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Title: BILE SALTS IN DIGESTION AND TRANSPORT OF LIPIDS

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Abstract

Because of their unusual chemical structure, bile salts (BS) play a fundamental role in intestinal lipid digestion and transport. BS have a planar arrangement of hydrophobic and hydrophilic moieties, which enables the BS molecules to form peculiar self-assembled structures in aqueous solutions. This molecular arrangement also has an influence on specific interactions of BS with lipid molecules and other compounds of ingested food and digestive media. Those comprise the complex scenario in which lipolysis occurs. In this review, we discuss the BS synthesis, composition, bulk interactions and mode of action during lipid digestion and transport. We look specifically into surfactant-related functions of BS that affect lipolysis, such as interactions with dietary fibre and emulsifiers, the interfacial activity in facilitating lipase and colipase anchoring to the lipid substrate interface, and finally the role of BS in the intestinal transport of lipids. Unravelling the roles of BS in the processing of lipids in the gastrointestinal tract requires a detailed analysis of their interactions with different compounds. We provide an update on the most recent findings concerning two areas of BS involvement: lipolysis and intestinal transport. We first explore the interactions of BS with various dietary fibres and food emulsifiers in bulk and at interfaces, as these appear to be key aspects for understanding interactions with digestive media. Next, we explore the interactions of BS with components of the intestinal digestion environment, and the role of BS in displacing material from the oil-water interface and facilitating adsorption of lipase. We look into the process of desorption, solubilisation of lipolysis products and formation of mixed micelles. Finally, the BS-driven interactions of colloidal particles with the small intestinal mucus layer are considered, providing new findings for the overall assessment of the role of BS in lipid digestion and intestinal transport. This review offers a unique compilation of well-established and most recent studies dealing with the interactions of BS with food emulsifiers, nanoparticles and dietary fibre, as well as with the luminal compounds of the gut, such as lipase-colipase, triglycerides and intestinal mucus. The combined analysis of these complex interactions may provide crucial information on the pattern and extent of lipid digestion. Such knowledge is important for controlling the uptake of dietary lipids or lipophilic pharmaceuticals in the gastrointestinal tract through the engineering of novel food structures or colloidal drug-delivery systems.

Contents

List of Abbreviations

Beta-lactoglobulin: BLG
Bile Acids: BA
- Cholic acid (CA)
- Chenodeoxycholic acid (CDCA)
- Glycocholic acid (GCA)
- Glycochenodeoxycholic acid (GCDCA)
- Taurocholic acid (TCA)
- Taurochenodeoxycholic acid (TCDCA)

Bile extract: BE
Bile salts: BS
- Cholate (C)
- Deoxycholate (DC)
- Chenodeoxycholates (CDC)
- Glycodeoxycholates (GDC)
- Taurocholates (TC)

Cellulose nanocrystals: CNC
Critical micelle concentration: CMC
Dietary fibre (DF)
- Methylcellulose (MC)
- Hydroxypropylcellulose (HPC)
- Hydroxypropylmethylcellulose (HPMC)

Diffusion coefficient (effective diffusivity, D_{eff})
Dynamic light scattering (DLS)
Fatty acids (FA)
Free fatty acids (FFA)
Gastrointestinal (GI)
Mean-square displacement (MSD)
Nanoparticles (NP)
Nuclear magnetic resonance (NMR)
Phospholipid (PL)
Phosphatidylcholine (PC)
Small-angle X-ray scattering (SAXS)
Whey Protein Isolate (WPI)

1. General introduction to BS

Bile is a very diverse physiological fluid, with a wide range of functions. It was recognised as early as 200 AD by Aelius Galen as one of the four humours of the body [1]. However, the plethora of functions of bile has only recently been identified. Bile is a complex mixture of many compounds, and some of them, e.g. BS, are surface active. In this article, we will review the synthesis, composition and path/fate of bile and the BS in the human body. However, the focus will be on the surfactant-related functions of bile salts in the gastrointestinal (GI) tract, such as interactions with food emulsifiers and dietary fibre, facilitating of the interfacial adsorption of lipase and colipase to the lipid substrate interface, and finally the role in intestinal transport of lipids through the participation in formation of mixed micelles and furthering colloidal transport in the intestinal mucus. These processes are illustrated schematically in Figure 1.

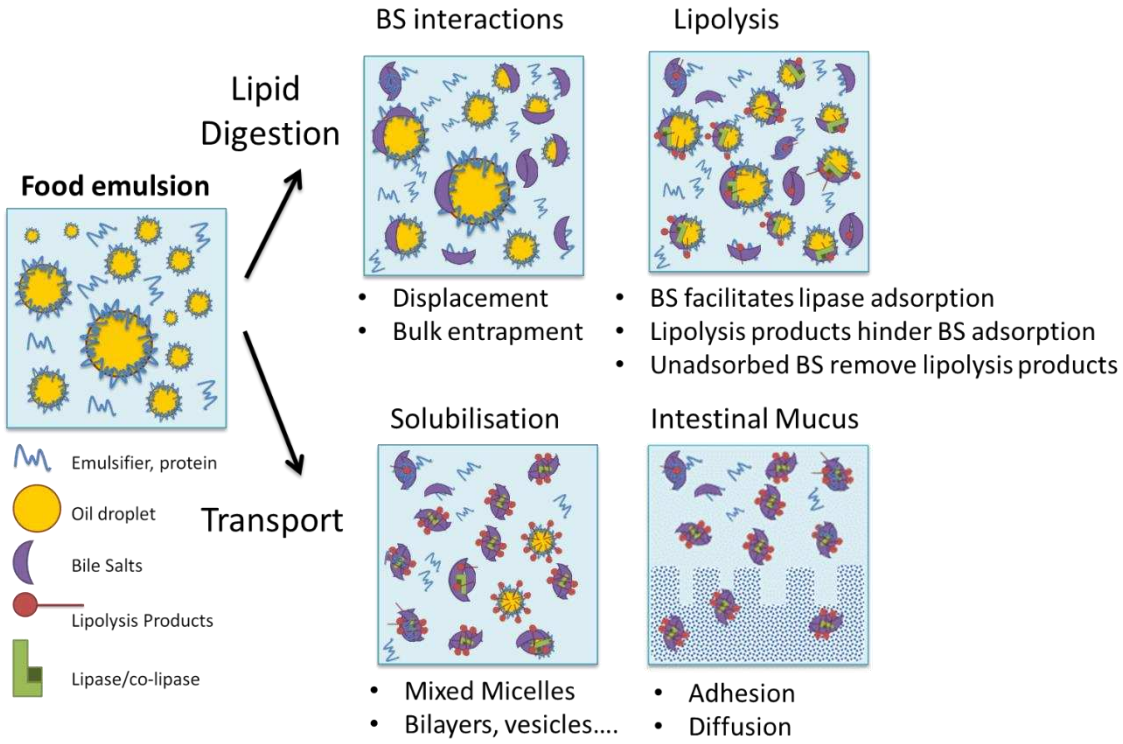


Figure 1. Schematic representation of the roles of bile salts (BS) in the intestinal digestion and transport of lipids. Only the main interactions, important for surfactant-related functions of BS, are shown.

1.1. Chemical structure

Bile is comprised of 95% water, in which there is a number of inorganic and organic constituents, including bile acids (40 mM), cholesterol (3 mM), sodium and potassium (145 mM and 4 mM, respectively), and phospholipids (7 mM), with a pH of 7.5-8.0 [2]. The colour of bile is determined by the concentration of bilirubin. Bile acids (BA) are physiologically planar surface active molecules, initially synthesised from cholesterol in the liver [3], as shown in Figure 2A. Those synthesised from cholesterol are generally known as primary BA, and in humans these are cholic acid (CA) and chenodeoxycholic acid (CDCA). The primary synthesis

begins with the hydroxylation of cholesterol with cholesterol 7α-hydroxylase, also known as the neutral (or classic) pathway [4], as shown in Figure 2B.

Most of the BA, before leaving the hepatocytes, are conjugated with glycine or taurine. These conjugated BA are glycocholic acid (GCA), taurocholic acid (TCA), glycochenodeoxycholic acid (GCDCA), and taurochenodeoxycholic acid (TCDCA). The conjugated BA are also known as BS, because the pK_a of conjugated BA is lower than the unconjugated form (pK_a of 3.9 for glycine conjugated vs 5.0 for unconjugated BA), and the BA exist in their deprotonated form in the duodenum [5]. For the purpose of this review, BA, when they have left the ampulla of Vater and entered the duodenum, are termed BS.

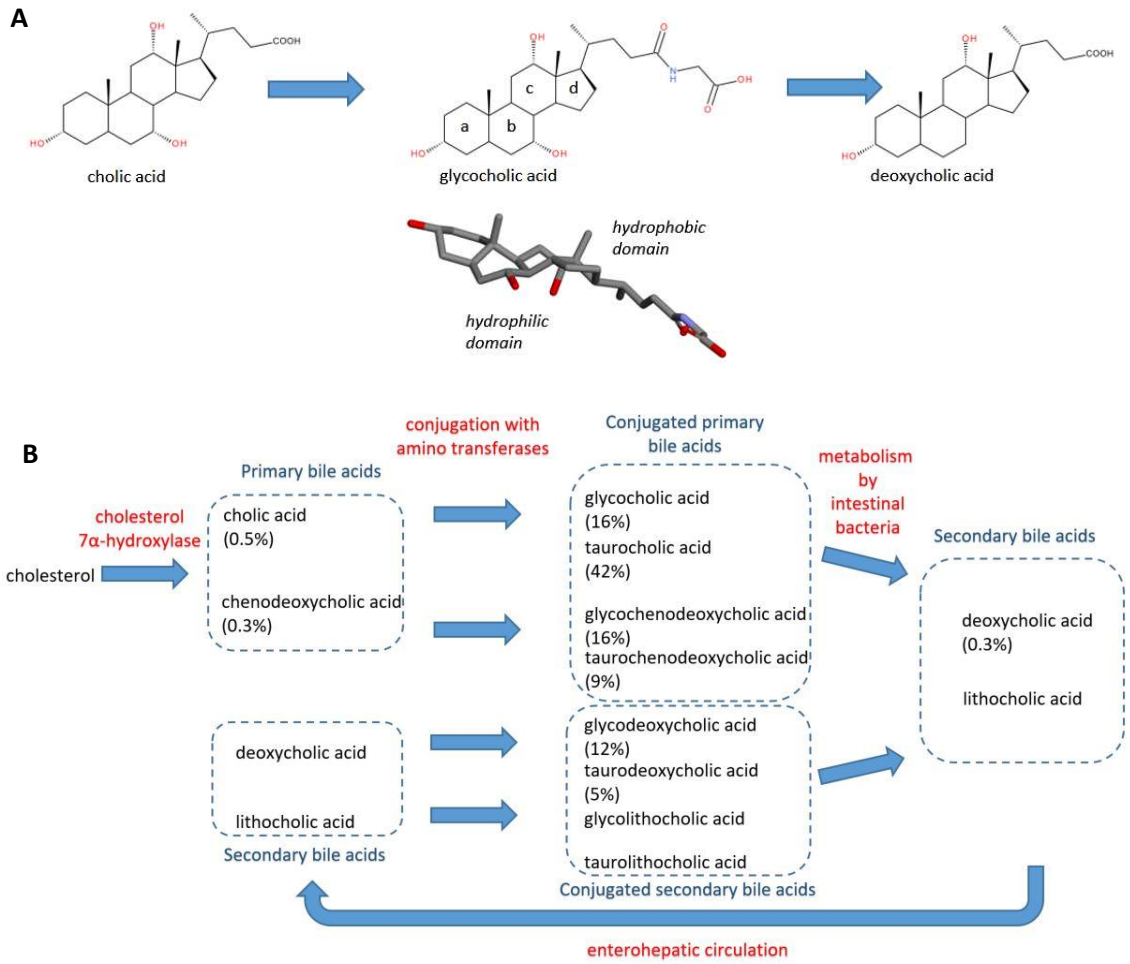


Figure 2. (A) Exemplary structures of primary, conjugated primary, and secondary bile acids (BA). The BA is comprised of three six-carbon rings (a, b, and c), as well as a five-carbon ring (d), with a flat structure depending on the cis- or trans- conformation between the first two rings (a and b). The concave side of the BA is hydrophilic due to the presence of the hydroxyl groups, whereas the convex side of the steroid skeleton is hydrophobic with a number of methyl groups. (B) Schematic representation of the path of formation of various primary, conjugated primary, secondary and conjugated secondary BA, including the recycling of the bile salts (BS) via enterohepatic circulation. The approximated duodenal BS composition is given in percentage (where available) [6].

