Carbohydrate Polymers **18** (1992) 139–142

The effects of polysaccharide chain-length in coating liposomes with partial palmitoyl hyaluronates

Yoshihiro Kawaguchi, Kuraya Matsukawa

Ishihara Chemical Co., Ltd, Tabata-shinmachi, Kita-Ku, Tokyo 114, Japan

Yasuo Gama & Yutaka Ishigami

National Chemical Laboratory for Industry, Higashi Tsukuba-City, Ibaragi 305, Japan

(Received 2 September 1991; accepted 30 September 1991)

High and low molecular weight hyaluronates (average mw, HA-Na:2 000 000; HA2-Na:20 000) were induced to partial palmitoyl derivatives (HA-P-Na and HA2-P-Na) to be water-soluble. Both derivatives coated the surface of liposomes, preventing the destruction of liposomes by phospholipase D (PL-D). The anchoring with palmitoyl chains was restricted in the presence of cholesterol in liposomes. Sufficient coating with HA2-P-Na needed more anchoring in the absence of cholesterol. It seems that conformations of polysaccharide chains on the surface of liposomes are largely different between HA-P-Na and HA2-P-Na.

INTRODUCTION

Hyaluronates (HA), which can act as a physical barrier to protect the cell surface, compose a pericellular coat surrounding many cells (Patterson *et al.*, 1975). For example, cell surface hyaluronate appears to prevent several different types of viruses from making contact with the synovial cells, and thereby prevents infection (Clarris *et al.*, 1974). In a similar fashion, the cell coat may block the access of activated macrophages and lymphocytes to the surfaces of some types of target cells and in so doing protect these cells from cytotoxicity (Forrester & Lackie, 1981). For the purpose of artificially reconstructing HA-coated cells and putting them to use for the study on the physiological role, partially palmitoyl hyaluronate as prepared and used as an assembly of an artificial cell wall on liposomes. Recently, some papers have described the usefulness of the polysaccharide-coated liposomes in drug delivery systems (Sunamoto & Iwamoto, 1986; Sunamoto *et al.*, 1986). The targeting effect that the constituent sugar of these polysaccharide produces is examined in their papers. Though 'the loop-train-tail conformation' of the polysaccharides on liposomal surfaces is assumed (Kobayashi, 1991), more conformational studies are necessary to clarify the surface function. For the latent utility of our HA derivatives, modification of liposomes with derivatives was attempted. In this study, it was shown that HA derivatives coated liposomes are resistant to the phospholipase-D (PL-D) activity.

MATERIALS AND METHODS

Preparation of partial palmitoyl hyaluronates

The hyaluronates used in this study were high molecular weight sodium hyaluronate (HA-Na, average mw above 2 000 000) and low molecular weight sodium hyaluronate (HA2-Na, average mw of 20 000). The average molecular weight was estimated by the method of Laurent *et al.* (Laurent *et al.*, 1960; Cleland *et al.*, 1968). HA2-Na was obtained by hydrolysis of HA-Na to be available commercially.

Hydrolysis of hyaluronates

A 2% solution of HA-Na was mixed with 2% NaOH equivalent. The mixture was stirred at 50°C until the HA-Na degraded to the desired molecular weight. The mixture was then neutralized with 1 M HCl, whereupon NaCl was added to reach 2 mol liter^{-1} and 3 volumes (600 ml) of ethanol were added to precipitate the

Carbohydrate Polymers 0144-8617/92/$05.00

hydrolyzate. After washing with ethanol, the precipitate was dried under reduced pressure at 40°C.

Acylation of hyaluronates

HA-Na and HA2-Na were esterified with palmitoyl chloride in *N,N*-dimethylformamide in the presence of pyridine. A typical procedure is described as follows: To a stirred suspension of HA-Na (0·5 g) in dry dimethylformamide (200 ml), palmitoyl chloride (4·5 g) and pyridine (1·6 g) were added at room temperature. The mixture was stirred for certain periods (2 weeks) at the same temperature. Insoluble products (product A and/or a (HA-P and/or HA2-P)) were filtered and washed with acetone, chloroform, and methanol. These products were sufficiently dried under reduced pressure at 40°C.

Modulation of acylation degree

Product A and/or a (HA-P and/or HA2-P) were water-insoluble because of sufficient esterification and the formation of intramolecular acid anhydride. Therefore, a good water-solubility was brought by a moderate alkaline treatment. A general procedure of the moderate alkaline treatment is described as follows: HA-P and HA2-P were suspended in chloroform and a few drops of 1 M NaOH or 28% ammonium solution was added to the suspension, then stirred for several hours. After the solvent had evaporated, the residue was collected by filtration and washed with acetone and chloroform. These materials were dissolved in water and neutralized by the addition of 1 M HCl. The insoluble parts were discarded through filtration. The filtrates were dried under reduced pressure at 40°C, and product B and/or b (HA-P-Na and/or HA2-P-Na) were obtained as water-soluble derivatives.

