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Effect of Surfactant−Bile Interactions on the Solubility of Hydrophobic Drugs in Biorelevant Dissolution Media

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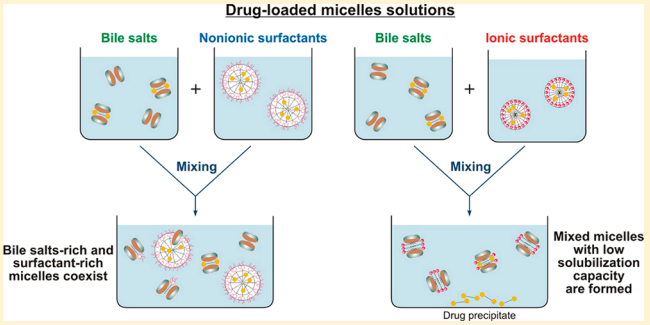
Supporting Information

ABSTRACT: Biorelevant dissolution media (BDM) methods are commonly employed to investigate the oral absorption of poorly water-soluble drugs. Despite the significant progress in this area, the effect of commonly employed pharmaceutical excipients, such as surfactants, on the solubility of drugs in BDM has not been characterized in detail. The aim of this study is to clarify the impact of surfactant−bile interactions on drug solubility by using a set of 12 surfactants, 3 model hydrophobic drugs (fenofibrate, danazol, and progesterone) and two types of BDM (porcine bile extract and sodium taurodeoxycholate). Drug precipitation and sharp nonlinear decrease in the solubility of all studied drugs is observed when drug-loaded ionic surfactant micelles are introduced in solutions of both BDM, whereas the drugs remain solubilized in the mixtures of nonionic polysorbate surfactants + BDM. One-dimensional and diffusion-ordered ^{1}H NMR spectroscopy show that mixed bile salt + surfactant micelles with low drug solubilization capacity are formed for the ionic surfactants. On the other hand, separate surfactant-rich and bile salt-rich micelles coexist in the nonionic polysorbate surfactant + bile salt mixtures, explaining the better drug solubility in these systems. The nonionic alcohol ethoxylate surfactants show intermediate behavior. The large dependence of the drug solubility on surfactant−bile interactions (in which the drug molecules do not play a major role *per se*) highlights how the complex interplay between excipients and bile salts can significantly change one of the key parameters which governs the oral absorption of poorly water-soluble drugs, *viz.* the drug solubility in the intestinal fluids.



KEYWORDS: *bile salt−surfactant interactions, drug solubility, precipitation, biorelevant dissolution media, DOSY*

INTRODUCTION

Drug solubility is one of the critical physicochemical characteristics of any new molecular entity (NME), as it provides valuable guidance about the possible issues in drug product formulation. In particular, the solubility of drugs in intestinal fluids is one of the factors that determines oral absorption.[1] However, the high-throughput methods for solubility screening employed in lead optimization are usually based on simple buffered solutions,[2] which can result in the underestimation or overestimation of intestinal drug solubility.[3] The latter is especially important for lipophilic drug molecules with low solubility in water, which are estimated to account for up to 90% of NME.[4] To solve this issue, a variety of dissolution media that contain diverse combinations of intestinal fluid components and properties (biorelevant media) have been developed.[5−10] If the simulated intestinal fluid medium is chosen according to the drug physicochemical properties (weak acid/base or nonionizable), the measured drug solubility is usually in good correlation with drug solubility in human intestinal fluids.[11,12] For this reason, the data for drug dissolution in biorelevant medium is used also as an input for in silico oral absorption prediction,[13] which is an integral part of the physiologically based pharmacokinetic (PBPK) modeling.[14,15]

Significant effort has been devoted to clarify the main parameters controlling drug solubility in the complex environment of a simulated intestinal fluid, which usually consists of one or several bile salts, phospholipids, lipid digestion products (fatty acids and monoglycerides), inorganic salts, and buffers.[7,9] Söderlind et al.[9] found that the bile acid conjugation and the degree of hydroxylation of the steroid ring have only a minimal effect on the solubility of 24 drugs. An empirical equation which correlates Log P/D and intrinsic aqueous solubility values with the solubility ratio for sodium taurocholate was proposed by Mithani et al.[16] and Glomme et al.[17] to estimate intestinal drug solubility. On the other hand, Zhou et al. studied systematically the effect of 8 factors on the solubility of 13 drugs and showed that the bile salt, oleic acid, and phospholipid content played an important role for the solubility of all drug molecules studied,

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whereas the pH was the dominant factor for the solubility of acidic drugs.[18] Grove et al. reported that increasing bile salt and phospholipid levels had a bigger effect on seocalcitol solubility than lipolysis products.[19] In contrast, lipid digestion products were shown to significantly increase the solubility of fenofibrate and cinnarizine, whereas the effect of bile salts and phospholipids was much smaller.[20] The solubility of danazol and griseofulvine was affected only slightly by the increasing concentration of bile salts, phospholipids and lipid digestion products.[20] Despite the accumulated data, there is still no clear understanding on how the drug molecular structure and properties determine the measured drug solubility at different biorelevant media compositions.

Given the complexity of the biorelevant media itself, it is not surprising that the influence of formulation excipients on drug solubility in biorelevant media is not yet systematically studied. The effect of classical surfactants is particularly interesting, as they can interact with the colloidal aggregates in the biorelevant media[21] and can impact drug solubility. Surfactants, such as polysorbates and sodium lauryl sulfate, are found in more than 100 FDA-approved oral pharmaceutical products[22] which emphasizes the importance of this issue in drug development.

The physicochemical interactions in binary mixtures (surfactant + pure bile salt) have been studied extensively.[23−28] Bile salts and cationic alkyltrimethylammonium bromide surfactants form mixed micelles due to strong electrostatic attraction between the oppositely charged head groups.[23−25] At low electrolyte concentrations, the nonionic polysorbate surfactants and bile salts also form mixed micelles with attractive interactions, due to decreased electrostatic repulsion between the similarly charged bile salt molecules in the mixed aggregates.[26−28] Different behaviors were observed for anionic surfactants, for which the nature of the interaction with the bile salts (attractive or repulsive) was found to depend on the specific molar ratio of the two components.[23]

Several studies highlight the effect of surfactants on the solubility of lipophilic molecules in biorelevant conditions.[29−32] In the context of lipid digestion, we showed that the dissolution kinetics of lipid digestion products (such as fatty acids and mono- and diglycerides) can be significantly increased, depending on the surfactant-to-bile ratio.[29] Madelung et al. studied the dissolution rate of two drugs from drug−surfactant discs and found that a low concentration of sodium lauryl sulfate had a strong impact on the rate of dissolution, while the bulk solubility was not affected.[30] The addition of polyoxyethylene(4) lauryl ether (Brij 30) to sodium cholate micelles decreased the solubility of rifampicin.[31] Similarly, the presence of sodium lauryl sulfate in fenofibrate immediate release tablets decreased drug solubility in a biorelevant media, composed of sodium taurocholate and lecithin, due to disruption of the bile salts−phospholipid vesicles.[32] Interestingly, such an effect was not observed for poloxamer surfactants in the same study. The described results demonstrate that the surfactants used as excipients in oral pharmaceutical products can have negative, positive, or no effect on drug solubility in biorelevant media, depending on the specific composition. However, no general rules are available to predict the effect, thus slowing down the optimization of drug formulations.

Therefore, the major aim of the current study is to clarify the impact of surfactant structure and surfactant-bile interactions on the solubility of hydrophobic drugs in biorelevant media. A range of 12 surfactants with different charge and hydrophobic chain length was studied. The model drug used in most experiments was fenofibrate, which was chosen because of its poor aqueous solubility and its sensitivity to biorelevant media composition.[20] Two additional drugs (danazol and progesterone) were also studied to verify the main trends observed with fenofibrate. To simulate the human intestinal fluids, we used a solution of porcine bile extract which contains a mixture of bile acids, phospholipids, cholesterol, and fatty acids. Previous studies have shown good agreement between the solubility of poorly water-soluble substances measured in an in vitro model based on porcine bile extract[33] and the in vivo behavior.[34] The main trends observed with the bile extract were verified by comparative experiments with one of the main bile salts in the human bile, sodium taurodeoxycholate (NaTDC). The aggregate size, composition, and interactions in the most interesting bile + surfactant solutions were determined by ^{1}H NMR DOSY experiments. In addition, the bile−surfactant interactions were assessed by comparing the critical micellar concentrations (CMCs) of the single components and the mixed systems.

