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Preparation and properties of new unilamellar non-ionic/ionic surfactant vesicles

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Abstract

Non-ionic surfactant vesicles (NSVs) were prepared from polysorbate 20 and cholesterol by means of two different methods: by direct sonication of an aqueous dispersion of the various components (bulk) or by solubilization of the components, evaporation of the organic solvent to form a film inside the vessel used for the preparation and then by sonication (film). The influence of the preparation technique on the properties of the obtained structures was studied. Vesicles with bigger dimensions and higher entrapment efficiency were obtained when sonication was carried out after the film formation.

Vesicle formation in the presence of ionic surfactants was investigated in order to evaluate the effect of charged components on vesicle dimensions, entrapment efficiency and stability. Dimethyldioctadecylammonium bromide (DDOA) and cetylpyridinium chloride (CPy) were used to introduce a positive charge in the vesicle structure, while dicetylphosphate (DCP) was used for a negative charge. Better resistance to osmotic stress and higher entrapment efficiency values were obtained with vesicles containing DCP and CPy. © 1998 Elsevier Science B.V.

Keywords: Polysorbate 20; Non-ionic surfactant vesicles; Ionic surfactants; Entrapment efficiency; Vesicle stability; Freeze-fracturing

1. Introduction

In recent years non-ionic surfactant vesicles (NSVs) (Florence, 1993; Uchegbu and Florence, 1995) received great attention as potential drug delivery systems for different routes of administration (e.g. intravenous, Rogerson et al., 1988; intraperitoneal, Uchegbu et al., 1994; oral, Yoshida et al., 1992, or topical, Schreier and Bouwstra, 1994), as well as in cosmetics (Handjani-Vila et al., 1993; Mazda et al., 1994).

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NSVs can be prepared both as multilamellar and unilamellar structures and can entrap hydrophilic, lipophilic and amphiphilic molecules (Ozer et al., 1991); moreover NSVs show, in most cases, numerous advantages over the liposomes such as lower costs and higher chemical stability; furthermore, vesicle aggregation and fusion usually occur at a remarkable lower rate for NSVs than for liposomes (Seras et al., 1992).

In a previous study (Santucci et al., 1996) we reported a simple and rapid method for the preparation of unilamellar NSVs from mixtures of Polysorbate 20 (Tween 20) and cholesterol.

Even if the critical packing parameter (CPP), (Uchegbu and Florence, 1995), for Tween 20 is well below 0.5 and the head group area above 0.5 nm^2 (about 1.5 nm^2) it was possible to obtain unilamellar vesicles when an equimolar amount of cholesterol was used (Fig. 5A, B).

This is in accordance with the observation that a cone shaped amphiphile plus a wedge shaped one (e.g. cholesterol) cooperate to form bilayer membranes in vivo (Israelachvili et al., 1980). The aim of the present work is to investigate in more detail the influence of the method of preparation and of the presence of ionic surfactants on size, stability and sodium calcein entrapment of the Tween 20-cholesterol vesicles.

2. Materials

Polysorbate 20 (TW20) and cholesterol (CHOL) were purchased from Merck (Darmstadt).

Dicetylphosphate (DCP), dimethyldioctadecylammonium bromide (DDOA), cetylpyridinium chloride (CPy), the lipophilic fluorescent probe diphenylhexatriene (DPH) and the hydrophilic fluorescent probe sodium calcein (CA) were obtained from Sigma (St. Louis, MO).

Sephadex G75 and G200 were Pharmacia (Upsala, Sweden) products.

Phosphate buffer (0.07 M, pH 7.4) was used.

3. Methods

3.1. Preparation of non-ionic surfactant vesicles

As reported in Table 1, in a typical group of experiments for vesicle preparation, CHOL had a constant concentration (15.0 mM) while the concentrations of TW 20 and DCP ranged from 1.0 to 15.0 mM, for an overall concentration of the last two substances that remained constant (i.e. 15 mM).

