J. Pharm. Pharmacol. 1985, 37: 863–868
Received August 29, 1985



# The preparation and properties of niosomes—non-ionic surfactant vesicles

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Vesicles were prepared on hydration of a mixture of a single or double alkyl-chain, non-ionic surfactant with cholesterol. These vesicles, or 'niosomes', are capable of entrapping and retaining water soluble solutes such as carboxyfluorescein, are osmotically active and can be formulated to release entrapped solute slowly. The physical characteristics of the vesicles were found to be dependent on the method of production and three such methods, based on liposome technology, are described. The vesicles have been characterized by photon correlation spectroscopy, freeze fracture electron micrography, measurement of solute entrapment efficiency, and solute release rates. Vesicular forms of the single chain surfactant which could be formed under certain conditions in the absence of cholesterol are also described.

The concept of carriers to deliver drugs to target organs and modify drug disposition has been widely discussed and is well documented (Gregoriadis 1981). The majority of such reports have concerned the use of phospholipid vesicles, or liposomes (Poste et al 1984). These exhibit certain disadvantages, one of which relates to their chemical instability. Because of their predisposition to oxidative degradation, phospholipids must be stored and handled in a nitrogen atmosphere. The cost and variable purity of natural phospholipids might be other considerations militating against adoption of liposomes as drug delivery vehicles. Alternatives to phospholipids are thus of interest from the technical viewpoint and could also allow a wider study of the influence of chemical composition on the biological fate of vesicles.

Many synthetic amphiphiles form vesicles (Fendler 1982), but as most are ionic and relatively toxic, they are generally unsuitable for use as drug carriers. Vesicle formation by some members of dialkyl polyoxyethylene ether non-ionic surfactant series was reported by Okahata et al (1981). Handjani-Vila et al (1979) reported that vesicular systems were formed when a mixture of cholesterol and a single-alkyl chain, non-ionic surfactant, synthesized by them, was hydrated. The resultant vesicles, which have been termed 'niosomes', can entrap solutes, are osmotically active and are stable (Baillie et al 1984). In addition, handling and storage of the surfactant requires no special conditions. Preliminary studies in this laboratory indicate that niosomes behave in-vivo, like liposomes, prolonging the circulation of entrapped drug and altering its organ distribution and metabolic stability (Azmin et al 1985). As with liposomes, the properties of niosomes depend both on the composition of the bilayer (Inoue 1974) and on the method of their production (Szoka & Papahadjopoulos 1980). The present study, which is confined to the investigation of encapsulation and retention of entrapped solute, compares various methods of niosome production as a prelude to further biological investigations.

During this work it was observed that stable vesicles could be formed in the absence of cholesterol; these displayed the general characteristics of niosomes, but were more permeable to entrapped solute.

Throughout this paper the terms niosomes and surfactant vesicles are used interchangeably to describe closed vesicular structures formed from non-ionic surfactants with or without the admixture of cholesterol or other lipids.

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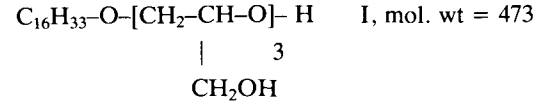
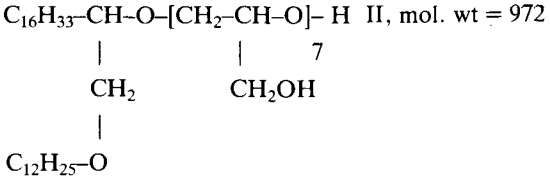
## MATERIALS AND METHODS

### *Materials*

5,6-Carboxyfluorescein (CF) (Eastman Kodak) was partially purified over activated charcoal before use. The non-ionic surfactants I and II were used as received from Synthelabo, France.



