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Formation of liposomes by resorcinolic lipids, single-chain phenolic amphiphiles from *Anacardium occidentale* L.

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Abstract

Resorcinolic lipids isolated from *Anacardium occidentale* nut oil extract (CNSL), unsaturated congeners of those isolated from bacterial and graminaceous sources, form at alkaline conditions liposomal structures alone as well as in the mixtures with cholesterol, fatty acids or phosphatidylethanolamine. Those vesicular structures show relatively high entrapment of the marker and stability of their size. The retention of the captured solute depends upon the type of resorcinolic lipid and on the temperature, but in general, is lower than control phospholipid liposomes.

Keywords: Liposome; Lipid vesicle; Resorcinolic lipid; Cardol; Methylcardol; Liposome encapsulation; Liposome stability

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 (e.g., [2]). In bacterial organisms, these compounds have been demonstrated as members of the cell membrane lipids that play the role, not yet explained, in membrane functioning, especially during the transition of cells from vegetative to dormant state (cyst) [4–7] that was clearly demonstrated in experiments presented by Reusch [7]. However, the role of resorcinolic lipids in plant cells is not yet elucidated.

The most prominent resorcinolic lipids (cardol and 2-methylcardol), as well as other phenolic lipids, are present in the oil obtained from extraction of the shell of cashew nuts, *Anacardium occidentale* (which contains up to 20% of resorcinolic lipids). The oily extract from the roasting of cashew nuts (cashew nut shell liquid-CNSL) is one of the most important sources of these compounds for chemical formaldehyde polymerization and resins in industry [3,8].

Cardol and methylcardol (trivial names) belong to the group of natural and easily isolated amphiphilic compounds [2] and are in fact homologs of orcinol (1,3-dihydroxy-5-methylbenzene) or methylorcinol (1,3-dihydroxy-2-methyl-5-methylbenzene) with C15 alkenyl side chain attached to the ring instead of 5-methyl group, that is predominantly di- and tri-

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enoic (double bonds at C8, 11, 14). These compounds due to their amphiphilic properties fast and effectively incorporate into the phospholipid bilayers exhibiting effects [9–15] that were dependent on their incorporation [14,16,17]. Additionally, Batrakov and his colleagues, using bacterial alkylresorcinols, showed that saturated-chain homologs could form stable black lipid membranes [18], especially at high pH (>7.5). Natural bacterial alkylresorcinols, when present in the mixtures with phospholipids, stabilized phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol black lipid membranes, with the extent of the stabilization dependent on the type of phospholipid [19]. A similar tendency has been reported for total saturated alkylresorcinols isolated from *Azotobacter* cysts [20]. Our studies were undertaken to extend the above-mentioned studies with special emphasis put on abilities of cardol and methylcardol (highly unsaturated C15 alkenylresorcinols) with respect to forming vesicular, liposomal structures.

In this work we demonstrated that resorcinolic lipids isolated from *A. occidentale* nut oil extract (CNSL), slightly different in their chemical nature from those isolated from bacterial and graminaceous sources [21–23], can form liposomal structures alone as well as in the mixtures with cholesterol, fatty acids or phosphatidylethanolamine. We have studied encapsulation efficiency, size and size stability and retention of the captured solute in vitro in buffer of liposomes of various resorcinolic lipids and lipid/resorcinolic lipid composition.

Table 1
Composition of liposomes used in the studies

Lipid composition	wt%	mol%
Cardol (C)	100	100
C:Chol	50:50	45:55
C:PA	50:50	55:45
C:OA	50:50	53:47
C:LA	50:50	53:47
Methylcardol (MetC)	100	100
MetC:Chol	50:50	46:54
MetC:PA	50:50	57:43
MetC:OA	50:50	54:46
MetC:LA	50:50	54:46
PE/PC	70:30	70:30
C:PE	50:50	30:70

Chol, cholesterol; PA, palmitic acid; OA, oleic acid; LA, linoleic acid; PE, phosphatidylethanolamine (bacterial); PC, phosphatidylcholine (egg).

