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Stability of liposomes containing bio-enhancers and tetraether lipids in simulated gastro-intestinal fluids

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ABSTRACT

The stability of egg phosphatidylcholine (EPC) and cholesterol (Chol) based liposomes and liposomes with the addition of the tetraether lipid glycerylcaldityl tetraether (GCTE) and the bio-enhancers cholyl-sarcosine, octadecanethiol and TPGS 1000 in Tris buffer pH 2, sodium taurocholate 10 mM and pancreatin was compared. At pH 2 all formulations released nearly 100% of the small hydrophilic fluorescent marker carboxyfluorescein (CF) within the first 10 min, whereas they were mostly stable in size as confirmed by dynamic light scattering (DLS) measurements. Also leakage of the macromolecule FITC-dextran 70 kDa over 60 min at pH 2 was at most 23.9%. After 20 min in 10 mM sodium taurocholate vesicles without GCTE showed a release of CF between 84.0% and 89.5%. In contrast, GCTE-stabilised formulations after 90 min in sodium taurocholate exhibited a CF release between 36.6% and 69.0% depending on the addition of bio-enhancers. Pancreatin had a minor influence on liposome stability in all assays. It is possible to form EPC/Chol vesicles containing different types of bio-enhancers and to stabilise them with GCTE against bile salts. This type of liposomes could be a versatile tool for the oral delivery of drug substances with poor stability in the GI tract and low permeability.

1. Introduction

Soon after their discovery in 1965, liposomes were used for the oral delivery of peptide and protein drugs (Bangham et al., 1965; Patel and Ryman, 1976; Patel et al., 1982; Chiang and Weiner, 1987; Fricker et al., 2010). Especially for proteins used for the treatment of chronic diseases, development of an oral dosage form is feasible, but rendered difficult. Common features of many proteins are their high molecular weight, hydrophilicity and susceptibility to degradation by proteases or low pH leading to a low oral bioavailability (Fricker and Drewe, 1996). Liposomes might help to stabilise proteins in the gastro-intestinal tract (GIT) and to improve their permeation through the intestinal mucosa. However, also liposomes show instabilities after oral application, especially against not only bile salts but also pancreatic enzymes and the acidic conditions in the stomach (Rowland and Woodley, 1980; Iwanaga et al., 1997, 1999). This leads not only to a reduction of intact liposomes reaching the intestinal mucosa but also to a strong leakage of liposomally encapsulated drugs into the GIT, where they are exposed to low pH or proteases. Several approaches were made to improve the stability of liposomes against the harsh conditions in the GIT. Vesicles can be coated with polymers such as chitosan, polyethylene glycol or pectin not only to improve the membrane integrity but also to provide a mucoadhesivity and to prolong the retention of the formulations in the gut (Takeuchi et al., 1994; Filipović-Grcić et al., 2001; Werle et al., 2009; Werle and Takeuchi, 2009). Liposomes made with phospholipids with a glass transition above body temperature or containing other stabilising lipids like gangliosides can survive the gastro-intestinal tract (Muramatsu et al., 1996; Han et al., 1997; Taira et al., 2004). Since their first description by Langworthy in 1977 naturally derived tetraether lipids (TELs) were used in liposomes to improve their stability and also their immunogenicity for vaccine delivery (Langworthy, 1977; Choquet et al., 1994; Fan et al., 1995; Komatsu and Chong, 1998; Patel and Chen, 2005). TELs are present in a great variety in both archaeal and bacterial membranes (Lo and Chang, 1990; Schouten et al., 2007). Their unique properties make them good candidates for the use in liposomes for oral drug delivery. They are less susceptible to hydrolysis and oxidation than normal phospholipids. Furthermore, TELs are membrane spanning and thus can stabilise bilayer membranes. Despite their rigid structure, they have a low glass transition temperature below 0 °C and are therefore easy to handle at room temperature compared to stabilising phospholipids like disteaorylphosphatidylcholine (De Rosa et al., 1986; Gliozzi et al., 1986; Gambacorta et al., 1995). Commonly, the so called archaeosomes are prepared by the polar lipid fraction obtained from archae and contain a mixture of

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bipolar TELs. In contrast, in the present study, we are the first to use only one single structure, the glycerylcaldityl tetraether (GCTE), for the stabilisation of liposomes. GCTE can be obtained after hydrolysis of the polar lipid fraction of Sulfurous acidocaldarius, followed by several purification steps (Lo et al., 1989). The use of a single chemical entity allows a more target-oriented change of liposomal properties and an easier adjustment of their stability in the intestine. Furthermore, a single structure has lower demands on analytical methods and leads to a higher batch to batch consistency in an industrial production process.

Even when intact liposomes reach the intestinal mucosa, uptake of encapsulated protein drugs or vesicles is usually very low (Whitmore and Wheeler, 1979; Rowland and Woodley, 1981a,b,c). Protection of the encapsulated drug by use of stabilised liposomes might not be sufficient for most protein drugs to achieve a reasonable bioavailability. Previous attempts to increase the uptake of liposomal carriers and their encapsulated drugs were among others the use of M-cell targeted liposomes or mucoadhesive vesicles (Takeuchi et al., 2005; Werle et al., 2010). Due to their high versatility concerning composition, liposomes are suitable drug carriers for the use of bio-enhancers. Chemically defined enhancers represent a cost effective way of bioavailability improvement and have already been widely investigated in the literature (Maher et al., 2008). The simultaneous delivery of enhancer and drug in one vehicle could allow a reduction of enhancer needed and thus also a reduction of possible toxic side effects. Unfortunately, most enhancers are surfactants and can destabilise liposomes making a better understanding of their influence on the stability of liposomes for peroral delivery desirable. In this study, we tested the stability of egg phosphatidylcholine (EPC) and cholesterol (Chol) based liposomes with and without GCTE and the bio-enhancers D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS), cholylsarcosine (CS) and octadecanethiol (OT). TPGS is a non-ionic surfactant and was originally used as a water-soluble vitamin E derivative (Authority, 2007). Beside the surfactant characteristics of TPGS, which mostly implies also permeation enhancing properties, the PEG chain can contribute to stabilisation of liposomes and TPGS is a known inhibitor of P-glycoprotein (Collnot et al., 2007). The anionic bile salt derivative CS, the sarcosine (N-methylglycine) conjugate of cholic acid, behaves very similarly to naturally occurring conjugated bile acids (Lillienau et al., 1992). Bile salts have been in use for a long time as permeation enhancers and there have already been studies with liposomal bile salt formulations (Degim et al., 2004; Song et al., 2005). One advantage compared to taurocholic acid, which has previously been used as an excipient, is lack of tumorgenicity of CS. Taurocholic acid may be metabolised by deconjugation and 7-dehydroxylation to deoxycholic acid, which has been reported to have a certain tumorigenic potential (Hill, 1978; Nair and Turjman, 1983; Kandell and Bernstein, 1991; Latta et al., 1993). However, due to methylation of the amide bond, CS cannot be deconjugated to form deoxycholic acid. In addition, this bile acid derivative has already been tested in humans with short bowel syndrome in the context of bile acid replacement therapies (Emmett et al., 2003; Kapral et al., 2004).

