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The relation between the adsorption behavior at the interface and the conformational changes in hyaluronates partially modified with various acyl chains

Yoshihiro Kawaguchi[a*], **Kuraya Matsukawa**[a] & **Yutaka Ishigami**[b]

[a]*Ishihara Chemical Co., Ltd., Nishi-Yanagihara-Cho, Hyogo-Ku, Kobe, Hyogo 652, Japan*
[b]*National Institute of Materials and Chemical Research, Higashi Tsukuba-City, Ibaraki 305, Japan*

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Partially acylated hyaluronates, respectively having a different acyl chain and polysaccharide chain, were prepared as water-soluble derivatives with substitution degrees of approximately 1/33. Conformational changes of polysaccharide chains with partial acylation were studied on the CD spectrum of the induced cotton effects of the derivatives–thiazine dye complexes. Significant cotton effects were observed in derivatives having the combination of hyaluronate molecular weight from 70,000 to 2,000,000 and a lauroyl or palmitoyl chain as the acetyl group. HA37-P, particularly, which introduced palmitoyl chains for hyaluronates of the molecular weight 370,000, exhibited typical cotton effects, and the kinetic viscosity of HA37-P solution lowered compared with that of unmodified HA37. These acyl derivatives exhibited significant conformational changes on the CD spectrum, and lowered the interfacial tension of *n*-octane to water. It was confirmed that HA37-P in particular lowers the interfacial tension by adsorbing oil droplets and contributes to improve the membrane fluidity by the fluorescent probe method. The formation of exiplexes under oil-free conditions suggested that the partial acyl hyaluronates having a hydrophobic–hydrophilic combination such as HA37-P might form a micellar polymer in water by themselves.

INTRODUCTION

Partial palmitoyl hyaluronates as we previously reported (Kawaguchi *et al.*, 1991) act as polysaccharide surfactants and adhere to the surface of oil droplets with significant conformational changes (Kawaguchi *et al.*, 1993). We also proposed that partial palmitoyl hyaluronates might have a different adsorption structure on the surface of liposomes corresponding to the length of the polysaccharide chain (Kawaguchi *et al.*, 1992).

It is generally expected that the form of polymer chains adsorbed at the interfaces is considerably different from the form in solution. There have been many studies elucidating the conformation of linear polyelectrolyte chains adsorbed at the interfaces using various experimental techniques (Kawaguchi *et al.*, 1987). However, the conformation of a single polyelectrolyte chain has not yet been elucidated.

In elucidating the adsorption form and behavior, our present object is the hydrophilic–hydrophobic polymer partially acylated to hyaluronates. It is known that native hyaluronates have an entangled form in water at an adequate concentration; however, no detailed analysis concerning the conformation of hyaluronate alone in water has ever been successful. We have already noted that the partial introduction of palmitoyl chains resulted in adsorption on the surface of oil droplets. It is expected that any conformational restriction of hyaluronates with partial palmitoylation will lead to this adsorption behavior. In our studies, it is convenient that hyaluronates have highly free conformation in water and that these conformational changes may be monitored by measurement of the induced cotton effects applying the binding property to basic dyes such as toluidine blue (TB).

In this study, we tried to confirm the conformational changes following acylation in addition to palmitoylation. These conformational changes exhibited signs of the induced cotton effects of HA derivative–TB complex on CD spectrum. Moreover, we attempted

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*To whom correspondence should be addressed.

analysis of the adsorption behavior dependent on the conformational changes in addition to the adsorption form on the oil droplets. The adsorption behaviour was examined by comparing the conformational changes with the ability to decrease interfacial tension measured using the spinning-drop method. The form of polymers at the interface was also studied by using rhamnolipid B-pyrenacyl ester (RB–PE) as the fluorescence probe (Suzuki *et al.*, 1989).

