ELSEVIER

Colloids and Surfaces A: Physicochem. Eng. Aspects 212 (2003) 227–234

COLLOIDS AND SURFACES A

www.elsevier.com/locate/colsurfa

Microcalorimetric studies on the physical stability of poly-ethylene glycol-grafted liposome

Der-Zen Liu [a,*], Yi-Ling Hsieh [a], Sheng-Yen Chang [a], Wen-Yih Chen [b]

[a] *Graduate Institute of Biomedical Materials, Taipei Medical University, Taipei, Taiwan, ROC*
[b] *Department of Chemical Engineering National Central University, Chung-Li, Taiwan, ROC*

Received 1 November 2001; accepted 24 June 2002

Abstract

Liposomes were prepared from mixtures of egg-PC, cholesterols and distearoyl-phosphatidyl-ethanolamine covalently attached poly-ethylene glycol (PEG) with a molecular of weight 2000 (DSPE–PEG_{2000}). In this work examines how PEG_{2000}-grafted lipids affect the surface properties of the egg phosphatidylcholine (egg-PC) liposomal bilayer membrane through zeta potential and interaction potential measurements using microcalorimetry. Experimental results demonstrate that the absolute value of the zeta potential of PEG_{2000}-grafted PC liposomes decreased from −19 to −8 mV when increasing DSPE–PEG_{2000} from 0 to 7 mol fraction, and the repulsive interaction potential of PEG_{2000}-grafted PC liposomes decreased compared with those liposomes without PEG-grafting. However, the phenomenon of fusion between the liposomes incorporated with PEG-grafted PC was reduced. In brief, this result of fusion is contrary to the expectation of the interaction potential measurement; therefore, we believe that the steric hindrance of the grafted PEG_{2000} molecules on the liposomal surface contribute to a major imposition on the approach between liposomal surface and the formation of inverted micelles which are suggested as the necessary steps of liposome fusion.

Keywords: Poly-ethylene glycol-liposome; Physical stability; Zeta potential; Interaction potential

1. Introduction

Liposomes (lipid vesicles) have a unique closed structure and special physical and chemical properties. As early as 1973, researchers recognized that liposomes could be an effective drug delivery vesicle [1]. The way the structure of liposomes minics that of cell membranes enables liposomes to serve a model for physical and biochemical studies of biomembranes [2–5]. However, in vitro and in vivo instability of liposomes has limited not only their widespread application but also some of their potential advantages. Recently, extensive research has sought to improve the stability of liposomes resulting in novel liposome systems with better in vitro and in vivo physical stabilities.

Liposome stability plays important roles at various stages of medical practice. However,

* Corresponding author. Tel.: +886-2-2739-0581
E-mail address: tonyliu@tmu.edu.tw (D.-Z. Liu).

0927-7757/02/$ - see front matter
PII: S0927-7757(02)00306-0

liposomes are somewhat biologically unstable as a parenteral drug delivery system owing to their rapid uptake and clearance from circulation by cells of the mononuclear phagocytic system (MPS) located mainly in the liver and spleen [6,7]. A major breakthrough occurred in 1990, as liposomal bilayer membranes containing lipids with covalently attached poly-ethylene glycol (PEG-lipid) by which the membrane surface steric inhibits protein and cellular interactions with liposomes drastically prolonging the blood circulation time when injected in animal models [8]. These vesicles are the so-called Stealth liposome [9] or sterically stabilized liposomes (SSL) [10]. The steric repulsion of the stealth liposome appears to not only stabilize liposome suspensions against aggregation but also inhibit the adsorption of various opsonins onto the liposomal surface and degrade biological interactions.