In contrast to the other two enhancers, OT has no surface active properties, but the thiol group shows a certain mucoadhesivity, a principle already successfully used for bioavailability improvement (Takeuchi et al., 2001).

To assure a sufficient protection of encapsulated protein, liposomes should maintain their vesicular form and exhibit no leakage of the protein. Furthermore, the influx of small molecules through the lipid bilayer should be minimal to avoid protein denaturation by protons or high salt concentrations. We tested the liposomal formulations for their stability under acidic conditions, in bile salts and in pancreatin. Change in size and size distribution was monitored to conclude on the vesicular shape of the particles. In addition, leakage of both fluorescein isothiocyanate-dextran (FITC-dextran) (70 kDa) and carboxyfluorescein (CF) was examined; the first as model for a large hydrophilic molecule and the latter to investigate the membrane permeability of small hydrophilic molecules.

2. Materials and methods

2.1. Materials

EPC was provided by Lipoid GmbH (Ludwigshafen, Germany) and GCTE by Bernina Plus GmbH (Planegg, Germany). TPGS 1000 was supplied by Eastman (Kingsport, TN, USA). Cholylsarcosine was obtained from Prodotti Chimici e Alimentari S. p. A. (Basaluzzo, Italy). Cholesterol, FITC-dextran (Mw 70,000 Da), pancreatin from porcine pancreas (8× U.S.P.), octadecanethiol and sodium taurocholate (minimum 95% TLC) were purchased from Sigma–Aldrich (Taufkirchen, Germany). 5(6)-Carboxyfluorescein was provided by Serva (Heidelberg, Germany). All other chemicals were obtained in the highest purity from the usual commercial sources.

Pancreatin mixture contains non-soluble components, which could disturb the fluorescence measurements. In order to remove these impurities, 1.25% (m/m) of pancreatin was dispersed in phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, K_2HPO_4·1.5 mM, Na_2HPO_4·$2H_2O$ 8.1 mM) and centrifuged at 15,000 × *g* for 1 h at 4 °C (Beckman J2-MC, Beckman Instruments GmbH, München, Germany). Finally, the supernatant was filtrated using a 0.45 μm sterile filter. The lipase activity was determined according to the assay described in Ph. Eur. 6.3. using a Dosimat E 412 (Metrohm Herisan, Metrohm GmbH, Filderstadt, Germany) for titration and a pH-Meter E 512 (Metrohm Herisan, Metrohm GmbH, Filderstadt, Germany) for pH measurement. The pancreatin solution was further diluted with PBS 1:4 to achieve a lipase activity of 300 U/ml and stored at −80 °C until use.

2.2. Preparation of liposomes

The different enhancers were mixed with EPC and cholesterol, whereby EPC was always 50% (mol/mol) of the lipid mixture and the enhancers either 10% (CS and OT) or 2.5% (TPGS). The liposomes were prepared by the film method according to Bangham et al. (1965). Therefore, the lipids were dissolved in chloroform/methanol (9:1) and mixed in a 5 ml glass vial in the desired ratio. The solution was dried under a nitrogen stream and kept under a high vacuum for 2 h to remove any solvent traces. The films were either hydrated with CF 50 mM in PBS or FITC-dextran 20 mg/ml in PBS or with PBS without any marker to achieve a final total lipid concentration of 10 mM (CF and PBS liposomes) or 100 mM (FITC-dextran liposomes). Subsequently the liposomes were extruded 21 times through a 200 nm polycarbonate membrane using a LiposoFast extruder (Avestin, Ludwigshafen, Germany). Size and polydispersity were checked by dynamic light scattering (DLS) using a Zetasizer 3000 HS (Malvern Instruments GmbH, Herrenberg, Germany) in the intensity mode.

2.3. Dynamic light scattering stability assay

Liposomes were diluted 1:10 with either Tris buffer pH 2 (Tris 50 mM, KCl 2.7 mM and NaCl 120 mM) or pancreatin in PBS or sodium taurocholate 11.11 mM in PBS – resulting in a final concentration of 10.00 mM sodium taurocholate – and incubated at 37 °C for 60 min, respectively 90 min. A 1:10 dilution of the liposomes in PBS was used as a control. After 10, 30, 60 and in case of the pancreatin and sodium taurocholate assay also after 90 min, a sample was withdrawn, 1:20 diluted and immediately two runs in the rapid mode were performed in the Zetasizer to determine size and polydispersity index (PI).

2.4. Carboxyfluorescein release

The non-encapsulated CF was separated from the liposomes by a Sephadex® G50 fine size exclusion chromatography. Release of the marker was determined at 37 °C using a Fluoroskan Ascent® (Thermo Fischer Scientific, Waltham, USA) after injection of the liposomes in Tris buffer pH 2, pancreatin in PBS or sodium taurocholate 11.11 mM in PBS resulting in a 1:10 dilution of the formulations. Increase of fluorescence was measured at 485 nm excitation and 520 nm emission wavelength. Because the fluorescence of CF is pH-dependent, the samples were neutralised after 2, 10, 30 and 60 min with Tris buffer pH 10 to achieve a final pH of 7.4. The emission of the liposomes in the mixture of the two different Tris buffers was set as zero release control and the fluorescence in Triton-X 1% in the Tris buffer mix as 100% release control. The emission of CF in the other two assays could be measured continuously and the emission in Triton-X 1% in pancreatin solution, respectively. Triton-X 1% in PBS was set as 100% release. The pancreatin solution has a certain quenching effect on the fluorescence, thus the emission in PBS was only used as a negative control for the test in sodium taurocholate. As the leakage of CF caused by pancreatin is an enzymatic reaction, a 0% release of the marker immediately after liposome injection can be hypothesized. All tests were performed in triplicate in Costar® 24 well plates (Corning, Kaiserslautern, Germany). In these type of wells the influence of the surface tension reduction on the fluorescence by the bile salt and Triton-X is less pronounced than in 96 well plates. The leakage of CF over the time was calculated as follows:

$$\%\ \ \mathrm{CF}\ \ \mathrm{release} = \frac{\mathrm{FE} - \mathrm{FE}_0}{\mathrm{FE}_{\mathrm{Trit}} - \mathrm{FE}_0} \times 100\%, \quad (1)$$

where FE is the fluorescence emission at the different time points, FE_0 is the emission of negative control and FE_{Trit} the emission of liposomes after destruction with Triton-X 1%.

