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Research Paper

Cyclodextrin-Mediated Drug Release from Liposomes Dispersed Within a Bioadhesive Gel

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Purpose. The aim of the present study was to design a new mucosal drug delivery system composed of liposomes dispersed within a bioadhesive hydrogel containing methyl-β-cyclodextrin (MeβCD) for controlled drug release.

Methods. A hydrophilic model molecule, inulin, was encapsulated within positively charged and PEG-ylated liposomes and its release was measured in the presence of MeβCD after vesicle dispersion within the bioadhesive Carbopol® 974P gel. Freeze-fracture electron microscopy (FFEM) was used to follow liposome morphological changes when dispersed within the hydrogel. Liposome-MeβCD interactions were investigated by turbidity monitoring during continuous addition of MeβCD to liposomes and by FFEM.

Results. Inulin diffusion within the gel was influenced by Carbopol® 974P concentration since no gel erosion occurred. When dispersed within the gel, positively charged liposomes displayed a higher stability than PEG-ylated vesicles. In the presence of MeβCD, higher amounts of free inulin were released from liposomes, especially in Carbopol®-free system. MeβCD appeared to diffuse towards lipid vesicles and permeabilized their bilayer allowing inulin leakage. Indeed, freeze-fracture experiments and liposome turbidity monitoring have shown that MeβCD behaved as a detergent behavior, resulting in lipid vesicle solubilization.

Conclusion. MeβCD is able to mediate, within a bioadhesive hydrogel, the release of a liposome-encapsulated molecule allowing further application of this delivery system for mucosal administration.

KEY WORDS: cyclodextrins; drug modified release; gel; lipid vesicle solubilization; liposomes.

INTRODUCTION

The mucosal route offers many advantages for drug delivery especially for peptides and proteins. Drug bioavailability is improved due to avoidance of degradation in the gastro-intestinal tract and hepatic first-pass metabolism. However, a short drug residence time (1), the presence of enzymes (2–4) and a limited permeability of the epithelial barrier (5–7) are the main drawbacks of mucosal administration. Different approaches can be proposed to overcome these limitations. The use of a bioadhesive hydrogel such as Carbopol 974P, can increase the drug contact time with the mucosa therefore reducing its rapid clearance (1,8,9). Encapsulation of sensitive drugs within liposomes allows their protection from degradation in the biological fluids. Furthermore, their large drug reservoir effect has been widely described in ophthalmic (10), vaginal (11,12) or buccal delivery (13). Finally, passage of drugs through the mucosal epithelium can be improved by the mean of penetration enhancers (12). Cyclodextrins, particularly methylated β-cyclodextrins (MeβCD), can enhance the mucosal absorption of peptides (14–20). Cyclodextrins are supposed to act as a penetration enhancer, transiently changing the mucosal permeability by removing membrane components, such as cholesterol (21), proteins (22) and phospholipids (23). Cyclodextrins are cyclic oligosaccharides of six, seven or eight D-glucopyranose units, named α-, β-, and γ-cyclodextrin, respectively. The three-dimensional ring structure of these compounds ressembles a truncated cone displaying an internal hydrophobic and an outer surface that is hydrophilic.

The main goal of our research is to design a novel mucosal drug delivery system that is bioadhesive due to the presence of Carbopol 974P hydrogels. The gel contains both liposomes for their reservoir and, if necessary, their protective effects and MeβCD as a drug penetration enhancer. Moreover, it could be considered that, MeβCD will also interact

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ABBREVIATIONS: CHOL, cholesterol; FFEM, freeze-fracture electron microscopy; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MeβCD, methyl-β-cyclodextrin; OD, optical density; PEG, poly(ethylene)glycol; PEG_{2000}-DSPE, 1,2 stearoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-methoxy[poly-(ethyleneglycol)-2000]; QELS, quasi-elastic light scattering; SA, stearylamine; SPC, soybean phosphatidylcholine.

with liposome membrane and consequently modulate the encapsulated-drug release making it available for absorption.

The release of a hydrophilic model molecule, inulin (5 kDa), from liposomes either dispersed within the bioadhesive Carbopol 974P gel or not and incubated in the presence of MeβCD was evaluated. Because the mechanism involved in the interactions between MeβCD and liposomes is a prerequisite to understand its ability to modulate drug release, MeβCD interactions with positively charged or PEG-ylated liposomes were investigated *in vitro*.

MATERIALS AND METHODS

Materials

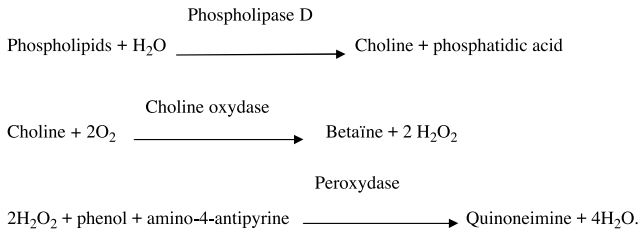
Randomly methylated β-cyclodextrin (MeβCD) BETA W7 (M_w = 1310 g mol^{-1}, D.S. = 1.8) was a gift from Wacker-Chemie GmbH (Burghausen, Germany), soybean phosphatidylcholine(SPC) was generously provided by Lipoid GmbH (Ludwigshafen, Germany). Cholesterol (CHOL) (grade > 99%), stearylamine (SA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) and inulin (Dahlia tubers) were purchased from Sigma Chemical (St. Louis, MO, USA). 1,2 stearoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-methoxy[poly-(ethyleneglycol)-2000] (PEG_{2000}-DSPE) was provided by AVANTI Polar Lipids (Birmingham, AL, USA). Carbopol 974P NF was a gift from Noveon Inc. (Cleveland, OH, USA). [^{3}H(G)]-Inulin, (Dahlia tubers, M_w = 5 kDa), specific activity of 360.0 mCi g^{-1} and scintillation liquid Ultimagold were purchased from Life Science Products Perkin Elmer (Boston, MA, USA). All other chemicals used were of analytical grade. All experiments were performed using a 0.22-μm-filtered 10 mM HEPES aqueous buffer containing 145 mM NaCl and adjusted to pH 7.4 with 0.1 N NaOH.

