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Mini Review: Recent advances in biosurfactant-based association colloids – self-assembly in water

1 - revised manuscript -

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16 **Keywords: rhamnolipid, saponin, glycyrrhizin, aescin, self-assembly, micelles, scattering**
17 **techniques**

18 **Abstract**

19 Recent studies of self-assembly in binary systems of bio-surfactants, either of microbial origin or
20 saponins extracted from plants, are reviewed. Saponins in water reported in the first section include
21 aescin, glycyrrhizin, and quillaja saponins, while rhamnolipids are discussed in the second section on
22 microbial surfactants. Studies of surface activities are a natural starting point of the characterization of
23 surfactants, but here we focus mainly on physico-chemical and structural properties of self-assembled
24 bulk structures in solution, often characterized by scattering techniques. When quantitative modelling
25 is performed, self-assembly parameters like aggregation numbers, head group areas, and resulting
26 shapes can be followed as a function of physical-chemical parameters like concentration, composition,
27 temperature, or pH. Morphologies include micelles and their structural evolution with addition of other
28 bio- or synthetic surfactants, co-surfactants, proteins or phospholipids.

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32 **1 Introduction**

33 Biosurfactants are structurally diverse amphiphiles stemming from natural resources. The mode of
34 production by plants or microorganisms is reflected by the classification into plant saponins and
35 microbial biosurfactants, produced by bacteria or yeast. Within saponins, molecules are usually named
36 after the plant used to produce them, although a chemical variety of biosurfactants may correspond to
37 the same name. Microbial biosurfactants are mainly glycolipids including trehalolipids, sophorolipids,
38 and in particular rhamnolipids, which serve us here as paradigm: produced by the bacterium
39 *Pseudomonas aeruginosa*, they possess outstanding surface-activity. In many cases biosurfactants
40 offer significant benefits compared to synthetic petrochemistry-based surfactants, especially in the
41 context of sustainability: bio-surfactants are made using mild production conditions (bio-reactors or
42 extraction from plants), they often have lower toxicity and enhanced biocompatibility, and saponins
43 possess pharmacological activity, e.g., antiviral [Bailly & Vergoten (2020)] or antimicrobial, justifying
44 the growing interest in their large-scale production. [Kitamoto et al. (2002)] Biosurfactant properties
45 are linked to their molecular hydrophilic/hydrophobic structure – which is often less clearly separated
46 as compared to synthetic molecules –, resulting in self-assembly in solution with sometimes surprising
47 properties. One may think of the spontaneous formation of so-called ISCOMs (immune-stimulating
48 complexes containing Quillaja saponins used as adjuvants to vaccines) [Morein et al. (1984), Sjolander
49 et al. (1998), Pedersen et al. (2012)], or of gelation induced by network formation of elongated micellar
50 structures [Saha et al. (2015)]. It is the purpose of this article to review important recent progress on
51 aggregate formation in (mixed) biosurfactant systems. Mixtures of biosurfactants with lipids have been
52 investigated due to their use in vesicle-based drug delivery (saponins) and also to test their haemolytic
53 activity which is necessary for their application in consumer care products. Moreover, in such products
54 synthetic surfactants are also present and their interaction with biosurfactants might be relevant.

55 Saponins occur in plants and are part of the defense against fungi and bacteria. Historically, the name
56 was due to foaming and emulsifying properties of, e.g. Quillaja bark saponin used for washing.
57 Nowadays, saponins are still mainly extracted from different plants (e.g. horse chestnuts, licorice,
58 soapbark tree *quillaja saponaria*, digitonin from common foxglove, oleandrin, gypsogenin from
59 *saponaria officinalis*, etc.). Saponins are of growing interest due to their pharmacological activity. They
60 exhibit typical surfactant properties, like a critical micellar concentration (CMC) [Geisler et al. (2019a),
61 Dargel et al. (2019)], or interfacial activity [Wojciechowski (2013), Góral & Wojciechowski (2020)].
62 All saponins have in common that the hydrophobic part is formed by a triterpenic or steroidal structure
63 with attached sugar moieties, enabling this group of molecules to stabilize emulsions and to solubilize
64 bulky hydrophobic molecules.