2.5. FITC-dextran release

Liposomes were diluted around 1:4 with PBS and centrifuged at 150,000 × *g* for 90 min at 4 °C (Himac CS 100FX, Hitachi Koki, Tokyo, Japan). Supernatant was removed and liposomes were redispersed in the initial volume of PBS and the centrifugation step was repeated. Directly before the assay, the pellet was dispersed in PBS to achieve a lipid concentration of approximately 50 mM. The formulations were incubated in Tris buffer pH 2, pancreatin in PBS and sodium taurocholate 11.11 mM in PBS at 37 °C for 60 min and 90 min, respectively. Immediately after the incubation the samples were applied on a Sepharose® CL-4B column, eluted with PBS and liposomes and free FITC-dextran were collected separately. The free marker and the liposomes were diluted 1:10 with Triton-X 1% in PBS. Untreated liposomes served as a control. After 1:10 dilution in PBS, they were also separated from any non-encapsulated FITC-dextran by size exclusion chromatography. To determine the recovery rate of the fluorescent marker after the chromatography, non-columned liposomes were diluted 1:10 with Triton-X 1% after 1:10 dilution in PBS. All samples were measured in triplicates in a black Costar® 96 well plate (Corning, Kaiserslautern, Germany). The percentage of encapsulation (*E*%) was determined by the following equation:

$$E\% = \frac{\mathrm{FE}_{\mathrm{lip}}}{\mathrm{FE}_{\mathrm{lip}} + \mathrm{FE}_{\mathrm{free}}} \times 100\%, \quad (2)$$

where FE_{lip} is the fluorescence emission of the liposome fraction and FE_{free} of the free marker fraction after correction of the dilution. The percentage of FITC-dextran remaining in the liposomes after incubation in the different buffers in comparison to untreated liposomes was calculated by the following equation:

$$\%\mathrm{FITC-dextran}_{\mathrm{remaining}} = \frac{E\%_{\mathrm{treated}}}{E\%_{\mathrm{control}}} \times 100\%, \quad (3)$$

where $E\%_{treated}$ is the percentage of encapsulation in the treated liposomes and $E\%_{control}$ in the non-treated liposomes. The recovery rate (RR) in % was calculated as follows:

$$\%\mathrm{RR} = \frac{\mathrm{FE}_{\mathrm{lip}} + \mathrm{FE}_{\mathrm{free}}}{\mathrm{FE}_{\mathrm{uncol}}} \times 100\%, \quad (4)$$

FE_{uncol} is the fluorescence emission of the uncolumned liposomes. Only samples with a recovery rate between 90% and 110% were taken into account for statistics.

2.6. Statistics

All values are presented as means ± SEM. Groups were compared by one-way Student's *t*-test. Differences were considered significant at $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Plots and statistics were made using the software Prism® (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Liposome stability at pH 2

The majority of the formulations were stable in size and polydispersity over 90 min in Tris buffer pH 2 (Fig. 1A and B). However, the formulations with EPC/GCTE/Chol and with EPC/GCTE/OT/Chol showed a strong growth in size over the time going together with an increase of PI. All formulations showed a nearly 100% release of CF after just 10 min at pH 2 (Fig. 1C). In contrast, the encapsulation efficiency of FITC-dextran remained at nearly 100% after 60 min compared to the control for most formulations (Fig. 1D). Only the EPC/GCTE/Chol liposomes were slightly less stable than the same formulation without GCTE.

3.2. Liposome stability in sodium taurocholate 10 mM

After a certain drop in the first minutes, the liposomes were stable in size in the bile salt solutions (Fig. 2A). Also the PI showed the biggest change in the first 10 min, whereas the two formulations without bio-enhancers and the vesicles with GCTE and CS were stable in their size distribution over the time (Fig. 2B). The least stable formulation concerning the PI were the EPC/CS/Chol liposomes with an increase of 3.75-fold. The CF release in bile salts allows a better discrimination of the vesicle stability (Fig. 2C). All liposomes without GCTE released nearly 100% CF in the first 10 min. GCTE vesicles with CS and OT released 67.2% (±4.4) and 69.0% (±1.3), respectively after 90 min, liposomes with GCTE and TPGS 40.6% (±9.3) and GCTE vesicles without enhancer 36.6% (±3.3). FITC-dextran encapsulation exhibited the same inclination of stability, though the release was always lower compared to CF (Fig. 2D). GCTE vesicles retained nearly all of the markers and also EPC/Chol liposomes were remarkably stable containing still 85.3% (±5.2) of FITC-dextran after 90 min. GCTE formulations with bio-enhancers were all significantly more stable than the corresponding formulations without the tetraether lipid.

3.3. Liposome stability in pancreatin 0.3% 8× USP

Influence of pancreatin on the stability of the liposomes was very low in all three assays (Fig. 3A–D). Only the CF release varies between the formulations. Most vesicles released less than 10% of the fluorescent dye, only the EPC/GCTE/Chol liposomes were less

