Physicochemical Stabilization of Lipid Microspheres by Coating with Polysaccharide Derivatives

Anders CARLSSON,† Toshinori SATO, and Junzo SUNAMOTO*,††
Department of Industrial Chemistry, Faculty of Engineering, Nagasaki University, Nagasaki 852
††Laboratory of Materials Science of Polymers and Artificial Cell Technology,
Department of Polymer Chemistry, Faculty of Engineering,
Kyoto University, Sakyo-ku, Yoshida Hommachi, Kyoto 606
(Received July 29, 1988)

A methodology to improve lipid microspheres (LM) as a carrier for lipophilic drugs and their physicochemical properties are described. The LM were prepared from glycerides, glycerol, and phospholipids. The method involves coating of the surface of the LM with a naturally occurring or chemically modified polysaccharide. Special emphasis will be made on cholesterol-bearing pullulan and amylopectin. This is the same approach as that adopted for the stabilization of liposomes. Turbidity measurement revealed that the coating effectively depressed the Ca^{2+}-induced aggregation of the LM. From fluorescence polarization measurements, it was concluded that the fluidity of the LM surface decreased with the polysaccharide coating. Furthermore, the coating effect was investigated by zeta-potential measurements. The coating reduced the negative zeta-potential of the LM to an apparently neutral value.

Lecithin emulsions, so-called lipid microspheres (LM) or microdroplets, have recently gained wide interest as a carrier for lipophilic drugs.[1-4] Liposomes are complementary to LM as a drug carrier system (DDS), especially for hydrophilic drugs. However, there are several advantages of LM over liposomes, such as a high colloidal stability, which makes it possible to store the emulsions for a long period (several months) at room temperature without any noticeable change in their physicochemical properties. Furthermore, they can be produced on an industrial scale, and maybe most importantly, LM can incorporate a relatively large amount of lipophilic substances. LM are oil-in-water emulsions where phospholipids are used as the emulsifying agent.[1] The oil phase can entirely consist of a substance with medicinal potency, such as methoxyflurane used as local anesthesia.[2] Alternatively, a neutral fat is used, e.g. soybean oil, in which lipophilic drugs are dissolved. Steroids[3] and prostaglandins[4] are examples of drugs which have been incorporated in LM and successfully employed in treatments of several diseases.

The methodology for using liposomes in DDS has been relatively well established, and their physicochemical properties and fate in biological systems also have been rather well characterized.[5,6] For liposomes, in addition, much effort have been made to improve their stability and cell-recognizability.[7] However, relatively few significant investigations have been made on LM.

Therefore, we investigated the colloidal stability of LM in the presence of salts and polysaccharides by turbidity measurements. The zeta-potential of the LM was determined by microelectrophoretic measurements in the presence and absence of the polysaccharides. Furthermore, an extensive fluorescence spectroscopic investigation was carried out. The spectral behavior and the fluorescence polarization of two different fluorophores encapsulated in the LM were monitored. The amphiphilic *N*-dansylhexadecylamine (DSHA) and the hydrophobic 1,6-diphenylhexatriene (DPH) were used as the fluorescent probes.

The employed polysaccharides were the same as those previously used for the stabilization of liposomes, namely cholesterol-bearing amylopectin and pullulan.[7] As a comparison, we also investigated the effects of unmodified dextran, amylopectin, and pullulan, respectively. In addition, we used ethyl(2-hydroxyethyl)cellulose (EHEC) which is a nonionic cellulose ether. EHEC is widely used, inter alia, as an emulsifier in foodstuffs and pharmaceutical formulations.[8]

Experimental

Materials. Lipid microspheres (LM) were kindly prepared by Dr. Kiyoshi Iwamoto, Eisai Co., Tsukuba, Japan.[9] One ml of an aqueous LM emulsion contained 100 mg of soybean oil, 12 mg of egg yolk phospholipids, and 25 mg of glycerol which makes the system isoosmotic in normal saline solution. The phospholipids employed (Asahi Chemical Industry Co., Japan) contained 67.0 mol% phosphatidylcholine, 18.6 mol% phosphatidylethanolamine, and 0.8 mol% lysolecithin. In addition, the phospholipids consisted of an unspecified amount of negatively charged lipids, such as phosphatidic acid and phosphatidylserine. The acid value was 17.1 mg/kg. The LM were sealed in glass ampoules under N_2 atmosphere and stored at 5 °C prior to use. All measurements were performed on LM diluted 1000 times (by volume) with water.