MATERIALS AND METHODS

Materials. A total of 12 surfactants with different surfactant charge, headgroup type, and chain length were used to investigate the impact of surfactant structure on drug solubility in biorelevant media. The properties of the studied surfactants and the used abbreviations are summarized in Table 1. The nonionic surfactants studied were 3 polysorbates with saturated hydrophobic chains (C_{12} to C_{18}) and one unsaturated polysorbate ($C_{18:1}$). We studied also a homologue series of anionic surfactants of the alkylsulfate type, with hydrophobic chain lengths of C_{12} and C_{14}. Additional anionic surfactants studied were ethoxylated alkylsulfates. The cationic surfactants we studied are a homologue series of alkyl trimethylammonium bromides with hydrophobic chain lengths of C_{14} and C_{16}. Although some of the cationic surfactants are toxic and rarely used in drug delivery, we included them in the current study to clarify the general effects of the surfactant charge.

Porcine bile extract (Sigma), which contains 50 wt % bile acids, 6 wt % phosphatidylcholine, less than 0.06 wt % Ca^{2+},[35] 1.2 wt % cholesterol, and 6.7 wt % FA,[34] was used to prepare the biorelevant medium in most of the experiments. The pure bile salt sodium taurodeoxycholate (Sigma, > 95%) was used in comparative solubility experiments, as well as in the model experiments, such as ^{1}H NMR investigations of the bile−surfactant interactions and surface tension measurements for determination of the CMCs of the mixed surfactant + bile solutions.

Most of the drug solubility experiments were performed with fenofibrate, whereas progesterone and danazol were used in several key experiments to check whether the trends observed with fenofibrate are general. All studied drugs have very low aqueous solubility (0.8 μg/mL for fenofibrate,[36] 1 μg/mL for danazol,[37] and 10 μg/mL for progesterone[38]) and belong to Class II of the Biopharmaceutical classification system.[39] The molecular structures and purity of fenofibrate, progesterone, and danazol are also included in Table 1.

Mobile phase solvents for HPLC analysis included methanol (HPLC grade, 99.9%) and deionized water, filtered through a 450 nm NYLON filter. All aqueous solutions and phases were prepared using deionized water from the water-purification system Elix 3 (Millipore, USA). An exception were the samples analyzed via ^{1}H NMR spectroscopy, which were prepared in D_2O (99.8 atom % D, TCI). 3-(Trimethylsilyl)propionic-

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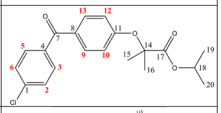
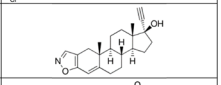
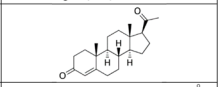
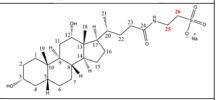
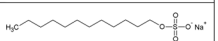
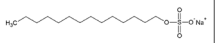
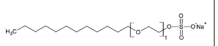
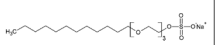
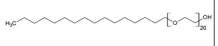
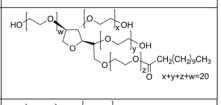
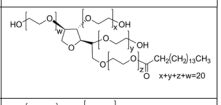
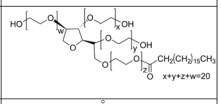
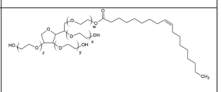
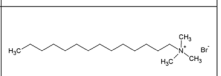
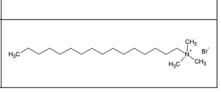
Table 1. Properties of the Studied Drugs and Surfactants

Name	Acronym used in text	Supplier, purity	Molecular mass, g/mol	Structure
Fenofibrate	-	TCI, 98 %	361	
Danazol	-	AlfaAesar, 98 %	338	
Progesterone	-	TCI, 98 %	315	
Sodium taurodeoxy-cholate	NaTDC	Sigma, 95 %	522	
Sodium lauryl sulfate	$C_{12}SO_4Na$	Arcos, 99 %	288	
Sodium tetradecyl sulfate	$C_{14}SO_4Na$	Merck, 95 %	316	
Sodium lauryl ethoxy-1 sulfate	$C_{12}E_1SO_4Na$	Stepan Co., 70 %	332	
Sodium lauryl ethoxy-3 sulfate	$C_{12}E_3SO_4Na$	Stepan Co., 70 %	420	
Brij 58	$C_{16}EO_{20}$	Sigma – Aldrich	1124	
Tween 20	T20	Sigma – Aldrich	1228	
Tween 40	T40	Sigma – Aldrich	1277	
Tween 60	T60	Sigma – Aldrich	1309	
Tween 80	T80	Sigma – Aldrich	1310	
Tetradecyl trimethyl ammonium bormide	$C_{14}TAB$	Sigma, 99%	336	
Cetyl trimethyl ammonium bromide	$C_{16}TAB$	Merck, 99%	364	

2,2,3,3-d4 acid sodium salt (TMSP-Na, 98 atom % D, Sigma) was used as an internal standard for the samples studied via ^{1}H NMR spectroscopy. Sodium and potassium chlorides (99%) were obtained from Merck.

Drug Solubility Determination. The equilibrium drug solubility at constant bile extract and surfactant concentration was determined by weighing fenofibrate (15 mg) and surfactant (50 mg) in a glass bottle and then adding 10 mL of freshly prepared solution of 10 mM bile extract, 137 mM NaCl, and 10 mM KCl. For the experiments at different bile salt-to-surfactant ratios and constant bile salt + surfactant total concentration, the following procedure was used: separate solutions of 10 mM bile extract, NaTDC, and surfactant in electrolyte (137 mM NaCl and 10 mM KCl) were prepared by stirring for 1 h at T = 37 °C, and then the required surfactant-to-bile ratio was obtained by mixing the appropriate volumes of these solutions in a glass bottle with preweighed drug (the total volume was constant at 10 mL). The concentrations of fenofibrate (1.5 mg/mL), danazol (1.5 mg/mL), and progesterone (10 mg/mL) used in all of the experiments were in large excess of the aqueous solubility of the drugs. The concentrations of the electrolytes were in the range of physiological Na^+ and K^+ concentrations in the human small intestine,[40] and the pH of the bile extract + surfactant mixtures was 6.0 ± 0.3.

The aqueous drug suspensions obtained by these procedures were stirred on a magnetic stirrer at 400 rpm for 24 h at 37 °C. After this period of incubation, the suspension was filtered through a 200 nm NYLON syringe filter to eliminate all undissolved particles. Finally, the concentration of the solubilized drug in the obtained clear aqueous phase was determined by HPLC (see Supporting Information for experimental details). Every step of the procedure (including filtration) was performed at T = 37 °C.

The drug solubilization efficiency was assessed by the measured solubility enhancement (SE), defined as SE = C/C_0, where C is the drug solubility in the surfactant, bile, or surfactant + bile solution, and C_0 is the intrinsic aqueous drug solubility.

Determination of CMC. The CMCs of the individual surfactants, NaTDC, and their mixtures were determined from the surface tension isotherms of the respective solutions. The surface tension at different surfactant or surfactant + NaTDC concentrations was measured on a K-100 instrument (Kruss, Germany) via the Wilhelmy plate method at T = 37 °C.

Study of Aggregate Size and Bile Salt−Surfactant Interactions by ^{1}H NMR Spectroscopy. NMR spectra were obtained on a Bruker Avance III HD 500 spectrometer (Rheinstetten, Germany). A Bruker broadband high-resolution probe (Observe) fitted with an actively shielded single axis Z-gradient was used. Experiments were conducted at T = 37 °C. The studied samples were prepared as described in the Drug Solubility Determination section, except that deuterium oxide (99.8 atom % D) was used instead of water to dissolve the surfactants and/or the bile salt (NaTDC). TMSP-Na-2,2,3,3-d4 was added to each sample as the internal standard (0 ppm) prior to measurement. The resolution of the obtained spectra is 0.001 ppm, and the standard error in the determination of the chemical shifts is <0.002 ppm. An LED experiment using bipolar gradients (ledbpgp2s) was used to obtain 2D DOSY spectra. The diffusion dimension was sampled in 64 steps by variation of the gradient pulse strength G in a linear ramp in the range from 5 to 70 or 90% (depending on the sample) of the maximum gradient output of the gradient unit (0.5 T/m). The diffusion delay (Δ) and gradient pulse length (δ) measurement parameters were optimized in order to collect representative relaxation spectra which allow proper data analysis and determination of the molecular diffusion coefficient. For most of the samples, diffusion delay Δ = 100 ms and gradient pulse length $\delta/2$ = 2 ms were used. The diffusion coefficient was extracted by regression fitting of the decay in peak intensity as a function of gradient pulse strength, and the aggregate size was calculated by using the Stokes−Einstein equation, $D = (kT)/(6\pi\eta r)$, where D is the diffusion coefficient, k is the Boltzmann constant, T is the absolute temperature, η is the viscosity of the medium, and r is the aggregate radius. The Topspin 3.5 pl7 software package (Bruker) was used for spectrum collection and data analysis.