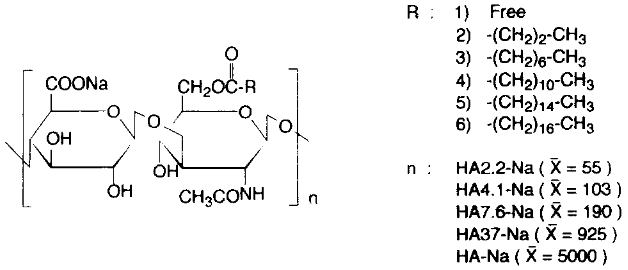
MATERIALS AND METHODS

Preparation of partially acylated hyaluronates

The partially acylated hyaluronates (Fig. 1) were prepared according to the method previously described (Kawaguchi *et al.*, 1991, 1992, 1993). The hyaluronates used in this study were high molecular weight sodium hyaluronates (HA–NA: average mol. wt above 2,000,000), medium molecular weight sodium hyaluronates (HA37-NA: average mol. wt 370,000 and HA7·6-NA: average mol. wt 76,000) and low molecular weight sodium hyaluronates (HA4·1-NA: average mol. wt 41,000 and HA2·2-NA: average mol. wt 22,000). HA37-NA, NA7·6-NA, HA4·1-NA and HA2·2-NA were prepared by hydrolysis of commercially available HA–NA, and these average molecular weights were estimated by the method of Laurent *et al.* (1960). The water-soluble partially acetylated hyaluronates were prepared through acylation and subsequent modulation of the acylation degree. The partially acylated hyaluronates prepared in this study were derivatives induced with substitution degrees of approximately 1/33 of butyroyl, octanoyl, lauroyl, palmitoyl and stearoyl esters.

Analysis of induced cotton effects on CD spectrum

The induced cotton effects of each partially acylated hyaluronate–TB complex were studied as previously reported. CD spectra were measured with a JASCO spectropolarimeter J-600 (Japan Spectroscopic Co., Ltd., Japan). The partially acylated hyaluronate–TB complexes were prepared according to the method described by Matsumura & Nakajima (1973). TB was recrystallized by the method of Pal and Schubert (1962) before use. Aqueous solutions of our derivatives and TB were prepared separately. The solutions were mixed together just before use. The final concentration of TB was 1×10^{-4} M. The concentration of each derivative was given as the equivalent ratio of anionic site of polymers to dye (P/D).



R : 1) Free
2) $-(CH_2)_2-CH_3$
3) $-(CH_2)_6-CH_3$
4) $-(CH_2)_{10}-CH_3$
5) $-(CH_2)_{14}-CH_3$
6) $-(CH_2)_{16}-CH_3$

n : HA2.2-Na ($\bar{X} = 55$)
HA4.1-Na ($\bar{X} = 103$)
HA7.6-Na ($\bar{X} = 190$)
HA37-Na ($\bar{X} = 925$)
HA-Na ($\bar{X} = 5000$)

Fig. 1. The acyl derivatives from sodium hyaluronates.

Determination of interfacial tensions

The interfacial tensions of *n*-octane to water solutions of partially acylated hyaluronates (20 mg/ml) were determined by the spinning drop method (Vonnegut, 1942; CID Japanese Committee, 1977). The vertical diameter (d: 10^{-2} cm) of the oil droplet for the rotary axis was measured, when it reached about one-third of horizontal diameter under adequate rotary speed (P: ms/rev) on the spinning drop type interfacial tensiometer Model 500 (Core Laboratories Co.). The difference in density (p: g/cc) between water and *n*-octane was estimated to be 0·297. The interfacial tension (∂: dyne/cm) was calculated by the following formula:

$$\partial = 0{\cdot}522 \times 106 \times p^3/P^2.$$

Measurement of kinetic viscosity

The kinetic viscosities of hyaluronates and their derivative solutions were measured with Ubbelohde's viscometer at 20°C. Each test solution was prepared at a concentration of 2×10^{-4} M in distilled water.

Analysis by the fluorescent probe method

To study the form of our hyaluronate derivatives in water and the adsorption behavior on oil droplets, the existence of the excimer formation was investigated with rhamnolipid B–pyrenacyl ester (RB–PE) as the fluorescent probe. RB–PE was previously dispersed in 1% hyaluronate derivative solution by ultrasonication (40 W, 2 min), and prepared at a final concentration of 1×10^{-5} M with no oil condition. On the other hand, 0·5 and 1·0% (w/v) emulsions of *n*-octane in 1% hyaluronate derivatives solution were prepared by ultra-sonication (40 W, 3 min), and RB–PE was also dispersed in these emulsions to a final concentration of 1×10^{-5} M. Each preparation was excited at 350 nm and the fluorescent spectrum was measured from 350 to 600 nm.

