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Co-existing colloidal phases in artificial intestinal fluids assessed by AF4/MALLS and DLS: A systematic study into cholate & (lyso-) phospholipid blends, incorporating Celecoxib as a model drug.

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Abstract

Colloidal phases (self-assemblies) in aqueous dispersions of selected binary bile salt/phospholipid blends were studied utilizing the combined analytical approach of asymmetrical flow field-flow fractionation (AF4) and multi-angle laser light scattering (MALLS) in order to resolve the co-existence of different colloidal assemblies. The binary blends were prepared by freeze-drying from tert-butanol / water co-solvent solutions. The blends contained one of two bile salts (sodium taurocholate (TC) or sodium glycodeoxycholate (GDX)) and a mono- or di-acyl phospholipid (lyso-phosphatidylcholine (L-PC) and phosphatidylcholine (PC), respectively). Bile salt and phospholipid (PL) concentrations and their respective ratios were varied systematically within the physiological range found in human intestinal fluids. Furthermore, the BCS class II drug Celecoxib was incorporated in selected blends to assess its potential impact on colloidal phases. To further investigate the smallest self-assemblies observed in AF4/MALLS analysis, dispersions of TC and GDX, respectively, were prepared and analyzed by dynamic light scattering (DLS).

AF4/MALLS analysis revealed that binary bile-salt/phospholipid blends form three distinct particle fractions, when the concentration of bile-salt was sufficiently high (≥ 3.5 mM). Those fractions were assumed to be very small pure bile-salt dimeric / oligomeric self-assemblies (Ø≈2-3 nm), mid-sized mixed micelles (Ø≈10 - 50 nm) and large liposomes/aggregates (Ø≈150 – 280 nm). If present, Celecoxib was found solubilized within the structures, but at the lowest TC concentration triggered the formation of an additional (vesicular) phase.

Abbreviations

AF4, asymmetrical flow field-flow fractionation; CF, crossflow; CPP, critical packing parameter; CXB, celecoxib; DLS, dynamic light scattering; dRI, differential refractive index; FaSSIF, fasted state simulated fluid; FeSSIF, fed state simulated intestinal fluid; GDX, sodium glycodeoxycholate; L-PC, lyso-sn-glycero-3-phosphocholine; HIF, human intestinal fluid; MALLS, multi-angle laser light scattering; NTU, nephelometry turbidity unit; PC, sn-glycero-3-phosphocholine; PBS, phosphate buffered saline; PDI, polydispersity index; PES, polyether sulfone; PL, phospholipid; Rg, radius of gyration; SIF, simulated intestinal fluid; t-BuOH, tertiary butanol; TC, Na taurocholate.
1 Introduction

Phospholipids (PLs) and bile-salts are amphiphilic biosurfactants that play a key role in solubilizing dietary fats within colloidal structures, primarily mixed micelles, which facilitate their digestion and absorption. Under physiological conditions, bile is emptied from the gall bladder into the small intestine where the bile-salt rich mixed micelles swell through solubilization of dietary lipids and their digestion products. The phase behavior of these physiologic colloidal systems may be complex due to the various lipidic constituents present in the small intestine. When looking at a simple, however physiologically relevant, binary system consisting of bilayer-forming PLs and micelle-forming bile salts, a phase transition is expected to occur between a lamellar and a mixed micellar phase depending on the molar ratio between PLs and bile-salts. Additionally, in excess of bile salts, the mixed micellar phase may co-exist with a phase consisting of small, simple bile salt micelles (for a comprehensive review see (Carey, 1985)). Similarly to dietary constituents, lipophilic drug molecules interact with lipidic colloidal phases in the intestine, which influences the solubilization and potentially absorption of the drug molecule. In cases where drug absorption varies with the prandial state, the variation in absorption is considered to correlate with the extent of interaction between the drug compound and the lipidic colloidal phases present in the intestine. To this end biomimetic in-vitro media for dissolution studies have been developed where the primary focus is on the solubilizing capacity of the media for a variety of poorly soluble drugs (Perrier et al., 2018) whereas the colloidal characteristics of the media seem of minor importance. The phase behavior of ternary model systems consisting of bile salt, PL, and aqueous medium has been extensively studied since the 1960s e.g. by Small et al. (Small et al., 1966) using e.g. polarized light microscopy as well as a variety of bulk techniques, e.g. X-ray diffraction, dynamic light scattering (DLS) and nephelometry (reviewed in [Carey & Small, 1972; Carey, 1985]). These techniques allowed the authors to develop an equilibrium phase diagram corresponding to the aqueous lipid compositions found in the upper small intestine of humans. Pouton and coworkers supplemented the phase diagram with lipids arising from lipid digestion (Birru et al., 2014). Although these studies indicate the likely phase behavior of bile in the intestine, their main limitation is that bulk techniques typically cannot fully resolve co-existing colloidal phases. In a previous study (Elvang et al., 2017) on fasted state simulated intestinal fluid (FaSSIF; consisting of a fixed amount of sodium taurocholate and lecithin), we have demonstrated that by combined asymmetrical flow field-flow fractionation (AF4) and multi-angle laser light scattering (MALLS) a deeper insight into co-existing lipid phases can be achieved. A subsequent AF4/MALLS study (Elvang et al., 2016) into aspirated human intestinal fluids (HIFs) revealed a broader diversity of co-existing colloidal phases as compared to the colloidal phases present in the simulated intestinal fluids (SIFs) FaSSIF and fed state simulated intestinal fluid (FeSSIF), likely due to the more complex bile-salt and lipid composition of HIF. Recently, Müllertz and co-workers and Riethorst and coworkers have used the image-based techniques cryogenic transmission electron microscopy (cryo-TEM) and a combination of cryo-TEM, negative stain TEM and cryogenic...
scanning electron microscopy (SEM), respectively, to elucidate the colloidal characteristics of HIFs (Müllertz et al., 2015) (Riethorst et al., 2016a). Also, Riethorst found that FeHIF contained a broad variety of colloidal structures, which were not present in FeSSIF. Despite the excellent resolution of these image-based techniques, the comparatively elaborate data analysis and the challenge of detecting very small and very large particles, represent major drawbacks of these techniques. Therefore, we have designed and carried out a systematic study into selected binary systems consisting of PL and bile-salt in physiologically relevant concentrations (referred to as artificial intestinal fluids) in the presence and absence of a model drug compound using AF4/MALLS to elucidate the capability of this technique to follow the phase behavior of this simple, but nevertheless relevant system. A better understanding of how these simple systems behave when analyzed by AF4/MALLS may aid the interpretation of AF4/MALLS data of complex HIF. Thereby, an insight into the interaction between drug compounds and colloidal phases present in the small intestine may be gained. For the binary systems, sodium taurocholate (TC) was chosen as model bile salt, because of its relative abundance in human bile as well as its widespread use in SIFs (Fuchs and Dressman, 2014). Also, sodium glycodeoxycholate (GDX) was chosen as model bile salt because in a nephelometric study carried out by Birru and coworkers, the PL-solubilizing behavior of GDX highly resembled the PL-solubilizing behavior of a mix of the five most abundant bile salts found in the small intestine of human (Birru et al., 2014). As model PLs, a phosphatidylcholine rich lecithin fraction (PC) and a lyso-phosphatidylcholine rich lecithin fraction (L-PC), representing PCs post-digestion counterpart, were chosen. Finally, celecoxib (CXB) was included as a lipophilic BCS class II model drug compound.