EXPERIMENTAL RESULTS

The fenofibrate solubility in surfactant, bile, and bile + surfactant solutions at constant surfactant and bile concentrations is presented in the Fenofibrate Solubility at Constant Surfactant

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and Bile Extract Concentration section. The effect of surfactant-to-bile ratio on the solubility of several hydrophobic drugs is described in the Effect of Bile-to-Surfactant Ratio on Drug Solubility section. Porcine bile extract was used as a bile salt source for the experiments in the Fenofibrate Solubility at Constant Surfactant and Bile Extract Concentration and Effect of Bile-to-Surfactant Ratio on Drug Solubility sections. Experiments with mixtures of surfactant and a single bile salt (NaTDC) were performed to check whether the observed general trends in solubility are due to surfactant−bile salt interactions (Bile Extract vs Sodium Taurodeoxycholate: Effect on Fenofibrate Solubility section). The mixed CMCs of the surfactant and NaTDC mixtures were determined in the Determination of CMC in Mixed NaTDC + Surfactant Solutions section. The composition and properties of the micelles, as well as the interactions between NaTDC and the surfactants, were studied by ^{1}H NMR spectroscopy (Investigation of Micelle Size, Composition, and Intramicellar Interactions by NMR section).

Fenofibrate Solubility at Constant Surfactant and Bile Extract Concentration. The SE of fenofibrate in solutions of 0.5 wt % surfactant +10 mM bile extract is presented in Figure 1.

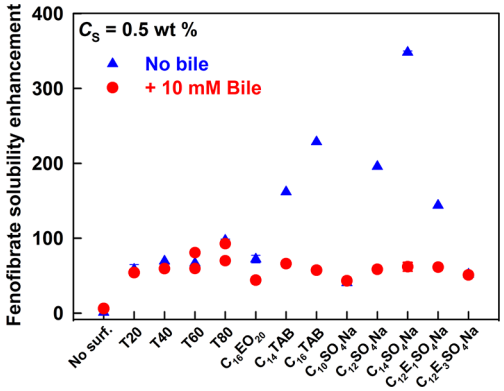

Figure 1. Fenofibrate SE, as a function of surfactant type, in aqueous (blue triangles) or in biorelevant (red circles) medium. Surfactant concentration is 0.5 wt %, and the concentration of bile extract in the biorelevant medium is 10 mM. Both the aqueous and the biorelevant medium contain 137 mM NaCl and 10 mM KCl. The results are presented as mean ± SD for n = 3−6 (the error bars can be smaller than the symbols). For n = 2, both experimental points are plotted.

The experimental data in surfactant-only solutions (no bile extract) is also presented for comparison. For the cationic and most of the anionic surfactants, fenofibrate SE decreased very strongly in biorelevant medium. For example, the SE for $C_{12}SO_4Na$ decreased from ≈200 in the absence of bile, to ≈60 in biorelevant medium. Such an effect was not observed for the nonionic surfactants, which had similar SE, both in the absence and in the presence of bile. The highest SE in biorelevant medium was measured for T80 (SE = 92), whereas all other surfactants had lower SE, in the range of 45−70.

In the absence of bile extract, the main reason for the different extent of fenofibrate solubilization by ionic surfactants on one side, and nonionic surfactant on the other side, is their different molecular mass. The ionic surfactants are in the range of 300−400 g/mol, whereas the nonionics are above 1000 g/mol; hence, as the comparison is made at constant weight concentration, the molar concentration of all ionic surfactants is 2 to 3 times higher than the nonionics. The effect of the surfactant structure on fenofibrate solubilization in the absence of bile extract was analyzed in detail in our previous work, which showed that the micellar solubilization capacity is higher for surfactants with a longer hydrophobic chain length and sulfate headgroup.[41]

Effect of Bile-to-Surfactant Ratio on Drug Solubility. Experiments at constant total molar concentration of bile + surfactant (10 mM) and different bile-to-surfactant ratios were performed to clarify better the role of bile−surfactant interactions on drug solubility. Four surfactants with different headgroup types and charges were studied, Tween 20 (nonionic), $C_{16}EO_{20}$ (nonionic), $C_{12}SO_4Na$ (anionic), and C_{14}TAB (cationic). For Tween 20, the fenofibrate solubility decreased linearly with increasing the molar fraction of bile extract, $f_{bile} = C_{bile}/(C_{bile} + C_{surf})$, see Figure 2A.

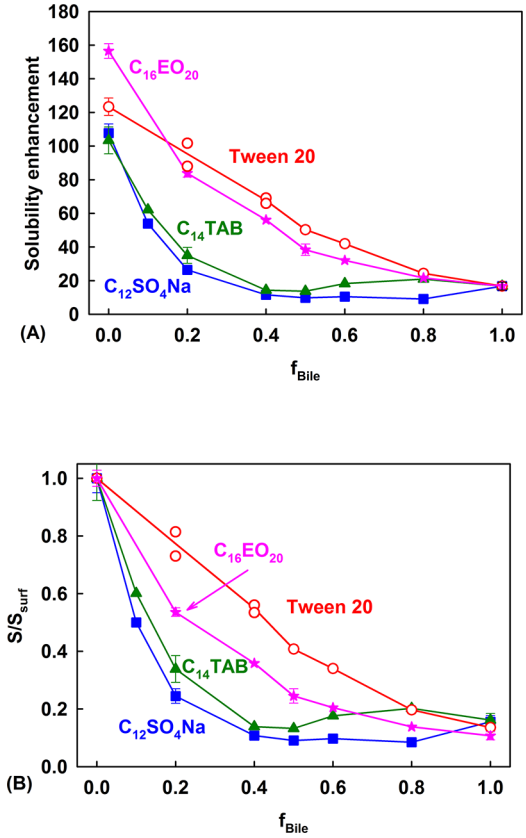

Figure 2. (A) SE of fenofibrate and (B) fenofibrate solubility in the surfactant + bile extract mixtures, scaled with the solubility in surfactant-only solutions, S/S_{surf}. The results are presented as a function of the molar fraction of bile salts in the surfactant + bile extract mixtures. The studied surfactants are C_{14}TAB (green triangles), Tween 20 (red circles), $C_{12}SO_4Na$ (blue squares), and $C_{16}E_{20}$ (pink stars). The total surfactant + bile concentration is fixed at 10 mM. All samples contain 137 mM NaCl and 10 mM KCl. The pH of the bile + surfactant mixtures was 6.0 ± 0.3. The results are presented as mean ± SD for n = 3−6 (the error bars can be smaller than the symbols). For n = 2, both experimental points are plotted.

In contrast, the presence of even a small fraction of bile (f_{bile} = 0.2) in the mixture resulted in a pronounced decrease in fenofibrate SE for the charged C_{14}TAB and $C_{12}SO_4Na$ surfactants: SE decreased from ≈110 for the surfactant-only systems to SE = 40 (for C_{14}TAB + bile) and to SE = 25 (for $C_{12}SO_4Na$ + bile), at f_{bile} = 0.2. Further increase of f_{bile} had no significant effect on fenofibrate solubility in $C_{12}SO_4Na$ + bile mixtures, whereas a shallow minimum was observed in the

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C_{14}TAB + bile solution at f_{bile} = 0.5. The nonionic $C_{16}EO_{20}$ displayed an intermediate behavior; the decrease in SE upon addition of bile was smaller than that of the ionic surfactants but bigger than that of Tween 20.

These different regimes of drug solubilization can be observed even more clearly when the results are scaled with the drug solubility in surfactant-only solutions, S/S_{surf}, where S is fenofibrate solubility in each bile extract + surfactant mixture, and S_{surf} is the solubility in the solution of the respective surfactant in the absence of bile (*viz.* at f_{bile} = 0), see Figure 2B.

