ELSEVIER

Biochimica et Biophysica Acta 1418 (1999) 253–260

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Dual effect of alkylresorcinols, natural amphiphilic compounds, upon liposomal permeability

Jerzy Gubernator, Maria Stasiuk, Arkadiusz Kozubek *

Department of Lipids and Liposomes, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Przybyszewskiego 63/77, 51-148 Wroclaw, Poland

Received 19 January 1999; accepted 5 February 1999

Abstract

The effect of 5-*n*-alkylresorcinols, natural amphiphilic compounds, upon properties of phospholipid vesicles depends on their localization asymmetry. A significant increase of the bilayer permeability is observed when the title compounds are present only in the external medium. When these amphiphiles are preincorporated into the bilayer during its formation, the resulting liposomes effectively encapsulate water-soluble solutes which still remain in liposomes after 25 h. Additionally, the size of liposomes made of alkylresorcinol-phosphatidylcholine mixtures after eight cycles of freezing and thawing only (180–200 nm) is severalfold smaller than the size of vesicles prepared in a similar way from phospholipids only and the resulting liposomes are more homogeneous. These liposomes modified with alkylresorcinols are also stable during 40 day storage at both 4°C and 20°C, in contrast to control liposomes that already strongly aggregate after 10 days.

Keywords: Liposome; Alkylresorcinol; Bilayer permeability; Liposomal encapsulation

1. Introduction

Resorcinolic lipids, the natural amphiphilic long-chain homologs of orcinol (1,3-dihydroxy-5-*n*-methylbenzene), were first demonstrated in Gingkoaceae and Anacardiaceae plants. They were isolated as bilobol and cardol, the unsaturated homologs of 5-*n*-pentadecylresorcinol [1–3]. The occurrence of these compounds and their derivatives has been demonstrated in an increasing number of plants and microbes (see e.g. [3–6]). Due to their amphiphilic character, resorcinolic lipids exhibit strong affinity for lipid bilayers and biological membranes [7–11]. Previous reports, in general, were focused on studying the effect of these compounds present in the external medium on the functional and structural properties of biological membranes. It was demonstrated that the incorporation of higher cardol and bilobol homologs (C_{17} to C_{25} side chain), isolated from cereal bran or *Azotobacter chroococcum*, into liposomal membrane induces an increase in their permeability for solutes [7,12]. In a cellular system this increase in permeability results in hemolysis of erythrocytes [9,13,14]. Preincorporation of long chain alkylresorcinols into the phospholipid bilayer induced a cholesterol-like rigidifying effect upon the bilayer fluidity, as shown by ESR, monolayer and DSC studies [15–17]. In this paper we present comparative studies on the effect of resorcinolic lipids (long chain homologs of cardol) on liposomal membrane when present

* Corresponding author.
E-mail: kozubek@angband.microb.uni.wroc.pl

0005-2736/99/$ – see front matter
PII: S0005-2736(99)00026-7

in the external medium and when preincorporated into the same membrane during its formation. It will be shown that the presence of these amphiphilic molecules in both halves of the bilayer stabilizes the membrane and results in the enhancement of liposomal entrapment as well as a decrease in the liposomal size.

2. Materials and methods

Resorcinolic lipids used in the studies were isolated chromatographically from rye bran acetone extract according to the procedure described earlier [18]. Normal phase chromatography on silica gel 60 using chloroform-methanol (85:15, v/v) was used first for isolation of purified alk(en)ylresorcinols – a mixture of their saturated, monounsaturated and diunsaturated homologs. For separation of the mixture according to the unsaturation argentation chromatography on silica gel impregnated with 12% $AgNO_3$ (w/w) and elution with the same as for normal-phase solvent were used. Separation of individual homologs present in each group (saturated, monounsaturated and diunsaturated) was achieved by isocratic reversed-phase HPLC on RP-18 silica gel and methanol-water (96:4, v/v) as a solvent system. Purity of the isolated material was checked by HPTLC. For the experiments 5 mM methanolic stock solutions of isolated homologs and a mixture of saturated-chain alkylresorcinols (C_{15}–C_{25} with average chain length equal to C_{18}), predominant components of initial mixtures (over 80% of total alk(en)ylresorcinols), were used. Cardol (a mixture of 5-*n*-pentadec-8*Z*-enyl, 5-*n*-pentadeca-8*Z*,11*Z*-dienyl and 5-*n*-pentadeca-8*Z*,11*Z*,14*Z*-trienyl resorcinol) was isolated by normal-phase chromatography on silica gel with chloroform-ethyl acetate (90:10, v/v) from technical cashew nut-shell liquid (CNSL), which was a gift from Cardanol Chemical (Ghent, Belgium). Egg phosphatidylcholine (PC) and egg phosphatidylethanolamine (PE), grade 1 purity, were from Lipid Products (Nutfield, UK), calcein, carboxyfluorescein and cholesterol were from Sigma (Poznan, Poland), nucleopore polycarbonate membranes were from Costar Europe (Badhoevedorp, The Netherlands). The remaining chemicals were of the best available purity from POCh (Gliwice, Poland).