Considering the interactive forces between liposomes, incorporating lipids with bulky polar headgroups increases the repulsive forces between lipid bilayers, as measured by X-ray, neutron diffraction, micro-pipette manipulation [11], and small-angle X-ray scattering (SAXS) [12]. Needham et al. [13] demonstrated that including 10 mol% of monosialogangliosides (G_{M1}) in PC bilayers increased the interbilayer separation more then two fold as compared to PC bilayers alone, while PC bilayers containing 5 mol% of DSPE–PEG_{1900} increased the separation even more. Furthermore, Kuhl et al. [14] focused on short-chain ethylene oxide headgroups grafted onto liposomal surfaces; according to their results, incorporating a slight amount of dielaidoylphosphatidylethanolamine - polyethylene oxide (DEPE–PEO_{45}) lipid greatly enhances liposome stability. Kuhl et al. contended that short polymer chains can provide a physical barrier or force buffer around the bilayer to prevent contact between liposomes. In addition, both Kenworthy et al. [15] and Lasic [16] proved that the attachment of PEG molecules to liposomal surfaces can strongly degrade the attractive forces and increase the repulsive steric forces imposed on each other between liposomes. However, no direct evidence or measurements have been presented on the effect of PEG-lipid on the interactive forces between liposomes. The effects of PEG-lipids on the interactive forces include the electrostatic force, van der Waal force, and even the short-range hydration force.

Therefore, as to the investigation aims of this study, we examined the interaction potential by measuring the dilution heat of PC liposomes incorporated with various amounts of DSPE–PEG_{2000} by isothermal titration calorimetry (ITC). The parameter of interaction potential for PC liposomes was then calculated from the empirical dilution heat data combined with a simple statistical thermodynamic model [17]. To sum up, aggregation and fusion behaviors are explained by the interaction potential between PC liposomes incorporated with varying amounts of DSPE–PEG_{2000}.

2. Materials and methods

2.1. Materials

Distearoyl-phosphatidylethanolamine (DSPE) covalently attached poly(ethylene-glycol) (DSPE–PEG_{2000}) was supplied by Shearwater Polymers, while egg-phosphatidylcholine (PC) ($\sim$99%), cholesterol, and α-tocopherol were purchased from Sigma (USA). Chloroform, methanol, Tris, and sodium chloride were obtained from Merk (Germany). All chemicals were used as received without further purification.

2.2. Preparation of stealth liposome suspension solution

Large multilamellar PEG_{2000}-grafted liposomes were prepared with the method reported by Bangham et al. [18]. Briefly, the lipid mixture, 0.59 mg ml^{-1} of α-tocopherol, egg PC (3.75 mg ml^{-1}), cholesterol (9.6 mg ml^{-1}), and various amounts of DSPE–PEG2000 (from 0 to 7 mol%) were dissolved in a chloroform/methanol mixture (2:1 volume ratio). The solvent was evaporated in a round-bottomed flask at 50 °C, and nitrogen was used to blow over the dried lipid film for approximately 30 min in order to remove traces of organic solvents. The lipid bilayer film was then hydrated in 10 ml of buffer solution composed of

20 mM Tris, 100 mM NaCl and 1 mM EDTA (with the pH being adjusted to 7.4 with HCl) at 323 K.

Frozen then thawed (FT) the MLVs liposomes in order to reduce lamellarity [19]. These liposomes were subjected to freezing (in boiling nitrogen) and thawing (in a water bath at 323 K) five times, and were then sonicated for 30 min using Bandelin HD-200 sonication probe at a power setting of KE 76/D under nitrogen to prepare small unilamellar vesicles (SUVs). The temperature was maintained at 323 K by circulating the water in round-bottomed flask. Then the final optically clear suspension was filtered through a 0.22-μm Millipore filter for three cycles.

In our experimental system, liposomes were added to the same amounts of a-tocopherol and cholesterol. α-Tocopherol is believed to act as an antioxidant in lipid membranes to avoid oxidative damage caused by interactions between free radicals and PC [20]. Therefore, pressure on the chemical stability was diminished.

2.3. Calculation of the number of liposomes

The number of PC lipids within a liposome can be estimated by calculating the ratio between the head-group surface areas of egg-PC (0.70 nm^2) [21], distearoyl-phosphatidylethanolamin (0.43 nm^2) [15], and cholesterol (0.28 nm^2) [21]. This calculation allows us to estimate the number of liposomes originating from 3.75 mg ml^{-1} of egg PC [22].

