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Liposomes incorporating hydrophobically modified glucose oxidase

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Abstract−Glucose oxidase (GOD) was hydrophobically modified by conjugating palmitic acids to the amino groups of the enzyme. The number of palmitic acid residues conjugated to GOD was determined by using trinitrobenzenesulfonic acid (TNBS). According to the result, six palmitic acids turned out to be covalently attached to one GOD molecule. The activity of the modified GOD was 75%-80% of that of the native one, and due to the conjugated alkyl chain, the modified GOD was more surface-active than the native one. The modified GOD was incorporated into the liposomal bilayer of dioleoylphosphatidylethanolamine (DOPE) and cholesteryl hemisuccinate (CHEMS) by a detergent removal method. According to the results of the pH-dependent release, no significant release was observed in 60 sec at pH 8.2. An appreciable amount of calcein released at pH 7.0. A marked amount released at pH 6.0. The degree of release became more extensive at pH 5.0 and pH 5.6. This is possibly because the liposome is disintegrated into hexagonal phases under acidic conditions.

Key words: Glucose Oxidase, Liposome, pH-sensitive Release

INTRODUCTION

Stimuli-sensitive liposomes have attracted many scientists' interests, since the liposomes are versatile as a carrier of drug. The specific functions of the liposomes are induced by direct interaction with targets sites [1,2], environmental pH [3,4] and temperature changes [5-11]. The sensitivity of liposomes to stimuli can be controlled by either altering the composition of membrane or modifying the surface. Temperature-sensitive liposomes were proposed by modifying the surface of liposomes with hydrophobically modified poly (*N*-isopropylacrylamide) (poly(NIPAM)) [7-11]. The polymer exhibits lower critical solution temperature (LCST) around 32 °C. It takes an expanded form below LCST, and attains a contracted form above the temperature. The interaction of the polymer and the lipid membranes give rise to the release of the materials entrapped in the inner aqueous phase of liposomes. On the other hand, pH-sensitive liposomes were prepared by combining dioleoylphosphatidylethanolamine (DOPE) and the copolymer of NIPAM and methacrylic acid [12]. Since the molecule of DOPE is conical and its packing parameter is greater than 1, the phospholipids tend to form a non-bilayer structure in a physiological condition. In order to form a bilayer, it needs complementary molecules which fill the space among the head groups of DOPE. The hydrophobically modified copolymers of NIPAM and methacrylic acid were used as pH-sensitive complementary molecules. It is a weakly acidic amphiphile and could stabilize DOPE bilayers at neutral pH. Among pH-sensitive complementary molecules, cholesteryl hemisuccinate (CHEMS) has been the most frequently employed [13-17]. It is also a weakly acidic amphiphilic molecule and could stabilize DOPE bilayers at physiological pH.

In this study, pH-sensitive liposomes composed of DOPE and CHEMS were prepared and the surface of liposomes was modified with glucose oxidase (GOD). In order to incorporate GOD into the liposomal membranes, GOD was hydrophobically modified by covalently attaching alkyl chains to the amino groups of the enzyme. The rationale for the incorporation of GOD in the liposomal membranes is to develop a glucose-sensitive carrier. The hydrophobically modified GOD (HmGOD) was characterized in terms of the enzymatic activity, the surface activity and the degree of modification. In addition, the pH-sensitive release of DOPE/CHEMS/HmGOD liposomes was investigated.

EXPERIMENTAL

1. Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Fluka. Deoxycholate (DOC), cholesteryl hemisuccinate (CHEMS), glucose oxidase (GOD) from *Aspergillus niger* Type X, palmitic acid *N*-hydroxysuccinimide ester, sodium phosphate, calcein, sodium acetate trihydrate, horseradish peroxidase, β-D-glucose, o-dianisidine dihydrochloride, sodium lauryl sulfate (SLS) and amino acid standard were purchased from Sigma Chemical Co. Trinitrobenzene sulfonic acid (5% methanol solution) was provided by PIERCE. All other reagents were in analytical grade.