The conjugation takes place within the hepatocytes with BA CoA-ligase, and BA-CoA:amino acid N-acyltransferase [7], occurring in both the endoplasmic reticulum and peroxisomes of the cells [8]. The process of conjugation decreases the hydrophobicity of BA, as shown in Table 1. A change in the ratio of glycine and taurine (normally 3:1) conjugation may be considered an indicator of liver disease [9,10], however it may also change with diet [11]. The decreased hydrophobicity, as well as the lower pK_a of the BA, limits re-adsorption of the BA (Boyer, 2013). The BA leave the hepatocytes by several ATP-driven export pumps into the canaliculus, a small space formed between adjoining hepatocytes. The BA then flow into bile ductules, and eventually into the common bile duct [5]. Once excreted, the BA become part of mixed micelles, which include also phospholipids (PLs); predominantly phosphatidylcholine (PC), and sterols (mostly cholesterol) [12]. As the BA travel through the biliary system, they pass by cholangiocytes. These release bicarbonate into the bile, thereby making the bile more alkaline. Finally, the bile is stored in the gallbladder [13]. The gallbladder will contract, after a meal is consumed, resulting in the release of bile into the duodenum.

Table 1 Changing hydrophobicity of various primary, conjugated primary and secondary bile acids. Data from Heumann et al. [14]

Bile acid	Hydrophobicity
Secondary conjugated lithocholic acid	
Secondary unconjugated deoxycholic acid	
Secondary conjugated deoxycholic acid	
Primary unconjugated chenodeoxycholic acid	
Primary conjugated chenodeoxycholic acid	
Unconjugated cholic acid	
Conjugated cholic acid	

BA have a wide range of critical micellar concentrations (CMCs) in water. The CMC of primary BA ranges between 5.9 and 17 mM, with a mean of 10.7 mM [15–19], whereas the CMC of conjugated primary BA ranges between 4 and 15 mM [20–22] with a mean of 6.7 mM, and that for secondary and conjugated secondary BA ranges between 2.8–7.5 mM and 1.9–8 mM, respectively, with means of 4.4 mM and 3.8 mM [20,23,24]. The data are presented in Figure 3.

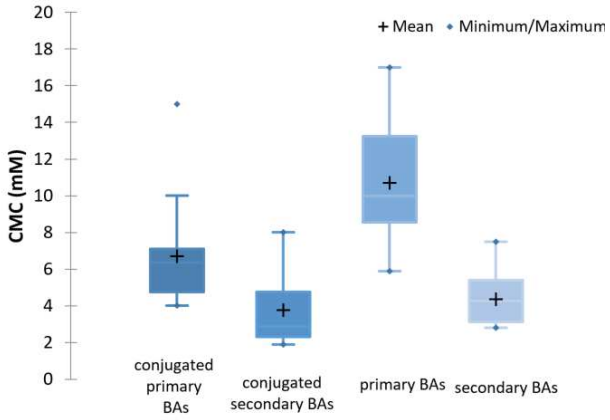


Figure 3. Comparison of the critical micelle concentration (CMC) values of various groups of bile acids (BA). Shown are the mean, minimum and maximum; and the bands represent the first and third quartiles of the CMCs. A total of 112 CMC values were collated.

On average, primary BA display a significantly higher average CMC than either conjugated primary, secondary, or conjugated secondary BA. The BS micelles have a very small aggregate number of 2 to 12 BA units [18]. Additionally, unlike traditional surfactants, the planar BA, have a low potential for solubilisation of hydrophobic molecules [25]. The solubilising potential of the micelles is enhanced by up to three times if the BA are mixed with PLs [26].

1.2. Physiological function of BS

BS and BA have a wide range of functions, from emulsification of dietary lipids, and facilitating their digestion and absorption, to signal transduction in cell proliferation, metabolism, and differentiation [27]. As a direct mode of action, BS form mixed micelles, which aids in the absorption of lipolysis products and lipid-soluble vitamins. Further details on the function of BS in lipid digestion will be discussed in the next sections. Indirectly, BA regulate their own synthesis through the farnesoid receptor X. This receptor is located through the biliary system, as well as in the ileal enterocytes. Activation of the farnesoid receptor X inhibits cholesterol 7α-hydroxylase. Impairment of BA formation results in a range of cholestatic diseases. These can lead to cirrhosis, fibrosis, liver failure, hepatocellular carcinoma, as well as cholangiocarcinoma. Lower than normal concentrations of BA in bile can result in supersaturation of cholesterol, which may lead to cholelithiasis [28].

At any given point in time, ca. 85-90% of the bile is in the small intestine, less than 1% is in the liver, and ca. 10-15% are stored in the gallbladder [13]. Intestinal BS are mostly conjugated (as shown in Figure 2B), and confer antimicrobial properties, by changing or disrupting the bacterial membrane, or by denaturing membrane proteins. This enables to control microbial overgrowth in the intestine [2,29]. Bacteria in the ilium and colon metabolise BS. Primary BA are first deconjugated and 7α-dehydroxylase transform them into secondary deoxycholic and lithocholic acids. A small amount of tertiary ursodeoxycholic acid is also produced by 7β-hydroxysteroid dehydrogenase [13]. This deconjugation of BS reduces their solubility and increases toxicity. However, about 95% of BS are reconjugated and readsorbed by enterocytes in the ileum. From there, the BA enter the portal blood, and are recycled in the liver. The remaining 5% (or ca. 0.5 g of the total BA) are removed from the body by faecal excretion.

2. Bulk interactions of bile salts with emulsifiers and dietary fibres

Once in the small intestine, BS have a number of functions to aid digestion. This section focuses on reviewing interactions between BS and a broad range of food emulsifiers and stabilisers in bulk. These interactions are relevant since they may interfere with the role played by BS in food digestion, and thus have an impact on the hydrolysis, transport and absorption of nutrients such as lipids and amino acids, which will be addressed specifically later on. The mechanisms of interactions will certainly depend on the nature of the molecules involved and the level of study (mesoscopic or molecular). At a meso-scale, physical entrapment of BS could be expected, for instance, if dealing with water-soluble high-molecular weight stabilisers that provide relatively high bulk viscosities. On the other hand, at molecular level, electrostatic and/or hydrophobic interactions may occur if ionic and/or non-ionic amphiphilic emulsifiers/stabilisers are present. In addition, these will depend on changes in solution and environmental conditions such as pH, ionic strength, solvent composition and temperature. All

these parameters will be taken into account when reviewing interactions between BS and individual dietary fibres, proteins and low-molecular weight surfactants of lipidic and non-lipidic nature. *In vitro* studies mimicking the physiological environment in the small intestine (pH, ionic strength, temperature, etc.) are of major interest here since *in vivo* studies usually only report on the BS content of excreted or serum samples, from which little or no details on the intermediate mechanisms of interactions can be elucidated [30,31]. In addition, defining interactions between BS and food emulsifiers/stabilisers in animal or human gastrointestinal (GI) contents is not trivial due to the complexity of the media [32]. Therefore, *in vitro* models seem appropriate to contribute building up the comprehensive knowledge on the role of BS in digestion.

2.1. BS and dietary fibres

The term dietary fibre (DF) was first postulated as indigestible carbohydrates of plant origin. This term was then restricted to indigestible plant carbohydrates with associated health benefits, comprising non-starch polysaccharides, lignin and other analogous polysaccharides. Later on, the definition has expanded to include oligosaccharides with properties similar as water-soluble DF, and indigestible resistant starches behaving as DF in the large intestine [33]. A broader definition also considers fibres of animal origin, such as chitosan, and modified or synthetic carbohydrate polymers. The interactions between BS and DF have increasingly received interest in the last decade due to the health benefits associated with the passage of the latter through the GI tract, despite their indigestible nature [33,34]. The physiologic effects of a particular type of DF within the GI tract are attributed to its physicochemical properties such as solubility, degree of viscosity, and fermentation in the large intestine [33]. Dietary fibres which are water-soluble, hereinafter termed as soluble DF, and either viscous or gel-forming under GI conditions, may delay gastric emptying and small bowel transit and retard the transport and absorption of glucose, triglycerides and cholesterol from the small intestine [34]. This delaying effect is reduced as the molecular weight of DF, and thus viscosity, decreases. Soluble DFs are also very accessible to bacterial enzymes, and therefore very rapidly fermented in the proximal colon, tending to have less impact on colonic transit. On the other hand, insoluble DFs do not affect the viscosity of gastric and intestinal contents, tending to accelerate small bowel transit, and have more marked laxative effect contributing to faecal bulking [35]. The healthy attributes of DFs are associated with their physiological effects within the GI tract. The reduction of blood cholesterol and hyperlipidaemia is thought to be linked with the interactions with BS, amongst other mechanisms. *In vivo* studies in humans have shown that 2- or 3-day-diet with increased content of DF induces increased bile excretion within the 24 h upon consumption [31,36]. One of the possible reasons for this is that DF has a bile sequestering capacity, leading to bile excretion. An alternative, but non-exclusive, explanation is that DF decreases the permeability of the intestinal mucus layer lining the surface of epithelium, and thus hindering bile absorption [37]. These two combined mechanisms may alter the bile reabsorption in the distal small intestine (ileum) into the enterohepatic circulation [38]. Therefore, more BA need to be synthesised *de novo* from cholesterol in blood in order to restore the bile pool. On the other hand, interactions between DF and BS may also lower the rate of lipid digestion and uptake, with subsequent reduced lipid concentration in plasma.

