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Conformational changes of hyaluronates with partial palmitoylation and the adsorption structures on the surface of oil droplets

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The palmitoyl derivatives from hyaluronates, which were useful as new polysaccharide surfactants, were used to form complexes with toluidine blue (TB) in a similar manner to native hyaluronates. However, the binding levels of TB decreased because the anionic sites of hyaluronates might be shielded by partially introduced palmitoyl groups. Nevertheless, the induced Cotton effects were greatly increased for the palmitoyl derivatives compared with the native polysaccharide, and reflected the conformational change of the main polysaccharide chains with palmitoyl groups. It seems that this conformational change contributes to the adsorption behavior of the palmitoyl hyaluronates on the surface of oil droplets.

INTRODUCTION

We previously reported on the emulsification activity of polysaccharide surfactants derived from hyaluronates (Kawaguchi *et al.*, 1991). In strong emulsification by ultrasonic treatment, the partial palmitoyl derivatives of high molecular weight hyaluronates induced the coalescence and coagulation of oil droplets in the emulsion.

We also reported that partial palmitoyl derivatives of high molecular weight and low molecular weight hyaluronates might have a different adsorption structure on the surface of liposomes (Kawaguchi *et al.*, 1992).

Polysaccharide surfactants like our hyaluronate derivatives are unusual, and their adsorption structure on the surface of oil droplets has not been examined. Recently, some papers have described liposome coating with polysaccharide derivatives (Sunamoto & Iwamoto, 1986; Sunamoto *et al.*, 1986). It is assumed that polysaccharides on liposomal surfaces maintain 'a loop–train–tail conformation' (Kobayashi, 1991). However, it is difficult to clarify the adsorption structure, because these polysaccharides are nonionic and analysis of these conformations is difficult. We analyzed the conformational changes of our hyaluronate derivatives and the adsorption structure using the fact that hyaluronates interact with thiazine dye (e.g. toluidine blue) and induce Cotton effects.

MATERIALS AND METHODS

Preparation of partial palmitoyl hyaluronates

The hyaluronates used in this study were high molecular weight sodium hyaluronates (HA-NA : average mol. wt. above 2 000 000 and HA37-NA : average mol. wt. 370 000) and low molecular weight sodium hyaluronates (HA7·6-NA : average mol. wt. 76 000 and HA2-NA : average mol. wt. 20 000). The average molecular weight was estimated by the method of Laurent *et al.* (Laurent *et al.*, 1960; Cleland *et al.*, 1968). HA37-NA, HA7·6-NA, and HA2-NA were obtained by hydrolysis of HA-NA and were commercially available. The acylation, the change in of the degree of acylation, and

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the degradation of intramolecular acid anhydride were continuously carried out according to our previous papers (Kawaguchi *et al.*, 1991, 1992). HA-NA, HA37-NA, HA7·6-NA, and HA2-NA were respectively esterified with palmitoyl chloride in *N,N*-dimethylformamide in the presence of pyridine. HA-P, HA37-P, HA7·6-P, and HA2-P, which were water insoluble palmitoyl derivatives, were converted to water soluble derivatives (partial palmitoyl hyaluronates : HA-P-NA, HA37-P-NA, HA7·6-P-NA, and HA2-P-NA, respectively) through a moderate alkaline treatment. The degree of esterification of each derivative was determined by ^{1}H-NMR. Derivatives with substitution degrees of approximately 1/33 were used in this study.

Formation of complexes of partial palmitoyl derivatives with toluidine blue (Fig. 1)

It is known that native hyaluronates form complexes with toluidine blue (TB) and induce a metachromatic shift with an increase of TB binding in the absorption spectrum. Complexes were prepared according to the method described by Matsumura and Nakajima (1973). Aqueous solutions of derivatives and TB were prepared separately. The solutions were mixed together just before use. TB was recrystallized by the method of Pal and Schubert (1962) before use. The pH was checked with a glass electrode and was adjusted with a small amount of hydrochloric acid where necessary. The final concentration of TB was 1×10^{-4} M. The concentration of each derivative was given as the ratio of anionic sites of polymers to dye (*P/D*). Absorption spectra and circular dichroism (CD) were measured with a Shimadzu spectrophotometer (Shimadzu Corporation, Japan) and a JASCO spectropolarimeter J-600 (Japan Spectroscopic Co. Ltd, Japan), respectively.