Methods

Liposome Preparation and Characterization

Positively charged SA (SPC:CHOL:SA 60:30:10 mol%) and PEG-ylated (SPC:CHOL:PEG_{2000}-DSPE 64:30:06 mol%) liposomes were prepared as followed: required amount of lipids in chloroform solutions were mixed and dried in a round bottom flask. Multilamellar liposomes were formed in the aqueous buffer by the film hydration method (24). Liposome dispersions were sequentially extruded through polycarbonate membranes of 0.4 μm and 0.2 μm pore size. Oligolamellar vesicles were formed.

SPC concentrations were determined by enzymatic assay (Phospholipides enzymatiques PAP 150, Biomerieux, Marcy L'Etoile, France) (25). The amount of lipids present in liposome suspensions was calculated by assuming that the lipid composition was unchanged during the different steps of liposome preparation. The enzymatic assay consists in the cleavage of phosphatidylcholine in choline by phospholipase D, which is then oxidized in betaine with the simultaneous production of hydrogen peroxide. The hydrogen peroxide which is produced quantitatively, oxidatively couples 4-amino-antipyrine and phenol. Peroxydation results in the generation of a coloured compound, quinoneimine, quantified by spectrophotometry at 505 nm (spectrophotometer Perkin Elmer Lambda 11) (Scheme 1).



Vesicle mean diameter was measured by quasi-elastic light scattering (QELS) (Nanosizer Coulter N4 Plus Coulter Coultronics, Margency, France).

^{3}H-Inulin Encapsulation Within Liposomes by Freeze-Thawing Method

^{3}H-Inulin was encapsulated within SA and PEG_{2000}-DSPE liposomes by the freeze-thawing procedure. Blank liposomes were mixed with an appropriate amount of a buffer solution containing both radiolabelled and non-radiolabelled inulin. The mixture was shaken with a vortex for 3 minutes. The suspension was then rapidly frozen in liquid nitrogen for 5 min followed by thawing at 37°C for 3 min. The procedure was repeated five times. Free inulin was separated from liposome-encapsulated inulin by ultracentrifugation (Beckman L7-55, Life Science, Boston, MA, USA) at 150,000 × *g* for 90 min at +4°C three times using a rotor 70iTi. After each ultacentrifugation, liposomes contained in the pellet were suspended in a required amount of aqueous buffer. The washing process was repeated three times. After the third centrifugation, the quantity of free inulin was negligible and the total lipid concentration was determined as described in the previous section.

Preparation of Gels Containing Free or Liposome-Encapsulated Inulin

An appropriate amount of Carbopol 974 PNF was slowly added to the aqueous buffer under constant stirring. When MeβCD was added to this solution, its final concentration was 2 or 5% w/v. Preparations were stored at +4°C during 12 h to ensure complete hydration of the polymer. After an overnight period, ^{3}H-inulin encapsulated-liposomes were then added to the Carbopol solutions in order to obtain a final lipid concentration of 2 or 10 mM and a final Carbopol concentration of 1.1% or 1.5% w/v. 1.1% w/v Carbopol solutions containing free inulin and MeβCD (0% or 5% w/v) were also prepared using the same inulin concentration as in the corresponding 2 or 10 mM lipid dispersions (3 mg of inulin/μmol of lipids).

In Vitro ^{3}H-Inulin Release from Gel Systems

In vitro release studies were carried out in a thermostated chamber maintained at 37°C using a membrane free release model (26). Aliquots (0.5 g) of free inulin in 1.1% w/v Carbopol solution with or without 5% w/v MeβCD or liposome-encapsulated inulin (2 mM and 10 mM of lipids)

dispersed within a Carbopol preparation (1.1% or 1.5% w/v) containing or not MeβCD (0%, 2%, and 5% w/v) were introduced in 6 ml vials. The preparations were then gelled with a 18% w/v NaOH solution. The aqueous buffer pre-equilibrated at 37°C was used as release medium. Five milliliters of the release medium were carefully layered over the surface of the gel to avoid mixing. At different time intervals (0, 1, 2, 4, 6, and 24 h), the entire release medium was removed and inulin released from the formulations was obtained by measuring ^{3}H-inulin radioactivity in an aliquot of this medium (150 μl) using scintillation counting after addition of 4 ml of scintillation liquid (Ultimagold). The percentage of inulin released P_R was calculated as the ratio of the ^{3}H-inulin in the release medium $[^3\text{H-inulin}]_R$ and the total amount of ^{3}H-inulin initially present in the preparation $[^3\text{H-inulin}]_{tot}$:

$$P_R = \left[^3\text{H-inulin}\right]_R / \left[^3\text{H-inulin}\right]_{tot} \times 100 \qquad (1)$$

In the case of liposomes dispersed within the gels, the amount of lipids present in the release medium was enzymatically quantified as described above. The release medium was previously freeze-dried and the residue was suspended in a minimum amount (600 μl) of purified water.

In Vitro ^{3}H-Inulin Release from Liposomes Dispersed in MeβCD Solutions

MeβCD solution (0.5 ml) in the aqueous buffer at 4.4% w/v was added to 0.6 ml SA or PEG_{2000}-DSPE liposome dispersions (3.7 mM total lipids) to reach final MeβCD and lipid concentrations of 2% w/v and 2 mM, respectively. In the same conditions, 0.5 ml of MeβCD solution at 11% w/v was added to 0.6 ml of SA or PEG_{2000}-DSPE liposome dispersions (18.3 mM total lipids) to reach final MeβCD and lipid concentrations of 5% w/v and 10 mM, respectively. Then the mixtures of liposomes with MeβCD were incubated at 37°C for 30 min and 24 h. Free ^{3}H-inulin released from liposomes was determined by measuring the radioactivity in the supernatant after ultracentrifugation of release medium aliquots (150,000 × *g*, +4°C, 90 min). As control, liposomes without MeβCD were incubated in the same conditions and free inulin was determined by using the same procedure.