65 The second group of bio-surfactants is produced by microorganisms. The most important low-
66 molecular weight representatives of this group are rhamnolipids, sophorolipids and trehalolipids, but
67 also surfactin [Lewinska et al. (2022)], and mannosylerythritol lipids [Kim et al. (2002)], which are of
68 interest in the context of bioemulsifiers, or lipopeptides and phospholipids. Their hydrophobic tail is
69 usually composed by one or several, possibly branched fatty acid chains, while their hydrophilic head
70 group can contain a phosphate group (phospholipids), a peptide loop (lipopeptides), or a sugar moiety
71 (glycolipids), e.g. based on the monosaccharide (“ose”) rhamnose, or disaccharides like sophorose,
72 trehalose, or maltose. The latter may also be dirhamnose, as opposed to monorhamnose [Chen et al.
73 (2010b)]. A summary of these compounds can be found in [Rodrigues (2015), Jahan et al. (2020)].
74 Other types of biosurfactants, such as the well-studied model sugar surfactants (alkyl polyglucosides,
75 abbreviated C_iG_j), which can be obtained by esterification of bio-sourced sugar head groups and fatty
76 acids, or microbial polymeric biosurfactants (high-molecular weight) exist, but are outside the scope
77 of this mini-review.

78 The shape of – pure or mixed – biosurfactant aggregates formed in water is controlled by the underlying
 79 chemistry: the spontaneous curvature of the hydrophilic-hydrophobic amphiphilic film reflects the
 80 interactions between each moiety. The bending energy to be paid for deviations from the spontaneous
 81 curvature defines the available shapes, as illustrated by some glycolipids which possess large
 82 hydrophilic sugar head groups, favoring high curvatures, and thus globular micelles. As the curvature
 83 is reduced, rod- and disk-like structures may be found. At higher concentrations, where competition
 84 for water molecules may induce a reduction of the head group, such aggregates may form liquid
 85 crystalline phases. In this short review we are mainly addressing the formation of micellar aggregates
 86 of saponins and microbial surfactants in water, respectively. Saponins are exemplarily discussed in
 87 section 2 for aescin, glycyrrhizin, and quillaja saponins, while we have focused on rhamno- and
 88 sophorolipids in the third section. Scattering techniques are ubiquitous to the field, and the interested
 89 reader is referred to ref. [Zemb & Lindner (1988)].

90 2 Binary systems of saponins in water: Aescin, Glycyrrhizin, and Quillaja saponins

91 Saponins are plant-derived biosurfactants. In applications, they serve as emulsifier in food foams, or
 92 in pharmaceutical or cosmetic formulations. Prominent representatives of this group like aescin
 93 (sometimes also named escin), glycyrrhizin, and quillaja saponins (for chemical structures see Figure
 94 1a-d), as well as their mixtures with synthetic surfactants are discussed below.

95 Aescin:

96 Aescin is one of the most studied saponins. It stems from the horse chestnut tree (*aesculus*
 97 *hippocastanum*) and is available commercially in a rather pure form. Its predominant component has
 98 the typical triterpenoid hydrophobic moiety with several polar groups attached one-sided, and a large
 99 hydrophilic head group of three saccharide groups carrying a single carboxyl group. The latter confers
 100 a possibly slightly ionic character to the molecule, at high pH.

