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Disintegration by surfactants of egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin

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Disintegration by surfactants of egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin was investigated by measuring the amount released of a marker dye from the vesicles. In solutions of pH around 7, anionic and nonionic surfactants caused vesicle disintegration at very low concentrations, while cationic surfactants produced a breakdown of the vesicles at rather high concentrations. Increase in the alkyl chain-length of surfactant molecules brought about decrease in the surfactant concentration at which vesicle disintegration starts. As the length of the polyoxyethylene chain in nonionic surfactant molecules increased, the tendency of vesicle disintegration to occur decreased. Both anionic and cationic surfactants gave clear solutions above their critical micelle concentrations when they acted on the phospholipid vesicles, whereas nonionic surfactants left ghost cell-like debris consisting of carboxymethylchitin molecules in their micellar solutions. The effect of pH on vesicle disintegration was notable for ionic surfactants but not for nonionic surfactants. Thus, anionic surfactants increased the degree of disintegration as pH increased, while cationic surfactants produced an identical vesicle disintegration curve below pH 8 above which the curve started to shift toward the lower concentration region of the agents. These findings were explained in terms of surfactant penetration into phospholipid bilayers and solubilization of phospholipid molecules by surfactant micelles.

Introduction

The preparation of vesicle membranes of enhanced stability is an important objective of current vesicle research. In order to achieve this goal, two major approaches have so far been proposed. One is to prepare polymerized phospholipid or surfactant vesicles. For instance, Ringsdorf and co-workers have succeeded in producing polymerized vesicles that keep a nearly spherical shape, even under the harsh dehydration and evacuation procedures needed in the preparation of samples in electron microscopy [1–6]. Another is made through the combined use of polymer and lipid. Seki and Tirrell, for example, have reported that poly(acrylic acid), poly(methacrylic acid) and poly(α-ethylacrylic acid) can be used to modify the properties of phospholipid vesicle membranes [7]. Some other works in this line are found in the literature [8–11].

On the other hand, we have succeeded in preparing egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin, a derivative of chitin which is poly(*N*-acetyl-D-glucosamine)

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Abbreviations: SDS, sodium 1-dodecyl sulfate; SOS, sodium 1-octyl sulfate.

[12]. Electron microscopic observations on the phospholipid vesicles have suggested that carboxymethylchitin molecules form a mesh-like structure over the entire vesicle surface [13]. Although increased stability is expected to result from a complexation of the phospholipid bilayer with the polymer, the detailed mechanism of the complexation and the structure of the vesicle membrane are not known.

This paper describes the interaction betwen egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin and surfactants. This sort of information would be useful in elucidating the formation and stability of the vesicle membranes.

Materials and Methods

Chemicals. Purified egg yolk phosphatidylcholine was a gift from Asahi Kasei Kogyo Co. (Tokyo). Carboxymethylchitin was obtained by treating chitin with sodium monochloroacetate as before [12]. Bromthymol blue, sodium 1-dodecyl sulfate (SDS), sodium 1-octyl sulfate (SOS), 1-dodecylpyridinium chloride (DPC) and 1-hexadecylpyridinium chloride (HPC) were purchased from Tokyo Kasei Kogyo Co. (Tokyo). Nonaoxyethylene 1-dodecyl ether (BL-9), polyoxyethylene 1-dodecyl ether (BL-21) and polyoxyethylene 1-hexadecyl ether (BC-15SS0) were gifts from Nikko Chemicals Co. (Tokyo). All of these surfactants were used without further purification. Dichloromethane was employed in this work as the solvent for the lipid. All other chemicals used were of reagent grade.

Vesicle preparation. Unilamellar vesicles [13] were prepared by using a two-step emulsification technique as described in the previous paper [13]. 20 ml of an aqueous saturated solution of Bromthymol blue, a marker, was dispersed as fine droplets in 20 ml of an egg yolk phosphatidylcholine solution in dichloromethane (5%) to yield a water-in-oil emulsion. The emulsion obtained was then dispersed in 200 ml of carboxymethylchitin solution in water (0.2%) to give a water-in-oil-in-water complex emulsion. After stirring the complex emulsion for 20 min, 200 ml of the aqueous carboxymethylchitin solution was poured into the emulsion under stirring to make the dispersed droplets much finer. Stirring was further continued until dichloromethane was completely evaporated-out at room temperature. The aqueous vesicle dispersion thus obtained was centrifuged to collect egg yolk phosphatidylcholine vesicles stabilized with carboxymethylchitin. The collected phospholipid vesicles (mean diameter 0.4 μm) were washed on the centrifuge with a phosphate buffer solution (pH 7.6, ionic strength 0.154) and redispersed in the same medium. The phospholipid concentration in a 1% (v/v) suspension of the vesicles was found to be 0.1 μg/ml as phosphorus content.