Analysis of emulsification activities of derivatives for soybean oil

The emulsification activities of our derivatives were examined as described in our previous report (Kawaguchi *et al.*, 1991). Soybean oil was used in the oil phase at a final concentration of 5% (w/v). Each derivative was added in the water phase at a final concentration of 5 mg/ml as an emulsifier. Polyoxyethylene sorbitan monooleate was used at the same concentration as a reference. The emulsification was performed with shaking (amplitude 5 cm; 80 cycles/min). After emulsification, each emulsion was quickly divided and each aliquot was poured into a test-tube. The test-tubes were left to stand at room temperature. The water phase (3 ml) in each test-tube was carefully removed from the bottom, and its transmittance at 600 nm was measured as a function of time.

Adsorption of TB to the surfaces of the emulsion particles

Soybean emulsions with our derivatives (5%) were prepared by sonication. Soybean oil (0·25 g) was added to the HA derivatives solution (25 mg in 4·5 ml of distilled water), and emulsified by ultrasonic treatment (40 W, 2 min.). Next, TB solution ($1{\cdot}25 \times 10^{-2}$ M, 0·5 ml) was added to each emulsion, and the mixtures were stirred for 10 min at room temperature. The mixtures were ultracentrifuged at 30 000 rpm for 30 min with a Beckman ultracentrifugator Model L8-80M and rotor SW-60 (Beckman Instruments Inc., USA), and separated into oil and water phase. Part of the water phase (0·2 ml) was taken from the bottom of the centrifuge tubes and diluted 20-fold with distilled water. The absorption spectra were measured with a Shimadzu spectrophotometer UV-3100. The TB concentration of the water phase was compared in each emulsion to reflect the nonadsorbed level on the surface of the emulsion particles.

RESULTS

Formation of complexes with TB and induction of metachromatic shift

Native hyaluronates (HA) formed with TB and induced a metachromatic shift in the absorption spectrum.

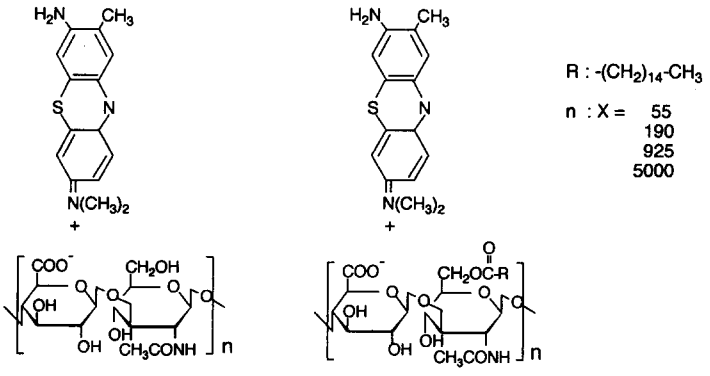

Fig. 1. The chemical structure of hyaluronate, esterified hyaluronate, and TB.

