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1-*O*-Alkylglycerol vesicles (Algosomes): their formation and characterization

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Abstract

1-*O*-alkylglycerols (ALKG) have exhibited several biological activities and a prominent effect on blood-brain barrier permeability. They have markedly improved brain uptake of cancerostatic agents. Since ALKG are amphiphilic, we explored their tendency to assemble into bilayer vesicles, which can be applied as carriers for drugs. Vesicles (Algosomes) were formed by film hydration method using ALKG (tetra-, penta-, hexa-, hepta-, octa- or nona-decylglycerols) in combination with cholesterol (CHOL) and dicetyl phosphate (DCP) (1-*O*-alkylglycerol:CHOL:DCP in 45:45:10 molar ratio). On microscopic examination, the algosomes were found to be conspicuously spherical and the dispersion was a mixture of multi-lamellar and small-unilamellar vesicles. Phase transition temperatures of 1-*O*-hexadecylglycerol (HXDG) and CHOL mixtures were tested by differential scanning calorimetry (DSC). The changes in phase transition temperatures indicate the vesicle forming tendency of ALKG in presence of CHOL. Alkyl chain length dependent variations in vesicle size, zeta-potential (ZP) and capture volume (CV) could not be observed. Vesicles of 1-*O*-tetradecylglycerol (TTDG) showed improvement in CV with increase in CHOL content from 15 to 55 mol%. However the vesicle size decreased. On challenging algosomes with hypertonic salt solution [potassium iodide (KI) in water], vesicle size decreased and thus algosomes were found to be osmotically sensitive. Algosome dispersions on addition of higher concentrations of KI (40–100 mM) brought about increases in vesicle size and at concentrations 60 mM and above showed aggregation. All vesicular dispersions were stable for only a few days.

Keywords: Vesicles; 1-*O*-alkylglycerols; Zidovudine (AZT); 1-*O*-alkylglycerol vesicles; Algosomes

Abbreviations: ALKG, 1-*O*-alkylglycerols; AZT, zidovudine which is chemically 3′-Azido-3′-deoxythymidine or azidothymidine [CAS-30516-87-1]; CHOL, cholesterol; CV, capture volume; DCP, dicetyl-phosphate; DSC, differential scanning calorimetry; HPDG, 1-*O*-heptadecylglycerol; HPLC, high performance liquid chromatography; HXDG, 1-*O*-hexadecylglycerol; KI, potassium iodide; NNDG, 1-*O*-nonadecylglycerol; OCDG, 1-*O*-octadecylglycerol; PNDG, 1-*O*-pentadecylglycerol; TTDG, 1-*O*-tetradecylglycerol; ZP, zeta potential.

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1. Introduction

1-*O*-Alkylglycerols (ALKG) exhibit a variety of biological actions. Prominent among them are antibacterial (Ved et al., 1984; Brisette et al., 1986) and antifungal (Hallgren et al., 1978) activities. The effects of alkyl glycerols in immunological stimulation, radiation prophylaxis and cancer treatment have been reported (Hallgren, 1983; Weber, 1988).

The ether glycero-lipids, rather than the corresponding acyl lipids, may render biological membranes more stable towards chemical and enzymatic attack (Paltauf, 1983). Enrichment of membranes with these stabilizing ether lipids was found when cultured Ehrlich ascites cells were supplemented with exogenous 1-*O*-hexadecylglycerol (HXDG) (Cabot and Synder, 1980). Short chain alkylglycerols (C_3–C_5) may be used for opening blood brain barrier to transport anticancer drugs into the brain at a high rate (Unger et al., 1984, 1985). Even long chain alkylglycerols (C_{16}–C_{19}) have improved the brain penetration of rifampicin (Rambhau et al., 2001)

Besides these biological activities, ALKG are amphiphilic and exhibit surface active properties (Apte et al., unpublished). Therefore it was interesting to exploit the amphiphilic properties of these lipids for variety of applications. This investigation was designed to explore the possibilities of converting ALKG of higher chain length (C_{14}–C_{19}) into bilayer vesicles, which can be applied as carriers for drug delivery.

