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# Microbial Biobased Amphiphiles (Biosurfactants): General Aspects on CMC, Surface Tension and Phase Behaviour.

Niki Baccile<sup>a,\*</sup>, Alexandre Poirier<sup>a</sup>

<sup>a</sup> Sorbonne Université, Laboratoire de Chimie de la Matière Condensée de Paris (LCMCP), UMR CNRS 7574, 4 place Jussieu, Paris, F-75005, France

E-mail: [niki.baccile@sorbonne-universite.fr](mailto:niki.baccile@sorbonne-universite.fr)

## **Abstract**

Biosurfactants of microbial origin are compounds obtained from the microbial fermentation of glucose and vegetable oils. Developed for their surface active properties combined to a high biodegradability and low toxicity, these molecules have a complex behaviour in water and addressing them as biobased amphiphiles, or bioamphiphiles, is more appropriate. This chapter illustrates the most important properties in solutions of microbial bioamphiphiles, from hydrophilic lipophilic balance (HLB), hydrophilic lipophilic difference (HLD), critical micelle concentration (CMC) and surface tension (ST), typically illustrating their surfactant character, to more complex self-assembly properties, including phase behaviour, rather illustrating their amphiphilic character. These data are critically discussed in the perspective of classical head-tail but also bolaform amphiphiles.

## **Keywords**

Critical Micelle Concentration, CMC, Surface Tension, Phase Diagram, Self-Assembly, Surfactant, Biosurfactants, Amphiphile, Bioamphiphiles, Hydrogels

## Introduction

Surfactants are a class of chemicals applied in a vast array of applications and markets, reaching production volumes of about 20 million tons per year,<sup>1,2</sup> with an economic weight of 43.7 billion dollars in 2017, projected to reach 66.4 billion dollars by 2025.<sup>3</sup> The word “surfactant” is the contraction of “SURFace ACTive AgeNT”, indicating their ability to adsorb at interfaces, with the property of lowering the surface tension of water. This behavior is attributed to their ‘amphiphilic’ nature defined as molecules with a hydrophilic (“water-loving”) and a hydrophobic (“water-hating”) part. Due to the widespread use and applications of surfactants, research on surfactants constitutes a scientific domain of its own.

Surfactants have played a decisive role in shaping the concepts of sustainability and green chemistry. Fatty acid soaps guarantee cleanliness and hygiene since time immemorial. Surfactants are involved in the environmentally friendly production of rubber, plastics, paints and adhesives in the aqueous phase. In the field of polymer synthesis, surfactants make this possible in water, thus lowering, or even eliminating, the risks of these processes, such as fire hazards. Toxic emissions are reduced towards zero and occupational safety is increased.

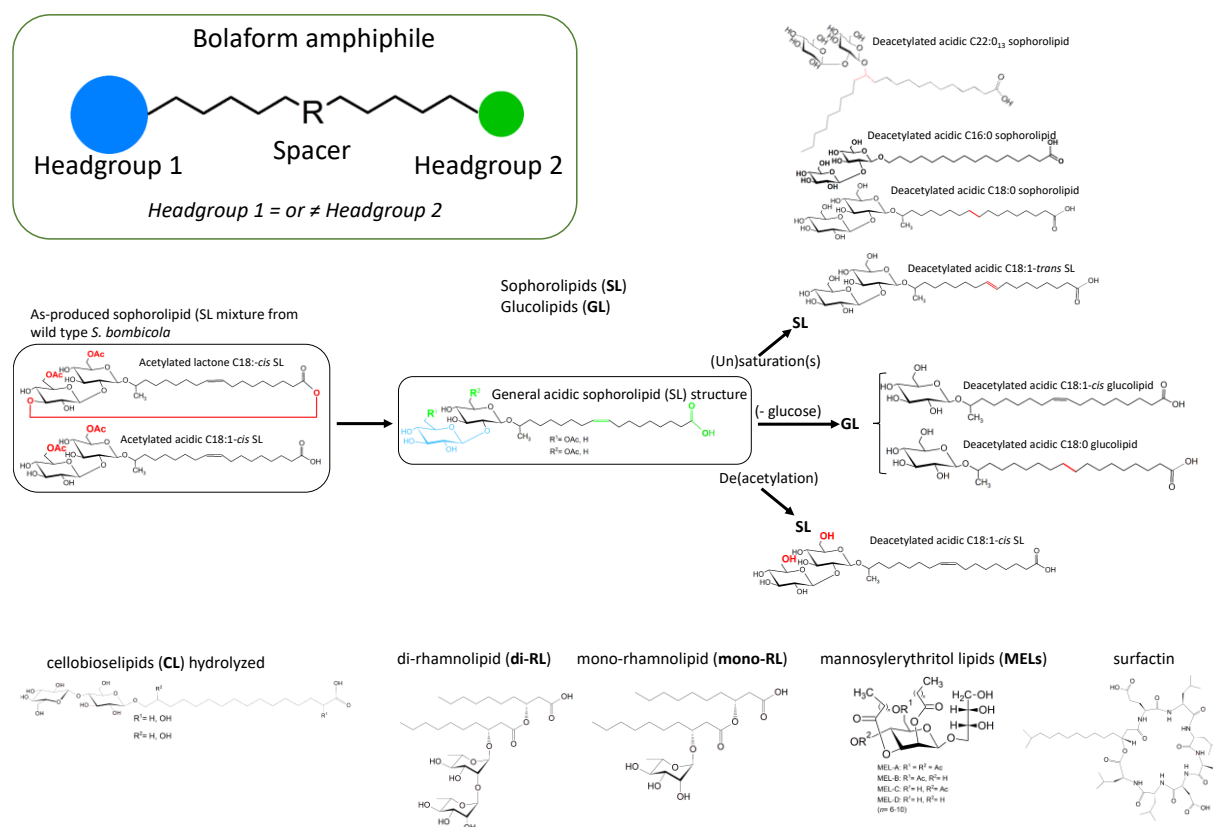
However, their ubiquitous use in our everyday lives also has some drawbacks. Surfactants have been associated with pollution problems, but also with dermatological issues such as skin irritation and even allergic reactions. Moreover, many of the produced surfactants are derived from petrochemical resources and associated with harsh and/or polluting production processes. Many products have already been banned for reasons of toxicity and/or pollution in the past 30 years and more are expected to follow. For these reasons, investigations aiming at finding non-toxic, benign, products and more specifically natural biobased alternatives to petrochemical surfactants started as a sub-field in surfactant’s science since the 1960’s, and developed as a field *per se* since the 1970’s, motivated by the oil crisis and raising of oil costs.<sup>4-6</sup> Employment of linear alkylbenzene sulphonates and methyl ester sulphonates instead of their branched counterparts, use natural fatty alcohol alternatives to synthetic alcohol polyglycoethers or sulphates; green fatty alcohol (or guerbet) alcohol polyglycol ethers, -ethersulphate, -phosphates, and -sulphosuccinate surfactants replaced alkylphenol polyglycoethers. These are some of the most common strategies employed by industry to develop more benign molecules.<sup>7,8</sup> The quest of more ecofriendly surfactants is then just a natural consequence of this long-date trend.