When positively charged DDOA or CPy were used, concentrations of these surfactants either above or below 1 mM remarkably reduced the yield in obtained vesicles; consequently only such concentration of these surfactants was actually used for our investigation, while TW20 concentration was 14 mM (Table 1, samples F and G).

Unilamellar vesicles were obtained by means of two different methods:

(a) Bulk (sample 1, Table 1)—direct sonication (3 min, 60°C) of a vortexed dispersion of the various components (in a typical experiment—i.e. sample 1 of Table 1—91.5 mg of TW20 and 29.0 mg of CHOL were used) in an aqueous phase (5.0 ml of phosphate buffer), that in several experiments contained also 0.1 M Na calcein; (b) Film (sample 2, Table 1)—the appropriate amounts of the various substances (in a typical experiment—i.e. sample C of Table 1—45.8 mg of TW20, 20.6 mg of DCP and 29.0 mg of CHOL were used) were dissolved in 3.0 ml of a $CHCl_3/CH_3OH$ (1/3)

Table 1
Composition of the samples (mM)

Sample	TW 20	CHOL	DCP	DDOA	CPy
1 & 2	15.0	15.0	—	—	—
A	14.0	15.0	1.0	—	—
B	11.0	15.0	4.0	—	—
C	7.5	15.0	7.5	—	—
D	5.0	15.0	10.0	—	—
E	1.0	15.0	14.0	—	—
F	14.0	15.0	—	1.0	—
G	14.0	15.0	—	—	1.0

TW 20: polyoxy-ethylene sorbitan monolaurate; CHOL: cholesterol; DCP: dicetylphosphate; DDOA: dimethyldioctadecylammonium bromide; CPy: cetylpyridinium chloride.

Table 2
Characterization of the vesicles

Sample	Mean diameter (nm)	p.i.	f.a.	e.e.
1	153 ± 57	0.30 ± 0.01	0.36 ± 0.01	0.47 ± 0.07
2	202 ± 58	0.30 ± 0.01	0.36 ± 0.01	0.67 ± 0.07
A	221 ± 60	0.20 ± 0.01	0.38 ± 0.02	0.56 ± 0.06
C	279 ± 57	0.20 ± 0.01	0.15 ± 0.01	0.90 ± 0.07
E	234 ± 58	0.40 ± 0.01	0.03 ± 0.02	0.10 ± 0.08

p.i.: Polydispersity index; f.a.: fluorescence anisotropy; e.e.: entrapment efficiency (mol/mol of surfactant). Each value is the mean ± SD of at least three experiments.

mixture in a test-tube. The organic phase was removed at room temperature under reduced pressure to form a thin film inside the test-tube. The residue of the organic solvents was then removed at 10^{-1} mmHg.

The dried film was hydrated by addition of an aqueous phase (phosphate buffer) that in several experiments contained also CA (0.1 M); the dispersion was vortexed for 10 min and then sonicated for 3 min at 60°C.

Sonication was carried out with a Soniprep 150-MSE, Crowley—equipped with an exponential microprobe operating at 23 kHz and an amplitude of 6 mm.

For DPH-loaded vesicles, the fluorescent probe was previously dissolved in CH_3OH and vacuum evaporated in the same vessel used for vesicle preparation. The final DPH concentration was 2×10^{-4} M.

3.1.1. Vesicle purification

The vesicle dispersion was transferred in a visking tubing (36/32 S.I.C.) and purified by exhaustive dialysis against phosphate buffer and then by gel-filtration on Sephadex G75 (for CA) or G200 (for DPH), using a glass column (50 × 1.8 cm) and phosphate buffer as eluent.

3.2. Vesicle characterization

3.2.1. Vesicle size measurement by dynamic laser light scattering

The vesicle dispersions were diluted about 100 times in phosphate buffer. Dust particles in the samples were eliminated by filtration through a 0.45 μm pore size filter.

