SERUM STABILITY OF NON-CATIONIC LIPOSOMES USED FOR DNA DELIVERY

M.M. MADY

Biophysics Department, Faculty of Science, Cairo University, Giza, Egypt

Abstract. It is well known, that serum components destabilize liposomal membranes. Therefore, most *in vitro* transfection protocols avoid serum, which make the extrapolation to *in vivo* situations difficult. In this study, we investigated the stability of different anionic liposomal formulations including AVEs (Artificial Viral Envelopes) in 100% fetal calf serum (FCS) by measuring the efflux of the entrapped carboxyfluorescein (CF). The results suggest that lysis of AVE liposomes in serum can be decreased by incorporating sphingomyelin (SM) in the liposomal membrane and using highly lipid doses.

Key words: Liposomes, sphingomyelin, stability, serum, carboxyfluorescein.

INTRODUCTION

Liposomes and lipid-based drug delivery systems have been used extensively over the last decade to improve the pharmacological and therapeutic activity of a wide variety of drugs. More recently, this class of carrier system has been used for the delivery of relatively large DNA and RNA-based drugs, including plasmids, antisense oligonucleotides and ribozymes. Non viral, lipid-based vectors have attracted particular attention in the field of gene therapy for a number of reasons, including safety, lack of antigenicity, versatility, and ease of handling [18, 19]. Müller *et al.* [25] have designed anionic liposomes, based on the composition of anionic retroviral envelopes [7] (AVEs), which display good transfection efficiency for cultured cells in the presence of serum. These AVE liposomes have special fusogenic properties, allowing them to transport encapsulated or associated drugs into cells. Also, AVE liposomes effectively transport protaminecomplexed-oligonucleotides into HepG2 cells [23, 35]. Liposomal gene delivery system with a net negative surface potential should exhibit less nonspecific tissue uptake and a better overall biocompatibility than cationic carrier systems [28]. However, the potential use of non-viral vectors as gene carriers by intravenous injection is limited by their low stability in bloodstream. Therefore, most *in vitro* transfection protocols avoid serum, which make the extrapolation to *in vivo* situations difficult.

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The stability of liposomes in the presence of serum can be thought as a first approximation to gain insight into the behavior of liposomes in biological fluids. To optimize the potential of AVE liposomes as gene carriers, it is important to characterize their stability in terms of controlled release of their contents *in vitro*, simulating physiological conditions *in vivo* [21]. In the present work, we have quantified the disruptive effects of 100% FCS on the tested liposomes and have studied whether modifications (phosphatidylcholine (PC), sphingomyelin (SM), and cholesterol (CHOL) content) in their phospholipid composition might produce liposomes with an increased carrier potential for application *in vivo*.

MATERIALS AND METHODS

1,2-dilauroyl-sn-glycero-3-phosphoethanolamine (DLPE), dioleoyl phosphatidyl serine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and Bovine sphingomylin (SM) were obtained from Avanti Polar-Lipids Inc. (Ottawa, Canada). Tris ultra pure buffer and sodium deoxycholate were obtained from ICN Biomedicals. 4(5)-carboxyfluorescein (CF) was purchased from Fluka (Germany). All reagents were of analytical reagent grade.

PREPARATION OF LIPOSOMES

AVE^{TM}-3 liposomes are composed of equimolar amounts of DLPE, DOPS and CHOL [35]. Liposomes were formulated according to the well-established method of extrusion [32]. In short, the appropriate phospholipid composition was mixed in organic solvent (chloroform) in a 50 ml round flask. The organic solvent was evaporated to dryness by a Mini-Rotavapor. The resulting thin lipid film was hydrated with tris buffered saline (10 mM, 140 mM NaCl) containing 100 mM CF at pH 7.4. The resulting lipid suspension was extruded through 200 nm nucleopore membranes, using a commercially available extruder device (Liposofast, Avestin Inc., Canada). Particle size measurement was done by dynamic laser light scattering and the size was approximately in the range of 200 ± 30 nm. Free CF was removed by dialysis in Tris buffered saline containing 10 mM Tris and 140 mM NaCl at pH 7.4 after extrusion [26].