2.1. Studies on the effect of external resorcinolic lipids

The effect of external resorcinolic lipids upon liposomal membrane were studied using unilamellar liposomes containing calcein which were prepared by FAT-VET technique (frozen and thawed vesicles by extrusion technique) [19–21]. Briefly, a dry film of 5–7 mg lipids was hydrated first by vortexing with 0.5 ml 35 mM calcein buffered in 10 mM Tris-HCl, pH 7.4, than subjected to eight freezing (at −70°C) and thawing (at +50°C) cycles. The FAT liposomes obtained were extruded 10 times through 100 nm nucleopore polycarbonate filters using a small-volume extrusion apparatus [21] to obtain VET liposomes. Calibrated liposomes were freed from non-encapsulated dye by molecular filtration on a Sepharose 4B column (1×20 cm) eluted with 10 mM Tris-HCl pH 7.4. The resulting liposomes (20 μmole of lipid) were incubated for 15 min with microliter amounts of studied lipids in the elution buffer at room temperature in the dark and the degree of calcein release was determined spectrofluorimetrically. Calcein fluorescence was excited at 490 nm and monitored at 520 nm. The total amount of calcein in liposomes was assessed after their lysis by addition of Triton X-100 to a final concentration of 0.1%.

Relative fluorescence of samples (F) was calculated as follows:

$$F = \frac{100(F_t - F_0)}{(F_\infty - F_0)}[\%]$$

where F_t is fluorescence of samples after incubation with phenolic lipid, F_0, initial fluorescence of samples (before incubation with phenolic lipid) and F_∞, maximal fluorescence of sample after lysis by Triton X-100.

Each determination was done in triplicate.

2.2. Studies on the effect of membrane preincorporated resorcinolic lipids

Liposomes were prepared in a way similar to that described above. Briefly, dry film of 2.5 mg lipid mixture containing cholesterol or resorcinolic lipid was hydrated first by vortexing with 0.5 ml of 100 mM carboxyfluorescein in 10 mM Tris-HCl, pH 7.4. Multilamellar liposomes were subjected to eight freezing and thawing cycles (−70°C to +50°C). These

FAT liposomes obtained were extruded 12 times through 100 nm nucleopore polycarbonate filters using a mini-extrusion apparatus. Calibrated liposomes were left for 12 h in the refrigerator and were then freed from non-encapsulated dye by molecular filtration on a Sepharose 4B column (1×20 cm) eluted with 10 mM Tris-HCl pH 7.4. Collected liposomes were diluted to 5 ml with the buffer and used for further studies. For some experiments liposomes obtained only by the freezing and thawing procedure were used.

Determination of the liposomal entrapment was done in a similar way as described above using 10 mM Patent Blue Violet as a marker instead of carboxyfluorescein and the liposomes were processed without extrusion. Liposomes were freed from non-encapsulated dye by molecular filtration on a Sepharose 4B column (1×20 cm) eluted with 10 mM Tris-HCl pH 7.4. The amount of marker dye entrapped in liposomes was determined colorimetrically at 635 nm after their lysis by addition of Triton X-100 to a final concentration of 0.1% and the amount of lipid was estimated by phosphorus determination in the sample.