$C_{16}H_{33}$–O–[$CH_2$–CH–O]– H  I, mol. wt = 473
|  3
$CH_2OH$



$C_{16}H_{33}$–CH–O–[$CH_2$–CH–O]– H  II, mol. wt = 972
|  |  7
$CH_2$  $CH_2OH$
|
$C_{12}H_{25}$–O

Cholesterol (CHOL) was purchased from Sigma, Dorset, UK. All other reagents were of analytical grade.

*Methods*

(a) *Ether injection.* 150 μmol of surfactant/CHOL mixture was dissolved in 20 ml diethyl ether and injected slowly (0·25 ml $min^{-1}$), through a 14-gauge needle, into 4 ml of aqueous phase maintained at 60 °C.

(b) *Hand-shaking.* 150 μmol surfactant/CHOL mixture was dissolved in 10 ml diethyl ether in a 50 ml round-bottomed flask, and the ether removed at room temperature (20 °C), under reduced pressure, in a rotary evaporator (Buchi). The dried surfactant film was hydrated with 5 ml aqueous phase at 50–60 °C with gentle agitation.

(c) *Sonication.* To 150 μmol surfactant/CHOL mixture in a 10 ml glass vial was added 2 ml aqueous phase. The mixture was probe sonicated at 60 °C for 3 min using an M.S.E. 150 W sonicator with a titanium probe; the power was set at approximately 10–15% of maximum output.

In each preparative method, the aqueous phase used for surfactant hydration was 200 mM CF solution or water to give, respectively, CF or 'empty' niosomes. In each method, the 150 μmol of material hydrated was either pure surfactant or a mixture of surfactant with CHOL.

*Separation of free from niosomal CF*

The aqueous dispersions of CF niosomes produced by each method were dialysed exhaustively in Cuprophane dialysis tubing, against pH 7·4 phosphate buffered (Sörensens $1·3 \times 10^{-2}$ M) 0·9% w/v saline (PBS).

*CF entrapment efficiency*

The concentration of CF within the internal niosomal aqueous compartment was assumed to be 200 mM, that is the concentration used to hydrate the surfactant/CHOL mixture. At this concentration CF is self-quenched and on complete vesicle disruption at a final concentration of 50% n-propanol or 0·1% Triton X-100, dilution in the extra-vesicular bulk volume gives an approximate $10^3$-fold increase in fluorescence. The released CF, in a suspension of known surfactant concentration, was determined spectrofluorimetrically using a Perkin Elmer Model 203 fluorescence spectrophotometer (excitation $\lambda$ = 486 nm, analyser $\lambda$ = 514 nm). Entrapment efficiency was expressed as litres of entrapped CF solution per mole surfactant.

*Freeze fracture electron micrographs*

Empty niosomes were passed through 0·8 μm polycarbonate membranes (Nucleopore) at 50 °C. Small samples (approx. 10 μl) of the filtrates were flash-frozen, at −190 °C, in liquid propane then fractured under vacuum ($4 \times 10^{-6}$ Torr). These preparations were shadowed using platinum/carbon at a 45° angle and then carbon backed, all at −100 °C under vacuum. The cleaned shadowed replicates were observed by transmission electron microscopy.

*Stability in buffer*

The stability of niosomes in buffer was determined by monitoring CF efflux by a method based on that of Allen & Cleland (1980). 25 μl of CF niosome suspension was diluted to 5 ml with PBS and this dilute suspension stored in the dark at a constant temperature with gentle agitation. At time intervals a 3 ml sample was removed into a 1 cm cuvette and the fluorescence measured as described above. Total CF entrapment was determined by measuring the fluorescence of 25 μl niosome suspension + 100 μl n-propanol after dilution to 5 ml with PBS; CF efflux was expressed as % of the total and as mol CF $mol^{-1}$ surfactant $min^{-1}$.