RESULTS

Induced cotton effect of each acyl hyaluronate complex with TB

The CD spectrum of various complexes are shown in Fig. 2(a), (b), (c), (d) and (e). The largest amplitude of the induced cotton effects in HA2·2 derivatives occurred with palmitoylation as shown in Fig. 2(a), and the cotton effect exhibited at 567 nm was unsatisfactory to be typi-

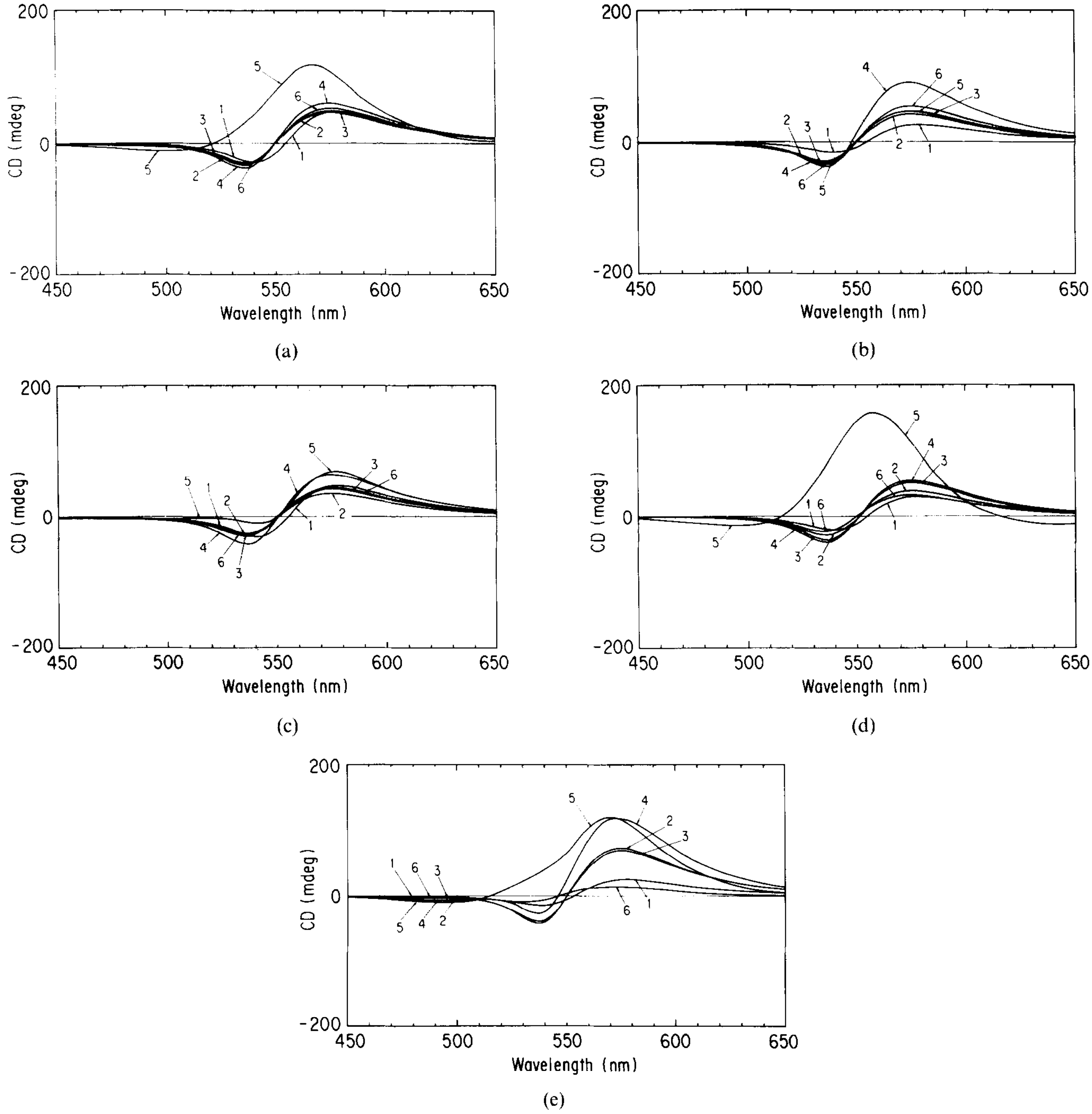


Fig. 2. CD spectra of the complexes of various hyaluronates and their acyl derivatives with TB. (a) HA2·2 and derivatives; (b) HA4·1 and derivatives; HA7·6 and derivatives, (d) HA37 and derivatives, (e) HA and derivatives; respectively 1: free; 2: butyroyl; 3: octanoyl; 4: lauroyl; 5: palmitoyl; 6: stearoyl.

cal. In HA4·1 derivatives, the induced cotton effect with palmitoylation was at 573 nm and was not typical, and the amplitude decreased significantly as shown in Fig. 2(b). HA7·6 derivatives did not cause any distinct induced cotton effects. Lauroyl and palmitoyl HA7·6 exhibited only a slight tendency of amplitude increase (Fig. 2(c)). Palmitoyl HA37 derivative exhibited the most typical induced cotton effects at 556 nm in all HA derivatives, as shown in Fig. 2(d). In HA and the acyl derivatives, the amplitude of the induced cotton effects increased gradually in the order butyloyl, octanoyl, lauroyl and palmitoyl derivatives (Fig. 2(e)). The cotton effects with lauroyl and palmitoyl HA, exhibited at 572 and 570 nm, respectively, were very typical.

The decreasing effects of the interfacial tension of *n*-octane to water