To gain more insight on the mechanisms that govern these trends, additional experiments at low surfactant concentrations in the absence of bile were performed. In Figure 3, the SE of fenofibrate in surfactant-only solutions is compared with the SE measured in the mixed bile + surfactant solutions. The results are plotted as a function of the concentration of surfactant in each solution, omitting the bile salts, which are present in the mixed bile extract + surfactant solutions. In agreement with the results described in the previous paragraph, the presence of bile had a minor effect on the fenofibrate solubility in Tween 20 solutions, whereas a very strong decrease was observed for $C_{12}SO_4Na$ and C_{14}TAB at the same surfactant concentration. For example, the fenofibrate SE measured in a solution of 6 mM $C_{12}SO_4Na$ was SE = 53, compared to only SE = 11 for a solution which contains 6 mM $C_{12}SO_4Na$, but it is in the presence of the 4 mM bile extract. The effect for C_{14}TAB was of similar magnitude: fenofibrate SE = 61 was measured for 6 mM C_{14}TAB, compared to SE = 14 for 6 mM C_{14}TAB + 4 mM bile extract. Therefore, the presence of bile extract in solutions of $C_{12}SO_4Na$ and C_{14}TAB significantly decreases fenofibrate solubilization.

Experiments with two additional hydrophobic drugs (danazol and progesterone) were performed to clarify whether the observed effects of surfactant type are valid also for drugs with different molecular structures. The solubilities of danazol and progesterone in bile + surfactant mixtures at different bile-to-surfactant ratios were measured and compared with fenofibrate. To account for the different solubilities of the drugs in the surfactant micelles, the results were again scaled with the drug solubility in the surfactant-only system (S/S_{surf}), see Figure 4.

The presence of a minimal fraction of bile extract in the solution (f_{bile} = 0.2) very strongly decreased the drug solubility for all studied drugs in the solutions of cationic C_{14}TAB and anionic $C_{12}SO_4Na$ surfactants. In contrast, a linear decrease of the drug solubility with the increase of bile extract fraction was observed in solutions containing the nonionic surfactant Tween 20 for fenofibrate and danazol. The solubility of progesterone also linearly decreased from f_{bile} = 0–0.6; however, further increase of the bile extract fraction had a small effect on progesterone solubility.

Bile Extract vs Sodium Taurodeoxycholate: Effect on Fenofibrate Solubility. All experiments presented so far were performed with porcine bile extract, which is a mixture of bile salts and other surface-active substances, such as phospholipids, fatty acids, and cholesterol. To check whether the observed strong effects on drug solubility are due mainly to the interactions between the surfactants and the bile salts, fenofibrate solubility was studied also in binary mixtures of surfactants with a single bile salt, NaTDC, which is one of the principal taurine-conjugated bile salts in human bile.[42]

A very good agreement between the results from the experiments with porcine bile extract and NaTDC was obtained for $C_{12}SO_4Na$, C_{14}TAB, and Tween 20, see Figure 5. All major trends observed with the bile extract were reproduced very well

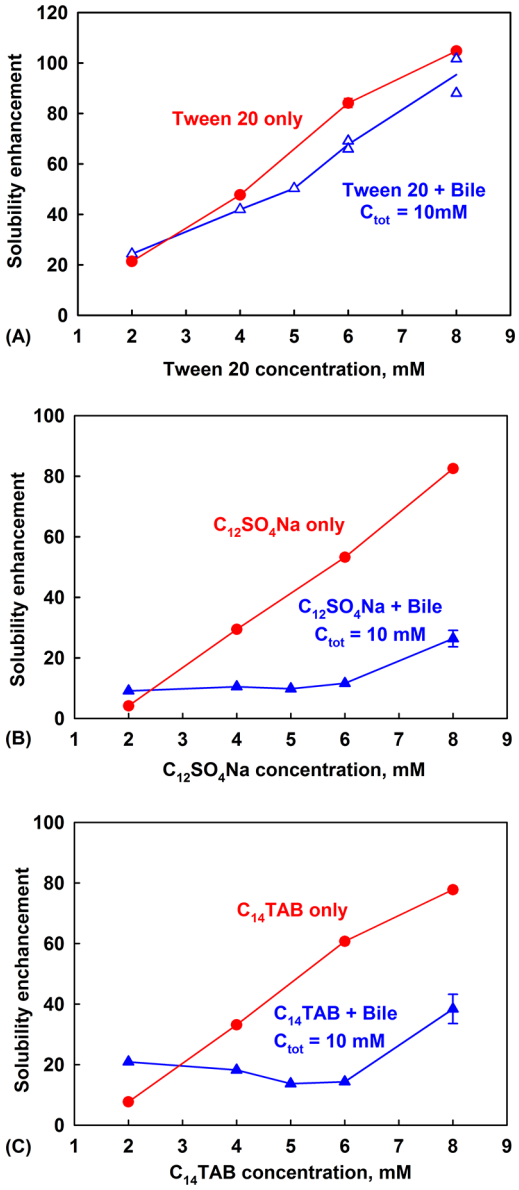

Figure 3. Fenofibrate SE as a function of the concentration of (A) Tween 20, (B) $C_{12}SO_4Na$, and (C) C_{14}TAB surfactants in the presence of surfactant only (red circles) or in mixed solutions of surfactant + bile extract (blue triangles). The total surfactant + bile concentration is fixed at 10 mM for the mixed surfactant + bile solutions (blue triangles). All samples contain 137 mM NaCl and 10 mM KCl. The pH of the bile + surfactant mixtures was 6.0 ± 0.3. The results for the bile + surfactant mixtures are presented as mean ± SD for n = 3–6 (the error bars can be smaller than the symbols), whereas for n = 2, both experimental points are plotted. The results for the surfactant-only solutions are from single experiments.

with the single bile salt. Small differences were observed only at high fractions of bile (f_{bile} > 0.5). For all systems studied, fenofibrate SE was slightly higher for the mixtures surfactant + porcine bile extract, compared to the mixtures surfactant + NaTDC. The latter is due to the presence of additional surface-active components in the bile extract (phopsholipids and fatty acids[34,35]). Also, the shallow minimum in fenofibrate SE observed around f_{bile} = 0.5 for the bile extract + C_{14}TAB mixtures was even more pronounced for NaTDC + C_{14}TAB: fenofibrate solubility could not be measured at f_{bile} = 0.5 and 0.6, as it was below the limit of detection of the HPLC method (3

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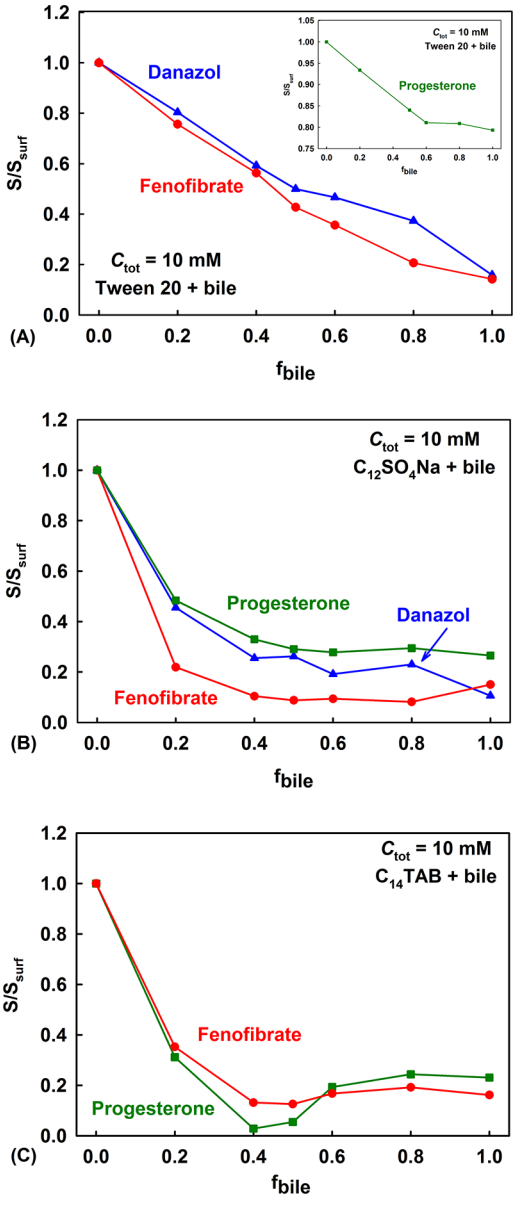

Figure 4. Drug solubility in mixtures of (A) Tween 20 + bile, (B) $C_{12}SO_4Na$ + bile, and (C) $C_{14}TAB$ + bile. The drug solubility is scaled with the solubility in surfactant-only solutions, S/S_{surf}, and the results are presented as a function of the molar fraction of the bile salts in the surfactant + bile extract mixtures. The results are obtained with the drugs progesterone (green triangles), fenofibrate (red circles), and danazol (blue squares). The total surfactant + bile concentration is fixed at 10 mM. All samples contain 137 mM NaCl and 10 mM KCl. The pH of the bile + surfactant mixtures was 6.0 ± 0.3.