2 Materials and methods

2.1 Chemicals

Lipoid E80 containing 83.2% (m/m) sn-glycero-3-phosphocholine and 8.6% (m/m) sn-glycero-3-phosphatidylethanolamine obtained from eggs, subsequently designated as PC, and Lipoid SLPC80 containing at least 80% (m/m) lyso-sn-glycero-3-phosphocholine obtained from soybeans, subsequently designated as L-PC, were kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Celecoxib (CXB) was purchased from abcr GmbH (Karlsruhe, Germany). Sodium-glycodeoxycholate (≥97 % m/m), monobasic sodium phosphate dihydrate (≥98 % m/m), sodium azide (≥99.5 % m/m), sodium chloride (≥99 % m/m), sodium hydroxide (≥98 % m/m) and sodium taurocholate (≥97 % m/m) were purchased from Sigma-Aldrich Denmark ApS (Brøndby, Denmark).

Highly purified water was obtained from a Milli-Q® integral water purification system (Milli-Q® Advantage A10®; MerckMillipore, Merck A/S, Hellerup, Denmark).
2.2 Preparation of artificial intestinal fluids

The binary bile salt-PL blends consisting of a fixed amount of either PC or L-PC, varied amounts of TC or GDX and in the presence or absence of CXB were prepared by freeze-drying from a tertiary butanol (t-BuOH) water co-solvent system. See Table 1 below for specified blend compositions.

For this, stock solutions of PC (6 – 13 mg mL⁻¹), L-PC (11 - 101 mg mL⁻¹) and CXB (3 – 11.5 mg mL⁻¹) were prepared in a 4:1 (w/w) t-BuOH:water co-solvent system. Furthermore, stock solutions of TC (sodium taurocholate monohydrate, 10.8 mg mL⁻¹) and GDX (sodium glycodeoxycholate 10 – 10.5 mg mL⁻¹) were prepared in a 1:1 (w/w) t-BuOH:water co-solvent system. Previously, the thermodynamic solubility of CXB (15.0 ± 3.5 mg mL⁻¹) and PC (>500 mg mL⁻¹) in t-BuOH was determined by Fong and co-workers (Fong et al., 2016). By optical evaluation, no solid material was observed in the stock solutions.

Aliquots of the stock solutions corresponding to the desired compositions were transferred to vials and 80:20 (w:w) t-BuOH:water was added to a final volume of 5 mL, obtaining a clear solution.

The blends were frozen overnight at -80°C and placed in the pre-cooled (-60 °C) Christ Gamma 2-16 LSC freeze dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany). Freeze-drying was conducted as previously described (Fong et al., 2015).

After completion of the freeze-drying program, the yield was determined to ensure complete desiccation, the vials were closed, sealed with Parafilm M® (Bemic Company Inc., Oshkosh, USA) and stored in a desiccator over CaCO₃ beads at RT until further use (not longer than 6 weeks). The blends were dispersed in phosphate buffered saline (PBS; see below) to yield 0.75 mM of (lyso-)PL, 1 to 20 mM of bile salt and 0.8 mM of drug (if indicated).

2.3 Preparation of pure bile-salt dispersions

Pure bile-salt dispersions of different concentrations were prepared by dilution from a 20 mM TC and a 20 mM GDX stock dispersion, respectively. The dispersions were allowed ~24 hours of equilibration time, at room temperature, prior to DLS measurements. As dispersing solvent, PBS was used, as specified in section 2.4.

2.4 Asymmetrical flow field-flow fractionation (AF4) / multi-angle laser light scattering (MALLS)

The AF4/MALLS instrumental setup was as described in a previous study (Elvang et al., 2016) with slight modifications. Here, the frit-inlet AF4 channel had been specifically applied to preserve the supramolecular assemblies, in accordance with literature (Elvang et al., 2017). The AF4 accumulation wall (ultra-filtration membrane) was always polyether sulfone (PES) with a molecular weight cut-off (MWCO) of 10 kDa. Static light scattering, UV-extinction signal (λ 205 nm) and differential refractive index (dRI) were recorded simultaneously during AF4 fractionation. As carrier liquid, 0.2% (m/m) sodium azide-preserved PBS (pH 6.5 ± 0.05) was used, which had the following composition: sodium chloride (50 mM), monobasic sodium phosphate dihydrate (15 mM), sodium
hydroxide (4 mM), yielding an osmolality of 135 ± 10 mOsm kg⁻¹. As stated earlier, the same PBS was used to disperse the freeze-dried samples prior to analysis. The temperature in the autosampler was set to 37 °C.

Briefly, AF4 separates particles by their hydrodynamic sizes. This is achieved by a laminar liquid flow, which transports particles along the channel, combined with an adjustable perpendicular crossflow (CF). Smaller particles oppose the downward CF force more efficiently than larger particles. I.e. due to the differences in Brownian motions of particles of different sizes, smaller particles equilibrate higher up in the channel where the liquid flow is faster (due to the parabolic flow-profile) as compared to larger particles. Excellent in-depth descriptions of the instrumentations and theory of AF4/MALLS are available in the literature (Wyatt, 1998) (Fraunhofer and Winter, 2004) (Wagner et al., 2014). A comprehensive description of the fractionation of PL vesicles is given in literature (Hupfeld et al., 2009b) (Hupfeld et al., 2009a) (Hupfeld et al., 2010).

Upon dispersion of a given freeze-dried blend, the blend was initially vortex-shaken for a few minutes, and afterwards allowed to equilibrate overnight. Prior to injection into the AF4 channel, the dispersions were syringe-filtrated (0.45 µm pore-size cellulose membrane, minisart®, Sartorius, Goettingen, Germany). As a preliminary experiment, consecutive injections of the same dispersions were carried out to assess potential colloidal changes over time. All blend variations were prepared in triplicate (for clarity reasons, not all replicates analyzed are shown in the fractograms presented in the result section).

### 2.5 Dynamic light scattering

DLS measurements were performed on a DelsaMax (Beckman Coulter, Denmark ApS c/o, Copenhagen, Denmark) at 25 °C in backscattering mode. The average particle size (\(Z_{av}\)) and polydispersity index (PDI) values were obtained from the instrument’s cumulant analysis. Each sample was measured multiple times with 10 acquisitions for each measurement. For a given sample, the \(Z_{av}\), PDI-values and the associated standard deviations (SD) were calculated from the last four consecutive acquisitions. All sizes in this manuscript are given as diameters.

### 2.6 1H-NMR

The NMR experiments of samples dissolved in PBS (pH 6.5) with 10 % D₂O were carried out on an Agilent dd600 spectrometer operating at a proton frequency of 599.622 MHz. Line positions are referenced to TMS via the deuterium resonance of the solvent.