The size distribution of liposomes was determined with dynamic light scattering at Contin mode (Zetasizer 5000, Malvern, UK). Release of marker dye

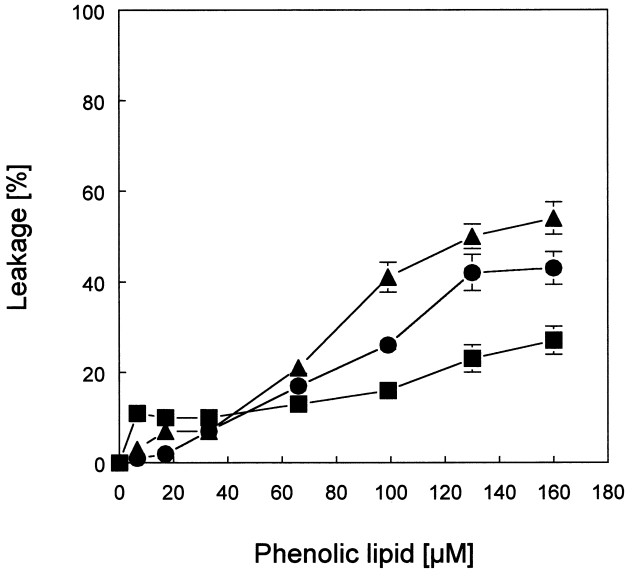

Fig. 1. Leakage of calcein from phospholipid vesicles induced by 5-*n*-pentadecylresorcinol. Liposomes were prepared from PC (●), PC/PE (80:20) (▲) and PC/CHOL (80:20) (■). For further details see Section 2.

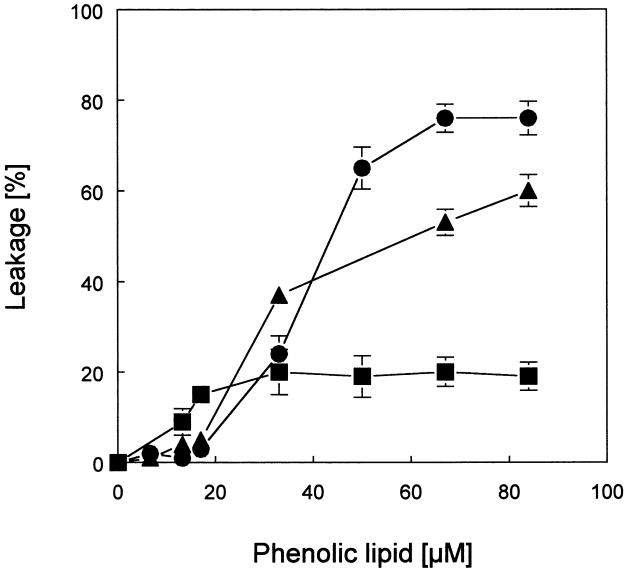

Fig. 2. Leakage of calcein from phospholipid vesicles induced by 5-*n*-tricosylresorcinol. Liposomes were prepared from PC (●), PC/PE (80:20) (▲) and PC/CHOL (80:20) (■). For further details see Section 2.

entrapped in the liposomes was determined spectrofluorimetrically. For fluorescence measurements excitation at 490 nm and emission at 520 nm were used. Maximal leakage was assessed by lysis of liposomes by addition of Triton X-100 to a final concentration of 0.1%.

Phosphorus was determined by the method of Rouser et al. [22] and alkylresorcinols with the method of Tluscik et al. [23].

3. Results and discussion

Resorcinolic lipids, both when preincorporated into the bilayer during its formation and after interaction in the solution with pure phospholipid membrane, significantly affect barrier properties of PC liposomes. Due to a high octanol/water partition coefficient (log $P_{o/w}$ in the range of 7–10, depending on the alkylresorcinol side chain length [11]), resorcinolic lipid molecules present in the aqueous medium will preferentially partition and incorporate into the phospholipid bilayer. During this process they induce increased permeability of the membrane and the leakage of liposomes.