Confirmation of the esterification

The introduction of esters was confirmed qualitatively with analysis by IR and the hydroxamic ferrate method (Synder & Stephens, 1959; Renkonen, 1961). The degrees of esterification of HA-P-Na and HA2-P-Na were shown as the ratio of the palmitoyl group to the N-acetyl group, as determined by ^{1}H-NMR. ^{1}H-NMR was measured at 200 MHz in D_2O using 3-(trimethylsilyl) propionic acid-d_4 sodium salt (TMSP) as the internal standard (Fig. 1).

Preparation of HA and HA2-coated liposomes encapsulated calcein

HA and HA2-coated liposomes were conventionally prepared by Vortex methods (Noro & Ishii, 1984*a*, *b*). A solution of calcein (4×10^{-4} mol liter^{-1}) was encapsulated in liposomes under a constant molar ratio (egg lecithin:cholesterol = 1:1). Non-encapsulated calcein was removed by gel filtration on a Sephadex G-75 column. Calcein-encapsulated liposomes prepared in this way were adjusted to a lecithin concentration of 0·8 μmol ml^{-1} determined with a commercially available test kit for phospholipid. HA and HA2-coated liposomes were prepared as follows: HA-P-Na and HA2-P-Na were respectively added to calcein-enclosed liposomes at the final concentration of 2–10 mg ml^{-1}, and the mixtures were stirred at 37°C for 1 h. In HA2 coating, cholesterol-free liposomes were additionally used.

Fluorescence measurement for calcein release by reaction with PL-D

Fluorescence measurements (Oku *et al.*, 1982; Sunamoto *et al.*, 1984) were carried out on a Shimazu fluorospectrophotometer, and calcein emits at 518 nm by excitation at 480 nm. The release of calcein from HA- or HA2-coated liposomes by reaction with PL-D was followed by monitoring the increase in the fluorescence in course of time. The reaction mixture consisted of HA- or HA2-coated liposomes (2 ml) and PL-D solution (23 units in 50 μl). The total amount of calcein encapsulated in liposomes was determined by destroying the liposome membranes with 50 μl of 10% (v/v) Triton X-100 solution for HA- or HA2-coated liposomes (2 ml).

Zeta potential on the surface of HA- and HA-2 coated liposomes

After the preparation of liposomes by Vortex methods without calcein, HA2- and HA-coated liposomes were prepared as described above. Non-coated liposomes, which were added with stearylamine (SA) and dicetylphosphate (DCP), were prepared as comparisons. The zeta potential was measured on Laser Zee Model 500 at pH 4, 6 and 8. Acetate–sodium acetate buffer (M/10, pH 4 and 6) and M/15 KH_2PO_4–Na_2HPO_4 buffer (pH 6 and 8) were respectively used.

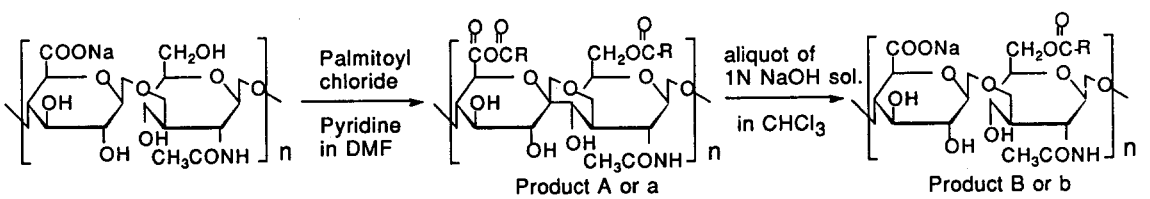

Fig. 1. Scheme of the esterification reaction.

Table 1. Analytical results by hydroxamic ferrate method and NMR

Product	mw of HA-Na	Solubility[a] in water	Hydroxamic ferrate method[b] (OD at 530 nm mg^{-1})	Degree of esterification by NMR (palmitoyl group/ N-acetyl group)
A-1	2 000 000	×	0·072	ND[c]
A-2		×	0·370	ND[c]
B-1		○	0·171	1/34
B-2		○	0·004	1/67
b-1	20 000	△	0·032	1/37
b-2		△	0·083	1/33

[a]×, Insoluble; △, considerably soluble; ○, soluble at 10 mg ml^{-1}.
[b]Colorimetric analysis for fatty acid esters.
[c]Not determined.