In general, direct interactions at a molecular level and/or reduction of bile diffusion by entrapment in polymer network are the two plausible mechanisms of interactions between DF and BS. Most of the investigations on the binding of BA to DF are based on centrifugation or dialysis methods, where the free bile content is measured in the supernatant or dialysate, respectively, and/or the bound bile is assayed in the pellet or dialysis sample, respectively [39–41]. Nevertheless, to date, scarce information is available in literature regarding specific molecular mechanisms of interactions between BS and DF to help elucidating the pathway or cascade of events related with the healthy attributes of dietary fibre. DFs occur in isolated, more or less soluble form or as a part of the more or less intact complex cell wall architecture in the diet, making it difficult to find the mechanisms behind their physiological effects [39]. Studies on DF retaining an intact cell wall structure from fruits, vegetables and cereals did not provide conclusive results on the specificity of interactions with BA or the effect of the composition (e.g. soluble versus insoluble) that could contribute to the extent of binding with BS [39]. For all these reasons, we will focus on the interactions of isolated soluble DF and BS. Insoluble DF has rather low cholesterol-reducing effect as compared to soluble DF [40], which may be linked to its rather different physiological effect within the GI tract, as pointed out above. In addition, isolated insoluble DF such as cellulose do not exhibit BA binding properties. Dongowski observed that isolated microcrystalline cellulose do not bind BA *per se* [39], which may be explained by the hydrophilic nature of this fibre composed of linear chains of several thousand glucose units. Therefore, the author attributes its role in BA binding when it stabilises the cell wall architecture after the digestion process.

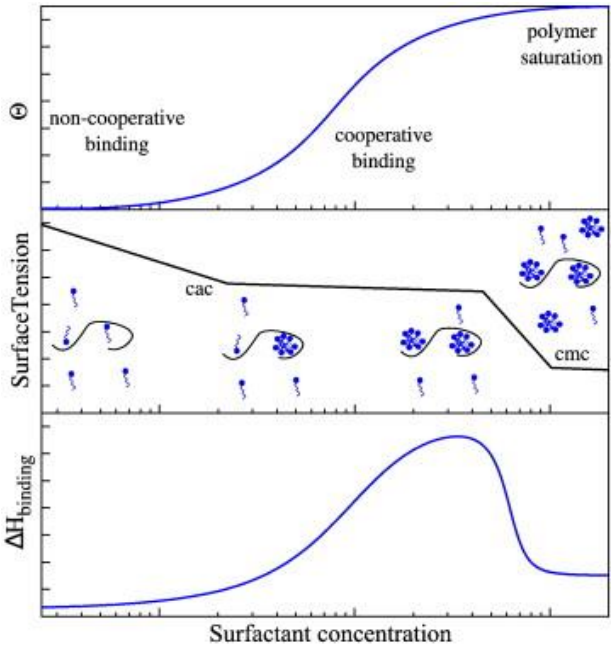


Figure 4. General trend of (from top to bottom) a binding isotherm, surface tension and enthalpy of binding in polymer-surfactant systems, as a function of surfactant concentration. Reprinted from [42]. Copyright (2015), with permission from Elsevier.

The interactions of BS with isolated soluble DFs depend on the physicochemical characteristics of fibres. Soluble DFs are composed, in principle, of biopolymers, of which backbone is based mainly on carbohydrates. These vary in the molecular weight, branching, type of substitution groups, distribution of substitution groups, surface charge and hydrophobic pockets, etc.,

which in turn depend on the pH and ionic strength conditions. In general, the interactions between BA and biopolymers can be described as a cooperative process and depend on the concentration regime of BA, which is the same as for any other surfactant. An example of a typical binding isotherm, trend in surface tension and binding enthalpy in surfactant/polymer systems is shown in Figure 4.

2.1.1. BS and cereal β-glucan/arabinoxylan

Cereal *β*-glucan consists of a linear backbone of glucosyl units. The consumption of 3 g/day of *β*-glucan present in fibre-rich meals from oats and barley has associated health benefits, such as reducing or maintaining normal blood LDL-cholesterol levels, amongst others [43,44]. This health claim may be more related to the interactions with BA, and not to the inhibition of cholesterol absorption or synthesis, as BA have been shown to be excreted to larger extent with the intake of high-molecular weight *β*-glucan [45]. Hydrophobic and electrostatic interactions between BA molecules and *β*-glucan are not expected due to the hydrophilic and non-ionic nature of the latter. Indeed, direct binding of BA onto *β*-glucan fractions was not observed by nuclear magnetic resonance (NMR) under physiological conditions of pH in the presence of GCA [46]. Therefore, other physical mechanisms, such as bile micelles entrapment by viscous solution of *β*-glucan may occur with this soluble dietary fibre. Similar mechanism has been hypothesised for arabinoxylan [39]. Arabinoxylan is a heteropolymer comprising a xylopyranose backbone with substituents of α-L-arabinofuranose. Arabinoxylan present in wheat and rye is thought to have similar cholesterol-reducing properties as *β*-glucan [47]. NMR-titration studies evidenced direct dynamic molecular contact between *β*-glucan and BS micelles of NaTCDC under physiological duodenal pH, as reflected in consistent chemical shifts in BS resonance in the presence of *β*-glucan, whereas no direct molecular interactions occurred between arabinoxylan and the same BS [48]. The reduced intensity in BS signal in the presence of arabinoxylan suggests that arabinoxylan forms local entangled polymer network reducing the mobility of BS micelles. Both mechanisms may explain the delayed passage of NaTCDC and porcine bile through dialysis membrane in the presence of the polysaccharides [49], the effect being greater at higher polymer concentration, and thus viscosity. These mechanisms of interaction between *β*-glucan or arabinoxylan and BS were further corroborated with another BS, NaGCDC, and the whole porcine bile by NMR spectra and small-angle X-ray scattering (SAXS), and were found to be combined depending on the source of the soluble DF and its molecular weight [50]. Namely, high molecular weight barley and oat *β*-glucan and low molecular weight wheat arabinoxylan and high molecular weight rye arabinoxylan showed the two combined mechanisms of interaction with BS.

2.1.2. BS and chitosan

Chitosan is a biopolysaccharide that is positively charged under acidic conditions and is frequently used in food processing. It is derived from chitin exoskeleton of arthropods by alkaline or enzymatic deacetylation, so that the linear molecular structure is composed of randomly distributed glucosamine and N-acetylglucosamine units. The interactions between oppositely charged biopolymers and surfactants have shown pronounced synergisms that lead to interesting assembly features [42]. Chitosan and the BS, NaTC, have been reported to bind by electrostatic interactions at pH 3, at which chitosan is positively charged, and a wide range

of temperature (10-40 °C) [51]. The technique used was isothermal titration calorimetry. It is likely that electrostatic interactions take place between the sulfated head-group of NaTC and ammonium ion in chitosan. The effect of salt addition (NaCl), which screened the electrostatic attraction between the BS and chitosan or the repulsion between bile molecules, supported this as a major driving force for BS binding. Also, the temperature dependence of the binding enthalpy at intermediate BS concentrations had the opposite trend to that of the demicellization enthalpy. This suggested that the BS binds to chitosan forming micelle-like clusters. Chitosan also possesses hydrophobic pockets along the backbone, which play a role in interacting with amphiphiles, such as BS, through the apolar steroidal backbone. Indeed, these interactions have led to the design of chitosan-BA structures for encapsulation of lipids [52] and for responsive drug release upon stimuli applied: an increase of temperature and salt concentration [53].

2.1.3. BS and cellulose ethers

Cellulose ethers are derived from cellulose by chemical modification. They are obtained by partially reacting the hydroxyl groups in each glucose ring in the native cellulose backbone. These cellulose derivatives are widely used in the food industry due to their functional properties, such as surface activity and thickening efficiency. Several studies have investigated the bulk interactions between cellulose ethers and BS, NaTDC and NaTC, by micro-differential scanning calorimetry and rheology under neutral pH conditions [54,55]. Hydrophobic association of BS and non-ionic cellulose ethers: methylcellulose (MC), hydroxypropylmethylcellulose (HPMC) and hydroxypropylcellulose (HPC), was hypothesised based on the bile-induced inhibition of the thermogelation properties of cellulose ethers. This effect was observed from the shift of the thermal transition to higher temperatures and the reduction of the transition enthalpy, as well as from the weakened gel strength in the presence on BS, which depended on the degree of hydrophobicity of the BS and cellulose ether [55]. Namely, the more hydrophobic BS (NaTDC) hindered to a larger extent the thermal structuring of all cellulose ethers tested, suggesting greater extent of adsorption onto fibre molecules and reducing their hydrophobic association. Examination of surface tension has also shown evidence of molecular association between the BS, NaDC, and ethylhydroxyethyl cellulose in aqueous basic solutions. This was reflected in the observation of a first plateau in the surface tension-log BS concentration curve, followed by a decrease in surface tension above the concentration of cellulose ether saturation by NaDC, and a second plateau corresponding to the formation of pure BS micelles [56]. This could be considered a typical trend, similar to that shown in Figure 4 (middle panel) for mixtures of polymers and surfactants. Pizones Ruiz-Henestrosa et al. also observed bulk interactions between HPMC of different molecular weights and hydrophobicity with the whole porcine bile extract (BE) in simulated intestinal media [57]. The association was assessed by measuring particle size and conductivity, and by comparing cloud point temperature. The BE aggregates formed at a physiological concentration dissociated in the presence of both types of HPMC. A more recent NMR study has proved interactions between a model BS, NaTC, and HPMC in simulated intestinal media[58]. Interactions between the BS and HPMC molecules were favoured in the simulated intestinal media mimicking the fasted state (lower BS concentrations) and might be driven by hydrophobic forces. On the contrary, BS self-association predominantly occurred in the simulated intestinal media mimicking the fed state (higher BS concentrations). Ionic strength

influenced the interactions between HPMC and NaTC, probably by screening the repulsion between BS molecules, but the ion type had a minimal effect.

2.1.4. BS and pectin

Pectin is a soluble fibre commonly present in fruits and vegetables and is used as a gelling agent in the food industry. It is a linear and branched polysaccharide comprising a backbone of linked galacturonic acid monomers and branches of monosaccharides: galactose, mannose, glucose and xylose. Interactions between pectin and BE or pectin and the BS, NaTC, have been reported [59,60]. It was found that BE promoted the aggregation of pectin, which was visualised by turbidity and optical microscopy, and isothermal titration calorimetry suggested the direct binding of bile onto pectin molecules. Electrostatic attraction between both anionic components, pectin and BS, at pH 7 are not expected, but hydrophobic interactions between the apolar steroidal backbone of BA and the methoxyl groups in the pectin molecules may occur. Interestingly, an early study showed that contaminants in commercial pectin samples were responsible for this binding, instead of the purified fraction of native high-molecular weight pectin. The authors analysed those possible interactions by NMR spectroscopy and dialysis experiments [61]. The BS NaTC also showed binding to complexes of pectin and *ε*-polylysine [60], which is a cationic biopolymer frequently used in the food industry as preservative. This is relevant since these kinds of complexes are likely to be present in real food matrices, and thus enter the GI environment, and binding of pectin with BS may be affected. However, these results showed the ability of a model BS to bind biopolymer complexes formed by electrostatic interactions. On the other hand, Singh et al. have recently modelled the interactions of several BA (unconjugated CA, DCA, CDCA, and their glyco-conjugated counterparts) with pectin through molecular docking, and provided the estimated strength of the binding energy [62]. They showed that GCA, GDCA and CA had a larger binding affinity than DCA, CDCA, GCDCA. It seems surprising that the more hydrophilic GCA (conjugated and trihydroxy BA) showed more favourable interactions than the more hydrophobic unconjugated and dihydroxy BA (DCA and CDCA). Thus, other important factors, such as hydrogen bonding, might be underestimated in molecular docking to accurately model experimental conditions.

