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Research paper

In vitro evaluation of liposomes containing bio-enhancers for the oral delivery of macromolecules

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ABSTRACT

The aim of this work was to develop a new type of liposomes containing bio-enhancers for oral delivery of hydrophilic macromolecules. The study focused on EPC/cholesterol-based formulations combined with TPGS 1000 and 400, cholylsarcosine (CS), cetylpyridinium chloride (CpCl) and stearylamine (SA) covering a broad range of different types of enhancers. Most of the tested liposomal formulations and enhancers showed neither influence on cell viability in the Alamar Blue® assay nor an increase in lactate dehydrogenase LDH release. But, at a concentration of 1 mM, CpCl exhibited a strong toxicity after 2 h, TPGS 1000 reduced the cell viability at the same concentration after 8 h significantly. Only one liposomal formulation with 25% CpCl led to a decrease in viability to 60.0% after 8 h at a total lipid concentration of 5 mM. In the Caco-2 Transwell® model, one formulation with 5% TPGS 400 improved the permeation of FITC-dextran 70 kDa 3.34 ± 0.62-fold, one with 10% CpCl 3.69 ± 0.67 and one with 10% CS and 2.5% SA 3.41 ± 0.51-fold without influencing the TER. Liposomes with 10% SA or 25% CpCl increased the permeation of FITC-dextran 29.02 ± 5.84, respectively 39.28 ± 2.10-fold, but led also to a strong reduction in the TER. Especially, the three formulations which enhanced the permeation of FITC-dextran around 3.5-fold without showing any cell toxicity or decrease in TER should be safe and effective candidates for the development of an oral delivery system for hydrophilic macromolecules.

1. Introduction

Oral application is by far the most convenient route for drug delivery, especially for long and repeated therapeutic use. But the development of formulations for the oral administration of BCS Class III drugs, especially macromolecules like proteins, heparin or oligonucleotides is rendered more difficult for several reasons. Macromolecules are mostly poorly absorbed due to their high molecular weight and hydrophilicity according to Lipinski's rule of five, and peptides may be degraded pre-systemically in the gastrointestinal tract (GIT) leading to a reduced fraction reaching the intestinal wall [1–3]. This usually results in a bioavailability of less than 1% [4]. In order to overcome these problems, several approaches were taken, for instance the use of absorption enhancers like surfactants and small molecule carriers, enzyme inhibitors and the use of particulate systems, mostly nanoparticles or liposomes [5–7].

The first approaches to use liposomes for oral delivery were not very encouraging mostly due to poor reproducibility of the results [8,9]. Nevertheless, liposomes have some important advantages over other delivery systems as they are well characterised, have good biocompatibility and high versatility [10]. It seems reasonable to combine liposomes with other mechanisms for permeation enhancement to further improve absorption of peptides, as it is possible to deliver protein drugs and enhancers together in one vehicle to the enterocytes. This would allow a reduction in the amount of permeation enhancers used and a decrease in toxic side effects. Unfortunately, bio-enhancers are often surfactants, which can interact easily with the liposomal membrane and are known to destabilise or even destroy phospholipid vesicles. But this effect is clearly dependent on the lipid composition and the type and concentration of the enhancers used, allowing theoretically the formation of stable liposomes containing surfactants.

In this study, we used various bio-enhancers with different characteristics concerning proposed mode of action, charge and chemical properties. D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS 1000) is a non-ionic surfactant and was originally used as a water-soluble vitamin E derivative [11]. TPGS 400 just differs in the length of the PEG chain and thus in the hydrophilic–lipophilic balance (HLB). Besides the surfactant characteristics of TPGS, which mostly imply also permeation enhancing properties,

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the PEG chain can contribute to the stabilisation of the liposomes and is known to have a certain mucoadhesivity [12]. The anionic bile salt derivative cholylsarcosine (CS), the sarcosine (N-methylglycine) conjugate of cholic acid, behaves very similarly to natural occurring conjugated bile acids [13]. This compound has originally been developed for bile acid replacement in patients with a malabsorption syndrome [14,15]. Bile salts are in use for a long time as permeation enhancers and there have already been studies with liposomal bile salt formulations [16,17]. However, here we present the first study using CS in liposomes. Cetylpyridinium chloride (CpCl) is a cationic surfactant mostly used as disinfectant but also with applications as bio-enhancer [18]. Furthermore, it can be easily incorporated into liposomal membranes [19]. Stearylamine (SA) is a cationic lipid and in contrast to the other enhancers used has just little surfactant properties. Incorporated in liposomal membranes, it leads to a positive surface charge and can therefore promote the cellular uptake of the particles [7].

In the present work, the ability of various enhancers to form liposomes with the host lipids egg phosphatidylcholine (EPC) and cholesterol (Chol) was examined and the possible use of the liposomes for oral drug delivery was assessed by investigating their toxicity and the permeation improvement of a dextran derivative in the Caco-2 Transwell® model. FITC-dextran 70 kDa used in this study is a stable macromolecule, which is no known substrate of any cellular transport mechanism and shows only low interactions with liposomal lipids [20,21]. Thus, it allows an investigation into the enhancement potential of the liposomal systems with little influence of possible drug/drug carrier interactions.

2. Materials and methods

2.1. Materials

EPC was provided by Lipoid GmbH (Ludwigshafen, Germany). TPGS 1000 and TPGS 400 were supplied by Eastman (Kingsport, TN, USA). CpCl was purchased from Roth (Karlsruhe, Germany). CS was obtained from Prodotti Chimici e Alimentari S.p.A. (Basaluzzo, Italy). Cholesterol, SA and fluorescein isothiocyanate-dextran (Mw 70000 Da) (FITC-dextran) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Culture media, fetal bovine serum (FBS) and supplements were purchased from Biochrom (Berlin, Germany). All other chemicals were obtained in the highest purity from the usual commercial sources.

2.2. Cell culture

Caco-2 cells were grown in T-75 flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acids, 1% pyruvate, 1% L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in an atmosphere with 5% CO_2 and in equilibrium with distilled water. The medium was changed every other day, and the cells were subcultured at 80% confluency. Cells were used in the experiments at passage number 35–45.

2.3. 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 and cholesterol summed up to the other half of the formulation. The liposomes were prepared by the film method according to Bangham et al. [22]. 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.