2. Materials and methods

Cardol and methylcardol were isolated by normal-phase chromatography on a silica gel column with chloroform/ethyl acetate (90:10, v/v) from technical cashew nut shell liquid (CNSL), which was a kind gift from Cardanol Chemical (Ghent, Belgium). Cholesterol, Patent Blue Violet, Sephadex G-50, Triton X-100 were from Sigma (USA), fatty acids from Merck (Germany), egg phosphatidylethanolamine and egg phosphatidylcholine from Lipid Products (Nutfield, UK). The remaining chemicals were of the best available purity from POCh (Gliwice, Poland).

2.1. Vesicle preparation

Vesicles (12 types) of different lipid compositions have been prepared (Table 1). Lipids from stock solutions in chloroform were mixed at the indicated molar ratio, dried in the vacuum evaporator and finally under a stream of nitrogen. Dry lipid films were hydrated by the addition of 1 ml of 1 mM solution of Patent Blue Violet in borate buffer (30 mM H_3BO_3, 70 mM KCl), pH 9.0 and 12.5, then vortexed and subjected to seven cycles of freezing liquid nitrogen and subsequent thawing at warm water bath (50–55°C). Liposomal suspensions were then extruded through polycarbonate filters: 10×400 nm and 10×200 nm, using a high-pressure extruder (PPH Marker, Wroclaw, Poland). Nonencapsulated marker dye was removed from the samples by gel filtration on a Sephadex G-50 column (1×20 cm).

2.2. Determination of liposomal entrapped volume

For determination of the lipid content of the liposomes, phosphate assay [24], cholesterol assay [25] (when it was necessary) and alkylresorcinolic lipids assays [26] were used. The Patent Blue Violet entrapped in liposomes was determined quantitatively by measurement of the absorbance at 635 nm of a samples taken from the vesicle preparation after ad-

dition of 0.1 ml 10% Triton X-100. The entrapped volume of the vesicles was calculated according to the equation:

$$\text{entrapped volume (mol/l)} = \frac{\dfrac{\text{[PBV] in 1 ml of liposomes}}{\text{[PBB] in 1 ml of water}}}{\text{[lipid] in 1 ml of liposomes}}$$

2.3. Determination of liposomal size

The mean diameters of the vesicles (Table 2) were determined by photon correlation spectroscopy using particle sizer Zetasizer 5000 (Malvern Instruments, UK) using the volume distribution curves.

2.4. Determination of liposomal solute retention

PBV-containing liposomes were incubated at 37°C or stored at 4°C. At various time points the content of PBV remaining in vesicles was determined by measurement of the absorbance at 635 nm of a diluted fraction of the vesicle preparation in the presence of 10% Triton X-100. The liposomal solute retention was calculated as PBV released from the liposomes (determined in percent retention) in comparison to the total PBV entrapped in the liposomes.

3. Results and discussion

In the present work we have shown that at pH 12.5 single-chain amphiphiles, resorcinolic lipids isolated from CNSL, form vesicular structures, which are able to entrap effectively aqueous solutions. We have also demonstrated that cardol and methylcardol can form liposomal vesicles in the mixtures with cholesterol and fatty acids (palmitic acid, oleic acid, linoleic acid) at pH 12.5. Additionally, cardol forms liposomal structures in the mixture with PE not only at pH 12.5 but also at pH 9.0. It was postulated