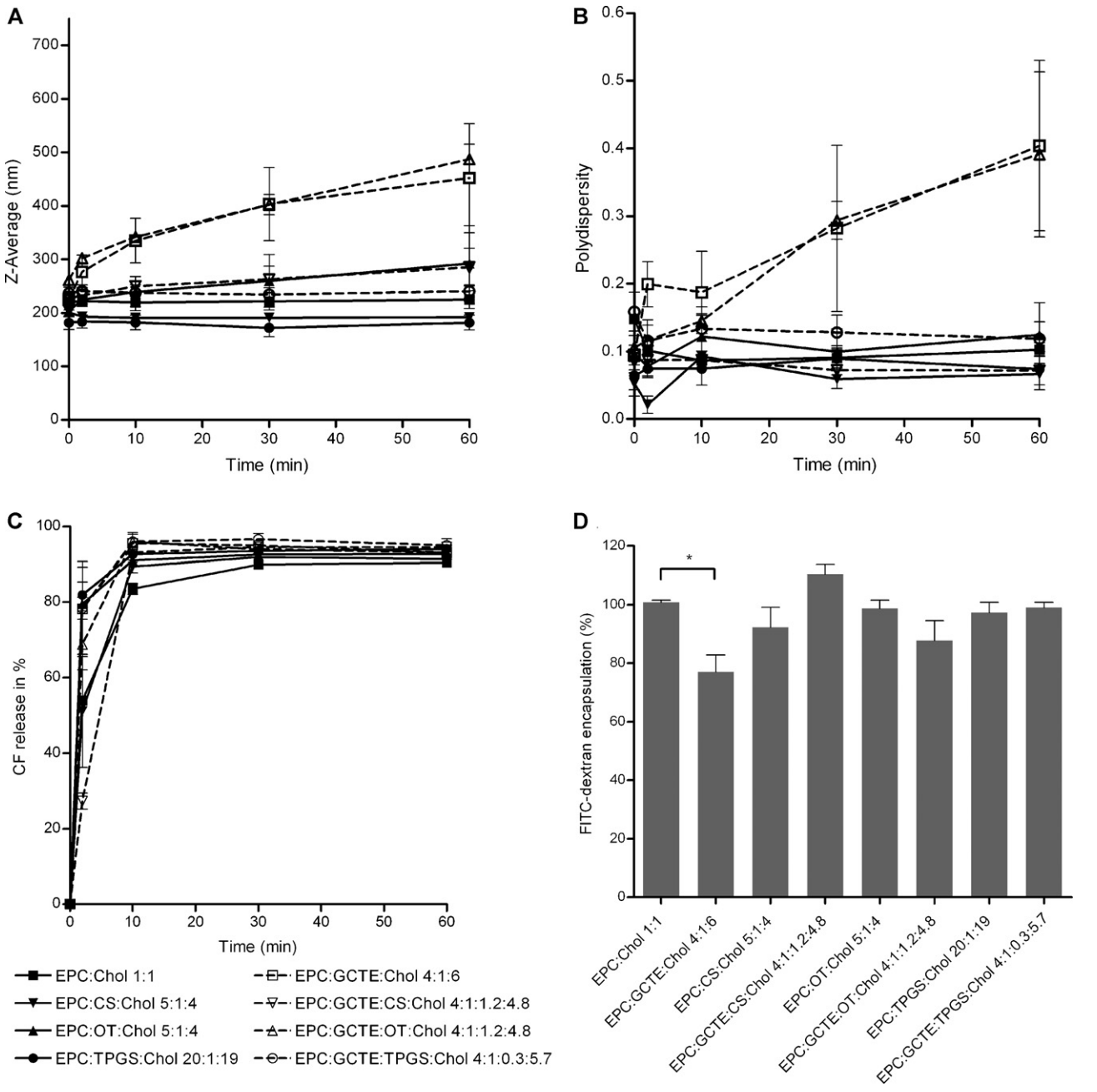

Fig. 1. Size (A), polydispersity index (B), CF release (C) and FITC-dextran 70 kDa encapsulation (D) after 60 min of liposomes in Tris buffer pH 2 at 37 °C. Values represent mean ± SEM with $n = 4$ (A and B) or $n = 3$ (C and D). Groups with and without GCTE in graph D were compared by one-way Student's t-test with $^*p < 0.05$, $p < 0.01$, $p < 0.001$.

stable by releasing 18.1% (±0.2) in 90 min, whereas TPGS seemed to have a certain stabilising effect on the CF release.

4. Discussion

In the present study, we compared the effects of the tetraether lipid glycerylcaldityl tetraether (GCTE) and various bio-enhancers on the stability of EPC and cholesterol based liposomes. We used a purified, single type TEL, GCTE, to avoid the disadvantages a mixture of TELs entails. It is likely, that the ratio of the different lipid components of archae membranes varies over the time, making a comprehensive analysis vital. Furthermore, the properties of lipid vesicles can be more easily adjusted and the amount of the TEL needed can be reduced by use of a defined and purified TEL. EPC liposomes with 50% of cholesterol were chosen as reference formulation since the stability of EPC vesicles increases with higher amount of cholesterol, whereas the transition temperature remains below body temperature (Schubert and Schmidt, 1988). Cholesterol formed crystalline structures in the membrane of EPC liposomes at a concentration of around 40%, visible in differential scanning calorimetry scans (data not shown). Thus, a concentration far above 40% is not expected to increase the stability of the vesicles any further, but will complicate handling of the lipid mixture.

Release of the two hydrophilic markers at pH 2 differed very significantly with their molecular weight, indicating that the vesicles were not totally destroyed but a leakage of the small molecule CF through the membrane could occur (see Fig. 1C and D). Also the DLS data indicate that the vesicles stayed intact over the time (see Fig. 1A and B). This was already found in previous studies, where liposomes stayed intact at low pH and leakage of macro-