Bio-based surfactants, or biosurfactants, are defined as molecules that are fully based on biomass such as sugars, plant oils, amino acids, etc. This field is characterized by two different production approaches. In the first, chemical, approach, biobased hydrophilic and hydrophobic molecules are covalently linked through organic<sup>9,10</sup> chemistry. In the second, biological, approach, biosurfactants are either extracted from plants or produced through biocatalytical (use of enzymes) or microbial processes. Although the frontiers between and within these approaches are sometimes blurry, a broad community agrees on employing the word “biosurfactants” in relationship to amphiphilic surface active agents produced by a microbial fermentation process. One then speaks of “*microbial biosurfactants*”.<sup>4,11,12</sup>

Research on microbial biosurfactants is known since the 1960’s,<sup>13,14</sup> but it is becoming a trendy topic since two decades, during which a large number of review papers and books have been published.<sup>11,12,15–27</sup> They commonly address the topic of microbial biosurfactants’ classification, the synthesis’ strategy, derivatization and genetic modification towards development of new chemistry,<sup>18,28,29</sup> their aqueous and antimicrobial properties and their application potential in various fields.<sup>4,12,15,17–19,30–33</sup>

The number of existing biosurfactants from microbial origin is quite impressive, as well as the number of microorganisms producing them.<sup>34</sup> However, only few can be produced in sufficient amount, with acceptable purity and homogeneity to be satisfactorily studied from a physicochemical point of view. Rhamnolipids (RLs), sophorolipids (SLs), cellobioselipids (CLs), mannosylerythritol lipids (MELs) and surfactin are broadly recognized as the most classical ones. Trehalolipids (TLs) are an interesting case. We are not aware of any specific study on the solution and interface properties of trehalolipids, despite some non-negligent work that has been done on this family of compounds since the mid 50’s.<sup>35</sup> In the meanwhile, chemical derivatizations of existing biosurfactants,<sup>18</sup> and more recent trends in the production of new biosurfactants from engineered strains,<sup>15,17,18,28,29,34,36–41</sup> constitute promising alternatives to expand the biosurfactant portfolio in the future. The availability of these new compounds since less than a decade and ready collaboration between researchers across disciplines has made their advanced characterization possible. Figure 1 summarizes the most important biosurfactants found in the literature. It also includes some derivatives, like glucolipids (GL) or stearic acid SL. The list is far from being exhaustive, as a number of new derivatives, may them be of chemical or biotechnological origin, are produced regularly.

We anticipate that, considering the latest research on the solution properties of the molecules given in Figure 1, the word “biosurfactant” is reductive and one should rather speak of bioamphiphiles, whereas a surfactant is an amphiphile with surface active properties. In fact, most of the molecules in Figure 1 only show surface active properties under specific conditions of pH and temperature and in some cases they do not show them at all. Addressing to them as biosurfactants only could then be erroneous in some cases. In the field of colloids science, the surfactant and lipid communities are generally distinct, although connected by many bridges. Property- and applicationwise, the same distinction should occur in this field. However, one must acknowledge that the word biosurfactant is nowadays largely employed and it would be quite tedious to introduce a newer terminology. We then try to identify when the molecules in Figure 1 behave as surfactants and when they behave as lipids.



**Figure 1 – Most important biosurfactants found in the literature.**

This chapter focuses on the physicochemical properties and phase behaviour of microbial biosurfactants in aqueous solution within the broader context of surfactants in solution. Please note that this chapter has been adapted from a recent review article written by the authors, to which the reader can address for a more tutorial presentation of the field.<sup>42</sup>

The chapter will end with some perspectives in colloids and materials science that are peculiar to this class of compounds.

### **Biosurfactants in solution**

This section recalls few major concepts of surfactants in solution, connecting properties with molecular structure. In parallel, the same ideas will be outlined for the major biosurfactants.

#### *Hydrophilic-lipophilic balance (HLB)*

The hydrophilic-lipophilic balance (HLB) and hydrophilic-lipophilic difference (HLD) are two widespread approaches to forecast the emulsification ability of surfactants.<sup>43-45</sup> The HLB was conceived to create an empirical relationship between the surfactants' properties (e.g., oil-in-water (o/w) or water-in-oil (w/o) emulsifier, wetting agent or detergent) and their molecular composition, whereas the latter is generally expressed in terms of the balance of the hydrophilic and hydrophobic portions of the molecule. Initially developed for polyoxyethylene-type surfactants, HLB has been widened to a much broader class of molecules by including the contribution of specific chemical groups, which have a strong influence on the properties. Despite its astonishing simplicity, the HLB method has been employed in the surfactant industry for years and it works nicely on well-established molecules, like non-ionic surfactants. Nonetheless, this method fails for a number of systems because it does not take into consideration the effect of temperature, electrolytes and ionic strength, impurities, and additives in general. Another drawback is certainly the plethora of existing methods to calculate the HLB method, which was actually by-passed by the Hydrophilic Lipophilic Difference (HLD) method, developed in the late 70's. HLD, developed by Salager,<sup>46-48</sup> is much less known but it constitutes an evolution of HLB because it includes external parameters such as temperature, salinity and the nature of the oil. In the end, both HLB and HLD revealed to be useful for few standard ionic and non-ionic surfactants, but they cannot easily be generalized to complex amphiphiles, like divalent, gemini, branched or bolaform (Figure 1) surfactants.

The HLB of major surfactants is well-known. For instance Tween derivatives have HLBs between 10 and 20 while Brij from 4 to 16, depending on the length of the PEO headgroup.<sup>49</sup> On the contrary, few scientific publications discuss the HLB of biosurfactants and one of the

latest was published on surfactin in 2005.<sup>50</sup> Using the Griffin formula ( $HLB = 20 \times (MW_H/MW_S)$ , with  $MW_H$  and  $MW_S$  respectively being the molecular weight of the hydrophilic part and of the whole surfactant), one finds values between 6 and 13 for MELs,<sup>51-53</sup> 21 for surfactin<sup>50</sup> and, on the basis of the chemical formulas, one can estimate values contained between 5 and 15 for SLs and RLs.<sup>54</sup> For many biosurfactants, the properties expected according to the calculation of the HLB are in agreement with the broad range of properties experimentally observed, like o/w emulsification and detergency.<sup>55,56</sup> However, calculated HLB for biosurfactants can be very broad, as in the case of TLs, or the expected properties may not correspond to the value of HLB, thus generating confusion and bad expectations.

Marqués *et al.* estimate an HLB of 11 for a TLs mixture so that o/w emulsion is expected, although w/o emulsion is obtained.<sup>57</sup> Acidic SLs are expected to be o/w emulsifiers, but in fact their bolaform nature make them poor emulsifying agents. To improve their emulsifying character, the hydrophobic character of the tail must be improved by chemical modification.<sup>58</sup> HLB also fails to predict the behavior in mixture of compounds with different HLBs. Some studies provide the HLB for a given biosurfactant, as reported for individual MELs, but calculated HLB fails to predict and understand the interfacial behavior of a mixture of MELs, which constitute the actual raw compound.<sup>51</sup> Finally, HLB becomes unsuitable to predict the behavior of polymeric and proteic biosurfactants like surfactin, because the HLB range expected by surface efficiency of surfactin<sup>59,60</sup> is far from the HLB calculated by emulsification method.<sup>50</sup> HLB of surfactin is varying with environmental conditions like pH, specific ions condensation and temperature and it is a source of debate.<sup>61-63</sup> These specificities render HLB useless and require more refined understanding of the biosurfactant behavior in solution and in oil and water mixtures.

### *Surface Tension (ST) and critical micelle concentration (CMC)*

The surface tension is a parameter of paramount importance in a number of physical phenomena like adsorption, wetting, catalysis, distillation and much more, with direct involvement in the conception of industrial products in coating, food, detergents, cosmetics and so on. Surface tension is defined as the energy required to create a unit area of interphase<sup>64</sup> and surfactants play a crucial role in lowering the surface tension of water at the water-air interface from about 70 mN/m to about 25 to 40 mN/m. Upon mixing micromolar amounts of a surfactant in water, the water-air interface is occupied by surfactant monomers,

pointing the hydrophilic headgroup towards water and the hydrophobic chain towards air. This phenomenon is at the origin of the reduction in surface tension and to the increase in surfactant packing at the interface.<sup>65</sup>

When the surfactant reaches the conditions of maximum packing, it will start aggregating into spheroidal aggregates, micelles, in the bulk solution. The concentration at which aggregation occurs is called critical micelle concentration, widely known as CMC,<sup>2</sup> and also referred to as CMC1, in opposition to CMC2, the concentration value above which micellar growth is rapidly implemented.<sup>66</sup> CMC is classically determined by the inflection point in surface tension vs. concentration experiments, although many other techniques, such as turbidity, self-diffusion NMR, solubilization, pyrene fluorescence and many others can be equally used. The typical CMC1 values for a broad set of surfactants settles in the order of the mM range, although the dispersion is broad (between  $10^{-5}$  and  $10^{-1}$  M) and it strongly depends on the chemical structure of the surfactant, where type of headgroup and chain length are critical parameters.<sup>67</sup>