The interfacial tension of *n*-octane to water (ITO: 51·68 mN/m at 20°C), was affected by various acyl hyaluronates as shown in Figs 3 and 4. It was observed that hyaluronate derivatives generally caused ITO to decrease. The introduction of rather short acyl chains was even more effective for decreasing ITO. It was shown that there is a tendency for ITO to rise with elongation of the acyl chain length. However, hyaluronates of medium molecular weight (HA7·6 and HA37) again caused ITO to decrease with palmitoylation (Fig. 3). The relation between ITO and the molecular weight of HA was examined for various palmitoyl derivatives (Fig. 4). The combination of acyl and polysaccharide chains in the molecule of hyaluronate derivatives which decrease ITO, is similar to that of the acyl hyaluronates showing remarkable cotton effects.

Decline of kinetic viscosity with palmitoylation

The constant value (*K*) of Ubbelohde's viscometer was estimated by assuming the kinetic viscosity of water to be 1·0038 at 20°C. In palmitoyl derivatives of high molecular weight hyaluronates, a remarkable change of kinetic viscosity was observed in comparison with the original hyaluronates. The palmitoyl HA37 solution, which exhibited the most typical induced cotton effects, had a kinetic viscosity of 237 cSt. In contrast, HA37 solution consisting of unesterified hyaluronates had a kinetic viscosity of 413 cSt.

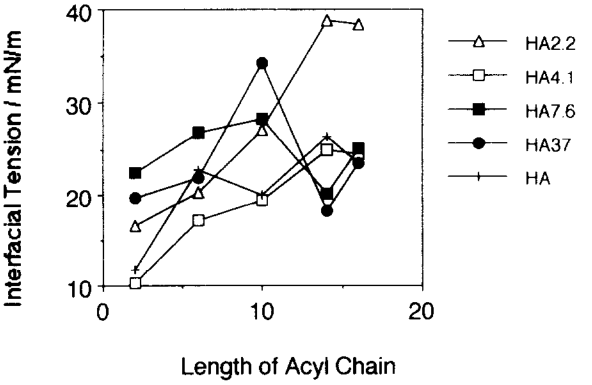


Fig. 3. The relation between interfacial tension of *n*-octane and the chain length of various palmitoyl hyaluronates.

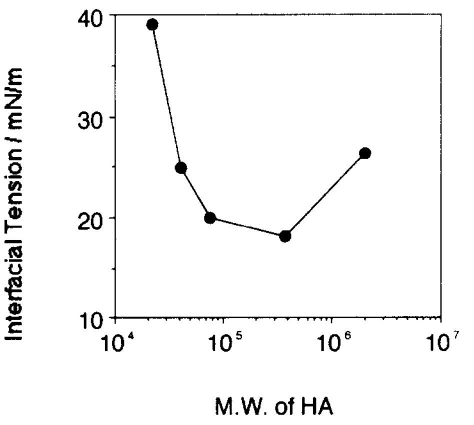


Fig. 4. The relation between interfacial tension of *n*-octane and the molecular weight of various palmitoyl hyaluronates.