*Photon correlation spectroscopy*

5–10 ml of empty niosome suspensions were fractionated over Sepharose 4B (column size 30 × 2·5 cm) using water as eluent and collected as 4 ml fractions. For each preparation, that fraction with the highest absorbance at 400 nm was selected, passed through a 1·2 μm (Millipore) filter at room temperature, and diluted between 10–25 fold with filtered (1·2 μm) distilled water. Vesicle diameter was then measured at a 90° scattering angle using a single-clipped correlation spectrometer (Malvern Instruments) in conjunction with an He/Cd 10 mW laser (Liconix) ($\lambda$ = 632·8 nm).

*Osmotic shrinkage*

Osmotic shrinkage of vesicles was investigated by monitoring reductions in vesicular mean hydrodynamic diameter following addition of small volumes (10–100 μl) of 1 M NaI solution to 2·2 ml volumes of empty niosome suspensions.

## RESULTS AND DISCUSSION

*Latency of CF fluorescence and entrapment efficiency.*

Each of the three preparative methods described herein produced closed, discrete structures as judged from the latency of CF fluorescence which could be

demonstrated microscopically and macroscopically on disruption of the niosome preparations, for example by n-propanol, and these results are analogous to those described for liposomes (Kirby et al 1980) as are the entrapment efficiencies (Table 1).

Table 1. Entrapment efficiency of CF solution by niosomes of different composition and different methods of preparation.

| Preparative method | Niosome composition (mol %) | Entrapment efficiency[a] (litre mol$^{-1}$ surfactant) |
|---|---|---|
| Handshaken | 100 I | 0·124 |
| | 50 I : 50 CHOL | 0·106 ± 0·065 |
| Ether injection | 100 I | 1·22 ± 0·120 (5) |
| | 80 I : 20 CHOL | 0·85 ± 0·090 (3) |
| | 70 I : 30 CHOL | 0·68 ± 0·080 (6) |
| | 60 I : 40 CHOL | 0·86 ± 0·130 (3) |
| | 50 I : 50 CHOL | 0·44 ± 0·060 (9) |
| | 50 II : 50 CHOL | 0·65 ± 0·24 (6) |
| Sonication | 50 I : 50 CHOL | 0·132 ± 0·090 |

[a] Mean value ± standard deviation, number of observations in parentheses.
Niosomal CF was determined spectrofluorimetrically after vesicle disruption.

Thus, those niosomes prepared by ether injection had entrapment efficiencies which were significantly higher than those for hand shaken and sonicated vesicles. Ether injection liposomes are known to have high entrapment efficiencies (Deamer & Bangham 1976). However, in general, entrapments here (Table 1) are lower than those described in the literature (Szoka & Papahadjopoulas 1980) for comparable liposome preparations.

The differences in entrapment efficiency between, for example, ether injection (50% I : 50% CHOL, 0·44 litre mol$^{-1}$) and hand shaken (50% I : 50% CHOL, 0·106 litre mol$^{-1}$) niosomes presumably reflect the type of vesicle formed by each method. It would be expected that the unilamellar structures (Fig. 1A) formed by ether injection represent a more efficient use of surfactant than the multilamellar structures (Fig. 1B) formed by hand shaking, and, despite having an approximately 3-fold smaller diameter, the unilamellar niosomes display a significantly greater entrapment efficiency. For a series of ether injection vesicles prepared from surfactant I with different CHOL content (Table 1), it was apparent that the cholesterol decreased the entrapment efficiency, the lowest value, 0·44 litre mol$^{-1}$, being obtained at 50 mol% cholesterol. The entrapment with surfactant I without cholesterol was significantly higher at 1·22 litre mol$^{-1}$ although niosomes of this composition prepared by hand shaking gave a poor entrapment at 0·124 litre mol$^{-1}$. These monocomponent non-ionic surfactant vesicles are novel and could not be formed using surfactant II alone.

However, admixture of CHOL and surfactant II allowed higher entrapments of CF than that achieved using comparable surfactant I : CHOL mixtures (Table 1) although this was still less than achieved with liposomes.