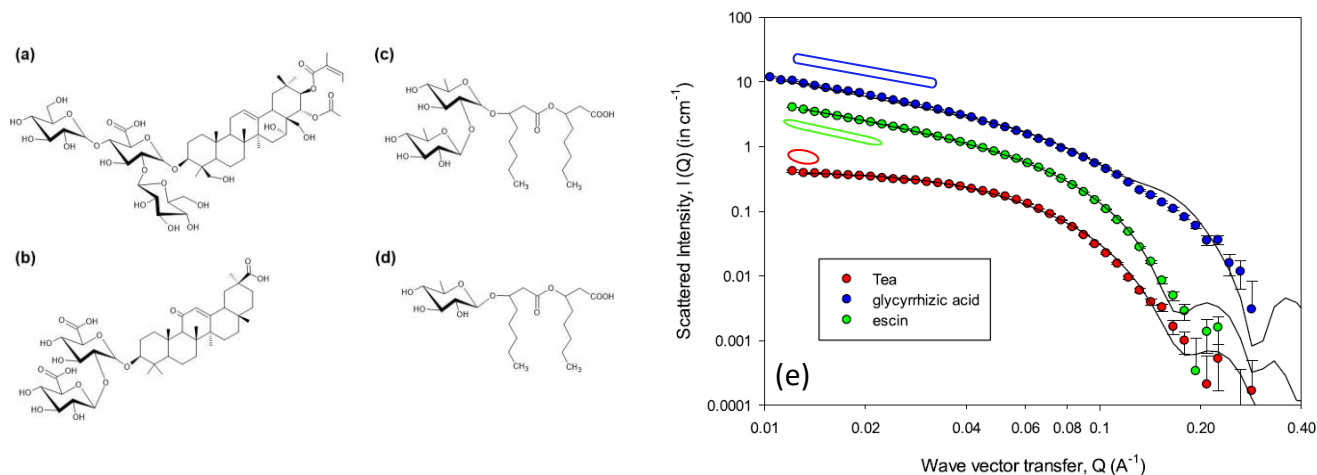
101 In pure water, this saponin behaves like an amphiphile. The determination of the CMC at fixed
 102 physiological pH 7.4, between 10 and 50°C, has been performed recently, using the autofluorescence
 103 of aescin. [Dargel et al. (2019)] Moreover, modeling of SAXS intensities shows that with increasing
 104 temperature, and only slightly affected by concentration, aescin micelles evolve from cylindrical
 105 (typical length 10 nm, radius 2 nm) into shorter, ellipsoidal shapes.

106 Aescin has a strong biological activity and interacts with 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine
 107 (DMPC) phospholipid membranes decomposing them to bicelles (or lipid nano-disks, with bilayer
 108 structure), by stabilizing edges [Geisler et al. (2019b)]. Similarly, when conventional surfactants are
 109 added to lipid bilayers, the formation of bicelles was found, in which the surfactant molecules form
 110 their highly curved edge. [Dürr et al. (2013)] Small-angle scattering and TEM with a combined analysis
 111 by indirect Fourier transformation [Fritz & Glatter (2006)] and simultaneous modelling of SAXS and
 112 SANS showed that the nanodisk diameter decreases from ca. 30 nm to 12 nm with increasing aescin
 113 content. Moreover, depending on the amount of aescin, the disks reassemble upon temperature increase
 114 from the gel to the fluid phase of the phospholipid. Later, the same group showed that aggregate
 115 morphology can evolve from nano-disks into larger ribbons and finally stacks of sheets [Geisler et al.
 116 (2021)].

117 Very recently, Tucker et al. have studied the influence of oxyethylene chain length of model nonionic
 118 surfactants (*n*-dodecyl polyglycol ether, C₁₂E₃ to C₁₂E₈) on the interactions with aescin by SANS
 119 [Tucker et al. (2022b)]. The limited compatibility between the oxyethylene and sugar headgroup
 120 controls the self-assembly, which encompasses elongated aescin micelles and intermediate planar

121 (vesicular or locally lamellar) morphologies. It is striking that the transition to the latter does not
 122 depend much on the curvature of the added nonionic surfactant. In this context, we note that the
 123 spontaneous formation of very small micro (or “nano”)-vesicles has been evidenced with synthetic
 124 surfactants by scattering before [Oberdisse et al. (1996), Oberdisse & Porte (1997), Gradzielski et al.
 125 (1997), Gradzielski (2003), Grillo et al. (2008), Bressel et al. (2010)]. Although such strong curvatures
 126 were believed to be impossible for bilayers, they have now shown to exist also in biosurfactant systems.