Specifically, the measured samples were a dispersion of 0.75 mM PC, 5 mM TC and 0.8 mM CXB, and the same sample diluted x 6 with PBS. The diluted sample was measured immediately when prepared and then consecutively over time (up until 18 hours).
3 Results

3.1 AF4/MALLS

By dispersing the freeze-dried blends in PBS, which yielded opalescent to milky dispersions, readily injectable samples for AF4/MALLS analysis were obtained. All results from AF4/MALLS analysis (Figure 1 – 9) are presented as the light scattering signal at a 90° detection angle as a function of time. Additionally, the crossflow (CF) profile used is depicted with a red, dashed line. In Table 3 MALLS size-data of AF4-fractionationated GDX dispersions are given. MALLS size-analysis of TC dispersions yielded no valid results. The preliminary AF4/MALLS studies showed that replicates of all dispersions included here were highly reproducible with respect to the resulting fractograms. Also, in a time-resolved manner, the fractograms of all dispersions were reproducible, except for the 1 mM [GDX PC] dispersion, where the time-resolved analysis revealed changes in the co-existing colloidal states present (See Figure 1). Figure 1 shows two distinct phases initially, ‘mixed micelles’ and ‘liposomes’, as indicated by the preferred fit functions (fit not shown). During the first three hours, the AUC of the ‘liposome’ fraction decreased whereas the AUC of the ‘mixed micelle’ fraction increased. After 18 hours, a measurement of the same sample revealed that the two colloidal phases, which were initially observed, had fused into one fraction leaving only a little shoulder of the ‘liposome’ fraction. This fused fraction likely was comprised of mixed micelles with a retention time in between the two phases that had been detected previously.

NMR spectroscopy is a strong technique for the determination of molecular composition of supramolecular assemblies, and thus ultrastructural changes in a system. Diffusion coefficients of such assemblies can be obtained as well, by employing self-diffusion NMR technique, which provides a means to create detailed phase-diagrams, as recently shown by (Vogtherr et al., 2015). In this study, we carried out preliminary H-NMR experiments where we diluted selected blends to the same extent as in the AF4-channel, which showed no changes in 1H-NMR spectra over a period of 18 hours (data not shown). This study was performed specifically to assess the stability of colloidal structures over a timeframe covering the data-collecting time for both AF4/MALLS and DLS measurements. Finding that the integrity of the colloidal species is preserved, agrees with our previous observations, where we compared particle sizes (DLS, MALLS) of both FaSSIF and FaSSIF-V2 in a diluted (after AF4 fractionation) and undiluted state (Elvang et al., 2017), and found them very similar. These results thus alleviate concerns on potential dilution-effects in the AF4 channel.

3.1.1 Artificial intestinal media containing sodium taurocholate

Figure 2 shows the fractograms of dispersions containing 0.75 mM PC (fixed amount) and varying concentrations of TC (1 - 20 mM). The CF profile, was the same in all fractionations of blends containing TC, except for the blend with 1 and 10 mM TC (depicted with the magenta and yellow graph, respectively). For these blends, an extended method was used causing a shift of the last peak (“release peak”) to a later retention time. The CF profile of this extended method is depicted
with a green, dashed line. The extended method was part of the method-development process, where attempts were made to appropriately fractionate the largest particles, eluting after the crossflow goes to zero, within the defined cross-flow gradient. As this turned out to only delay the co-elution of the largest particles, a shorter method was selected, in the interest of saving time. In Figure 2, two distinct particle fractions can be distinguished, assigned ‘TC micelles’ and ‘mixed micelles’. Furthermore, a third population, which elutes when the CF drops close to zero (0.02 mL min\(^{-1}\)), is observed. At this low CF, any particles remaining in the channel elute rapidly, i.e. this is not a third properly separated fraction but merely indicates the presence of particles larger than the earlier, narrowly distributed fraction, here depicted as ‘liposomes/aggregates’. The graph-insert in the top-right corner of Figure 2 is a zoom-in, where an ‘elution peak’ (0 – 0.3 min.) and the first real peak ‘TC micelles’ (0.7 – 2.0 min.) can be distinguished. The ‘TC micelles’ peak elutes right after the injection peak at the highest CF (2 mL min\(^{-1}\)). For this peak, the light scattering signal is only detected for the two highest TC concentrations (10 & 20 mM). A distinct UV-signal for this peak is seen at the two highest and for the 3.5 mM TC-blend (data not shown). Both, the onset and the AUC of the second ‘mixed micelles’-peak vary slightly between the different samples.

Figure 3 shows the fractograms of similar dispersions (i.e. containing 0.75 mM PC and TC at varied concentrations) in the presence of 0.80 mM CXB. In the presence of CXB, the light scattering intensities and the elution times of the ‘TC-micelles’ and ‘mixed micelles’ were similar to the corresponding blends without CXB (Figure 2, note the different scaling) for all dispersions, except for the dispersion containing the lowest amount of TC (1 mM). Here, incorporation of CXB gave rise to a change of the fractogram: a large, splitted peak is seen. Concurrently with the emergence of these larger assemblies, the peaks of the smaller assemblies (‘TC-micelles’ and ‘mixed micelles’), which are observed at the higher TC concentrations, are absent.

Figure 4 shows the fractograms of dispersions containing 0.75 mM L-PC and TC at varied concentrations. Similar to the sample series containing PC, which is shown in Figure 2, two distinct particle fractions can be distinguished (‘TC-micelles’ and ‘mixed micelles’) in Figure 4. Also, the ‘TC micelle’ peak elutes early at the highest CF and the light scattering signal is only detected for dispersions with higher TC concentrations (5, 10 & 20 mM). Again, slight variations in both onset and AUC of the ‘mixed-micelles’ peak are observed. When comparing the ‘mixed micelles’ containing PC (Figure 2) to the ‘mixed micelles’ containing L-PC (Figure 4), the L-PC containing ‘mixed micelles’ elute at earlier retention times at all TC concentrations.

Analogous to Figure 3, Figure 5 shows the fractograms of dispersions containing 0.75 mM L-PC (fixed amount) and TC at varied concentrations in the presence of 0.80 mM Celecoxib. The fractograms in the presence of CXB resemble those in Figure 4, where CXB is not present. However, a substantial difference can be observed for the ‘TC micelles’ fraction at 20 mM TC compared to the corresponding dispersion. Here, the light scattering intensity is considerably
higher (approximately 8 times). Also, the light scattering intensity of the mixed micelles fraction is doubled as compared to the corresponding dispersion.

### 3.1.2 Artificial intestinal media containing sodium glycodeoxycholate

Figure 6 shows the fractograms of dispersions containing 0.75 mM PC (fixed amount) and varied concentrations of GDX (3.5 – 20 mM). Here, three particle-fractions were distinguished assigned ‘GDX micelles’, ‘mixed micelles’ and ‘liposomes/aggregates’, in contrast to the bimodal distribution observed for the dispersion containing 1 mM GDX, which is presented in section 3.1. The first fraction, ‘GDX micelles’, is more prominent at higher bile-salt concentrations, the ‘mixed micelle’ fraction is present in all dispersions whereas the ‘liposome/aggregates’ fraction is only slightly observable in the dispersion containing 3.5 mM GDX compared to the dispersions containing 10 and 20 mM GDX.