Determination of marker release. The degree of vesicle disintegration by surfactants was estimated by the amount released of Bromthymol blue. The particle concentration of vesicle suspension was adjusted to be 1.0% (v/v) by the addition of the buffer solution. All surfactants were dissolved in the same buffer solution at various concentrations. 3 ml each of surfactant solutions was added to the equal volume of the vesicle suspension. After the mixtures were incubated for 1 h at 37°C, they were immediately centrifuged at $25\,000 \times g$ to remove the undisintegrated vesicles, and 4 ml each of the supernatants was pipetted-off and the concentration of the marker in it was determined spectrophotometrically at 615 nm.

The degree of vesicle disintegration was expressed in terms of the amount of Bromthymol blue released from the vesicles as follows:

$$\text{Degree of disintegration}\,(\%) = (B - B_0)/(B_t - B_0) \times 100$$

where B_0 is the marker concentration in the control supernatant of the vesicle suspension in the absence of surfactant (the release of the marker after incubation was always less than 5%), B_t the total marker concentration in the supernatant obtained after treating the vesicle suspension with concentrated surfactant solution, and B the marker concentration in the sample supernatant in the presence of surfactant. It is evident that the degree of marker release corresponds to the degree of vesicle disintegration.

The leakage of the marker from the vesicles was also examined at different pH in the absence and presence of surfactant in the same manner as above. The buffer solutions used were citrate (pH 3.8), phosphate (pH 5.4, 6.5 and 7.6), Tris-HCl

(pH 7.6 and 8.6) and carbonate (pH 9.6 and 10.4) buffer solutions. The ionic strength of these buffer solutions was kept at 0.154 by the addition of NaCl.

Zeta-potential measurements. Zeta potential of the vesicles was measured at room temperature by a Laser Zee Meter 500 (Pen-Kem Inc., U.S.A.), a microelectrophoresis apparatus equipped with a cubic prism which rotates at a speed controlled by the operator to keep the image of the moving particle stationary. The particle concentration of vesicle suspension was adjusted to 0.1% (v/v) with a phosphate buffer solution (pH 7.6, ionic strength 0.0154). 10 ml each of solutions of surfactants at different concentrations in the buffer was added to the equal volume of the vesicle suspension. After the mixtures were allowed to stand for 1 h at room temperature, they were used as samples for zeta-potential measurement.

Experimental Results

Fig. 1 shows the degree of vesicle disintegration estimated from the amount leaked of Bromthymol blue as a function of surfactant concentration in the phosphate buffer (pH 7.6, ionic strength 0.154). SDS and BL-9 caused the leakage of the marker at very low concentrations, while 1-dodecylpyridinium chloride produced the marker release at concentrations much higher than those for the anionic and nonionic surfactants. As the three surfactants have an alkyl chain of the same length in their molecules, this result indicates that the sign of electric charge on the hydrophilic headgroup of surfactant affects vesicle disintegration to a great extent.

At concentrations higher than the critical micelle concentrations for SDS and 1-dodecylpyridinium chloride (0.63 and 5.0 mM, respectively), the release of the marker was complete and the resultant solutions were visually transparent. In the case of BL-9, even the mixtures prepared using micellular solutions were slightly turbid and ghost cell-like debris were observed, microscopically, to remain.

The alkyl chain-length of surfactant was found to have a remarkable effect on the phenomenon of vesicle disintegration. A typical example is shown in Fig. 2 where the leakage of the marker is seen to start and become complete at much lower concentrations for 1-hexadecylpyridinium chloride

