmed by IR spectra, that was equatorial C-O-S showed the peak at 820 cm^{-1} while SO_2OH at 1200-1300 cm^{-1}. Degree of substitution per unit (DS) was calculated by elementary analysis, by taking the ratio of the percentage of sulfur to percentage of nitrogen. The value of DS was found to be varied from less than 1 to 2 as shown in Table 1. This suggested that the substitution was occurred at one or more positions of hydroxyl groups of HA unit. Table 2 showed the ^{13}C-NMR chemical shift of the products from DS = 0.3 to 2.0.

Table 2. ^{13}C-NMR chemical shifts of Sodium Hyaluronates and Hyaluronate derivatives[a]

Sample	β-D-Glucuronate unit						β-D-N-Acetyl galactosamine unit						
	C-1	C-2	C-3	C-4	C-5	COO⁻	C-1	C-2	C-3	C-4	C-5	C-6	CH_3
HA-Na [b] (Reference 5)	104.4	73.8	74.8	81.2	75.5	175.2	101.7	55.6	84.1	69.7	76.6	61.7	23.7
HA-Na	106	75	76	82	79	177	103	57	85	71	77	63	25
S-HA-Na (DS=0.3)	106	75	76	83	78	178	104	57	85	71	77	63s[c] 70w[c]	25
S-HA-Na (DS=0.8)	105	75	76	83	79	178	104	57	85	71	78w[c] 75s[c]	63w[c] 70s[c]	25
S-HA-Na (DS=2.0)	105	75m[c] 82m[c]	76m[c] 83m[c]	82	79	178	103	57	85	78s[c] 71w[c]	75	70	25

a) Condition : 0.5% LiCl in D_2O at 25 °C. Chemical shifts (PPM) are given from internal sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS).
b) Measured in D_2O at 30 °C.
c) s : means strong, m : means medium, w : means weak peaks.

By comparing the chemical shift values to that of sodium hyaluronate,[5] the sulfonated position was confirmed as follows. In the case of DS = 0.3 and 0.8, sulfonation was occurred only at hydroxyl group of C-6 of N-acetylgalactosamine unit, while neighboring C-5 was affected to shift downfield for 2 ppm. On the other hand in the case of DS = 2.0, sulfonation was occurred predominantly at C-6 of N-acetylgalactosamine unit, and partially at C-4 hydroxyl group of N-acetylglucosamine unit and also at C-2, C-3 hydroxyl groups of glucuronate unit (Fig. 1).

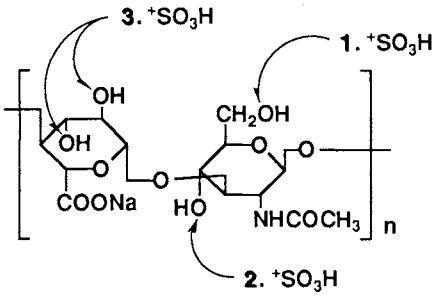

Fig. 1. Reactivity of hydroxyl groups in sulfonation.

Degree of sulfonation per unit (DS) and the reaction conditions were summarized in Table 1. At reaction temperature 90 °C, DS was maximized at 2.0 in reaction time for 1 h, and less than 1.6 for longer reaction time. At 60 °C, DS was drastically reduced, showing the highest value of DS at 1.1 for 1 h of reaction time (Run 1) and 0.6 for 3 h. However, in the case of Run 1, limiting viscosity value was as high as 7.6 dL/g. It suggested to avoid the degradation of HA backbone. Under hard conditions, such as at reflux for 1-2 h, DS was reduced to as small as 1.3 and the limiting viscosity was reduced to 0.85 dL/g.

The conjugation of nucleic acid moiety with hyaluronic acid by ester bonding was achieved by the reaction of 1,2-O-ethano (cyclic) derivatives of nucleic acid compound. Reaction of cyclic nucleic acid base compound with various kinds of organic reagents at room temperature gave a series of pyrimidine derivatives without any catalyst.[6] One of typical reactions involves esterification with acetic acid in bulk to afford the corresponding acetate. When the reaction is applied to polymeric acids, such as poly (acrylic acid), poly (methacrylic acid), and poly (L-glutamic acid), the polymers containing uracil or thymine are obtained in high yield. Thus, this reaction of cyclic nucleic acid base with polymeric acid was applied to synthesis of hyaluronic acid derivatives containing nucleic acid base. 5-Bromouracil is known to have a antitumor activity, thus, it was also concerned on the conjugation with hyaluronic acid backbone. In this case, it was expected to provide a macromolecular prodrug with reduced side-effects.

The procedure was as follows; to the suspension of sodium sulfonated HA (**2**, DS = 2.0, 1.2 mmol) in 50 mL of dried N, N'-dimethylformamide, 2.3 mmol of 1,2-O-ethano-5-bromouracil (**3a**) [6] was added and stirred at

Table 3. The conjugation of nucleic acid bases with sulfonated hyaluronate

Run	S-HA-Na : 1,2-O-Ethano-5-BrUra / 1,2-O-Ethano-Thy (molar ratio)	DS of (2) [a]	Content of 5-BrUra or Thy [b] (%)	Solubility in H_2O
1	1.0 : 1.5 (5-BrUra)	2.0	24	Soluble
2	1.0 : 1.0 (5-BrUra)	2.0	22 (21) [c]	Soluble
3	1.0 : 1.0 (5-BrUra)	0.8	18 [c]	Insoluble
4	1.0 : 1.0 (Thy)	2.0	15	Soluble
5	4.0 : 3.0 (Thy)	2.0	6.1	Soluble

Experimental conditions; reaction time, 5 days in 20 mL of DMF.

a) Degree of sulfonation per unit was determined from elementary analysis, see Table 1 and 2.

b) Determined from UV spectra at 282 nm (5-BrUra) and 270 nm (Thy).

c) Content of 5-BrUra which was determined from elementary analysis.