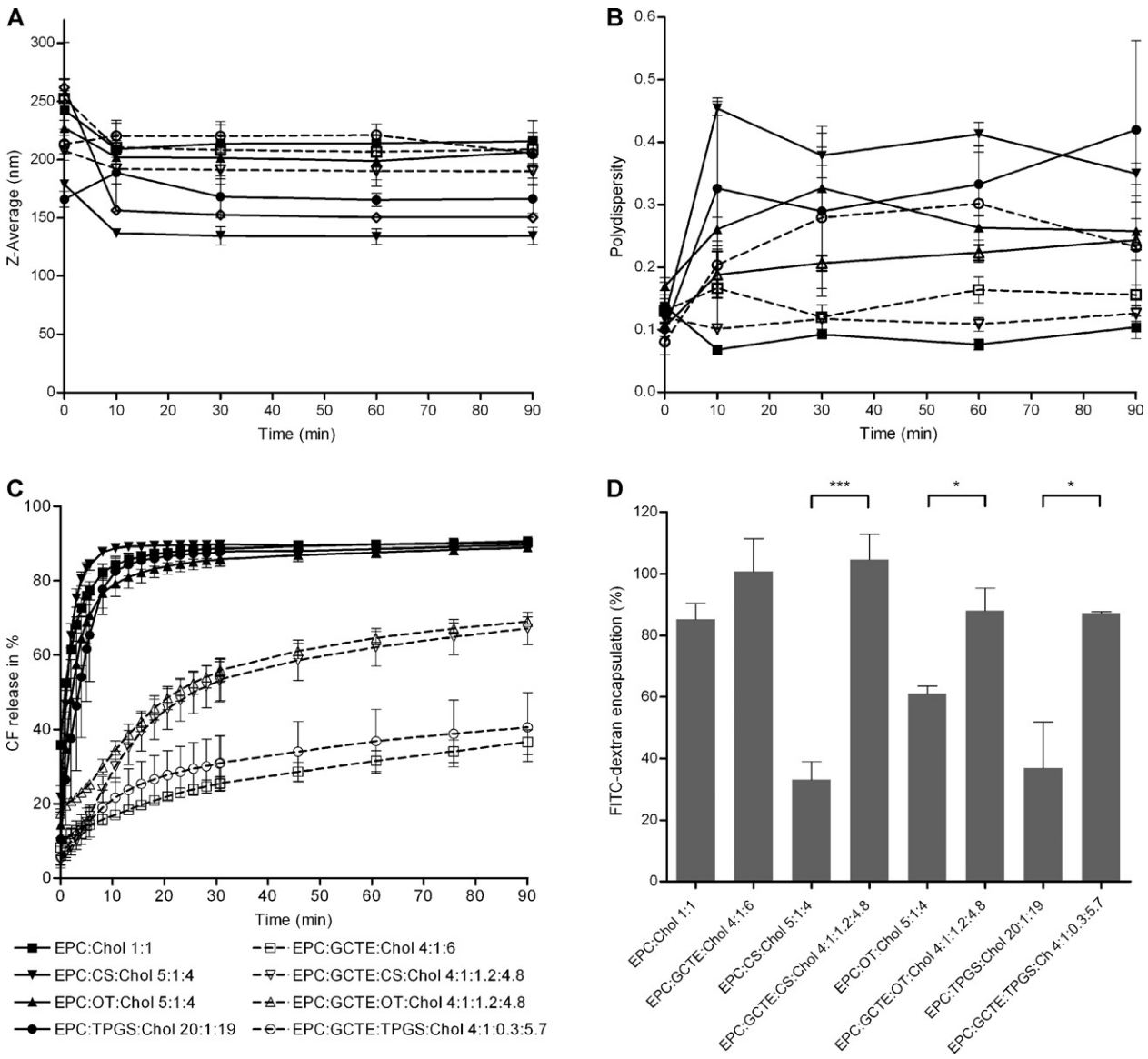

Fig. 2. Size (A), polydispersity index (B), CF release (C) and FITC-dextran 70 kDa encapsulation (D) after 90 min of liposomes in sodium taurocholate 10 mM at 37 °C. Values represent mean ± SEM with $n = 4$ (A and B) or $n = 3$ (C and D). Groups with and without GCTE in graph D were compared by one-way Student's t-test with $^{*}p < 0.05$, $p < 0.01$, $^{***}p < 0.001$.

molecules was not very pronounced (Patel et al., 2000; Taira et al., 2004). TELs are known to increase the stability of membranes at low pH, but the EPC/GCTE/Chol formulation showed the highest instability under those conditions and this effect was reduced by the addition of the two surfactant bio-enhancers TPGS and CS (Elferink et al., 1994). It seems likely, that the high rigidity of GCTE promotes membrane defects at high proton concentrations and that the fluidising effect by surfactants helps to reduce the leakage of small molecules (Cavagnetto et al., 1992; Gabriel and Chong, 2000). Aramaki et al. (1993) found a CF release in pH 2 of around 60% for different EPC/Chol liposomes. However, the liposomes examined in Aramaki's study were multilamellar vesicles (MLV) in contrast to the extruded, unilamellar vesicles (ULV) used in the present study. MLV can be generally considered as more stable because the inner aqueous core of the liposomes is protected by multiple layers of membrane. The surprisingly high leakage of CF in this study might be also related to the method used. Before fluorescent measurement, the samples had to be neutralised, which led temporarily to different proton concentrations inside and outside the liposomes, assuming that the internal pH decreased during incubation time. At low pH inside the vesicles CF is non-ionic and thus better membrane-permeable, leading to a facilitated permeation of the marker to the outside, where the counter permeation is hindered due to the anionic charge of the fluorescent marker at neutral pH. This effect may also help to explain the somehow higher leakage for phospholipid-based vesicles found here compared to previous studies, where hydrophilic non-ionic marker like sucrose or polyvinylpyrrolidone was used (Rowland and Woodley, 1980; Choquet et al., 1994). Still, the proton gradient can only play a role in CF leakage, when protons can diffuse into the inner aqueous compartment of the vesicles during the incubation in pH 2 buffer. Even if a macromolecule remains inside the liposomes, it is exposed to a high proton concentration, which could cause for example denaturation in terms of peptide drugs.

Human bile is described to contain predominantly the 5 bile salts chenodeoxycholic and cholic acid in equimolar concentration (40%), deoxycholic acid (15%) and lithocholic and ursodeoxycholic acid (together 5%). The bile salt concentration in the small intestine remains relatively constant at around 5–20 mM in duodenum and jejunum and decreases in the ileum due to reabsorption of