2. Materials and methods

1-*O*-Tetradecylglycerol (TTDG), 1-*O*-pentadecylglycerol (PNDG), 1-*O*-hexadecylglycerol (HXDG), 1-*O*-heptadecylglycerol (HPDG), 1-*O*-octadecylglycerol (OCDG), and 1-*O*-nonadecylglycerol (NNDG) were gifts from Institut für Biochemie und Technologie der Fette, Bundesanstalt für Getreide- Kartoffel- und Fettforschung, Münster, Germany. Zidovudine which is chemically 3′-Azido-3′-deoxythymidine or Azidothymidine [CAS-30516-87-1] (AZT) was donated by Burroughs Wellcome Research Laboratories, Research Triangle Park, NC and paracetamol was a gift given by Parke Davis, Hyderabad, India. Cholesterol (CHOL) and dicetyl phosphate (DCP) were obtained from Sigma, St. Louis, MO. Ammonium acetate and acetic acid of analytical grade were purchased from s.d. Fine Chemicals, Boisar, India. Chloroform of analytical grade was purchased from Ranbaxy Fine Chemicals, S.A.S. Nagar, India. Methanol (HPLC grade) was procured from Qualigens Fine Chemicals, Mumbai, India.

2.1. Algosome preparation

Algosomes were prepared with 200 μmol of lipid mixture (ALKG:CHOL:DCP in 45:45:10 molar ratio) dissolved in 9 ml of chloroform and 1 ml of methanol in a round bottom flask which was kept under reduced pressure in rotary evaporator (Rotavapor R-114, Buchi Lobortechnik AG, Flawil, Switzerland) till it formed a thin dry film on the walls of the flask at 60 °C. The dried thin lipid film was hydrated with 10 ml of phosphate buffered saline (PBS, pH 7.4) containing AZT (10 mg/ml) maintained at the same temperature. The vesicles were then sonicated for 1 min using ultrasonicator (LABSONIC L, B. Braun Biotech International, USA) on which power was set at 50% of maximum output.

2.2. Characterization of vesicles

2.2.1. Photomicrography

Photomicrographs of algosomes were taken using LABORLUX S microscope, (Leitz Wetzlar, Germany) fitted with camera at various magnifications.

2.2.2. Size and size distribution

The mean size and size distribution of algosome dispersions was determined by photon correlation spectroscopy (PCS) using Zetasizer 3000HSA (Malvern Instruments, Malvern, UK). Each sample was diluted to a suitable concentration with filtered distilled water. Analysis was performed at 25 °C with an angle of detection of 90°. The mean size ($\pm$S.D.) was directly obtained from the instrument.

2.2.3. Zeta potential measurement

The zeta potential (ZP) of algosomes was determined using Zetasizer 3000HSA (Malvern instruments, Malvern, UK) using a suitable diluting medium. Charge on vesicles and their mean ZP values (±S.D.) were obtained directly from the instrument.

2.2.4. Capture volume

The capture volume (CV) of algosomes encapsulating AZT was assessed by ultrafiltration using Centrisart I (20 000 Da cut-off, Sartorius AG, Gottingen, Germany). Centrisart consists of two tubes an inner (floater) which freely slides in an outer (centrifuge) tube. The bottom of the inner tube has a filtering membrane (cut-off 20 000 Da).

The algosome dispersion (2 ml) was introduced in outer centrifuge tube and floater was inserted slowly into it. This complete tube set was centrifuged ($2500 \times g$) for 5 min using laboratory centrifuge (R8A, Remi Equipments, Mumbai, India). Unentrapped portion of AZT present as solution in the dispersion medium of algosome separated in the inner tube which was immediately pipetted out, diluted suitably and analyzed for the drug content. This was taken as unentrapped drug in the preparation. Encapsulation efficiency was calculated by subtracting unentrapped AZT from total AZT and was expressed as CV in μl/μmol of lipid.

2.2.5. Latency studies

Changes in CV of algosomes with time were measured through latency studies. The algosomes were studied over a period 12 h for changes in CV using Centrisart I (20 000 Da cut-off, Sartorius AG, Gottingen, Germany).

2.2.6. Effect of osmotic gradients on algosomes

Since electrolytes can bring about osmotic gradients and aggregation effects a preliminary study was necessary to find out which of the concentration range of electrolyte (KI) is suitable to study osmotic gradient effects. This study was initially conducted on TTDG vesicular suspension and was latter extended to all other algosomes using appropriate concentrations of KI.

Osmotic sensitivity and the aggregation tendency of TTDG vesicles was investigated by monitoring changes in vesicle diameter using Zetasizer 3000HSA. Algosome suspension (1 ml) was challenged with 1 ml KI solution containing 5, 10, 15, 20, 40, 60, 80 or 100 mM of KI. The mixture was allowed to stand for 1 h and the vesicle size was measured.

2.2.7. Differential scanning calorimetry studies

Mixtures of HXDG with varied concentrations of CHOL were studied by differential scanning calorimeter (Mettler DSC-30, Mettler-Toledo, Germany) to understand the thermotropic properties and phase transition behaviour. DSC studies also provide an insight into bilayer-vesicle formation capability of this lipid mixture. For this analysis every time about 2 mg of mixture was used with heating rate of 5 °C/min.