Freeze-Fracture Electron Microscopy (FFEM)

Freeze-fracture replica of SA and PEG_{2000}-DSPE liposomes dispersed within 1.1% w/v Carbopol gel were examined by transmission electron microscopy. Freeze-fracture was also used to investigate the effect of 5% w/v MeβCD on liposome dispersions after 24 h of contact. The same liposomal dispersion without MeβCD was used as reference. A drop of liposome dispersion or liposomes dispersed within gel containing 20% glycerol, as a cryoprotectant, was deposited in a small gold cup and rapidly frozen in liquid nitrogen. Fracturing, freeze etching and shadowing with Pt-C were performed at −100°C in a shadowing equipment (Balzers BAF-400) fitted with a freeze-fracture and etching units. The replica were examined with a JEOL (JEM-100SX) transmission electron microscope operating at 80 kV accelerating voltage.

Turbidity Measurements

Continuous turbidity measurements were carried out as previously described (27,28). Briefly, MeβCD solution (152.5 mM) in the aqueous buffer was added continuously by using a 1 ml precision syringe (Hamilton, Bonaduz, Switzerland) to 1.3 ml of liposome preparation at different initial lipid concentrations (0.5, 0.75, and 1 mM) and placed in an optical quartz cell equipped with a paddle stirrer. Turbidity at 400 nm was continuously monitored at 37°C using a Perkin Elmer Lambda 2 double spectrophotometer (Boston, MA, USA).

Theoretically, at each step of vesicle solubilization, the total MeβCD concentration ($[\text{MeβCD}]_{tot}$ mM) is related to the total lipid concentration ($[\text{lip}]_{tot}$ mM) according to the following linear relationship (29–31):

$$[\text{MeβCD}]_{tot} = [\text{MeβCD}]_{bulk} + \left[R_e \times [\text{lip}]_{tot}\right] \qquad (2)$$

where $[\text{MeβCD}]_{bulk}$ is the concentration of MeβCD molecules (mM) which are not associated to lipids, and R_e is the molecular ratio of MeβCD to lipid in the mixed aggregates ($[\text{MeβCD}]_{ag}/[\text{lip}]_{ag}$). Characteristic steps of the solubilization process can be depicted by turbidity measurements and correspond to breaks in optical density (OD) variation as a function of cyclodextrin addition. At these points of the solubilization curves, linear plots of total MeβCD concentration ($[\text{MeβCD}]_{tot}$) *vs.* total lipid concentration ($[\text{lip}]_{tot}$) give values of the solubilization parameters from Eq. (2): the slope of the lines is equal to the composition of the lipid-MeβCD mixed aggregates ($[\text{MeβCD}]_{ag}/[\text{lip}]_{ag} = R_e$) and their extrapolation to zero total lipid concentration ($[\text{lip}]_{tot} = 0$) gives the concentration of MeβCD molecules ($[\text{MeβCD}]_{bulk}$) which are not associated to lipids. The apparent partition coefficient K of cyclodextrin molecules between the aggregates and the aqueous phase can be defined as (32):

$$K = (R_e/(R_e + 1)) \times \left(1/[\text{MeβCD}]_{bulk}\right) \qquad (3)$$

RESULTS

SA and PEG_{2000}-DSPE liposomes were characterized by QELS and their mean hydrodynamic diameter was of 160 ± 48 and 185 ± 49 nm ($n = 9$), respectively. Polydispersity index was always lower than 0.15, indicating that liposome population was homogeneous in size.

In vitro release of free ^{3}H-inulin and liposome-encapsulated ^{3}H-inulin both dispersed in the bioadhesive gel was first studied. Surprisingly, as shown in Fig. 1A, diffusion of free inulin was hindered by the presence of MeβCD. In contrast, when inulin was entrapped into liposomes, addition of MeβCD promoted its release. The encapsulation of inulin within SA liposomes led to a lower release after 24 h (29 ± 3% and 66 ± 16% for SA and PEG_{2000}-DSPE 2 mM liposomes, Fig. 1B) compared to a free inulin reference gel (74 ± 1%, Fig. 1A) showing the influence of liposome composition. SA liposomes retained inulin in the gel more than PEG_{2000}-DSPE liposomes did, especially in the absence of MeβCD (Fig. 1B). No liposome-entrapped ^{3}H-inulin was found in the receptor medium as well as no lipid traces, indicating that only free inulin was quantified in the release medium. Because the release studies were carried out in an open medium, liposome on the surface of the gel could have diffused into the medium. However, when assayed, lipids in the medium