2.2. BS and emulsifiers

2.2.1. BS and Proteins

Less attention has been paid in scientific literature to interactions between proteins and BA, [63,64], despite their relevance to determining not only the bioaccessibility and bioavailability of amino acids, but also the ascribed cholesterol-lowering effect [65]. Indeed, interactions between protein and BS can be a determining factor used in protein purification based on the predominant role played by hydrophobic interactions [64].

In vitro studies on protein isolates are the focus here to account for the sole contribution of peptides in the interactions with BS. Soy protein isolate and wheat gluten have been reported to have a BA binding capacity of 17% and 12%, respectively, under duodenal pH conditions. Cholestyramine, a BA binding resin, was considered a reference of 100% BA binding capacity in this study [66]. The authors used a mixture of BA formulated with 75% tauro-conjugated and

418 25% glyco-conjugated C, CDC and DC BA. On the other hand, acid-soluble lupin protein isolate
419 and corresponding hydrolysates have been shown to bind different BA to a greater extent than
420 soy protein counterparts with no particular trend among different BA [67]. Protein
421 hydrolysates need to be considered as well since proteins are digested by GI enzymes,
422 although the extent of digestion will depend on the native protein molecular structure. No
423 differences between BA binding capacity of undigested protein isolate (91% purity) and
424 hydrolysates (≤20% degree of hydrolysis) were found in the latter study. This contrasts with
425 the decreased BA binding capacity of lentil protein hydrolysates as compared to the
426 undigested lentil protein concentrate [68]. The higher degree of hydrolysis in the latter study
427 (>25%) may account for these differences, as well as the lower protein purity of the lentil
428 protein concentrate (ca. 80%). All these earlier studies were based on centrifugation technique
429 and the BA binding capacity was assayed from the free bile content in the supernatant.
430 Therefore, no details on molecular mechanisms can be elucidated from this approach.

431 The use of UV-Vis absorption, fluorescence and circular dichroism have shed light on the
432 interactions between zein and NaTC at submicellar BS concentration. The combination of
433 techniques showed that protein undergoes conformational changes in the secondary and
434 tertiary structure in the mixed protein-BS aggregate above certain NaTC concentration [69].
435 The experimental procedure was carried out at pH 4, at which electrostatic binding is likely to
436 occur between positively charged zein nanoparticles (NPs) (IP at pH 6.2) and negatively
437 charged NaTC. Nevertheless, hydrophobic binding may also contribute, and eventually have a
438 similar unfolding effect under neutral pH conditions, which are usually typical of the small
439 intestinal lumen. In addition, BS are often present at higher concentrations (above the CMC) in
440 the more realistic conditions within the small intestine, thus the effect observed in the latter
441 study at submicellar BS concentration may be also envisaged. The conformational changes in
442 the protein secondary structure induced by BS [70,71] may explain the enhancement of
443 protein digestion for some dietary proteins (*β*-lactoglobulin (BLG), bovine serum albumin,
444 myoglobin and a commercial dietary protein supplement) observed by Gass et al. in the
445 presence of a mixture of conjugated BA [72], and their antimicrobial effect [71]. Indeed, BS
446 may bind to the hydrophobic pockets of protein, inducing changes in the native structure and
447 exposing hidden cleavage sites for enzymatic action. Martos et al. also observed and increased
448 *in vitro* duodenal digestibility of hen's egg ovalbumin in the presence of BS [73]. In a more
449 realistic environment of the gut, endogenous PLs vesicles are also present, and have been
450 shown to affect duodenal proteolysis in the presence of BS in different ways. For instance,
451 ovalbumin digestion by pancreatic enzymes was increased to a larger extent [73], whereas BLG
452 was protected from the enzymatic degradation [74]. The phospholipids vesicles did not induce
453 any significant conformational change in the secondary structure of both proteins as observed
454 by circular dichroism [70,73], suggesting that specific interactions between protein and
455 phospholipids, such as short-range hydrophobic, are not involved. Nevertheless, non-specific
456 interactions between protein and PLs (e.g. long-range electrostatic) are not ruled out and BS
457 may also mix with the PLs vesicles reducing the amount of BS monomers available to interact
458 with the protein. The last mechanism only accounts for the protective effect against BS-
459 induced denaturation that can explain the observations for BLG but not for ovalbumin.
460 Therefore, other protein-specific mechanisms are likely to occur.

Recent work has also studied interactions between BLG and BE, by combining transmission electron microscopy, dynamic light scattering (DLS), ζ-potential and conductivity measurements [75]. The bile aggregates that form upon an excess in BE concentration, evolve to a different supramolecular structure in the presence of low protein concentration, and eventually dissociate in the presence of high protein concentration. Another study has focused on the molecular interactions between *β*-casein micelles and two BS, NaC and NaDC (at concentrations below the CMC) by using fluorescence spectroscopy techniques and DLS [76]. At submicellar concentrations and neutral pH, the more hydrophobic NaDC interacts to a larger extent with β-casein micelles than NaC, forming larger mixed aggregates. This suggests that the driving forces in the formation of these possible mixed micelles are hydrophobic and hydrogen bonding.

2.2.2. BS and low-molecular weight surfactants

The role of BS in lipid digestion, and in particular in the formation of mixed micelles with lipids to facilitate nutrient transport, is well known and it will be further discussed in Section 4. Mixed micelles of BS and lipids involve endogenous lipids, secreted during GI digestion, such as PLs, and dietary lipids from the lipolysis of triglycerides, such as monoglycerides and free fatty acids (FFA). Many reports are available in literature regarding self-assembly of lipids derived from triglycerides, but less on their mixtures with BA. One example is the recently published work by Sadeghpour et al. [77], where SAXS was used to explore the molecular bulk arrangement of a mixture of lipids (monoglycerides: monoolein/monolinolein) in excess of water in the presence of two BS, NaC and NaDC. The biosurfactants coexisted with the lipids reducing the negative curvature of lipid membrane upon increasing their concentration to the point of transition from bicontinuous cubic lyotropic liquid crystals to lamellar vesicles with positive curvature. This observation suggests that BS molecules tend to occupy the head-group region of the lipid molecules inducing positive average interfacial curvature. The size of the vesicles is reduced at higher BS concentrations, suggesting a more efficient disruption by BS. This behaviour agrees well with that reported previously by Gustafsson et al., between NaC/NaTC and monoolein in excess of water or 0.9 wt% NaCl [78]. BS molecules were inserted perpendicular to the surface of lipid bilayers in lamellar phases. This was inferred from the estimation of the average molecular interfacial area occupied by BS, cholate (C). They also reported that the BS incorporation in small mixed micelles was rather different, namely they were positioned flat on the micelle surface. In addition, Salentinig et al. studied by SAXS the addition of a mixture of glyco- and tauro-conjugated BS, along with PC and cholesterol, to mixed monoolein and FFA (oleic acid) in phosphate-buffered saline at pH 6.8 [79]. Increasing the concentration of BS led to a decrease in the negative curvature of the self-assembled structures, evolving from a coexistence of inverted hexagonal and bicontinuous cubic structures to vesicles.

Vinarov et al. explored further the interactions between various types (i.e. ionic, non-ionic) of surfactants and porcine BE or NaTDC to explain the different mechanisms of drug solubility and the role of surfactant charge [80]. NMR and surface tension measurements indicated that ionic surfactants interact to a larger extent with BS at a 1:1 ratio, forming mixed micelles of lower solubilisation capacity. On the other hand, non-ionic surfactants interact to a lesser extent with BS at a 1:1 ratio, forming surfactant-rich and BS-rich micelles coexisting together. The size of

non-ionic surfactant-rich micelles is larger than BS-rich micelles and mixed micelles of ionic surfactant and BS, allowing a better solubilisation capacity. Patel et al. have investigated the interactions of non-ionic Triton X-100 with the BS, NaC and NaDC, by DLS, small-angle neutron scattering, turbidity and viscosity, and for varying parameters such as pH and salt concentration [81]. Since pH is not static within the gastric or the intestinal compartment, one could expect variations within the proximal small intestine (duodenum) immediately after gastric emptying. These variations may be more pronounced for the late phase of gastric processing of food, when the gastric pH has achieved low values. The incorporation of acidic, gastric chyme into the duodenum will locally alter the neutral pH and this may certainly affect the mixed micelle behaviour of anionic BS. At acidic pH values, BS are in a protonated form (below their pK_a). This neutralises the effective negative charge and imparts more hydrophobicity to BA molecules, promoting a growth of mixed micelles, as reported for Triton and NaC/NaDC; the effect being greater in the case of the more hydrophobic NaDC. Namely, the mixed micelles change from nearly spherical at neutral pH to prolate ellipsoidal at acidic pH and the BS concentrations above the CMC.

Finally, interactions between polymeric surfactants from the family of block copolymers, Pluronics, and BS have been also recently addressed. Pluronics F68 and F127, with a central polypropylene oxide hydrophobic block and two lateral polyethylene oxide hydrophilic blocks, are approved for oral intake by the US Food and Drug Administration, and research work has demonstrated the ability of these emulsifiers to control the lipid digestion rate of oil-in-water emulsion-based delivery systems *in vitro* [82] and *in vivo* in rats [83]. The effect was greater *in vitro* for the emulsions stabilised with Pluronics of larger molecular weight F127 than the low-molecular weight homologue F68 [84]. The attributed mechanisms, for the sustained lipolysis induced by Pluronics, are not only interfacial, as it will be discussed below, but also bulk-related [85]. Micro-differential scanning calorimetry was used to investigate the bulk binding between these Pluronics and a model BS, NaTDC, based on how the micellization enthalpy of Pluronics was decreased and the micellization temperature shifted to higher temperatures by the increasing concentration of BS [86]. Hydrophobic association is the main driving force for the interactions between non-ionic Pluronic and anionic BS to occur, corroborated by the larger extent of interaction with the relatively more hydrophobic Pluronic F127, and additional experiments performed under high ionic strength conditions, screening electrostatic interactions of NaTDC [87]. Later studies performed with Pluronic P123 micelles and two BS, NaDC and NaTC, have corroborated the greater intensity of the interactions with the more hydrophobic NaDC [88]. In addition, the combination of DLS and spectroscopic techniques used in this study shed light on how BS molecules penetrate into the core of the Pluronic micelle, increasing the polarity at low BS concentrations. At higher BS concentrations, mixed micelles are formed and two types of complexes are formed: the copolymer-rich or the BS-rich. This behaviour at higher BS concentration resembles the one reported above for non-ionic surfactants by Vinarov et al. [80], and for HPMC [58].