Pullulan-50 (M.W. 50000) and amylopectin-112 (M.W. 112000) were kind gifts from Hayashibara Biochemical Laboratories, Inc., Okayama, Japan. Dextran-40 (M.W. 40000) was purchased from Tokyo Kasei Co., Tokyo, and dextran-176 (M.W. 176000) from Nakarai Chemicals Ltd., Kyoto. Ethyl(2-hydroxyethyl)cellulose-146 (EHEC-146; M.W.

† On leave from Physical Chemistry 1, Chemical Center, Lund University, P.O.B. 124, S-221 00, Lund, Sweden.

146000 with 2,12-hydroxyethyl groups and 0.9 ethyl group per glucose unit) was kindly presented by Berol Kemi AB, Stenungsund, Sweden. Cholesterylpullulan (CHP) and cholesterylamylopectin (CHAp) were prepared according to the procedure described elsewhere.[10] CHP was synthesized from pullulan-61 (M.W. 61000) in Dojindo Laboratories, Kumamoto, Japan. This sample contained 1.0 cholesteryl group per 100 glucose units (analyzed by ^{1}H NMR) and is coded as CHP-61-1.0. Another CHP sample was synthesized in this laboratory (by the courtesy of K. Sakai) from pullulan-50 and contained 1.9 cholesteryl groups per 100 anhydroglucose units. This is coded as CHP-50-1.9. CHAp was synthesized from amylopectin-112 and it contained 1.8 cholesteryl groups per 100 glucose units and is coded as CHAp-112-1.8.

Both *N*-dansylhexadecylamine (DSHA) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were the same as those used in previous studies.[11,12] All other reagents were of analytical grade and used without further purification. Membrane-filtrated water (Milli-Q, Millipore Corp., Bedford, USA) was used in all experiments and all glass wares were washed in dichromate–sulfuric acid and rinsed carefully with pure water.

Turbidity Measurements. An appropriate amount of electrolyte solution was added to a cuvette cell containing the LM emulsion diluted 1:1000 and preincubated at a given temperature (adjusted within ±0.5 °C). The turbidity (the optical density increase) was recorded at 600 nm as a function of time on a Hitachi 200-10 recording spectrophotometer, equipped with a thermoregulated cell compartment as previously described.[13] No stirring of the emulsion was performed. The results are presented as turbidity change $\Delta\tau$, calculated from $\tau_t-\tau_0$, where τ_t is the turbidity at time t and τ_0 is the turbidity of the LM emulsion at time zero without salt (τ_0 was typically 0.2 absorbance unit at λ=600 nm).

Zeta-Potential Measurements. Microelectrophoretic measurements were carried out on a Laser Zee Meter model 501 (PenKem, Inc., New York). In general, for charged particles, the zeta-potential ζ (V) is related to the electrophoretic mobility v ($m^2V^{-1}s^{-1}$) by the following equation:[14,15]

$$\zeta = v\eta/\{\varepsilon_r\varepsilon_0 f(\kappa, R)\} \quad (1)$$

where η is the viscosity of the medium, ε_r is the relative permittivity of the medium, ε_0 is the permittivity of free space, κ is the Debye–Hückel parameter, and R is the particle radius. For spherical particles at relatively low ionic strength, $f(\kappa, R)$=2/3 and thus Eq. 1 becomes the following:

$$\zeta = 3v\eta/(2\varepsilon_r\varepsilon_0) \quad (2)$$

In an aqueous solution at 20 °C Eq. 2 reduces to

$$\zeta = 2.126 \times 10^6 v \quad (3)$$

Actually, the instrument reads out ζ directly as calculated automatically from ζ=1.417×10^6 v, assuming that the measurement is carried out in an aqueous solution at 20 °C and that $f(\kappa, R)$ is unity. However, the latter assumption is only valid at relatively high ionic strength. In fact, we performed most of the measurements without salt or at low ionic strength (e.g. 5 mM $CaCl_2$). This means a transformation is necessary.[15]

The measurements of ζ were made on LM diluted 1:1000 over a temperature range of 20—24 °C. Since both ε_r and η are temperature dependent, the ζ-potential was corrected to 20 °C by the relation: $\zeta_{corr.}=\zeta_{meas.}$ [1−0.02(t−20)], where t (°C) is the actual temperature at which the measurement was made.