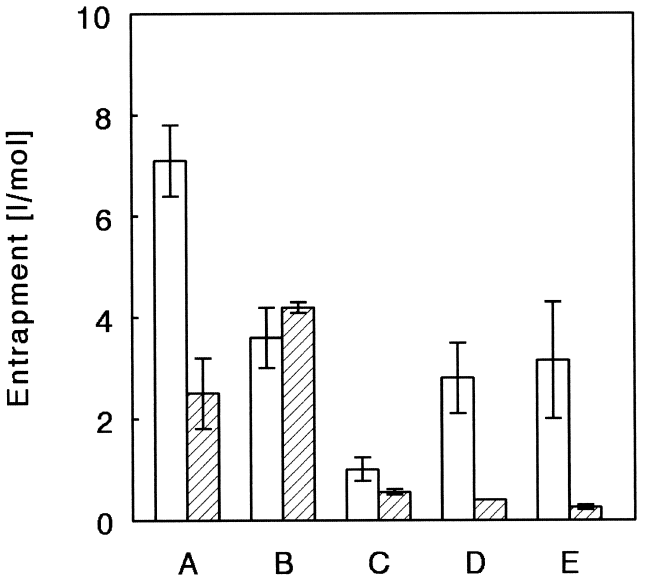


Fig. 1. Encapsulation efficiency of liposomes containing resorcinolic lipids. A, Cardol (methylcardol) liposomes; B, C(MeC):Chol liposomes; C, C(MeC):PA liposomes; D, C(MeC):OA liposomes; E, C(MeC):LA liposomes. Hatched bars, methylcardol; open bars, cardol.

Table 2
Size and size stability of liposomes extruded through 200-nm membrane during 9-month storage at 4°C and 20°C

Liposome	pH	Initial size (nm)	Size (nm) storage at 4°C	Size (nm) storage at 20°C
Cardol	12.5	108 ± 2	123 ± 5	700 ± 7[b]
Methylcardol	12.5	145 ± 7	169 ± 6	320 ± 5[c]
C:Chol	12.5	186 ± 5	149 ± 10	222 ± 4
C:PA	12.5	102 ± 2	780 ± 10[a]	700 ± 10[a]
C:OA	12.5	145 ± 2	135 ± 3	127 ± 4
C:LA	12.5	159 ± 2	133 ± 3	137 ± 2
PE/PC	9.0	185 ± 2	181 ± 7	173 ± 6
PE/PC	12.5	150 ± 1	152 ± 3	155 ± 1
C:PE	9.0	59 ± 10	226 ± 0.4[b]	278 ± 4[b]
C:PE	12.5	57 ± 5	135 ± 3[b]	238 ± 5[b]

[a]One-month storage.
[b]Three-month storage.
[c]Six-month storage.

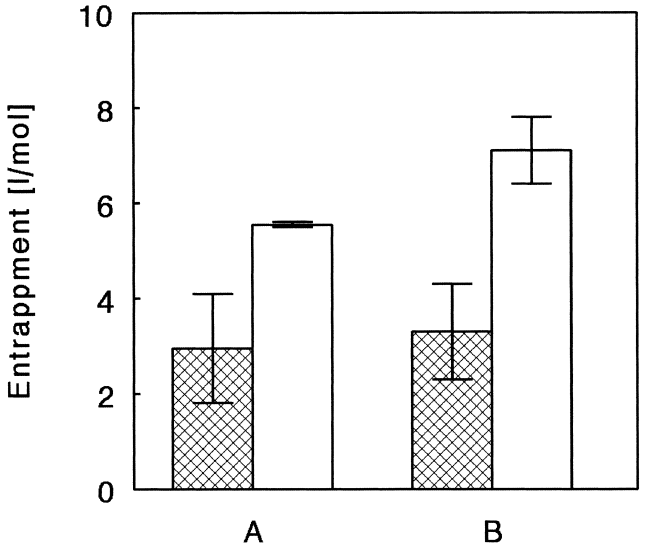
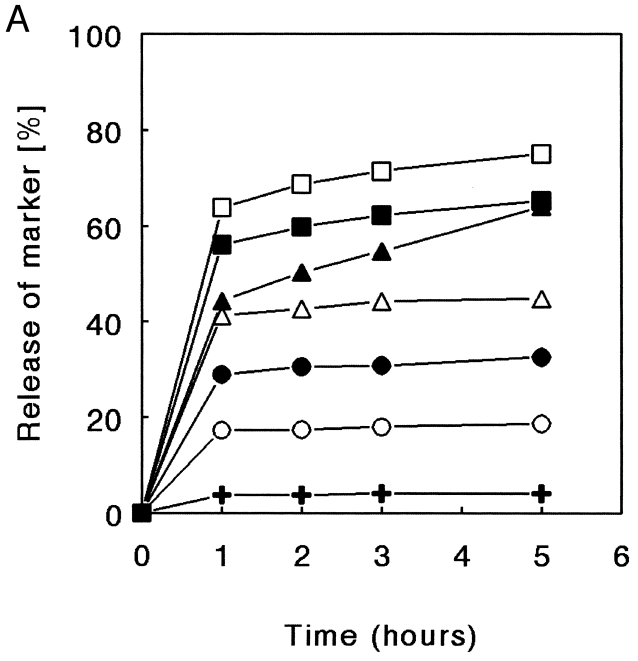