LIPOSOME STABILITY ASSAY

Liposome stability is defined as their capability to retain the structural integrity of the lipid bilayer and prevent leakage of their aqueous contents [38]. The release experiments were run immediately after the separation of free CF from encapsulated CF in liposomes. The liposomes were incubated with 100% FCS at 37 °C. At indicated time points, 10 µl of the suspension were diluted 200 times with Tris buffered saline and the fluorescence intensity (F; excitation at 470 nm,

emission at 520 nm) was measured on Spectrofluorometer LS 50B. To lyse liposomes completely, 20 µl of 10% sodium deoxycholate was added and the total fluorescence F_{total} (corresponding to 100% release) was measured. The percentage of CF release was calculated by dividing F by F_{total}. A plot of % CF release versus time was linear until approx. 20–30% CF remained trapped in liposomes, indicating that the initial 70–80% of leakage is a single exponential process. Estimates of half-time $t_{½}$ (time at which 50% of CF is still encapsulated) was made from the initial linear portion of the curve [20].

RESULTS AND DISCUSSION

The half time for 100 mM CF from osmotically stable liposomes in the presence of buffer or serum at 37 °C was examined and the results are presented in Table 1. Comparison between liposomes incubated with 100% FCS or Tris buffered saline showed that serum promotes CF release from liposomes. The $t_{½}$ were 23.85 hrs and more than 451 hrs for DLPE/SM/PS/CHOL (1:1:1:1) liposomes in 100% FCS and Tris buffered saline, respectively (Table 1). This is true in other tested samples, that the release is very slower in Tris buffer saline than in 100% FCS (Table 1). These results agree with previous data [2, 9] and might be due to the transfer of lipids from liposomes to high-density lipoproteins (HDL), which is a major factor responsible for the disruptive effect of serum on the integrity of liposome bilayer. The process of this lipid transfer seems to be mediated by apolipoproteins [14, 29, 34]. Moreover, liposomes are eliminated from circulation by association with certain plasma proteins, called opsonins, which promote macrophage uptake, or by direct adsorption to the cell surface and ultimate phagocytosis by macrophages in liver and spleen [24]. Activated complement components also induce liposome leakage by forming pores in the liposomal membrane [13, 17].

Table 1

Half-time of leakage for CF from liposomes measured at 37°C in 100% FCS and in Tris-buffered saline (10 mM Tris and 140 mM NaCl at pH 7.4).

	Half-leakage times ($t_{½}$) hrs	
Sample	100% FCS	Tris-Buffer
DLPE+PC+PS+CHOL (1:1:1:1) 1mg/ml	13.32 ± 0.55	58.77 ± 1.26
DLPE+PC+PS+CHOL (1:1:1:1) 0.33 mg/ml	12 ± 0.23	55.5 ± 0.53
DLPE+PC+PS+CHOL (1:2:1:1) 1mg/ml	18.92 ± 0.7	104.57 ± 0.1
DLPE+PC+PS+CHOL (1:2:1:1) 0.33 mg/ml	14.8 ± 0.24	79.06 ± 0.3
DLPE+SM+PS+CHOL (1:1:1:1) 0.25 mg/ml	23.85 ± 7.7	> 451
DLPE+PS+CHOL (1:1:1) 0.33 mg/ml	12.5 ± 5	127.6 ± 23.2
DLPE+SM+PS (1:1:1) 1 mg/ml	4 ± 1	
DLPE+SM+PS (1:1:1) 0.33 mg/ml	1.916 ± 0.02	45.8 ± 0.8
DLPE+PS (1:1:1) 0.33 mg/ml	0.97 ± 0.19	20.386