127 The same group in the UK has also investigated the properties of mixtures of glycyrrhizic acid, aescin,
 128 and tea saponin [Tucker et al. (2021b)]. This is an interesting series due to the increase in saccharide
 129 groups from two to four. As shown in Figure 1e, tea saponins form globular structures due to the largest
 130 headgroup, and this is also the preferred morphology in any mixture including tea saponins. Blends
 131 without tea saponin have been found to keep the elongated structure as seen by the low- q slope, with a
 132 subtle interplay between glycyrrhizin and aescin curvatures.



133

134 **Figure 1:** Chemical formulas of (a) Aescin, (b) Glycyrrhizin, (c) di-Rhamnolipid and (d) mono-Rhamnolipid.
 135 e) SANS intensities for tea, aescin (shifted vertically x8), and glycyrrhizic acid saponin (shifted x16), at 5 mM
 136 with 0.1 M NaCl. The solid lines are model fits, the corresponding geometries are sketched in the same color,
 137 and parameters are reported in the original article. Figure reprinted from ref. [Tucker et al. (2021b)] with
 138 permission from Elsevier.

139 It is noteworthy that in all these studies small-angle scattering with detailed quantitative modelling
 140 (namely core-shell) is needed to characterize each population of aggregates, and their coexistence. In
 141 the last study, e.g., the absence of the structure factor gives access to the form factor, permitting a
 142 trustworthy determination of average aggregation numbers of about 80 for tea saponins. This is similar
 143 to that observed in most globular micelles formed by conventional surfactants, whereas elongated
 144 structures reach 300. Increasing the head group size leads to smaller spherical micelles, thus following
 145 the well-known packing criteria of Israelachvili and Ninham [Israelachvili et al. (1976)]. However,
 146 in detail, the packing requirements are more sophisticated due to the much more complex structure of
 147 saponins and biosurfactants [Tucker et al. (2021b)].

148 Glycyrrhizin:

149 Glycyrrhizin (or glycyrrhizic acid, known for its sweet taste) is extracted from licorice roots
 150 (*glycyrrhiza glabra*). This saponin is based on a triterpenoid tail, and the hydrophilic part is a
 151 disaccharide carrying carboxyl groups. As opposed to simpler molecules like dodecyl maltoside, which
 152 forms globular micelles in agreement with its conical packing, the large hydrophobic group favors a
 153 lower spontaneous curvature. It is now established that glycyrrhizin forms elongated structures as seen

154 from the q^{-1} -power law behavior of the SAXS data (and also directly in TEM or AFM) [Saha et al.
 155 (2015), Matsuoka et al. (2016)], which presumably connect to form gels in water after a heating-
 156 cooling cycle. Matsuoka et al. have followed the CMC as a function of pH by fluorescence and light
 157 scattering. Tucker et al have studied the aggregate morphology by SANS [Tucker et al. (2021a)]. In
 158 pure D₂O, detailed modelling provides the lateral dimensions (typically 3-4 nm core, plus a 2 nm thick
 159 hydrated shell) of elongated (length ca. 27 nm) aggregates, with aggregation numbers of 150. Different
 160 salts then induce moderate micellar growth, presumably due to reduction in head group interaction.
 161 Note that there has been a debate about the existence of a CMC, and on the size of glycyrrhizin
 162 micelles; different values have been reported under different experimental conditions, in particular pH.
 163 Due to the presence of carboxyl groups on some saponins, it is important to take pH modifications into
 164 account, or use buffers. The carboxyl groups (especially the one at the C20 position) are protonated up
 165 to a pH value of 6. Below this value glycyrrhizin is amphiphilic with CMC [Matsuoka et al. (2016)]
 166 and self-assembly [Dargel et al. (2021)].