There are four main families of classical head-tail surfactants and they are classified on the basis of their headgroup: cationic, anionic, non-ionic and zwitterionic. Whichever the chemical nature of the head group, the CMC decreases with increasing the length of the alkyl chain, where the decrease is more pronounced for non-ionics than for ionics, respectively a factor 3 and 2 upon addition a methylene group in the aliphatic chain. The CMC values of non-ionic surfactants are about two orders of magnitude lower than the values of ionic surfactants. Interestingly, among ionic surfactants, the difference in CMC is milder, with cationics having higher CMC values than anionics, while among non-ionics, CMC slightly increases with bulkiness of headgroup. Other parameters have an important influence on CMC such as the valency of counterions for ionic surfactants (the higher the valency, the lower the CMC), branching, unsaturation, cosolutes. Temperature is also an important parameter, which however has a much stronger impact on the surfactant's solubility itself through the Kraft phenomenon. The Kraft point is defined as the temperature below which the surfactant is insoluble and above which solubility experiences an exponential increase.<sup>67</sup>

One last remark concerns the estimation of CMC for bolaamphiphiles (bolas). Bolas have attracted a lot of attention in the past years,<sup>68</sup> but they have been studied in a less rational manner than single chain surfactants. For these reason, to the best of our knowledge, no general experimental trend in their CMC has been reported so far. Nonetheless, Nagarajan



has calculated, and compared two types of experiments; the CMC values for bolaform surfactants and found that higher values than single-head amphiphiles are expected, the second headgroup in bolas improving the monomer solubility in water. Depending on the nature of the headgroup (ionic or non-ionic), he gives values in the order of  $10^{-2}$  M.<sup>69</sup>

ST and CMC have been extensively reviewed in the past for many biosurfactants.<sup>12,16,19,31</sup> However, as shown hereafter, a rationalized comparison of ST and CMC across biosurfactants is not easily possible. For this reason, the purpose of the following paragraphs is a critical overview of ST and CMC in the context of biosurfactants, rather than list of hardly-to-compare values.

ST experiments on biosurfactants started already in the 60's,<sup>70-72</sup> although thorough ST measurements were only carried out from the 80's onward, when low-molecular weight glycolipid biosurfactants, like RLs, SLs or TLs, appeared to have a better market potential in view of replacing petroleum-based surfactants.<sup>73-75</sup> In the meanwhile, constant improvements in developing both structural variety and increasing production rates contributed to promote ST studies later on.<sup>76,77</sup> Interestingly enough, even if some biosurfactants were discovered in the 50's, like CLs, the study of their interfacial properties only started half a century later.<sup>78,79</sup>

Biosurfactants have similar concentration-dependent ST profiles as reported for synthetic surfactants, but the mechanism of surface stabilization depends on their molecular weight. Low-molecular weight (LMW) biosurfactants, like RLs, SLs or MELs (< 1 kDa), follow the classical adsorption/desorption mechanism at the air-water interface considered to be at thermal equilibrium ( $\sim kT$ ). High-molecular weight (HMW) biosurfactants, like surfactin, emulsan or alasan ( $1 < Mw / kDa < 500$ ), on the contrary, follow a colloidal interfacial adsorption behavior, considered to be irreversible in the range of  $kT$ . In the literature, the former are generally referred to as biosurfactants and the latter bioemulsifiers.<sup>30,80</sup>

Table 1 - Minimal surface tension of common biosurfactants in aqueous media. RL: rhamnolipids, SL: sophorolipids, TL: trehalolipids; CL: cellobioselipids; MEL: mannosylerythritole lipids

BS	ST (mN/m)	Inferior limit	Superior limit	Ref.
RLs	25.0 – 37.4	Mixture from <i>P. aeruginosa</i> 44T1.	Di-RL from <i>P. aeruginosa</i>	81-86
SLs	34.2 – 48.0	Mixture from <i>S. bombicola</i> NRRL Y-17069.	Mixture from <i>C. bombicola</i> ATCC 22214.	19,56,87

TLs	19.0 – 43	Succinoyl TL from <i>Rhodococcus sp.</i> SD-74.	TL dicorynomycolates from <i>R. erythropolis DSM43215</i> .	75,88,89
MELs	24.2 – 33.8	Purified mono acetylated MEL-C from <i>P. graminicola CBS10092</i> .	Purified acylated MEL-A from <i>P. antarctica T34</i> .	52,77,90 -92
CLs	34.7 – 41.9	Sodium salt of CL mixture from <i>C. humicola JCM 10251</i> .	Mixture from <i>C. humicola JCM 1461</i> .	79,93,94
Surfactin	27.0 – 30.0	Surfactin from <i>B. subtilis IAM 1213</i> in 100mM NaHCO <sub>3</sub> .	Industrial Surfactin from <i>Wako pure chemical Ind, Japan</i> .	59,70,95, 96
Emulsan	27 – 46.4	Emulsan from <i>A. calcoaceticus PTCC 1318</i> .	Emulsan from <i>A. calcoaceticus RAG-1 (ATCC 31012)</i> .	97,98
Alasan, liposan	Good emulsifying capability			12,99- 101

Table 1 gives the range of minimal surface tension for LMW<sup>102–104</sup> and HMW biosurfactants.<sup>105–107</sup> For all compounds, ST varies between 50 and 20 mN/m, although large disparities can be found for the same compound, as it is the case of trehalolipids, for which a range of 19 to 43 mN/m could be found in the literature. Overall, these values are comparable to the ST of classical surfactants: anionic surfactants, like sodium hexadecyl sulfate (SHS) and sodium dodecylsulfate (SDS), reduce the surface tension to 36 and 38 mN/m, respectively while nonionic surfactants like triton X-100, -114 and -165 have a minimal surface tension of 33, 30 and 39 mN/m.<sup>108</sup> Similar values are also reported for more exotic cationic gemini surfactants, of which the ST in water in the order of 33 – 41 mN/m according the carbon chain length.<sup>109</sup> Even alkyl polyglucosides, synthetic glycosidic amphiphiles, like lauryl glucoside sulfosuccinate and  $\beta$ -D-octyl, decyl and dodecyl glucoside have a minimal surface tension contained between 30 and 40 mN/m.<sup>110,111</sup> According to the above, one can conclude that biosurfactants display classical values of minimal surface tension and it is then hard to attribute a non-ionic or an ionic character to these molecules on such basis. However, the efficiency of biosurfactants to reduce ST, and in particular their absolute values, should be interpreted with caution, and the key limiting factors will be discussed later.

CMC is classically measured for biosurfactants and relative data is abundant in the literature.<sup>12,16,19,31</sup> For this reason, Table 2 reports only the typical range of CMC for each biosurfactant and the reader is encouraged to refer to previous literature for a more extensive list of CMC values.

Table 2 - CMC in aqueous media of common biosurfactants in aqueous media. Literature reports CMC values in both mM and wt:vol units. To allow direct comparison, we convert the reported values using reported or calculated values of the molecular mass, Mw. Superscripts in the Mw column refer to the specific values used to convert concentration units across studies: a) Mw= 503 g/mol (mainly mono RL);<sup>15</sup> b) given in the corresponding article ; c) Mw= 689 g/mol (weighted average);<sup>15</sup> d) Mw= 705 g/mol (main acetylated acidic form);<sup>15</sup> e) Mw= 870 g/mol (weighted average);<sup>57</sup> f) Mw= 2542 g/mol (calculated from Mw of trehalose and mycolic acid); g) Mw= 1354 g/mol;<sup>75</sup> h) Mw= 1212 g/mol (weighted average);<sup>57</sup> i) Mw= 648 g/mol (MEL-A2);<sup>112</sup> j) Mw= 490 g/mol (mono acyl MEL A);<sup>52</sup> k) Calculated after Ref. <sup>94</sup>; l) Mw= 750 g/mol;<sup>79</sup> m) Mw= 780 g/mol (calculated after Ref. <sup>94</sup>); n) Mw= 1036 g/mol (most used).