The bulk interactions of different compounds with BA contribute, in combination with interfacial mechanisms, to delaying lipid digestion in emulsion-based systems, as described in more detail below.

3. Interfacial Interactions of BS with emulsifiers

Lipids consumed in a diet are either already emulsified within a food product, by emulsifiers used during the food manufacture (e.g. milk proteins, PLs, etc.), or undergo progressive emulsification caused by the combined action of peristaltic motion and physiological surfactants (e.g. BS and PLs secreted with bile, or monoglycerides produced from triglycerides during the gastric and small intestinal lipolysis). The gastric environment can promote significant structural reorganisation of emulsified lipids, involving flocculation or coalescence of emulsion droplets, and the mechanism often depends on the susceptibility of the original emulsifier to factors such as low pH or hydrolysis by gastric enzymes [89–91]. The gastric lipolysis of ingested lipids accounts for the release of only 10-25% of FFA from the triglyceride substrate [92]. Therefore, the majority of consumed lipids are hydrolysed in the small intestine, which they enter after the gastric pre-treatment. Interactions between the intestinal BS and emulsifiers at the oil-water interface can ultimately determine the interfacial composition, which in turn can impact on the degree and rate of the small intestinal lipolysis. Usually, in the first step, BS adsorb onto the oil droplet displacing existing -surface active material and promoting the adsorption of pancreatic lipase and colipase. Then, the lipase hydrolyses triglycerides, into FFA and monoglycerides, which remain at the interface, competing with BS adsorption. Finally, BS facilitate the removal of the lipolysis products from the interface by desorbing and forming mixed micelles, which solubilise those products [70]. There have been a number of studies dealing with the interfacial activity of BS and interactions with other emulsifiers, as reviewed recently by Pilosof [93]. This section will focus on the adsorption of BS in order to prepare the foundations of the next section addressing specifically interfacial lipolysis.

3.1. Interfacial properties of BS: adsorption, desorption, self-assembly

The investigation of interfacial activity of pure BS is a fundamental step in understanding the role of BS in lipolysis. However, adsorption curves and interfacial dilatational rheology of pure BS are still scarce in the literature, and only recent studies reveal differences between different BS species at the interface. Scientific literature agrees on the fact that BS reduce the interfacial tension rapidly but to a lesser extent than conventional surfactants [70,94]. This is due to their peculiar molecular structure, containing a rigid steroid ring system instead of a liquid-like linear hydrocarbon that is typical for conventional surfactants. This rigidity is responsible for the directional and specific nature of the intermolecular hydrogen bonds between several hydroxyl groups of the BS molecule. Accordingly, BS are very effective in covering the free interfacial area but do not create a very cohesive network consistent with the relatively high final interfacial tension attained [95]. Some types of BS reduce the interfacial tension in a stepwise manner, suggesting the existence of conformational regimes and preferential adsorption orientations. The orientation of BS upon adsorption has been debated in the literature and recently addressed with computer simulations by Euston et al. [94]. Results show a complex distribution of lateral tilt angles upon molecular adsorption where BS can adopt different orientations from flat to upright depending on the nature of the molecule and on the conditions (pH, ionic strength, concentration, etc.). In fact, Euston et al. point out the possible relation of this dynamic adsorption state with the reported ability of BS to displace adsorbed material, relevant to their biological role in lipid digestion.

Recent works also report data on the desorption profiles of pure BS, which are more susceptible to the molecular differences between BS [95,96]. These data will later relate to transport and solubilisation of lipolytic products. Maldonado-Valderrama et al. reported on the adsorption–desorption profiles of two different BS at the air-water interface by means of surface tension. The study included dilatational rheology experiments, and revealed differences between NaTC and NaGDC, which originated from different complexation properties, relevant to the digestion process [95]. NaTC presents faster adsorption rates and various conformational regimes in contrast to NaGDC, which adsorbs slower and in a single conformation. Desorption profiles reveal that NaGDC fully desorbs upon buffer rinsing whereas NaTC adopts an irreversibly adsorbed form at high surface coverage. Along similar lines, Parker et al. reported on the adsorption of six different BS (C, DC and CDC conjugated with taurine and glycine) onto solid hydrophobic surfaces, using dual polarisation interferometry and atomic force microscopy [96]. DC and CDC adsorbed more rapidly and desorbed to a greater extent following buffer rinsing than C, whereas taurine/glycine did not influence the behaviour.

This peculiar, bifacial amphiphilicity of BS plays also a significant role in the self-assembly of BS and the structure and morphology of BS micelles. Recent molecular simulations have been applied to identify these structures, and provided evidence of the formation of flexible and disordered structures that corroborate two models based on experimental findings. Namely, the formation of primary micelles, owing to hydrophobic interactions, and secondary micelles through hydrogen bonding, and also the formation of disk-like structures, owing principally to hydrophobic interactions between the hydrophobic faces of BS molecules oriented to the centre of the micelle are all representative BS micelles [94]. The molecular interactions between BS at interfaces, the conformational states, the interfacial orientations and the self-assembly have an important role in understanding the molecular features that control the physiology of BS, but those research areas still hold large knowledge gaps and open questions.

3.2 Interfacial interactions of BS: competitive adsorption and displacement

The interactions between emulsion components and BS, in the absence of lipase, can determine the interfacial composition where lipase adsorbs, hence influencing the rate and extent of lipolysis, as reviewed recently by Pilosof [93]. There are many experimental evidences, which correlate the presence of a cohesive interfacial layer of emulsifier that resists the action of BS with a reduction in the FFA release, measured for similar emulsion systems upon lipolysis [97,98]. Accordingly, there are a number of works investigating interactions of BS with surface active agents/emulsifiers in model systems that do not contain lipase, and using various experimental approaches such as examining interfacial tension, surface coverage of emulsion droplets (ζ-potential, droplet size) and microscopy.

Consider an interfacial layer of emulsifier, which forms an adsorbed network, with addition of BS into the bulk solution will result in BS adsorbing into defects at the interfacial layer and growing into clusters. Hence, BS adsorb at the interface compressing the network and displace the adsorbed emulsifier by means of orogenic displacement. This was visualised for BLG adsorbed layers at air-water and oil-water interfaces with Atomic Force Microscopy, and measured with interfacial tension techniques [99]. The mechanism of orogenic displacement

from the air-water interface was also visualised for the milk phospholipid and phospholipid−protein monolayers [100]. Sarkar et al. compared the interactions of different protein-stabilised emulsions with BS, and corroborated the orogenic mechanism but obtained less displacement for cationic-lactoferrin compared to that seen for anionic-BLG coated droplets. In this case, electrostatic binding of BS to the lactoferrin interfacial layer induced more adsorption of cationic lactoferrin. Hence, interaction of the protein-stabilized interfaces with BS depends somehow on emulsifier type [101]. More recently, the impact of BS on protein-coated gold NPs has also been described by the orogenic mechanism, where islands of aggregated proteins appeared, surrounded by larger regions of BS [102]. He et al. provide new details for the molecular level of the displacement mechanism by identifying a lag-burst kinetics in the displacement of surfactants from liquid crystal-water interface, which is BS specific, depending on the number and position of the hydroxyl group in the BS molecule [103]. In particular, the lag time and burst rate are largely correlated with the BS hydrophobicity. Hence, the more hydrophobic mono- and dihydroxy BS show shorter lag time and faster burst rate as compared to the more hydrophilic trihydroxy BS. Furthermore, the authors also visualized, with polarizing optical microscopy, a liquid crystal transition from homeotropic to tilted interfacial orientation, which followed the displacement.

Interfacial tension offers an interesting platform for carrying out model studies and assessing the interaction of BS with different emulsifiers. BS lower the interfacial tension, and the dilatational modulus can be easily assessed *in vitro* as the BS penetrate into the adsorbed layer, and hence promote displacement [99]. Using that experimental approach, Torcello-Gómez et al. demonstrated that Pluronics (F68) are more resistant to displacement by BS than other types of surfactants, such as lecithin [104]. Emulsion studies supported this trend as BS destabilised emulsions of Pluronic F68 and lecithin above a critical BS concentration, which was higher when the emulsifier was the Pluronic [105]. Similarly, Bellesi et al. demonstrated that soy protein is more resistant than BLG to displacement by BS from the oil-water interface. Soy protein displays a larger dilatational modulus proving the existence of favourable molecular interactions, which promote partially resistance to BS displacement [106]. The strong interactions occurring between BS and fibres in bulk can also reflect in alteration of the interfacial activity of BS, and this has received increasing attention lately [54,57,93,106]. Authors agree about a strong resistance of HPMC against BS adsorption at the oil-water interface due to binding of cellulose fibres to BS in bulk, which was discussed in detail in Section 2. BS are sequestered by hydrophobic interactions with HPMC fibres, which can be dependent on the methyl/hydroxypropyl ratio [57] and the type of BS [55,107]. Similar results have been reported for BS micelles binding to pectin molecules, promoting aggregation through hydrophobic interactions [59].

4. Role of BS in small intestinal lipolysis

In the GI tract, interactions of BS with digestive enzymes and products occur simultaneously and take place both in bulk and at the interface of emulsions droplets. This comprises a complex scenario and in order to understand all the different phenomena, lipid digestion is mimicked in the literature *in vitro* by looking into isolated interfaces and into emulsified systems and the encountered phenomena are analysed at different scales [93,108]. Unravelling the role of BS in lipid digestion at the interfacial level requires evaluating the role

of each of the major components of lipolysis. Accordingly, this section looks first into the interaction of BS with digestive enzymes, and then explores more complex systems, composed of BS, emulsifiers and lipase/colipase. Several studies on the desorption/displacement of lipolysis products, leading to release of FFA and monoglycerides from emulsified systems, are also summarised.

4.1. Interfacial interactions of BS with digestive enzymes

Scientific reports on BS and lipase interacting in the absence of other emulsifiers are scarce. The interaction of BS with lipase at an oil-water interface readily complicates the system since it includes the formation of FFA as the lipolysis proceeds. Accordingly, the interpretation of the results at the interfacial level needs to establish a different interfacial response of each compound [109]. Figure 5 shows a comparison of the adsorption/desorption profiles of BS, lipase and BS+lipase mixtures at the olive oil-water interface in a duodenal buffer, highlighting the differences encountered. BS lower the interfacial tension rapidly and practically recover the value of the clean interface after desorption, i.e. 25-28 mN/m (Figure 5). The lipase adsorbs irreversibly onto the olive oil–water interface as the interfacial tension remains unchanged after the subphase exchange. The mixed BS+lipase system reduced the interfacial tension to a larger extent than the individual components, suggesting the formation of a complex interface composed of BS, lipase, monoglycerides, and FFA (the latters produced *in situ*). Upon desorption, the value lies in between that of BS and lipase, owing to a possible competition between the solubilisation of lipolysis products by BS and the presence of FFA anchored at the interface (Figure 4). Analysis of the dilatational elasticity values of these interfaces can show more detail on the composition and role of each compound in lipolysis [109].