Fluorescent analysis on the adsorption behavior of acyl hyaluronates on oil droplets

RB–PE (Fig. 5) used as fluorescent probe, exhibited a radiation spectrum indicating the excimer formation on the adsorption layer of acyl hyaluronates on oil droplets. Figure 6 shows that the radiation (at 385 nm) of RB–PE monomer disappears and excimer radiation (excimer-1 at 510 nm and excimer-2 at 455 nm) appears in the emulsion with palmitoyl hyaluronates (HA37-P). Excimer-1 was detected in the cases of no oil or limited oil in excess of surfactants. The appearance of typical excimer-1 is shown in Fig. 6. Excimer-1 in the case of no oil was remarkably exhibited in the emulsion with HA37-P, followed by palmitoyl HA7·6 (HA7·6-P). Along with the addition of *n*-octane, excimer-1 shifted to excimer-2 indicating that the membrane fluidity of emulsion particles was being enhanced.

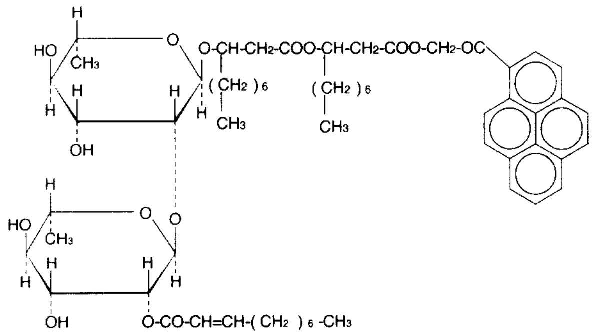


Fig. 5. The chemical structure of rhamnolipid B-pyrenacyl ester (RB–PE).

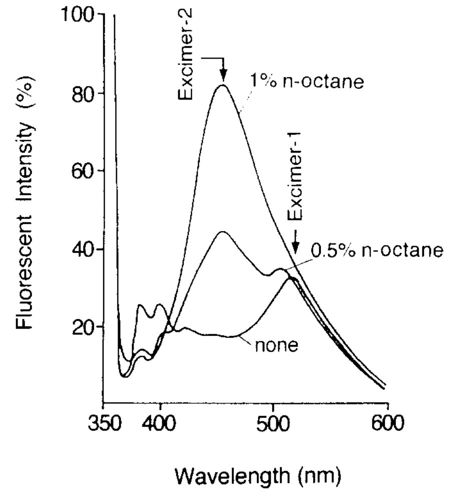


Fig. 6. The relation between the emulsion stability and the membrane fluidity within the surface adsorbed layer of the emulsion particle prepared by HA37-P-NA.

DISCUSSION

We previously described that polysaccharide surfactants were induced to be water-soluble from hyaluronates by partial palmitoylation (Kawaguchi *et al.*, 1991, 1993). These partial palmitoyl hyaluronates have a conformational variation depending on the length of the main polysaccharide chain, and function in emulsification and in liposome coating (Kawaguchi *et al.*, 1992). These conformational changes were confirmed by analysis of the induced cotton effect using TB. Therefore, it should be considered that the hydrophobic–hydrophilic combination in the giant molecule like a series of acyl hyaluronates might have influence on the behavior at the interface. We studied the induced cotton effects with the acyl hyaluronates having various hydrophobic–hydrophilic combinations. As shown in a series of CD spectra, the conformational changes were observed in the appropriate combination of the length of polysaccharide chains and acyl chains. The appropriate range of the molecular weight of polysaccharide chains was broad, above 70,000 and below 2,000,000. Lauroyl and palmitoyl groups were suitable with respect to acyl chains. In order to cause conformational changes of the polysaccharide chain with acylation, the acyl chains sufficient number and length must be introduced to a hyaluronate molecule.