Figure 7 shows the fractograms of dispersions containing 0.75 mM L-PC (fixed amount) and varied concentrations of GDX (1 – 20 mM). Similar to the corresponding PC dispersions (Figure 6), three fractions can be distinguished here. The ‘mixed micelles’ fractions elute earlier as compared to the ‘mixed-micelles’ in the corresponding dispersions containing PC. The ‘GDX micelles’ peak, which is observable at all GDX concentrations for the corresponding PC dispersions, is first detected at GDX concentrations of 10 mM. The ‘liposome/aggregates’ fraction is observable at all GDX concentrations (down to 1 mM GDX).

When comparing Figure 8 with Figure 6, showing fractograms of corresponding PC containing dispersions in the presence (Figure 8) and absence of 0.8 mM CXB (Figure 6), the ‘GDX micelles’ and ‘mixed micelles’ fractions do not differ substantially. However, the ‘liposomes/aggregates’ fractions showed drastic changes in the scattering signal for all dispersions. The most pronounced increase in scattering intensity of the ‘liposome/aggregates’ fraction was observed for the dispersion containing 1 mM GDX (orange).

Analogous to Figure 8, Figure 9 shows the fractograms of dispersions containing L-PC (fixed amount), GDX and 0.8 mM CXB. Comparing Figure 9 to Figure 7, which shows the corresponding L-PC-GDX dispersions in the absence of CXB, similar changes regarding the colloidal diversity as seen when incorporating CXB into the corresponding PC-GDX dispersions are revealed, i.e. the most pronounced difference is the substantially increased light scattering signal of the ‘liposomes/aggregates’ fraction.

### 3.2 DLS

Table 2 gives the DLS results, in terms of $Z_{av}$ values and associated PDI's, for pure bile salt dispersions in PBS at different concentrations. For all TC dispersions, no valid results were obtained. For the GDX dispersions, a $Z_{av}$ value of 3-4 nm was obtained for GDX concentrations down to 10 mM. However, below 10 mM GDX valid measurements could not be obtained.
In Table 3 size data of AF4-fractionated GDX dispersions obtained from off-line DLS analysis are given and compared to MALLS size data. TC dispersions are not included here, since valid size data from DLS analysis were scarce.

4 Discussion

Artificial intestinal media in the form of binary PL-bile salt blends, which contained PC or L-PC and TC or GDX in physiologically relevant concentrations, with and without the BCS class II model compound CXB, were prepared by freeze-drying. Dispersing the lyophilizates in PBS provided opalescent to milky dispersions readily injectable for AF4/MALLS analysis. By varying the ratio between the selected bile salts and PLs, we aimed at mapping potential colloidal changes over a large biorelevant range of artificial intestinal fluids. To see potential influences of poorly soluble, lipophilic drug compounds on the colloidal phases present, we further compared dispersions with and without the BCS class II model drug compound, CXB, incorporated. AF4/MALLS enabled us to separate distinct colloidal fractions according to their hydrodynamic sizes and derive particle sizes via on-line MALLS-analysis. DLS was applied to obtain size-data of particle-fractions collected from AF4, primarily where size-analysis by MALLS was not feasible and to compare with MALLS size data. For the wavelength applied here, the theoretical lower limit for MALLS specifications is approximately 30 nm in particle diameter.

Generally, we were able to follow the incorporation of PC and L-PC into mixed micelles with TC or GDX. Additionally, at certain bile salt to PL ratios, we were able to detect colloidal phases, which co-existed with the mixed micelles, i.e. small bile salt rich micelles (‘TC micelles’ and ‘GDX micelles’) and larger liposomes/aggregates.

When discussing co-existing colloidal phases and particle sizes, the dynamic nature of these physiologically relevant systems should be considered. The colloidal species as well as their sizes can be affected by various experimental conditions. When considering the method presented here, AF4/MALLS, the colloidal phases may undergo changes during fractionation, especially due to the up-concentration during the ‘focus-mode’, which is inevitable for conventional AF4-channels, or the dilution in the AF4-channel. To avoid structural changes, a ‘frit-inlet’ AF4-channel was selected here. The frit-inlet channel replaces the ‘focus-mode’ with a ‘hydrodynamic relaxation’ step (Moon et al., 1997). However, the dilution of the dispersions during fractionation cannot be avoided. When considering the off-line DLS analysis of the dilute fractions obtained from AF4-fractionation, changes in the colloidal phases may occur over time after fractionation. Hence, DLS measurements were carried out immediately after fractionation, typically resulting in < 30 min. of time in a diluted state.

As these experimental conditions potentially influence the integrity of colloidal structures, considerations on the morphology of pure bile-salt micelles and mixed-micelles proposed by Small and Mazer (Carey and Small, 1972) (Mazer et al., 1980), should be taken into account here. Carey
and Small proposed the formation of very small ‘primary aggregates’ for pure bile-salt dispersions above their CMC, with aggregation numbers varying from 2-20 (Carey and Small, 1972). This was based on the conformation of bile-salt molecules. The conformation of all bile salts is similar with regards to how the hydroxy-groups are situated in space relative to the rest of the molecule. Due to the ‘kinked’ spatial structure, that is cholesterol, the hydroxyl-groups are located close to each other on one of the planar sides. This renders the bile salt molecules hydrophilic at the concave side and hydrophobic at the convex side. The hydrophobic interactions between the convex sides, resulting in the formation of the micelle, were confirmed by H-NMR analysis. With certain bile salts, secondary micellar aggregates may arise via hydrogen-bonds. Due to these structural properties, bile salt micelles differ in their ultrastructure as compared to micelles formed from other amphiphilic compounds (i.e. surfactants) with a hydrophobic tail and hydrophilic head-group, which typically form spherical micelles. Mixed micelles comprising e.g. PLs and a given bile-salt, achieve the thermodynamically most favorable arrangement by maintaining the PLs in a bilayered formation that is surrounded by bile-salt molecules, whose hydrophobic convex side is orientated towards the PLs’ aliphatic hydrocarbon chains. With increasing size, the ultrastructure of mixed PL-bile salt micelles is thus becoming more disc-shaped. Based on experimental data, the structural model of mixed micelles proposed by Small was later further refined (Mazer et al., 1980). This ‘mixed-disc’ model proposed that inverse bile-salt dimers also are located within the lipid bilayer of the disc-shaped mixed micelles. The ‘mixed-disc’ model was derived from experiments following the change in hydrodynamic radius ($R_h$) with increasing ratios of phosphatidylcholine to bile salt (PC:BS) at fixed temperatures and NaCl concentrations. Theoretically, the disc-radius was suggested to be proportional to the total amount of PC bilayer in solution divided by the total amount of bile salt available to coat the perimeter of the micelles (eq. 1).

$$r = \frac{2\rho}{\sigma} \frac{C_L}{C_{BS} - IMC}$$  \hspace{1cm} (eq. 1)

Where $r$ is micelle radius, $\rho$ is number of bile-salt molecules per unit length of disc perimeter, $\sigma$ is number of PC molecules per unit area in the micelle bilayer, $C_L$ is molar concentration of PC, $C_{BS}$ is molar concentration of bile-salt and IMC represents the inter-micellar bile salt concentration.