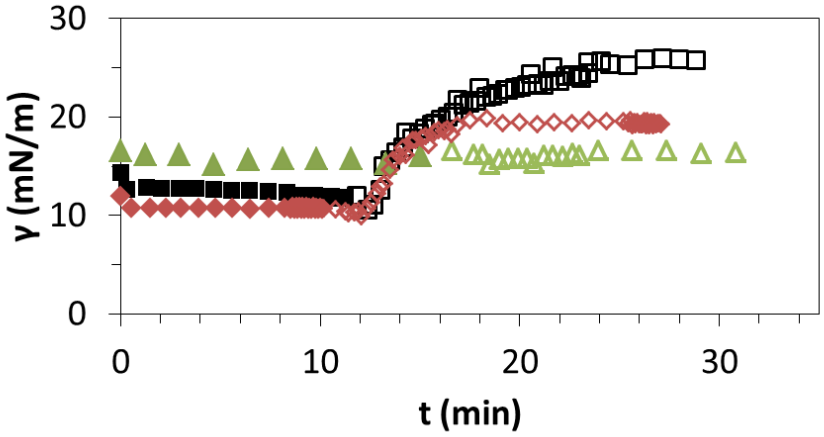


Figure 5: Adsorption (closed symbols) and desorption (open symbols) profiles of bile salts (BS, black squares), lipase (green triangles) and BS+lipase (red diamonds) at the olive oil-water interface. The desorption curves were recorded after the exchange of subphase with duodenal media (37 °C, pH 7, NaCl 150 mM and $CaCl_2$ 10 mM). The BS is a mixture of 52.7% NaTC and 47.3% NaGDC to a total 1 mM and lipase 0.16 g L^{-1}. Values are means of three replicate measurements, with SD < 2%. More experimental details can be found in [109].

Torcello-Gómez et al. have analysed the role of calcium in the interaction of BS and lipase at the oil-water interface. In the absence of BS, calcium complexes with FFA at the oil−water interface forming a relatively strong viscoelastic network of calcium soaps over time. The presence of BS disrupts the network of calcium soaps promoting the formation of calcium soap islands at the interface. As a result, Torcello-Gómez et al. proposed that the faster rate of lipolysis in the presence of calcium is due to FFA complexation with calcium and desorption rather than reduced electrostatic repulsion between lipase and BS. [110]. An interesting work by Parker et al. addresses the adsorption/desorption profiles of mixed BS/colipase onto solid hydrophobic surfaces [96]. When comparing different BS, simultaneous adsorption of colipase shows a greater cooperativity with NaTC and less desorption upon buffer rinsing than with NaGDC. The authors hypothesize that less desorption promotes the adsorption and residence time of the colipase-lipase complex while more desorption favours the displacement and solubilisation of lipolytic products from the interfacial region into the micellar phase [96]. This is in agreement with the faster and more extensive desorption profile of individual GDC as compared to C BS discussed above. This supports the idea that cooperative adsorption occurs between certain BS and colipase to facilitate the adsorption and activity of pancreatic lipase.

4.2. Interfacial approaches to studying lipolysis: emulsion studies

We have already explained that interfacial lipolysis involves adsorption, conformational changes, enzymatic processes and desorption of the lipolysis products. In this complex scenario, the presence of emulsifiers can alter the process of lipolysis by either promoting or decreasing the activity of lipases, as emulsifiers compete with lipases and BS for the interface and interact with the enzymes and BS in the bulk solution. On the one hand, resisting displacement by BS might alter lipolysis by preventing the access of lipase through competitive adsorption [93]. The presence of lipolytic products at the interface also prevents further access of lipase [111]. On the other hand, bulk interactions that entrap lipase promote hydrophobic interactions, which reduce interfacial activity of lipase or directly inhibit the lipolytic site through specific interactions [112]. Ionic surfactants can also denature lipase, resulting in the reduced enzymatic activity, while non-ionic surfactants micelles can interact with lipase, hindering adsorption of the enzyme. ref [113].

The specific role of interfacial coverage and lateral packing on lipolysis has been assessed by means of monitoring changes in the interfacial tension of isolated interfaces [108], and complemented with emulsion microstructure studies [93,97,98]. Polar lipids with larger head-groups (galactololipids) provide a decreased lipolysis rate in emulsions accompanied by a higher resistance to displacement by BS, which confirms the influence of interfacial molecular packing of the lipids at the oil-water interface on lipolysis [114]. Similarly, the length of hydrophilic/hydrophobic chains in Pluronics (F68 and F127) affects the rate of lipolysis [115] Specifically, *in vitro* digestion occurring at oil water interfaces stabilised by Pluronic suggests that in the presence of a steric barrier, the susceptibility to lipolysis depends on the interfacial coverage as controlled by the interfacial tension, apart from the steric bulky layer remaining in the aqueous phase. Pluronics can inhibit lipolysis owing to steric hindrance at the oil-water interface caused by their hydrophilic tails, which hinders the adsorption of BS+lipase at the droplet interface. [85]. Speranza et al. generalise this trend by stating that the bioaccessibility of FFA increases with the hydrophobicity of the surfactant and decreases with the length of

aliphatic chain [116]. Lipolysis of multi-layered protein/pectin films also showed that the combined action of BS and lipase is ruled by BS with lipase co-adsorbing and generating lipolytic products [117]. This was further confirmed by Bellesi et al. who correlated the susceptibility to BS displacement of different emulsifiers to FFA release upon lipolysis [118]. These findings have been recently corroborated by measuring the dynamics of the emulsion interface during *in vitro* digestion by Fluorescence Resonant Energy Transfer [119]. This technique allows measuring *in situ* structural changes of emulsion droplets stabilised by lecithin upon simulated digestion providing changes in the intermolecular spacing between the two different fluorescent probes labelled emulsifier. Results confirm that BS and lipase disrupt the adsorbed layer. Furthermore, addition of a second adsorbed layer of chitosan or ε-Polylysine reduces considerably the disruption of the interface and the release of FFA. The role of BS in lipolysis with regard to the interfacial activity of BS is also highlighted in the studies concerning the available interfacial area, where experimental results confirm the intensification of lipolysis as the available interfacial area increases, that is the oil droplet size is reduced [106,120–122].

Great attention has been directed lately to the use of NPs as emulsifiers and their impact on lipolysis [98]. Most studies agree that NPs delay/impede lipid digestion owing to a strong an irreversible adsorption which inhibits displacement by BS. Tzoumaki et al. report a lower release of FFA from emulsions stabilised by chitin NPs compared to proteins [123]. Sarkar et al., compared the barrier promoted by microgel particles and heat-treated fused microgel particles, and found that the fused network hindered adsorption of BS, and hence delayed lipid digestion [124]. The specific role of BS on lipolysis has been also addressed by Marefati et al., which compared physical stability of quinoa starch granules Pickering emulsions during *in vitro* lipolysis, showing larger changes in samples in the presence of BS and less destabilization of heated emulsions upon lipolysis [125]. Cellulose nano crystals (CNCs) are a novel ingredient, which has been shown to impact on the microstructural stability of emulsions under *in vitro* lipolysis. Sarkar et al. demonstrated that adding high concentrations of CNCs decreases the rate and degree of lipolysis of protein-coated lipids. This is principally due to the binding of CNCs to BS, which prevents BS adsorption to the oil-water interface, and hence the displacement and later solubilisation [126]. However, as in the case of emulsions, the available interfacial area could also be determinant here, as the presence of CNCs induces flocculation of protein coated droplets reducing the overall area available to adsorption[126]. The pivotal role played by the interfacial area in Pickering emulsions has been also noted by Shah et al. [127]. A recent review deals specifically with Pickering emulsions, and analyses their stability to various biophysical environments [98].

The type of oil can also alter the lipolysis profile of emulsified systems. In this regard, Marze et al. established the importance of solubility of individual FA in the digestibility of different oils [128]. Ye et al. corroborated the importance of oil composition by analysing duodenal digestion of three representative oils emulsified with whey protein isolate (WPI), and ascribing the different lipolysis degree to different FA compositions and triglyceride profiles. According to the researchers, BS played a key role in displacing WPI and acting as a lipase activator and a lipase-assisted adjuvant [129]. The interactions of BS and adsorbed protein depended on BS structure, and hence on the hydrophobic forces acting between BS and protein and between BS and lipid, and resulting in different abilities of BS to displace WPI from different oil phases.

The influence of the physical state of lipid was also assessed, by comparing lipolysis and β-carotene transfer from canola oil emulsions and Solid Lipid NPs [130]. Nik et al. found a more substantial degree of lipolysis and β-carotene transfer from canola oil emulsions, which correlated with a greater surfactant displacement by BS from the liquid lipid surface [130]. Thus, as recently stated by Corsten et al., hindering adsorption of BS is one of the main approaches to control lipolysis [131]. A recent work by Wilde et al. also highlights the importance of food structure, apart from composition, in digestibility of oat-based food matrices [132]. The presence of phytosterols affected the stability and physical-chemical properties of mixed micelles promoting the disappearance of the smaller micelles following digestion. Smaller micelles are likely comprised only by BS, which can exchange rapidly with those in solution and adsorbed at the interface, removing lipolytic products. Their results suggest that BS bound more effectively into larger complex structures, which may bind lipophilic compounds hence reducing lipid hydrolysis.

The self-assembly structures formed during lipid digestion have only recently been studied by scattering techniques, which showed that the type of self-assembly organisation depended strongly on BS [79,133,134]. Salentinig et al. studied *in situ*, with time-resolved SAXS, the *in vitro* digestion of a triolein emulsion by pancreatic enzymes in the presence of different concentrations of BS mixture. In general, they observed that the original emulsion, with no internal structure, transitioned during the lipolysis to an emulsified microemulsion, inverse micellar cubic (Fd3m) liquid crystalline phase, inverted hexagonal liquid crystalline phase, and finally lamellar vesicles at pH 7. The presence of higher BS concentrations accelerated the formation of vesicles containing the lipolysis products. The use of depolarised DLS in comparison to normal DLS and cryo-Transmision Electron Microscopy confirmed the presence of anisotropic particles based on bilayer structures, such as bicelles (crossover between bilayer and micelle). All these studies were focused on long-chain lipids, with more than 12 carbons in the aliphatic chain. The formation of intermediate lyotropic liquid crystals cannot be usually observed in medium-chain and short-chain lipid systems in the presence of simulated intestinal juice containing BS, phosphatidylcholine and cholesterol, since there is a direct transition to vesicles and micelles [79,135]. This is likely related to their higher solubility in water, and associated with lower solubilisation capacity as compared to long-chain lipids. In addition, these interactions will certainly depend on the pH, due to different protonation states of lipids, and on the ionic strength, due to changes in electrostatic interactions. Namely, higher pH and ionic strength accelerate structure formation and transitions. To our knowledge, liquid crystalline phases, such as cubic or hexagonal, have not been reported in the limited *ex vivo* studies available in the literature. The reason for this may be that these phases are formed transiently at the surface of a triglyceride droplet being digested, and require surface-specific techniques for their identification with increased time resolution [136]. Moreover, the specific role of BS in the formation of these structures has not been addressed so far systematically and literature works highlight some controversy. For example, Salentinig et al. suggest vesicles as the dominating final structures of lipolysis [79], whereas Marze et al. suggest that the major assembly formed is a mixed micelle with a lower proportion of vesicles [133]. Interestingly, these works differ fundamentally in the concentration of BS used, which might explain the discrepancy. Vesicles only appear at high concentrations of BS, whereas inverse micelles or liquid crystalline phases appear at low concentration of BS or in their

absence. At the physiological concentrations, micelles and vesicles coexist, with the micelles being the major type of assembly. According to Marze et al., the emulsifier used in emulsion preparation seems to be less determinant than the type of oil in the self-assembly. However, the influence of complex emulsifiers, NPs or fibres has not been addressed specifically with this type of experiments yet.