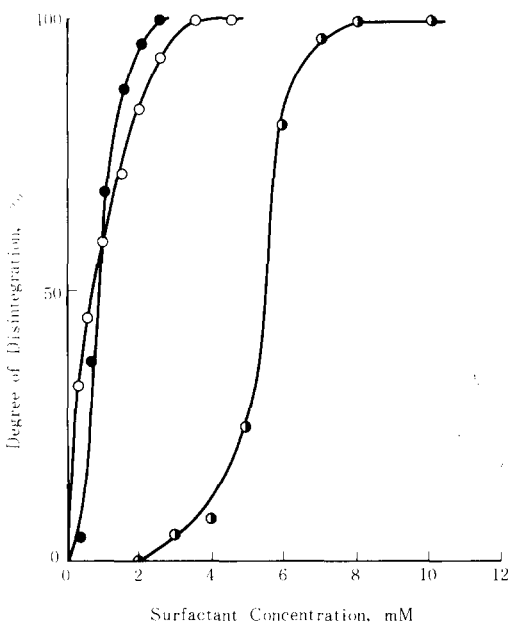

Fig. 1. Disintegration curves for the phosphatidylcholine vesicles in solutions of different surfactants at pH 7.6. Surfactant: SDS (○); 1-dodecylpyridinium chloride (◑); BL-9 (●).

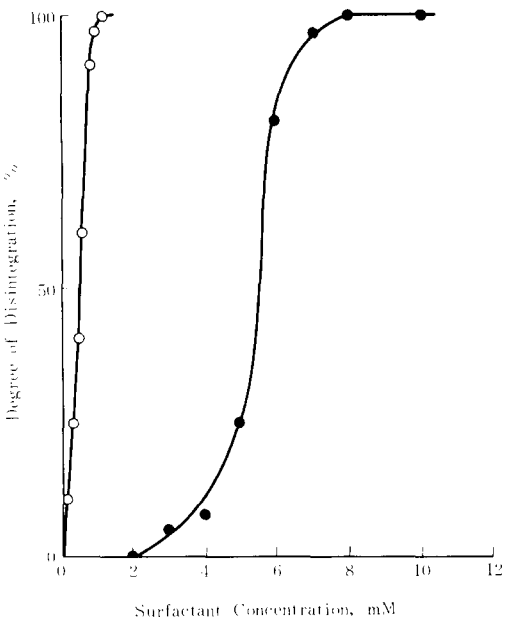

Fig. 2. Disintegration curves for the phosphatidylcholine vesicles in solutions of cationic surfactants at pH 7.6. Cationic surfactant: 1-hexadecylpyridinium chloride (○); 1-dodecylpyridinium chloride (●).

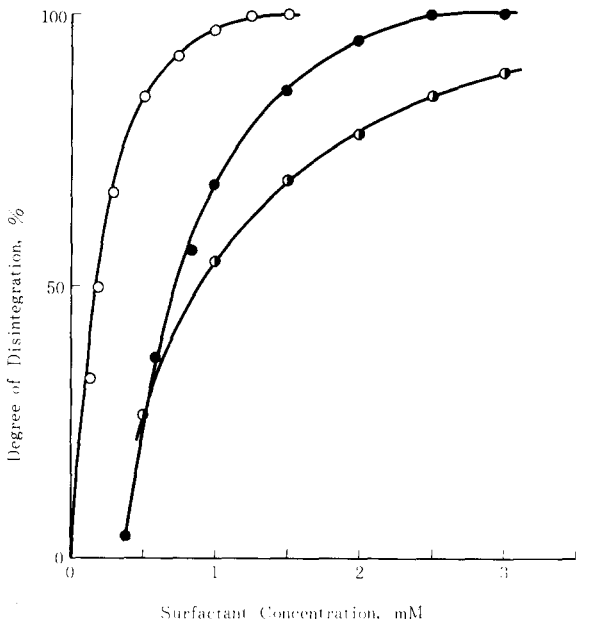

Fig. 3. Disintegration curves for the phosphatidylcholine vesicles in solutions of nonionic surfactants at pH 7.6. Nonionic surfactant: BC-15SS (○), BL-9 (●), BL-21 (◑).

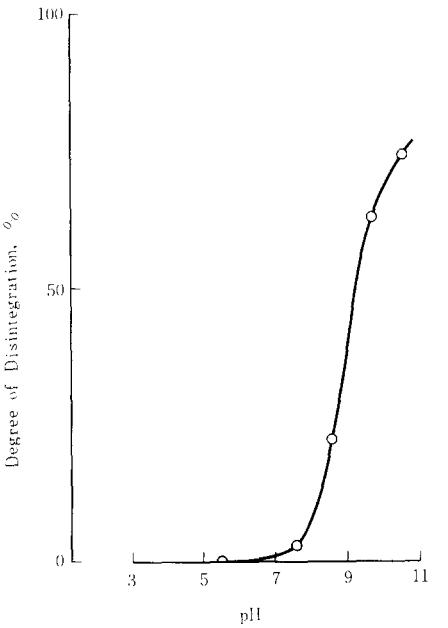

Fig. 4. Effect of pH on the stability of the phosphatidylcholine vesicles.

than for 1-dodecylpyridinium chloride. This would be caused by the higher penetrability and solubilizing power of 1-hexadecylpyridinium chloride toward the phospholipid vesicles.