The SM containing liposomes were more stable than other tested liposomes. The $t_{½}$ in 100% FCS were 23.85 hrs, 14.8 hrs, 12 hrs and 12.5 hrs for DLPE /SM/PS/CHOL (1:1:1:1), DLPE/PC/PS/CHOL (1:2:1:1), DLPE/PC/PS/CHOL (1:1:1:1), and DLPE/PS/CHOL (1:1:1:1), respectively (Fig. 1). Including sphingomyelin in liposomes decreased the rate of transfer of lipid to HDL, leading to the increased stability of these liposomes. This can be interpreted as being due to intermolecular hydrogen bonding between the sphingosine back-bones of the sphingomyelin molecule [1]. Sphingomyelin (SM) has a molecular shape and hydration properties similar to PC, but, in the presence of cholesterol, SM is thought [22, 37] to be involved in domain formation and be enriched in detergentresistant membranes or “rafts” [30, 33]. SM and CH (1/1) complexes form stable, well-packed lamellar structures [12].

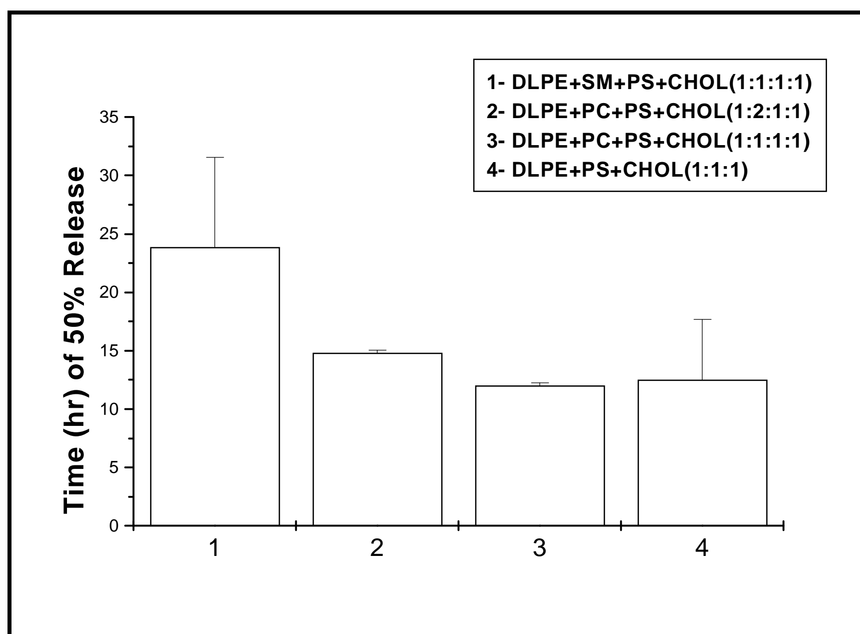

Fig. 1. – Half-leakage times ($t_{½}$) for CF from different liposomal formulations incubated with 100% FCS at 37 °C.

Like PC, SM has phosphocholine as its zwitterionic polar headgroup. In PC, both hydrocarbon chains are ester-linked to a glycerol backbone, the sn-1 chain usually is saturated (e.g. palmitate or stearate), and the sn-2 chain usually contains one or more cis double bonds. In contrast, in SM, the sphingoid base serves the dual role as both interfacial backbone and nonpolar hydrocarbon chain. Structural features that may impact on SM’s ability to form specialized membrane environments include an abundance of long, saturated acyl chains that sometimes provide marked intramolecular chain-length asymmetry and interfacial functional groups that can donate and accept hydrogen bonds with neighboring lipids [6].

Cholesterol is likely to act as a phosphocholine headgroup spacer because the sterol polar headgroup is small compared to SM/PC's phosphocholine.

There is intermolecular hydrogen bonding between cholesterol's 3-OH group and SM's amide group [5]. The C-N bond of an amide has enhanced ionic character and diminished rotational capacity compared to the C–O bond of an ester [36]. This difference may help stabilize interfacial hydrogen bonds between cholesterol and SM. SM is one of the few naturally occurring membrane lipids whose fluid-gel coexistence region is poised close to mammalian physiological temperature.