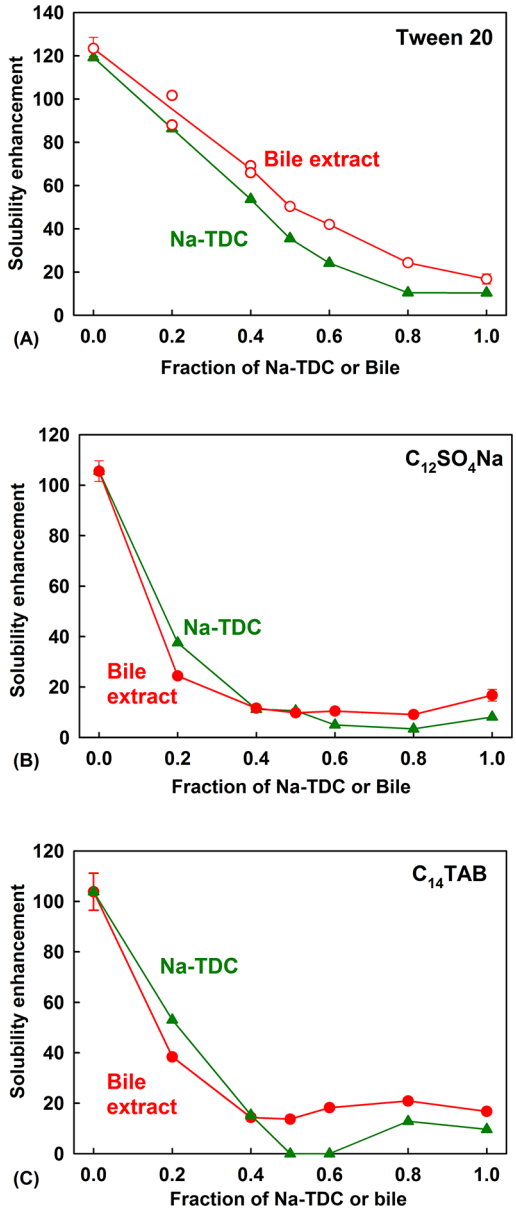

Figure 5. Fenofibrate SE as a function of the molar fraction of bile salts in surfactant + bile mixtures for (A) Tween 20, (B) $C_{12}SO_4Na$, and (C) $C_{14}TAB$ surfactants. The bile salts source is porcine bile extract (red circles) or NaTDC (green triangles). The total surfactant + bile concentration is fixed at 10 mM. All samples contain 137 mM NaCl and 10 mM KCl. The error bars can be smaller than the symbols. The results for the bile + surfactant mixtures are presented as mean ± SD for n = 3–6 (the error bars can be smaller than the symbols), whereas for n = 2, both experimental points are plotted. The results for the NaTDC + surfactant mixtures are from single experiments.

μg/mL). Formation of liquid droplets was visually observed in these samples.

Determination of CMC in Mixed NaTDC + Surfactant Solutions. The good reproducibility of the main trends in fenofibrate SE when the porcine bile extract was replaced by pure NaTDC provided an opportunity to study in more detail the surfactant–bile salt interactions, which play such an important role in drug solubility in biorelevant media. One of the classic approaches to assess these interactions is to measure the CMC of the mixed NaTDC + surfactant solutions and check if there is a minimum in the CMC, compared to the CMC of the individual amphiphiles.

The CMCs of the individual surfactants, NaTDC, and the binary surfactant + NaTDC mixtures at a ratio of 1:1, as determined from the surface tension isotherms (see Figure S1 in the Supporting Information), are compared in Figure 6.

One sees, that the mixtures of NaTDC + charged surfactants displayed a pronounced minimum in the CMC, whereas a shallow minimum was observed for the nonionic polysorbate surfactant + NaTDC mixture. The observed pronounced minima in the mixtures of NaTDC and ionic surfactants indicate a strong attraction between the molecules of the two components (NaTDC and surfactant), irrespectively of the

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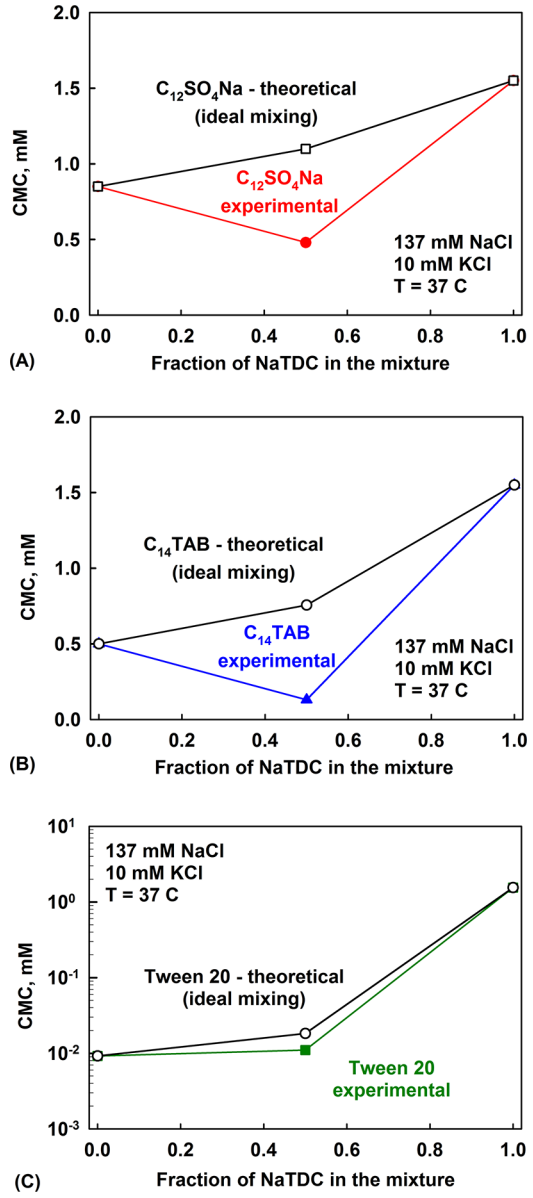

Figure 6. CMCs as a function of the molar fraction of NaTDC in surfactant + NaTDC mixtures for (A) $C_{12}SO_4Na$, (B) $C_{14}TAB$, and (C) Tween 20. The black lines represent the calculated mixed CMC of the surfactant + NaTDC mixtures when ideal mixing is assumed. All samples contain 137 mM NaCl and 10 mM KCl. The results are obtained from surface tension isotherms, measured at T = 37 °C.

charge, positive or negative, of the surfactant headgroup. These results motivated us to study in more detail the intermolecular interactions inside the micelles, as described below.

Investigation of Micelle Size, Composition, and Intramicellar Interactions by NMR. The properties of the colloidal aggregates in surfactant and NaTDC solutions were studied by ^{1}H NMR spectroscopy. Diffusion-ordered NMR spectroscopy (DOSY) allowed simultaneous determination of micelle size and molecular composition, whereas the shift of the peak positions in the standard one-dimensional NMR spectra was used as a tool to investigate the interactions between the surfactant and NaTDC molecules inside the mixed micelles. This approach was used for solutions of $C_{12}SO_4Na$, Tween 20, Tween 60, $C_{16}EO_{20}$, and their 1:1 mixtures with NaTDC. The experiments were performed at a total concentration of 10 mM, similarly to the solubility measurements. The peaks in the ^{1}H NMR spectrum of NaTDC and fenofibrate were assigned to their respective atoms on the basis of literature data[43] and simulated ^{1}H NMR spectra (ChemBioDraw Ultra 11.0 software, Cambridge soft).

Micelle Size and Composition As Determined via DOSY. The output from a DOSY experiment can be presented as a 2D map in which each spot is characterized by the ^{1}H chemical shift and the diffusion coefficient of the respective molecule. As most of the surfactant and NaTDC molecules are incorporated within micellar aggregates (due to the low CMC values and, hence, low concentration of the free monomers), the size and the composition of the aggregates formed in the respective solutions can be determined.

The results obtained for the mixture of Tween 20 and NaTDC after solubilization of fenofibrate are presented in Figure 7. One sees that all ^{1}H signals corresponding to Tween 20 molecules are characterized with one diffusion coefficient, which is different from the diffusion coefficient associated with the signals for the NaTDC molecules. Therefore, the majority of Tween 20 and NaTDC molecules are not present in the same (mixed) aggregates. Instead, these molecular species are incorporated in separate colloidal aggregates with different sizes.