In terms of the cytotoxicity studies, the film was hydrated with Krebs–Ringer–Buffer (KRB) (NaCl 142 mM, KCl 3 mM, $K_2HPO_4 \cdot 3H_2O$ 1.5 mM, HEPES 10 mM, D-glucose 4 mM, $MgCl_2 \cdot 6H_2O$ 1.2 mM and $CaCl_2 \cdot 2H_2O$ 1.4 mM) to a final concentration of 150 μmol/ml lipid dispersion and the vesicle were extruded five times through an 800-nm membrane and 15 times through a 200-nm membrane using a Lipex™extruder (Northern Lipids, Burnaby, BC, Canada).

For the permeation assay, the lipid film was hydrated with FITC-dextran 20 mg/ml in KRB to a lipid concentration of 200 μmol/ml. The dispersion was then sonicated in a bath type sonicator for 2 h (Elmasonic S 300 H, Elma®, Singen, Germany) and extruded 41 times through a 100-nm membrane using a LiposoFast extruder (Avestin, Ludwigshafen, Germany). The liposomes were separated from the non-encapsulated marker over a Sepharose® CL-4B column, and the amount of the encapsulated FITC-dextran was determined by its fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520 nm in a Fluoroskan Ascent® plate reader (Thermo Fischer Scientific, Waltham, MA, USA) against a calibration curve. The liposomes were further diluted to a FITC-dextran concentration of 0.5 mg/ml.

The final lipid concentration of the liposomes was determined by HPLC. Size and polydispersity were checked by photon correlation spectroscopy (PCS) using a Zetasizer 3000 HS (Malvern Instruments GmbH, Herrenberg, Germany).

2.4. HPLC-lipid analysis

The liposomes were diluted 1:10 in methanol, preincubated at 35 °C for at least 30 min and then injected by an autosampler (35 °C) in a Dionex UltiMate® 3000 system (Dionex, Idstein, Germany) with a UV PDA detector and an Acclaim® 120 C18 5-μm column (4.6 mm · 250 mm) at 45 °C. The mobile phase consisted of following solvents: water + 0.05% trifluoroacetic acid (TFA) (phase A), methanol + 0.05% TFA (phase B) and acetonitrile + 0.05% TFA (phase C). The flow was kept at 1.2 ml/min throughout the run. The gradient was programmed as shown in Fig. 1. The concentration of the substances was determined by comparing the UV absorption at 215 nm to a calibration curve.

2.5. Cytotoxicity assays

Cytotoxicity of liposomes and bio-enhancer solutions was investigated using the Alamar Blue® assay (AbD Serotec, Oxford, UK) and by determination of the release of lactate dehydrogenase (LDH) with a test kit (Sigma–Aldrich, Taufkirchen, Germany). The Alamar Blue® assay is based on the ability of mitochondrial dehydrogenase to cleave the tetrazolium rings of a blue MTT derivative (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide),

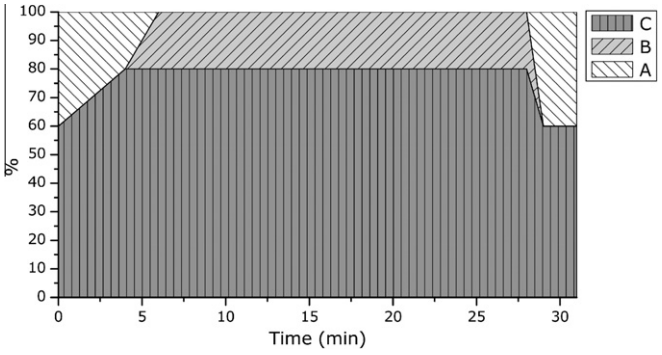

Fig. 1. HPLC gradient with following mobile phases: water + 0.05% TFA (phase A), methanol + 0.05% TFA (phase B) and acetonitrile + 0.05% TFA (phase C).

whereby a pink-coloured formazan product is formed. The LDH assay relies on the reduction of NAD by LDH, which releases from the cells due to cell membrane damage. The formed NADH in turn reduces a tetrazolium salt to a red-coloured product which can be determined photometrically.

Caco-2 cells were seeded onto rat tail collagen (Roche, Mannheim, Germany) coated 96-well plates at a density of 65,000 cells/cm^2 and grown for 14 days under the conditions described above. Twelve hours prior to experiments, the growth medium was changed to serum, antibiotics and phenol red-free DMEM, and the cells were finally washed twice with KRB.

In terms of the Alamar Blue® assay, groups of eight wells were incubated with the solutions of the bio-enhancers in five concentrations (1 μM, 10 μM, 100 μM, 1 mM and 10 mM) and the liposomal formulations in three concentrations (0.5 mM, 5 mM and 50 mM total lipid) in KRB for 2, 4 and 8 h at 37 °C. Due to its poor water solubility, SA was dispersed in KRB using a tip sonicator (Soni 130, G. Heinemann, Schwäbisch Gmünd, Germany) for 5 min at 130 W and only tested in three concentrations (1 μM, 10 μM and 100 μM). After incubation, the cells were washed twice with KRB and an Alamar Blue® solution in KRB (1:80) was added. After 4 h at 37 °C, the colour change of the dye was determined at an excitation wavelength of 530 nm and an emission wavelength of 590 nm in a Fluoroskan Ascent® plate reader. Cells incubated with KRB were used as untreated control, and cells exposed to 1% Triton X-100 were used as positive control. Cell viability was expressed as follows:

$$\%\ \text{Cell viability} = \frac{\text{Sample value}}{\text{Untreated control}} \times 100\% \quad (1)$$

For the LDH release assay, cells were treated similar as described for the Alamar Blue® cytotoxicity assay with little changes. A 25-μl sample was withdrawn from the cell supernatant after 2, 4 and 8 h, diluted with the same volume of KRB and was incubated with 50 μl of the assay mixture for 30 min in the dark as recommended by the manufacturer. The reaction was stopped by the addition of 10 μl 1N HCl and subsequently, the colour change of the dye was measured by a Tecan microplate absorbance reader (Sunrise, Tecan, Grödig, Austria) at a wavelength of 490 nm and a reference wavelength of 700 nm. To eliminate the influence of the liposomes and the bio-enhancers on the absorbance, a blank was measured of the formulations with a concentration equal to the final assay concentration. Cell viability was calculated by the following equation:

$$\%\ \text{Cell viability} = \frac{A_{Triton1\%} - (A_{sample} - A_{blank})}{A_{Triton1\%}} \times 100\% \quad (2)$$

where $A_{Triton1\%}$ is the absorbance after incubation with Triton X-100%1, A_{sample} the absorbance after incubation with the test solutions, and A_{blank} the absorbance of the test solutions without the addition of the assay dye mixture.