Figure 2 shows that the presence of cholesterol in liposomes decreases the liposome leakage and consequently, increases their half-life times. The $t_{½}$ were 23.85 hrs, 1.916 hr for DLPE/SM/PS/CHOL (1:1:1:1), DLPE/SM/PS (1:1:1), respectively. Also, the $t_{½}$ were 12.5 hrs, 0.97 hr for DLPE/PS/CHOL (1:1:1) and DLPE/PS (1:1), respectively. It is known, that cholesterol inhibits the phospholipid exchange of liposomes with HDL [8, 15, 17] and association with serum proteins that facilitates the uptake of liposomes by the reticuloendothelial system (RES) [3, 4]. Incorporation of cholesterol in liposomes is known to pack the phospholipid molecules above their phasetransition temperature and reduce permeability to solutes [27]. It has been suggested that the increased packing of phospholipids by cholesterol can also prevent their removal by plasma high-density lipoproteins and preserve liposomal stability in the presence of serum [11].

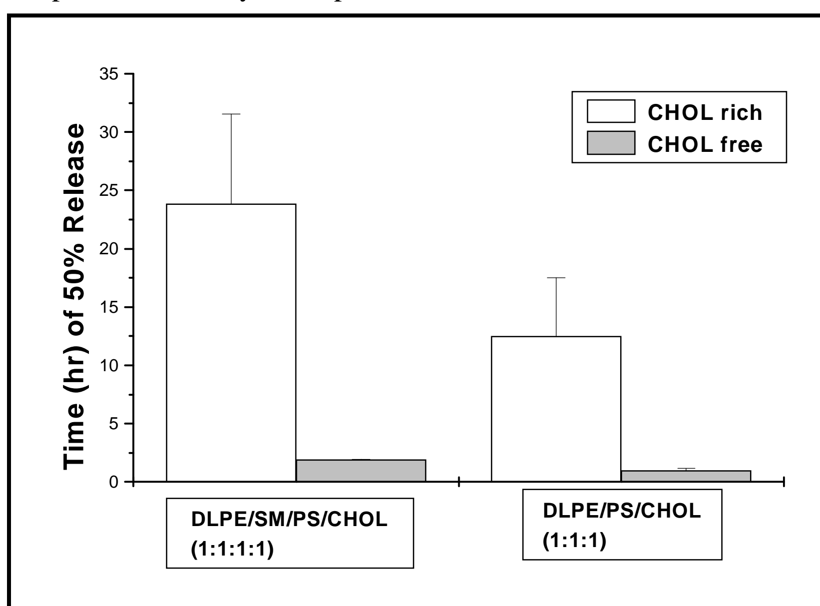

Fig. 2. Effect of CHOL on % CF release from different liposomal formulations, incubated with 100% FCS measured at 37 °C.

Longer circulation times of liposomes *in vivo* were observed with higher lipid doses and this is due to saturation of the reticuloendothelial system (RES) uptake or a decrease in serum protein levels on the surface of the liposome that can then lower the probability of liposome uptake by RES [20, 21, 31]. The $t_{½}$ were 4 hrs and 1.916 hr for DLPE/SM/PS (1:1:1) sample of lipid concentrations 1 mg/ml and 0.33 mg/ml, respectively, after incubation with 100 % FCS at 37 °C (Fig. 3). Also, the $t_{½}$ were 18.92 hrs and 14.8 hrs for DLPE+PC+PS+CHOL (1:2:1:1) sample of lipid concentrations 1 mg/ml and 0.33 mg/ml, respectively (Fig. 3).