*Electron micrographs*

The micrographs in Fig. 1 provide, in the first instance, confirmation of the formation of vesicular structures using each of the preparative methods and vesicle formation with pure surfactant I preparations (Fig. 1D). The replicates of the vesicles produced after hand shaking (Fig. 1B), which are essentially surfactant analogues of Bangham's original liposomes (Bangham et al 1965), are similar in appearance to electron micrographs of phospholipid vesicles. Thus they are large vesicles with a mean hydrodynamic diameter (Table 2) of some 900 nm (50% I : 50% CHOL) and an obvious multilamellar

Table 2. Niosome size, composition and method of preparation.

| Preparation | Composition (mol %) | Niosome diameter (m × 10$^{-9}$) Hydrodynamic[a] | Electron microscopic[b] |
|---|---|---|---|
| Hand shaken | 100 I | 986·9 ± 123·2 | 490·0 ± 378·0 |
| | 50 I : 50 CHOL | 894·7 ± 129·5 | 460·0 ± 323·0 |
| | 47·5 I : 47·5[c] | 964·6 ± 127·2 | n.d. |
| Ether injn. | 100 I | | n.d. |
| | 50 I : 50 CHOL | 335·1 ± 93·6 | 306·0 ± 178·0 |
| | 50 II : 50 CHOL | 302·1 ± 81·5 | n.d. |
| Sonicated | 50 I : 50 CHOL | 127·8 ± 89·9 | 152·5 ± 81·6 |

n.d. Not determined.
[a] Hydrodynamic diameters were determined by photo correlation spectroscopy at a 90° scattering angle.
[b] Electron microscope sizes were determined by direct measurement of 100 vesicles in electron micrographs of freeze fracture replicates. All sizes shown are mean values ± standard deviation.
[c] Vesicles contained 5% diacetyl phosphate.

structure. Although the mean diameter of 460 nm obtained by direct measurements on the electron micrographs appears low, it must be remembered that these suspensions were passed through an 0·8 μm polycarbonate membrane which effectively imposes an upper limit on vesicle size. The size of the much smaller niosomes prepared by ether injection (Fig. 1A), or sonication (Fig. 1C), would not be affected by this filtration and there is good agree-

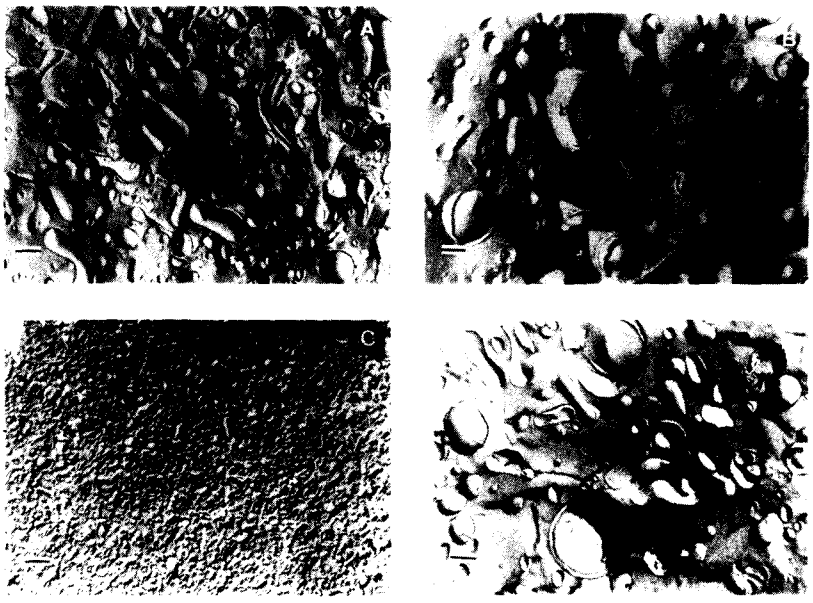



FIG. 1. Freeze fracture replicates of surfactant vesicle suspensions prepared by A, ether injection; B and D, hand shaking and C, sonication. Vesicle composition was A, B and C, 50 : 50 mol% surfactant I : cholesterol and D, 100 mol% surfactant I and in all cases the suspensions were extruded through a 0·8 μm pore membrane before freezing. Vesicles comprising 50 : 50 mol% surfactant II : cholesterol and prepared by the appropriate method had the same appearance as the replicates shown in A, B and C. Bar = 500 nm.

ment (Table 2) between the hydrodynamic and electron micrograph diameters for both of these niosome types. This is in spite of the fact that photon correlation spectroscopy emphasizes the contribution of large structures to the mean diameter. As might be expected, all of the niosome preparations were heterogeneous in size distribution.