To determine accurately the diffusion coefficient and, hence, the size of the aggregates, regression analysis of the ^{1}H peak intensity decay with increasing gradient strength was used (see the Materials and Methods section for more details). The aggregate size in surfactant + NaTDC mixtures after fenofibrate solubilization is presented in Figure 8A. In the solution of $C_{12}SO_4Na$ + NaTDC, both the surfactant and the bile salt were present in micelles with a diameter of 4.1 ± 0.3 nm. In contrast, in the Tween 20 + NaTDC solution, the nonionic surfactant molecules were incorporated in micelles with d = 6.7 ± 0.2 nm, compared to d = 4.4 ± 0.7 nm for the micelles composed of bile salt molecules. Similar behavior was observed also in the other mixtures of NaTDC + nonionic surfactants (Tween 60 and $C_{16}EO_{20}$): in all cases, the nonionic surfactant and the NaTDC molecules were not incorporated in the same micellar aggregates. Instead, NaTDC formed micelles with diameters between 4 and 6 nm, whereas the nonionic surfactants formed bigger micelles with diameters ranging from 7 to 9 nm.

The micelle size in solutions of the individual surfactants were also measured, see Figure 8B. The diameter of the NaTDC-only micelles is 2.8 ± 0.2 nm, which is significantly smaller than the size of NaTDC aggregates measured in the NaTDC + surfactant mixtures (d_H = 4−6 nm). In contrast, the size of the micelles measured in solutions of the individual nonionic surfactants was bigger than the size of the aggregates in which the corresponding molecules were found in the NaTDC + surfactant mixtures. For example, the size of Tween 20-only micelles was 9.1 ± 0.9 nm, compared to 6.7 ± 0.2 nm for the same molecules in the mixed Tween 20 + NaTDC solution. A similar effect was observed also for Tween 60 and $C_{16}EO_{20}$. These results are discussed in the Discussion section below, after presenting the complete set of experimental data.

Comparison of the size of the empty and drug-loaded micelles showed that fenofibrate solubilization has no significant effect on the diameter of $C_{12}SO_4Na$ micelles (d = 3.6 nm), see Figure S2 in the Supporting Information. The diameter of NaTDC and Tween 20 micelles increased slightly after solubilization of fenofibrate, from 2.6 to 2.8 for NaTDC and from 8.1 to 9.1 for Tween 20.

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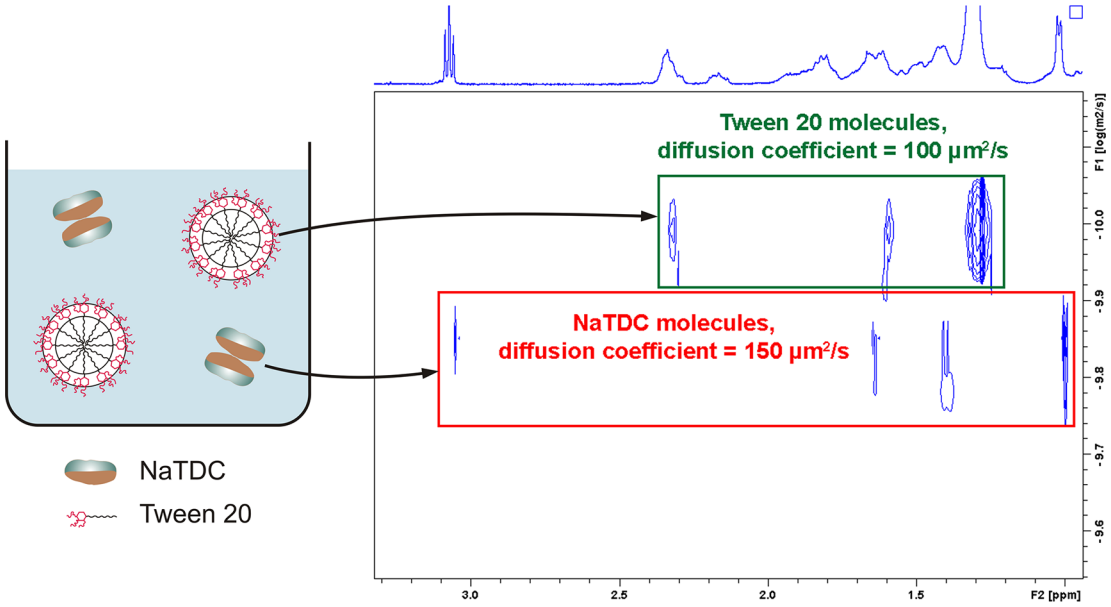

Figure 7. Schematic presentation of the separate NaTDC and Tween 20 micelles, coexisting in the mixed solutions (left-hand-side) and related typical results from ^{1}H DOSY NMR experiments (right-hand-side). The molecules of NaTDC and Tween 20 were identified by their characteristic, nonoverlapping ^{1}H NMR spectrum peaks.

Interactions between NaTDC, Surfactant, and Fenofibrate Molecules Studied via Chemical Shifts and NOESY. The precise location of the ^{1}H peaks in the NMR spectra of a given molecule can be significantly influenced by other groups (*viz.* molecules) which are located close by. This results in a shift of the ^{1}H NMR spectrum toward smaller or bigger ppm values due to the "shielding" or "de-shielding" action on the studied ^{1}H nuclei, exerted by the atoms of the neighboring molecule.[44] The so-called "shielding" is explained with higher electron density around the respective ^{1}H nuclei due to presence of nearby electron-donating groups, whereas "de-shielding" occurs when the electron density around the nucleus is reduced due to electron-withdrawing groups. Hence, spatial proximity and interactions between the molecules can be assessed when the chemical shifts in a reference sample are compared to the chemical shifts in the sample of interest.[43,45,46]

The change in the chemical shifts of NaTDC and surfactant molecules in their mixed solutions were calculated as $\Delta = \delta_{mix} - \delta_{ref}$, where δ_{mix} is the chemical shift of a molecule in the mixed NaTDC + surfactant solutions, and δ_{ref} is the chemical shift measured in a solution of the same molecules in D_2O.

The biggest changes in the NMR spectrum were observed for NaTDC molecules in the presence of the anionic $C_{12}SO_4Na$ (Table 2). For example, the NaTDC triplet at C-25 (next to the amido group) was transformed into a doublet of triplets in the presence of $C_{12}SO_4Na$. Such an effect was not observed in any of the NaTDC + nonionic surfactant mixtures. Furthermore, the NaTDC protons at C-1, C-21, and C-26 experienced significant positive shifts (>0.020 ppm) in the mixed $C_{12}SO_4Na$ + NaTDC solutions, whereas the shift was either much smaller (at C-1 and C-21) or in the opposite direction (negative shift at C-26) in all nonionic surfactants + NaTDC solutions. All nonionic surfactants induced a negative shift of the protons at C-18 and C-14β, whereas such an effect was not observed for $C_{12}SO_4Na$.

The changes in the proton chemical shifts of surfactant molecules induced by NaTDC were relatively small (<0.015 ppm), see Table S1 in the Supporting Information. In general, the protons of the anionic $C_{12}SO_4Na$ surfactant molecules experienced negative shifts, whereas positive shifts were observed for all nonionic surfactants.

To further investigate the significant interactions observed in the mixed NaTDC + $C_{12}SO_4Na$ solution (*viz.* peak splitting), NOESY experiments were performed. This NMR technique is used to identify protons that are in close proximity, at less than 0.5 nm.[47] However, the results showed that there are no protons in NaTDC that are closer than 0.5 nm to any of the protons in $C_{12}SO_4Na$.

The fenofibrate molecules solubilized in the bile and surfactant micelles can be considered as a probe which could provide additional information about the properties of the micelles. Several proton peaks associated with the aromatic structure of fenofibrate were detected, and the change of their chemical shifts under different conditions was evaluated (see Figure S3 and Table S2 in the Supporting Information). The biggest changes in the chemical shifts of fenofibrate aromatic protons were measured for the $C_{12}SO_4Na$ + NaTDC mixture (when compared to $C_{12}SO_4Na$-only solution), whereas the effects were much smaller for the Tween 20 and 60 surfactants. Intermediate behavior was observed for the $C_{16}EO_{20}$ system.