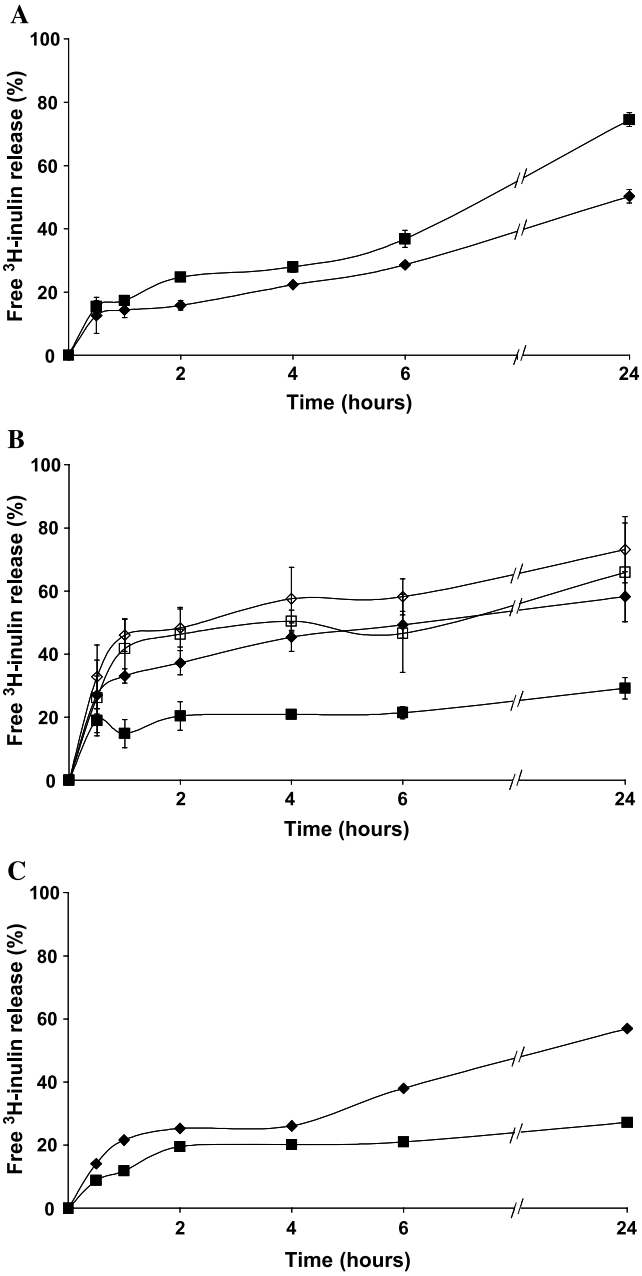


Fig. 1. Comparison of the inulin release kinetic from gels containing free inulin (A) solubilized in a 1.1% w/v Carbopol 974 PNF gel without MeβCD (■) or with 5% w/v MeβCD (♦); 2 mM liposomes dispersed within 1.1% w/v (B) or 1.5% w/v (C) Carbopol 974 PNF gels. ■ SA liposomes without MeβCD; ♦, SA liposomes with 5% w/v MeβCD, □, PEG_{2000}-DSPE liposomes without MeβCD, ◇, PEG_{2000}-DSPE liposomes with 5% w/v MeβCD.

were not detectable and their amount was lower than the detection level of the enzymatic assay. It is therefore assumed that only lipid traces could have diffused from the gel.

Inulin release decreased when carbomer concentration increased from 1.1% to 1.5% w/v especially at short times and 5% w/v MeβCD (Figs. 1A and B).

In vitro release of ^{3}H-inulin depended on MeβCD concentration (Fig. 2). Indeed, at the highest MeβCD concentration tested (5% w/v), inulin released from 10 mM SA liposomes reached 54 ± 6% instead of 34 ± 9% without

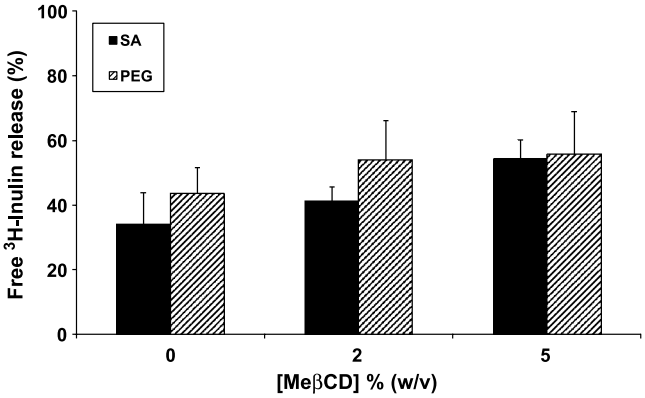


Fig. 2. Effect of MeβCD concentration on the release kinetic of inulin from 10 mM SA or PEG_{2000}-DSPE liposomes dispersed within a 1.1% w/v Carbopol gel during 24 h of incubation.

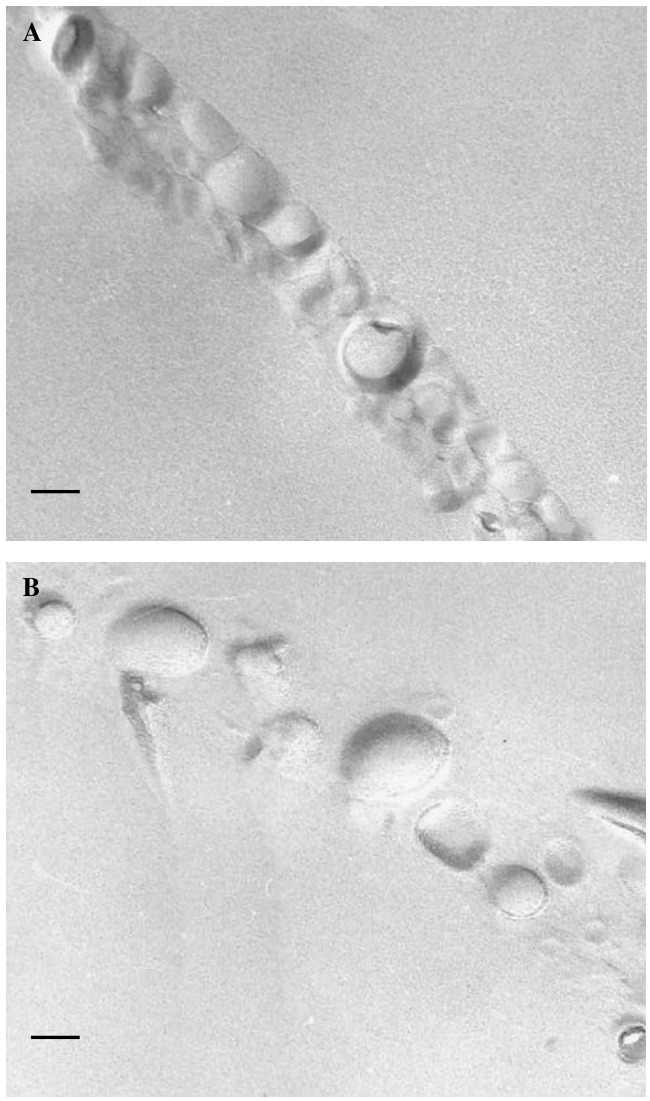


Fig. 3. Transmission electron micrographs of freeze-fractured PEG_{2000}-DSPE liposomes (10 mM) dispersed within a Carbopol 974 PNF gel (1.1% w/v) without MeβCD (A) or with 5% MeβCD (B). Bar: 200 nm.