A disproportionally large increase in micelle size was found when the amount of PC relative to bile salt was increased. The immediate explanation, when considering eq. 1, is that the bile salt available to solubilize the PC is much less than $C_{BS} - IMC$, inferring that the bile salt molecules must also be dispersed within the bilayers of the disc-micelle (see Figure 10 for illustrations of the structures discussed above). Theoretically, the bile salts could also exist as pure bile salt micelles, however it was shown that these were very unlikely to exist at the given PC:BS-ratio.
Also, the same study showed that the average micelle size increased when the total lipid concentration was lowered by dilution and that thereby the phase-boundary\(^4\) between pure bile-salt micelles and mixed micelles shifted to lower PL:BS ratios. However, the increase in micelle size upon dilution was neglectable when dilution was conducted with a solvent containing a bile salt concentration equaling the IMC. This suggested, that mixed-micelle growth behavior is governed by the equilibrium between the bile salt monomers in solution and the bile salts within the mixed-micelles. Since the IMC must be re-established when diluting with e.g. pure water, bile salts have to diffuse out of the mixed micelles. This effectively increases the PL:BS ratio, which has been shown to increase the average micelle size. However, a timeframe for these phenomena to take place is not provided.

### 4.1 Artificial intestinal media containing sodium taurocholate

In Figure 2 and Figure 4, two particle fractions could be distinguished. The first fraction (eluting at CF 2 mL min\(^{-1}\)) likely represents very small, pure TC micelles or very small mixed TC-PC/L-PC micelles containing high amounts of TC. The high content of TC in this fraction was supported by a relatively high UV-extinction signal at \(\lambda = 205\) nm, the wavelength at which TC has its absorption maximum (data not shown). The second fraction likely consists of comparably larger mixed PC/L-PC-TC micelles, showing strong light scattering, along with moderate UV-absorption. The third population of particles, which eluted after the CF has reached its minimal set-point and was not fractionated completely, also showed strong light scattering, but no or marginal UV-absorption. They likely represent PC rich assemblies (Figure 2) or L-PC rich assemblies (Figure 4).

It was not possible to derive particle sizes of the TC-micelle peak from the MALLS data, since the light scattering signals did not show sufficient angular dependence. This is in agreement with published DLS data indicating TC micelles with a hydrodynamic diameter of only 2 to 3 nm (Mazer et al., 1979) and an average aggregation number of 5 ± 3 (Carey, 1985).

With increasing TC concentrations, a larger light scattering peak as well as UV-extinction peak are observed for this fraction. Since the TC micelle peaks occur at the same elution time (i.e. CF) at all investigated TC concentrations, the micelles are likely of the same size. The increase in AUC at higher TC concentrations implies that more pure TC micelles per unit volume are formed from TC molecules not used for solubilization of the PLs. This observation fits with published DLS data (Mazer et al., 1979) indicating that TC-micelles show a concentration independent self-associate (micelle) size. For the 3.5 mM TC dispersion, only a UV-peak and no light-scattering peak was observed, which may be explained by the poorer sensitivity of the light-scattering detector for these very small particles. For the 1 mM TC dispersion, no MALLS or UV peak was observed at the retention time, where TC-micelles should elute. It remains unclear, whether the sensitivity of the detectors employed is insufficient or whether there are no pure TC-micelles formed. When

\(^4\) The phase-boundary is where the system changes from mixed micelles only, to mixed-micelles co-existing with liposomes.
considering published inter-micellar TC-concentrations of comparable blends (determined by dialysis), it is regarded unlikely that pure TC-micelles form under this condition (1mM TC / 0.75 mM PC; (Carey, 1985)).

To reveal the particle-sizes of the colloidal assemblies too small for MALLS analysis, AF4 fractions from the TC dispersions were collected and analyzed off-line using DLS. However, valid results could not be obtained. In an attempt to analyze a 20 mM pure TC dispersion with AF4/MALLS (injection volume of up to 300 µL), no distinct light scattering peak was observed in contrast to the binary PL TC dispersion containing 20 mM TC. Obviously, the presence of other lipids influences the bile salt aggregation-numbers and thus micelle sizes, as shown by Mazer and coworkers (Mazer et al., 1976) with SDS as a model amphiphilic compound. Also, Small and co-workers showed that the bile-salt CMC is effectively lowered by addition of PC to a given bile salt solution (Carey and Small, 1972).

The second fraction in Figure 2 likely represents comparably larger mixed PC-TC micelles showing strong light scattering, along with moderate UV-absorption. As proposed by Mazer (Mazer et al., 1980) in the ‘mixed disc’ model, these mixed PC TC micelles can structurally be described as bilayer discs, surrounded by TC molecules on their perimeter. Depending on the TC/PC-ratio, total lipid-concentration, salt-concentration, temperature and other factors, the discs may vary in size. The variations in onset of the second fraction as seen in Figure 2, indicate small variations in particle size. The size-range observed here (Ø ≈ 10 to 50 nm) agrees with literature data. An unambiguous correlation between the TC-concentration and the onset and/or AUC of the mixed micelle peak was not observed. Still, there was a tendency towards smaller mixed micelles (earlier peak onset, smaller AUC) with higher TC-concentrations. At this very low PC-concentration studied here (0.75 mM), small TC-micelles appear to co-exist with mixed micelles at all studied molar PC/TC-ratios (up to ≈ 0.2), which is in agreement with experimentally derived phase diagrams (Mazer et al., 1980).

The third population of particles in Figure 2, which was not completely fractionated, likely represent PC-rich assemblies. The occurrence of this third particle population, which is assumed to be PC-rich and shows substantially larger diameters is unexpected for the PC/TC-ratios studied here (0.04 to 1.3), when considering literature data. In essence, for binary dispersions of PC and TC (concentration range of 3.5 to 20 mM), AF4/MALLS indicated three co-existing particle species (small TC micelles, mixed micelles of intermediate size and large PC assemblies) in a reproducible manner. This observation contradicts published phase diagrams (Mazer et al., 1980) of binary blends of bile salts and swelling amphiphiles like lecithin-constituents, where three separate regions with clear phase boundaries are postulated: (1) co-existing simple and mixed micelles (2) mixed micelles (3) bilayers and liposomes. Since published phase diagrams are based on DLS- and nephelometric studies, the discrepancy may be explained by the fact that AF4/MALLS is able to resolve co-existing species in contrast to these traditional bulk methods. This is supported by the
fact that Cohen and Carey (Cohen and Carey, 1990) produced a phase diagram of a ternary blend containing PC, cholesterol and TC by means of DLS upon high performance gel filtration, where they were able to separate pure TC micelles from mixed TC-PC micelles.