2.6. Permeation studies

Caco-2 cells were seeded at a density of 75000 cells/cm^2 onto rat tail collagen-coated 12 Transwell® polyester filters with 0.4-μm pore size (Corning, Kaiserslautern, Germany) and grown for 21 days under the conditions described above. Twelve hours prior to experiments, the growth medium was changed to serum, antibiotics and phenol red-free DMEM and the cells were finally washed twice with KRB. The transport experiments were performed in a device (cellZscope®, Nanoanalytics, Münster, Germany) monitoring automatically the transepithelial electrical resistance (TER) and the cell capacitance (C_{cl}) of 24 filters in parallel by measuring the frequency-dependent impedance of the cell layer. The theoretical background of the impedance measurement is described in detail by Wegener et al. [23]. The filter inserts were placed inside the cellZscope®, and 0.6 ml KRB in the apical (A) and 1.0 ml KRB in the basolateral (B) compartment were added. The cells were allowed to equilibrate for 2 h before the first measurement of TER and C_{cl}, which were monitored throughout the entire experiment every hour. Directly after the first measurement, the KRB on the A-side was replaced with the liposomal dispersion or the solution of the free FITC-dextran. Samples of 200 μl were withdrawn every hour from the B-side, replaced with KRB and transferred in a black 96-well plate (Corning, Kaiserslautern, Germany). The concentration of the FITC-dextran was determined as described above. The apparent permeation coefficient (P_{app}) of dextran was calculated following the equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{A \cdot C_0} \left(\frac{\text{cm}}{\text{s}}\right) \quad (3)$$

where C_0 is the concentration of FITC-dextran on the A-side (μg/cm^3) at time point zero, and A is the total surface area of the filter (cm^2). dQ/dt was calculated by the slope of the linear range of the permeation rate of FITC-dextran (μg/s).

2.7. Calculation of the liposome encapsulation efficiency

In order to compare the performance of the different liposomes depending on their encapsulation efficiency, a virtual permeation coefficient for the liposomes was calculated by relating the apparent permeation coefficient of the FITC-dextran to the encapsulation efficiency of the liposomes. The apparent permeation coefficient ($P_{app/lipid}$) was calculated as follows:

$$P_{app/lipid} = P_{app} \cdot \frac{C_0}{C_{lipid}} \left(\frac{\text{cm g}}{\text{s mol}}\right) \quad (4)$$

where C_0 is the concentration of the dextran on the A-side (μg/cm^3), and C_{lipid} is the total lipid concentration on the A-side (μmol/cm^3).

2.8. Statistics

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

3. Results and discussion

3.1. Liposome properties

Size and polydispersity of the liposomes are shown in Figs. 2 and 3. All formulations were in size between 110 nm and 190 nm and showed a narrow distribution indicating the successful formation of liposomes. Although the vesicles for the toxicity studies were extruded through a 200-nm membrane, they were mostly similar in size to the liposomes for the transport studies, probably due to the higher flow rate of the Lipex™extruder [24].

Fig. 4 shows the ratio of the marker FITC-dextran to the total lipid amount in the liposomes after the purification step. To achieve a final marker concentration of 0.5 mg/ml, the liposomes were finally diluted to a total lipid concentration between 15 and 40 mM depending on the encapsulation efficiency of the liposomal formulation.

Whereas SA is used rather often in liposomes to obtain a positive surface charge, there is little use of CpCl and to our knowledge, no use of TPGS and CS in liposomes [10,25–28]. However, there are at least two studies using other bile salts in liposomes

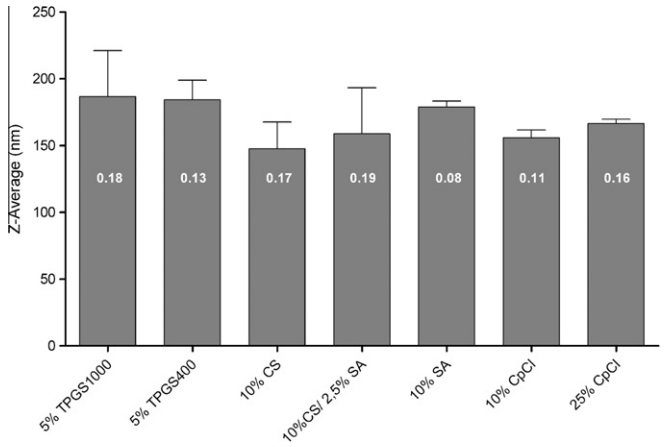

Fig. 2. *Z*-Average and polydispersity index (marked in white on the size bars) of the liposomal formulations for the permeation studies. The size is expressed in nm and given as means ± SEM with $n = 3$.

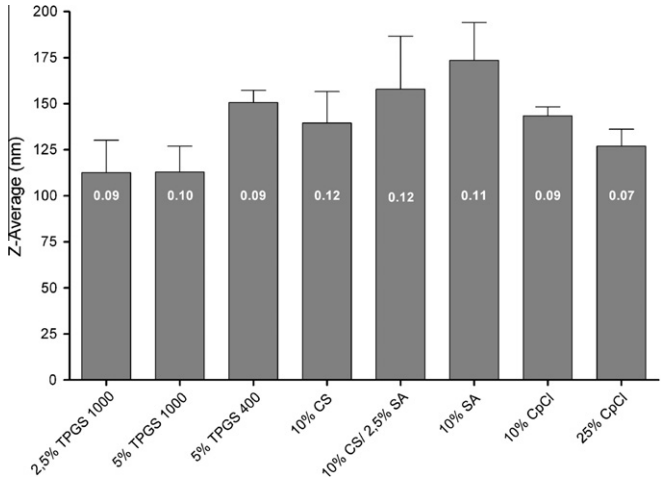

Fig. 3. *Z*-Average and polydispersity index (marked in white on the size bars) of the liposomal formulations for the toxicity studies. The size is expressed in nm and given as means ± SEM with $n = 3$.