BS	Mw (g/mol)	CMC		Inferior limit	Superior limit	Ref.
		(mM)	(mg/L)			
RLs	475 – 677 <sup>113</sup>	0.004 <sup>a</sup> – 0.36 <sup>b</sup>	2 <sup>b</sup> – 181.1 <sup>a</sup>	Mono-RL from <i>P. spec. DSM 2874</i>	Mono-RL from <i>P. aeruginosa</i>	73,81–86,114,115
SLs	621 – 707 <sup>113</sup>	0.009 <sup>c</sup> – 0.97 <sup>d</sup>	6 <sup>b</sup> – 680 <sup>b</sup>	Mixture from <i>Candida bombicola ATCC 22214</i>	Acidic SL from <i>C. bombicola ATCC 22214</i> .	19,56,87,95,116
TLs	870 <sup>e</sup> – 2500 <sup>f,57,89</sup>	0.0005 <sup>g</sup> – 0.039 <sup>h</sup>	0.7 <sup>b</sup> – 34 <sup>b</sup>	TL dicorynomycolates from <i>R. erythropolis DSM43215</i> .	TL tetraester from <i>R. erythropolis 5177</i>	57,75,88,89
MELs	490 – 916 <sup>51,52</sup>	0.0017 <sup>b</sup> – 0.36 <sup>b</sup>	1.1 <sup>i</sup> – 176.4 <sup>j</sup>	Tri-acetylated MEL (MEL-A2) from <i>P. churashimaensis OK96</i> .	Mono acylated MEL-A from <i>P. antarctica T34</i> .	52,77,90,91,112
CLs	750 – 780 <sup>k</sup> 79,94	0.02 <sup>b</sup> – 0.41 <sup>b</sup>	15 <sup>l</sup> – 319.8 <sup>m</sup>	CL mixture from <i>C. humicola 9-6</i>	CL mixture from <i>C. humicola JCM 1461</i>	79,93,94
Surfactin	1022 – 1088 <sup>117–119</sup>	0.009 <sup>b</sup> – 0.096 <sup>b</sup>	9.3 – 99.5 <sup>n</sup>	Industrial Surfactin from <i>Wako pure chemical Ind, Japan</i> .	Surfactin from <i>B. subtilis YB7</i> .	59,63,70,95,120

Emulsan	1000000 <sup>71</sup>	$2 \times 10^{-5} - 6 \times 10^{-5}$	20 <sup>b</sup> – 58 <sup>b</sup>	Emulsan from <i>A. venetianus</i> RAG-1 (ATCC 31012).	Emulsan from <i>A. calcoaceticus</i> RAG-1 (ATCC 31012).	97,98,106
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Most biosurfactants have a bolaform, double hydrophilic, structure and, according to the CMC predictions for bolaamphiphiles by Nagarajan,<sup>69</sup> one could expect better solubility and higher CMC than head-tail surfactants. He reports CMCs in the order of 1-10 mM and in the range 0.1 – 10 mM for, respectively, cationic and nonionic bolaamphiphiles. From Table 2, the CMC for biosurfactants are rather in the  $\mu\text{M}$  than in the mM range, that is between one and up to three orders of magnitude smaller than what is predicted for bolaamphiphiles, thus confirming the fact that the behavior of biosurfactants in aqueous solution cannot be easily predicted on the sole basis of their gross molecular structure. Interestingly, the CMC of biosurfactants are also smaller, on average, than classical head-tail ionic surfactants and rather in the order of non-ionic surfactants. For instance, the CMC range corresponding to short ( $x=6$ ) and long ( $x=18$ ) chain cationic  $\text{C}_x\text{TAB}$  is contained between 1008 mM and 0.26 mM,<sup>121</sup> between 136.1 mM ( $x=8$ ) and 0.16 mM ( $x=18$ ) for the anionic  $\text{C}_x\text{SO}_4\text{Na}$ ,<sup>122,123</sup> and between 10 mM ( $x=8$ ) and 0.5  $\mu\text{M}$  ( $x=16$ ) for nonionic  $\text{C}_x\text{E}_8$ .<sup>67,124</sup> In addition, the CMCs of alkyl polyglucosides (APGs) with an alkyl chain varying between  $\text{C}_8$  and  $\text{C}_{14}$  were reported to be in the range 1.7 – 25 mM, 1.2 mM, 0.8 – 2.2 mM, 0.19 – 0.30 mM at RT and 0.27  $\mu\text{M}$  at 50°C for the  $\text{C}_{14}$  derivative.<sup>111,125</sup>

At a first glance, the CMC of biosurfactants is comparable to the CMC of non-ionic surfactants with long tails rather than to the CMC of ionic surfactants. However, an appropriate comparison is very risky, because the CMC for biosurfactants is extremely variable among different molecules and even for a given molecule. The highest CMC range, between few  $\mu\text{M}$  and up to the mM, corresponds to SLs, RLs, CLs and MELs, while the lowest ranges are reported for TLs, surfactin and emulsan (below the  $\mu\text{M}$ ). Furthermore, values between 0.008 and nearly 1 mM are reported for SLs, just to cite one example, but similar variations are reported for RLs or MELs (Table 2). If, the effect of pH on biosurfactants is very important and it partially explains different values for the same molecule (from pH 7 to 9 CMCs of mono- and di-RL are respectively 2 and 1.6 times higher),<sup>86</sup> it cannot explain such a systematic,

impressively wide, range of CMC for a given molecule. More explanations are suggested below.

Finally, although a crucial parameter, CMC is largely insufficient to study the aggregation behavior of surfactants in general and biosurfactants, in particular. The wide range of CMC values available in the literature for biosurfactants makes this parameter unreliable and of practical poor use.

#### *Surface tension and CMC data dispersion*

Table 1 and Table 2 show a dispersion of ST and CMC across biosurfactants and within a given BS family. We believe that several factors could explain such different results.

Phase behavior under dilute conditions could explain such incoherent values. Biosurfactants have a rich phase behavior, even under dilute conditions. For instance, MELs were not reported to have a CMC but rather a critical aggregation concentration (CAC), because no micellar phase was observed between the free molecular state and the first aggregated structures, found to be vesicles at a first CAC and sponge phase at a second CAC for MEL-A.<sup>126-128</sup> In fact, formations of more complex phases than micellar are classically observed for many biosurfactants. Different self-assembled structures can be obtained at low concentrations with SLs, RLs or MELs according to pH. As for peptidic biosurfactants, Ishigami *et al.* have shown that surfactin has a specific capability to form  $\beta$ -sheet structure by self-assembling in aqueous media.  $\beta$ -sheet formation associates with the high aggregation number, suggesting a rod-shape micelle at basic pH.<sup>120</sup> However, at neutral pH, it has been shown by Shen *et al.* a ball-like structure with remarkably low aggregation number.<sup>119</sup>

Molecular purity and batch uniformity are undoubtedly another problem to consider for biosurfactants. Impurities are well-known factors influencing the value of ST and CMC in petrochemical surfactants, as the well-known case of dodecanol, a hydrolysis byproduct in SDS formulations.<sup>67</sup> On the other hand, batch uniformity is specific to biosurfactants and it was recently shown to play an important role on the phase behavior of SLs.<sup>129</sup> Batch homogeneity depends on many factors, including biosurfactants production processes, and in particular the kind of microorganism (Table 1, Table 2) but also the carbon source (soybean oil, olive oil, rapeseed oil).<sup>55,87,104,130</sup> In all cases, many congeners can be produced at the same time at different ratios from one process to another, thus influencing the final property. This is known for many systems including RLs, SLs,<sup>113</sup> TLs,<sup>131</sup> MELs,<sup>51</sup> and CLs<sup>132</sup> and often include

the number of acetylation, the unsaturation of the tail, or the number of glucosidic moieties. For instance, a large minimal ST range is observed with two homologues of purified TL (19.0 – 43 mN/m). In the case of more or less complex batches, synergistic effects, also known for chemical surfactants,<sup>1</sup> can strongly influence both ST and CMC, as shown by Hirata *et al.*, according to whether the natural ratio between lactonic and acidic SLs provide the lower minimal ST.<sup>95</sup>