When polymer was added, the system was sonicated with a probe-type sonicator for 1 min (20 W) at 0 °C. The final polymer concentration in the cuvette cell was 0.1 wt%. This means that one can discard the effect of added polymer on the viscosity of the sample emulsion (cf. Eqs. 1 and 2). When salt was added, normally 3-4 measurements were made on a freshly prepared LM-salt sample and the whole procedure (from the addition of salt to the recording of ζ) was completed within 4 min.

Fluorescence and Fluorescence Polarization Measurements. The procedures were essentially the same as those previously adopted for the characterization of liposomes.[11–13,16,17] A small amount of the fluorescent probe dissolved in THF was added to the LM emulsion (diluted 1:1000), which also contained appropriate amounts of salt and/or polymer. The resulting mixture was then sonified on a Tomy UR-200P probe-type sonicator (20 W, duty 40 on the meter of the instrument) for 2 min at 60—70 °C under N_2 atmosphere. The final concentrations of DSHA and DPH in the LM were 1×10^{-6} M (M=mol dm^{-3}) and 2.5×10^{-8} M, respectively. The molar ratio of the fluorescent probe to the phospholipid was 1/15 for DSHA and 1/615 for DPH, respectively.

Spectral measurements were performed on a Hitachi 650-10S fluorescence spectrophotometer equipped with a thermoregulated cell compartment. Fluorescence polarizations were measured out on a Union Giken FS-501S fluorescence polarization spectrophotometer equipped with a thermoregulated cell compartment and a magnetic stirrer. A Sord Microcomputer M 200 Mark II system was combined with the instrument to control the measurement conditions and collect the data. DSHA was excited at 340 nm and its fluorescence was detected using a sharp cut-off filter Y-46 (Hoya Glass Works, Tokyo) to shut out the light of wavelengths less than 440 nm. DPH was excited at 360 nm and an L-39 filter was employed to cut the light below 370 nm. The degree of fluorescence polarization p was calculated by the following equation:[18]

$$p = (I_{VV} - C_f I_{VH})/(I_{VV} + C_f I_{VH}) \quad (4)$$

where I is the fluorescence intensity and subscripts V and H refer to the vertical and horizontal orientations of the excitation (first subscript) and analyzer (second) polarizers, respectively. The factor C_f ($=I_{HV}/I_{HH}$) corrects for artifacts arising from mismatched detectors.[19] All the I-values were taken as an average of at least 20 runs, with a gate time of 1 second. Before the computation of p, the intensity data were corrected by subtracting the intensity from a conventional LM emulsion. This was done in order to avoid artifacts arising from light scattering and fluorescent impurities which otherwise would yield a too high p-value.[19]

Table 1. Zeta-Potentials for LM at 20.0°C and Different Ca^{2+}- and Na^{+}-Ion Concentrations

	C_{salt}/mM	ζ/mV[a]
Without salt		−62.9±4.2
Ca^{2+}	0.5	−28.1±2.5
	1.0	−14.7±1.8
	2.0	−7.5±1.4
	3.0	0
Na^{+}	15	−29.4±2.9
	30	−20.7±1.5
	60	−9.4±1.3
	90	0

a) Average of at least 6 measurements. Error limits correspond to single standard deviations.

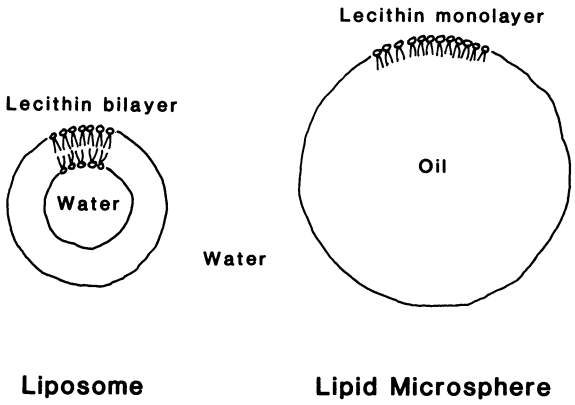

Fig. 1. Schematic representation of the structures of liposome and lipid microsphere.