2.4. Determination of particle size distribution of liposomes by PCS

Liposome size distribution was determined by photon correlation spectroscopy (PCS) that employed a zetasizer 3000 (Malvern Instruments, UK). The samples (liposome suspension solution) were diluted in a buffer solution until their concentrations were low enough to avoid hydrodynamic and electrostatic interactions between vesicles but high enough that reliable autocorrelation functions from PCS measurements could be achieved. All PCS measurements were taken at 298 K and the viscosity of the sample solution was 1 cp. The liposome suspension solution was placed in a sample holder, and laser light (helium-neon laser of wavelength 633 nm and 5 mW) was focused on the sample. Scattered light was detected at 90° to the incident beam using a photomutiplier tube. Five replicate measurements were made of each liposome suspension, and the mean average particle diameter and polydispersity index were obtained using Malvern software supported comulant analysis.

2.5. γ-potential measurements of liposome

The electrophoretic mobility and zeta potential of liposome suspension solutions were determined by laser Doppler electrophoretic mobility measurements with the zetasizer 3000 (Malvern Instruments).

2.6. Dilution heat by ITC and interaction potential of PEG-grafted liposomes

The deviation of a solution from ideal behavior due to two-body interactions in a suitable concentration of solution can described by vant Hoff's law [23]. Therefore, it is desirable to obtain the value of a second virial coefficient and b_2/B_0 as the interaction potential (ε) index between liposomes. This study extend own development of measuring second virial coefficient of the colloidal system by isothermal titration calorimetry to the liposome system [17,24].

PEG-grafted liposome suspension solution dilution heat was determined by applying isothermal titration calorimetry (ITC). Briefly, 2 ml of liposome suspension solution was placed in an ampoule. A series of 150 μl buffer solutions was then titrated into the dispersion liposomes suspension solution using a Hamilton microliter syringe at 30-min intervals after the ampoule and the heat sink reached thermo-equilibrium. The liposome suspension solution was titrated five times per experiment. All experiments were performed at temperatures of 298 and 310 K, respectively.

Dilution heats of PEG-grafted liposome suspension solutions with the number density of the liposome suspension solution can be generally expressed as a function of a second order poly-

nomial in Eq. (1):

$$d\frac{q}{Nk_BT}\Big/\frac{d\rho}{} \cong d\frac{E}{Nk_BT}\Big/\frac{d\rho}{} = b_2 + b_3\rho + b_4\rho^2 \quad (1)$$

For an open liquid system, pressure changes and titration volume are neglected in the system total volume. The observed dilution heat of each titration is comparable to the internal energy change. Furthermore, the internal energy change can be expressed by the virial coefficient and the number density in Eq. (2):

$$\frac{E}{Nk_BT} = \frac{3}{2} - T\sum_{i=1}^{\infty}\frac{1}{i}\frac{dB_{i+1}}{dT}\rho^i \quad (2)$$

Comparing Eq. (1) with Eq. (2) reveals that the coefficient of the dilution heat polynomial can be affiliated with the virial coefficients as in Eq. (3):

$$b_2 = -T\frac{dB_2}{dT} \quad (3)$$

According to statistical mechanics, the relation between the second virial coefficient and the interaction potential energy function $U(r)$ is

$$B_2(T) = -2\pi\int_0^{\infty}\left[\exp\left(-\frac{U(r)}{k_BT}\right) - 1\right]r^2dr \quad (4)$$

and if a square-well energy potential function is picked and plugged into Eq. (4), then Eq. (3) can be rewritten as:

$$b_2 = -T\frac{dB_2}{dT} = -B_0(\lambda^3 - 1)e^{\varepsilon/k_BT}(\varepsilon/k_BT) \quad (5)$$

$$b_2/B0 = (\lambda^3 - 1)e^{\varepsilon/k_BT}(\varepsilon/k_BT) \quad (6)$$

where $B_0 = (16/3)\pi R_{HS}^3$, $\lambda = (2R_{HS} + \sigma)/2R_{HS}$ and σ, ε denotes the width and depth (or energy barrier) of the square-well energy potential function.