Fig. 3 shows a very similar trend exhibited by BC-15SS and BL-9 or BL-21 in the effect of alkyl chain-length on vesicle disintegration. Fig. 3 also shows that the degree of vesicle disintegration is higher for BL-9 than for BL-21 when compared at the same concentration.

In Fig. 4 is shown the effect of pH of the medium on vesicle disintegration in the absence of surfactant. The phospholipid vesicles were very stable until pH reached a value of 8 above which they lost abruptly their stability due presumably to the increased electrostatic repulsion between the anionic groups of egg yolk phosphatidylcholine and carboxymethylchitin as a result of their complete dissociation.

Vesicle disintegration by ionic surfactants was notably dependent on pH of the medium. Figs. 5 and 6 show the effect of pH on vesicle disintegration for SDS and 1-dodecylpyridinium chloride, respectively. The degree of vesicle disintegration increased as pH increased, in the case of the anionic surfactant. On the contrary, the cationic surfactant produced an identical vesicle disintegra-

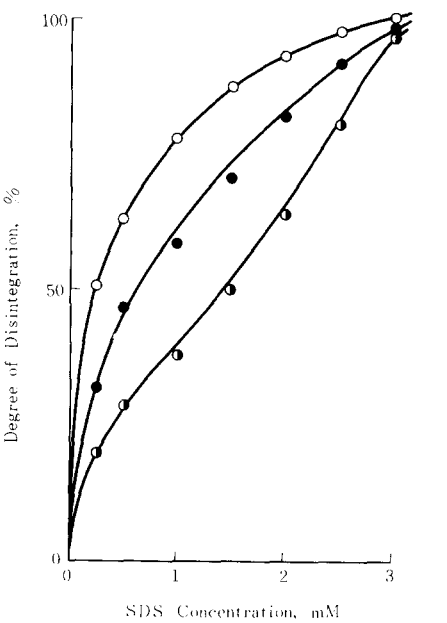

Fig. 5. Effect of pH on vesicle disintegration by SDS. pH: 8.6 (○), 7.6 (●), 6.5 (◑).

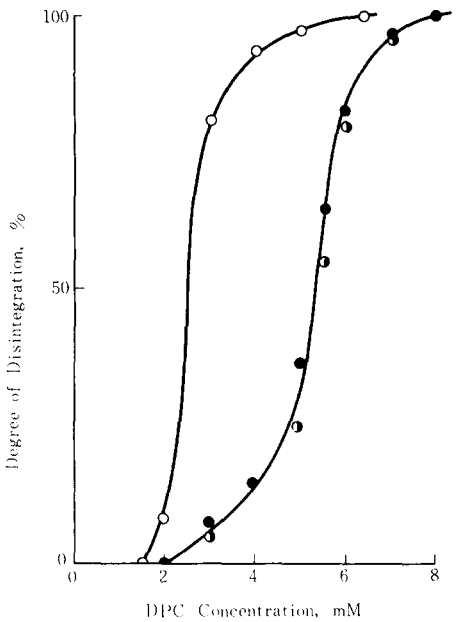

Fig. 6. Effect of pH on vesicle disintegration by 1-dodecylpyridinium chloride (DPC). pH: 8.6 (○), 7.6 (●), 6.5 (◑).

tion curve below pH 8 at which the curve started to shift toward the lower concentration region of the agent. Nonionic surfactants were not affected by pH in the development of their action on the vesicles, showing the importance of electrostatic interaction between the vesicle membrane and surfactant molecules in the phenomenon of vesicle disintegration.

Conventional vesicles prepared from egg yolk phosphatidylcholine were found comparable in the stability to surfactant with the phospholipid vesicles stabilized with carboxymethylchitin, though the former was much more fragile to pH change than the latter. The chitin-coated vesicles were also resistant to globulins and fibrinogen and no leak of the marker was detected in high concentrations of the proteins.