DISCUSSION

The experimental results obtained in the current study (see Figures 1−4) show clearly that the extent of drug SE in simple aqueous solutions of the studied surfactants can be much higher than that measured for the same drug + surfactant system, but in biorelevant media. The drug molecular structure did not have a significant effect on the observed trends, which were the same for the two types of steroidal drugs (danazol and progesterone) and one nonsteroidal drug (fenofibrate). Comparison between the results obtained with porcine bile extract and with a single bile salt (NaTDC) demonstrated that bile salts are the main component in the biorelevant media which governs the drug solubility, whereas the other surface active components present in the bile extract (6% phospholipids and 7% fatty acids[34,35]) have only a minor effect on the solubilization of the studied set of drug molecules (Figure 5). Hence, the interactions between the

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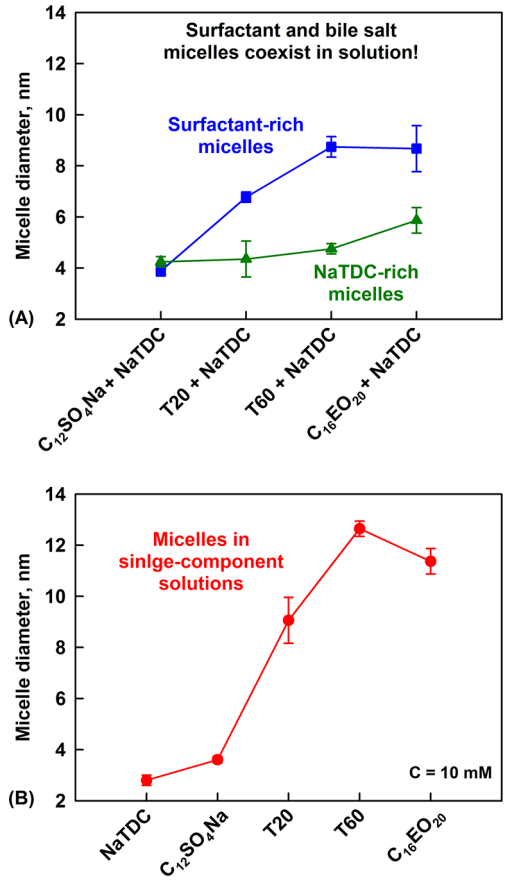

Figure 8. Diameter of the drug-loaded micelles, as determined by ^{1}H DOSY NMR. (A) Surfactant-rich (blue squares) and NaTDC-rich (green triangles) micelles coexist in the binary 1:1 surfactant + NaTDC mixtures, C_{tot} = 10 mM. (B) Micelle size in individual surfactant solutions, C = 10 mM (red circles). The different molecular species in the micelles were identified by their characteristic ^{1}H NMR spectrum peaks. Regression analysis of the decay of at least two characteristic, nonoverlapping ^{1}H NMR peaks per molecule was used to determine the average micelle diameter ($\pm 3\sigma$, n = 3–6). All samples contain 137 mM NaCl and 10 mM KCl. All solutions are studied after solubilization of fenofibrate at T = 37 °C and are measured at the same temperature.

surfactants and the bile salts are the key factors which govern the drug solubility in these complex mixtures.

The significant decrease in drug solubility for the ionic surfactant + bile mixtures can be explained by the formation of mixed micelles with low drug solubilization capacity. The latter hypothesis is supported by the direct measurement of the micelle size and composition by ^{1}H DOSY, which showed that the NaTDC and $C_{12}SO_4Na$ molecules are present in aggregates with the same size ($d_H \approx 4$ nm) in the mixed NaTDC + $C_{12}SO_4Na$ solution (Figure 8). However, the question stands whether NaTDC and $C_{12}SO_4Na$ micelles with the same size coexist separately in the solution, or these are, in fact, mixed micelles. The measured change in the chemical shifts of the ^{1}H NMR spectrum of NaTDC in the mixed NaTDC + $C_{12}SO_4Na$ solution (see Table 2) provides evidence that the surfactant and bile salt molecules are incorporated in the same aggregates. In particular, the transformation of a triplet to doublet of triplets at C-25 and the significant downfield shift at C-26 of NaTDC both demonstrate that the presence of $C_{12}SO_4Na$ changes profoundly the environment of the NaTDC molecules in the micelles, which could only occur when mixed micelles are formed. The downfield shift and the peak splitting are most likely caused by spatial proximity of the hydrophilic tauro-moiety of NaTDC and the sulfate headgroup of $C_{12}SO_4Na$. However, since both headgroups are similarly charged, the electrostatic repulsion prevents them from being in very close contact, as evidenced also by the NOESY spectra. Most likely, the sulfate group of $C_{12}SO_4Na$ is close to the amido-group of NaTDC, which causes the observed changes at C-25 and C-26 of the ^{1}H NMR spectrum of NaTDC. Increased penetration of water between the bile salt and the surfactant head groups in the mixed micelles is also possible, but cannot account for the observed effects: the NMR spectrum at submicellar concentration of NaTDC (1 mM), where its molecules are fully hydrated, shows only a slight positive shift at C-25 and C-26 (see Figure S4 in the Supporting Information).

The significant changes in the chemical shifts of fenofibrate aromatic protons when NaTDC is mixed with $C_{12}SO_4Na$ are due to the changed environment of the solubilized fenofibrate in the micelles. These shifts also support the explanation that mixed micelles are formed in this system. The pronounced minimum in the CMC of the ionic surfactant + NaTDC mixtures (Figure 6) provides additional evidence for the formation of mixed micelles with a strong attraction between the NaTDC and $C_{12}SO_4Na$ molecules.[48,49] Mixed micelle formation in binary NaTDC + C_{16}TAB and NaTCDC + $C_{12}SO_4Na$ mixtures was reported before by Jana et al.[23] We can thus conclude that the ionic surfactants and bile salts form mixed micelles with low solubilization capacity for hydrophobic drugs.

The specific effect of surfactant charge (positive or negative) was minor and was manifested mainly as a shallow minimum in the drug solubility around f_{bile} = 0.5 for the mixtures of cationic

Table 2. Change in the Chemical Shifts of ^{1}H Peaks of NaTDC Molecules in Surfactant + NaTDC Mixed Solutions, after Solubilization of Fenofibrate[a]

	change in the chemical shifts of NaTDC molecules (ppm)			
NaTDC carbon number	$C_{12}SO_4Na$	$C_{16}EO_{20}$	Tween 20	Tween 60
1	+0.021	+0.021	+0.011	+0.012
14-b	overlap	−0.034	−0.029	−0.033
18	no shift	−0.011	−0.013	−0.014
21	+0.023	+0.022	+0.012	+0.012
25 (triplet)	peak splitting (triplet → doublet of triplets)	−0.007	−0.006	−0.007
26 (triplet)	+0.030	−0.019	−0.014	−0.017

[a]The total surfactant + NaTDC concentration is 10 mM at a ratio of 1:1. The reference ppm values used for the calculation of the change in the chemical shifts were determined from the solution of NaTDC (at C = 10 mM) after fenofibrate solubilization. Shifts bigger than 0.001 ppm are considered as significant.

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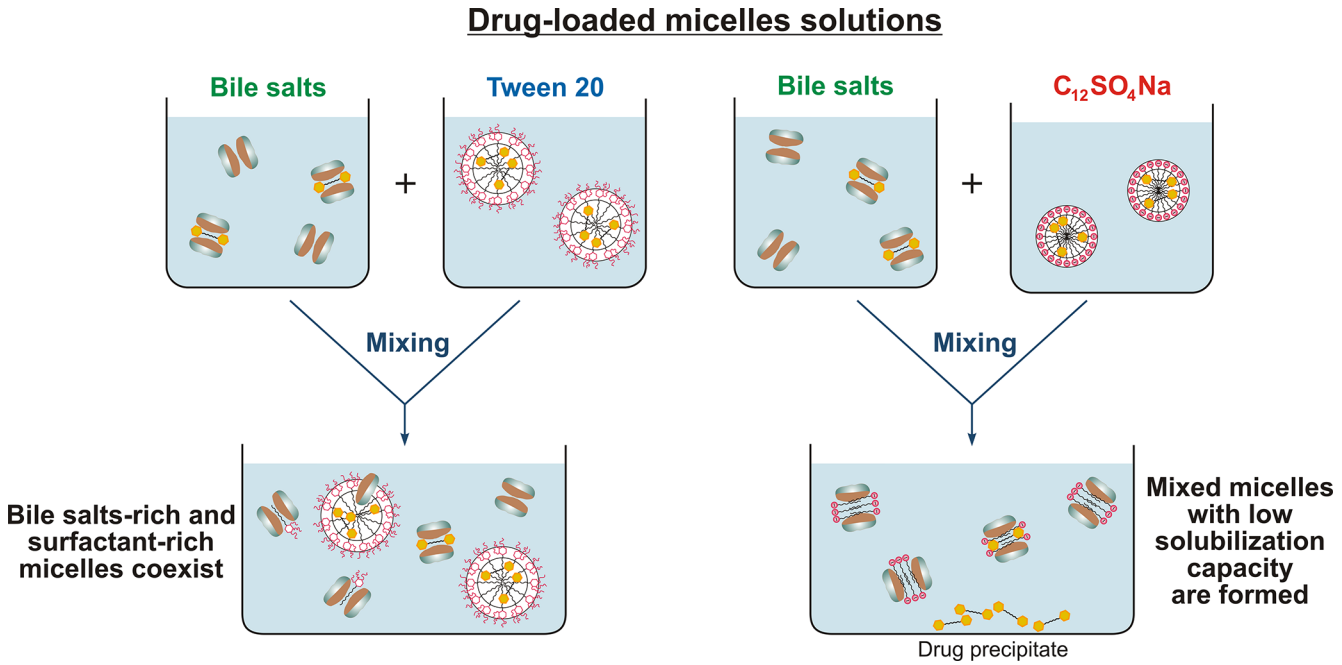