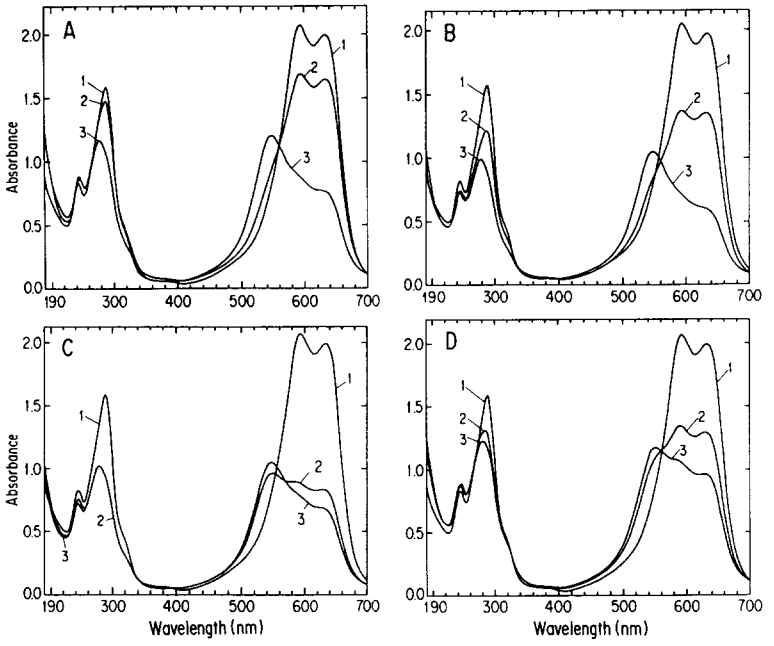

Fig. 2. Absorption spectra of complexes of various hyaluronates and these palmitoyl derivatives with toluidine blue. (A) HA; (B) HA37; (C) HA7·6; (D) HA2. 1, TB; 2, partial palmitoyl hyaluronates/TB complex; 3, hyaluronate/TB complex.

There was a suppression of the absorption maxima (spectrum 1 in Fig. 2) due to the free dye (alpha peak at 633 nm and beta peak at 593 nm) and an appearance of a metachromatic band (gamma peak at around 550 nm: spectrum 3 in Fig. 2). However, partial palmitoyl hyaluronates had a moderate metachromatic shift due to a small amount of binding dye (spectrum 2 in Fig. 2). These facts were found in hyaluronates (HA-NA, HA37-NA, HA7·6-NA and HA2-NA) of different molecular weights and their derivatives (HA-P-NA, HA37-P-NA, HA7·6-P-NA and HA2-P-NA).

Induced Cotton effects of complexes with TB

The Cotton effects induced in the CD, corresponding to the visual absorption maxima, are shown in Fig. 3. The amplitude of the induced Cotton effect of the HA37-P-NA/TB complex at 557 nm, was extremely large. HA-P-NA/TB and HA2-P-NA/TB complexes exhibited typical secondary effects, while those of HA7·6-P-NA/TB complexes were slightly atypical. All native hyaluronates (HA)/TB complexes exhibited atypical effects.

Emulsification activity of derivatives for soybean oil

Each partial palmitoyl hyaluronate could emulsify soybean oil with mild treatment by shaking (Fig. 4). It seems that the emulsion activity depends upon the molecular weight of the hyaluronates, and the derivative from low molecular weight hyaluronates has excellent activity and stability.

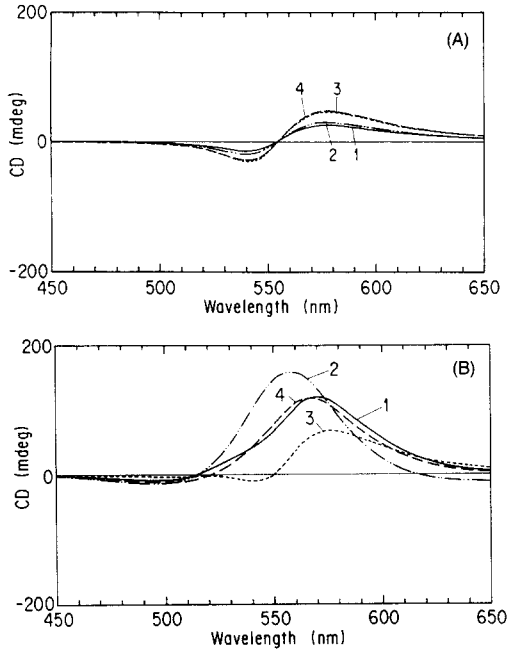

Fig. 3. CD spectra of the complexes of native hyaluronates and partial palmitoyl hyaluronates with TB. (A) Native hyaluronates: 1, HA-NA; 2, HA37-NA; 3, HA7·6-NA; 4, HA2-NA. (B) Partial palmitoyl hyaluronates: 1, HA-P-NA; 2, HA37-P-NA; 3, HA7·6-P-NA; 4, HA2-P-NA.