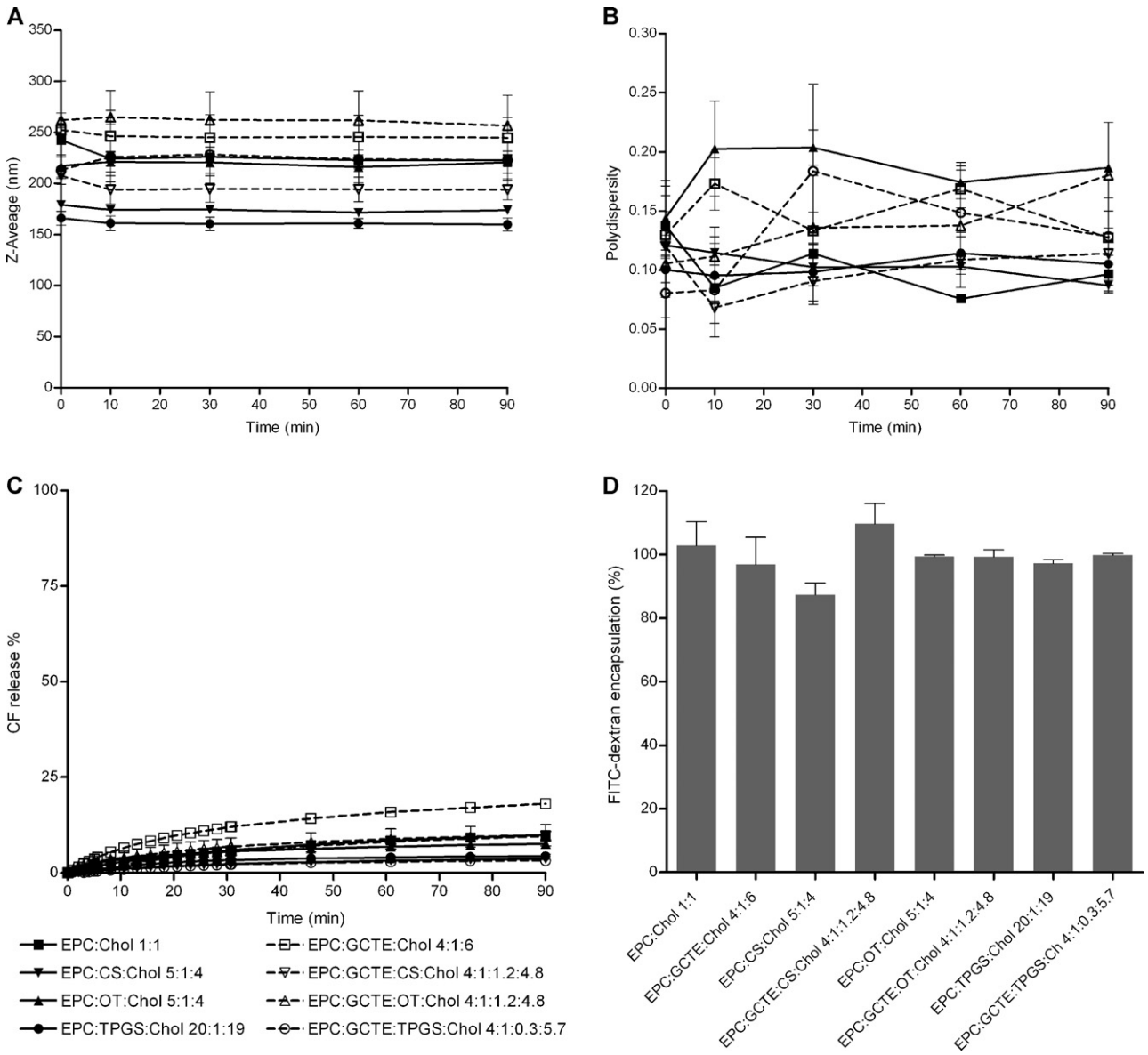

Fig. 3. Size (A), polydispersity index (B), CF release (C) and FITC-dextran 70 kDa encapsulation (D) after 90 min of liposomes in pancreatin 0.3% 8× USP at 37 °C. Values represent mean ± SEM with $n = 4$ (A and B) or $n = 3$ (C and D). Groups with and without GCTE in graph D were compared by one-way Student's t-test with $p < 0.05$, $p < 0.01$, $p < 0.001$.

the bile salts (Borgstrom, 1957; Hofmann, 1989; Kararli, 1995). To facilitate the assay and to assure that the bile acid is fully dissolved, 10 mM sodium taurocholate was used in this study instead of the physiological mixture. This concentration is above the critical micelle concentration of sodium taurocholate. Thus, it should interact rapidly with the liposomes forming mixed vesicles and finally mixed micelles (Walde et al., 1987). This might cause a total leakage of CF, which could indeed be observed for the EPC based liposomes (see Fig. 2C). However, the DLS data did not show any indication for the formation of micelles since size of vesicles was stable over the time and only the change in PI revealed some instability of the liposomes (see Fig. 2A and B). FITC-dextran release of GCTE-free liposomes suggests either the transient formation of membrane pores large enough for the macromolecule to pass or the solubilisation of vesicles into mixed micelles and a continuous disturbance of membrane integrity (see Fig. 2D). Cholesterol is known to hamper forming of pores and membrane solubilisation, which is in good agreement with the low FITC-dextran release of EPC/Chol liposomes (Schubert and Schmidt, 1988). In contrast, surfactants make the membrane more susceptible to sodium taurocholate. Also OT, which is not surface active, reduced the membrane stability, and this may be due to the lower amount of cholesterol in this formulation compared to EPC/Chol liposomes or the low melting point (24–31 °C) of OT. The stabilising effect of GCTE might be related both to its membrane spanning structure increasing the intermolecular forces in the membrane and to its rigidity hindering the insertion of sodium taurocholate into the membrane. Altogether, the stability results in bile salts suggest, that GCTE-stabilised liposomes remain with their membrane integrity and are likely to protect encapsulated drugs from degradation.

Ether lipids are known to be less susceptible against pancreatin, moreover Burns et al. (1981) described a competitive inhibition of phospholipase A_2 by diether lipids (Choquet et al., 1994). Taira et al. (2004) found a CF release from conventional EPC/Chol liposomes in pancreatin of around 10% after 90 min, which is in good agreement with our results. The somewhat higher leakage of the EPC/GCTE/Chol formulation might be related not only to an enzymatic degradation of the lipids but also to protein/membrane

interaction causing a destabilisation of the liposomal membrane (see Fig. 3C). The failure to improve stability against pancreatin of the TEL could be due to the fact that the amount of ester phospholipids is still 36% in the formulations giving phospholipase A_2 enough targets. The stabilising effect of TPGS on the liposomes is probably due to sterical hinderance of the pancreatic enzymes by the PEG chain. Considering the size stability and the very low FITC-dextran release the CF release in pancreatin is likely caused by small membrane defects and not by a rupture or total disintegration of some vesicles. Some of the effects could also be associated to membrane/protein interactions and not to enzymatic processes.

5. Conclusions

Liposomes containing both the stabilising tetraether lipid GCTE and bio-enhancers could be a versatile tool for oral delivery of proteins or other drug substances, which have a low oral bioavailability due to gastro-intestinal degradation and low permeation. In the present study, we could show that GCTE can improve stability of liposomes against sodium taurocholate and especially could reduce the destabilising effect of bio-enhancers in the liposomal membrane. However, it has to be clarified in further studies, if GCTE might reduce a possible positive effect of bio-enhancers in liposomes on the bioavailability of encapsulated drugs and thus would diminish its benefit concerning stability increase. This should be dependent from the mode of action of the respective enhancer. Especially the performance of enhancers, which increase permeation of a drug by fluidising the cell membrane, could be reduced. The influence of GCTE might be less significant for mucoadhesive substances or those opening tight junctions.