167 **Quillaja saponins:**

168 This group of saponins can be extracted from *Quillaja Saponaria Molina*, the soap bark tree. The
 169 hydrophobic part is again a steroid or triterpenoid. The sugar head group illustrates the great chemical
 170 variability of saponins. Its saccharides may be glucose, galactose, xylose, rhamnose, arabinose, and
 171 glucuronic acid, substituted at up to three positions in the glycone (mono-, bis, and tri-desmosides,
 172 respectively). More than sixty different saponins have been identified in *Quillaja Saponaria* by mass
 173 spectroscopy [Nord & Kenne (1999)]. The surface activity of Quillaja saponins is suspected to be
 174 involved in their cytotoxic and haemolytic properties, and it has been investigated by Wojciechowski
 175 et al. by surface tension and neutron reflectometry [Wojciechowski et al., (2014a)]. In a second article,
 176 dynamic light scattering showed that aggregation properties depend on both the origin of the molecules
 177 and on time [Kezwon & Wojciechowski (2014)]. Initially small globular micelles (5 to 7 nm) evolve
 178 into objects of hundreds of nanometers. The same surface techniques have been applied to characterize
 179 the penetration of phospholipids []. Very recently, a comparative study of different saponins has also
 180 been conducted, including their effect on model lipid monolayers [Wojciechowski et al. (2021)].

181
 182 The incorporation of different molecules into Quillaja micelles has attracted quite some interest,
 183 including mixtures with conventional surfactants [Tucker et al. (2022a)]. Toppel et al. have studied the
 184 uptake of lutein (a carotenoid), resulting in micellar growth [Toppel et al. (2016)]. In a structural
 185 investigation including SANS, Peixoto et al. have investigated mixtures of mate saponin, and in
 186 particular benchmarked them against Quillaja saponins in terms of uptake of two model drugs,
 187 carbamazepine and flurbiprofen [Peixoto et al. (2011)]. Kezwon and Wojciechowski have studied
 188 interactions of Quillaja saponin with food-related proteins (hen egg lysozyme, bovine β -lactoglobulin
 189 and β -casein) by surface rheology [Kezwon & Wojciechowski (2014)]. The authors explain the
 190 differences observed for saponins of different origins by different acidities, modifying electrostatic
 191 interactions with proteins. The biological activity of saponins may be due to their interaction with
 192 cholesterol embedded in phospholipid membranes. Adding cholesterol to Quillaja saponin has been
 193 shown using SAXS and SANS to trigger a transition from globular to spherohelical micelles, followed
 194 by a reentrant phase transition [Liu et al. (2013)].
 195

196 **3 Binary systems of microbial surfactants in water: rhamnolipids**

197 Microbial biosurfactants possess intrinsically the same advantages as saponins. Their most important
 198 macroscopic physico-chemical manifestation of amphiphilicity is the strong surface activity leading to

199 foaming. E.g., very low concentrations of surfactin (a cyclic lipopeptide produced, among others, by
200 *Bacillus Subtilis*) or rhamnolipids lower the surface tension of the water/air interface by more than a
201 factor of two [Rodrigues (2015)]. Rhamnolipids favor the formation of bacterial biofilms, they possess
202 a CMC, and form micelles above it. All (bio-)surfactants have the capacity to penetrate and possibly
203 disrupt membranes or phospholipid systems. Although this may be vital for any pharmacological
204 activity, we focus on the bulk behavior through the formation of biosurfactant aggregates in water.