To study the effect of resorcinolic lipids on the membrane barrier properties, the effect of these com-

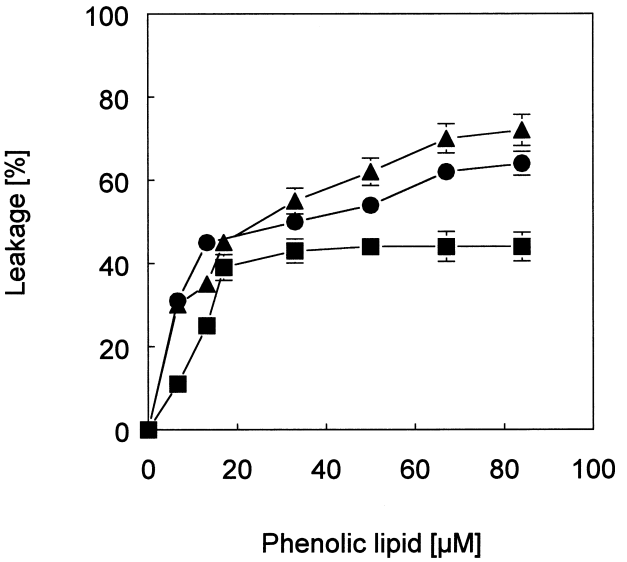

Fig. 3. Leakage of calcein from phospholipid vesicles induced by cardol (unsaturated congener of 5-*n*-pentadecylresorcinol). Liposomes were prepared from PC (●), PC/PE (80:20) (▲) and PC/CHOL (80:20) (■). For further details see Section 2.

pounds on the increase in liposomal membrane permeability which resulted from the interaction of the bilayer with resorcinolic lipids present in the external medium was analyzed. For these studies three types of alk(en)ylresorcinolic compounds have been used: 5-*n*-pentadecylresorcinol and 5-*n*-tricosylresorcinol, the short and long chain homologs isolated from graminaceous material and cardol (natural monounsaturated, diunsaturated and triunsaturated homologs of 5-*n*-pentadecylresorcinol) isolated from the cashew nutshell liquid (CNSL), the main commercial source of resorcinolic lipids. Upon injection into the liposomal suspension all compounds studied induced dose-dependent leakage of the marker dye from liposomes. The extent of the leakage was dependent both on the inducing agent and the lipid composition of the bilayer. The strongest effect of 5-*n*-pentadecylresorcinol was observed for PC:PE and PC liposomes, where 56% and 42% release was observed, respectively (Fig. 1). The alkylresorcinol-induced leakage from PC:CHOL reached a level of 25% at the highest concentration of 5-*n*-pentadecylresorcinol tested. The 5-*n*-tricosylresorcinol (Fig. 2), the homolog with the longest side chain, was most effective upon liposomes made of PC (80% release of the content) and PC:PE (60% release). Liposomes prepared form PC:CHOL showed approx. 20% release, similar to the data obtained for 5-*n*-pentadecylresorcinol.

Among the compounds studied, cardol was the most effective in enhancement of the bilayer permeability (Fig. 3). This mixture of the unsaturated congeners of 5-*n*-pentadecylresorcinol (cardol) induced approx. 80% release of the marker dye from PC:PE liposomes. Similarly high leakage was observed for PC liposomes. Liposomal membranes consisting of PC:CHOL were again more resistant to the membrane-disturbing effect of resorcinolic lipid, although the leakage was almost twice as high as that observed for 5-*n*-pentadecylresorcinol. The last set of data indicates the importance of the unsaturation of the side chain in the detergent-like properties of alkylresorcinol molecules and confirms previous observations made on erythrocytes [9,12]. The lack of complete release of the liposomal content and its stabilization at a constant level even at increasing concentrations of amphiphiles (see Figs. 2 and 3, curve for PC:CHOL) suggested the possibility of resealing of the permeabilized bilayer when the number of alk(en)ylresorcinol molecules in the bilayer exceeds some critical value. Above this value some of