Results and Discussion

As reported so far by Sunamoto and his co-workers,[7] liposomes can be effectively stabilized by coating the surface with a naturally occurring polysaccharide which bears cholesteryl groups in part. As we mentioned under Experimental the LM contain anionic lipids. Therefore, the surface of the LM employed in this work is negatively charged (Table 1), and the LM easily aggregate and are salted-out upon the addition of small amounts of electrolytes (Fig. 2). If one realizes the structural similarity of the LM surface, which is considered to be covered by a lipid monolayer, to that of liposomes as schematically represented in Fig. 1, it seems possible to also coat the LM with polymers in order to improve their structural stability. Therefore we investigated the colloidal stability of LM after coating the surface with different polysaccharides, particularly focusing on the effect of salt addition.

Due to the large particle size of the oil droplets and the difference in the refractive index between water and oil, the LM emulsion scatters light when irradiated. This feature was utilized to monitor the aggregation of LM in the presence of salt and different water-soluble polymers. Electrolytes were added up to the concentration corresponding to that under the physiological conditions. (In general, for mammalian the extra cellular fluid contains 145 mM Na^{+}, 5 mM K^{+}, 2.5—5 mM Ca^{2+}, and 1—2 mM Mg^{2+}.) The polymer concentration was arbitrarely chosen to be approximately

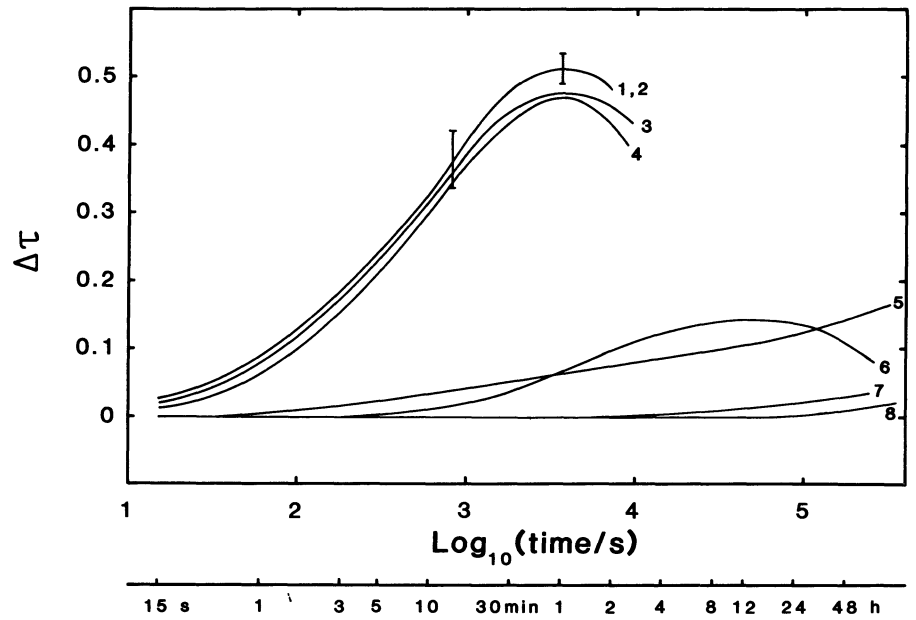

Fig. 2. Turbidity change ($\Delta\tau$) vs. time for LM (diluted 1:1000) after addition of 5 mM aqueous $CaCl_2$ at 37.0°C. 1: Without polysaccharide; 2: dextran-40 (0.96 mg ml^{-1}); 3: amylopectin-112 (0.95 mg ml^{-1}); 4: dextran-176 (0.95 mg ml^{-1}); 5: EHEC-146 (0.92 mg ml^{-1}); 6: pullulan-50 (0.95 mg ml^{-1}); 7: CHP-50-1.9 (0.87 mg ml^{-1}); 8: CHAp-112-1.8 (0.95 mg ml^{-1}).