for permeation enhancement [16,17]. The cautious use of surfactants in lipid vesicles might be related to their potentially destabilising effect on liposomes. Besides the packing constraints of the lipid bilayer, steric conditions and the curvature of the vesicles, this is mostly dependent on the critical micelle concentration (CMC) of the surfactant, whereas electrostatic interactions have an inferior influence [29]. The CMC values for the different enhancers at room temperature found in the literature are as follows: CpCl 0.98 mM, CS between 10 and 11 mM, TPGS 1000 around 0.02 mM and TPGS 400 around 1.5 mM [13,30,31]. For SA, no CMC could be found in the literature. Usually, when the CMC of a surfactant increases, also the concentration needed to form mixed micelles with phospholipids is increased [29]. Surprisingly, TPGS 400 has a higher CMC compared to TPGS 1000 even though it has a lower HLB (8.3 vs 13.2) [32]. However, the smaller size and the lower encapsulation efficiency of the formulation with 5% TPGS 1000 indicate that indeed TPGS 1000 in the chosen concentration forms not just mixed vesicles but also mixed micelles. This does not apply to TPGS 400 or at least to a lower extent apparent by the bigger size of the liposomes formed by the LiposoFast extruder. The CS-containing vesicles for the cytotoxicity studies were remarkably smaller than the others. Despite the high CMC of CS, the smaller size and the very poor encapsulation efficiency of both of the CS-containing formulations imply again the formation of mixed micelles. It is unlikely that the small FITC-dextran/lipid ratio is only caused by the negative charge of CS, since there is no difference in the ratio for the formulation containing both the positively charged SA and CS. In terms of the manually extruded vesicles, the liposomes with 10% SA were slightly bigger compared to the other formulations. This effect was also found by Zschrönig et al. [33]. The liposomes with 25% CpCl showed the highest encapsulation efficiency though they were comparable in size to the other formulations. This may indicate that the cationic CpCl interacts with the carboxylic groups of the fluoresceine isothiocyanate and binds to some extent the fluorescent marker to the lipid bilayer.

Summarising the results of the liposome characterisation, the data show that it is possible to form liposomes containing surfactants in different ratios. However, the influence of temperature changes, dilution of the formulations and other in vivo conditions were not examined in this study.

3.2. Cytotoxicity studies

Most of the tested enhancers and all of the liposomal formulations with one exception showed no toxicity in the tested concentrations in both assays. Altogether, the assays led to similar results, whereas the LDH release after incubation with the CpCl solution in all concentrations seemed to be lower as the release after incubation with KRB (data not shown), it is likely that the free CpCl can inhibit the enzymatic reaction and finally the formation of the red tetrazolium dye. On the other hand, this effect could not be observed for the two liposomal formulations containing CpCl, probably because the amount of free CpCl is very low in case of the liposomal dispersions.

Cell viability for the formulations, which showed a toxic effect, is displayed in Figs. 5 and 6. In the Alamar Blue® assay, CpCl exhibited at a concentration of 1 mM already a strong cytotoxicity and TPGS 1000 reduced the cell viability at a concentration of 10 mM after 8-h incubation to a minimum, whereas no effect could be observed after 2-h incubation time. This was also confirmed by the LDH release. The liposomes with 25% CpCl had a strong toxicity in a total lipid concentration of 50 mM after 4-h incubation. However, by reducing the CpCl amount to 10%, any toxic effect in the tested concentration could be avoided, although the total CpCl amount of 50 mM liposomes with 10% CpCl is higher than for the 5 mM liposomes with 25% CpCl, indicating that the toxic effect is not just correlated with the total amount of the surfactant but also with its concentration in the liposomal membrane. The SA/CS liposomes led in all tested concentrations after 8 h to a slightly higher LDH release compared to the KRB control. This effect was not visible in the Alamar Blue® assay.

The cytotoxicity of surfactants is dependent on their charge, chemical characteristics and their CMC, but also on the cells and the assay used for determining the toxicity [34–36]. Often cationic compounds show a higher toxicity compared to uncharged or negatively charged molecules. As a matter of fact, CpCl was found to be the most toxic of the investigated enhancers. Burgalassi et al. found in their study with two different corneal cell lines a decrease in the cell viability of 50% after 1-h incubation with CpCl in a concentration of around 10 μM [37]. In a fibroblast cell line, the concentration of CpCl leading to 50% survival after 30 min was determined at around 0.19 mM with two different assays [38]. In a further study, the cytotoxicity of CpCl was comparable to that of the anionic surfactant sodium dodecyl sulphate concerning its haemolytical activity in erythrocytes and protein leaching of nasal mucosa [18]. The somehow lower toxicity found in the present study might be related to a lower susceptibility of Caco-2 cells to the cationic surfactant. The LD_{50} was determined at 200 mg/kg in rats and at

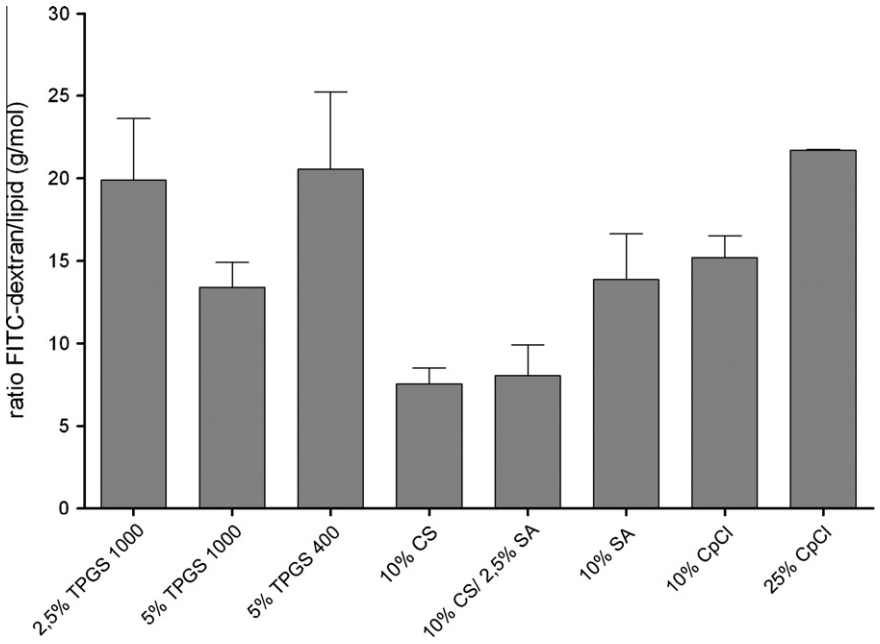

Fig. 4. Ratio of FITC-dextran to the total lipid amount in the liposomes after purification. The ratio is expressed in mass FITC-dextran/total lipid amount (g/mol) and given as means ± SEM with $n = 3$.