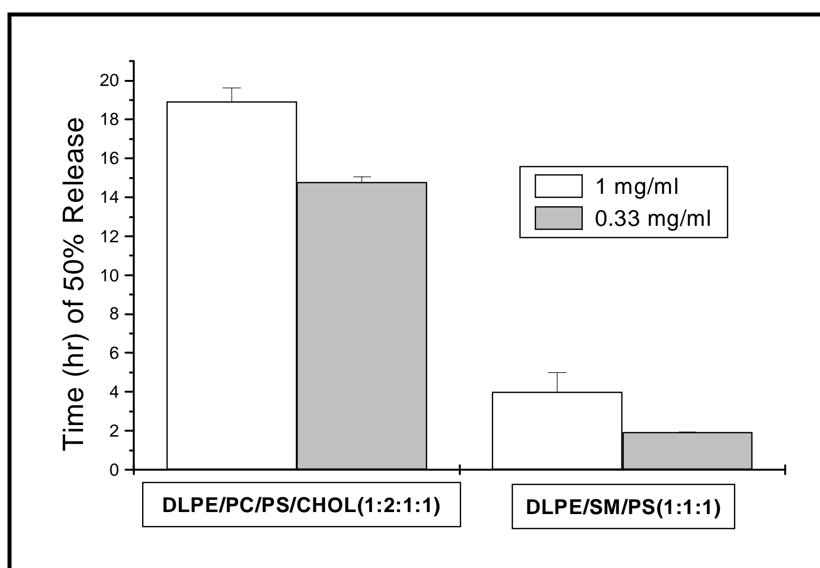

Fig. 3. Effect of lipid concentration on % CF release from different liposomal formulations, measured at 37 °C in 100% FCS.

CONCLUSION

Because of the fast progress of nucleic acid-based technologies in the treatment of diseases, the call for appropriate delivery vehicles becomes increasingly important. One of the problems confronting researchers who wish to use liposomes in the treatment of disease is the rapid removal of liposomes *in vivo*. The ideal vehicle should avoid immediate uptake by the mononuclear phagocyte system and have prolonged circulation in blood, thus increasing the probability of reaching the desired targets. In addition, the vehicle should be able to deliver its contents efficiently into the cell cytoplasm, avoiding lysosomal degradation [32].

There are biological reasons for the lower stability of some AVE liposome compositions compared to the stability of conventional liposomes. These reasons include for example, that viral envelopes, which were the basis for the development

of AVE, are more prone to be attacked by complement system, because the body may have been exposed earlier to this type of viral membranes. One of the lipid components of AVE is a negatively charged lipid, and it is known, that negatively charged liposomes bind to and are endocytosed by cells to a greater extent than neutral liposomes [10, 14]. For example, PS-containing liposomes are taken up by scavenger receptors [16]. We found that DLPE/SM/PS/CHOL (1:1:1:1) liposomal formulation is the most stable formulation in FCS among the examined liposomal formulations.

In conclusion, the high lipid doses and the $t_{½}$ values of DLPE/SM/PS/CHOL (1:1:1:1) liposomes may be sufficient for the targeting of these liposomes to special cell types using receptor-sensitive ligands thereby enhancing localized uptake of drugs into cells of interest, especially if one considers the rapid blood circulation in mammals.