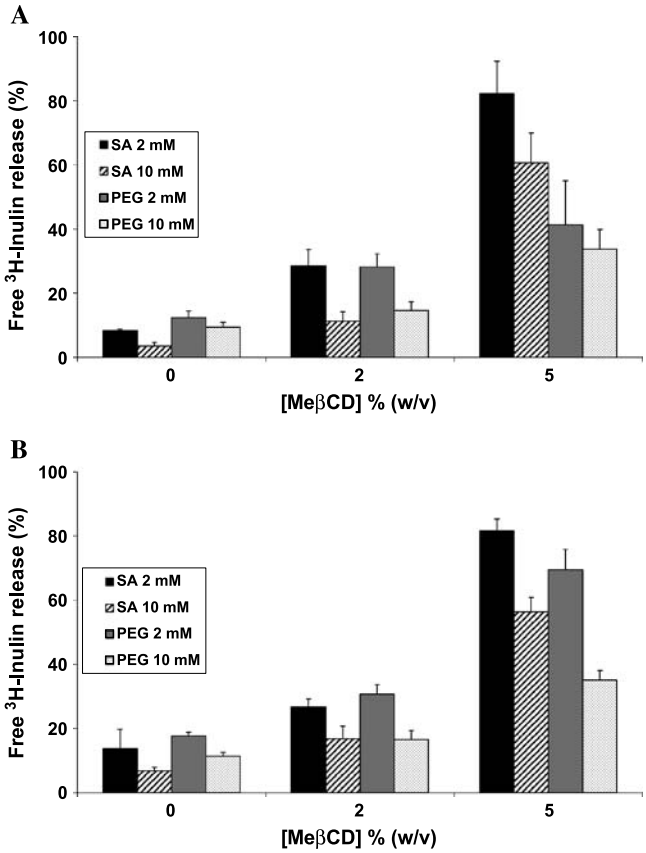


Fig. 4. Free ^{3}H-inulin released (%) from SPC:CHOL:SA and SPC:CHOL:PEG_{2000}-DSPE at 2 and 10 mM lipids after incubation with 2% and 5% MeβCD for 30 min (A) and 24 h (B).

MeβCD after 24 h (Fig. 2). Inulin retention within the gel was lower with increasing cyclodextrin concentration.

For SA liposomes, lipid concentration did not influence inulin diffusion (Figs. 1 and 2). On the contrary, for PEG_{2000}-DSPE liposomes, a lower overall release was observed at high lipid concentration.

PEG_{2000}-DSPE liposomes dispersed within 1.1% w/v Carbopol gel were characterized by FFEM which has shown vesicles of around 200 nm mean diameter (Fig. 3). No morphological changes of these liposomes were observed even after 24 h of contact with 5% w/v MeβCD (Fig. 3).

In order to consider only liposome-MeβCD interactions, we performed inulin release studies in systems devoided of Carbopol. For each lipid composition studied, the released amounts of ^{3}H-inulin from liposome suspensions incubated at 37°C with MeβCD, depended on both MeβCD and total lipid concentration (Fig. 4). 5% w/v MeβCD induced a significant ^{3}H-inulin release, especially at lipid concentration of 2 mM. The amount and the profile of ^{3}H-inulin release were also influenced by lipid composition. After 30 min, 2 mM SA liposomes incubated with 5% w/v MeβCD showed a dramatic release of entrapped inulin (80%) (Fig. 4A), whereas for PEG_{2000}-DSPE liposomes (2 mM), the release was clearly lower (Fig. 4A). However, after 24 h, the same amount of inulin was released from SA and PEG_{2000}-DSPE liposomes. Lipid concentrations of 10 mM did not exhibit such release differences (Fig. 4B).

Finally, FFEM analysis of both liposome formulations showed a significant decrease of number of liposomes after 24 hours of contact with 5% w/v MeβCD (Fig. 5).

To elucidate the mechanism of MeβCD action on liposomes containing either SA or PEG_{2000}-DSPE, turbidity

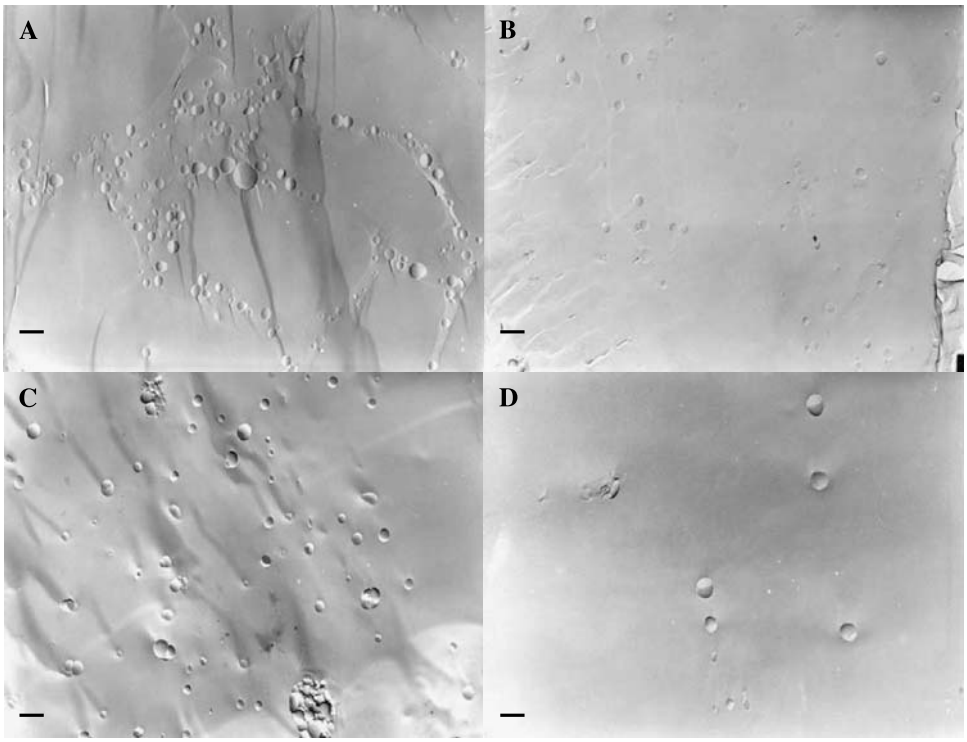


Fig. 5. Transmission electron micrographs of freeze-fractured samples. (A) SA 10 mM vesicles without MeβCD. (B) SA 10 mM vesicles with 5% MeβCD after 24-h contact. (C) DSPE-PEG_{2000} 10 mM vesicles without MeβCD. (D) DSPE-PEG_{2000} 10 mM vesicles with 5% w/v MeβCD after 24-h contact. Bar: 400 nm.