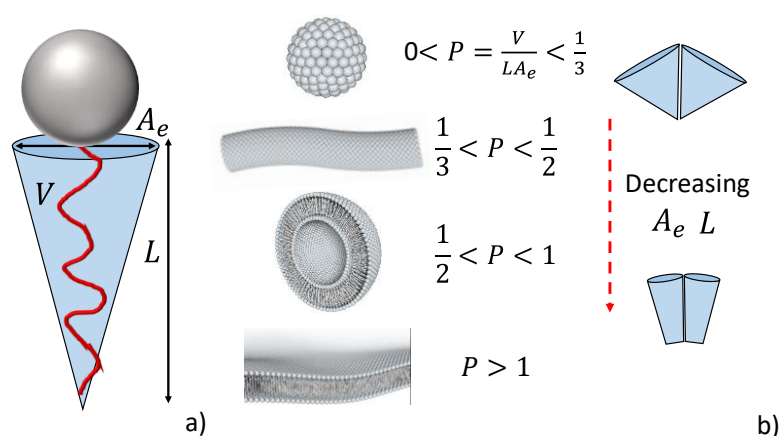
Physicochemical conditions like ionic strength, type of ions and pH of course play an important role because, if most alkylpolyglycosides are neutral surfactants, biosurfactants have a chargeable chemical group like COOH. Some studies focusing on mono- and di-RLs show no variation of the minimal surface tension with addition of NaCl < 1 M for di-RL, and a very weak decrease for mono-RL. However, a visible increase in phosphate buffer at the same pH is observed, indicating the stronger influence of hydrogenophosphate ions. Moreover, the weak NaCl-dependence about surface tension is an indication of a non-ionic surfactant.<sup>86,133</sup> From an acidic to a more basic pH, minimal ST increases for both mono- and di-RL. Furthermore, the higher CMC at basic pH provides information about the assumption of molecules negatively charged, due to the carboxylate group.<sup>86,134</sup>

In summary, the complex structure and dual neutral/charged nature of most biosurfactants push towards a more detailed structural study of the aggregation behavior in water, thus making the study of CMC or ST poorly informative. In the next section, we will focus on the advanced self-assembly behavior of biosurfactants.

### *Self-assembly and phase diagrams*

Beyond CMC, amphiphiles assemble into more or less complex morphologies as a function of concentration, temperature and molecular shape. The combined work of Tanford<sup>135</sup> and Israelachvili, Mitchel and Ninham (IMN)<sup>136</sup> across the years supported a thermodynamic description of amphiphile self-assembly in solution, which could explain, and even predict, the morphology of the aggregates and the phase diagram. The idea behind the packing parameter,  $PP$ , is extremely simple and powerful, as it relies three geometric (but also thermodynamic) parameters to a given shape,  $PP = \frac{V}{LA_e}$ ,<sup>136</sup> with  $L$  and  $V$ , the length and volume of the aliphatic chain, respectively, and  $A_e$ , the equilibrium surface area of the hydrophilic headgroup. Making the hypothesis that the hydrophobic region of the micelles is liquid, meaning to have a similar density as hydrocarbon liquids, then one expects that the aliphatic chain of the

amphiphile homogeneously fills the micellar core. The consequence is stronger than it seems as one can now state that the volume of the micellar core is equal to the volume of a single aliphatic chain, well-known or easy to estimate, times the aggregation number,  $N$  (number of molecules constituting the aggregate). The core volume can then be written as  $V_{core} = \frac{4}{3}\pi R^3 = NV$  ( $R$ , the micellar radius), while the surface area will be  $A_s = 4\pi R^2 = NA_e$ . If, according to the model, one imposes that  $R$  cannot exceed  $L$ , that is  $R \equiv L$ , one finds the condition that  $\frac{V}{LA_e} = \frac{1}{3}$ , where  $\frac{1}{3}$  is the maximum value that the quantity  $\frac{V}{LA_e}$  can assume. Figure 2b summarizes the classical limits of the  $PP$  for the major morphologies found in amphiphilic systems: spheres, cylinders and bilayers.



**Figure 2 – a) Scheme of an amphiphile molecule and its main geometrical parameters,  $A_e$ = equilibrium surface area of the headgroup,  $L$ = length of the tail,  $V$ = volume occupied by the tail. b) Typical values of the packing parameter,  $PP$ , and corresponding amphiphile morphology.**

If the  $PP$  model could predict the morphology and phase transition of a large number of amphiphiles, it has some drawbacks. For instance, the exact interpretation of the molecular parameters has been often limited to their simple geometry, such as considering  $A_e$  as equivalent to the steric hindrance of the surfactant's headgroup,<sup>137</sup> while the notion of  $A_e$  is thermodynamic. The value of  $A_e$  depends on the ionic strength in ionic surfactants (the higher the ionic strength, the smaller the surface area), but also on the binding affinity of counterions,<sup>138,139</sup> the hydration of counterions (chaotropic/kosmotropic effects),<sup>138,139</sup> the hydration of the headgroup, the hydrophobicity of some headgroups. The homogeneity of the tail's density is another underestimated parameter in the  $PP$  model.<sup>137</sup> Entropy is not considered, although it has an important role,<sup>140</sup> and so on. The  $PP$  model, for instance, can neither describe microemulsions and explain the existence of a second CMC (CMC2),<sup>141</sup> nor

explain the formation of ribbons with specific counterions.<sup>142</sup> Since the late 80's, several authors have developed complementary, and even alternative, models in the effort to build a generalized theory of self-assembly: Svenson has reviewed the major ones some time ago<sup>143</sup> and we address the more experienced reader to the works of Eriksson (1985 and onward),<sup>144,145</sup> Blankenshtein (1990 and onward),<sup>146</sup> Nagarajan (1991 and onward)<sup>147-149</sup> and, more recently, Bergström (2000 and onward),<sup>150,151</sup> who tried to build a general micelle model.<sup>141,152</sup> A general, less technical, discussion on the self-assembly of surfactants can be found in several books<sup>1,2</sup> and a more general consideration on the limits of modelling the self-assembly of amphiphiles is presented in ref. <sup>146</sup>.

An important remark should be done. The *PP* model and the structures presented in Figure 2 help understanding the difference between a surfactant- and a lipid-like behaviour. Amphiphiles forming spherical and cylindrical micelles are generally considered to be surfactants (e.g., CTAB, SDS, TWEEN...), while amphiphiles forming bilayer vesicle or flat membranes generally fall in the description of lipids (phospholipids, sphingolipids...). As discussed below, biosurfactants present both properties according to the molecular structure and physicochemical conditions and, in this sense, one should rather speak of bioamphiphiles with surfactant-like or (phospho)lipid-like behaviour.

The models to understand and predict the self-assembly properties of amphiphiles have mainly been developed on the basis of head-tail amphiphiles, for which the number of existing phase diagrams is countless, but generally following the predictions of the *PP* model for classical ionic surfactants. Extrapolations could induce believing that the same models should be valid for more complex amphiphiles, such as gemini surfactants or bolaamphiphiles,<sup>66</sup> although this is not the case.<sup>143</sup> In fact, considering the specific structure of the latter, which often requires a multi-step organic synthesis scheme, thus limiting their commercial potential, the amount of theoretical work associated to complex structures is clearly less abundant.

For bolaamphiphiles, for instance, Nagarajan has shown that the theory of self-assembly and the packing parameter approach are applicable.<sup>69</sup> If part of his predictions are interesting and verified for some specific examples available in the literature,<sup>153</sup> the broader amount of work published on the self-assembly of bolaamphiphiles along the years has demonstrated that these molecules have a much richer, and more complex, phase behavior,<sup>68,154</sup> depending on a broader number of parameters, which have never been rationalized so far. In particular, many bolas have a spontaneous tendency to form



semicrystalline fibers or lipid nanotubes,<sup>68,154</sup> which are morphologies that are not predicted by any of the theoretical models. This illustrates the limits of *PP* model to satisfactorily describe the self-assembly of bolas in particular, and of complex amphiphiles in general.<sup>143</sup> In a more recent work,<sup>149</sup> Nagarajan evokes the use of refined models or complex DNA, peptide or polyoxometallate amphiphiles (but not bolaamphiphiles), and Bergström has shown the validity of the general micelle model to gemini surfactants.<sup>152</sup> However, the amount of available data where refined models were successfully applied to a broad range of new amphiphiles is still too small to generate a trustable and generalize picture of the structure-property relationship in more advanced systems. Failure of the packing parameter approach depends on many variables,<sup>143</sup> of which a tentative non-exhaustive list can be additional simultaneous inter- and intra-molecular interactions due to the more complex structure of the amphiphile;<sup>155</sup> strong binding effects of the counterions as well as counterion-induced assembly and orientation;<sup>142</sup> presence of stimuli-responsive chemical groups which react with physicochemical parameters like pH, light, temperature, etc...;<sup>153</sup> kinetic effects.