2.3. Estimation of total AZT content in algosome

The total AZT content in the algosome was estimated by mixing 0.2 ml of the preparation with 1 ml of methanol followed by vortexing (Cyclomixer, Remi Motors, Mumbai, India). The lysed preparation was then diluted with sufficient quantity of distilled water. The AZT content was estimated using HPLC.

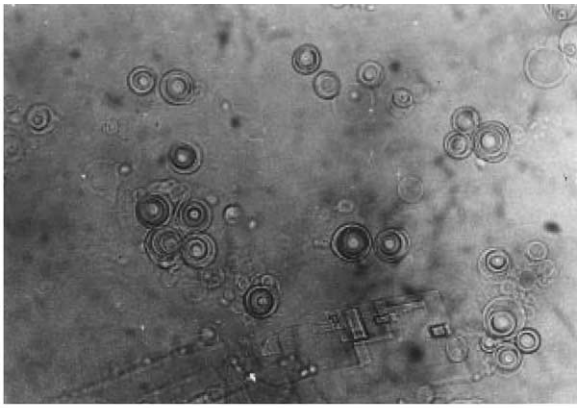
2.3.1. High-performance liquid chromatography of AZT

AZT content in samples was analyzed by high-performance liquid chromatography (HPLC) method previously reported (Gopinath et al., 2001a).

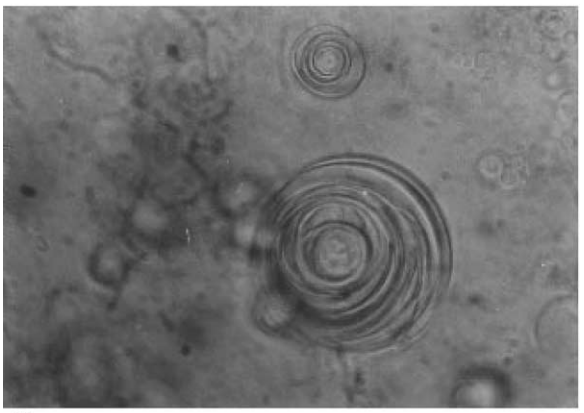
A HPLC system (Shimadzu Corporation, Kyoto, Japan) equipped with SCL-6A system controller, LC-6A solvent delivery unit, SPD-6AV variable UV spectrophotometric detector, C-R4A chromatopac data processor and Injector (Rheodyne) fitted with 20 μl capacity loop was used for the analysis. An octadecylsilane reverse phase stainless steel analytical column (25 cm × 4.6 mm) was employed for chromatographic separation.

Mobile phase: Methanol–water (20:80) to which ammonium acetate was added to make a final

concentration of 10 mM. The pH was adjusted to 4.0 with acetic acid. Flow rate: 1 ml/min. UV detection at 267 nm and separation was at ambient temperature. The retention times were 3.4 and 6.2 min for internal standard (Paracetamol) and AZT, respectively. The concentrations of AZT in unknown samples was determined using a standard graph.



(a)



(b)

Fig. 1. Photomicrograph of unsonicated algosome dispersion formed with HXDG:CHOL:DCP (45:45:10). (a) Under 450 × magnification. (b) Under 1500 × magnification.

3. Results and discussion

3.1. Formation of algosomes

Formation of vesicles was confirmed by light microscope at 450 × and 1500 × magnifications. The majority of vesicles were multi-lamellar and had a heterogeneous size distribution (Fig. 1).

All ALKG (C_{14}–C_{19}) in presence of 45 mol% of CHOL and 10 mol% of DCP formed bilayered vesicles on hydration with phosphate buffered saline (pH 7.4) containing 10 mg/ml of AZT (Table 1). In absence of CHOL and the charge inducer DCP vesicles could not be formed. All vesicular dispersions showed stability for only a few days.

3.2. Vesicle size and zeta potential

Vesicle size measured by photon correlation spectroscopy showed that the vesicles ranged from 350 to 800 nm (Table 1). The ZP of all the vesicular systems were negative. Vesicle size and ZP did not exhibit any chain length dependency. Vesicles of TTDG showed decrease in vesicle size with increasing concentration of CHOL. All ALKG vesicles, excepting the one prepared with TTDG showed almost similar ZP values, which were ranging between −50 and −58 mV. This could be because of the common polar head group that these amphiphiles possess. The higher ZP value for TTDG vesicles could be because of relatively higher polarity of glycerol head group due to shorter hydrophobic chain length, which might have favored interaction with a greater number of water dipoles compared to other materials.