400 mg/kg in rabbits [39]. Moreover, there are mouthwashes with CpCl in the market and throat lozenges with a daily maximum intake of 10 mg (Dobendan Strepsils®), which are approved in Germany. Furthermore, by comparing the toxicity of the 10% CpCl liposomes with the free substance, a reduction in toxicity of around 50-fold can be observed.

In an animal study, rats were fed daily amounts of around 37.7 mg/kg SA for two years without causing any side effects [40]. Several studies investigating the cell toxicity of liposomes containing SA can be found [27,41,42]. In one study, a growth inhibition of 50% was shown by 10% SA liposomes in Caco-2 cells already at a total lipid concentration of 0.05 mM. However, in this study, the cells were incubated already one day after seeding for 6 days. In this early state, the cells are more susceptible to growth inhibition because they are not organised in a tight cell layer. Furthermore, the long incubation time differs from our methods explaining the different findings.

The higher toxicity of TPGS 1000 compared to TPGS 400 might be again related to the lower CMC of TPGS 1000. As described before, a lower CMC implies a higher cytotoxicity. Collnot et al. found in their study in Caco-2 cells in a LDH release assay for TPGS 400 no toxic effect up to a concentration of 10 mM and for the TPGS 1000, a toxicity starting at a concentration of 625 μm, which is in good conformity with our results [43]. In the literature, a LD_{50} of >7000 mg/kg rat is described, and a daily intake of 1000 mg/kg of TPGS 1000 and more than 1000 mg/kg of TPGS 400 is considered as safe, suggesting a very good safety profile [11,44].

In a previous study of our working group, we found no cytotoxicity for CS in a WST-1 transformation assay and just a slight LDH release in a concentration of 10 mM in Caco-2 cells confirming the results found in the present study [45]. Several in vivo studies in rodents and also humans suggest a good safety of CS [13,46–48]. Especially, the low N-demethylation in the sarcosine group going along with a marginal dehydroxylation indicates a low carcinogenic potential.

To a certain extent, a relation between the efficacy of an enhancer and its toxicity can be assumed and is also described in the literature [36,49]. However, for daily therapeutical use, toxic side effects have to be avoided without diminishing the desired enhancing effect. Depending on overall toxicity and mode of action of the enhancer type, the safe but effective concentration range can be reached more or less reliable. Especially, the two TPGS derivatives used in the present study are orally well tolerated, and the maximum daily intake suggested by the authorities is far above the amount necessary for the use as excipient and more related to their originally intended application for treatment of vitamin E deficiency. On the other hand, the use of the more toxic cationic enhancers in oral delivery systems has to be carefully considered until more well-founded data concerning a safe maximum daily intake are available. Still, the advantage of liposomal systems containing enhancing substances is the ability to reduce the amount of enhancer necessary to achieve the desired effect, as they are co-delivered with the drug to the mucosa.

3.3. Permeation studies

The permeation rate of FITC-dextran and the development of the TER and the C_{Cl} for the different formulations are shown in Fig. 7.

For most of the formulations, an increase in the TER over the time could be observed, whereas the capacitance was stable during the experiment. The vesicles with 25% CpCl reduced the resistance nearly down to zero after 3 h and increased the C_{Cl} around fivefold. Considering the cell toxicity of the CpCl liposomes found in the toxicity assays, the change in the TER is probably the result of a toxic effect of the vesicles due to an interaction of the cationic surfactant with the cell membrane causing a disintegration of the membrane structure and a detachment of the cells, visible in the strong increase in the capacitance. This was also confirmed by microscopical examination of the filter inserts after the experiment. The formulation with 10% SA, which showed no toxicity, had a similar influence on the TER and the C_{Cl}, but to a much lower extent. Since the surface area of the apical membrane is much greater than that of the basolateral membrane, the capacitance of the total cell layer is mostly dominated by the latter. The doubling of the capacitance can be explained by the opening of the tight