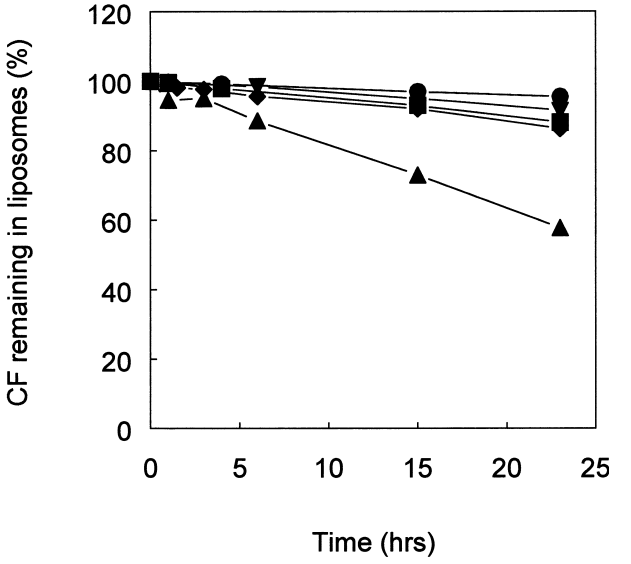

Fig. 4. Leakage of the marker from vesicles prepared from phospholipid-alkylresorcinol mixtures. ●, PC/CHOL (70:30); ▲, PC/pentadecylresorcinol (80:20); ◆, PC/pentadecylresorcinol (70:30); ■, PC/Alkylresorcinols C_{15}–C_{25} (80:20); ▼, PC/alkylresorcinols C_{15}–C_{25} (70:30). For further details see Section 2.

the alk(en)ylresorcinolic molecules might be relocated and enter the inner monolayer, thereby decreasing the packing constraint otherwise present in the outer layer. This also may suggest the possibility of different effects of alkylresorcinols upon their incorporation into both halves of the bilayer during preparation of liposomes.

Therefore in further experiments the barrier properties of the liposomes prepared from phospholipid-resorcinolic lipid mixtures were studied.

The leakage of carboxyfluorescein from liposomal vesicles prepared from PC-pentadecylresorcinol and PC-alk(en)ylresorcinol mixtures was compared with the leakage observed for liposomes prepared from PC-CHOL mixture. The smallest release of marker dye was observed for control (PC-CHOL) vesicles. During a 24 h experiment at 23°C, pH 7.4, only approx. 5% of the dye was released from the liposomes (Fig. 4). Liposomes containing resorcinolic lipids were more permeable and the release of carboxyfluorescein was more pronounced than in control liposomes. However, this enhanced liposomal permeability was decreased with an increase in the amount of resorcinolic lipid in the lipid mixture from which liposomes were prepared. For PC-pentadecylresorcinol liposomes release was almost halved when the amount of pentadecylresorcinol was raised from 20% (w/w) to 30% (w/w) (Fig. 4). A more pronounced effect was observed for PC liposomes modified by natural alkylresorcinol mixture. Liposomes containing 20% of the above modifier after 24 h released less marker than those modified by 20% of pentadecylresorcinol. After raising its membrane concentration to 30%, the release from liposomes was even more suppressed. It is worth noting that the leakage of carboxyfluorescein from liposomes containing 30% (w/w) of alk(en)ylresorcinols was comparable to the release from control PC-CHOL vesicles (7% vs. 5%). The above described effect was also observed for other 5-*n*-alkylresorcinol homologs such as 5-*n*-nonadecylresorcinol and 5-*n*-tricosylresorcinol (data not shown). It should be noted that the time scale and the concentrations of alkylresorcinols in both experiments were different. Even assuming 100% partition of alkylresorcinol molecules into the bilayer during their incubation with liposomes, their membrane concentration will be lower than in the experiment in which they were preincorporated during liposome preparation.