Fig. 2. Encapsulation efficiency of liposomes prepared from PE and cardol/PE mixtures at various pH. A, PE:PC liposomes; B, cardol:PE liposomes. Hatched bars, pH 9.0; open bars, pH 12.5.

earlier [20] that in the phospholipid–resorcinolic lipid mixtures a structural network of molecules is formed that is held together by intermolecular hydrogen bonding between the alkylresorcinol's hydroxyl groups and the polar head-groups of the phospholipids.

We have studied various types of liposomes (Table 1) in terms of liposomal encapsulation efficiency. Fig. 1 shows the changes of entrapped volume of pure cardol or methylcardol vesicles resulting from the addition of cholesterol or fatty acids. It was demonstrated that the entrapped volume of pure cardol liposomes is 3-fold higher than for pure methylcardol liposomes. The presence of the other lipids in the cardol bilayer results in a decrease of encapsulation efficiency. The addition of cholesterol, oleic acid or linoleic acid decreases this amount by about 50%, and palmitic acid by about 85%. Contrary to this, in the case of methylcardol vesicles, the presence of cholesterol increases their entrapped volume 2-fold. However, the presence of fatty acids decreases encapsulation efficiency of these vesicles by 75–90%.

Fig. 2 shows the encapsulation efficiency for pure PE liposomes and PE:cardol liposomes at pH 9.0 and 12.5. It was observed that studied liposomes show a higher entrapped volume at pH 12.5 than



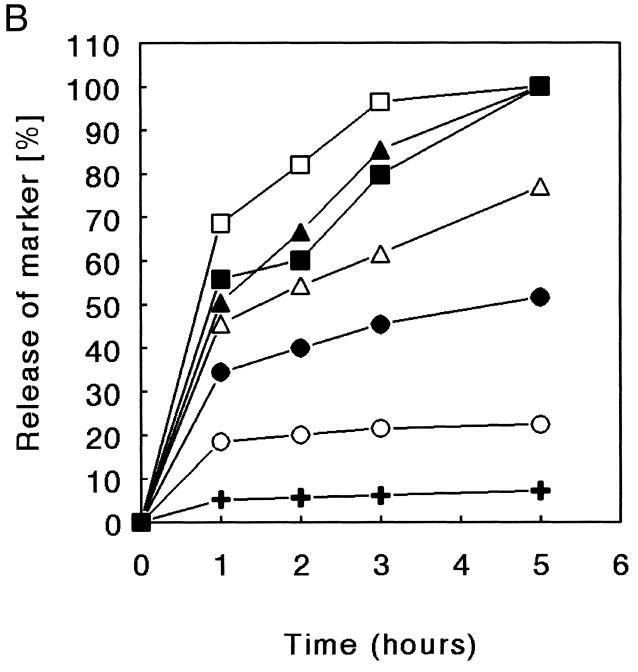


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Fig. 3. (A) Release of marker from liposomes prepared of pure cardol (□), methylcardol (■), C:Chol (○), C:PA (●), C:OA (△), C:LA (▲) and control PC:Chol (+) during storage in 4°C. (B) Release of marker from liposomes prepared of pure cardol (□), pure methylcardol (■), C:Chol (○), C:PA (●), C:OA (△), C:LA (▲) and control PC:Chol (+) during storage at 37°C.