In our experimental system, the value of b_2/B_0 can be described qualitatively from the interaction potential (ε) between PEG_{2000}-grafted PC liposomes.

3. Results and discussion

3.1. Zeta potential measurements

This study measured the zeta potential (ζ-potential) of PEG-grafted PC liposomes incorporated with varying amounts of DSPE–PEG_{2000}. The data (shown in Table 1) correspond to the results of Carrion et al. [25] and McLaughlin et al. [26] indicating that there is a weak electrostatic repulsive force between PC liposomes at pH 7.4. This experiment also demonstrated that the incorporation of DSPE–PEG_{2000} into the PC bilayer decreases the negative zeta potential. The absolute value of the zeta potential of PEG_{2000}-grafted liposomes decreased from −19 to −8 mV as the concentration of DSPE–PEG increased from 0 to 7 mol%. This observation can be explained by the exposed PEG chains shielding the negatively charged liposomal surface, thus decreasing the absolute zeta potential of the liposome [27]. Moreover, Woodle et al. [28] believed that the phenomenon was attributable to an increase in the hydrodynamic radius from the steric effects of PEG. Furthermore, Kostarelos et al. [29] reported that adsorption or incorporation of the tri-block copolymer (PEO–PPO–PEO) shifted the shear plane away from the liposomal surface, and, subsequently, reduced the absolute values of the zeta potential of the liposome.

3.2. Interactions between phosphatidylcholine liposomes

Although the interactive forces between liposomes have been thoroughly discussed [30,31],

Table 1
Values of zeta potential of PEG-grafted PC liposomes with various amount of incorporated DSPE–PEG_{2000} (values are the mean ± standard deviation, $n = 3$)

Mole fraction of DSPE–PEG (mol%) [DSPE–PEG/PC + VitE + Chol]	Zeta potential (mV) (298 K)
0	−18.8 ± 1.6
3	−13.2 ± 1.3
5	−10.3 ± 1.2
7	−8.7 ± 0.7

they have not yet been able to directly measure them. For that, this study proposed a thermodynamic approach to examine how PEG_{2000}-grafted lipids affect the interaction potential, not the interaction forces, of intervesicle interactions between PEG_{2000}-grafted liposomes. The interaction potential can be used to explain the stability of PEG-grafted PC liposomes thermodynamically. The isothermal titration calorimetry was utilized to measure the dilution heat of PC liposomes without and with various levels of added DSPE–PEG_{2000} and to derive values of b_2 and b_2/B_0 from the dilution heat measurements (as discussed in Section 2). Differences in depth (ε) and location of the interaction potential energy minimum between any two liposomes caused by adding different amounts of DSPE–PEG_{2000} are discussed herein.

The ITC experiments of this study show that the system of PC liposomes with or without added DSPE–PEG_{2000} has a positive b_2 value as indicated in Table 2. This finding reveals that the overall net interactive forces and potential between any the two PC liposomes is repulsive, thus preventing aggregation or fusion and enhancing the liposomal stability. Previously, Needham et al. [32] confirmed that incorporating DSPE–PEG_{1900} increases both the magnitude and the range of the repulsive pressure between adjacent SOPC/PC bilayers. They attributed the repulsive pressure to the steric repulsive pressure since it did not result from increasing the electrostatic, undulation, or hydration repulsive forces. However, in our experimental system, the value of b_2/B_0 (Table 2) decreased when adding DSPE–PEG_{2000} to PC liposomes; this observation shows that the repulsive interaction potential (ε) is negative (as an energy barrier), and the repulsive interaction potential of PEG_{2000}-grafted liposome is decreased (lower values of $|\varepsilon|$ and σ). These results indicate that the net interactive repulsive potential or energy barrier decreases when DSPE–PEG_{2000} is incorporated into PC liposomes. Possible explanations are that the electrostatic repulsive forces between PEG_{2000}-grafted PC liposomes decrease and hydrophilic interactions between PEG-headgroups increase