In Figure 4, L-PC and TC are shown to form small supramolecular assemblies (second fraction) with retention times even shorter than those found for PC and TC (refer to the second fraction in Figure 2). This indicates that the ‘mixed micelles’ containing L-PC are comparably smaller than their PC counterpart. Theoretically, when dispersed in aqueous medium, L-PC forms micelles rather than liposomes, which can be explained by the well-known concept of critical packing parameter (CPP), a dimensionless number suggesting the ultrastructure of surfactant aggregates. The variables used to calculate the CPP are the surfactant’s head area, tail-volume and tail length. Exemplary, a large head area compared to tail-volume will result in the formation of high-curvature micelles. Predicting the ultrastructure solely based on the CPP is, however, not always adequate, since the effective values of head-areas and tail-volumes can change with temperature and ionic strength of the medium.

Also in the case of the mixed TC-PC/L-PC micelles (second fraction in Figure 2 and 4), no particle sizes could be derived from the MALLS data and attempts to derive particle sizes of collected AF4 fractions with DLS yielded no valid results. In contrast, the GDX dispersions yielded satisfying DLS and MALLS measurements and size-analysis (see below). An explanation might be that the colloidal assemblies formed with TC may disassemble and re-assemble in a way/rate that renders light-scattering measurements difficult. However, a rough particle size estimate can be obtained by comparing with particle-sizes obtained with GDX blends for fractions eluting at similar retention times and cross flows.

4.1.1 Addition of Celecoxib

In Figure 3, the same type of dispersions (containing TC and PC), as in Figure 2 are presented, but with the addition of CXB. Interestingly, at the lowest TC concentration (1 mM) a major change in the colloidal phases is observed. The supramolecular assemblies have become much larger and the MALLS peak (third peak) likely represents a blend of ‘swollen’ mixed micelles and larger vesicular structures. A peak-split is observed, indicating an incomplete separation between two particle fractions of different morphology and sizes. The difference in morphology is supported by the subsequent size analysis, where the first half of the split-peak (10-16 min) fits with the Zimm equation (typically applied for small to mid-sized particles i.e. radius of gyration \( R_g \) = 10 – 100 nm; Assuming particles being of similar density and volume, at the point of measurement). The second half of the fraction fits very well with the hollow-sphere model whereas it does not comply with the Zimm equation. The interested reader is referred to (Elvang et al., 2016) for fitting examples. Sizes of the ‘swollen’ mixed micelles and the vesicular structures were here found to range between 30 – 60 nm and 140 – 300 nm in diameter, respectively. This finding illustrates how the diversity of a colloidal system apparently can change when incorporating an API, depending on the ratios of the
dispersions’ molecular constituents. This leads us to propose that CXB acts as ‘lipid’ material and thus influences the types and relative distribution of co-existing colloidal assemblies formed from intestinal bile salt and PL.

As Figure 3, Figure 5 shows the fractograms of dispersions containing TC and L-PC, with the addition of CXB (corresponding dispersions without CXB are presented in Figure 4). For the L-PC dispersions, the most prominent change in terms of light scattering signals, is observed for the dispersion containing 20 mM TC. Here, the scattering signals are increased significantly for peaks representing TC-micelles and mixed-micelles. Since there is no shift to later retention times (indicating a notable increase in particle size), the particles must have increased in number or changed in refractive index (composition). Thus, it can be hypothesized that CXB behaves like a ‘lipid’ that is solubilized by L-PC and TC, creating more (and/or differently composed) particles of similar sizes.
4.2 Artificial intestinal fluids containing sodium glycodeoxycholate

In vivo, GDX is present in comparable amounts to TC (Riethorst et al., 2016b) and is among the most abundant bile salts found in human bile (Birru et al., 2014). A variety of bile salts exist in vivo differing in chemical structure (functional groups) and, importantly, differing in solubilization capacity towards PLs. This results in differences in the colloidal phases formed from binary PL-bile salt systems, as shown by Birru and co-workers (Birru et al., 2014). By means of nephelometry, Birru et al. produced a phase-diagram showing the nephelometry turbidity unit (NTU) as a function of bile salt mass fraction of PL content. From this they postulated a system consisting of either only micelles or a system, where micelles and liposomes co-existed. Using DLS, they assigned sizes of 3 to 7 nm to the micelle population and 300 to 700 nm to the liposome population. The binary GDX PL dispersions studied here are in the same range of PL:GDX mass ratios. Furthermore, they compared the phase behavior of binary bile salt-PL systems, where they used six different bile salts; taurodeoxycholate, glycocholate, glycochenodeoxycholate, taurocholate, taurochenodeoxycholate and glycodeoxycholate. Also, they compared the PL solubilization in these systems to a system containing a mixture of the five bile salts. The phase behavior of the binary system containing GDX resembled the phase behavior of the mixed system very closely, whereas the phase behavior of the binary system containing TC did not resemble the mixed system at all. This recommends careful selection of bile salt(s) when attempting to mimic in vivo conditions with respect to colloidal assemblies forming and drug solubilization. It has already been shown how the apparent solubility of drug compounds can be affected by the variety of colloidal structures present in the system (Kossena et al., 2003) (Kleberg et al., 2010).

For the binary GDX-PL dispersions with 3.5, 10 and 20 mM GDX, AF4/MALLS resolved three distinct colloidal populations (Figure 6 and Figure 7). Hence, in general the colloidal phases observed with GDX were quite similar to those observed with TC. In analogy to the TC/PL-systems, the three particle-species were designated GDX micelles, mixed micelles and liposomes/aggregates. The MALLS-derived sizes along with sizes obtained by off-line DLS analysis of the separate fractions are summarized in Table 3.

As previously observed with the TC-dispersions, a fraction emerged right after the elution peak at the highest CF. With increasing GDX concentrations, the light scattering intensity of this peak increased. This population is assumed to represent pure GDX micelles. In contrast to the TC micelles, which could be separated from the other fractions using a maximal CF of 2 mL min⁻¹, the GDX micelles were only separated when the initial CF was increased to 3 mL min⁻¹. This separation method allowed to separate all three particle-fractions (GDX micelles, mixed-micelles, liposomes/aggregates). Furthermore, corresponding size data could be obtained from fitting of the
MALLS data and/or off-line DLS analysis, which was not satisfactorily possible for the TC blends. The co-existence of three colloidal populations, (1) simple GDX-micelles approximately 3 nm in size, (2) mixed micelles in the size-range 15 to 20 nm and (3) liposomes in the size-range 150 to 300 nm for binary GDX/PC-blends contradicts the conclusions drawn by Birru and co-workers from their nephelometric and DLS-studies (Birru 2014). They postulated two distinct populations for GDX/PC-blends, a micelle population (3 to 12 nm in size) and a liposome population (mostly 300 to 400 nm in size). Again, this discrepancy may be explained by the higher sensitivity of AF4/MALLS to resolve co-existing populations as compared to bulk methods.