3.3. Capture volume and latency

CV dependency on chain length of ALKG could not be detected (Table 1). CVs were almost similar for all the vesicles (0.84–0.99 μl/μmol). However the CV increased with increase in CHOL concentration for TTDG vesicles. Several others reported improvement of CV with increase in CHOL concentration for niosomes (Yoshioka et al.,

Table 1
Effect of composition on vesicle size, zeta potential and capture volume of algosomes

Composition	Vesicle size (nm)	Zeta potential (mV)	Capture volume (μl/μmol)
ALKG:CHOL:DCP (mol%)	(AVG ± S.D.)		
TTDG-75:15:10	770.8 ± 7.5	−59.1 ± 3.5	0.715 ± 0.05
TTDG-55:35:10	529.3 ± 8.8	−58.8 ± 2.2	0.740 ± 0.12
TTDG-45:45:10	468.5 ± 7.1	−64.8 ± 6.2	0.842 ± 0.08
TTDG-35:55:10	422.4 ± 6.3	−60.2 ± 1.9	0.893 ± 0.09
PNDG-45:45:10	382.7 ± 4.3	−56.6 ± 0.6	0.968 ± 0.15
HXDG-45:45:10	375.6 ± 1.6	−58.4 ± 0.6	0.986 ± 0.20
HPDG-45:45:10	420.0 ± 3.7	−58.5 ± 0.5	0.854 ± 0.05
OCDG-45:45:10	370.1 ± 5.8	−57.8 ± 1.9	0.953 ± 0.08
NNDG-45:45:10	400.4 ± 2.2	−50.3 ± 0.5	0.995 ± 0.19

1994; Kiwada et al., 1985; Uchegbu and Florence, 1995).

We conducted a short duration (12 h) latency study on ALKG vesicles. Latency and alkyl chain length did not exhibit any relation (Fig. 2). Interestingly CVs of HXDG and HPDG vesicles were higher than others. The CV of TTDG vesicles with varying concentrations of CHOL were stable over 12 h indicating that the latency did not depend on concentration (Fig. 3). In general CV fluctuated within the first four hours after preparation indicating that sufficient time is necessary for vesicle dispersion to get stabilized.

3.4. Osmotic and aggregation effects of KI on algosomes

The effect of varying concentrations of KI on vesicle diameter of TTDG vesicles is shown in Fig. 4. At 5–15 mM concentration of KI, vesicle size decreased. Thereafter it steadily increased up to 40 mM concentration and then remained almost

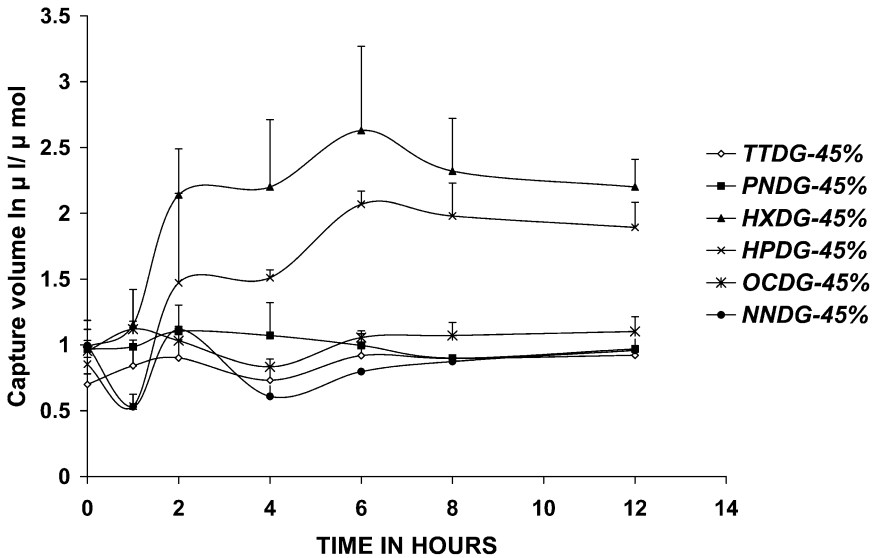


Fig. 2. Effect of alkyl chain length of 1-*O*-alkylglycerols on latency of algosomes containing ALKG:CHOL:DCP (45:45:10). Each point represents the mean ± S.D. ($n = 3$).