3.3. Changes of liposomal particle size

Experimental results show that the initial liposomal size decreased when the amount of DSPE–PEG_{2000} of the liposome composition was increased (Fig. 1). This result is considered due to a steric repulsion among PEG chains exposed from the outer and inner leaflet of the liposomal bilayer membrane. PEG chains exposed from the outer leaflet of the bilayer menbrane will increase the liposome particle curvature, whereas the PEG chains exposed to the inner leaflet do the opposite. In fact, more DSPE–PEG_{2000} exists in the outer leaflet than in the inner leaflet of liposomal bilayer membranes [27]. Therefore, adding DSPE–PEG_{2000} reduces the initial liposomal size.

Fig. 1 displays size changes of PC liposomes with or without added DSPE–PEG_{2000} at 298 K as a function of incubation time. The experimental results imply that PC liposomes can hold the electrostatic repulsive forces to temporarily pause in the secondary minimum state [33] (or in reversible aggregation) from the interaction poten-

Table 2
Values of second-order polynomial b_2 and the B_0 of virial coefficients of PEG-grafted PC liposomes with various amount of incorporated DSPE–PEG_{2000}

Reaction temperature	Mole fraction of DSPE–PEG (mol%) [DSPE–PEG/PC+VitE+Chol]	b_2 (nm^3)	B_0 virial coefficient (nm^3)	b_2/B_o
298 K	0	7.5×10^{12}	1.7×10^{6}	4.4×10^{6}
	3	1.1×10^{12}	9.9×10^{5}	1.1×10^{6}
	5	5.2×10^{11}	7.2×10^{5}	7.2×10^{5}
	7	3.8×10^{11}	6.6×10^{5}	5.8×10^{5}

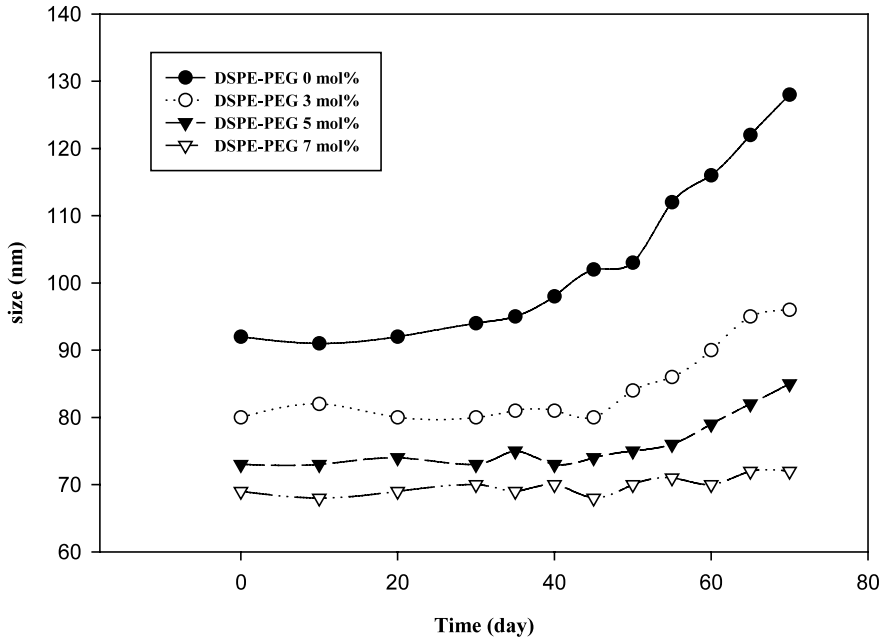

Fig. 1. Sizes of PC liposomes with various amount of incorporated DSPE–PEG_{2000} at 298 K with time. The number density of the liposome suspension solution was 10^{13} liposomes ml^{-1} (each point value is the average of three experiments).

tial perspective. Finally, PC liposome will reach the primarily minimum state (or irreversible aggregation) due to the effective collisions among liposomes, and the effective collisions may be promoted by the oxidation of lipids.