There was no indicaton in the ether injection preparation (Fig. 1A) of the multilamellar structures which can be seen in the hand-shaken vesicles with (Fig. 1B), or without (Fig. 1D), cholesterol. Phospholipids produce unilamellar vesicles when prepared by ether injection (Deamer & Bangham 1976). What is unusual in the present work is the high proportion of non-spherical vesicles; these have not been described in similar liposome preparations.

The smallest surfactant vesicles were obtained after sonication (Fig. 1C) and in the field shown the largest structure represents a vesicle of about 300 nm diameter. These larger structures, as expected, exhibit a multi-lamellar structure. The smallest structures visible in Fig. 1C, which presumably are the surfactant vesicle equivalent of phospholipid small unilamellar vesicles, have diameters of between 20 and 30 nm. Prolonged sonication could be expected to convert a greater proportion of the vesicle population to the unilamellar form.

*Stability in buffer*

Efflux of CF from niosomes was biphasic with an initial period of rapid release which, for most preparations, gave way after about 4 h to a period of slower release (Fig. 2). This second phase continued for at least 24 h. Cholesterol markedly decreased CF efflux during both release phases, which is in accord with the membrane stabilizing activity of this lipid (Demel & De Kruyff 1976). There was no significant difference between surfactant I and II niosomes, both containing 50% CHOL, in terms of CF efflux. The rate of efflux in these samples suggests that a substantial amount of entrapped CF would be retained under long-term storage conditions. However, vesicles prepared from 100% surfactant I were more permeable to the CF solute.

The rate of CF efflux, was calculated for the initial 4 h period (Fig. 2) and the values (mol CF $mol^{-1}$ entrapped CF $mol^{-1}$ surfactant $min^{-1}$) obtained were 100% surfactant I, $2{\cdot}1 \times 10^{-3}$; 50% surfactant I, $5{\cdot}9 \times 10^{-4}$; 50% surfactant II, $5{\cdot}4 \times 10^{-4}$. That is, addition of 50% CHOL reduced vesicle permeability to CF by a factor of 10.

*Osmotic shrinkage*

Addition of hypertonic salt solution to suspensions of empty niosomes causes an absorbance increase.

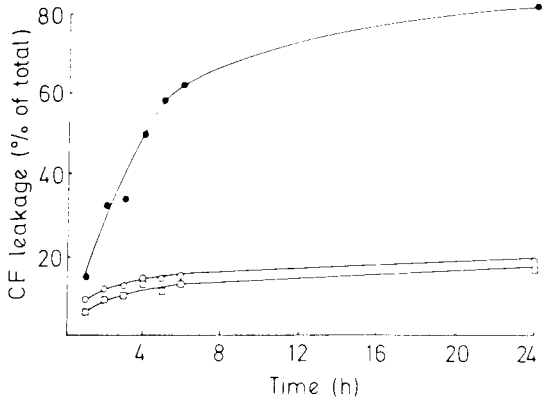



FIG. 2. Time course of carboxyfluorescein (CF) leakage from suspensions of ether injection surfactant vesicles in pH 7·4 phosphate buffer at 37 °C. CF efflux is expressed as a percentage of the total CF entrapped per mol of surfactant. Total entrapped CF was determined after propanol disruption of the vesicles. Vesicle composition, ●—●, 100% surfactant I; ○—○, 50% surfactant I; □—□, 50% surfactant II. 50% surfactant I or II refers to vesicles comprising the appropriate surfactant and 50 mol % cholesterol.