2. Modification of Glucose Oxidase

GOD was hydrohpobically modifies following a previous report [18]. DOC of 0.24 g, was dissolved in 12 ml of phosphate buffer (pH 8.8, 0.16 M). And then, GOD of 0.048 g was dissolved in the solution. In parallel, palmitic acid *N*-hydroxysuccinimide ester of 0.0228 g was dissolved in 2 ml of dry dioxane. The ester solution of 0.4 ml was added to the GOD solution in a manner that 4 portions

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of 0.1 ml were added every 2 h. Therefore, the molar ratio of GOD to the palmitic acid ester was 1 : 40. The reaction was done at 30 ºC for 10 h while gently stirring. To remove insoluble byproducts, the reaction mixture was filtered through a syringe filter (0.45 μm). The filtrate was dialyzed against 1 L of phosphate buffer (pH 8.0, 0.02 M) for 12 h with 4 time-exchanges of the buffer. Further dialysis was performed for 12 h with 2 time-exchanges of phosphate buffered saline (pH 7.4, 0.15 M). Finally, the dialyzed solution was filtered through a syringe filter (0.22 μm) and lyophilized for 3 days.

3. Determination of Number of Palmitic Acid Residues Conjugated to GOD

TNBS method was employed [19]. SLS of 0.025 g was dissolved in 50 ml of phosphate buffer (pH 8.9, 0.01 M). TNBS solution (0.03%) was prepared by adding 0.5 ml of trinitrobenzene sulfonic acid to 83 ml of the SLS solution. 1 ml of SLS solution, 1 ml of TNBS solution and 0.5 ml of HmGOD solution (3.55 mg/ml) were combined in a 10 ml vial and the reaction was done at 50 ºC, for 1 hr. And then, 1.9 ml of HCl solution (0.21 M) was added to the reaction and the vial was kept at room temperature for 30 minutes. Finally, the absorbance of the reaction was measured at 335 nm. A standard curve was prepared following the same procedure as described above, except that various concentrations of the amino acid standard solutions were employed instead of HmGOD. The number of palmitic acid conjugated to GOD was determined by comparing the absorbance of the enzyme solutions with the standard curve.

4. Determination of Enzymatic Activity

0.173 ml of glucose solution in distilled water (10%) and 0.827 ml of o-dianisidine dihydrochloride solution in distilled water (0.21 mM) were mixed together. And then, 0.033 ml of horseradish peroxidase solution in distilled water (0.33 mg/ml, 60 purpurogallin units/ml) was added to the solution. Finally, 0.1 of enzyme solution in sodium acetate buffer (0.023 mg/ml) was added to the previously prepared solution. The degree of enzymatic reaction was measured by determining the absorbance at 500 nm with time. As a control, sodium acetate buffer free of enzyme was employed. The activity was expressed in units/mg.

5. Surface Tension Measurement

The surface tensions of native GOD solution and HmGOD solution were measured by a ring method using a surface tension analyzer (SEO, DST 60, South Korea). The concentrations were adjusted to 0, 0.000625, 0.00125, 0.0025, 0.005, 0.01, 0.02, and 0.04 mg/ml.

6. Preparation of DOPE/CHEMS Liposome Incorporating HmGOD

DOPE solution in chloroform (10 mg/ml) and CHEMS solution in chloroform (6.5 mg/ml) were put into a 50 ml round bottom flask so that the molar ratio of DOPE to CHEMS was 7 : 3 and the total amount of lipid was 20 mg. The solvent was evaporated in a rotary evaporator at a reduced pressure to obtain a mixed dry film of DOPE and CHEMS. The dry film was dispersed into 2 ml of HEPES (pH 8.2) containing calcein (50 mM), and DOC (0.09%). HmGOD was added to the dispersion so that the molar ratio of lipid to HmGOD was 20,000 : 1. The mixture was sonicated for 20 min with a bath-type sonicator (Ultrasonic processor, VCX 500, SONIX, USA). The liposomal suspensions were allowed to stand at room temperature for 4-5 h. DOC and unentrapped calcein was removed by a gel permeation chromatography by using Sephadex G-50 column (1.6 cm×40 cm). The final concentrations of lipid were adjusted to 2 mg/ml.

7. pH-Dependent Calcein Release

The calcein-containing liposome suspensions of 0.15 ml, 2 mg lipid/ml, in HEPES (pH 8.2), were injected into a fluorescence cell containing 2.6 ml of HEPES, pre-adjusted to pH ranging from 5 to 8. The change in fluorescence was monitored at 524 nm with excitation at 494 nm. The percent release of calcein was determined as follows.

$$\% \text{ release} = (F_t - F_i)/(F_f - F_i) \times 100$$

where F_t is the intensity of fluorescence at a given pH and F_i is the initial intensity at pH 8.2. F_f is the total fluorescence after adding DOC so that the final concentration is 0.2%. Since the intensity of the fluorescence strongly depends on pH, F_t was corrected by using calibration curves.