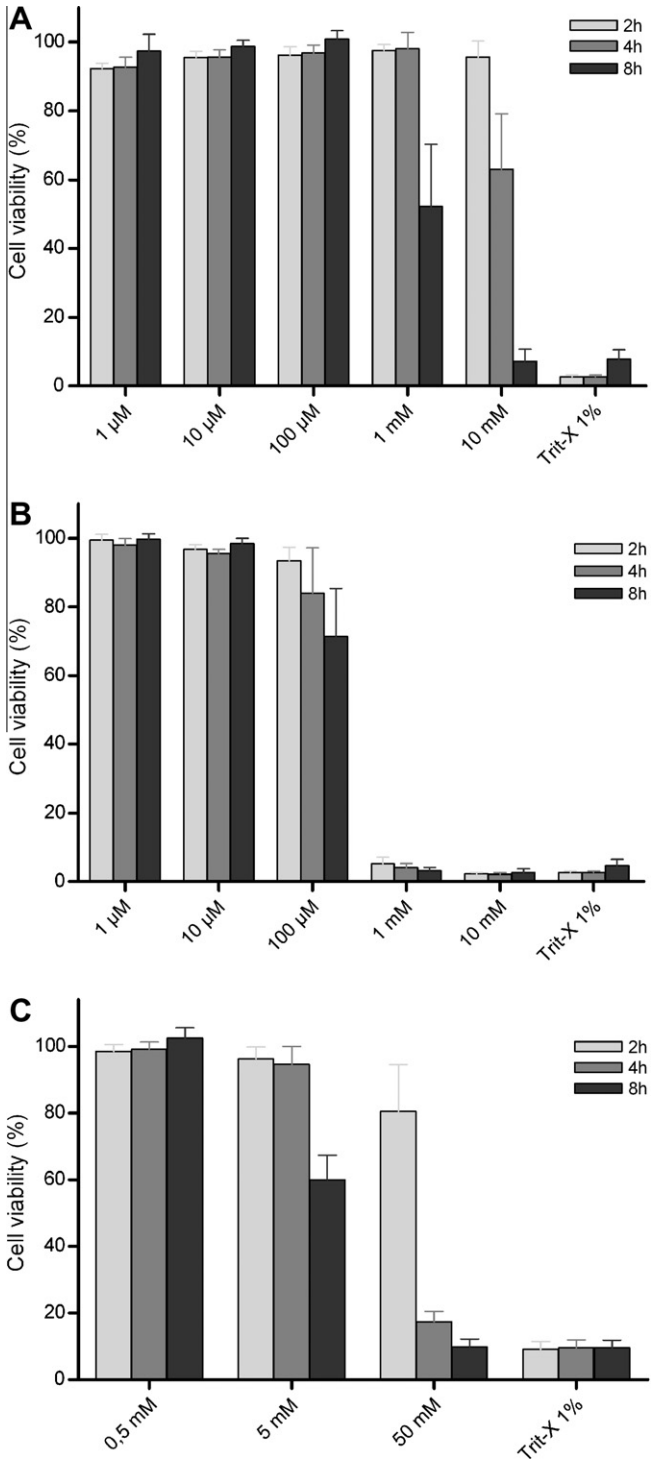

Fig. 5. Cell viability in the Alamar Blue® assay after 2-, 4- and 8-h incubation with (A) TPGS 1000 in KRB, (B) CpCl in KRB and (C) liposomes with 25% CpCl in KRB. Bars represent cell viability in % and are given as means ± SEM with $n = 3$.

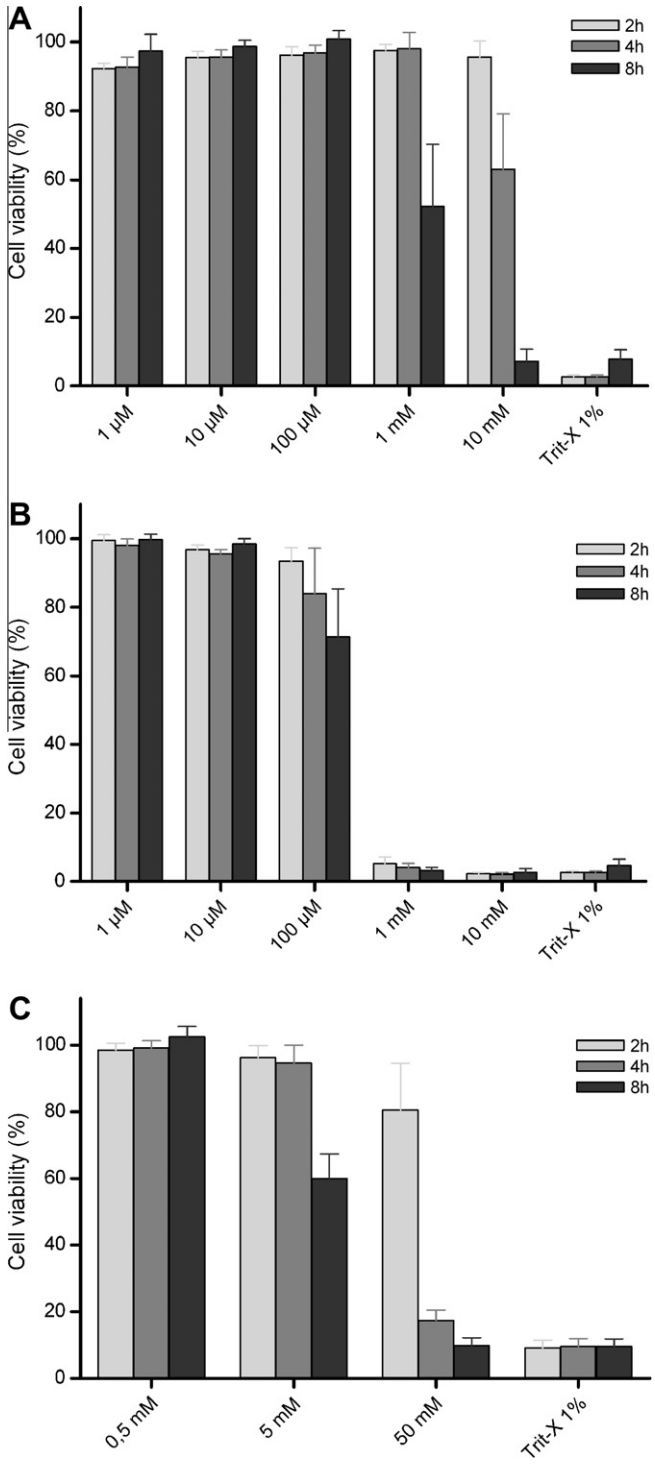

Fig. 6. Cell viability in the LDH assay after 2-, 4- and 8-h incubation with (A) TPGS 1000 in KRB, (B) liposomes with 10% CS and 2.5% SA in KRB and (C) liposomes with 25% CpCl in KRB. Bars represent cell viability in % and are given as means ± SEM with $n = 3$.

junctions and an enlargement of the membrane at the basolateral side [50].

The permeation rate of FITC-dextran was linear for all the formulations but for the two influencing the TER. For the 10% SA, two linear ranges, one from 0 h to 5 h and one from 6 h to 8 h, could be observed. The permeation for the liposomes with 25% CpCl was linear beginning after 2 h.