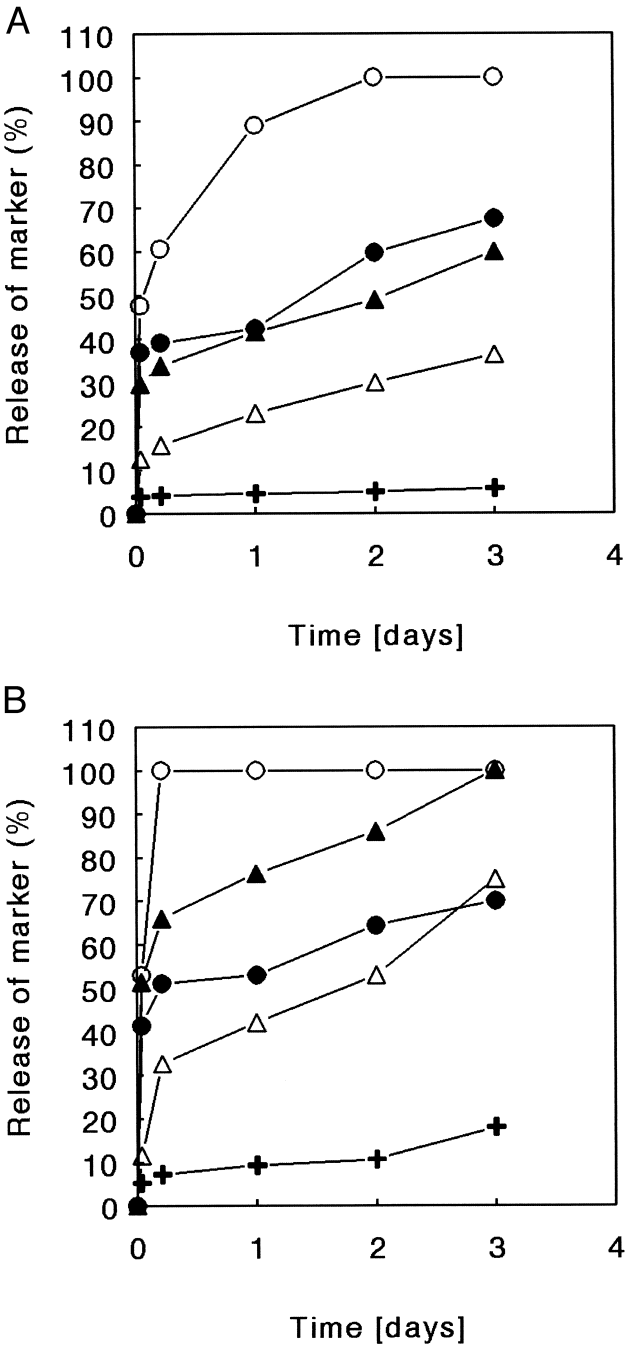

Fig. 4. (A) Release of marker from liposomes prepared of pure PE (△, pH 12.5 and ▲, pH 9.0), C:PE (○, pH 12.5 and ●, pH 9.0) and control PC:Chol (+, pH 7.4) during storage at 4°C. (B) Release of marker from liposomes prepared of pure PE (△, pH 12.5 and ▲, pH 9.0), C:PE (○, pH 12.5 and ●, pH 9.0) and control PC:Chol (+, pH 7.4) during storage at 37°C.

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at pH 9.0. The differences of entrapped volume, at pH 9.0, between pure PE vesicles and PE vesicles modified by cardol, are minimal. The presence of cardol in PE bilayer, at pH 12.5, results in an increase (by 40%) of entrapped volume.

Table 2 shows the results from the measurements of size and its size of liposomes stored at 4°C and 20°C. It was demonstrated that cardol liposomes modified by addition of cholesterol or fatty acids (excluding PA) show higher average diameters. The presence of cardol in PE bilayer causes a 3-fold decrease in the vesicle's average size. The storage of liposomes at −20°C results in strong aggregation of vesicles and dramatic increase in their average size as well as polydispersity of the suspension. For all liposomal formulation (excluding C:PA and C:PE vesicles) we have observed very good size stability at 4°C. In the case of liposomes containing cardol and oleic or linoleic acid, the average size did not change also during storage at 20°C.