0.9 mg per ml of LM emulsion (diluted 1:1000).

Presented in Fig. 2 are the turbidity measurements for LM in 5 mM aqueous $CaCl_2$ at 37 °C as a function of time. In the polymer-free system, a relatively rapid increase in the turbidity was observed, and after about 1 h $\Delta\tau$ reached its maximum. Due to precipitation of the LM aggregates, no further measurements were made after $\Delta\tau_{max}$. The same turbidity increase was recorded also at 25 °C. An almost clear solution was obtained if the samples were kept for 1—2 days in the presence of salts. Though not presented here, the effect of 150 mM Na^+-ion was less than that of 5 mM Ca^{2+}-ion. This was reflected through a slower increase in $\Delta\tau$ and a lower $\Delta\tau_{max}$. Even the addition of Na^+-ion caused a precipitation of the LM aggregates and led to a cleared supernatant. However, this was not observed until one week later. In the absence of salts, no significant change in turbidity was observed even after several months. These results suggest to us that we have to seriously consider the colloidal stability of LM in the blood stream when LM are employed as a carrier for lipophilic drugs, especially at higher concentrations.

However, if either CHAP-112-1.8, CHP-50-1.9, EHEC-146, or pullulan-50 was added to the LM before the addition of Ca^{2+}-ion, the aggregation behavior was dramatically altered (Fig. 2). This was reflected on the almost invariant $\Delta\tau$ values. Especially, the cholesterol-bearing polymers were superior in supressing the Ca^{2+}-induced aggregation, For example, the CHAP-112-1.8-coated LM showed no significant turbidity change even after 48 h. This means that these polysaccharides adsorb to the LM and effectively coat the surface, thereby largely preventing the salt-induced aggregation through steric stabilization.[20,21] The effect of pullulan-50 was noteworthy compared to the other three unmodified polysaccharides (amylopectin-112, dextran-40, and dextran-176). The three latter polymers completely lacked the ability to prevent LM aggregation. Even a three-fold increase in the concentration of amylopectin-112 did not significantly affect the result. Pullulan is a rather linear polymer and adsorbs more effectively to the LM surface than the other branched polymers.[13] The bulkyness of a branched polymer leads to a less surface coverage than that of a linear one.

Presented in Table 1 are the zeta-potential data for LM diluted 1:1000 at 20 °C as a function of the Ca^{2+}- and the Na^+-ion concentrations. The zeta-potential of the conventional LM employed in this work was approximately −63 mV. As expected, the addition of Na^+- or Ca^{2+}-ion led to a significant reduction in ζ, with the divalent cation substantially more effective than the monovalent,[22] as seen from the effective salt concentrations for to become zero. Thus, when the electrostatic double-layer repulsion is diminished, the colloidal particles can easily aggregate. No measurements were performed on systems in which both Ca^{2+}- and Na^+-ions were simultaneously present at the physiological concentration. If the Ca^{2+}-ion concentration is greater than 3% of the Na^+-ion concentration, the surface potential is solely determined by the Ca^{2+} ions.[22] Here, it should be pointed out that the obtained ζ-values probably are overestimated at the highest salt concentrations since $f(\kappa, R)$ in Eq. 1 appproaches unity at high ionic strength.[15] However, the measurements gave a qualitative picture of the changes of the surface potential upon the salt addition, and no efforts were made to correct the data.

Table 2. Zeta-Potentials for LM–Polysaccharide Systems[a] at 20.0 °C

Polymer	Concentration/mg ml^{-1}	ζ/mV
Dextran-40	1.00	−47.5
Dextran-176	1.01	−45.2
Pullulan-50	0.98	−32.6
EHEC-146	0.97	−13.5
CHP-61-1.0	0.98	−25.3
CHP-50-1.9	0.87	−18.8
CHAp-112-1.8	0.95	−11.3

a) The LM were diluted by water 1000 times (by vol.) prior to the addition of polysaccharide solution.