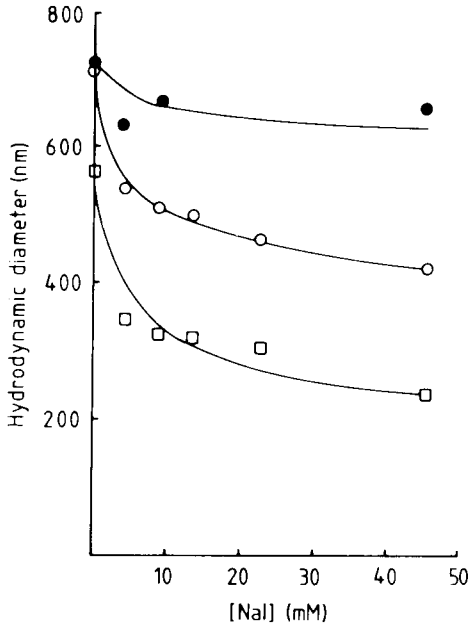



FIG. 3. Osmotic shrinkage of empty surfactant vesicles induced by challenge with NaI solution. Size was determined by photon correlation spectroscopy of suspensions of the multilamellar hand shaken vesicles and is expressed here as a mean hydrodynamic diameter. Vesicle composition as in Fig. 2.

similar to that described for liposomes by Blok et al (1975), who interpreted this to be due to an osmotically induced reduction in vesicle diameter. The osmotically induced reduction in niosome hydrodynamic diameter achieved by addition of NaI solution is shown in Fig. 3.

CHOL-free surfactant I niosomes were osmotically insensitive in contrast to vesicles containing this lipid. This apparent resistance to osmotic shrinkage presumably indicates high permeability to NaI, which is in general agreement with the results for CF efflux from these niosomes (Fig. 2), and also with the membrane stabilizing activity of cholesterol.

It must be assumed that, with the observed NaI-induced diameter reduction, there is a concomitant water efflux in response to the osmotic insult, and it is possible to quantify this efflux as follows.

For the 50% I:50% CHOL vesicle preparation, after addition of the first 10 μl of 1 M NaI solution there was a change in mean hydrodynamic diameter from 712·3 to 539 nm (Fig. 3), which assuming spherical vesicles, represents a change in volume of 56·61%. The same change in the volume of 0·106 litre (Table 1) entrapped by 1 mol surfactant is 0·06 litre. This volume is equivalent to 3·33 moles $H_2O$, so that the water efflux per mole of vesicular materials was 3·33 mol.

Similarly for 50% II:50% CHOL niosomes the water efflux per mole of vesicular material was calculated to be 4·52 mol. To estimate the true water flux through a single surfactant bilayer requires further vesicle characterization.

The observed osmotically induced volume changes occurred over about 1 min so that the water efflux rate is effectively 3·33 and 4·52 mol $min^{-1}$ per mol of surfactant I and II vesicles, respectively. These values are some $10^4$ greater than those for CF efflux.

*Conclusions*

From these results and the previous data from this laboratory (Azmin et al 1985) it can be concluded that niosomes are a promising vehicle for drug delivery. Being non-ionic they are likely to be less toxic than vesicles produced from ionic amphiphiles. Vesicles prepared from the mono- and di-alkyl surfactants appear here to have similar permeability properties in-vitro, although there may well be significant differences between mono-alkyl and di-alkyl niosomes in-vivo and in cell culture.

*Acknowledgements*

We thank L'Oreal, France for financial support (for GM), for gifts of surfactant and for permission to publish this paper; Mr J. S. Whittaker, Dr G. Vanlerberghe and Dr R. M. Handjani-Vila for helpful discussions and support; Mr Lawrence Tetley, Department of Zoology, University of Glasgow for expert technical assistance in production of the freeze fracture electron micrographs.

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