8. Dynamic Light Scattering

The pH-dependent changes in the size were measured with a particle size analyzer (ZetaPlus 90, Brookhaven Instrument Co., USA). The content of liposome in the suspension was adjusted to 0.03%. pH was varied from 5.0-8.2 with 1 mM NaOH and 1 mM HCl solutions.

RESULTS AND DISCUSSION

1. Determination of Number of Palmitic Acid Residues Conjugated to GOD

Fig. 1 shows the amino acid standard curve. The curve is expressed as A_{335}=0.572 M+0.0062 (R^2=0.9994), where A_{335} is absorbance at 335 nm and M is μmole of amino acids. Since TNBS method detects reactive amino groups, the absorbance of enzyme solutions is directly related to the number of reactive lysine residues of the enzymes. Under our experimental conditions, the absorbance of native GOD was 0.165, corresponding to 25.02 reactive amino groups

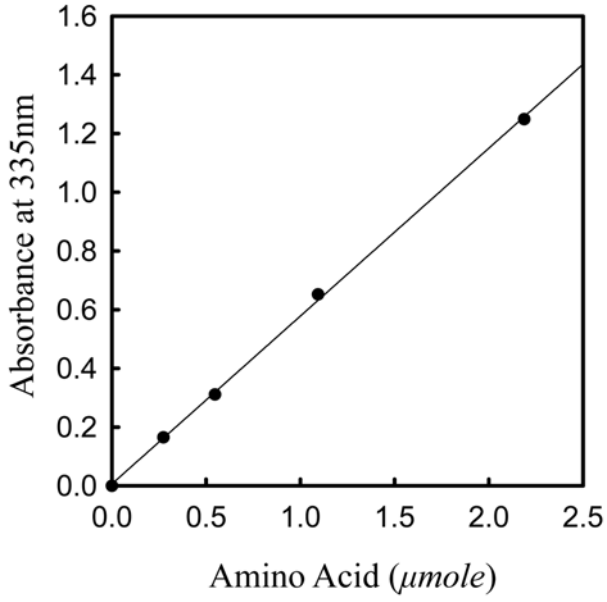

Fig. 1. Standard curve of amino acids.

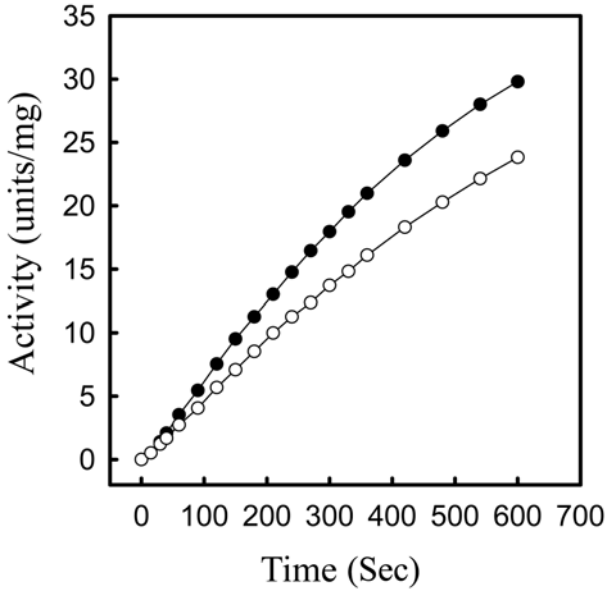

Fig. 2. Enzymatic activity of native GOD (●) and HmGOD (○).

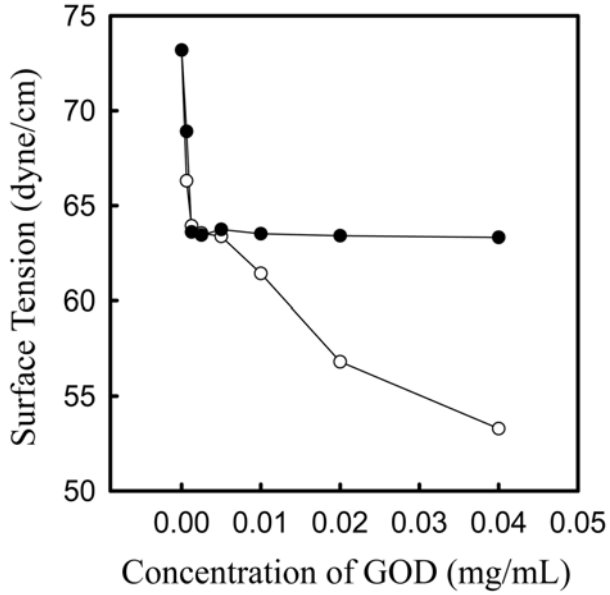

Fig. 3. Surface tensions of native GOD solution (●) and HmGOD solution (○).