There are two continuous steps for liposomes to be fused, as suggested: (i) liposomes have to be aggregated, and (ii) intermediate structures such as inverted micelles or inverse hexagonal structures have to occur after close juxtaposition and local perturbation in the liposomal bilayer packing of the aggregated liposomes [34]. In our experiment, results show that incorporating DSPE–PEG_{2000} into the PC bilayer decreases the repulsive interaction potential between PEG-grafted liposomes according to the data of zeta potential and the b_2/B_0 value obtained. But, fusion between PEG-grafted PC liposomes was inhibited compared to that for PC liposomes without PEG-grafting at the temperature of 298 K (Fig. 1). Simply speaking, incorporating DSPE–PEG_{2000}. into the PC bilayer decreases the liposomes interaction energy barrier compared with those liposomes without added DSPE–PEG_{2000} from the perspective of interaction potential. An explanation for this apparent inconsistency is that the steric hindrance of the grafted PEG_{2000} molecular on the liposomal surface imposes resistance to the approach between the liposomal surface and the formation of inverted micelle which was suggested as a necessary step for liposome fusion.

4. Conclusions

This research used the interaction potential to show the physical stability of DSPE–PEG_{2000} liposome. In this study, we demonstrate that the incorporation of a small amount of DSPE–PEG_{2000} into the PC liposomal bilayer decreases the absolute value of the zeta potential and the repulsive interaction potential. These results indicate that the decrease in the repulsive potential can be attributed to a decrement in the electrostatic

potential and an increment in hydrophilic attractive interactions occurring between PEG_{2000}-grafted PC liposomes. However, the fusion taking place between PEG_{2000}-grafted PC liposomes is reduced as a small amount of DSPE–PEG_{2000} is incorporated into the PC liposomal bilayer. Therefore, it is our strong belief that the phenomenon does not result from repulsive forces between PEG_{2000}-grafted PC liposomes. Instead, the major contribution is due to PEG_{2000} grafted onto the liposomal surface that acts as a steric barrier inhibiting the close approach of liposomal bilayers between liposomes. Furthermore, our work has proven the feasibility of isothermal titration calorimetry for studies of liposomal interactions and provides more information for discussions of liposomal interaction mechanisms.

Acknowledgements

The authors would like to thank the Bureau of Animal and Plant Health Inspection and Quarantine Council of Agriculture no. 91AS-3.1.3-BQ-B1.

Appendix A: Nomenclature

q	dilution heat of DSPE–PEG_{2000} egg-PC liposome suspension solution (mJ)
E	internal energy (mJ)
N	number of liposomes
k_B	Boltzmann constant (J K^{-1})
T	absolute temperature (K)
ρ	number density of liposome suspension solution (number ml^{-1})
b_2	second virial coefficient of the fitting polynomial of the dilution heat of a DSPE–PEG_{2000} egg-PC liposome suspension solution
B_0	virial coefficient
R_{HS}	the radius of liposome (nm)
σ	the width of the square-well potential function
ε	the depth of the square-well potential function (or energy barrier)