Table 2 presents the zeta-potentials of LM when various polysaccharide were added to the LM. The addition of all polymers caused a decrease in the zeta-potential. When a polymer adsorbs to the LM surface the charge density remains the same. However, due to the adsorbed polymer layer, the slipping plane is now moved to a point further out from the surface and therefore a lower ζ-potential is recorded. The effect of the two dextran derivatives was considerably less than that of the others. The results suggest that the coating efficiency by dextran is relatively low. In all cases in which the polymers were added, the addition of Ca^{2-}-ion to a concentration of 5 mM completely abolished the surface potential of the LM.

Clearly from the results the stability of the LM was largely affected by salts. The LM were especially sensitive to a divalent cation even at low concentration such as 5 mM Ca^{2+} (which is approximately the physiological concentration of the ion). The aggregation of the LM and the subsequent precipitation were directly observed as changes in the turbidity of the LM emulsions (Fig. 2).

The fluorescence polarization technique has frequently been used for the determination of the microviscosity of a molecular assembly, such as lipid bilayer membranes.[23] An important advantage of this technique is that the polarization data in terms of fluidity can be interpreted without further mathematical treatment. Presented in Fig. 3 are the p-values (as defined from Eq. 4) of DPH (top) and DSHA (bottom) in LM diluted 1:1000 as a function of temperature. Irrespective of the temperature, the p-

values of DPH in the hydrophobic core[23] of the LM were lower than those obtained in liposomal membranes.[12,16] This indicates that the fluidity of the interior core of the LM is higher compared to that of the lipid bilayers. Lipid bilayer membranes provide a more confined hydrophobic domain which leads to a more restricted motion of the fluorescent probe. The *p*-value of DPH in the LM decreased with an increase in the temperature, which indicates an increase in the fluidity of the interior core of the LM at higher temperatures. The fluorescence emission maximum (E_{max}) of DPH in the LM was 429 nm and constant over the range of 15—50 °C. The same emission maximum has been obtained when DPH was dissolved in both benzene and liposomes.[16,23] This indicates that DPH is locating in a very hydrophobic domain of the LM.

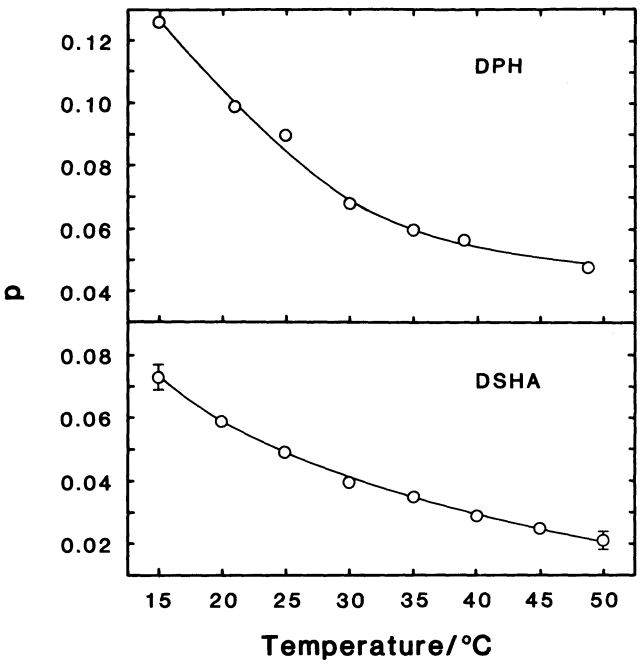

Fig. 3. Fluorescence polarization (*p*) of DPH (top) and DHSA (bottom) in LM (diluted 1:1000) as a function of temperature. The concentrations of DPH and DSHA in the LM were 2.5×10^{-8} M and 1×10^{-6} M, respectively.