The bonding sites of TB generally increase with increase of the molecular weight of hyaluronates. It is thought that TB would be attracted to the hydrophobic area near the acyl chains. It is estimated that the TB bonding level of lauroyl and palmitoyl HA is more than that of palmitoyl HA37, however, the induced cotton effects in palmitoyl HA37 are more remarkable than those in the others. This suggests that partial conformational changes of the HA37 molecule may effectively occur at every introduction of a palmitoyl chain, and effectively accumulate and grow to be enormous. This conformational change might account for the kinetic viscosity of palmitoyl HA37 being lower than that of HA37.

Thus it is thought that palmitoyl HA37 has a conformation in water similar to micellar polymers as suggested by Bader *et al.* (1984). Figure 7 shows the scheme of the conformation of our derivatives in the presence and absence of oil in water. The hyaluronates with partial acylation may form micellar polymers and these conformations may be involved in the adsorption behavior at the interface. As shown in Figs 3 and 4, the interfacial tension of *n*-octane (ITO) was reduced by the palmitoyl derivatives which have significant conformational changes. Palmitoyl HA2·2 did not necessarily lower ITO, though it exhibited remarkable induced cotton effects. This probably means that palmitoyl HA2 does not form the micellar polymer in the molecule itself, and the remarkable cotton effects may depend on the interaction between several molecules. The fact that palmitoyl HA (HA-P) did not lower ITO compared with palmitoyl HA7·6 (HA7·6-P), suggests that the HA-P molecule is too enormous to form the micellar polymer though HA-P has the conformational change reflected on the CD spectrum. RB–PE as fluorescent probe formed exiplexes as exhibited by excimer-1 on the micellar polymer of palmitoyl HA37. The formation of excimer-2 resulted in pyrenacyl groups of RB–PE forming the optically excited dimer by arranging face-to-face in the interfacial adsorption layer. Excimer-2 was distinctly formed in the emulsification of *n*-octane. This suggests that excimer-1 shows the micellar polymer to assume the form of random coils in water in no oil conditions, and that excimer-2 shows the micellar polymer to form the loop–train–tail type conformation on oil droplets similar to that on liposomes (Ishigami *et al.*, 1991). It is presumed that intramolecular acyl chains of the micellar polymer link to produce weak hydrophobic surroundings because these acyl chains cannot anchor in oil phase under such conditions that the polymer micellar assumes the form of random coils in water in the absence of oil. Consequently, it is considered that RB–PE disperses irregularly in the weak hydrophobic surroundings and forms the exiplex which fluoresces in the long wavelength range. It is presumed that the

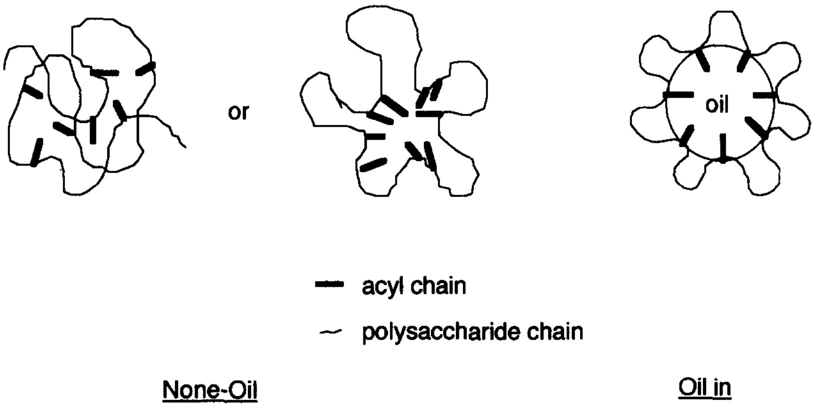


Fig. 7. Conformation of acyl hyaluronates in water and in emulsion.

formation of the exiplex depends on the behavior and the molecular size of polysaccharide chains conformationally swaying in water.

We believe our studies on partial acyl hyaluronates will contribute to designing a useful molecule in the chemical modification of biopolymers like polysaccharides. Because the conformational characteristics of polymers may be altered by chemical modification, the selection of polymer size is important to create various functional polymers derived from nature and it is also necessary to produce artificial cell walls in the biomimetic chemistry for clarifying vital reactions.

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