The apparent permeation coefficient of FITC-dextran as free control and encapsulated in the different liposomal formulations is shown in Fig. 8. Corresponding to the drop in the TER, the 25% CpCl could increase the permeation by 39.28 ± 2.10-fold. For the time between 6 and 8 h, the vesicles with 10% SA led to a similar enhancement, whereas the formulation had nearly no effect in the first 5 h, indicating a strong correlation between the resistance of the cell layer and the permeation of the marker [51,52]. Unfortunately, this good enhancement went along with a strong toxicity, indicated by the change in TER and C_{Cl}, making those formulation

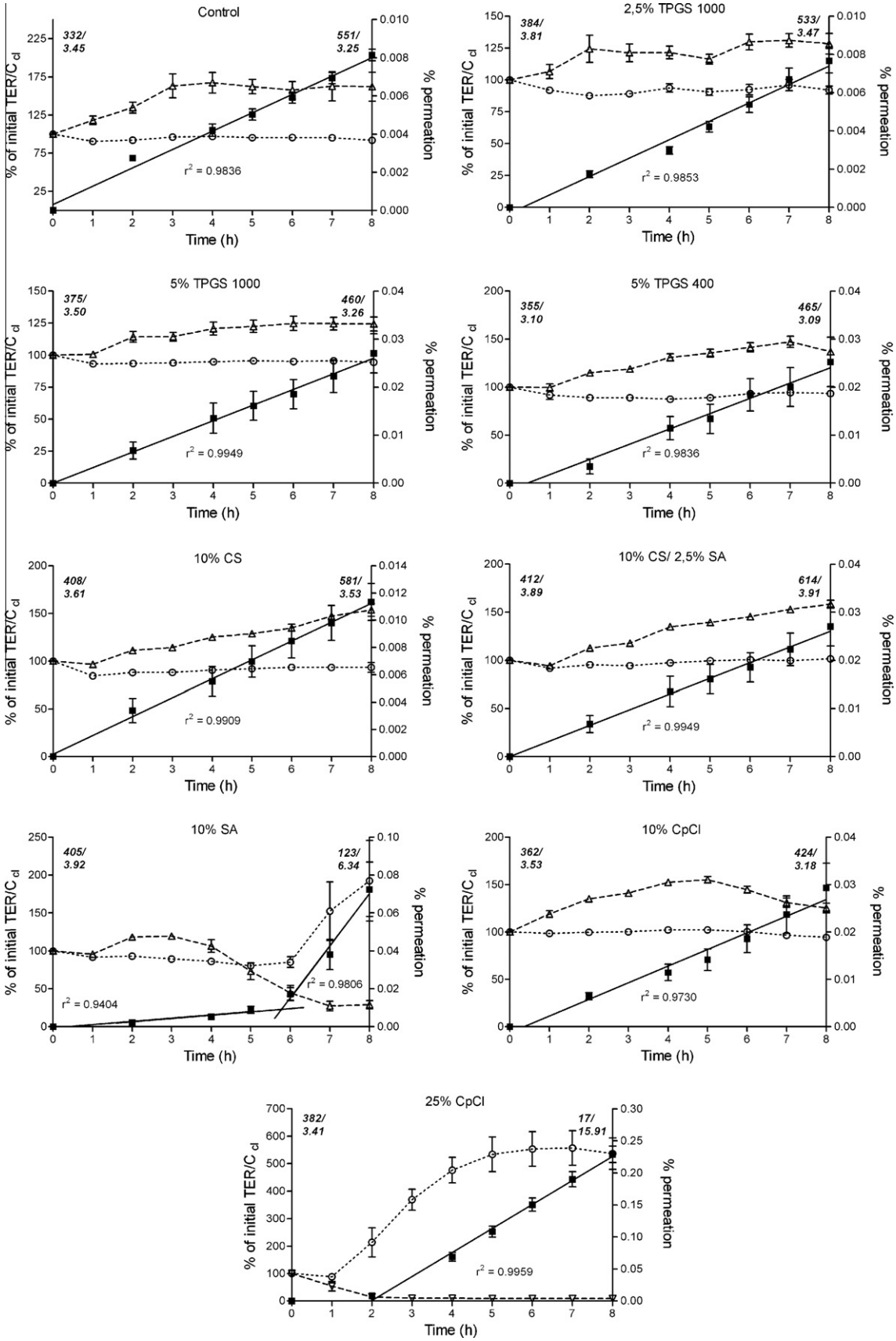

Fig. 7. The graph shows the development of TER (open triangles, left axis) and C_{Cl} (open circles, left axis) in % of the initial value and the permeation of FITC-dextran (closed squares, right axis) in % of the applied dose on the apical side. The values are given as means ± SEM with $n = 8$. Absolute values of TER in $\Omega\ cm^2$ (first value) and C_{Cl} in $\mu F/cm^2$ (second value) at the beginning of the experiment (left side) and the end (right side) are labelled in the graphs.

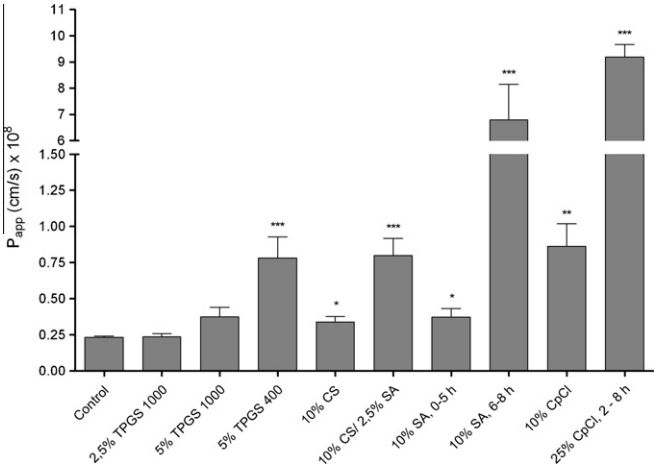

Fig. 8. P_{app} of FITC-dextran as free control and encapsulated in liposomes. The P_{app} is expressed in $(\mathrm{cm\ s^{-1}})\times 10^{8}$ and given as means ± SEM with $n = 8$. Control and treatment groups were compared by one-way Student's t-test with $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