To the best of our knowledge, the first report on the solution phase behavior of biosurfactants can be traced back to 1987, when the aggregation of RLs was explored as a function of pH.<sup>96</sup> Within the same context, the first study on surfactin is published in 1995, of MELs in 2000,<sup>127</sup> and SLs and CLs in 2004<sup>156</sup> and 2012,<sup>157</sup> respectively. For comparison, the self-assembly properties of lipids and surfactants are studied since the 50's and their rationalization has occurred in the 70's with the work of Tanford<sup>135</sup> and Israelachvili and coworkers.<sup>136,158</sup> This shows that a gap of at least 10 years, but more realistically 25-30 years, exists between the development of fundamental concepts in surfactants' science and their employment in the field of biosurfactants.

Several reasons explain such gap. In particular, the chemical structure of biosurfactants is more complex than common head-tail surfactants; biosurfactants are often bolaform and in many cases they have ionizable chemical groups like carboxylic acids for most glycolipids or lipopeptides. In this regard, external stimuli like pH, ionic strength or temperature are particularly affecting their phase behavior. This multi-functionality strongly favors additional weak interactions in the self-assembly process, such as hydrogen bond or pi-pi stacking at the same time as ionic, steric, van der Waals and entropic forces. For this reason, straightforward predictions of the morphology of biosurfactants' aggregates and phase behavior in water often fails. The packing parameter theory based on molecular shape can sometimes be used

to explain biosurfactants' aggregation, but it is not adapted for this class of functional compounds, for which the notion of clearly distinct hydrophilic and hydrophobic regions is sometimes dim, as clearly stated for surfactin.<sup>119</sup>

Most of the work dealing with the self-assembly and phase behaviour of biosurfactants has been carried out at concentrations below about 10 wt%, while typical phase diagrams for surfactants and lipids are studied at least up to 70-80% and for a large range of temperatures. One of the reasons to explain this gap is the lack of a recurrent and abundant sources of biosurfactants. So far, only specialized laboratories could produce these molecules and at a purity which was hardly reproducible. For this reason, most studies have been done under semi-diluted conditions. At the same time, diluted conditions are compatible with variations in the physicochemical environment, like pH or ionic strength. Below, we discuss the main results obtained in the study of the self-assembly and phase behaviour of selected biosurfactants. An overview is given in Table 3, while a more extensive description is given in Ref. <sup>42</sup>.

**Table 3 – Phase behaviour of the most important biosurfactants. Concentrations above 10 wt% for MELs are underlined.**

Name	Micellar (L <sub>1</sub> )		Fiber	Vesicle		Lamellar		Coacervate (L <sub>3</sub> )	Cubic (V <sub>2</sub> )	Ref.
	sphere	cylinder		SUV	MLV	Flat	Condensed			
										
<b>Nonacetyl. acidic (C18:1-cis)</b>	<b>10 &lt; pH &lt; 4, C &lt; 5 wt%</b>	<b>pH 4.5, 5 &lt; C/wt% &lt; 20</b>	<b>Mix with C18:0 SL pH &lt; ~7</b>							129,156,159–162
<b>Acetylated acidic (C18:1-cis)</b>	<b>7 &lt; pH &lt; 2</b>									38,159
<b>Lactonic (C18:1-cis)</b>				<b>Neutral (1 &lt; C/mM &lt; 5)</b>						159
<b>Symmetrical bola C16:0 sophorosides</b>	<b>T &gt; 28°C</b>		<b>T &lt; 28°C</b>							163
<b>Acidic (C18:0)</b>	<b>pH &gt; 7.5</b>		<b>7.5 &lt; pH &lt; 3</b>							160,162,164–166

<b>Acidic (C16:0)</b>	<b>pH&gt; 7.5</b>		<b>7.5 &lt; pH &lt; 3</b>							167
Acidic (C18:3-cis)				<b>Neutral pH</b>						168
<b>Acidic (C22:0<sub>13</sub>)</b>	<b>pH&gt; 7.5</b>				<b>pH &lt; ~7</b>		<b>pH &lt; 4</b>			169
<b>Glucolipid (C18:0)</b>	<b>pH&gt; 7.5</b>			<b>7 &lt; pH &lt; 4.5) T &gt; T<sub>m</sub></b>		<b>Neutral/Acidic (pH &lt; 7.8)</b>	<b>pH &lt; 4</b>			160,170-172
<b>Glucolipid (C18:1-cis)</b>	<b>pH&gt; 7.5</b>	<b>6.5 &lt; pH &lt; 7.5</b>		<b>7 &lt; pH &lt; 4.5</b>	<b>(pH 3 →) pH 6 (Lam to MLV phase change)</b>	<b>7 &lt; pH &lt; 4.5, T &lt; T<sub>m</sub></b>	<b>pH &lt; 4</b>			160,170
<b>Cellobioselipids Hydrolyzed</b>	<b>pH&gt; 7.5</b>		<b>pH &lt; ~7</b>							160
<b>di-RL</b>	<b>pH&gt; 6.8</b>				<b>pH &lt; ~7</b>	<b>pH &lt; 6</b>				86,96,115,133,173- 176

					and pH 9 ( C > 20-40 mM)					
<b>Mono-RL</b>	<b>pH &gt; 6.8</b>				<b>pH &lt; ~7 and pH 9 ( C &gt; 20-40 mM)</b>	<b>pH &lt; 6</b>				86,96,115,133,173-176
<b>Surfactin Cyclic</b>	<b>pH &gt; 7.5</b>	<b>6.5 &lt; pH &lt; 7.5</b>		<b>pH &lt; 6.5</b>	<b>Ba<sup>2+</sup> (pH 7.5)</b>	<b>pH &lt; 5.5</b>	<b>Ba<sup>2+</sup> (pH 7.5)</b>			119,177
<b>MEL-A</b>				<b>Neutral, &lt; CAC2 (2 x 10<sup>-5</sup> M)</b>		<b>L<sub>α</sub> Neutral, C &gt; ~ 65 wt%</b>		<b>Neutral, CAC2 (2 x 10<sup>-5</sup> M) &lt; C &lt; ~ 55 wt%</b>	<b>55 &lt; C/wt% &lt; 65</b>	126-128,178
<b>MEL-B</b>				<b>Neutral, &lt; CAC (6 x 10<sup>-6</sup> M)</b>	<b>Neutral pH, CAC (6 x 10<sup>-6</sup> M) &lt; C &lt; ~ 60 wt%</b>	<b>L<sub>α</sub> Neutral, C &gt; ~ 60 wt%</b>				126-128,179-181

MEL-C				Neutral	Neutral pH	L <sub>α</sub> Neutral (C not defined)				92,127,182,183
MEL-D					Neutral pH C < ~ 60 wt%	L <sub>α</sub> Neutral, C > ~ 60 wt%				179,181,184

### *Micelles in solution*

Micelles are certainly the most common self-assembled morphology observed for classical head-tail surfactants under dilute conditions. The corresponding packing parameter for spherical micelles is  $0 < P < 0.33$  and for elongated, rod- until worm-like micelles, is  $0.33 < P < 0.5$  (Figure 2). From a structural point of view, micelles are very well characterized by SAXS/SANS (spherical micelles: lack of  $q$ -dependency followed by  $-4$   $q$ -dependency of the intensity; cylindrical micelles:  $-1/-4$   $I(q)$  dependency, always in log-log scale) but important morphological information could be obtained by cryogenic transmission electron microscopy. Micelles are also characterized by lack of birefringency using optical microscopy under crossed polarizers. The micellization process can also be studied by many other concentration-dependent experiments, like light scattering, pyrene probe solubilization, surface tension.