Figure 9. Illustration of the model experiment, aimed to clarify the effect of the biorelevant medium (and of the bile salts present in it) on the solubilization capacity of the surfactant micelles. Drug-loaded micelles of surfactants and bile salts were prepared separately and then mixed at an 1:1 ratio. The solution of Tween 20 + bile remains clear, whereas precipitation is observed in the mixtures of bile salts with ionic surfactants. Counterions are not displayed in order to improve the clarity of the illustration.

surfactant + bile, whereas such minimum was not observed for the anionic surfactant + bile mixtures. The latter difference could be explained by the formation of ionic liquid between the oppositely charged cationic surfactant and bile salt molecules, which decreases the number of mixed micelles in the solution and thus negatively affects drug solubilization. In support of this hypothesis, formation of a phase-separated liquid was experimentally observed for the mixtures of C_{14}TAB + bile extract and C_{14}TAB + NaTDC at bile salt-to-surfactant ratio of 1:1. On the other hand, the small effect of the type of surfactant charge on the general dependence of drug solubility on f_{bile} is most likely due to the high ionic strength in the studied solutions (I = 147 mM), which screens the long-range electrostatic charge−charge interactions.[50]

In contrast to the ionic surfactants solutions, for which a drop in drug solubility was observed upon addition of bile salts, drug solubility decreased in a near-ideal fashion while increasing the bile salt fraction in the bile + nonionic polysorbate surfactant mixtures (Figures 2 and 4). In this case, the ^{1}H DOSY study of micelle composition and size clearly showed that separate NaTDC and polysorbate micelles coexist in these mixed solutions. However, the measured diameters of the micelles in the individual and mixed solutions were not identical (Figure 8); the size of the NaTDC micelles increased in the presence of polysorbate surfactant molecules, whereas the opposite was observed for the polysorbate micelles in the presence of NaTDC. These results could be explained only by assuming a partial mixing of NaTDC and polysorbates in the two types of micelles, which coexist in the mixed solutions. The bigger micelles are composed primarily of polysorbate molecules (surfactant-rich micelles) and contain a small fraction of NaTDC molecules, whereas the smaller micelles contain mainly NaTDC (bile salt-rich micelles) and a small fraction of polysorbate molecules. As the hydrophobic moiety of NaTDC (rigid steroid backbone) is very different from the straight and flexible alkyl chain in the polysorbates, it hinders the packing of the molecules in the micelles and decreases the micelle size. On the other hand, it is reasonable to expect that the incorporation of even a few big polysorbate molecules (M_W > 1200 g/mol) in a micelle composed of NaTDC (M_W = 522 g/mol) should increase the micelle size. In both cases, the fraction of foreign molecules in the micelles should be low (i.e., < 10%), as they are not resolved in the DOSY experiments. The change in the chemical shifts of NaTDC protons in the presence of polysorbates (Table 2) are small, which is in agreement with the proposed mechanism. The near-ideal behavior of the mixed Tween 20 + bile solutions is confirmed also by the relatively good agreement between the CMC values calculated assuming ideal mixing and the experimentally determined ones (Figure 6).

To verify further the mechanisms proposed to explain the solubilization behavior of the studied surfactant + bile mixtures, an additional experiment was performed. Separate solutions of Tween 20, $C_{12}SO_4Na$, C_{14}TAB, and bile, which were saturated with fenofibrate, were prepared, following the standard experimental procedure. In this way, clear solutions containing drug-loaded micelles for each of the 4 amphiphiles were obtained. Then, the bile solution was mixed at an 1:1 ratio with each of the 3 surfactant solutions. Precipitation was observed immediately after the drug-loaded micelles of the charged surfactants ($C_{12}SO_4Na$ and C_{14}TAB) were introduced in the biorelevant media, whereas the solution obtained by mixing of Tween 20 + bile remained clear (see Figure 9 for a schematical representation).

The latter results are in agreement with the proposed mechanisms and confirm that (i) the micelles of $C_{12}SO_4Na$ and C_{14}TAB are disrupted by the bile salt molecules, (ii) the ionic surfactant + bile salt mixed micelles have much lower solubilization capacity than the single-component $C_{12}SO_4Na$ and C_{14}TAB micelles, and (iii) the nonionic surfactant micelles are not significantly affected by the presence of bile.

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CONCLUSIONS

The effect of surfactants on the solubility of poorly water-soluble drugs in biorelevant dissolution media was studied in surfactant, bile, and bile + surfactant solutions at different bile and surfactant concentrations, mimicking those in the human intestinal tract. The role of bile salt−surfactant interactions on drug solubility was confirmed by experiments with mixtures of surfactant and a single bile salt (NaTDC). NMR spectroscopy provided additional information about the micelle size and intramicellar interactions between the surfactant and bile salt molecules. The main conclusions of the study are summarized as follows:

- Addition of bile salts to ionic surfactant solutions leads to drug precipitation due to the formation of mixed bile + surfactant micelles with drug low solubilization capacity. In contrast, the drug remains solubilized in nonionic surfactant + bile salt mixtures.
- The different behaviors of the mixed nonionic surfactant + bile solutions are due to the formation of separate surfactant-rich and bile salt-rich micelles, as evidenced by ^{1}H DOSY.
- Strong interactions between the headgroup of the alkylsulfate surfactant and the amido group of NaTDC explain the formation of mixed micelles for these similarly charged surfactants.
- Electrostatic attraction between the oppositely charged trimethylammonium bromide surfactants and the bile salts drives the formation of ionic liquids at ratios close 1:1 and of mixed micelles at the other ratios.

An essential conclusion of the study is that drug solubility is affected very significantly by specific bile−surfactant interactions, in which the drug molecules do not play a major role *per se*. Hence, the similar effects, demonstrated in the current study with fenofibrate, danazol, and progesterone, should be valid for other poorly water-soluble drugs, as long as the drug solubilization has a small effect on the properties of the mixed surfactant + bile micelles.

The large impact of surfactant−bile interactions on the drug solubility highlights the role of the complex interplay between commonly employed pharmaceutical excipients and the typical components of the human intestinal fluids. These interactions can significantly change one of the key parameters which governs the oral absorption of poorly water-soluble drugs. The obtained mechanistic understanding could be implemented in existing oral absorption simulators and PBPK models, with the aim to improve the in silico predictions and to facilitate the early drug development.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.8b00884.

^{1}H NMR chemical shifts of fenofibrate solubilized in surfactant and NaTDC micelles; determination of micelle size by DOSY; change in the ^{1}H chemical shifts of surfactant molecules in surfactant + NaTDC solutions; change in the chemical shifts of fenofibrate aromatic protons in surfactant + NaTDC mixtures; determination of the diffusion coefficient and aggregate size from different ^{1}H signals in a DOSY spectrum; ^{1}H NMR spectra of fenofibrate after solubilization in surfactant, NaTDC, and NaTDC + surfactant solutions; ^{1}H NMR spectra of NaTDC at concentration above CMC, below CMC, and in mixture with $C_{12}SO_4Na$; diameters of the empty and drug-loaded micelles of NaTDC, $C_{12}SO_4Na$, and Tween 20; fit of the ^{1}H peak intensity decay with increasing gradient strength; surface tension isotherms used for CMC determination; pH of bile extract + surfactant and NaTDC + surfactant mixtures (PDF)

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