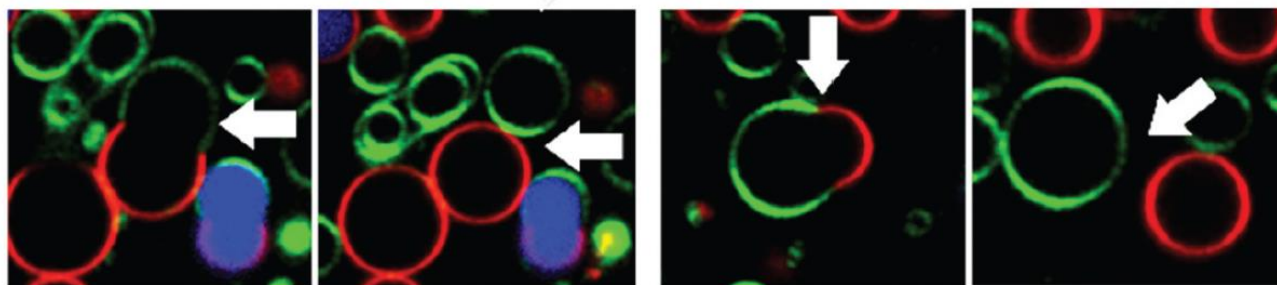
205 In a continuous effort to understand the phase behavior of blends of anionic surfactants as used in
206 detergency applications with biosurfactants, the Oxford group has published a series of articles starting
207 about a decade ago, in collaboration with I. Grillo (ILL), an expert of determination of aggregate
208 morphology. Chen et al. have studied the adsorption and bulk properties of mono- and dirhamnolipids
209 and their blends [Chen et al. (2010b)], of their mixtures with the anionic surfactant sodium dodecyl 6-
210 benzene sulfonate (LAS) [Chen et al. (2010a)], and of different types of sophorolipids (lactonic or
211 acidic, varying hydrophobicity) [Penfold et al. (2011)]. Using neutron reflectometry and small-angle
212 scattering with ca. 90% deuterated biosurfactant provided a detailed view of aggregate morphology.
213 While the phase diagrams of these systems are usually complex, the underlying tendencies can be
214 rationalized by simple rules: sugar head groups are bulky and mostly nonionic, lowering electrostatic
215 charge density, spontaneous curvature, and introducing steric repulsion, thus preventing the complex
216 formation. In a follow-up, the tolerance of surfactant mixtures including rhamnolipids and LAS to
217 precipitation induced by calcium has been investigated [Chen et al. (2013)]. Whereas synthetic anionic
218 surfactants employed in detergent formulations are highly sensitive to hard water, with possibly
219 disastrous effects on their function, the addition of rhamnolipids, which are mostly nonionic, somewhat
220 increase the tolerance towards multivalent ions. Depending on the surfactant system, ions of high
221 valency promote micellar growth and sometimes morphological transition to bilayer phases –
222 demonstrating the influence of electrostatics on spontaneous curvature. Incorporating nonionic
223 surfactants, and in particular biosurfactants, increases minimal ion concentration for precipitation. In
224 this study of morphologies before precipitation, the influence of the calcium ions is characterized by
225 the change in aggregation number and surface micellar charge. Pure rhamnolipids are found to show
226 practically superimposed scattering curves with and without Ca^{2+} (2 mM), indicating that CaCl_2 has
227 little impact on the self-assembly. In contrast, the anionic surfactant and its mixtures with rhamnolipids
228 show a complex response to Ca^{2+} addition with a tendency to form planar phases.

229
230 More recently, the same group has again performed small-angle scattering on blends of rhamnolipids
231 with the nonionic model surfactant C_{12}E_8 (*n*-dodecyl octaethylene glycol ether), LAS, and yet another
232 ionic one, sodium dioxyethylene monododecyl sulfate [Liley et al. (2017)]. The influence of the
233 rhamnolipids is to favor the formation of bilayer (vesicular) phases at high rhamnolipid and LAS
234 content. Coexistence of the two morphologies is observed as superimposed contributions in SAXS,
235 with increased low-*q* scattering including first- and second-order Bragg peaks characteristic of lamellar
236 phases, presumably multilayer vesicles or onions. In other phase regions, small interacting micelles
237 with internal composition equal to the nominal one are formed.

238 In very recent work, Palos Pacheco et al. [Pacheco et al. (2021)] have tuned the self-assembly of nature-
239 inspired (mono-)rhamnolipids via the shape of the hydrophobic moiety. These compounds rely on a
240 remarkable effort in organic synthesis, circumventing thus the usual lack of pure compounds. The
241 effect of asymmetry between the two lipid tails has been highlighted by surface tension measurements,
242 dynamic light scattering, and fluorescence quenching. The combination of a long (C_{14}) tail with a
243 second shorter one (C_6 to C_{14}) gives access to a range of molecular packings in-between conical or
244 cylindrical shapes, and thus more globular or more planar aggregates. Surface tension shows a strong
245 dependence on chain asymmetry. DLS results are difficult to interpret due to superposition of different

246 populations; a q-resolved scattering technique (SAXS/SANS) might have given further insight into
 247 local structures. The location of the pyrene fluorescence probe, finally, provides molecular
 248 understanding of the observed changes: the more asymmetric the tails, the less polar the core and the
 249 higher the free volume, allowing accommodation of the probe molecule. The same group has also used
 250 numerical approaches to understand the phase behavior of rhamnolipids better [Eismin et al. (2017)].