References

[1] G. Gregoriadis, FEBS. Lett. 36 (1973) 292.
[2] M. Costuleanu, E. Brailoiu, C.M. Filipeanu, O. Baltatu, S. Slatineanu, L. Saila, L.M. Nechifor, D.D. Branisteanu, Eur. J. Pharmacol. 281 (1995) 89.
[3] R.M. Fielding, Clin. Pharmacokinet. 21 (1991) 155.
[4] D.D. Lasic, H. Strey, M.C. Stuart, R. Podgornic, P.M. Frederic, J. Am. Chem. Soc. 119 (1997) 832.
[5] C. Karau, M. Petszulat, P.C. Schmidt, Int. J. Pharm. 128 (1996) 89.
[6] P.L. Beaumier, K.J. Hwang, Biochim. Biophys. Acta 731 (1983) 23.
[7] C.R. Alving, N.M. Wassef, J. Liposome Res. 2 (1992) 383.
[8] T.M. Allen, C. Hansen, F. Martin, C. Redemann, A. Yau-Young, Biochim. Biophys. Acta 1066 (1991) 29.
[9] M.C. Woodle, D.D. Lasic, Biochim. Biophys. Acta 1113 (1992) 171.
[10] M.C. Woodle, Adv. Drug Deliv. Rev. 32 (1998) 139.
[11] D.D. Lasic (Ed.) Liposome: from Physics to Application, Elsevier, Amsterdam, 1993.
[12] G. Blume, J. Liposome Res. 2 (1992) 355.
[13] D. Needham, K. Hristova, T.J. McIntosh, M. Wu, N. Dewhirst, D.D. Lasic, J. Liposome Res. 2 (1992) 411.
[14] T.L. Kuhl, D.E. Leckband, D.D. Lasic, J.N. Israelachvili, Biophys. J. 66 (1994) 1479.
[15] A.K. Kenworthy, K. Hristova, D. Needham, T.J. McIntosh, Biophys. J. 68 (1995) 1921.
[16] D.D. Lasic, F. Martin, Stealth Liposome, CRC Press, Boca Raton, FL, 1995.
[17] W.Y. Chen, C.S. Kuo, D.Z. Liu, Langmuir 16 (2000) 300.
[18] A.D. Bangham, M.M. Standish, J.C. Watkins, J. Mol. Biol. 13 (1965) 238.
[19] W. Zhang, E.C.A. Van Winden, J.A. Bouwstra, D.J.A. Crommelin, Cryobiology 35 (1997) 277.
[20] T. Koga, J. Terao, Biosci. Biotechnol. Biochem. 60 (1996) 043.
[21] F. Szoka, D. Papahadjopoulos, Annu. Rev. Biophys. Bioeng. 9 (1980) 467.
[22] R. Xu, Langmuir 14 (1998) 2593.
[23] W. Bruns, Macromolecules 29 (1996) 2641.
[24] D.Z. Liu, W.Y. Chen, L.M. Tasi, S.P. Yang, Colloids Surf. A 172 (2000) 57.
[25] F.J. Carrion, A..D. Maza, J.L. Parra, J. Colloid Interface Sci. 164 (1994) 78.
[26] A. McLaughlin, W.K. Eng, G. Vaio, T. Wilson, S. McLaughlin, J. Membr. Biol. 76 (1983) 183.
[27] A. Yoshida, K. Hashizaki, H. Yamauchi, H. Sakai, S. Yokoyama, M. Abe, Langmuir 15 (1999) 2333.
[28] M.C. Woodle, L.R. Collins, E. Sponsler, N. Kossovsky, D. Papahadjopoulos, F.J. Martin, Biophys. J. 61 (1992) 902.
[29] K. Kostarelos, Th.F. Tadros, P.E. Luckham, Langmuir 15 (1999) 369–376.

[30] J.N. Israelachvili, Intermolecular and Surface Forces, Academic Press, New York, 1992.
[31] T.L. Kuhl, D.E. Leckb, D.D. Lasic, J.N. Israelachvili, Biophys. J. 66 (1994) 1479.
[32] D. Needham, T.J. McIntosh, D.D. Lasic, Biochim. Biophys. Acta 1108 (1992) 40.
[33] S. Nir, J. Bentz, J. Wilschut, N. Duzgunes, Prog. Surf. Sci. 13 (1983) 1.
[34] P.M. Frederik, A.J. Verkleij, M.C.A. Stuart, Biochim. Biophys. Acta. 979 (1989) 275.