5. Role of bile salts in small intestinal transport

5.1. Mixed micelles

As part of the digestion process, a range of physiological surfactants are secreted into the gut lumen. These include PLs such as PC, which is present in the gastric compartment [137] and is a significant component of the bile secreted into the proximal small intestine [138]. As discussed in previous sections, bile also contains a range of charged BS [139]. In addition to facilitating the interfacial lipolysis of triglycerides, BS also have the ability to solubilise the products of lipolysis (FFA and monoglycerides) a process essential for effective absorption of dietary lipids by the intestinal epithelium. The process of formation of mixed micelles often involves incorporation of other lipids, such as lipid-soluble vitamins or cholesterol. The process of solubilisation of cholesterol, is, in fact, the major mechanism of cholesterol removal from the gut [26].

The relatively high surface activity of the lipolysis products means that their accumulation at the oil-water interface during digestion can render the triglyceride substrate inaccessible to pancreatic lipase. *In vitro* studies [140] showed that monoglycerides can be very efficient in expelling the enzyme from the interface, although the FFA, which also formed during digestion, did not have enough interfacial activity to exert a similar effect. Under physiological conditions, the inhibition of digestion caused by interfacial accumulation of lipolysis products is prevented by BS and PLs, which remove the products from the interface by solubilising them into mixed micelles. Triglyceride oil droplets coexist with mixed micelles until the lipid substrate is exhausted. Early light-scattering studies [26] showed that the size and structure of micelles depend on PL (lecithin) to BS molar ratio (PL/BS). At PL/BS < 0.6, small mixed micelles (hydrodynamic radius up to 3.5 nm) coexisted with simple BS micelles. Increasing the ratio over 0.6 resulted in formation of mixed micelles only, with hydrodynamic radii up to 30 nm and a disk-like shape, in which BS and PLs formed a mixed bilayer. This bilayer disk model [26] has since superseded previous models [141].

As summarised in previous sections, modulating the adsorption of BS is one of the most promising approaches to control the rate of lipolysis. However, apart from the physiological role of adsorbed BS in displacing other material form the interface, a major importance of unadsorbed BS has also been pointed out [111]. Sarkar et al. suggested that, compared to adsorbed BS, the presence of unadsorbed BS had more impact on the lipolysis they studied. Those BS present in mixed micelles might facilitate lipid digestion by an efficient removal of the lipolysis products from the interface. The researches stated that structural information about the mixed micelles formed in an aqueous phase would be needed in the future in order to confirm that hypothesis [111].

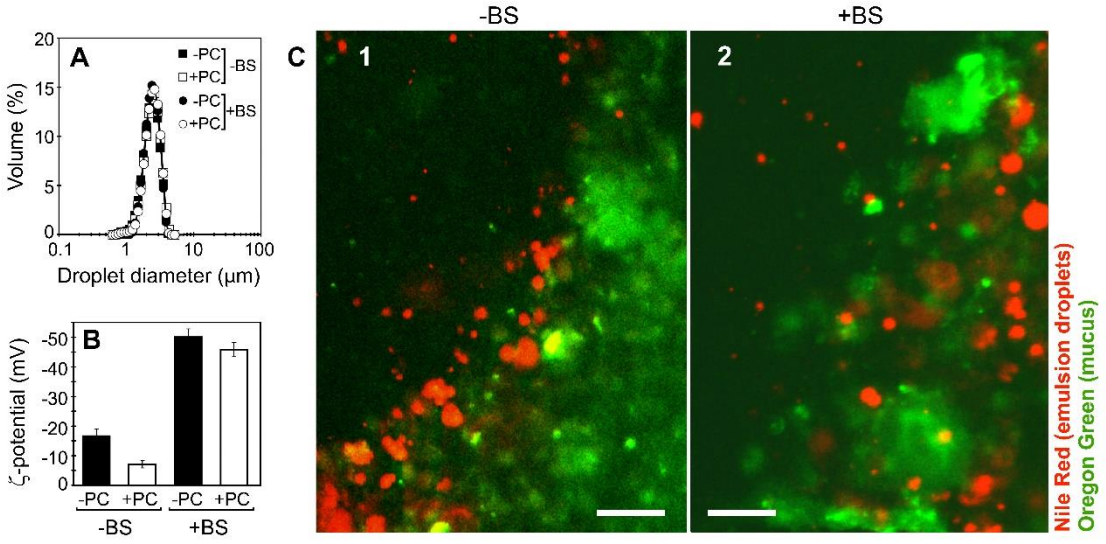
The formation of mixed micelles of BS and surfactants can also affect the degree and rate of lipolysis. Vinarov et al. studied the effect of surfactant concentration and charge on the lipolysis of oil-in-water emulsions in the presence of porcine BE [142]. The researchers found optimum solubilisation capacities of mixed micelles formed by bile and either non-ionic (Tween 20 and Tween 80), cationic (cetyltrimethylammonium bromide) or anionic (α-olefinsulfonate, and sodium lauryl ether sulfate) emulsifiers at various surfactant-to-bile molar ratios. The maximum ratio at which the complete lipolysis was observed depended on the nature of surfactant, and increased in the following order: non-ionic<cationic<anionic. This experimental parameter was inversely related to the solubilisation capacity of the mixed micelles to incorporate the lipolysis products. The lower the surfactant-to-bile molar ratio was required to achieve complete lipolysis, the lower the amount of surfactant was needed at a constant bile concentration. The non-ionic surfactants had the highest solubilisation capacity, followed by cationic and then anionic. The different behaviours depended on the charge of the surfactants, however additional research is needed to explain detailed molecular mechanisms of interactions. The mechanisms of drug solubilisation reported for similar surfactants in the presence of BS, previously discussed in Section 2, may help explain the different solubilisation capacity of lipolysis products [80].

5.2. Transport through intestinal mucus

The formation of mixed micelles is usually described as the process completing lipid digestion, which is then ultimately followed by absorption across enterocytes [143,144]. However, the transport of solubilised FFA and monoglycerides, and any other lipid molecules incorporated into the mixed micelles (e.g. cholesterol, lipid-soluble vitamins, hydrophobic pharmaceutical substances, etc.), towards the epithelium requires the micelles to diffuse through the protective barrier of intestinal mucus. The small intestinal mucus is a complex colloidal system that coats the entire intestinal epithelium, thus protecting it from direct exposure to luminal contents [145]. It acts as a natural sieve, allowing passage of nutrients, including the solubilised products of lipolysis, so that they can reach enterocytes and get absorbed. At the same time, the mucus prevents the epithelium from direct contact with pathogenic microorganisms, etc. [146,147].

Intestinal mucus is a physiological, gelled secretion that is highly hydrated and contains a range of organic compounds. Amongst them, there are two major, gel-forming biopolymers, MUC2 mucin glycoprotein [148] and extracellular DNA [149], which form a coherent network. The mucin is actively secreted by goblet cells located in the intestinal epithelium, whereas the DNA can originate from apoptotic epithelial cells [150], and its substantial amounts in the small intestinal mucus have been proposed to result from the fast turnover of the intestinal epithelium [149]. The mucus gel is produced through entanglement of the biopolymers and forms an adherent, unstirred layer. The gel strength and viscoelasticity are determined by the level of entanglement, the size of pores and “channels” in the network, and ultimately by the concentration of mucin and DNA. Thickness of the mucus layer can thin in the presence of shear, such as that induced by peristalsis. This creates a slippery plane, which has the ability to lubricate the peristaltic motion. The thinned mucus can maintain the coherence of the unstirred layer and its penetration is only possible by diffusion [146].

Particles in the GI tract lumen, including emulsified fat, cellular material and bacteria, are exposed to physiological surfactants that can adsorb to the particle surface and change the surface properties, and thus mucus interactions. This is one reason why the selective nature of the intestinal mucus barrier is not well understood. Whilst there is a body of published work looking at the micro-rheological properties of a range of different types of mucus [151–153], evaluation of the effect of physiological surfactants on the ability of colloidal particles to penetrate the mucus has not been extensively studied. We showed previously that interfacial adsorption of BS to model particles, fluorescent latex beads (0.5 – 2 μm in diameter), allowed them to penetrate into small intestinal mucus [154]. The BS imparted high negative surface charge to the particles, but not the mucus. Adsorption of BS to the surface of particles was assumed to significantly change their electrostatic interactions with the mucus network, which was also negatively charged, and hence prevent mucoadhesion of particles. In contrast, mucoadhesion of model bacteria (non-flagellated *Escherichia coli*) was observed despite the presence of BS in the system. This was linked to the fact that BS did not modify the weak negative charge of the bacterial cells, which might otherwise provide sufficient electrostatic repulsion between the bacteria and the mucus, and allow penetration. Importantly, similar studies were also conducted for model food emulsions that had been put through the *in vitro* gastrointestinal proteolysis in the presence or absence of PC and BS [155]. The emulsions were initially stabilised with a food-grade protein sodium caseinate, crosslinked by transglutaminase. The protein was eventually displaced from the emulsion droplets by the biosurfactants throughout the course of digestion. The BS adsorption to emulsion droplets enhanced their negative surface charge under the simulated small intestinal conditions. This was not expected for zwitterionic PC, and their co-adsorption did not significantly change the electrostatic properties of droplets. As for the latex particles [154], the high negative charge provided by adsorbed BS allowed the post-digestion emulsion droplets to penetrate into the mucus (Figure 6). The results of those two studies imply that the ability to penetrate intestinal mucus is not only limited to small molecules or nanometre-scale particles, such as mixed micelles, but may also be expected for much larger particles under physiological conditions of the gut. This, in turn, suggests that the lipolysis of fat droplets may not only take place in the lumen but also much closer to the intestinal epithelium, after the droplets have penetrated into the mucus.