The high release of CF at pH 2 is most likely related to small membrane damages but not to a disintegration of the liposomes indicated by a low FITC-dextran release and size stability. Nevertheless, not only a release of encapsulated drug but also damage by the high proton concentration during stomach passage has to be taken into account. To overcome this stability problem a freeze-drying of liposomes and encapsulation in enteric-coated capsules will be investigated in further studies.

References

Aramaki, Y., Tomizawa, H., Hara, T., Yachi, K., Kikuchi, H., Tsuchiya, S., 1993. Stability of liposomes in vitro and their uptake by rat Peyer's patches following oral administration. Pharm. Res. 10, 1228–1231.

Authority, E.F.S., 2007. D-Alpha-tocopheryl polyethylene glycol 1000 succinate (TPGS) in use for food for particular nutritional purposes. EFSA J., 1–20.

Bangham, A.D., Standish, M.M., Watkins, J.C., 1965. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 13, 238–252.

Borgstrom, B., Dahlqvis, T.A., Lund, H.G., Sjoval, L.J., 1957. Studies of intestinal digestion and absorption in the human. J. Clin. Invest. 36, 1521–1536.

Burns, R.A., Friedman, J.M., Roberts, M.F., 1981. Characterization of short-chain alkyl ether lecithin analogues: 13C NMR and phospholipase studies. Biochemistry 20, 5945–5950.

Cavagnetto, F., Relini, A., Mirghani, Z., Gliozzi, A., Bertoia, D., Gambacorta, A., 1992. Molecular packing parameters of bipolar lipids. Biochim. Biophys. Acta 1106, 273–281.

Chiang, C., Weiner, N., 1987. Gastrointestinal uptake of liposomes. II. In vivo studies. Int. J. Pharm. 40, 143–150.

Choquet, C.G., Patel, G.B., Beveridge, T.J., Sprott, G.D., 1994. Stability of pressure-extruded liposomes made from archaeobacterial ether lipids. Appl. Microbiol. Biotechnol. 42, 375–384.

Collnot, E.-M., Baldes, C., Wempe, M.F., Kappl, R., Hüttermann, J., Hyatt, J.A., Edgar, K.J., Schaefer, U.F., Lehr, C.-M., 2007. Mechanism of inhibition of P-glycoprotein mediated efflux by vitamin E TPGS: influence on ATPase activity and membrane fluidity. Mol. Pharm. 4, 465–474.

De Rosa, M., Gambacorta, A., Gliozzi, A., 1986. Structure, biosynthesis, and physicochemical properties of archaebacterial lipids. Microbiol. Rev. 50, 70–80.

Degim, Z., Unal, N., Essiz, D., Abbasoglu, U., 2004. The effect of various liposome formulations on insulin penetration across Caco-2 cell monolayer. Life Sci. 75, 2819–2827.

Elferink, M.G., de Wit, J.G., Driessen, A.J., Konings, W.N., 1994. Stability and proton-permeability of liposomes composed of archaeal tetraether lipids. Biochim. Biophys. Acta 1193, 247–254.

Emmett, M., Guirl, M.J., Santa Ana, C.A., Porter, J.L., Neimark, S., Hofmann, A.F., Fordtran, J.S., 2003. Conjugated bile acid replacement therapy reduces urinary oxalate excretion in short bowel syndrome. Am. J. Kidney Dis. 41, 230–237.

Fan, Q., Relini, A., Cassinadri, D., Gambacorta, A., Gliozzi, A., 1995. Stability against temperature and external agents of vesicles composed of archael bolaform lipids and egg PC. Biochim. Biophys. Acta 1240, 83–88.

Filipović-Grcić, J., Skalko-Basnet, N., Jalsenjak, I., 2001. Mucoadhesive chitosan-coated liposomes: characteristics and stability. J. Microencapsul. 18, 3–12.

Fricker, G., Drewe, J., 1996. Current concepts in intestinal peptide absorption. J. Pept. Sci. 2, 195–211.

Fricker, G., Kromp, T., Wendel, A., Blume, A., Zirkel, J., Rebmann, H., Setzer, C., Quinkert, R.-O., Martin, F., Müller-Goymann, C., 2010. Phospholipids and lipid-based formulations in oral drug delivery. Pharm. Res..

Gabriel, J.L., Chong, P.L., 2000. Molecular modeling of archaebacterial bipolar tetraether lipid membranes. Chem. Phys. Lipids 105, 193–200.

Gambacorta, A., Gliozzi, A., Rosa, M., 1995. Archaeal lipids and their biotechnological applications. World J. Microbiol. Biotechnol. 11, 115–131.

Gliozzi, A., Paoli, G., Pisani, D., Gliozzi, F., De Rosa, M., Gambacorta, A., 1986. Phase transitions of bipolar lipids of thermophilic archaebacteria. Biochim. Biophys. Acta 861, 420–428.

Han, M., Watarai, S., Kobayashi, K., Yasuda, T., 1997. Application of liposomes for development of oral vaccines: study of in vitro stability of liposomes and antibody response to antigen associated with liposomes after oral immunization. J. Vet. Med. Sci. 59, 1109–1114.

Hill, M., 1978. Epidemiology and etiology of colon cancer. In: Grundmann, E. (Ed.), Colon Cancer Cancer Campaign, vol. 2, pp. 115–127.

Hofmann, A.F., 1989. Enterohepatic circulation of bile acids. In: Handbook of Physiology, Section 6: The Gastrointestinal System III, pp. 567–595.

Iwanaga, K., Ono, S., Narioka, K., Kakemi, M., Morimoto, K., Yamashita, S., Namba, Y., Oku, N., 1999. Application of surface-coated liposomes for oral delivery of peptide: effects of coating the liposome's surface on the GI transit of insulin. J. Pharm. Sci. 88, 248–252.

Iwanaga, K., Ono, S., Narioka, K., Morimoto, K., Kakemi, M., Yamashita, S., Nango, M., Oku, N., 1997. Oral delivery of insulin by using surface coating liposomes improvement of stability of insulin in GI tract. Int. J. Pharm. 157, 73–80.

Kandell, R., Bernstein, C., 1991. Bile-salt acid induction of DNA damage in bacterial and mammalian-cells—implications for colon cancer. Nutr. Cancer 16, 227–238.