The *p*-values of DSHA in LM (diluted 1:1000) as a function of temperature are given in Fig. 3 (bottom). DSHA is relatively amphiphilic and the dansyl moiety of DSHA is believed to locate in polar region close to the membrane surface in liposomal membranes.[11] Consequently, it is assumed that when DSHA is added to LM the polar dansyl group is in the region close to the microsphere-water interface and is surrounded by phospholipids. As for DPH, the *p*-value of DSHA also decreased on heating. This reflects that the fluidity of the phospholipid monolayer of the LM is also temperature dependent. The present data suggest either that the LM surface is more fluid compared with that of a liposomal surface, or that the probe is locating in a more fluid region close to the oil phase. For example, we obtained a *p*-value of 0.03 at 37 °C, while for egg L single-walled liposomes the *p*-value was 0.06 at the same temperature.[11] Over the temperature range studied, the emission maximum of DSHA was constant at 483 nm. This is 30—35 nm lower than that in single-walled liposomes,[11,12] and 7 nm lower than the value in water, where DSHA molecules self-aggregate.[11] This blue shift in E_{max} of DSHA in LM indicates that the probe is in a less polar environment compared with that in bilayer membranes of liposomes.

Emission maxima and *p*-values of DSHA at 37 °C are summarized in Table 3. The addition of Ca^{2+}- and Na^{+}-ions to concentrations of 5 and 150 mM, respectively, did not lead to any significant change in the *p*-value of DSHA. On the basis of this result, we did not investigate the salt effect on the DPH polarization.

Table 3. Fluorescence Emission Maxima and *p*-Values of DSHA (1×10^{-6} M) in LM (diluted 1:1000) at 37.0 °C

	Concentration		E_{max}/nm	*p*
	Salt/mM	Polymer/mg ml^{-1}		
No additives			483	0.031
Ca^{2+}	1		480	0.034
	5		479	0.029
	50		477	0.030
Na^{+}	150		480	0.029
Dextran-40		0.99		0.036
Dextran-176		1.00		0.032
Dextran-176+Ca^{2+}	5	1.00		0.030
Pullulan-50		1.01	482	0.038
Pullulan-50+Ca^{2+}	5	1.01	478	0.030
EHEC-146		1.0	478	0.041
EHEC-146+Ca^{2+}	5	1.0	478	0.038
CHP-61-1.0		1.00	482	0.046
CHP-61-1.0+Ca^{2+}	5	1.00	480	0.043
CHAp-112-1.8		1.00	478	0.057
CHAp-112-1.8+Ca^{2+}	5	1.00		0.061

However, a slight blue shift in E_{max} (3—6 nm) was observed when salts were present, probably because of the movement of the probe into a more hydrophobic domain upon the dehydration around the polar head group. The shift was more pronounced by adding 50 mM Ca^{2+}-ion. The addition of dextran-40 and -176, pullulan-50, and EHEC-146 showed no or little effect on the p-value of DSHA. However, the cholesterol-bearing polysaccharides (CHP-61-1.0 and CHAp-112-1.8) considerably increased the polarization of DSHA. Especially the addition of the latter one led to a two-fold increase in the p-value. This suggests that when these two polymers adsorb onto the LM surface, the cholesteryl group behaves as a hydrophobic anchor in the surface lipid layer. This would result in a lower fluidity of the surface of the LM. Though data are not shown, the difference in the p-values between the two groups of polymers was more pronounced at lower temperatures (15—30 °C). Even the addition of Ca^{2+} to a concentration of 5 mM to the LM-polymer samples did not significantly change the p-value.

After the addition of DSHA, the LM-EHEC-146 sample was sonicated under milder conditions (3 short flashes at 40 °C under N_2 atmosphere) in order to avoid phase separation during this procedure. Of the polymers employed in this work, EHEC-146 is the most hydrophobic one. This is evident from a lower critical solution temperature of an aqueous EHEC solution.[24] The change in the procedure for encapsulating the probe did not significantly alter the p-value of DSHA in a polymer-free LM sample (Fig. 3).

Judging from the results through the present investigation, we could conclude that the LM can effectively be coated by cholesterol-bearing polysaccharides similarly to liposomes. The coating of the LM surface with the polysaccharide derivatives led to a reduction of the divalent metal ion-induced aggregation of the LM. This is very promising for LM to be utilized as an improved carrier for lipophilic drug.

The stay of A. Carlsson in Japan was supported by a grant from the Swedish National Board for Technical Development. Comments on this work from Prof. Björn Lindman and Dr. Björn Bergenståhl are gratefully acknowledged. The instrument for microelectrophoretic measurements was kindly put at our disposal by Tsukuba Research Laboratories, Eisai Co. This research was financially supported by a Grant-in-Aid (No. 62850151) from the Ministry of Education, Science and Culture.