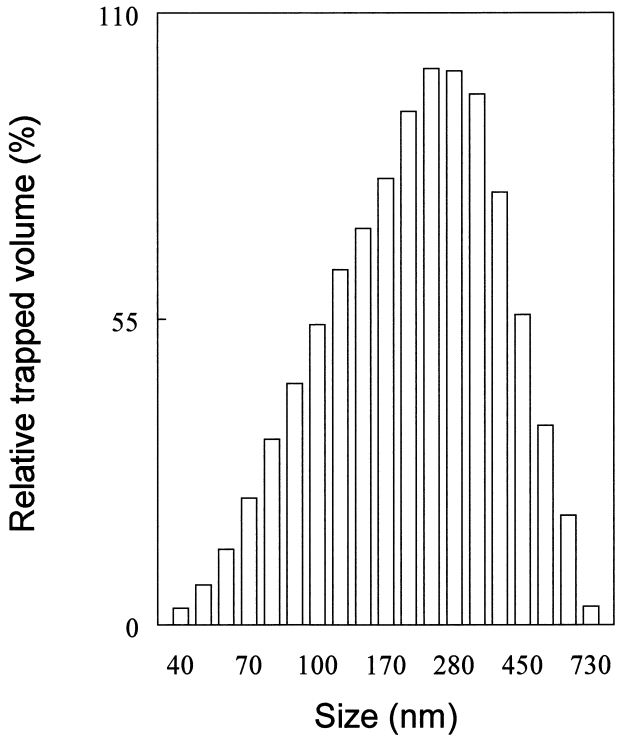

Fig. 6. Size distribution analysis (Contin) of PC-C_{19} alkylresorcinol (70:30) FAT liposomes.

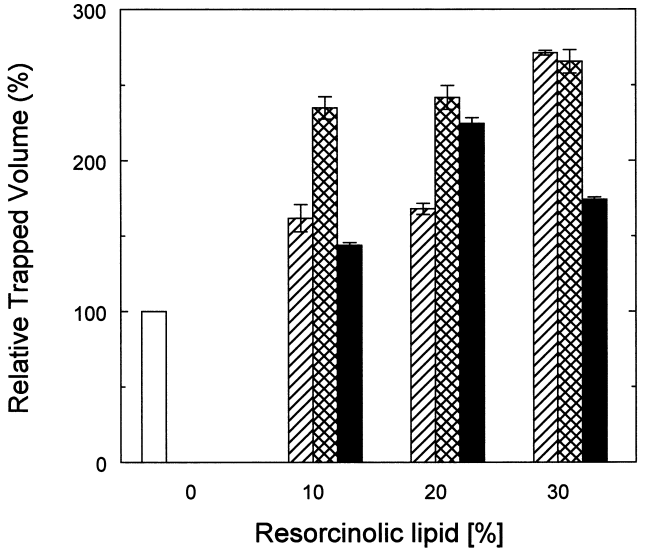

Fig. 5. The effect of the amount and type of alkylresorcinol on trapped volume of PC-alkylresorcinol FAT liposomes. Diagonally hatched bars, C_{15} alkylresorcinol; cross-hatched bars, C_{19} alkylresorcinol; black bars, C_{23} alkylresorcinol

Fig. 7. Freeze-fracture electron microscopy of vesicles prepared by FAT procedure from a PC-alkylresorcinol (70:30) mixture. Bar represents 100 nm.

These results indicate that the presence of alkylresorcinol molecules in both halves of the bilayer results in a less effective increase in membrane permeability. The observation that alk(en)ylresorcinols can form stable liposomal vesicles also in mixtures with PE alone (data not shown) suggests that the bilayer resealing effect of these compounds might be related to the compensation of the molecular shape within the membrane. These data are in agreement with the observation of Bitkov et al. demonstrating the ability of bacterial alkylresorcinols to stabilize black lipid membranes prepared from bacterial PE [24].

The molecular shape of an alkylresorcinol molecule is rather conical; therefore one might expect that presence of this type of modifier would affect the lamellarity and the size of the liposome. It was demonstrated that alkylresorcinols (a mixture of homologs or isolated individual homologs), when present in the lipid mixture, induced a significant increase in the captured volume of PC liposomes after subjecting them to several cycles of freezing and thawing. This increase was dependent both on the length of the alkylresorcinol side chain and its percent amount in the lipid mixture (Fig. 5). The C_{19} homolog was almost equally well effective in all lipid concentrations studied, homolog C_{23} was more effective at 20%, whereas the short chain homolog (C_{15}) was more effective at 30%. Additionally, size distribution analysis by PCS showed that after application of the FAT procedure (freezing and thawing) a population of vesicles with an average diameter of 180–200 nm was obtained (Fig. 6) which confirmed that the presence of those modifiers induces/facilitates a process of decreasing the liposomal diameter.