Kapral, C., Wewalka, F., Praxmarer, V., Lenz, K., Hofmann, A.F., 2004. Conjugated bile acid replacement therapy in short bowel syndrome patients with a residual colon. Z. Gastroenterol. 42, 583–589.

Kararli, T.T., 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. Biopharm. Drug Dispos. 16, 351–380.

Komatsu, H., Chong, P.L., 1998. Low permeability of liposomal membranes composed of bipolar tetraether lipids from thermoacidophilic archaebacterium Sulfolobus acidocaldarius. Biochemistry 37, 107–115.

Langworthy, T.A., 1977. Long-chain diglycerol tetraethers from Thermoplasma acidophilum. Biochim. Biophys. Acta 487, 37–50.

Latta, R., Fiander, H., Ross, N., Simpson, C., Schneider, H., 1993. Toxicity of bile-acids to colon-cancer cell-lines. Cancer Lett. 70, 167–173.

Lillienau, J., Schteingart, C., Hofmann, A., 1992. Physicochemical and physiological properties of cholylsarcosine—a potential replacement detergent for bile acid deficiency states in the small intestine. J. Clin. Invest. 89, 420–431.

Lo, S.L., Chang, E.L., 1990. Purification and characterization of a liposomal-forming tetraether lipid fraction. Biochem. Biophys. Res. Commun. 167, 238–243.

Lo, S.L., Montague, C.E., Chang, E.L., 1989. Purification of glycerol dialkyl nonitol tetraether from Sulfolobus acidocaldarius. J. Lipid Res. 30, 944–949.

Maher, S., Brayden, D.J., Feighery, L., McClean, S., 2008. Cracking the junction: update on the progress of gastrointestinal absorption enhancement in the delivery of poorly absorbed drugs. Crit. Rev. Ther. Drug 25, 117–168.

Muramatsu, K., Maitani, Y., Nagai, T., 1996. Dipalmitoylphosphatidylcholine liposomes with soybean-derived sterols and cholesterol as a carrier for the oral administration of insulin in rats. Biol. Pharm. Bull. 19, 1055–1058.

Nair, P., Turjman, N., 1983. Role of bile acids and neutral sterols in familial cancer syndromes of the colon. Dis. Colon Rectum 26, 629–632.

Patel, G.B., Agnew, B.J., Deschatelets, L., Fleming, L.P., Sprott, G.D., 2000. In vitro assessment of archaeosome stability for developing oral delivery systems. Int. J. Pharm. 194, 39–49.

Patel, G.B., Chen, W., 2005. Archaeosome immunostimulatory vaccine delivery system. Curr. Drug Deliv. 2, 407–421.

Patel, H., Ryman, B.E., 1976. Oral administration of insulin by encapsulation within liposomes. FEBS Lett. 62, 60–63.

Patel, H.M., Stevenson, R.W., Parsons, J.A., Ryman, B.E., 1982. Use of liposomes to aid intestinal absorption of entrapped insulin in normal and diabetic dogs. Biochim. Biophys. Acta 716, 188–193.

Rowland, R.N., Woodley, J.F., 1980. The stability of liposomes in vitro to pH, bile salts and pancreatic lipase. Biochim. Biophys. Acta 620, 400–409.

Rowland, R.N., Woodley, J.F., 1981a. The uptake of distearoylphosphatidylcholine/cholesterol liposomes by rat intestinal sacs in vitro. Biochim. Biophys. Acta 673, 217–223.

Rowland, R.N., Woodley, J.F., 1981b. Uptake of free and liposome-entrapped 125I-labelled PVP by rat intestinal sacs in vitro: evidence for endocytosis. Biosci. Rep. 1, 399–406.

Rowland, R.N., Woodley, J.F., 1981c. Uptake of free and liposome-entrapped insulin by rat intestinal sacs in vitro. Biosci. Rep. 1, 345–352.
Schouten, S., Van Der Meer, M.T.J., Hopmans, E.C., Rijpstra, W.I.C., Reysenbach, A.-L., Ward, D.M., Sinninghe Damste, J.S., 2007. Archaeal and bacterial glycerol dialkyl glycerol tetraether lipids in Hot Springs of Yellowstone National Park. Appl. Environ. Microbiol. 73, 6181–6191.
Schubert, R., Schmidt, K.H., 1988. Structural changes in vesicle membranes and mixed micelles of various lipid compositions after binding of different bile salts. Biochemistry 27, 8787–8794.
Song, K., Chung, S., Shim, C., 2005. Enhanced intestinal absorption of salmon calcitonin (sCT) from proliposomes containing bile salts. J. Control. Release 106, 298–308.
Taira, M., Chiaramoni, N., Pecuch, K., Alonso-Romanowski, S., 2004. Stability of liposomal formulations in physiological conditions for oral drug delivery. Drug Deliv. 11, 123–128.
Takeuchi, H., Matsui, Y., Sugihara, H., Yamamoto, H., Kawashima, Y., 2005. Effectiveness of submicron-sized, chitosan-coated liposomes in oral administration of peptide drugs. Int. J. Pharm. 303, 160–170.
Takeuchi, H., Yamamoto, H., Kawashima, Y., 2001. Mucoadhesive nanoparticulate systems for peptide drug delivery. Adv. Drug Deliv. Rev. 47, 39–54.
Takeuchi, H., Yamamoto, H., Niwa, T., Hino, T., Kawashima, Y., 1994. Mucoadhesion of polymer-coated liposomes to rat intestine in vitro. Chem. Pharm. Bull. 42, 1954–1956.
Walde, P., Sunamoto, J., O'Connor, C.J., 1987. The mechanism of liposomal damage by taurocholate. Biochim. Biophys. Acta 905, 30–38.
Werle, M., Hironaka, K., Takeuchi, H., Hoyer, H., 2009. Development and in vitro characterization of liposomes coated with thiolated poly(acrylic acid) for oral drug delivery. Drug Dev. Ind. Pharm. 35, 209–215.
Werle, M., Makhlof, A., Takeuchi, H., 2010. Carbopol–lectin conjugate coated liposomes for oral peptide delivery. Chem. Pharm. Bull. 58, 432–434.
Werle, M., Takeuchi, H., 2009. Chitosan-aprotinin coated liposomes for oral peptide delivery: development, characterisation and in vivo evaluation. Int. J. Pharm. 370, 26–32.
Whitmore, D.A., Wheeler, K.P., 1979. The fate of liposomes in the rat small intestine [proceedings]. Biochem. Soc. Trans. 7, 929–931.