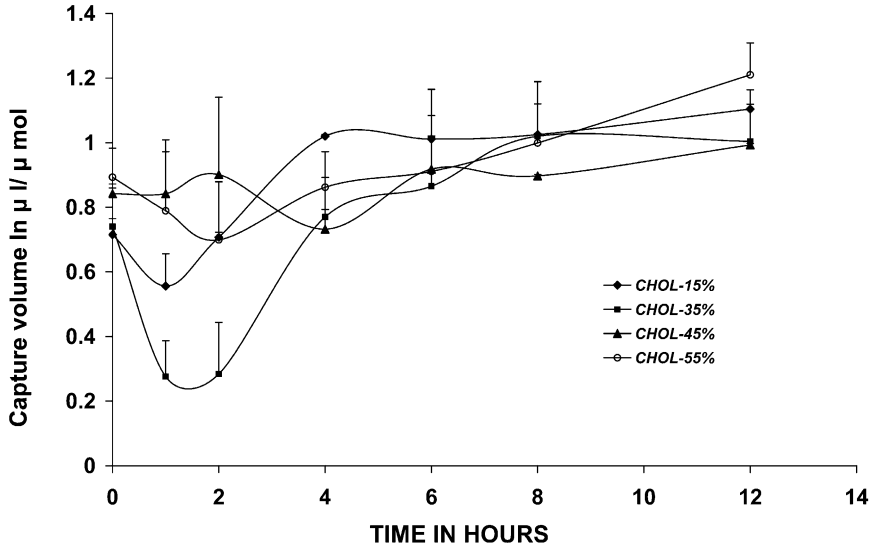


Fig. 3. Effect of cholesterol concentration on latency of 1-*O*-tetradecylglycerol vesicles. Each point represents the mean ± S.D. ($n = 3$).

constant up to a concentration of 100 mM. The substantial decrease in vesicle size due to 5–15 mM of KI confirms that algosomes are osmotically sensitive. At higher concentrations (20 mM onwards) although osmotic shrinkage is inevitable, the increase in size is obviously due to aggregation induced by KI. Presence of phosphate head groups of DCP and glycerol head groups of TTDG on the vesicle surfaces should invoke ionic and dipole barriers respectively in the double layer region. At sufficient concentrations of KI, ionic and dipole charges should be suppressed due to the counter ion effect of K^+, and the salting out effect respectively. This should lead to vesicle–vesicle interaction and consequently aggregation. From 60 mM onwards perceptible aggregation was

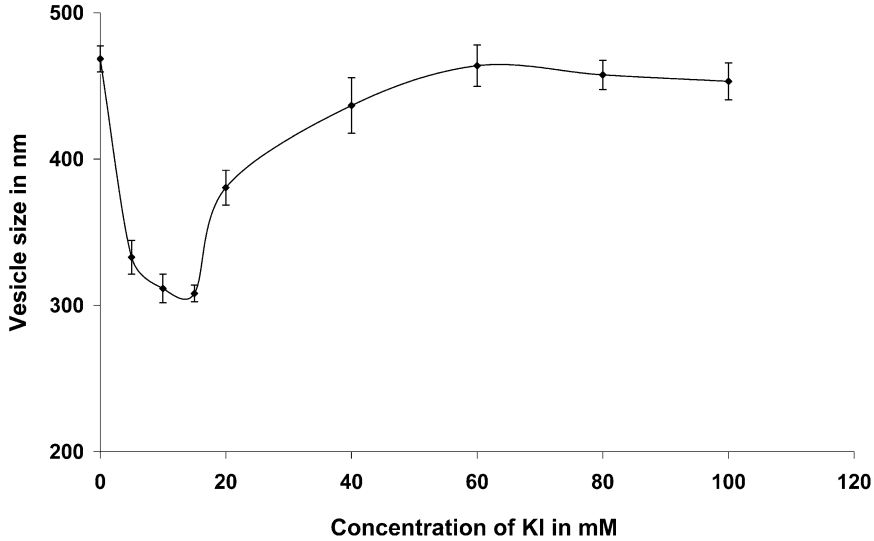


Fig. 4. Osmotic sensitivity of 1-*O*-tetradecylglycerol vesicles containing TTDG:CHOL:DCP (45:45:10). Each point represents the mean ± S.D. ($n = 3$).