Vesicle size distribution was measured on a Nicomp Model 370 Submicron Particle Sizer at 25°C, with a scattering angle of 90.0° and a refractive index of 1.210. The polydispersity index (p.i.) was then calculated (Table 2).

3.2.2. Fluorescence anisotropy measurements

Steady-state fluorescence anisotropy measurements, for DPH loaded vesicles, were carried out with a Perkin-Elmer LS5 spectrofluorometer, at the excitation (Ex) and emission (Em) wavelengths of 400 and 425 nm respectively. Although it is still controversial (Lentz, 1989) whether it is quite appropriate to calculate DPH fluorescence anisotropy for the evaluation of membrane fluidity, the various tested samples showed remarkable differences of anisotropy values that allowed us to distinguish among the various aggregated structures.

3.2.3. Entrapment efficiency

Entrapment efficiency (e.e.) was calculated by means of the quantitative evaluation of CA released by the purified vesicles after their complete disruption by addition of an equal volume of isopropanol, and a further dilution (1/10) with the phosphate buffer.

The concentration of the fluorescent probe was spectrofluorometrically (Ex/Em 490/520 nm) determined and e.e. was expressed as moles of entrapped CA per mole of surfactant. For calculations, the actual TW20 concentration was determined by the spectrophotometric method proposed by Kato et al. (1989): treatment with a cobalt thiocyanate solution, extraction with CH_2Cl_2 and determination of the absorbance of

the organic phase was determined at 620 nm. An appropriate calibration curve was used as a reference. To evaluate the amount of DCP that was actually present in the vesicle structure the spectrophotometric method proposed for the dosage of phosphorus, as soluble orthophosphate, by the IRSA-CNR was used (IRSA-CNR, 1994): the purified vesicles were treated with a mixed reagent containing ammonium molybdate (0.6% w/v), sulfuric acid (0.9% w/v), ascorbic acid (1% w/v) and antimonium-potassium tartrate (0.014% w/v). After 10 min the absorbance of the sample was determined at 885 nm. A calibration curve at standard DCP concentrations was used. The amounts of DDOA and CPy entering the vesicle bilayer were not calculated because of their small initial concentration with respect to TW20.

When necessary, the actual DPH entrapment was evaluated by fluorescence measurements (Ex/Em 325/400 nm) after disruption of the vesicles.

3.2.4. Physical stability

For the evaluation of vesicle physical stability, expressed as the resistance to an osmotic stress, 1.0 ml of CA loaded NSV samples were added to 10 ml of a Krebs–Ringer solution (Allen and Cleland, 1980), and the release of calcein was determined as a function of time by spectrofluorometric measurements. During the experiments, the temperature was maintained at 4, 25, 37 or 60°C by means of a thermostated water bath.

For an appropriate comparison of the physical stability of the different types of vesicles, the most significant results were obtained at 25 and 37°C.

In fact, at 60°C all the vesicles were disrupted in about 1 h, while at 4°C they were stable for more than 2 months.

It must be pointed out that, although no preservatives were added to the samples, no sign of microbiological alteration could be macroscopically detected during the storage at room temperature of the various preparations.

Vesicle physical stability was also estimated in non-stressed conditions (i.e. in phosphate buffer) by means of the determination of CA released from the aggregated structures as a function of time. For this purpose 5 ml of purified vesicles were dialysed against 150 ml of buffer solution and sodium calcein concentrations were detected by fluorescence measurements at fixed time intervals, the temperature was maintained at 25°C.

For a further evaluation of vesicle stability, high performance gel exclusion chromatography was carried out on vesicles maintained in the phosphate buffer at 4, 25, 37 or 60°C in a thermostated water bath.