Difference of absorption of TB on surfaces of emulsion particles

The emulsification by ultrasonication was employed for the purpose of maximizing the surface area of the emulsion particles. However, excess ultrasonic treat-

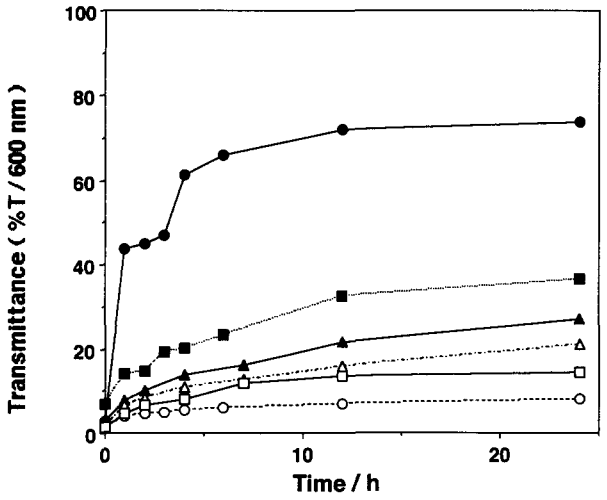

Fig. 4. The emulsion activity for 5% soybean oil in water. ●, none; ■, POE sorbitan monoileate; ▲, HA-P-NA; △, HA37-P-NA; □, HA7·6-P-NA; ○, HA2-P-NA.

ment (above 3 min at 40 W) was avoided as it caused the coalescence and coagulation of oil droplets, as shown in our previous paper (Kawaguchi *et al.*, 1991).

The absorption spectra of the water phase containing unadsorbed TB are shown in Fig. 5. Emulsion particles prepared by high molecular weight derivatives could bind the greater part of TB, although those prepared by low molecular weight derivatives (HA2-P-NA) could not. Blank 1 refers to the case of TB only, and Blank 2 refers to the case of the absence of HA derivatives. TB was not transported to soybean oil in Blank 2.

There are no HA derivatives to be isolated in the water phase, because no significant gamma peak (at around 550 nm) showing the formation of a complex with TB was detected on spectra 3, 4, 5, or 6 in Fig. 5.

DISCUSSION

Our HA derivatives are useful as new polysaccharide surfactants (Kawaguchi *et al.*, 1991). However, the

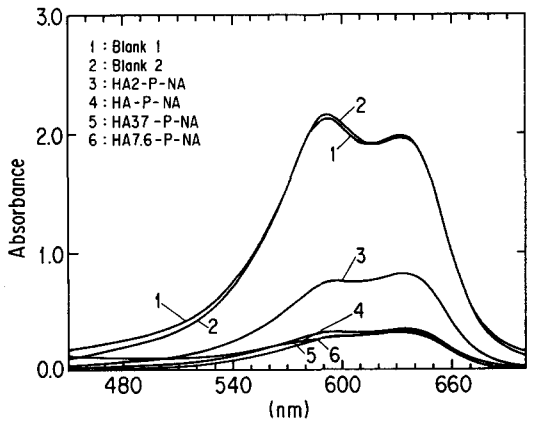

Fig. 5. The adsorption of TB to the surface of emulsion particles prepared with hyaluronate derivatives.

adsorption form of these giant molecules on oil droplets has not yet been examined. We have studied the change in the secondary structure of the HA chain with palmitoylation, and examined the relation between the conformation in water and the adsorption movement on oil droplets.

It is known that hyaluronates form complexes with TB and induce a metachromatic shift in the absorption spectrum with increase in TB binding (Matsumura & Nakajima, 1973). We also examined the appearance of the metachromatic shift with the formation of palmitoyl hyalruronate/TB complexes. The appearance of a gamma peak, to show multicomplexes of TB, was suppressed with palmitoylation. The spectra of Fig. 2(A)-2 and Fig. 2(B)-2 show that the levels of the gamma peak are small in the case of palmitoylated derivatives (HA-P-NA and HA37-P-NA) from high molecular weight hyaluronates, which can bind more TB levels than low molecular weight hyaluronates. This fact indicates that the binding level of TB on palmitoyl hyaluronate molecules decreases due to stereochemical obstruction with a long acyl chain.