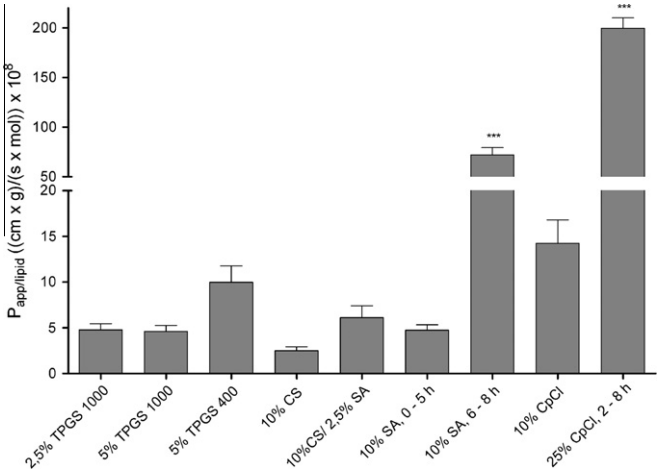

Fig. 9. Virtual $P_{app/lipid}$ of the total lipid amount. The $P_{app/lipid}$ is expressed in $(cm\ g)\times(\mathrm{s\ mol})^{-1}\times 10^{8}$ and given as means ± SEM with $n = 8$. Values were compared by one-way ANOVA test with $p^{*} < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

less suitable as drug delivery systems. However, the liposomes with 5% TPGS 400, with the mixture of CS and SA and the formulation with just 10% of CpCl could improve the permeation by 3.34 ± 0.62-fold, respectively by 3.41 ± 0.51 and by 3.69 ± 0.67-fold, with only small or no influence on TER or C_{Cl}. Balda et al. described that it is possible to influence the paracellular permeation of an aqueous marker without changing the electrical resistance of the cell layer [53]. A second explanation could be an endocytosis of the liposomes themselves. As Caco-2 cells lack caveolae, an uptake over clathrin-coated pits or clathrin- and caveolin-independent pathways is conceivable [54,55]. Theoretically, a fusion of the liposomes with the cell membrane could also contribute to the uptake and permeation of the macromolecule [56]. Furthermore, the direct uptake of the FITC-dextran into the cells could contribute to the permeation through the cell layer, but this event would be far more likely for the free marker compared to the liposomal encapsulated [4,57].

The bio-enhancing properties of TPGS are mostly referred to the inhibition of P-glycoprotein (P-gp) and the ability to act as a solubiliser of poorly water-soluble drugs [32,58,59]. However, the P-gp inhibition as the mechanism of enhancement is controversially discussed and might be just one among several modes of action [59]. Furthermore, FITC-dextran is known to be transported only passively, and the inhibition of P-gp should not influence the permeation of the dextran [20,21]. It is also discussed whether TPGS 1000 rigidises or fluidises cell membranes [59,60]. Swenson et al. mentioned that the apical membrane of enterocytes especially in the microvilli is rich in glycolipids and cholesterol leading to a high transition temperature of the lipid bilayer slightly over the physiological body temperature [34]. A change of a gel-state bilayer towards a liquid-crystalline-state bilayer or the other way around always leads to membrane defects during the transition. As no membrane transporters are involved in the permeation of dextran, the TPGS 400 liposomes probably act over an interference with the lipid bilayer of the cells leading to a facilitated uptake of the FITC-dextran or a higher fusion affinity of the liposomes with the cell membrane. The lack of efficacy of the TPGS 1000 in both concentrations might be related to the chain length of the PEG and a possible steric hinderance of the liposome–cell interaction.

In several reviews, the mechanism of the absorption enhancement by bile salts is described as the chelation of calcium ions in lower concentrations and the solubilisation of membrane lipids at higher bile salt concentrations, thus influencing both the paracellular and the transcellular route [34,35,61]. Interestingly, CS alone in liposomes could not change the permeation of FITC-dextran, whereas in combination with the cationic lipid SA it could. The ζ-potential of the 10% CS liposomes was negative (−3.93 mV ± 0.37), but already 2.5% SA changed the potential to a slightly positive value (3.42 mV ± 1.58). It seems likely that a negative surface charge of the liposomes makes a direct membrane interaction more difficult and that the enhancing effect of the formulation with CS and SA is not due to the SA itself but due to the positive surface charge of the vesicles allowing the CS to interact more efficiently with the Caco-2 cells. SA used alone could only enhance the permeation, when the TER was reduced, which could not be observed with this formulation. As mentioned above, an increase in the paracellular transport by an opening of tight junctions is not always correlated with a change in TER. This means that both pathways of permeation enhancement are theoretically possible for this formulation.

Also the liposomes with 10% CpCl did not change the TER significantly but could increase the permeation of the marker. CpCl showed in previous studies good enhancing effects on both small and large molecules, but the detailed mechanism of enhancement is not clarified yet [18,62]. Due to the positive charge of the liposomes, an interaction with cell membranes is facilitated. Again, a change in the properties of the membrane bilayer of the Caco-2 cells as mode of action seems likely.

To include the encapsulation efficiency of the vesicles into the analysis of their performance, a virtual permeation coefficient for the lipids was calculated (Fig. 9). Whereas the liposomes containing the mixture of SA and CS were superior to the formulations with just CS and TPGS 1000 concerning the P_{app} of FITC-dextran, their advantage was diminished regarding the $P_{app/lipid}$ due to their poor encapsulation efficiency. On the other hand, the liposomes with CpCl, which could encapsulate the FITC-dextran very efficiently, needed less lipid to deliver the same amount of marker. However, this effect is linked to the used marker and could be different for other encapsulated substances making a prediction of a possible superiority of the vesicles with CpCl in an industrial scale production difficult.

4. Conclusions

In this study, several bio-enhancers were used in liposomes to improve the permeation of dextran through a Caco-2 cell layer. It

was possible to form liposomes in good quality with all the tested enhancers. The cytotoxicity of the surfactants differed with their properties like charge and CMC but was always reduced in a liposomal formulation. In the Transwell® model, the formulations with 5% TPGS 400, 10% CS and 2,5% SA and 10% CpCl had an enhancing effect without influencing the TER or the C_{CI} suggesting a good safety profile. However, an increase in the P_{app} around 3- to 4-fold would be hardly sufficient for an oral application of most of BCS Class III drugs, which often have a bioavailability below 1%.

The Caco-2 model is the most commonly used in vitro model for oral delivery studies but it has some serious disadvantages like the lack of caveolae or the missing of intestinal fluids and a mucus layer [63,64]. In addition, the liposomes should not just enhance the permeation of proteins but also protect them from the harsh conditions in the intestine. It can be hypothesised that the liposomal formulations would give a better performance in comparison with a free protein in an in vivo model, where the stabilising effect and the interaction with the mucus layer are more important compared to the cell model. During this study, it was possible to identify some promising candidates for further in vivo experiments for the oral delivery of hydrophilic macromolecules.

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