References

1) S. S. Davies, C. Washington, P. West, L. Illum, G. Liversidge, L. Sternson, and R. Kirsh, "Biological Approaches to the Controlled Delivery of Drugs," ed by R. L. Juliano, *Ann. N. Y. Acad. Sci.*, **507**, 75 (1987).

2) D. H. Haynes and A. F. Kirkpatrick, *Anesthesiology*, **63**, 490 (1985).

3) Y. Mizushima, T. Hamano, and K. Yokoyama, *Ann. Rheum. Dis.*, **41**, 263 (1982).

4) Y. Mizushima, A. Yanagawa, and K. Hoshi, *J. Pharm. Pharmacol.*, **35**, 666 (1983).

5) F. Szoka and D. Papahadjopoulos, *Ann. Rev. Biophys. Bioeng.*, **9**, 467 (1980).

6) P. Machy and L. Leserman, "Liposomes in Cell Biology and Pharmacology," John Libbey & Co., London (1987).

7) J. Sunamoto and K. Iwamoto, "CRC Critical Reviews in Therapeutic Drug Carrier Systems," **2**, 117 (1986).

8) G. K. Greminger and K. L. Krumel, "Handbook of Water-Soluble Gums and Resins," ed by R. L. Davidson, McGraw-Hill, New York (1980), Chap. 3.

9) The mean diameter of the LM used in this work was approximately 270 nm with a standard deviation of 60 nm (determined by a Coulter N4 Sub-micron Particle Analyzer, Coulter Electronics, Inc., Hialeah, Florida).

10) J. Sunamoto, T. Sato, M. Hirota, K. Fukushima, K. Hiratani, and K. Hara, *Biochim. Biophys. Acta*, **898**, 323 (1987).

11) K. Iwamoto and J. Sunamoto, *Bull. Chem. Soc. Jpn.*, **54**, 399 (1981).

12) K. Iwamoto, J. Sunamoto, K. Inoue, T. Endo, and S. Nojima, *Biochim. Biophys. Acta*, **691**, 44 (1982).

13) J. Sunamoto, K. Iwamoto, H. Kondo, and S. Shinkai, *J. Biochem.*, **88**, 1219 (1980).

14) E. G. Richards, "An Introduction to Physical Properties of Large Molecules in Solution," IUPAB Biophysics Series, Cambridge University Press (1980), Chap. 9.

15) P. Sennett and J. P. Olivier, *Ind. Eng. Chem.*, **57**, 33 (1965).

16) J. Sunamoto, K. Iwamoto, K. Inoue, T. Endo, and S. Nojima, *Biochim. Biophys. Acta*, **685**, 283 (1982).

17) K. Iwamoto and J. Sunamoto, *J. Biochem.*, **91**, 975 (1982).

18) E. N. Hudson, "Fluorescence Spectroscopy," ed by A. J. Pesce, C.-G. Rosén, and T. L. Pasby, Marcel Dekker, New York (1971), Chap. 4.

19) T. L. Pasby, in Ref. 18, Chap. 6.

20) W. Norde, "Microspheres and Drug Therapy. Pharmaceutical, Immunological and Medical Aspects," ed by S. S. Davies, L. Illum, J. G. McVie, and E. Tomlinson, Elsevier Science, Amsterdam (1984), Chap. 3.

21) B. Bergenståhl, "Gums and Stabilizers for the Food Industry 4," ed by G. O. Phillips, D. J. Wedlock, and P. B. Williams, IRL Press, Oxford, Washington D. C. (1988), p. 363.

22) J. N. Israelachvili, "Intermolecular and Surface Forces," Academic Press, London (1985), Chap. 12.

23) M. Shinitzky and Y. Barenholz, *Biochim. Biophys. Acta*, **515**, 367 (1978).

24) A 0.1% water-EHEC-146 solution has a cloud point of 75 °C. A. Carlsson, G. Karlström, B. Lindman, and O. Stenberg, *Colloid Polym. Sci.*, **266**, 1031 (1988).