RESULTS

Preparation of water soluble derivatives

As shown in Fig. 1, the reaction of sodium hyaluronate with palmitoyl chloride gave product A and/or a (HA-P and/or HA2-P). Product A and/or a (HA-P and/or HA2-P) contained an intramolecular acid anhydride (absorption at 1810cm^{-1}) together with an ester group (absorption at 1745cm^{-1}), shown on the IR spectrum. The solubility of product A and/or a (HAP and/or HA2-P) depended on the degree of esterification, and it was basically insoluble in water (Table 1). Product A and/or a (HA-P and/or HA2-P) was converted to product B and/or b (HA-P-Na and/or HA2-P-Na) by the moderate alkaline treatment shown in Fig. 1. The absorption at 1810cm^{-1} disappeared on the IR spectra of product B and/or b (HA-P-Na and/or HA2-P-Na). This indicated that the intramolecular acid anhydride groups were removed from product A and/or a. The absorption at 1745cm^{-1} remained, however, confirming the existences of ester groups. HA-P-Na and HA2-P-Na were analysed by protons derived from the palmitoyl group at 0·9 and 1·28 ppm. The proper degree of esterification, to be water-soluble, was about 1/33 below (palmitoyl group/N-acetyl group), as shown in Table 1.

The tolerance of HA- and HA2-coated liposomes to PL-D activity

Partial palmitoyl hyaluronates (product B and/or b (HA-P-Na and/or HA2-P-Na)) used in this study have a favorable water-solubility to apply as coating

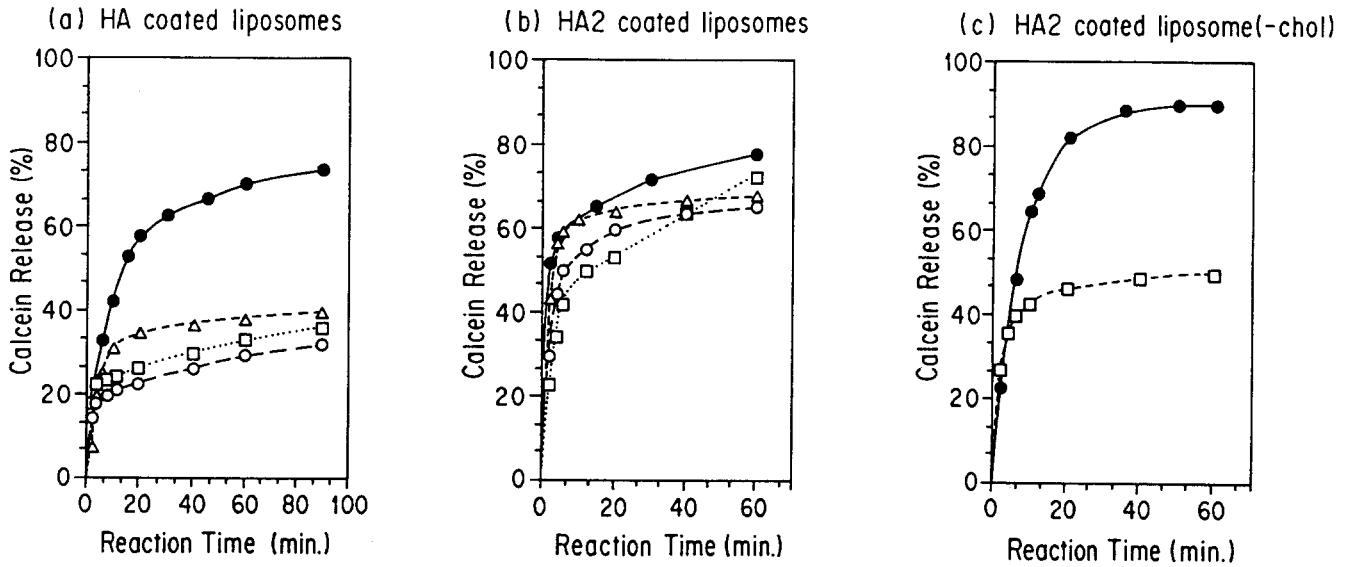

Fig. 2. Release of calcein from HA- and HA2-coated liposomes by reaction with PL-D. Each liposome solution (2 ml) and PL-D solution (23 units in 50 μl) was mixed and incubated at 25°C, and fluorescence was measured for the emission at 518 nm by excitation at 480 nm. The degree of calcein release was represented as the percentage of each fluorescent intensity measured to total intensity by an addition of 10% Triton X-100. ——●——, None coated; – – –△– – –, coated at 2 mg ml^{-1}; ·····□·····, coated at 5 mg ml^{-1}; — —○— —, coated at 10 mg ml^{-1} of HA derivatives, respectively.