per native GOD molecule. The total number of lysine residues in the dimeric GOD is 30 [20]. The difference in the number of amino groups is possibly because some of lysine residues are sequestered in the conformation of the enzyme and thus they are inactive. On the other hand, the absorbance of HmGOD was 0.127, calculated to be 19.03 reactive amino groups per the modified GOD molecule. This means that six palmitic acids were covalently attached to one GOD molecule through the amide bond with the reactive lysine residues. In previous report where GOD from *Aspergillus niger* (Type VII-S) was hydrophobized by using palmitic acid *N*-hydroxysuccinimide ester, the number of reactive amino groups in the native GOD was reported to be around 24 and the average number of covalently attached alkyl chains per GOD molecules was around 5 [18].

2. Determination of Enzymatic Activity

Fig. 2 shows the enzymatic activity of native GOD and HmGOD. The products of the enzymatic reaction are gluconic acid and hydrogen peroxide. The hydrogen peroxide oxidizes o-dianisidine dihydrochloride to the reduced form with aid of catalytic action of horseradish peroxidase. The characteristic wavelength of the reduced form is around 500 nm. Thus, the absorbance is a measure of the enzymatic activity. Since the amount of hydrogen peroxide increases with the reaction time, the absorbance was proportional to the time elapse. The absorbance was converted to units/mg enzyme by using the millimolar extinction coefficient of oxidized o-dianisidine at 500 nm. According to the data, the activity of HmGOD was 75-80% of that of native GOD. The covalent attachment of alkyl chains to the lysine residues would have a great effect on the enzymatic activity, possibly because of the deformation of the enzyme by the modification. Furthermore, since lysine residue exists in the active pocket site of GOD, the conjugation with amino groups of the amino acid residues could be a critical factor to deteriorate the activity.

3. Surface Tension Measurement

Fig. 3 shows the surface tensions of native GOD solution and HmGOD solution. The surface tension decreased to 63.7 dyne/cm when the concentration of native GOD increased to 0.005 mg/ml (Fifth point in Fig. 3). When the concentration was greater than 0.005 mg/ml, no significant change in the surface tension was observed with increasing concentration. The surface tensions of HmGOD solutions were almost the same as those of GOD solution when the concentrations were less than 0.005 mg/ml. Above the concentrations, however, the tension decreased with the concentration. For example, in case the concentration increased from 0.005 mg/ml to 0.04 mg/ml, the tension decreased from 63.4 dyne/cm to 53.2 dyne/cm. This means that HmGOD exists at the air/water interface more than native GOD does. That is, HmGOD is more surface-active than native GOD, because the hydrophobic alky chains are covalently

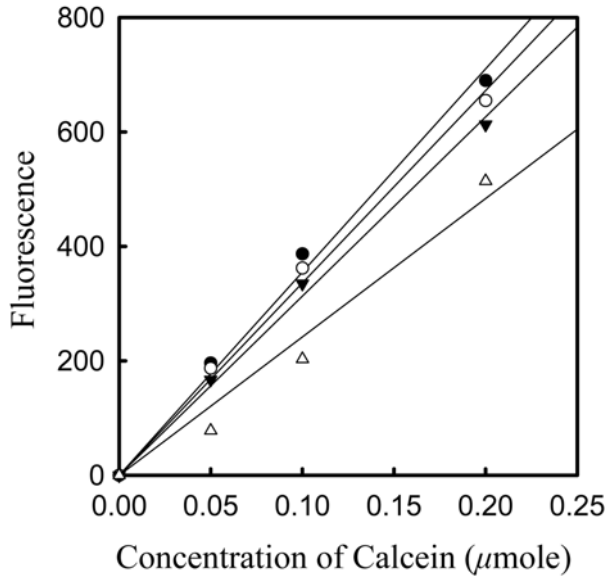

Fig. 4. Calibration curve of calcein fluorescence at various pHs. pH 5.0 (△), pH 5.6-6.0 (▼), pH 7.0 (○), pH 8.2 (●)

attached to GOD and they tend to bring GOD to the air/water interface.