monitoring of vesicles were undertaken (Fig. 6). Continuous addition of 152.5 mM MeβCD solution to vesicles led to a progressive decrease in sample OD until transparent solutions were obtained. By analogy with previous studies of vesicle-to-micelle transition, such decrease in turbidity evidenced the ability of MeβCD molecules to gradually damage the liposome structure, the ultimate stage being the complete solubilization of the lipid membranes into micelle-like aggregates (29). The solubilization profiles in Fig. 6 showed numerous break points revealing different stages resulting from liposome structure transformations or phase transitions which characterize the solubilization process. The intercept of the tangents to the OD curves (see insets) allowed to determine the total concentrations of MeβCD and lipids in the medium at the characteristic break points. Plotting the MeβCD concentrations *versus* lipid concentrations at each break point gave straight lines from which the MeβCD concentration in the aqueous phase ($[MeβCD]_{bulk}$) and the composition of aggregates (R_e) were determined according to Eq. (2). Apparent partition coefficients K of MeβCD between aqueous phase and lipid aggregates can be also calculated from Eq. (3). Results obtained for both liposome formulations are reported in Table I. The SA liposome solubilization profiles revealed four break points noted I to IV (Fig. 6A). At the first step (break point I), MeβCD molecules were already associated to lipid vesicles

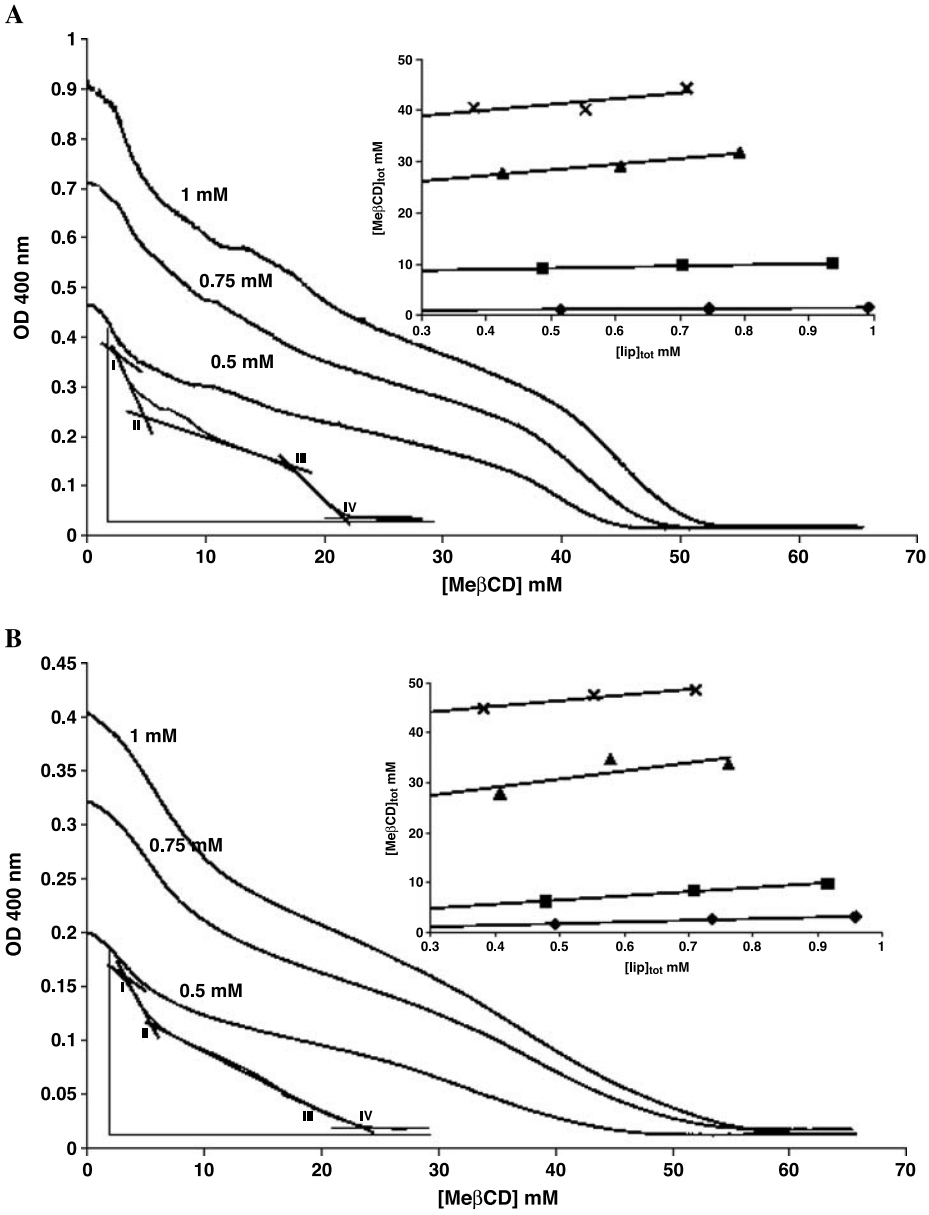


Fig. 6. Variation of optical density (OD) measured at 400 nm during the continuous addition of MeβCD to liposome formulations with initial lipid concentration of 1 mM, 0.75 mM and 0.5 mM. (A) SA liposomes. (B) DSPE-PEG_{2000}. Bottom inset: determination of characteristic break points corresponding to the intercept of the tangents to the curves. Top inset : linear relationship between total MeβCD ($[MeβCD]_{tot}$) and total lipid concentration ($[lip]_{tot}$) at each break point (I : ♦; II : ■ III : ▲; IV : ×) of the OD variation curves.

Table 1. Solubilization Parameters of Liposomes at the Characteristic Break Points of the Turbidity Curves

Lipid composition	$[MeβCD]_{bulk}$ (mM)	R_e	K (mM^{-1})
SPC:CHOL:SA 60:30:10 mol%			
Points I	0.82	0.694	0.498
Points II	8.11	2.21	0.085
Points III	22.87	11.04	0.040
Points IV	35.57	11.22	0.026
SPC:CHOL:PEG_{2000}-DSPE 64:30:06 mol%			
Points I	0.065	3.21	11.711
Points II	2.244	8.35	0.398
Points III	22.52	16.41	0.042
Points IV	40.99	11.85	0.023

($[MeβCD]_{bulk}$ = 0.82 mM, R_e = 0.694). Further points were characterized by increasing values of R_e until a MeβCD amount of 11 molecules per one molecule of lipid required to achieve total lipid vesicle solubilization (Table I).