951

952 **Figure 6.** Post-digestion emulsion droplets and their interactions with porcine small intestinal mucus. (A)
953 Size distributions and (B) ζ-potential values of the emulsion droplets used for experiments on diffusion
954 in the intestinal mucus. The droplets were obtained after *in vitro* gastro-duodenal proteolysis (+/-
955 phosphatidylcholine (PC) in the gastric phase of digestion; +/- bile salts (BS) in the duodenal phase of
956 digestion) of transglutaminase-crosslinked, sodium caseinate-stabilised emulsions (Na-Cas 1mg/mL; 18%
957 triglyceride oil, w/w). Subsequently, fine emulsion droplets (A) were separated from partially
958 destabilised, post-digestion emulsions and fluorescently stained before diffusion experiments. (C)
959 Confocal micrographs of the post-digestion emulsion droplets interacting with the *ex vivo* intestinal
960 mucus in the (1) absence or (2) presence of BS adsorbed onto the surface of the droplets (the droplets
961 were obtained from emulsions digested in the absence of PC and +/- BS). Top-left, dark areas in both
962 images show diluted post-digestion emulsions where the diffusion of oil droplets towards the mucus
963 layer took place from (15-min incubation at 37 °C). Image 1 shows droplets (red) accumulated at the
964 surface of the mucus layer (green) and unable to penetrate into the mucus in the absence of BS. Image 2
965 shows emulsion droplets penetrating into the mucus layer in the presence of BS. The scale bars
966 correspond to 15 μm. Adapted with permission from Macierzanka A. et al., Langmuir 2012;28:17349–62
967 [155]. Copyright 2012 American Chemical Society.

968 The above examples of *in vitro* studies suggest that the presence of negatively-charged BS may
969 play a vital role in determining passive transport of colloidal particles, including partially
970 digested fat droplets, through the intestinal mucus under physiological conditions. However,
971 the concentration of BS can vary significantly in the small intestine. In the postprandial adult
972 human intestine, it can range from 5.2 to 14.5 mM [156,157] as the bile is secreted in response
973 to meal consumption, whereas in the fasted state of digestion, the BS concentration is much
974 lower, with a typical range of 0.8–5.5 mM [6,156,158]. Low concentrations of BS in the small
975 intestinal lumen can also be found in human infants due to immaturity of the BS metabolism
976 and turnover [159–161]. In new-born infants, decreased BS synthesis, degree of conjugation,
977 and excretion rate have been reported [162–164], the latter caused by a lower gallbladder
978 ability to concentrate bile, compared to adult humans. This is further accompanied by an
979 impaired BS intestinal uptake [160]. The total BS concentration in the postprandial small
980 intestine of full-term infants (3-15 days after birth) was found to range from 0.4 to 1.5 mM
981 [165].

982

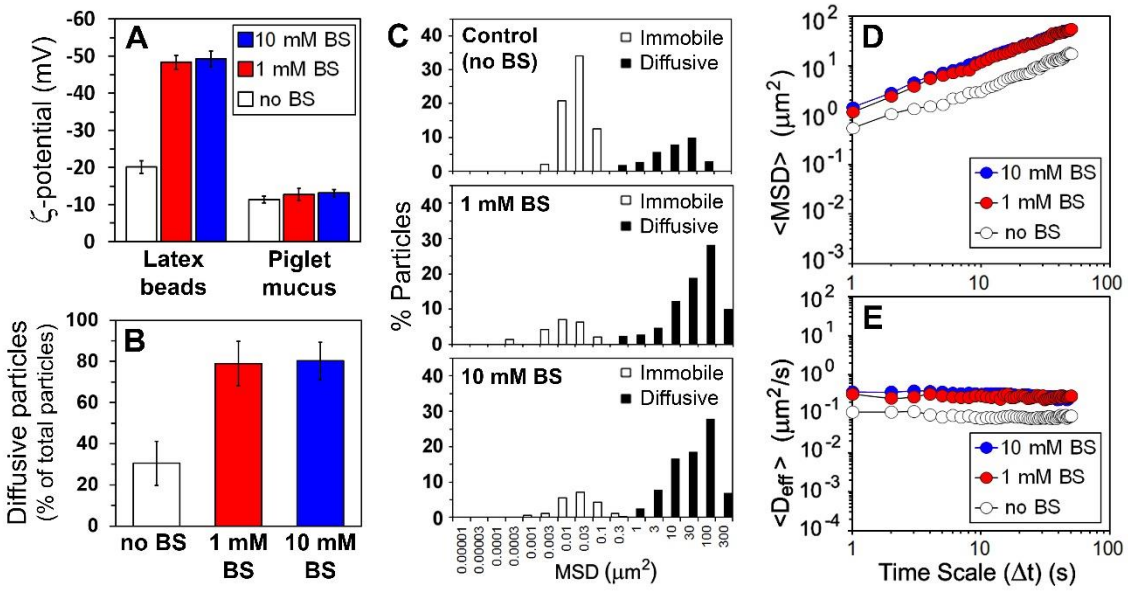


Figure 7. Effect of bile salt (BS) concentration on diffusion of 500-nm latex beads in the piglet small intestinal mucus gel, collected from jejunal mucosa**.** Impact of pre-incubation with different BS concentrations on (A) the electrostatic properties of particles and (B-E) the transport rates and distributions of latex beads in the mucus. (A) ζ-potential of the latex beads and the mucus dispersed in PBS buffer with/without BS (means ± SD, n = 5). (B) Proportions of diffusive beads in the collected, undiluted mucus gel (means ± SD, n = 5). (C) Distributions of mean-square displacement (MSD) values obtained for individual beads in the mucus gel at the time scale Δt = 50 s. (D) Ensemble mean-square displacements (<MSD>) for diffusive fractions of beads in the mucus gel as a function of time scale (Δt), and (E) ensemble diffusivities ($<D_{eff}>$) for diffusive fractions of beads in the mucus gel as a function of Δt (n = 5, with 100–150 beads per experiment). All measurements were done at 37 ± 0.1 °C. The data obtained for control samples (i.e. with no BS added) have been adapted from Reference [149].

We have investigated the extent to which changing BS concentration within the physiological range can impact on the permeability of the intestinal mucus to sub-micron sized particles, such as partially digested food particles or colloidal delivery systems, and their transport in the mucus. Fluorescent, carboxyl-modified, 500-nm latex beads were used as model particles in the experiment simulating the passage of particulate material from the lumen of the small intestine into the mucus layer overlaying the mucosal epithelium. The results are presented in Figure 7. The probe particles had been incubated with either 1 mM or 10 mM BS before they were subjected to multiple-particle tracking experiments in the mucus in order to mimic contrasting physiological concentrations of BS in the human small intestinal lumen [92,166]. A mixture of two BS, comprising equimolar quantities of NaTC and NaGDC, was used. Control samples, with no BS added, were also analysed. We used mucus collected from freshly excised, proximal small intestines of 2-week old piglets as a substitute for human mucus. Incubation of latex beads with BS caused a significant increase in their negative charge, from ca. -20 mV in the absence of BS, to ca. -50 mV with BS (Figure 7A). However, there was almost no difference in the ζ-potential value upon increasing the BS concentration from 1 mM to 10 mM. In contrast, the presence of BS had almost no effect on the net charge of the piglet mucus, with the ζ-potential values ranging from -11 mV to -13 mV before and after incubation with BS (Figure 7A). This suggests that there was very limited interaction of the biosurfactant with the mucus. In the next step, dispersions of latex beads were brought in contact with the mucus

samples placed in an optical cell, and the particles allowed sufficient time to diffuse into the mucus. As revealed by multiple-particle tracking confocal microscopy, the pre-incubation with BS, and the resulting increase in the negative surface charge of the latex beads, had a profound effect on their diffusion in mucus. The number of particles able to diffuse in the piglet mucus increased to ca. 80% in the presence of 1 mM BS, from ca. 30% recorded for the control conditions (Figure 7B), where the particles had significantly reduced ζ-potential in the absence of BS. Thus for the latter, the majority of particles (ca. 70%) were found immobilised by the mucus structure over the time-scale of the experiment. However, increasing the BS concentration, from 1 mM to 10 mM, had no effect on the overall number of diffusive particles. As shown in Figure 7C, the distances travelled by individual diffusive beads varied largely after the 50-s time-scale examined, with mean-square displacement (MSD) values ranging from 0.3 μm^2 to 300 μm^2. This pattern was consistent across the different experimental conditions tested. The ensemble MSD (<MSD>) for individual populations of diffusive particles in the function of time (Figure 7D) showed that the concentration of BS had almost no effect on the average distance travelled by the beads in mucus. The MSD data obtained from trajectories of individual beads have been converted to diffusion coefficients (effective diffusivities, D_{eff}) and ensemble diffusion coefficients ($<D_{eff}>$) calculated for families of diffusive particles, using the procedure described before [149]. The diffusive particles showed free diffusion, expressed by constant $<D_{eff}>$ values in time (Figure 7E). The diffusion rate did not seem to be affected by the BS concentration ($<D_{eff}>$ = 0.27 ± 0.03 $\mu m^2\ s^{-1}$ and 0.29 ± 0.04 $\mu m^2\ s^{-1}$ for 1 mM and 10 mM BS, respectively). However, the pre-incubation of the latex beads with BS gave rise not only to higher numbers of diffusive particles as compared to the control conditions (Figure 7B) but also increased the rate of diffusion in mucus by a factor of ca. 3.5. The $<D_{eff}>$ value recorded for the latex beads diffusing in the absence of BS was 0.08 ± 0.01 $\mu m^2\ s^{-1}$ (Figure 7E). Thus, although the presence of BS had a significant impact on the net surface charge of particles and enhanced their diffusion in the mucus, increasing the concentration from 1 mM to 10 mM had no effect. This might have been due to the molecular structure of BS and the flat conformation they adopt when adsorbed at interfaces, which allows a relatively large surface area to be occupied by a single BS molecule [70]. The similar electrostatic properties of the beads incubated at the two different BS concentrations suggest that the total surface area of the beads was fully saturated with BS already at the 1 mM concentration. If the mean molecular area of adsorbed BS at 37 °C is ca. 100 $Å^2$ per molecule [167], then at 1 mM BS there was enough BS molecules available to cover an area three orders of magnitude larger than the total surface area of the latex beads used in the experimental set-up. Thus, increasing the concentration to 10 mM might not have significantly changed the BS surface density. Since the net surface charge of the mucus was almost unaffected by the variations in BS concentration, this can explain similar diffusion characteristics of latex beads for both BS concentrations tested.

6. Conclusions

Fundamental understanding of the mechanisms underlying the role of BS in digestion and the transport of lipids will enable the development of strategies for improving food and pharmaceutical formulations with targeted digestibility. BS are key ingredients in the processes

of assembling molecules into functional micro- and nanostructures, which transport lipophilic nutrients in the gut. However, there is a need for understanding the role of BS in biophysical transformations of lipids in the digestion process. The bulk and interfacial interactions of BS with lipids and the self-assembly are directly related to lipolysis, and the resulting solubilisation and transport. The interaction of BS with dietary fibre and food emulsifiers can influence those processes in several ways. Namely, physical entrapment of BS can hinder the BS adsorption and the hydrolysis of lipids. Also, electrostatic and/or hydrophobic interactions may occur depending on the nature of the emulsifier, altering the lipolysis rate. Additionally, there is a need to further explore the interactions of adsorbed BS with other physiological surfactants such as PC, FFA, cholesterol and unadsorbed BS. And finally, there is growing evidence concerning the crucial role of BS in the transport of lipids through intestinal mucus. However, in this area too, there is a need for more research that would address, for example, a possible role of BS in preventing mucoadhesion of other substances. Thus, unravelling all the roles that BS may play in the digestion and transport of nutrients/bioactives requires collaborative research through multidisciplinary approaches, in which fundamental colloidal studies are carried out to accurately reflect complex *in vivo* scenarios.

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