Matsumura *et al.* noted that their complexes showed induced Cotton effects (Matsumura & Nakajima, 1973). We found that palmitoyl hyaluronate/TB complexes showed more significant and typical Cotton effects than native hyaluronate/TB complexes in the CD spectrum of Fig. 3(A) and (B). This fact indicates that the introduction of a palmitoyl group brings on conformational changes of polysaccharide chains. The 'twisting' of polysaccharide chains with palmitoylation induces 'swaying' of the sequence of TB on these chains, resulting in increased chirality.

On this occasion, the degrees of the appearance of the gamma peak in the spectra of Fig. 2(A)-2 and Fig. 2(B)-2 were small in large molecular weight palmitoyl derivatives such as HA-P-NA and HA37-P-NA. However, the enlargement of 'twisting' construction with palmitoylation induced the increase in chirality, although the binding levels of TB are small. Consequently, the HA37-P-NA/TB complex exhibited the most significant Cotton effects. It is probable that HA37-P-NA may assume the form of polymer micelles developed from random coils, as in the model of the micellar structure of water-soluble polymers described by Bader *et al.* (1984). On the other hand, because HA-P-NA has an enormous polysaccharide chain, it seems that any palmitoyl chains partially introduced cannot sufficiently effect the overall conformation of the polysaccharide. Therefore, the induced Cotton effects of the HA-P-NA/TB complex are smaller and more atypical than those of the HA37-P-NA/TB complex.

The overall conformation change may be small in HA7·6-P-NA, having a low molecular weight polysaccharide chain. Nevertheless, HA2-P-NA has a low molecular weight polysaccharide chain, and the amplitude of the Cotton effects for the HA2-P-NA/TB

complex increased. It seems that the closing complexes may be interacting. It is considered that the hydrophobic interaction occurs among complexes binding TB, because HA2-P-NA has only one-half of the palmitoyl chain in one hyaluronate molecule and TB binding on the anionic site is not obstructed.

As we showed previously (Kawaguchi *et al.*, 1991), these partial palmitoyl hyaluronates had emulsification activities and a tendency for more favorable emulsification with decreasing molecular weight of polysaccharide chains. At the same time, it was found that coagulation occurred in these emulsions when a large molecular weight derivative was treated to excess emulsification. These facts may indicate that the conformation of polysaccharide chains on oil droplets is closely related to the emulsification stability.

The degree of TB binding on the surface of emulsion particles prepared by various partial palmitoyl hyaluronates was compared. It is shown in Fig. 5 that the TB binding level immediately decreases in HA2-P-NA, having a polysaccharide chain with an average molecular weight of 20 000. This supports the view that differences in the efficiency of levels of anionic sites for binding TB exist among hyaluronate derivatives having polysaccharide chains of different molecular weights, as we reported for liposomal surfaces (Kawaguchi *et al.*, 1992). We consider that a significant length of polysaccharide chains may be required for the constitution of 'the loop-train-tail conformation' in the external water-phase. The introduction of palmitoyl chains to hyaluronates brings the conformational change in water according to the polysaccharide chain length. This conformational change may affect the absorption structure and the stability on the surface of the emulsion particles.

The relationship between these conformational changes and the adsorption behavior on oil droplets should be investigated in the future. A combination of palmitoyl groups and hyaluronates having a molecular weight of 370 000 and offering the polymer micellar form of the derivative in water was required for the formation of 'the loop-train-tail conformation' on the surface of the emulsion particles. The total balance of large molecules such as our derivatives may be regulated with changes in the length of the acyl chains and polysaccharide chains, and this regulation may be different from the theory of the hydrophile-lipophile balance (HLB) in ordinary surface-active agents.

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