All HPLC analyses were performed with a Perkin-Elmer 250 liquid chromatography apparatus, equipped with a Perkin-Elmer LS5 spectrofluorometer as detector, a 50 μl Rheodyne injector and a computer integrating apparatus. The column was a Tosohaas TSK G6000 PW (30.0 cm $\times$ 7.5 mm I.D.); the mobile phase was phosphate buffer; the flow rate was 0.15 ml/min; the temperature was maintained at 25°C, and a 1 mm flow-cell was used for the detection of the vesicles at 600/600 nm (i.e. the turbidity of the eluate). Again, more significant results were observed for vesicles maintained at 25 and 37°C and the collected data were in accordance with those obtained by fluorescence measurements.

All reported data represent the mean values of at least three separate experiments that always presented a good reproducibility, as indicated in Table 2.

3.2.5. Freeze-fracture electron microscopy

Vesicles were also examined by means of the freeze-fracture microscopy technique. The samples were impregnated in 30% glycerol and then frozen into partially solidified Freon 22, freeze-fractured in a freeze-fracture device (-105°C, 10^{-6} mmHg) and replicated by evaporation from a platinum/carbon gun.

The replicas were extensively washed with distilled water, picked up onto Formvar-coated grids and examined with a Philips CM10 transmission electron microscope.

4. Results and discussion

4.1. Influence of the preparation method

The unilamellar vesicles, prepared according to the two methods described above (samples 1/bulk

and 2/film in Table 1), have the same composition, but showed different characteristics in dimensions, entrapment efficiency and stability.

NSVs prepared without the preliminary 'film' formation had smaller dimensions and lower entrapment efficiency for Na calcein than the others obtained by means of the 'film' technique even if the fluorescence anisotropy values were the same in both cases (Table 2).

On the other hand, as reported in Fig. 1 that show the relative increase of CA released as a function of time in the Krebs–Ringer solution, sample 1 was more stable than sample 2 at the temperatures of 25 and 37°C; thus indicating that,

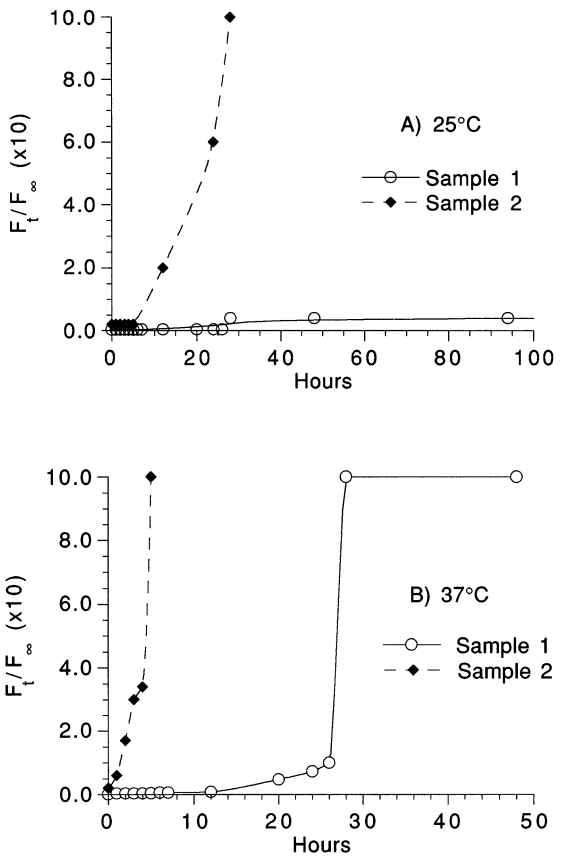

Fig. 1. Comparison of vesicle stability in the Krebs–Ringer solution at 25°C (A) and 37°C (B), according to the method used for the preparation technique: i.e. bulk or film (samples 1 and 2 of Table 1). The plot reports the release of CA as a function of time, expressed as increase of the relative fluorescence (F_t/F_∞).

besides the actual formulation, the technique used for the preparation of the vesicles has a fundamental role in determining the overall properties of NSVs.

Due to these differences, all comparisons of vesicle properties reported below refer to NSVs that were prepared by means of the same technique (i.e. film).