Most biosurfactants form micellar solutions in their ionized form at neutral-basic pH. This is the case for SLs, RLs, GLs but also surfactin and CLs (Table 3). Whenever studied, micelles have a classical ellipsoid of revolution shape and their cross diameter roughly corresponds to the size of the molecule, as expected for bolaamphiphiles.<sup>69</sup> The presence of a negatively-charged group introduces strong electrostatic repulsions between adjacent molecules and induce high curvatures, thus stabilizing spheroidal objects, which is in quite good agreement with the *PP* model. This is also verified for amino-derived biosurfactants, but at acidic pH values, when the amine is positively-charged.<sup>185</sup> In the case of a commercial deacetylated, lactone-free, C18:1 SLs, micellar solutions are generally observed for the neutralized compound, at acidic pH, for the COOH derivative, and at basic pH, for the aminyl derivative. We believe that this is due to the presence of polyunsaturated impurities in typical commercial, and non-commercial, batches. A more detailed overview of this aspect is given in Ref. <sup>42</sup>.

### *The fiber phase*

Fibrillation of amphiphiles and proteins is a well-known crystallization phenomenon occurring in soft matter and responsible for many living processes, like bacterial motility through actin<sup>186</sup> or neuronal degeneration through the protein Tau. Fibrillation is not uncommon for low-molecular weight bola<sup>68,187,188</sup> and peptides-based<sup>189</sup> amphiphiles, and several reports show similar processes in biosurfactants. The *PP* model does not describe fibrillation, because the model is based on the hypothesis of a liquid hydrocarbon core, which

is not the case for crystalline objects. In terms of characterization, flat and cylindrical fibers respectively provide a  $q^{-2}$  and  $q^{-1}$  (log-log) dependence of the scattered intensity,  $I(q)$ , in SAXS/SANS experiments. However, polydispersity, fiber aggregation and formation of spherulites generally provide a non-integer exponent, to be rather interpreted as a fractal dimension. In any case, optical microscopy and/or cryo-TEM should be employed for a clear-cut attribution of the shape and type of twist (ribbon, helical). In the case of crystalline flat fibers, a diffraction peak should be observed at distances in the order of the size of the molecule.

Unexpectedly, the fiber phase is very common for microbial amphiphiles with large headgroups. Logically, when fibers form, these compounds should then not be considered as surfactants anymore. Table 3 shows that the fiber phase is observed for saturated, C18:0 and C16:0 SLs but also hydrolyzed CLs. Deacetylated C18:1 SLs could also form this phase,<sup>156</sup> but some doubts still exist about its origin for this compound, whether it is a real phase or coming from C18:0 SLs impurities.<sup>129</sup> From a structural point of view, fibers are characterized by a flat semi-crystalline morphology, of which the cross section is in the order of 10-20 nm. They are generally infinitely long, although shorter fibers and spherulites can form under appropriate conditions of synthesis.<sup>190</sup>

Fibers are generally observed in the neutral form of the bioamphiphiles, that is at acidic pH for the COOH and basic pH for the NH<sub>2</sub> derivatives,<sup>185</sup> but they have been reported as a possibly thermodynamic phase even for charged compounds, in the specific case of C18:1<sub>trans</sub> SLs.<sup>166</sup> Symmetrical bolaform SLs, and other more exotic SLs derivatives, also seem to form fibers.<sup>163,191</sup> Fiber-like structures, but rather being described by a columnar phase, have also been reported for porphyrinic SLs.<sup>192,193</sup>

At the moment, we must acknowledge that only bioamphiphiles with large di-glucose headgroups (sophorose or cellobiose) form fibers. The actual reason is not clear, but it could be a combination of melting temperature,  $T_m$ , above RT, directional hydrogen and crystallization. Most of the fibers being twisted (ribbons or helices), one could invoke the presence of chiral centers. However, this may not be the case, and steric hindrance between adjacent molecules packed at a nonzero angle one with respect to the other could explain the twist, as proposed by others for the formation of lipid nanotubes.<sup>194</sup>



## Membranes

When speaking of membranes in the field of amphiphiles, one generally refers to bilayer membranes in the form of vesicles, multilamellar vesicles or lamellar phases, although sponge and other more complex phase can occur. In the case of microbial amphiphiles, membranes have been often observed, thus confirming that the term biosurfactant is not appropriate in this case. One should then rather employ the term bioamphiphile, as for the case of fibers. Vesicles, generally a metastable phase, are unique objects having the capability to encapsulate, transport and deliver a cargo (hydrophobic drugs, macromolecules, nanoparticles) with strong benefit in many domains from medicine to cosmetics. Lamellar phases are of major importance in living organisms. Phospholipids, not discussed in this review, are the major component of biological membranes. Lamellar phases have different notations according to their order, hydration or lipid tilt. These phases are an important topic of research in biophysics, for the better understanding of living organisms. The packing parameter for vesicles is  $0.5 < PP < 1$  while for lamellar phases  $PP= 1$ . As a general rule, both vesicle and lamellar phases are characterized by a typical  $q^{-2}$  dependence of the scattered intensity in SAXS/SANS experiments. In the case of multilamellar structures, at least two diffraction peaks should be observed, one being the harmonics of the other (1:2 ratio in position). Lamellar phases with poor long-range order, or swollen systems, make exception to these rules. If the  $q^{-2}$  dependence is generally always observed, the diffraction peaks may either be broad or even not appear at all. Polarized light microscopy, or cryo electron microscopy on diluted samples, should be generally employed as complementary techniques for a more clear-cut attribution.

A warning on the notation. Vesicles and lamellar phase have a number of notations, some only speaking to experts. One could refer to the encyclopedia of colloids science for more information,<sup>195</sup> or to Ref. <sup>42</sup> for a short summary within the context of bioamphiphiles. Hereafter, we report a broad overview of membrane structures found for bioamphiphiles, but a more critical discussion is given in Ref. <sup>42</sup>.

Membranes have been observed in the shape of vesicles and within the context of bioamphiphiles since 1987, in the case of RLs at acidic pH.<sup>96</sup> Ever since, a number of systems does show the formation of membranes, RLs (confirmed by further studies), surfactin, C22 derivative of SLs, GLs, MELs (Table 3). May them be multilamellar vesicles or lamellar phases,

membranes have been observed both under diluted and concentrated conditions, whereas the only reliable work performed at concentrations above 10 wt% has been reported for MELs.<sup>178,181,184,196</sup>

Differently than what it is found for phospholipids, the structure of the membranes prepared from microbial bioamphiphiles is generally interdigitated, with a total membrane thickness (between 3 and 4 nm) roughly corresponding to the size of a single molecule. This is generally explained by the bolaamphiphilic nature of most of these compounds. Most of the membranes are observed when the acidic group is neutralized, generally at acidic pH below 7, or sometimes below 6. In reality, in the pH range between 4 and 7, the membrane is composed by a difficult-to-evaluate mixture of charged ( $\text{COO}^-$ ) and uncharged ( $\text{COOH}$ ) derivatives, which guarantee the low membrane curvature and the colloidal stability at the same time. It is by the way not uncommon to observe, for RLs or GLs, for instance, a precipitation in a lamellar phase at pH below 4. More insight in the vesicle and lamellar phases are given in Ref. <sup>42</sup>.