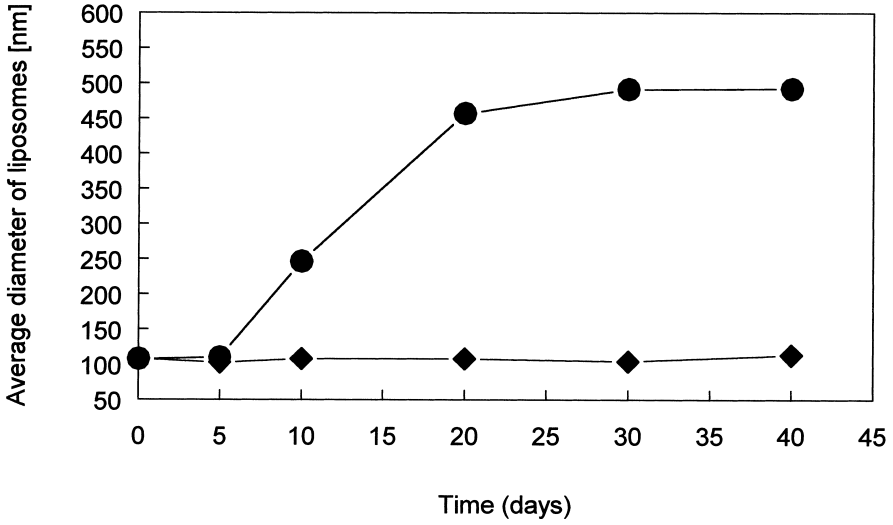

Fig. 8. Stability of PC/CHOL (70:30, w/w) liposomes extruded through 100 mn membrane during long term storage at 4°C (♦) and 20°C (●).

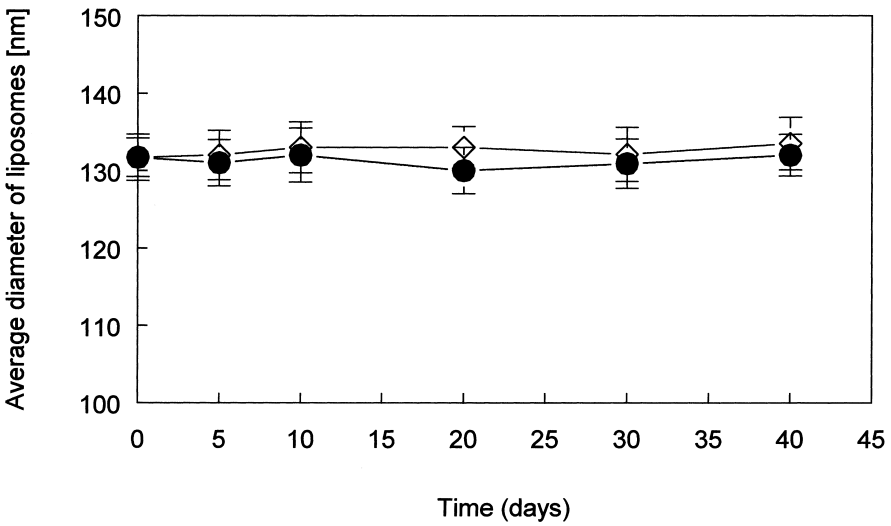

Fig. 9. Stability of PC/alkylresorcinols (70:30, w/w) liposomes extruded through 100 mn membrane during long term storage at 4°C (◇) and 20°C (●).

Freeze-fracture electron microscopy revealed that already freezing and thawing of multilamellar alkylresorcinol-PC liposomes led to formation of unilamellar and oligolamellar vesicles (Fig. 7). Modified with alkylresorcinols liposomes were more susceptible to the extrusion process and after 12 times passage through 100 nm pores 132 ± 3 nm unilamellar vesicles were obtained. To verify whether the observed effect is permanent and the liposomal size remained stable, the stability of modified and control (PC-CHOL) liposomes was studied during a 40 day experiment in which liposomes were kept at 4°C and 20°C (Figs. 8 and 9). A PC-alkylresorcinol liposome mixture (70:30, w/w) did not change the size of the liposomes when kept at both temperatures, whereas control vesicles were stable only when kept at 4°C. When PC-CHOL liposomes were kept at 20°C, a strong aggregation was observed resulting in the dramatic increase in their average size (Fig. 8) and polydispersity of the suspension.