agents for liposomes. Non-coated liposomes freely leaked calcein through the reaction with PL-D, and the level of released calcein reached to 75% of the enclosed total amount after 90 min. On the other hand, the calcein release from HA-coated liposomes occurred more slowly with an increase of HA-P-Na concentration and these levels after 90 min were 40%, 36% and 32% in coating at 2, 5, and 10 mg ml^{-1} respectively (Fig. 2*a*). Although the coating with HA2-P-Na could not scarcely supress the calcein release caused by PL-D (Fig. 2*b*), it reduced the releasing level in the cholesterol-free liposomes (Fig. 2*c*). The surface of the liposomes was surrounded by polysaccharide chains with anchor effects of palmitoyl chains into the bilayer, and obtained the tolerance to PL-D. The zeta potential of liposomes coated with HA-P-Na increased more than non-coated ones (Table 2).

DISCUSSION

It seems that the bulky polysaccharide chain of HA-P-Na can effectively surround the liposome surface. HA2-P-Na required more anchoring under cholesterol-free conditions for adequate surrounding. Cholesterol free liposomes coated with HA2-P-Na did not show an increase in the zeta potential. This suggests that the conformation like 'loop-train-tail' of polysaccharide chains on the liposomal surface are different between HA-P-Na and HA2-P-Na.

It could be considered that the composition of liposomes and the molecular size of HA should be selected in order to control the characteristics of HA on a liposomal surface. It is expected that the liposomes coated with these HA derivatives are available as a chemical sensor and a drug carrier and the liposomes may be useful in studying the function of cell surface hyaluronates.

Table 2. Zeta potentials of liposomes coated with hyaluronate derivatives

Additives[a]	pH 4[b]	pH 6[b]	pH 6[c]	pH 8[c]
None	−15·1	−22·6	—	−34·1
HA-P-Na	−26·6	−30·4	—	−48·1
HA2-P-Na	−17·6	−18·1	—	−29·6
HA2-P-Na(-Chol)	−22·3	−23·2	−24·9	−26·1
Stearyl amine	+24·8	+22·6	+28·4	+3·1
Dicetyl phosphate	−27·1	−33·8	−37·3	−39·0

[a]Molar ratio of lecithin/cholesterol is 1:1 except (HA2-P-Na(-Chol).
[b]M/10 Acetate-Na acetate buffer.
[c]M/15 KH_2PO_4-Na_2HPO_4 buffer.

REFERENCES

Clarris, B. J., Fraser, J. R. E. & Rodda, S. J. (1974). *Ann. Rheum. Dis.*, **33**, 240–2.

Cleland, R. L., Cleland, M. C., Lipsky, J. J. & Lyn, V. E. (1968). *J. Am. Chem. Soc.*, **90** (12 June) 3141.

Forrester, J. V. & Lackie, J. M. (1981). *J. Cell. Sci.*, **50**, 329–44.

Kobayashi, K. (1991). *Yukagagu*, **40**(5), 37–41.

Laurent, T. C., Ryan, M. & Pietruszkiewicz, A. (1960). *Biochim. Biophys. Acta*, **42**, 476.

Noro, T. & Ishii, F. (1984*a*). *J. Prac. Pharm.*, **35**(6), 53–9.

Noro, T. & Ishii, F. (1984*b*). *J. Prac. Pharm.*, **35**(7), 59–62.

Oku, N., Kendall, D. A. & Macdonald, R. C. (1982). *Biochim. Biophys. Acta*, **691**, 332–40.

Patterson, R. L., Patterson, D. A., Dienhardt, F. & Howard, F. (1975). *Proc. Soc. Exp. Bio. Med.*, **149**, 594–8.

Renkonen, O. (1961). *Biochim. Biophys. Acta*, **54**, 364.

Snyder, F. & Stephens, N. (1959). *Biochim. Biophys. Acta*, **34**, 244.

Sunamoto, J., Iwamoto, K. & Takeda, M. (1984). In *Polymers in Medicine*, ed. Emo Chiettini & Paolo Glusti. Plenum Publishing Corporation, New York, pp. 157–68.

Sunamoto, J. & Iwamoto, K. (1986). *CRC Critical Reviews in Therapeutic Drug Carrier Systems*, **2**(2) 117–36.

Sunamoto, J., Kondo, H. & Sato, T. (1986). *Rep. Asahi Glass Found. Ind. Technol.*, **48**, 231–9.