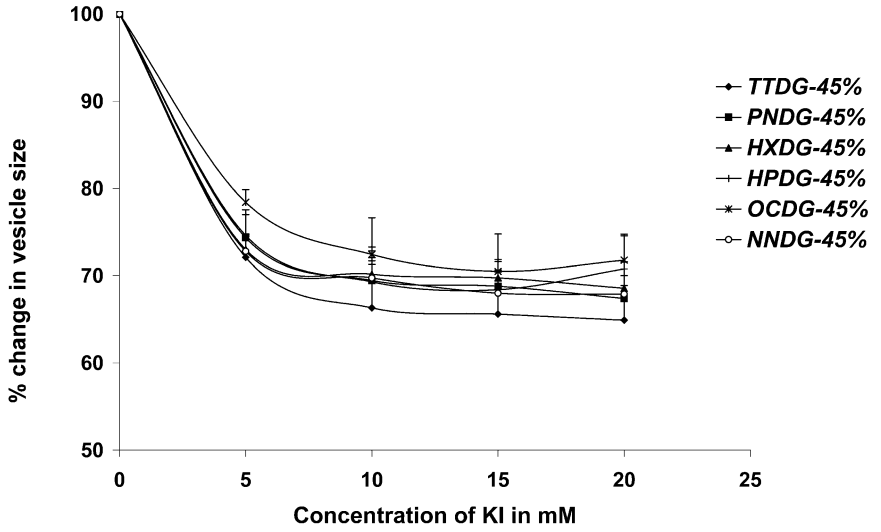


Fig. 5. Osmotic sensitivity of algosomes containing ALKG:CHOL:DCP (45:45:10). Effect of alkyl chain length of 1-*O*-alkylglycerols. Each point represents the mean ± S.D. ($n = 3$).

observed visually. Although all ALKG vesicles were osmotically sensitive, chain length had no effect on this property (Fig. 5).

Recently, formation of polyhedral niosomes with hexadecyldiglycerol ether and solulan C_{24} (91:9), and spherical niosomes with hexadecyldiglycerol ether, CHOL and solulan C_{24} (49:49:2) was reported (Arunothayanum et al., 1999). When these niosomes were challenged with 0.154, 1 or 2 M NaCl the vesicle diameter decreased. No aggregation was reported. This could be possible if a large dilution of vesicles is made with NaCl solution. Another possible reason could be the availability of large number of small polar head groups of glycerol and small number of long polyoxyethylene chains which could have resisted the salting out effect of electrolyte consequently suppressing aggregation.

3.5. Differential scanning calorimetry studies

HXDG which formed good vesicles and retained high CV for longer time was studied by DSC. The DSC scans of mixtures of HXDG and CHOL of varying compositions were obtained by increasing temperature at a rate of 5 °C/min.

HXDG and CHOL have exhibited sharp endothermic peaks at 59.2 and 150.7 °C, respectively (Fig. 6A and B). A 70:30 mol% mixture of HXDG and CHOL resulted in the abolition of CHOL peak (Fig. 6C). In addition a broad peak appeared between 55 and 65 °C. One of these peaks is very close to the phase transition temperature of HXDG (59.2 °C). Among the two additional shoulder peaks one which is conspicuous, E1, could be a eutectic mixture. Upon further decreasing the proportion of HXDG in the mixture (50:50) the two eutectic mixture peaks E1 and E2 became distinct and in addition a new peak K appeared at 70 °C (Fig. 6D). This additional peak K also persisted even when HXDG concentration is further reduced in the mixture (HXDG:CHOL = 30:70) (Fig. 6E).

Based on our preliminary DSC scan data the additional endothermic peaks at 70 °C for 50:50 and 30:70 mixtures of HXDG and CHOL indicates that these two lipids undergo molecular association to form structures which may be responsible for the formation of bilayer vesicles at such compositions. In consonance with this 50:50 mixture of HXDG and CHOL formed good bilayer vesicles.

Several amphiphiles with ether linkages have been the reported to form bilayered vesicles (Handjani-villa et al., 1979; Vanlerberghe et al., 1989; Baillie et al., 1985; Uchegbu and Florence,