4. pH-Dependent Calcein Release

The fluorescence intensity at the various pHs should be converted to the intensity at pH 7.4, since the liposomes were prepared at pH 7.4 but the release experiment was performed at various pHs, which is different from pH 7.4. The calibration curves of calcein fluorescence were obtained at various pHs, where the degree of release was observed (Fig. 4). The slope of each curve decreased with decreasing pH, which means that the intensity of fluorescence is suppressed at acidic pHs. With these curves, the fluorescence intensities were corrected.

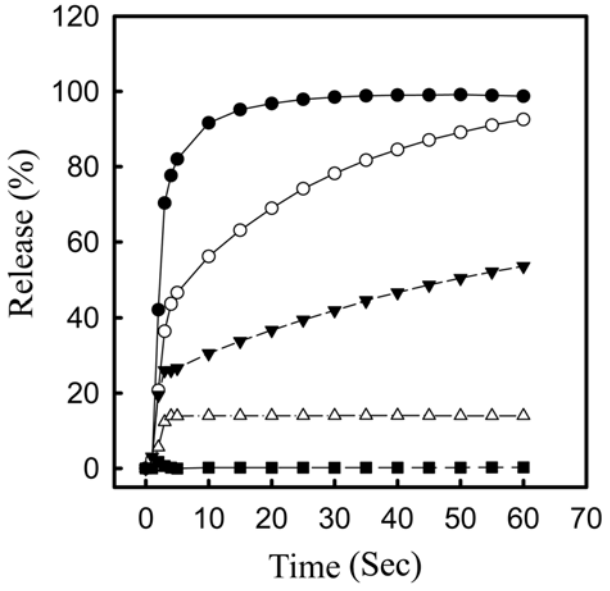

Fig. 5. pH-dependent calcein release from liposome composed of DOPE/CHEMS /HmGOD.
pH 5.0 (●), pH 5.6 (○), pH 6.0 (▼), pH 7.0 (△), pH 8.2 (■)

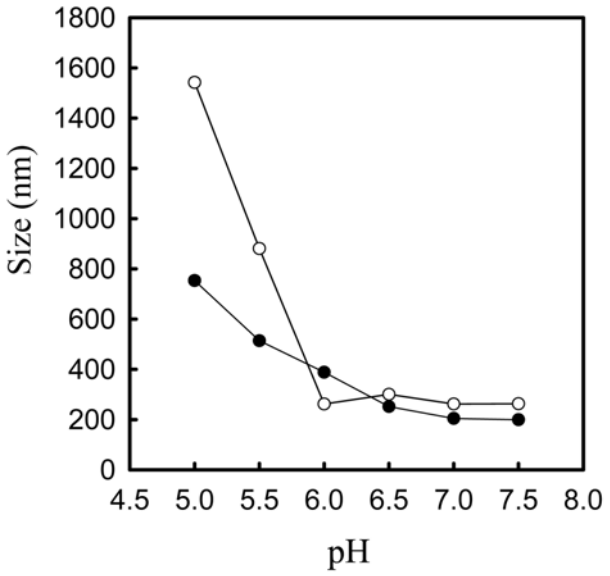

Fig. 6. Change in the size of DOPE/CHEMS liposome (○) and DOPE/CHEMS/HmGOD liposome (●) with pH.

Fig. 5 shows the pH-dependent calcein release from liposome composed of DOPE/CHEMS/HmGOD (7/3/0.00035 in molar ratio). At pH 8.2, no significant release was observed in 60 sec. At pH 7.0, an appreciable amount of calcein released. A marked amount released at pH 6.0. The degree of release became more extensive at pH 5.0 and pH 5.6. Since the pK value of carboxylic acid of CHEMS is 5.8, the carboxylic acid would be ionized above the pH, such as pH 7.0 and 8.2, so that the head group was large enough to stabilize DOPE bilayers. On the other hand, when the pHs were below the pK value, such as pH 5 and pH 5.6, the carboxylic acid would be deionized and thus the size of head group of CHEMS decreased. Accordingly, the DOPE bilayer would be destabilized into non-bilayer structures, giving rise to a higher release.