Fig. 3A,B show the leakage of entrapped marker dye (Patent Blue Violet) from the investigated (pH 12.5) and control PC:Chol (pH 7.4) liposomes. For vesicles containing cardol and cholesterol we observed higher solute retention than that determined for remaining vesicles during 5-h storage at 4°C (Fig. 3A) and 37°C (Fig. 3B). These liposomes released about 20% of encapsulated Patent Blue Violet. The liposomes consisting of C:PA and C:OA showed 30% and 40% leakage of PBV at 4°C, respectively, but also higher permeability of bilayers at 37°C (45% and 75% release of PBV, respectively). Other vesicles – C:LA, pure cardol and pure methylcardol liposomes – were less stable, and stronger leakage of PBV was observed at 37°C (complete release of PBV after 5-h storage at 37°C) (Fig. 3B).

Fig. 4A,B illustrate the effect of temperature on the leakage of Patent Blue Violet from PE vesicles and C:PE vesicles prepared at pH 9.0 and 12.5. Phosphatidylethanolamine liposomes show higher solute retention than PE liposomes modified with cardol during 3-day storage at 4°C (Fig. 4A) and 37°C (Fig. 4B). Higher permeability, leakage of PE bilayer at pH 9.0 than at pH 12.5 was observed and the opposite effect for PE liposomes containing 50 wt% of cardol. Pure PE vesicles show, at 4°C,

30% release of PBV at pH 12.5 and 2-fold higher release of PBV at pH 9.0 (Fig. 4A). The presence of cardol admixture makes the PE bilayer more permeable (complete release of PBV during 2-day storage at 4°C), but at pH 9.0 results in increased liposomal solute retention by 40% (Fig. 4A). Similar effects and higher leakage of Patent Blue Violet (70–100%) from the investigated liposomes were observed at 37°C (Fig. 4B).

Data presented in this paper indicate that oligoenoic-chain resorcinolic lipids from *A. occidentale* form, at pH 12.5, liposomal structures that are able to entrap effectively aqueous solutions. This ability may be related to the increase of the polar portion of the molecule due to ionization of hydroxylic groups at high pH, therefore altering the noncylindrical shape to more cylindrical and gaining the ability of formation of bilayer and vesicular structures. The presence of the additional compounds (cholesterol or fatty acids) in the resorcinolic bilayer results in an increased average diameter of liposomes, but also in a higher stability of liposomal size and solute retention. Additionally, at particular molar fraction in the mixture, alkylresorcinolic molecules (due to their noncylindrical shape, as suggested by ^{31}P-NMR data [9]) would be responsible for an increased curvature of the phospholipid bilayer and subsequently, a decrease in the vesicle size. Results obtained for pure PE and PE:cardol liposomes are in good agreement to our previous observations [2]. Bilayer and vesicle-modulating properties of single-chain phenolic amphiphiles described in this paper may also play a role in in vivo alteration of the cellular membrane properties during senescence or transition to a metabolically dormant state in, e.g., bacteria or plant seeds. The high-pH prepared liposomes may also have potential applications in delivery of some drugs that are not easily encapsulated into the liposomes at neutral pH, as was shown recently for atovaquone by Cauchetier et al. [27]. It should be mentioned that, contrary to the results presented in this paper, liposomes containing saturated resorcinolic lipid homologs or their semisynthetic derivatives are more stable at neutral pH and increase liposomal effectiveness in encapsulating such drugs as doxorubicin, mitoxantron or methotrexate (A. Kozubek et al., in preparation). Resorcinolic lipids liposomes (AR-osomes) and resorcinolic lipids derivative-based vesicles (PLAR-osomes) may be of potential application as the compounds used for their preparation do not exhibit any direct toxic effect upon rats and cells in culture (A. Kozubek, J. Gubernator, J. Rejman, unpublished results).

Acknowledgements

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