For PEG_{2000}-DSPE liposomes, the OD profiles presented similar shape. Four break points could also be observed on the solubilization curves (Fig. 6B). At the first step (break point I), roughly all MeβCD molecules were associated to lipids ($[MeβCD]_{bulk}$ = 0.065 mM, R_e = 3.21) (Table I). In the same way, MeβCD molecules were gradually assembling with lipids as the solubilization was taking place to reach a value of 11.85 molecules of MeβCD per one molecule of lipid in the final aggregates (Table I). At break point I, the apparent partition coefficient K of MeβCD between aqueous phase and lipid aggregates was significantly higher for PEG_{2000}-DSPE liposomes (K = 11.7 mM^{-1}) than for SA liposomes (K = 0.498 mM^{-1}). However, the coefficient K decreased for both liposome formulations, along the different transition steps of the aggregates before reaching a value of about 0.02 mM^{-1} when total lipid solubilization occured (Table I).

DISCUSSION

The main objective of this study was to design a new mucosal drug delivery system consisting of a bioadhesive Carbopol 974P liposomal gel containing MeβCD. Addition of MeβCD in the system had two main roles: one was to enhance drug penetration and the other was to interact with lipid vesicles in order to modulate release of liposome-entrapped molecules. In release studies, inulin was used as a model hydrophilic molecule and release through Carbopol formulations (with or without liposomes, or from liposomes alone) was investigated using a membrane free model (26). In all gel formulations, erosion did not occur under these experimental conditions, even after 24 h. Carbopol concentration (1.1 or 1.5 % w/v) affected inulin release showing that inulin efflux from the gel was more pronounced at low Carbopol concentration. Indeed, diffusion was facilitated as the Carbopol 974P concentration decreased due to the reduced viscosity.

Surprisingly, concerning gels without liposomes, free inulin diffusion within the gel matrix was hindered in the presence of MeβCD. This may be explained by the formation of hydrogen bonds between both molecules that impeded inulin diffusion in the gel. Indeed, inulin complexation by cyclodextrins was unlikely, considering the hydrophilic character of inulin.

When liposomes were dispersed within the gel, no lipids were detected in the receptor medium indicating that inulin was first released from the vesicles and was spreading within the gel. Due to their size and short duration of the experiments, liposomes were not able to diffuse through the gel so they remained in the matrix. Addition of PEG_{2000}-DSPE liposomes to the gel led to a significant increase in inulin release compared to Carbopol-free suspensions (66 ± 16% and 27 ± 9% of inulin released after 24 h, respectively). However, morphology of liposomes composed of PEG_{2000}-DSPE was not affected (Fig. 3). Therefore, it could be hypothetized that acrylic acid polymer affected vesicle permeability due to a lower hydration of PEG chains which is crucial for liposome stability. Indeed, water molecules could be mainly mobilized to interact with acrylic acid polymer for its unfolding, reducing water binding to PEG. The lack of hydration could induce a destabilization of PEG containing lipid vesicles (33). This phenomenon could be responsible of the weak stability of PEG-ylated liposomes within the Carbopol gel. The poor stability of PEG_{2000}-DSPE liposomes within the gel did not allow to characterize the effect of MeβCD on these vesicles.

In contrast, Carbopol affected only slightly SA liposome permeability allowing to preserve their reservoir effect. However, the permeability of SA liposomes was strongly enhanced by the presence of MeβCD. MeβCD diffused through the gel and interacted with lipid membranes permitting the release of the encapsulated inulin. Inulin in the free form was consequently able to diffuse through the gel.

The enhanced release effect of MeβCD on lipid vesicles was evaluated by measurements of ^{3}H-inulin leakage in a gel-free system. Both liposome formulations displayed inulin release when incubated with MeβCD. However, PEG-ylated liposomes retained more inulin than SA liposomes, even after 24 hours and especially for high lipid concentration. The steric hindrance provided by PEG chains could impede the diffusion of MeβCD molecules towards vesicle surface as this was shown for some proteins (34,35).

MeβCD behavior toward biological membranes is widely accepted to be related to cholesterol complexation and lipid depletion properties (22,36–39). In order to explain the mechanism involved in MeβCD interaction with liposomes, we have performed turbidity experiments. Whatever the lipid composition, continuous addition of MeβCD to vesicles led to a progressive decrease in sample turbidity. By analogy with previous studies of vesicle-to-micelle transition (29), such decrease in turbidity evidenced the ability of MeβCD molecules to gradually damage liposome structure by a solubilization process. The decrease of liposome concentration on freeze-fracture replica confirmed the solubilization effect of MeβCD on both formulations. These results pointed out that MeβCD behaved as a detergent. Indeed, lipid vesicle solubilization by a detergent obeys to the Lichtenberg's three stage model (29). According to this model, during the first stage of the solubilization process, the detergent partitions between the aqueous phase and the lipid bilayer. After a gradual solubilization, a progressive enrichment of the lipid bilayer by detergent molecules occurs until membrane

saturation. The second stage corresponds to the coexistence of MeβCD-saturated lipid vesicles and lipid-saturated micelle aggregates. Finally, the last stage corresponds to total liposome solubilization and their conversion into lipid-MeβCD mixed micelle-like aggregates.

Along the different stages of SA liposome solubilization process, MeβCD was located rather in the bulk phase than in the lipid bilayer (Table I). This location could be explained by the weak affinity of MeβCD to bind this lipid membrane as shown by the weak values of the apparent partition coefficient K (Table I). However, this did not reduce the MeβCD solubilization capacity. In contrast, the behavior of PEG_{2000}-DSPE liposomes was quite different. Compared to SA liposomes, more MeβCD molecules partitioned within the lipid bilayer and much more MeβCD molecules were associated to lipids (R_e = 3.21; K = 11.7 mM^{-1} at break point I) (Table I). This result suggested binding of MeβCD to PEG liposomes as consequence of an interaction with the hydrophilic chains of PEG which did not promote vesicle solubilization.