5. Dynamic Light Scattering

Fig. 6 shows the change in the size of liposome with pH. In case of DOPE/CHEMS liposome free of HmGOD, the size was almost constant with respect to pH when pH decreased from 8.0 to 6.0 and the value was around 250-300 nm. When pH decreased to 5.0, the size markedly increased and it reached values greater than 1,000 nm. The deionization of CHEMS in the acidic condition would result in the destabilization of DOPE liposomal membrane, leading to large clusters. Following previous reports, the DOPE liposomes are known to destabilize into hexagonal phase when the complementary molecules lose their function of stabilization [21,22]. Accordingly, the increased size would be due to hexagonal phases. Similarly to the pH-dependent change in size of DOPE/CHEMS liposome, the size of liposome of DOPE/CHEMS/HmGOD increased with decreasing pH. The size increment, however, was not as much as the case of DOPE/CHEMS liposome. Following the results of the release experiment, more than 90% released from DOPE/CHEMS/HmGOD. It means that the liposome was completely destabilized into a non-vesicular hexagonal phase. Nevertheless, the size did not increase as much as the size of DOPE/CHEMS liposome did. HmGOD is a kind of surfactant and it would act as a dispersant since it has hydrophobic alkyl chains. Hence, it could prevent liposomal lipids from clustering into large particles. The change of the closed vesicle to non-vesicular hexagonal phase could account for the pH-sensitive release described in the section of pH-dependent calcein release. Obviously, pH 5, pH 5.6 and pH 6.0, where calcein released markedly, are in accordance with pHs, where the size increased significantly.

CONCLUSION

Glucose oxidase was hydrophobically modified and the modified enzyme was incorporated into the bilayer membrane of DOPE/CHEMS liposome. The liposomes retained their contents at neutral condition and they released extensively at acidic condition. Therefore, the pH-sensitive liposome incorporating hydrophobically modified glucose oxidase could be used as a glucose-sensitive carrier.

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REFERENCES

1. E. O. Lee, J. G. Kim and J. D. Kim, *J. Biochem.*, **112**, 671 (1992).
2. A. Huang, Y. S. Tsao, S. J. Kennel and L. Huang, *Biochim. Biophys. Acta*, **716**, 140 (1983).
3. M. J. Choi, H. S. Han and H. Kim, *J. Biochem.*, **112**, 694 (1992).
4. M. Maeda, A. Kumano and D. A. Tirrell, *J. Am. Chem. Soc.*, **110**, 7455 (1988).
5. M. B. Yatvin, J. N. Weinstein, W. H. Dennis and R. Blumenthal, *Science*, **202**, 1290 (1978).
6. J. N. Weinstein, R. L. Magin, R. L. Cysyl and D. S. Zaharko, *Cancer Res.*, **40**, 1388 (1980).
7. H. Hayashi, K. Kono and T. Takagishi, *Biochim. Biophys. Acta*, **1280**, 127 (1996).
8. J. C. Kim, S. K. Bae and J. D. Kim, *J. Biochem.*, **121**, 15 (1997).
9. K. Kono, R. Nakai, K. Morimoto and T. Takagishi, *Biochim. Biophys. Acta*, **1416**, 239 (1999).
10. K. Kono, A. Henmi, H. Yamashita, H. Hayashi and T. Takagishi, *J. Control. Release*, **59**, 63 (1999).
11. J.-C. Kim, M.-S. Kim and J.-D. Kim, *Korean J. Chem. Eng.*, **16**, 28 (1999).
12. M. Zignani, D. C. Drummond, O. Meyer, K. Hong and J. C. Leroux, *Biochim. Biophys. Acta*, **1463**, 383 (2000).
13. R. M. Straubinger, N. Düzgünes and D. Papahadjopoulos, *FEBS Lett.*, **179**, 148 (1984).
14. D. Liu and L. Huang, *Biochim. Biophys. Acta*, **1022**, 348 (1990).
15. D. C. Litzinger and L. Huang, *Biochim. Biophys. Acta*, **1113**, 201 (1992).
16. H. Ellens, J. Bentz and F. C. Szoka, *Biochemistry*, **23**, 1532 (1984).
17. P. R. Cullis and B. De Kruijff, *Biochim. Biophys. Acta*, **559**, 399 (1979).
18. A. Baszkin, M. M. Boissonnade, V. Rosilio, A. Kamyshny and S. Magdassi, *J. Colloid Interface Sci.*, **190**, 313 (1997).
19. G. Hermanson, *Bioconjugate technigues*, Academic Press, San Diego, California (1996).
20. M. Kriechbaum, H. J. Heilmann, F. J. Wientjes, M. Hahn, K. D. Jany, H. G. Gassen, F. Sharif and G. Alaeddinoglu, *FEBS Lett.*, **255**, 63 (1989).
21. J. C. Kim and J. D. Kim, *Colloid Surf. B: Biointerfaces*, **24**, 45 (2002).
22. J. C. Kim, M. S. Kim and J. D. Kim, *Korean J. Chem. Eng.*, **16**, 28 (1999).