REFERENCES

1. ALLEN, T.M., L.G. CLELAND, Serum-induced leakage of liposome contents, *Biochim. Biophys. Acta*, 1980, **597**, 418–426.
2. ALLEN, T.M., A study of phospholipid interactions between high-density lipoproteins and small unilamellar vesicles, *Biochim. Biophys. Acta*, 1981, **640**, 385–397.
3. ALLEN, T.M., A. CHONN, Large unilamellar liposomes with low uptake into the reticuloendothelial system, *FEBS Lett.*, 1987, **223**, 42–46.
4. ALLEN, T.M., C. HANSEN, J. RUTLEDGE, Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues, *Biochim. Biophys. Acta*, 1989, **981**, 27–35.
5. BITTMAN, R., C.R. KASIREDDY, P. MATTJUS, J.P. SLOTTE, Interaction of cholesterol with sphingomyelin in monolayer and vesicles, *Biochemistry*, 1994, **33**, 11776–11781.
6. BROWN, R.E., Sphingolipid organization in biomembranes: what physical studies of model membranes reveal, *J. Cell Sci.,* 1998, **111**, 1–9.
7. CHANDER, R., H. SCHREIER, Artificial viral envelopes containing recombinant human immunodeficiency virus (HIV) gp. 160, *Life Sci.,* 1992, **50**, 481–489.
8. CHOBANIAN, J.V., A.R. TALL, P.I. BRECHER, Interaction between unilamellar egg yolk lecithin vesicles and human high density lipoprotein, *Biochemistry*, 1979, **18**, 180–187.
9. DAMEN, J, J. REGTS, G. SCHERPHOF, Transfer and exchange of phospholipid between small unilamellar liposomes and rat plasma high density lipoproteins, *Biochim. Biophys. Acta*, 1981, **665**, 538–545.
10. FRALEY, R., R.M. STRAUBINGER, G. RULE, E.L. SPRINGER, D. PAPAHADJOPOULOS, Liposome-mediated delivery of deoxyribonucleic acid to cells: enhanced efficiency of delivery related to lipid composition and incubation conditions, *Biochemistry*, 1981, **20(24)**, 6978–6987.
11. GREGORIADIS, G., C. KIRBY, A. MEEHAN, J. SENIOR, Targeting of liposomes: some prerequisites for success, in *Liposomes, Drugs and immunocompetent Cell Function*, C. Nicolau, A. Paraf eds., Academic Press, 1981, New York, p. 29.
12. HAQUE, MD.E., T.J. MCINTOSH, B.R. LENTZ, Influence of lipid composition on physical properties and PEG-mediated fusion of curved and uncurved model membrane vesicles: „Nature's Own" fusogenic lipid bilayer, *Biochemistry*, 2001, **40**, 4340–4348.
13. HARASHIMA, H., Y. OCHI, H. KIWADA, Kinetic modelling of liposome degradation in serum: effect of size and concentration of liposomes *in vitro*, *Biopharm. Drug Disposit.*, 1994, **15**, 217–225.