Discussion

Our view that penetration of surfactant ions takes place when solution of ionic surfactant is mixed with suspension of phospholipid vesicles is supported by the observed changes in the zeta potential of egg yolk phosphatidylcholine vesicles in the presence of SDS and 1-dodecylpyridinium chloride (Figs. 7 and 8). The zeta potential of the vesicles decreases first and then levels-off as SDS concentration increases. As the number of dodecyl sulfate anions penetrated into the phospholipid bilayer increases, the zeta potential of the vesicles becomes more negative to raise the lateral pressure of the bilayer. However, the number of dodecyl sulfate anions in the bilayer cannot be increased indefinitely owing to electrostatic repulsion between dodecyl sulfate anions and the anionic groups on molecules of the phospholipid. This should bring about levelling-off of the zeta potential of the vesicles as seen in Fig. 7. In such a situation, the lateral pressure of the bilayer would be maximum, and hence, the vesicles would be so vulnerable as to allow the leakage of the marker into the surrounding medium.

Although no change was observed in the zeta potential of the vesicles when BL-9 molecules were added to the vesicle suspension (Fig. 7), it is possible for molecules of the nonionic agent to penetrate into the phospholipid bilayer by virtue of their long alkyl chains to cause an increase in the lateral pressure of the bilayer. The lateral pressure increase would be large even after penetration by a small number of molecules of the nonionic agent, because their hydrophilic polyoxyethylene chains are likely to be bulky enough to raise the lateral pressure to a critical point at which the leakage of the marker starts. This may

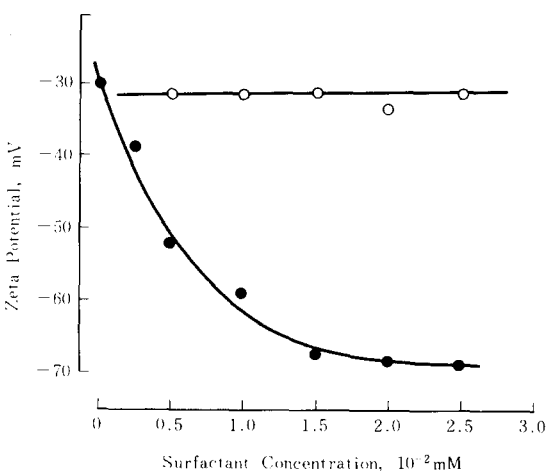

Fig. 7. Zeta potential of the phosphatidylcholine vesicles as a function of surfactant concentration. Surfactant: SDS (●); BL-9 (○).

explain the reason why BL-9 permits the marker to leak out through the vesicle membrane at very low concentrations. Longer polyoxyethylene chains of BL-21 will make molecules of the nonionic agent difficult to penetrate into the bilayer, thus retarding the release of the marker.

The change in the zeta potential of the vesicles caused by 1-dodecylpyridinium chloride is shown in Fig. 8 which makes a sharp contrast to that by SDS. With increasing concentration of 1-dodecylpyridinium chloride, the zeta potential increases, starting from a negative value, passing through the point of zero and changing the sign. This clearly demonstrates that dodecylpyridinium cations penetrate into the phospholipid bilayer to neutralize its negative charge and even to give a positive charge to it. The penetration of dodecylpyridinium cations will stop when the lateral pressure of the bilayer reaches a critical value for the start of marker leakage, at which the zeta potential levels-off.

It has been found that the addition of a small amount of cationic surfactant stabilizes conventional phospholipid vesicles (Stenius, P., private communication). This is consistent with the finding in the present work that the concentration of 1-dodecylpyridinium chloride at which the leakage of the marker becomes appreciable is much higher than that of SDS. Neutralization of the negative charge of phospholipid bilayers with the positive charge of surface-active cations would contribute to the stabilization of the bilayers. On the contrary, surface-active anions like dodecyl sulfate ions would have no such stabilizing action on phospholipid bilayers, since both surface-active anions and phospholipid molecules bear the same sign of electric charge at pH around 7.