251 The impact of mono- and dirhamnolipids on giant unilamellar phospholipid vesicles has been studied
 252 by the group of Winter [Herzog et al. (2020), Herzog et al. (2021)]. The formation of daughter vesicles
 253 upon addition of either RhaRhaC10C10, RhaC10C10 (corresponding to the di- and monorhamnolipids
 254 as illustrated for RhaRhaC8C8 and RhaC8C8 in Figure 1c-d), and the sugar-free precursor C10C10
 255 has been imaged as shown in Figure 2, and is interpreted in terms of the different spontaneous
 256 curvatures.



257

258 **Figure 2:** Confocal fluorescence microscopy of giant unilamellar vesicles showing budding and fission of
 259 domains after addition of RhaC10C10. Reproduced from reference [Herzog et al. (2021)] with permission from
 260 the Royal Society of Chemistry.

261 The group of J.S. Pedersen has studied the interaction of biosurfactants with proteins in water, via the
 262 formation of mixed self-assembled aggregates. The authors underline that changes in enzymatic
 263 activity caused by biosurfactants may occur, depending on the supramolecular structure of the latter.
 264 Binding to proteins may be favored or hindered by electrostatics, and may be cooperative or individual,
 265 thereby affecting the interactions. A light scattering and SAXS-study of rhamnolipid aggregates has
 266 been performed, followed by a comparison of synthetic sodium dodecyl sulfate (SDS) to rhamnolipid
 267 aggregates incorporating α -lactalbumin and myoglobin [Mortensen et al. (2017)]. Pure rhamnolipids
 268 form small prolate micelles containing some eleven molecules. The failure of modelling the scattering
 269 intensity by simple addition of individual contributions, and a detailed analysis of the scattering data
 270 by indirect Fourier transform shows that the mixed SDS/rhamnolipid aggregates harbor only a single
 271 protein, with little change of the overall shape and size of the micelle for lactalbumin, whereas
 272 myoglobin induces pairwise micellar association. This contrasts with SDS, which in pure solution has
 273 a larger core. Upon addition of α -lactalbumin, the micellar shape changes from oblate into prolate, with
 274 up to three proteins.

275 4 Summary and Outlook

276 We have reviewed recent progress in the determination of biosurfactant self-assembly in water. The
 277 precise measurement of physico-chemical properties of biosurfactants in water is still in its infancy, in
 278 spite of the growing importance of biosurfactants for a sustainable economy. This is presumably due
 279 to the large chemical variety – making the understanding of mixtures even more relevant. Clearly, the
 280 most quantitative techniques are small-angle and dynamic light scattering, because they enable precise
 281 measurements of biosurfactant aggregate morphologies.

282 The surfactant properties of biosurfactants and conventional surfactants are comparable. For example,
 283 the cmc of rhamnolipids (RhaRhaC10C10, RhaC10C10) is close to the one of the standard surfactant
 284 LAS, of about 10^{-1} mmol⁻¹. Similarly, the minimum surface tension value is comparable (30 mNm⁻¹)
 285 [Chen et al. (2010a)], increasing as expected with the number of rhamnose units due to the increasing
 286 bulkiness of the biosurfactants [Chen et al. (2010a)]. A major difference of especially saponins with
 287 conventional surfactant is the stronger pH-dependence of the surfactant properties, sometimes up to
 288 absence of micellization.

289 We anticipate biosurfactants to be of growing importance in the progressive replacement and
 290 improvement of synthetic surfactant mixtures by sustainable and biodegradable molecules in many
 291 applications, from pharmacology to detergency. Besides being used as “simple” surfactant, saponins
 292 also offer a large potential in pharmacy, and especially the antiviral activity of glycyrrhizin remains to
 293 be fully exploited e.g. in treatment of SARS-Cov2.

294 **5 Conflict of Interest**

295 The authors declare that the research was conducted in the absence of any commercial or financial
 296 relationships that could be construed as a potential conflict of interest.

297 **6 Author Contributions**

298 JO, TS, and TH have planned and written the article together.

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