14. HEATH, T.D., N.G. LOPEZ, D. PAPAHADJOPOULOS, The effects of liposome size and surface charge on liposome-mediated delivery of methotrexate-aspartate to cells *in vitro*, *Biochim. Biophys. Acta*, 1985, **820**, 74–84.
15. JONAS, A. G.T. MAINE, Kinetics and mechanism of phosphatidylcholine and cholesterol exchange between single bilayer liposomes and bovine serum high density lipoprotein. *Biochemistry*, 1979, **18**, 1722–1728.
16. KAMPS, J.A., H.W. MORSELT, G.L. SCHERPHOF, Uptake of liposomes containing phosphatidylserine by liver cells *in vivo* and by sinusoidal liver cells in primary culture: *in vivo – in vitro* differences, *Biochem. Biophys. Res. Commun.*, 1999, **256(1)**, 57–62.
17. KIRBY, C., J. CLARKE, G. GREGORIADIS, Effect of the cholesterol content of small unilamellar liposomes on their stability *in vivo* and *in vitro*, *Biochem. J.*, 1980, **186(2)**, 591–598.
18. LASIC, D.D., *Liposomes: From Physics to Applications*, Elsevier Science Publishers, NewYork, 1993.
19. LASIC, D.D, *Liposomes in Gene Delivery*, CRC Press, Boca Raton, 1997.
20. LELKES, P., H.B. TANDETER, Studies on the methodology of the carboxyfluorescein assay and on the mechanism of liposome stabilization by red blood cells *in vitro*, *Biochim. Biophys. Acta*, 1982, **716**, 410–419.
21. LELKES, P., P. FRIEDMANN, Stabilization of large multilamellar liposomes by human serum *in vitro*, *Biochim. Biophys. Acta*, 1984, **775**, 395–401.
22. LENTZ, B.R., M. HOECHLI, Y. BARENHOLZ,, Acyl chain order and lateral domain formation in mixed phosphatidylcholine-sphingomyelin multilamellar and unilamellar vesicles, *Biochemistry*, 1981, **20**, 6803–6809.
23. MADY, M.M., M.M. GHANNAM, W.A. KHALIL, R. REPP, M. MARKUS, W. RASCHER, R. MÜLLER, A. FAHR, Efficient gene delivery with serum into human cancer cells using targeted anionic liposome, *J. Drug Targeting*, 2004, **12**, 11–18.
24. MOGHIMI, S.M., H.M. PATEL, Differential properties of organ-specific serum opsonins for liver and spleen macrophages, *Biochim. Biophys. Acta*, 1989, **984**, 379–383.
25. MÜLLER, K., T. NAHDE, A. FAHR, R. MÜLLER, S. BRÜSSELBACH, Highly efficient transduction of endothelial cells by targeted artificial virus-like particles, *Cancer Gene Therapy*, 2001, **8**, 107–117.
26. OLSON, F., C.A. HUNT, F.C. SZOKA, W.J. VAIL, D. PAPAHADJOPOULOS, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, *Biochim. Biophys. Acta*, 1979, **557**, 9–23.
27. PAPAHADJOPOULOS, D., K. JACOBSON, S. NIR, T. ISAC, Phase transitions in phospholipid vesicles: fluorescence polarization and permeability measurements concerning the effect of temperature and cholesterol, Biochim. Biophys. Acta, 1973, **311,** 330.
28. ROERDINK, F., N.M. WASSEF, E.C. RECHARDSON, C.R. ALVING, Effects of negatively charged lipids on phagocytosis of liposomes opsonized by complement, *Biochim. Biophys. Acta*, 1983, **734**, 33–39.
29. SCHERPHOF, G., J. DAMEN, J. WILSCHUT, *Targeted drug delivery and biological interactions*, Vol. Vol. 3, CRC Press, Boca Raton, Fl., 1984.
30. SCHROEDER, R., E. LONDON, D. BROWN, Interactions between saturated acyl chains confer detergent resistance on lipids and glycosylphosphatidylinositol (GPI)-anchored proteins: GPI-anchored proteins in liposomes and cells show similar behavior, *Proc. Natl. Acad. Sci. U.S.A.,* 1994, **91**, 12130–12134.
31. SEMPLE, S.C., A. CHONN, P.R. CULLIS, Interactions of liposomes and lipidbased carrier systems with blood proteins: Relation to clearance behavior *in vivo*, *Adv. Drug Deliv. Rev*. 1998, **32**, 3–17.
32. Shepushkin, V.A., S. Simoes, P. Dazin, M.S. Newman, L.S. Guo, M.C. Pedroso de Lima, N. Düzgünes, Sterically stabilized pH-sensitive liposomes. *J. Biol. Chem.,* 1997, **272**, 2382–2388.

33. SIMONS, K., E. IKONEN, Functional rafts in cell membranes, *Nature* 1997, **387**, 569–572.
34. WEINSTEIN, J.N., R.D. KLAUSNER, T. INNEARITY, E. RALSTON, R. BLUMENTHAL, Phase transition release, a new approach to the interaction of proteins with lipid vesicles: application to liposomes, *Biochim. Biophys. Acta*, 1981, **647**, 270–284.
35. WELZ, C., W. NEUHUBER, H. SCHREIER, M. METZLER, R. REPP, W. RASCHER, A. FAHR, Nuclear transport of oligonucleotides in HepG2-cells mediated by protamine sulfate and negatively charged liposomes, *Pharm. Res.,* 2000, **17**, 1206–1211.
36. WIBERG, K.B., K.E. LAIDIG, Barriers to rotation adjacent to double bonds: 3. The C–O barrier in formic acid, methyl format, acetic acid, and methyl acetate: the origin of ester and amide “resonance”, *J. Am. Chem. Soc*., 1987, **109,** 5935–5943.
37. XU, X., E. LONDON, The effect of sterol structure on membrane lipid domains reveals how cholesterol can induce lipid domain formation, *Biochemistry*, 2000, **39**, 843–849.
38. YAMADA, M., H. HARASHIMA, H. KIWADA, Kinetic analysis of the interaction between liposomes and the complement system in rat serum: Re-evaluation of size-dependency, *Biol. Pharm. Bull*., 1998, **21(9)**, 964–968.