Table 2 gives the particle sizes of the 5 – 20 mM pure GDX dispersions as measured by DLS. This bile salt, which has a defined CMC (Reis et al., 2004), allowed us to assess if small pure bile salt micelles can be detected by our dynamic and static light scattering instrumentations. In this case, micelle hydrodynamic sizes of Ø 3-4 nm could be measured down to a GDX concentration of 5 mM by DLS. To compare, Cozzolino and co-workers (Cozzolino et al., 2006) measured the size of pure GDX micelles as a function of GDX concentration and ionic strength of the medium using DLS. At a comparable ionic strength and GDX concentration, they obtained a hydrodynamic diameter of 3.98 $\pm$ 0.6 nm where we measure 3.15 $\pm$ 0.05 nm. Below a GDX concentration of 5 mM, a correlation curve could not be obtained, and thus no reliable size values could be derived. High PDI values encountered during DLS measurements do not necessarily result from a high sample polydispersity but may be due to particles being very small and relatively few in number (particle numbers are reduced with concentration when assuming micelle size is always the same) producing a low count of scattered photons.

In contrast to the pure TC dispersions, injection of pure GDX dispersions into the AF4-channel produced a distinct MALLS peak where particles eluted at the highest CF (data not shown). Two important conclusions can be drawn from this observation: Firstly, it is possible to obtain signals from these very small structures with MALLS. Secondly, and more importantly, the GDX micelles are stable upon dilution in the AF4-channel, within the time-frame from injection to elution. If the micelles were not stable in the AF4-channel, the free GDX monomers would have been washed out through the ultrafiltration membrane.

4.2.1 Addition of Celecoxib

Incorporating CXB into the GDX blends had, in all cases, a profound effect on the fractograms. The fractions assigned to ‘liposomes/aggregates’ increased significantly in scattering intensity (see Figure 8 and 9). Based on DLS size-data of the collected ‘liposome/aggregates’ fractions, the sizes of this fraction did not differ substantially in the presence or absence of CXB. Thus, we assume that the particle number had increased significantly and/or the scattering characteristics (refractive index) had changed when CXB was incorporated. A satisfactory MALLS fit for the

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\[ \text{Cozzolino: Ionic strength} = 0.160, [\text{GDX}] = 20 \text{ mM} \]
\[ \text{This study: Ionic strength} = 0.156, [\text{GDX}] = 20 \text{ mM} \]
'liposome/aggregates' fraction could not be obtained with any model applied. The particles were so large that they eluted only after the CF dropped to zero and thus were not fractionated (i.e. particles of different sizes co-eluted) and the MALLS analysis was compromised.

5 Conclusions

AF4/MALLS technique has here proven suited for mapping of ultrastructural changes in biomimetic media in the form of binary bile salt-phospholipid systems, which contained bile salt and PL in physiologically relevant concentrations.

The current study revealed that binary bile salt-PL dispersions, over the whole concentration range studied, form at least two co-existing colloidal phases in a reproducible manner: (1) mixed micelles (in the size range of approx. 15 – 50 nm diameter) and (2) larger assemblies, likely vesicles, the specific sizes of which could not always be resolved due to incomplete fractionation. Furthermore, at and above bile salt concentrations of 3.5 mM (GDX) and 10 mM (TC), the dispersions contained very small (< 5nm) colloidal structures, likely bile-salt oligomeric self-assemblies. The pattern of colloidal phases was found highly reproducible and stable over typical analysis time periods except for the 1 mM [GDX PC] blend, which upon dispersion in aqueous medium initially formed two separate colloidal phases, which after 18 hours of equilibration time fused into one phase.

These findings indicate that by employing AF4/MALLS, a higher resolution of the (co-existing) particle phases can be achieved and thereby add to our current understanding of the phase behavior of dilute binary PL / bile salt blends. Obviously, independently of the type of bile salt or PL, at the very low concentrations and at all ratios studied here, binary bile salt / (lyso-)phospholipid blends formed two or three co-existing colloidal phases, depending whether the inter-micellar bile salt concentration (IMC) was below or above the micelle concentration. Below this concentration, a distinct mixed bile salt-PL micelle phase appears to co-exist with a population of larger lipid associates, which in many cases could be assigned to PL vesicles (liposomes). When the IMC of the bile salt was high enough, a third, distinct phase of very small bile salt micelles occurred. Increasing bile salt concentrations caused a decrease in size of the mixed micelles and a shift from mixed micelles to both a fraction of bile salt micelles and a population of larger lipid associates (liposomes).

Furthermore, in the artificial intestinal fluids containing TC, the model drug CXB was found to be incorporated without major impact on the phase behavior, except for one case (the lowest TC concentration), where CXB triggered the formation of an additional (vesicular) phase. In the artificial intestinal fluids containing GDX, CXB lead in all cases to a pronounced increase in light scattering intensity of the third particle population.

It remains to investigate, whether the phase behavior mapped here by using binary blends of selected lipids reflects the situation in complex aspirated human intestinal fluids of comparable bile salt and PL concentrations. Finally, it would be of interest to the pharmaceutical scientist to study
whether the co-existing colloidal phases described here behave differently with respect to the solubilization and release of poorly soluble drugs from colloidal states into the intestinal lumen, as this may have an impact on drug-absorption.

6 References


Figure 1:

Fractograms, over time, of blend [GDX PC] containing 1 mM GDX and 0.75 mM PC. The orange colored y-axis is associated with the fractogram (orange) obtained for the blend after it has been stored in the autosampler for 18 hours.

Figure 2:

Fractograms of blends [TC PC] containing 0.75 mM PC (fixed amount) and varied concentrations of TC.

Figure 3:

Fractograms of blends [TC PC CXB] containing 0.75 mM PC (fixed amount), CXB 0.80 mM (fixed amount) and varied concentrations of TC.

Figure 4:

Fractograms of blends [TC L-PC] containing 0.75 mM L-PC (fixed amount) and varied concentrations of TC.

Figure 5:

Fractograms of blends [TC L-PC CXB] containing 0.75 mM L-PC (fixed amount), CXB 0.80 mM (fixed amount) and varied concentrations of TC.

Figure 6:

Fractograms of blends [GDX PC] containing 0.75 mM PC and varied concentrations of GDX

Figure 7:

Fractograms of blends [GDX L-PC] containing 0-75 mM L-PC (fixed amount) and varied concentrations of GDX
Figure 8:
Fractograms of blends [GDX PC CXB] containing 0.75 mM PC (fixed amount), CXB 0.80 mM (fixed amount) and varied concentrations of GDX.

Figure 9:
Fractograms of blends [GDX L-PC CXB] containing 0.75 mM L-PC (fixed amount), CXB 0.80 mM (fixed amount) and varied concentrations of GDX.