4.2. Influence of ionic surfactant

The inclusion of ionic surfactants in the formulation is generally used to stabilize NSVs by means of an increase of their ζ potential (Grit and Crommelin, 1993).

Moreover, it has been reported (Bangham et al., 1965) that the thickness of the aqueous compartment in ovolecithin bilayers increases as a direct function of the surface charge (Verwey and Overbeek, 1948) and that the presence of a charge in the membrane structure can increase water uptake within the double layer (Abramson, 1971).

In our previous studies (Santucci et al., 1996) we verified that if the ratio surfactant:cholesterol was above or below 1:1, it was very difficult to prepare vesicles and it was also found that the best concentration for a maximun yield in obtained vesicles was 15 mM.

Thus, in the present work we used such concentrations for the cholesterol and for the surfactant mixture (i.e. as TW20 concentration decreased, ionic surfactant concentration increased accordingly, for an overall 15 mM concentration—Table 1).

In our experiments we verified that the different ratios of the two surfactants influenced vesicle dimensions, entrapment efficiency and stability.

The most interesting formulation seemed to be sample C that had the same concentration of TW20 and DCP (7.5 mM).

Among the various tested formulations, containing DCP, sample C vesicles were bigger than the others (samples A, B, D, E); they also showed the maximum value of entrapment efficiency (Table 2) and greater resistance in the Krebs-Ringer solution that was particularly evident at the temperature of 25°C. In Fig. 2 the stability in the stressed conditions of the various formula-

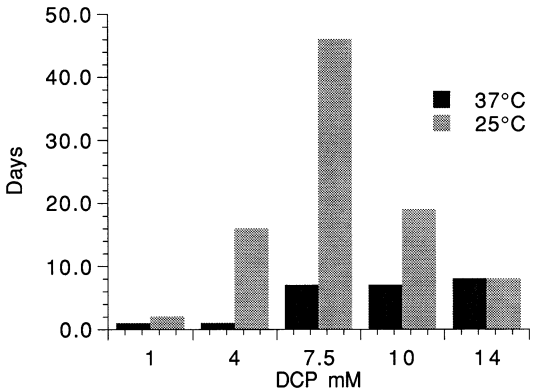

Fig. 2. Comparison of vesicle stability in the Krebs–Ringer solution at 25 and 37°C related to the amount of DCP present in the formulation (samples A–E of Table 1). The plot reports the time required to reach the maximum fluorescence value (F_∞).

tions, prepared with different amounts of DCP and containing CA, is expressed as days needed to reach the complete release of calcein (i.e. maximum CA fluorescence values).

According to Wan and Lee (1974), surface free energy at an interface decreases as hydrophobicity of the surfactant increases. This statement, that can be applied also to NSVs (Yoshioka et al., 1994), is in accordance with the results obtained from vesicle size evaluations (Table 2): the presence of an anionic surfactant such as DCP, that shows a high degree of hydrophylicity, led always to vesicles that were larger than the uncharged ones, reaching a maximum value for sample C (i.e. the most stable vesicles).

The higher stability in the Krebs–Ringer solution observed for sample C (Fig. 2) can be related to an optimized ion–dipole interaction between the hydroxyl group of cholesterol and the ionized phosphate group (Shah and Shulman, 1967). On the other hand a further increase of negative charges (sample E) did not yield more stable vesicles probably because of an induced remarkable repulsion within the double layers (Fig. 2). Accordingly, entrapment efficiency of sample C vesicles was remarkably higher than that obtained with sample E (Table 2).

It must be pointed out that since the presence of DCP in the formulation led to charged structures, a corresponding increase of the hydration of the bilayers should be obtained (Abramson, 1971). This effect was actually confirmed by the comparison of DPH fluorescence anisotropy of DCP charged vesicles and that of uncharged NSVs: as reported in Table 2, sample C showed a lower fluorescence anisotropy value with respect to the uncharged samples 1 and 2; but again, a further increase of DCP content (sample E) did not correspond to a lower fluorescence anisotropy value. This last result can be related to the hydrophobicity of DPH that can be confined only within the fewer uncharged sites of the vesicle, thus leading to a remarkable decrease of entrapped DPH in this highly charged vesicular structure (actually, sample E entrapped about 30% less DPH than sample C).