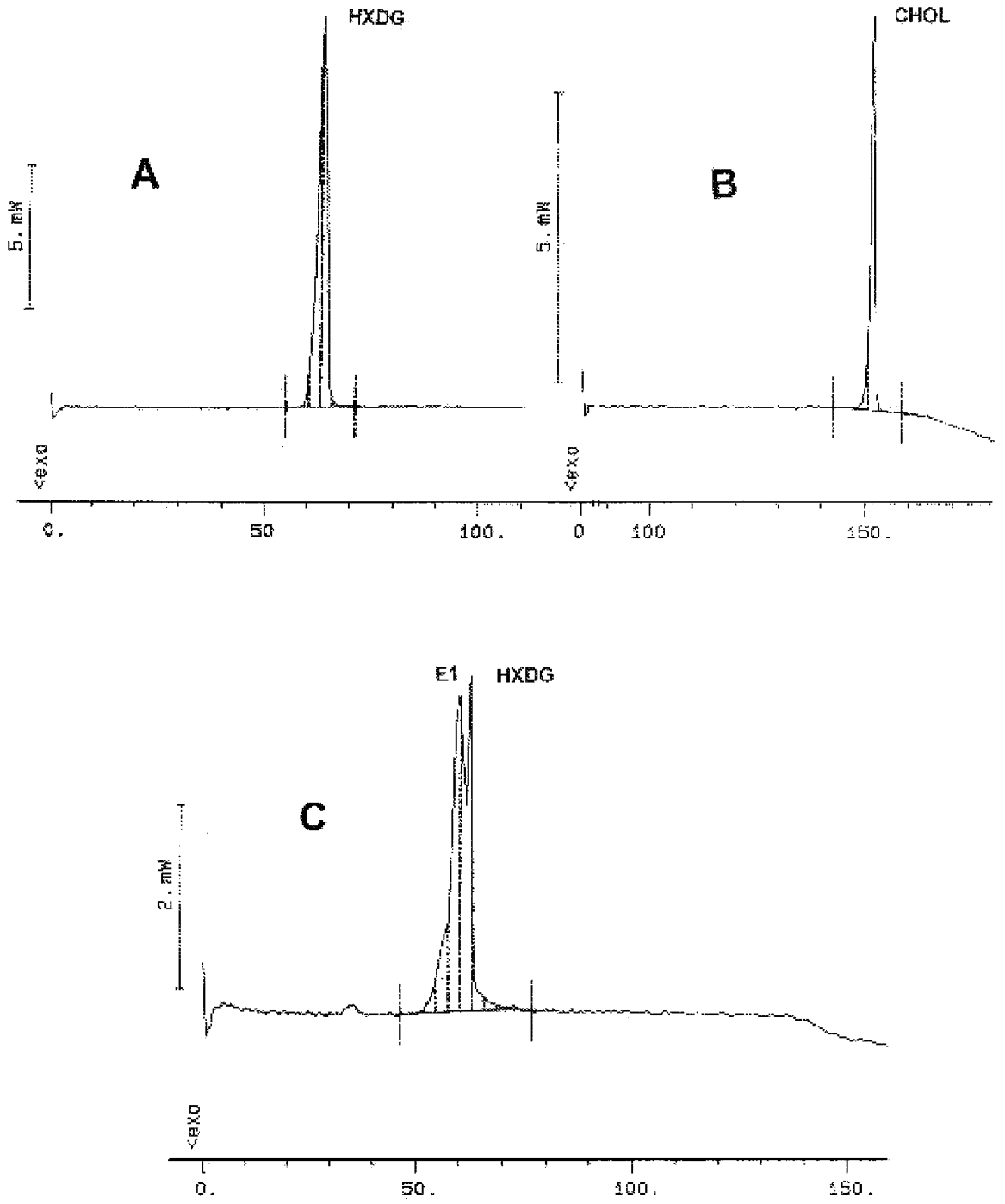


Fig. 6. DSC thermograms of 1-*O*-hexadecylglycerol and cholesterol mixture. (A) 1-*O*-Hexadecylglycerol (HXDG), (B) cholesterol (CHOL), (C) 1-*O*-hexadecylglycerol:cholesterol mixture (70:30), (D) 1-*O*-hexadecylglycerol:cholesterol mixture (50:50), (E) 1-*O*-hexadecylglycerol:cholesterol mixture (30:70). E1 and E2 are eutectic mixtures and K is molecular association complex.