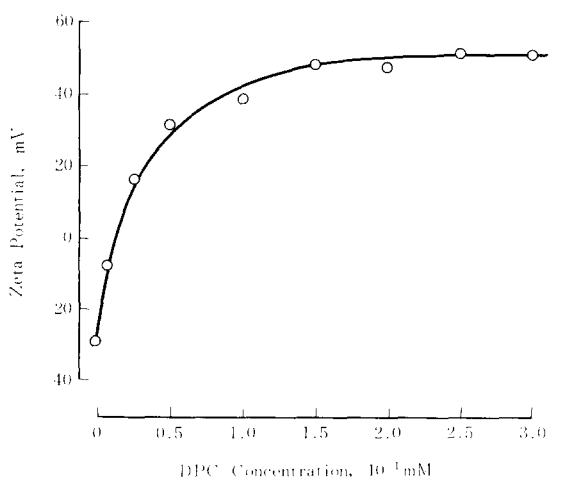

Fig. 8. Zeta potential of the phosphatidylcholine vesicles as a function of 1-dodecylpyridinium chloride (DPC) concentration.

Rise in pH of the medium should weaken the vesicle membrane, since the degrees of dissociation of both carboxyl groups of carboxymethylchitin molecules and phosphodiester headgroups of phosphatidylcholine molecules increase with increasing pH, whereby electrostatic repulsion between them is increased to make the membrane unstable. This leads to high degrees of vesicle disintegration on the alkaline side of pH (Fig. 4). It is suggested, therefore, that hydrogen bounding between undissociated carboxyl groups of the polyglucosamine and phosphodiester headgroups of the lipid plays an important role in stabilizing the structure of the vesicle membrane.

Facilitated vesicle disintegration by SDS at high pH can be interpreted as resulting from a combined effect of penetration of DS anions into the phospholipid bilayer to increase its lateral pressure and strong electrostatic repulsion between the highly ionized constituents of the membrane. As mentioned before, DP cations are capable of retarding vesicle disintegration even above pH 8 by reducing electrostatic repulsion through their penetration into the negatively charged bilayer. Bulky hydrophilic groups of the nonionic surfactant molecules penetrated into the bilayer would screen the electrostatic interaction between the components of the vesicle membrane to a considerable extent. This may be a major factor in preventing the vesicles from disintegrating at high pH in solutions of low concentrations of BL-9.

At high concentrations of ionic surfactants, there are a large number of micelles in solution. Hence, the molecules of the phospholipid liberated from the vesicles will readily be solubilized by the micelles and those of the polyglucosamine will be dispersed easily with the aid of surfactant ions. In the case of nonionic surfactants, ghost cell-like fragments consisting of the polyglucosamine are likely to be left in solution due to their insufficient dispersing action.

References

1 Hub, H.H., Hupfer, B., Koch, H. and Ringsdorf, H. (1980) Angew. Chem. Int. Edn. Engl. 19, 938–940

2 Akimoto, A., Dorn, K., Gros, L., Ringsdorf, H. and Schupp, H. (1981) Angew. Chem. Int. Edn. Engl. 20, 90–91

3 Bader, H., Ringsdorf, H. and Skura, H. (1981) Angew. Chem. Int. Edn. Engl. 20, 91–9

4 Gros, L., Ringsdorf, H. and Scupp, H. (1981) Angew. Chem. Int. Edn. Engl. 20, 305–325

5 Folda, T., Gros, L. and Ringsdorf, H. (1983) Makromol. Chem. Rapid Commun. 3, 167–174

6 Hub, H.H., Hupfer, B., Koch, H. and Ringsdorf, H. (1981) J. Macromol. Sci. Chem. A15, 701–705

7 Seki, K. and Tirrell, D.A. (1984) Macromolecules 17, 1692–1698

8 Takada, M., Yuzuriha, T., Katayama, K., Iwamoto, K. and Sunamoto, J. (1984) Biochim. Biophys. Acta 802, 237–244

9 Regen, S.L., Shin, J.-S. and Yamaguchi, K. (1984) J. Am. Chem. Soc. 106, 2446–2447

10 Aliv, K.V., Ringsdorf, H., Sclarb, B. and Leister, K.-H. (1984) Makromol. Chem. Rapid Commun. 5, 345–355

11 Bader, H., Dorn, K., Hupfer, B. and Ringsdorf, H. (1985) Adv. Polym. Sci. 64, 53–55

12 Kato, A., Arakawa, M. and Kondo, T. (1984) J. Microencap. 1, 105–112

13 Wehrli, E., Kondo, T., Kato, A., Izawa, H. and Kreuter, J. (1984) J. Microencap. 1, 329–333