As far as the positively charged structures are concerned, as previously pointed out, vesicles were obtained only when a 1 mM concentration of the cationic surfactants was used (samples G and F, Table 1).

The behaviour of sample G, containing 1 mM CPy, was similar, as far as entrapment efficiency (0.065 ± 0.007 mol/mol) and membrane fluidity (fluorescence anisotropy 0.15) are concerned, to that of sample C, that actually had a DCP concentration of 7.5 mM. This effect can be related to the different steric hindrance of CPy and DCP molecules. The lower e.e. value (0.022 ± 0.006 mol/mol) obtained with the other cationic surfactant, DDOA (sample F), supports this hypothesis.

In Fig. 3 vesicle resistance of samples F and G in comparison with that of the uncharged sample 2, is reported. Results are given as Na calcein released as a function of time in the Krebs–Ringer solution. Experiments were carried out at 25 and 37°C, respectively. It is possible to observe, at both temperatures that sample G was more stable than samples F and 2.

In Fig. 4 Na calcein released from samples 2, C, F and G in phosphate buffer at 25°C (i.e. in non-stressed conditions) is reported. The remarkably faster release observed for samples C and G, that actually correspond also to the most stable structures in the Krebs–Ringer solution (i.e. stressed conditions) and, at the same time, to the most 'hydrated' vesicles, is significant for a better

understanding of NSVs behaviour. The higher resistance to osmotic stress is related to the hydration and fluidity of the membrane that becomes consequently more 'deformable'; on the other hand CA release in the phosphate buffer can be related to a permeation of the hydrophilic probe through the more 'hydrated' bilayer that acts like a 'sieve' through which small water soluble molecules can rapidly diffuse.

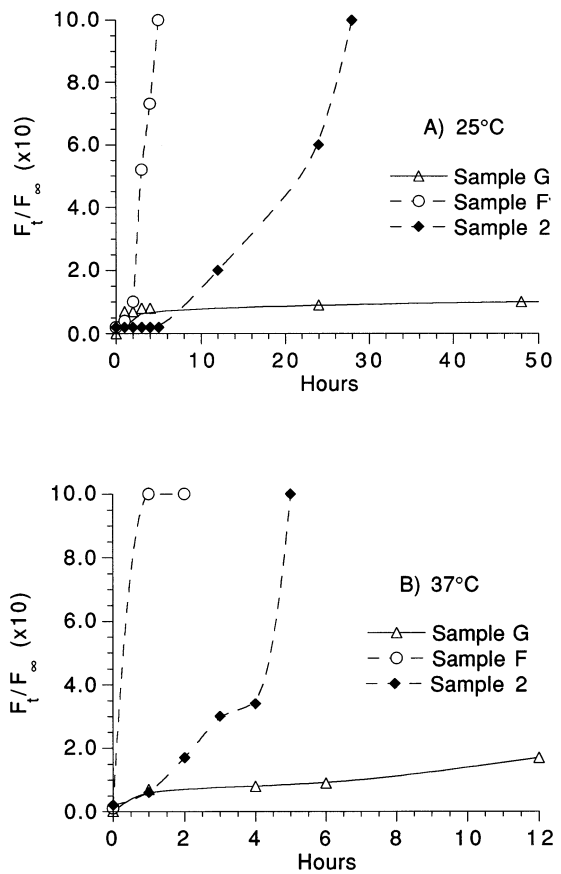

Fig. 3. Comparison of vesicle stability in the Krebs–Ringer solution at 25°C (A) and 37°C (B), according to ionic surfactant present in the formulation (samples G and F of Table 1). The behaviour of the reference without ionic surfactant is also given (sample 2 of Table 1). The plot reports the release of CA as a function of time, expressed as increase of the relative fluorescence (F_t/F_∞).