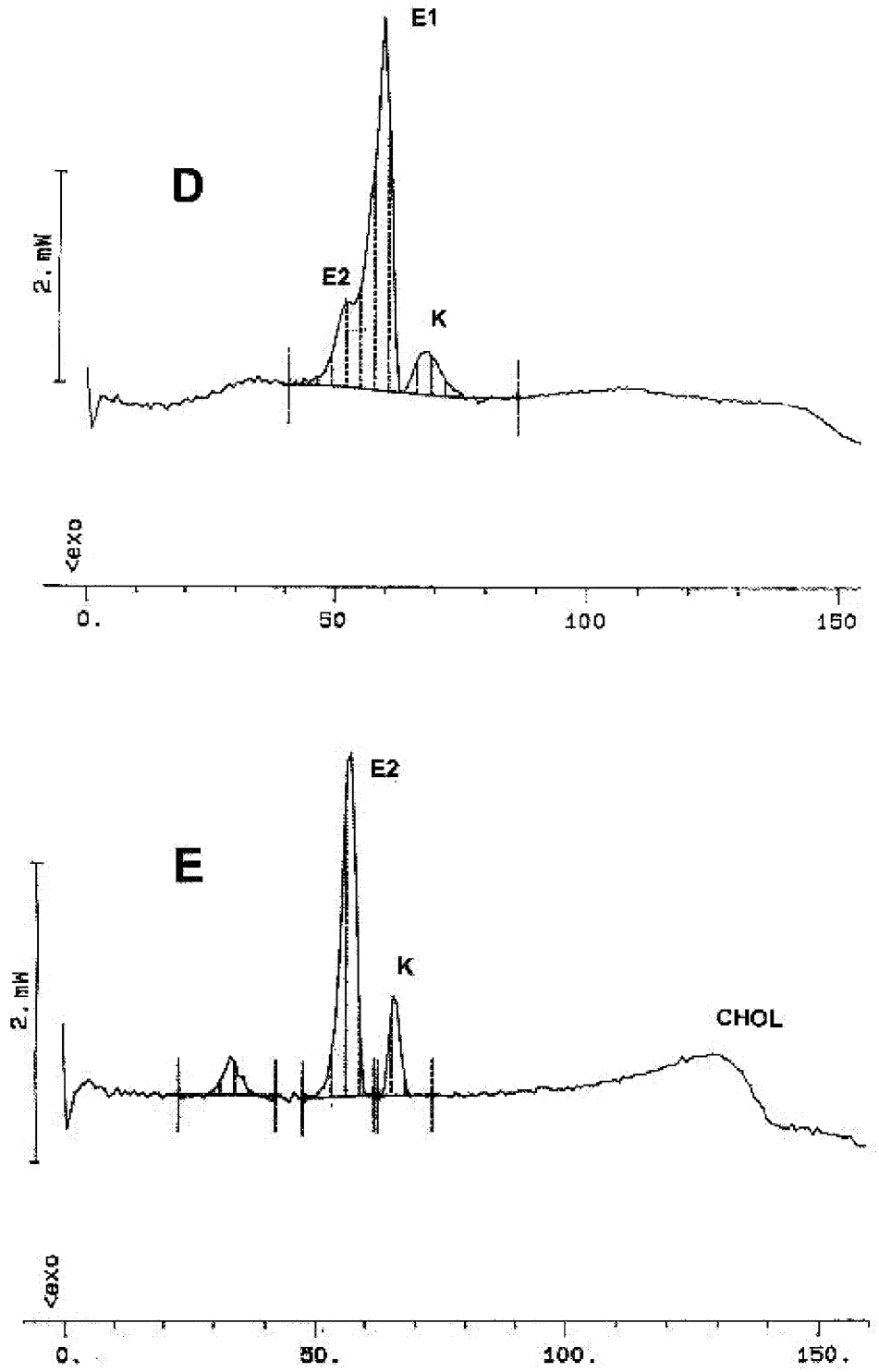


Fig. 6 (*Continued*)

1995). However ALKG are the simplest among these and have not previously been explored for vesicle formation.

From the vast literature on vesicles, it appears that not much attention was focused on exploring the possibility of converting the amphiphilic moieties that are biologically active, or can serve as targettable ligands, into bilayer vesicles. However as such a very few materials with dual qualities such as amphiphilicity and biological activity may be available. Hence, it would be instructive to synthesize such amphiphilic materials which can be converted into bilayer vesicles. The recent work of Uchegbu (1998) is on these lines. They have synthesized palmitoyl muramic acid and *N*-palmitoyl glucosamine and converted them into niosomes. The former is useful to improve the immuno-adjuvant effect and the latter improved targeting to malignant tissues. We have converted ascorbyl palmitate into vesicles (Gopinath et al., 2001b) and have synthesized some more amphiphiles which exhibit prominent antioxidant property. These may have potential applications in the treatment of disorders implicated with reactive oxygen species such as cancer and HIV.

While working on ALKG vesicles one important issue pertaining to the structural features of vesicle forming materials and their influence on vesicle formation arose. We made a preliminary attempt to examine this issue. In OCDG and glyceryl monostearate the polar head group and hydrophobic chain length are common. The only difference is, the former is an ether and the latter an ester. Interestingly the ether could form the vesicles whereas the ester could not. This provides some evidence that structural features of an amphiphile seem to play a key role in the formation of bilayer vesicles in addition to physicochemical properties such as optimum hydrophobicity, hydrophobic chain length and polar head group area, etc.

4. Conclusions

Algosomes are spherical vesicles and are capable of encapsulating drugs in the aqueous regions of the bilayer. They are osmotically sensitive, vulnerable to electrolytic destabilization and are stable for only a few days.

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