The solubilization experiments allow, for given MeβCD to lipid ratios, to determine the domains of vesicle-to-mixed aggregates transition. These experiments were carried out for weak lipid concentration (from 0.5 to 1 mM). However, it was reasonable to extrapolate the linear relationship between MeβCD and lipid concentrations to higher lipid concentrations (2 and 10 mM). Figure 7 shows the linear relationship between $[MeβCD]_{tot}$ and $[lip]_{tot}$ in solubilization experiments correlated to the MeβCD concentrations used in inulin release study. In the region under the curve of break points I only intact vesicles existed in the medium, whereas above curve of break points IV only lipid-MeβCD mixed micelle-like aggregates were present. These two curves delimited a region where there was a coexistence of both open lipid-MeβCD mixed aggregates and mixed vesicles. In the release experiments, as SA liposomes at 2 or 10 mM of lipids were incubated with 2% w/v (15 mM) (points 1 and 3) and 5% w/v (38 mM) (points 2 and 4) MeβCD, some lipid vesicles were then still intact coexisting with lipid-MeβCD mixed micelle-like aggregates (Fig. 7A). This means that MeβCD already affected partially liposome structure and could induce pronounced amounts of released inulin (Figs. 4A and B). On the other hand, when 10 mM of PEG_{2000}-DSPE liposomes were in contact with either 2% w/v (15 mM) or 5% w/v (38 mM) MeβCD, their vesicular structure remained entirely preserved explaining low amounts of inulin release, even after 24 h of incubation (Figs. 4B and 7B). High inulin release reflected the conversion of most of vesicles in lipid-MeβCD mixed aggregates. This occurred mainly after long incubation time of liposomes with MeβCD. In conditions of inulin release study, total lipid vesicle solubilization was not achieved. This explains that 100% inulin released from liposomes was never reached. Nevertheless, for 2 mM of lipids, liposomes were supposed to be already permeabilized by MeβCD. It is therefore assumed that permeabilization of lipid vesicle membrane by MeβCD was due to the formation of transient pores probably due to lipid and cholesterol solubilization as shown in a previous study (40). Indeed, transient holes could allow only to small amounts of inulin to be released. This phenomenon has been previously demonstrated in the case of solubilizing concentrations of non-ionic detergent, octyl glucoside, onto lipid vesicles. The transient

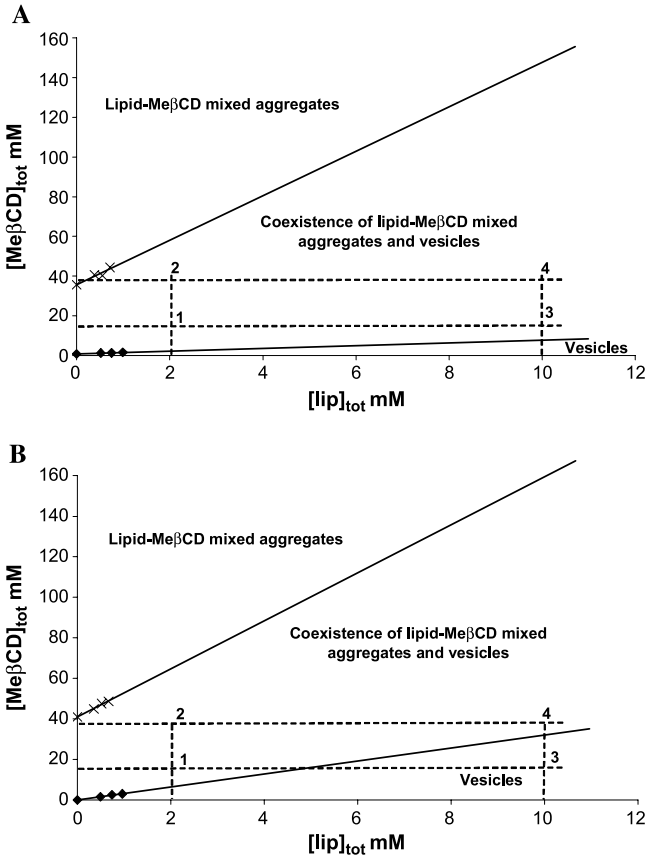


Fig. 7. Diagrams representing the correlation of *in vitro* inulin release from (A) SA and (B) DSPE-PEG_{2000} liposomes to their solubilization steps by MeβCD. The curves correspond to the linear relationship existing between $[MeβCD]_{tot}$ and $[lip]_{tot}$ at break points I (♦) and IV (×) obtained in the 0–1 mM range lipid and are extrapoled to higher lipid concentrations (10 mM). 1) 2 mM of lipids with 2% MeβCD (15 mM); 2) 2 mM of lipids with 5% MeβCD (38 mM); 3) 10 mM of lipids with 2% MeβCD; 4) 10 mM of lipids with 5% MeβCD (38 mM).

pores are large enough to allow the passage of 13 nm-particles (41). In addition, since the experimental conditions were not the same in inulin leakage and liposome solubilization, a different behavior of MeβCD could occur. Indeed, in inulin release study the entire MeβCD solution is added in the incubation medium, whereas in solubilization experiments MeβCD solution is added continuously for 3 hours until achieving total liposome solubilization. Kinetically-governed processes implied in MeβCD interactions with lipid bilayers should not be excluded, and prompts us to undertake a more careful comparison. Further studies should be performed now to examine the kinetics of the solubilization process of liposomes by MeβCD and the influence of PEG chains on the rate of the bilayer permeabilization.

CONCLUSIONS

In the current study, we have shown that a bioadhesive gel containing SA liposome-encapsulated inulin can be prepared without affecting lipid vesicle integrity. Addition

of MeβCD permits to modulate drug release due to interactions with liposomes resulting in an increase in their permeability. The mechanism involved in this phenomenon is shown to be a solubilization process where MeβCD acts as true detergent. Taking into account their stability, SA liposomes seem more appropriate than PEG_{2000}-DSPE liposomes. MeβCD-liposome interaction kinetics could be modulated using higher Carbopol concentration to slow down MeβCD diffusion. The lipid concentration did not display an important role in the inulin release, but in terms of drug concentration, the 10 mM lipid formulation will be more relevant. However, the release of a liposome-entrapped molecule could be easily mediated by MeβCD depending on the MeβCD-lipid ratio. It is particularly true in domains where MeβCD-lipid mixed micelle-like aggregates coexist with intact vesicles.

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