80 °C for 5 days to give the homogeneous solution. The solution was concentrated under reduced pressure and poured into excess of acetone. The product was dissolved in 30 mL of water and dialyzed for 10 days. Resulting solution was poured into excess of acetone to obtain light yellow product (**4a**) [7] in 78% yield. The same procedure was carried out in case of conjugation of thymine base [8] by the reaction of 1,2-O-ethanothymine (**3b**) [6] with sodium sulfonated HA (**2**, DS = 2.0).

Content of nucleic acid bases was determined from UV absorption at 283 nm (λ_{max} of 5-bromouracil), 270 nm (λ_{max} of thymine) and ^{1}H-NMR. In the case of 5-bromouracil, molar ratio of sulfonated HA (DS = 2.0) and 1,2-O-ethano-5-bromouracil were varied 1:1 and 1:1.5, as shown in Table 3. The conjugation of 5-bromouracil was achieved quantitatively around 20% in either case of molar ratio. The obtained derivatives were soluble in water, which was possible to study the interaction with polynucleotides or DNA. In the case of DS = 0.8 of sulfonated HA, the product was insoluble in water, while DS = 2.0 gave the water soluble analog. This suggested that water solubility was contributed to degree of sulfonation of HA.

In the case of thymine, molar ratio was varied to maximize the content of thymine base. In this case, molar ratio of sulfonated HA (DS = 2.0) and 1,2-O-ethanothymine were varied 1:1, 1:1.5, and 4:3. The conjugation of thymine was achieved 15% in 1:1 and 1:1.5. However, in the case of 4:3 molar ratio, the content of thymine base was drastically reduced to 6.1%.

We are focusing on properties of these water soluble nucleic analogs and potential applications in our further study.

References

1) T. Matsuda, H. Miwa, M. J. Moghaddam, and F. Iida, *ASAIO J.*, **39**, M327 (1993); N. Yui, T. Okano, and Y. Sakurai, *J. Controlled Release*, **26**, 141 (1993); J. W. Kuo, D. A. Swann, and G. D. Prestwich, *Bioconjugate Chem.*, **2**, 232(1991); M. Lyon, *Biochem. Biophys. Acta*, **881**, 22 (1986).
2) Y. Inaki and K. Takemoto, *Current Topics in Polymer Science.*, **1**, 79 (1987).
3) T. Wada, S. Chirachanchai, N. Izawa, Y. Inaki, and K. Takemoto, *J. Bioact. Compar, Polym.*, in Press.
4) R. E. Drzymala, P. A. Liebman, and G. Romhayi, *Histochemistry*, **76**, 363 (1982).
5) S. M. Bociek, A. H. Darke, D. Welti, and D. A. Rees, *Eur. J. Biochem.*, **109**, 447 (1980).
6) Y. Inaki, H. Futagawa, and K. Takemoto, *J. Polym. Sci., Polym. Chem. Ed.*, **18**, 1847 (1980); Y. Kita, H. Futagawa, Y. Inaki, and K. Takemoto, *Polym. Bull.*, **2**, 195 (1980); E. Mochizuki, Y. Inaki, and K. Takemoto, *Nucleic Acids Res. Symp.*, **10**, 125 (1981).
7) ^{1}H-NMR (in D_2O, at 25°C, ppm) : 2.01(3.0H, s, CH_3), 3.24(0.48H, t, C-2' of 5BrUra), 3.48(2.0H, d, C-6), 3.56(1.0H, dd, C-2), 3.63(1.0H, d, C-5), 3.66(1.0H, d, C-1), 3.71(0.48H, t, C-1' of 5-BrUra), 3.73(1.0H, dd, C-3), 3.74(1.0H, dd, C-4), 3.99(1.0H, s, C-1'), 4.08(1.0H, d, C-5'), 4.12(1.0H, dd, C-2'), 4.15(1.0H, dd, C-3'), 4.30(1.0H, dd, C-4'), 7.56(0.24H, s, C-6 of 5BrUra).
8) ^{1}H-NMR (in D_2O, at 25°C, ppm) : 1.82(0.5H, s, CH_3 of Thy), 2.01(3.0H, s, CH_3), 3.24(0.3H, t, C-2' of Thy), 3.48(2.0H, d, C-6), 3.56(1.0H, dd, C-2), 3.63(1.0H, d, C-5), 3.66(1.0H, d, C-1), 3.71(0.3H, t, C-1' of Thy), 3.73(1.0H, dd, C-3), 3.74(1.0H, dd, C-4), 3.99(1.0H, s, C-1'), 4.08(1.0H, d, C-5'), 4.12(1.0H, dd, C-2'), 4.15(1.0H, dd, C-3'), 4.30(1.0H, dd, C-4'), 7.52(0.2H, s, C-6 of Thy).

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