Data presented in this paper indicate that the effect of amphiphiles such as alkylresorcinols strongly depends on the conditions under which they are interacting/incorporating into the lipid bilayer. The compounds present in the external solution would tend to incorporate into the membrane and preferentially localize in the outer monolayer. When the number of alkylresorcinolic molecules in this layer exceeds some limiting value, the formation of non-bilayer structures [8] would be triggered and subsequently an increase in permeability would be observed. If the system were kept long enough to reach equilibrium, a further rearrangement of molecules would result in translocation of bilayer modifying molecules into the adjacent monolayer and their 'dilution'. In such a situation, the local concentrations required for an effective increase in permeability would be lowered. Thus, the 'resealing' effect should be observed. This resealing, however, would not be complete as some of the transiently formed non-bilayer structures as well as packing defects would still be present, resulting in an overall higher permeability of the modified membrane. Due to the conical shape, alkylresorcinolic molecules would be responsible for an increased curvature of the bilayer and subsequently a decrease in the vesicle size. On the other hand, introduction of alkylresorcinols enhances the rigidity and stability of the bilayer which is in good agreement with our previous observations indicating a 'cholesterol-like' effect of alkylresorcinols [15,16,25].

Acknowledgements

This work was supported by State Committee for Scientific Research grants No. 6 P04A 038 10 and 4 P05F 013 13.

References

[1] D.-S. Han, Soul Uidae Chapchi 5 (1964) 281–290.
[2] H.J. Backer, N.H. Haack, Rec. Trav. Chim. Pays Bas 60 (1941) 661–677.
[3] D. Wasserman, R. Dawson, J. Am. Chem. Soc. 70 (1948) 3675–3679.
[4] J.H.P. Tyman, in: Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, vol. 9, Elsevier, Amsterdam, 1991.
[5] J.H.P. Tyman, Synthetic and Natural Phenols, vol. 52, Elsevier, Amsterdam, 1996.
[6] A. Kozubek, J.H.P. Tyman, Chem. Rev. 99 (1999) 1–26.
[7] A. Kozubek, R.A. Demel, Biochim. Biophys. Acta 603 (1980) 220–227.
[8] A. Kozubek, R.A. Demel, Biochim. Biophys. Acta 642 (1981) 242–251.
[9] A. Kozubek, Z. Naturforsch. 39c (1984) 1132–1136.
[10] A. Kozubek, Z. Naturforsch. 40c (1985) 80–84.
[11] A. Kozubek, Acta Biochim. Pol. 42 (1995) 247–252.
[12] A. Kozubek, Acta Biochim. Pol. 34 (1987) 357–367.
[13] A. Kozubek, Acta Biochim. Pol. 34 (1987) 387–394.
[14] M. Stasiuk, A. Kozubek, Cell. Mol. Biol. Lett. 1 (1996) 189–198.
[15] A. Kozubek, A. Jezierski, A.F. Sikorski, Biochim. Biophys. Acta 944 (1988) 465–472.
[16] A.B. Hendrich, A. Kozubek, Z. Naturforsch. 46c (1991) 423–427.
[17] S. Gerdon, S. Hoffmann, A. Blume, Chem. Phys. Lipids 71 (1994) 229–243.
[18] A. Kozubek, Acta Aliment. Pol. 9 (1985) 185–198.
[19] L.D. Mayer, M.J. Hope, P.R. Cullis, A.S. Janoff, Biochim. Biophys. Acta 817 (1985) 193–196.
[20] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 161–168.
[21] R.C. MacDonald, R.I. MacDonald, B.Ph.M. Menco, K. Takeshita, N.K. Subbarao, L.-R. Hu, Biochem. Biophys. Acta 1061 (1991) 297–303.
[22] G. Rouser, A.N. Siakotos, S. Fleisher, Lipids 1 (1966) 85–86.
[23] F. Tluscik, A. Kozubek, W. Mejbaum-Katzenellenbogen, Acta Soc. Bot. Pol. 50 (1981) 645–651.
[24] V.V. Bitkov, V.A. Nenashev, N.N. Pridachina, S.G. Batrakov, Biochim. Biophys. Acta 1108 (1992) 224–232.
[25] A. Kozubek, M. Nietubyc, A.F. Sikorski, Z. Naturforsch. 47c (1992) 41–46.