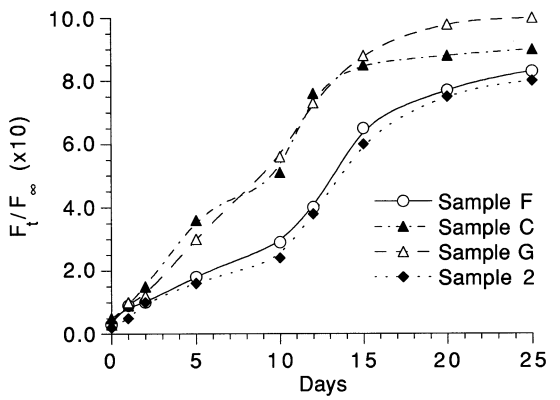

Fig. 4. Release of CA as a function of time from samples 2, F, G and C at 25°C. Results are expressed as increase of the relative fluorescence (F_t/F_∞).

In Fig. 5 it is possible to observe the electron micrographs of empty vesicles (A, B) and vesicles containing Na calcein, (hydrophilic-C), or DPH, (lipophilic-D, E, F). In Fig. 5D and F some wrinkled vesicles provide evidence for the presence of the lipophilic probe in the membrane structure.

5. Conclusions

The obtained results show first of all that different techniques used for the preparation of NSVs lead to vesicles with remarkably different properties. Furthermore, the amount and the molecular structure of the ionic surfactant can affect the behaviour of the vesicles and suggest that the presence of a net charge, whether negative or positive, can increase water uptake within the double layer. Such hydration leads to an increase, with respect to uncharged vesicles, of loaded hydrophilic molecules that can probably be located within the bilayer as well as in the core of the aggregated structures. Finally, vesicle stability in stressed and non-stressed conditions can be remarkably different according to the type and steric hindrance of the ionic surfactant used in the formulation.

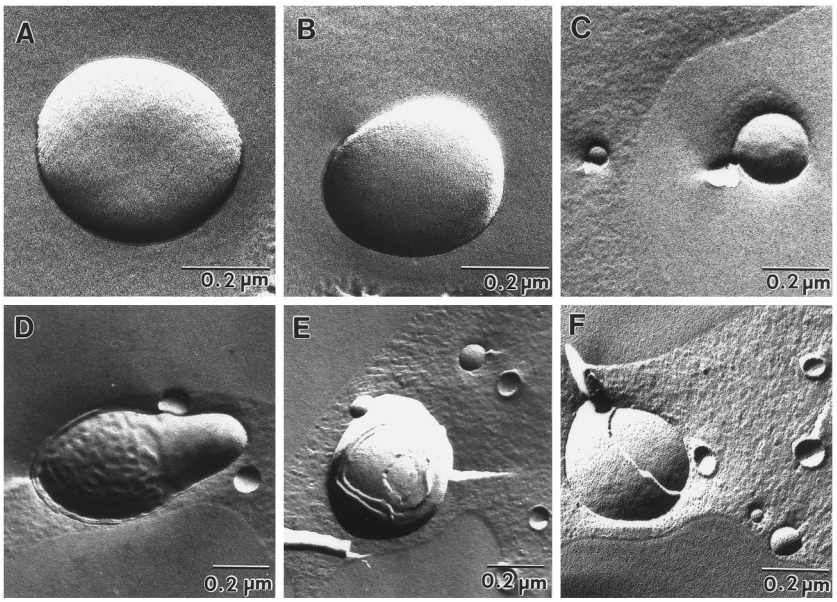

Fig. 5. Trasmission electron micrographs of sample 2 after freeze-fracture: A and B empty vesicles; C vesicles containing CA; D, E and F vesicles containing DPH.

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