Figure 10:
Illustrations of theoretical models of bile salt micelles and mixed micelles. (A) primary structure of a bile salt micelle, (B) secondary structure of a bile salt micelle, (C) small mixed micelle, (D) large/swollen mixed-disc micelle.
Table 1: Molecular composition and amount for the different blends prepared by freeze-drying. The “sample name” gives the bile-salt concentration in mM of either TC or GDX + which lipid species is present and if celecoxib is included or not.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Content (mg)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CXB</td>
<td>PC</td>
<td>L-PC</td>
<td>NaTC-H2O / NaGDX</td>
</tr>
<tr>
<td>1 mM TC/GDX PC</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td>3.5 mM TC/GDX PC</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>10 mM TC/GDX PC</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>26.8</td>
</tr>
<tr>
<td>20 mM TC/GDX PC</td>
<td>-</td>
<td>2.9</td>
<td>-</td>
<td>53.6</td>
</tr>
<tr>
<td>1 mM TC/GDX PC CXB</td>
<td>1.5</td>
<td>2.9</td>
<td>-</td>
<td>2.7</td>
</tr>
<tr>
<td>3.5 mM TC/GDX PC CXB</td>
<td>1.5</td>
<td>2.9</td>
<td>-</td>
<td>9.5</td>
</tr>
<tr>
<td>10 mM TC/GDX PC CXB</td>
<td>1.5</td>
<td>2.9</td>
<td>-</td>
<td>26.8</td>
</tr>
<tr>
<td>20 mM TC/GDX PC CXB</td>
<td>1.5</td>
<td>2.9</td>
<td>-</td>
<td>53.6</td>
</tr>
<tr>
<td>1 mM TC/GDX L-PC</td>
<td>-</td>
<td>-</td>
<td>1.1</td>
<td>2.7</td>
</tr>
<tr>
<td>3.5 mM TC/GDX L-PC</td>
<td>-</td>
<td>-</td>
<td>1.1</td>
<td>9.5</td>
</tr>
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<td>-</td>
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<td>13.5</td>
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<td>-</td>
<td>-</td>
<td>1.1</td>
<td>26.8</td>
</tr>
<tr>
<td>20 mM TC/GDX L-PC</td>
<td>-</td>
<td>-</td>
<td>1.1</td>
<td>53.6</td>
</tr>
<tr>
<td>1 mM TC/GDX L-PC CXB</td>
<td>1.5</td>
<td>-</td>
<td>1.1</td>
<td>2.7</td>
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<tr>
<td>3.5 mM TC/GDX L-PC CXB</td>
<td>1.5</td>
<td>-</td>
<td>1.1</td>
<td>9.5</td>
</tr>
<tr>
<td>5 mM TC L-PC CXB</td>
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<td>-</td>
<td>1.1</td>
<td>13.5</td>
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<tr>
<td>10 mM TC L-PC CXB</td>
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<td>-</td>
<td>1.1</td>
<td>26.8</td>
</tr>
<tr>
<td>20 mM TC/GDX L-PC CXB</td>
<td>1.5</td>
<td>-</td>
<td>1.1</td>
<td>53.6</td>
</tr>
</tbody>
</table>
Table 2: DLS derived average $Z_{av}$ values and associated PDI’s for GDX and TC ($n = 4$ *).

<table>
<thead>
<tr>
<th>Bile-salt</th>
<th>Concentration (mM)</th>
<th>$Z_{av}$ - diameter (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GDX</td>
<td>20</td>
<td>3.15 ± 0.05</td>
<td>0.241 ± 0.014</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.35 ± 0.05</td>
<td>0.232 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>(4.30 ± 0.57)**</td>
<td>(0.571)</td>
</tr>
<tr>
<td>TC</td>
<td>20</td>
<td>(2.5)**</td>
<td>(0.571)</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>n.a.***</td>
<td>0.571</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>n.a.***</td>
<td>0.571</td>
</tr>
</tbody>
</table>

* Here, $n$ refers to technical replicates from which the mean values and SD’s were calculated.

** Obtained values were unreliable

*** n.a. = not available:
Table 3: MALLS and DLS size data for all PC-GDX blends. The fraction names refer to the labels given in the respective fractograms (Figure 5, 6 and 8).

<table>
<thead>
<tr>
<th>Bile-salt conc. (mM) / Blend-type</th>
<th>Fraction</th>
<th>MALLS Astra best fit model</th>
<th>D$_2^*$ (nm)</th>
<th>DLS $Z_{av}$ - diameter $^\text{**}$ (nm)</th>
<th>PDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 / [GDX PC]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>28.9 ± 0.40</td>
<td>0.038 ± 0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>Hollow sphere</td>
<td>200</td>
<td>195.4</td>
<td>0.300</td>
</tr>
<tr>
<td></td>
<td>Swollen mixed micelles</td>
<td>Zimm</td>
<td>44</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3.5 / [GDX PC]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>17.68 ± 0.08</td>
<td>0.066 ± 0.012</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>Hollow sphere</td>
<td>283</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10 / [GDX PC]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>16.85 ± 0.11</td>
<td>0.071 ± 0.014</td>
<td></td>
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<tr>
<td></td>
<td>Liposomes</td>
<td>Hollow sphere</td>
<td>207</td>
<td>211.0 ± 3.81</td>
<td>0.230 ± 0.015</td>
</tr>
<tr>
<td>20 / [GDX PC]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>20.25 ± 0.33</td>
<td>0.203 ± 0.008</td>
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<tr>
<td></td>
<td>Liposomes</td>
<td>Hollow sphere</td>
<td>164</td>
<td>157.18 ± 7.40</td>
<td>0.272 ± 0.038</td>
</tr>
<tr>
<td>1 / [GDX PC CXB]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>3.5 / [GDX PC CXB]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>15.10 ± 0.07</td>
<td>0.090 ± 0.017</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>-</td>
<td>93.35 ± 1.97</td>
<td>0.252 ± 0.020</td>
<td></td>
</tr>
<tr>
<td>10 / [GDX PC CXB]</td>
<td>Mixed micelles</td>
<td>-</td>
<td>17.30 ± 0.19</td>
<td>0.133 ± 0.017</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>-</td>
<td>105.20 ± 2.80</td>
<td>0.243 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>20 / [GDX PC CXB]</td>
<td>GDX micelles</td>
<td>-</td>
<td>2.8</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed micelles</td>
<td>-</td>
<td>17.65 ± 0.23</td>
<td>0.205 ± 0.013</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liposomes</td>
<td>-</td>
<td>238.05 ± 7.18</td>
<td>0.571</td>
<td></td>
</tr>
</tbody>
</table>

$^*$ $D_2$ is derived from the peak on the same time-stamp as collected for DLS measurements. Single values are given.

$^\text{**}$ $Z_{av}$ is derived from a 1 mL sample taken from the respective peak maximum (1 min collection time).
Graphics Abstract
Figure 1
Figure 2

The diagram illustrates the crossflow (mL min⁻¹) and light scattering intensity at 90° detection angle (V) as a function of time (min) for different concentrations of TC (20 mM, 10 mM, 3.5 mM, 1 mM). The peaks represent different micellar structures: mixed micelles, TC micelles, and liposomes/aggregates. The inset shows a close-up view of the light scattering intensity for the 20 mM TC concentration.
Figure 5

Light scattering intensity at 90° detection angle (V)

Crossflow (mL min⁻¹)

Time (min)

- TC micelles
- Mixed micelles
- Liposomes/aggregates

Lines for different concentrations:
- 20 mM TC
- 10 mM TC
- 5 mM TC
- 3.5 mM TC
- 1 mM TC

Crossflow
Figure 7
Figure 8
Figure 9

Light scattering intensity at 90° detection angle (V)

Crossflow (mL min⁻¹)

- 1 mM GDX
- 3.5 mM GDX
- 10 mM GDX
- Crossflow

GDX micelles
Mixed micelles

Liposomes/aggregates