#### *Less common structures*

A number of other structures have been reported. Some of them being minor secondary structures, and others being major but unique for a given compound under specific conditions: 1) wormlike micelles have been reported for SLs, GLs and possibly surfactin;<sup>156,160,161,177</sup> 2) nanoplatelets were reported for SLs, CLs and GLs;<sup>160,162,169</sup> 3) a columnar phase was reported for porphyrinic derivatives of SLs;<sup>192,197,198</sup> 4) a coacervate phase was reported for MEL-A at low concentrations;<sup>126,128</sup> 5) cubic structures (Ia3d or Pn3m) have been observed at high concentration for MELs and surfactin;<sup>126,128,178,196</sup> 6) ill-defined structures were reported by several authors on SLs and RLs.<sup>38,159,162,199,200</sup>

#### *Comments*

The packing parameter, *PP*, concept has been discussed by several authors in relationship to the self-assembly of biosurfactants. It was employed to discuss, understand and tentatively explain the self-assembly properties of RLs,<sup>86,115,133,175</sup> SLs,<sup>159,160</sup> GLs,<sup>160</sup> and surfactin.<sup>119,175,196</sup> However, a deeper look at the existing data shows that this approach, classically used for standard head-tail surfactants, does not satisfactorily explain the experimental evidence and in this regard it cannot be employed in a straightforward manner

to predict the self-assembly of biosurfactants. We have estimated that in about 50% and 25% of the studies on biosurfactants, the *PP* model either fails or it is only partially verified.<sup>42</sup>

Predictions of the self-assembly and phase behaviour through the *PP* model partially works, with exceptions, for many biosurfactants under alkaline conditions. It generally fails for saturated systems under acidic conditions, while the discussion is open for surfactin, of which the role of the peptidic part, may it be linear or cyclic, is an open question. The reason why the *PP* theory is unreliable to explain and predict the self-assembly of most biosurfactants has been addressed. Most biosurfactants fall in the category of bolaamphiphiles, a class of compounds which has been rationalized by Nagarajan in the late '80s.<sup>69</sup> According to his work, calculation of *PP* for bolaamphiphiles relies on twice the headgroup surface area and half the chain length per molecule. Despite such a discrepancy, the upper and lower end-values of *PP* defining the shape of the aggregate do not change between one-headed and bolaamphiphiles. This fact rules out possible errors between the calculated *PP* and experimental morphology observed for bolaamphiphiles structures. The poor agreement between the *PP* theory and biosurfactants self-assembly must then be found elsewhere. A list of possible problems is given hereafter, although a more detailed description is given in Ref. <sup>42</sup>. a) The hypotheses behind the *PP* model theory may not be respected for BS, for instance the liquid-like nature of the hydrophobic tail, especially when fibrillation occurs; b) calculation of *PP* for biosurfactants may be erroneous; c) effect of pH is important as pH controls the amount of ionic, neutral and mixture of ionic and neutral species; d) hydrogen bonding and sugar headgroup configuration may have an underestimated role; e) effect of impurities and mixture of congeners, due to the fact that biosurfactants are produced by microorganisms; f) coexistence of multiple phases; g) fast kinetics vs. thermodynamic equilibrium, especially during variations of pH or temperature; h) strong impact of physicochemical parameters; i) possible effects of chirality and/or bulkyness of headgroup.

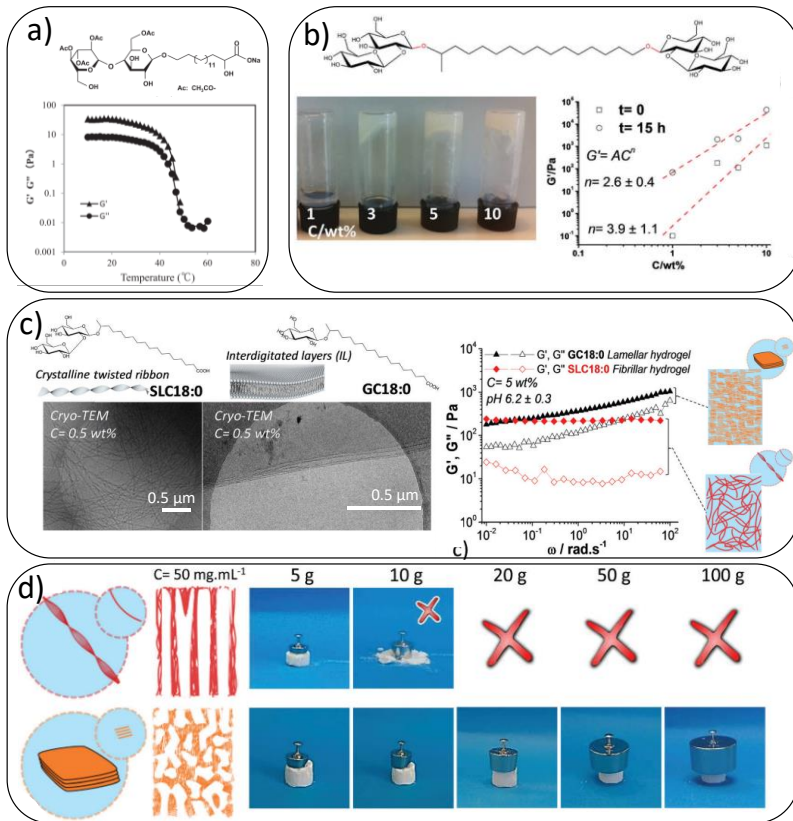
#### *Unique macroscale properties of biomphiphiles*

Synthetic and natural amphiphiles may have a broad range of different chemical structures, each characterized by an associated phase behaviour and, eventually, macroscale properties. The general trend is that a specific family of amphiphiles is characterized by well-defined properties. For instance, surfactant wormlike micelles can form micellar hydrogels, often found in liquid soaps and shampoos. Phospholipids stabilize vesicles, useful for

encapsulation, while peptide amphiphiles generally form fibers and fibrillar hydrogels. Of course, some permeability across boundaries exist, especially if these classes of molecules are mixed together. The astonishing, and actually undervalued aspect of biological amphiphiles, is their ability to mix nano- and macroscale properties within a relatively narrow range of molecular portfolio and physicochemical conditions. This is mainly due to the combined properties of the fatty acid, sugar and free carboxylic end-group. Although the combination of these aspects is not well-understood yet, the final effects are quite surprising, with a number of unexpected macroscale behaviours. Hereafter we illustrate just a few of them. In the following, we report the case of hydrogels, although more examples can be found in Ref. <sup>42</sup>.

Hydrogels can be obtained by colloids, polymers and low molecular weight gelators (LMWG). Surfactants can also form hydrogels, but generally in their wormlike micelle phase. In this regard, bioamphiphiles behave more as peptide amphiphiles than as surfactants. LMWG are a well-known class of molecules, which, upon cooling, self-assemble into fibers and stabilize a solvent into a gel. If the solvent is organic, they are referred to as organogelators, while in the case of water, they are referred to as hydrogelators.<sup>201,202</sup> Several reports demonstrate the hydrogelling effect of microbial bioamphiphiles, like CLs and various derivatives of SLs, in their fiber phase (Figure 3a-c).<sup>93,157,163,190</sup> More interestingly, new mechanisms of gelation have been described. The strength of C18:0 SL-based hydrogels was shown to be dependent on the rate of pH variation, from basic to acidic,<sup>190</sup> while this correlation was never reported for LMWG before. More interestingly, the C16:0 SL does not show this correlation at all, but it rather shows a dependency of the gel strength on the final pH,<sup>167</sup> as classically reported for other LMWG.<sup>203</sup>

An even rarer hydrogelling event has been reported for C18:0 GLs, which form very special lamellar hydrogels at less than 10 wt% in water (Figure 3c),<sup>172,204</sup> where lamellar hydrogels are extremely rare in the literature and only reported for specifically modified phospholipid membranes.<sup>205,206</sup> Processing of lamellar hydrogels from GLs into solid foams is possible via a controlled directional freezing method. Solid lamellar foams with 3D architecture show Young moduli in the order of 20-30 kPa in both the axial and equatorial directions, explained by the isotropic orientation of the macropores within the material, which can withstand up to 1000 times its own weight (Figure 3d). This result is particularly unexpected considering the strong anisotropic growth of ice and never reported before for materials obtained with standard amphiphiles.



**Figure 3 – Low-molecular weight biosurfactant-only gelators in water. a) Temperature evolution of  $G'$ ,  $G''$  for CLs (17) hydrogels (reproduced from Ref. <sup>93</sup>, copyright Japan Oil Chemical Society). b) Concentration evolution of  $G'$ ,  $G''$  for C16:0 SL-hydrogels (reproduced from Ref. <sup>163</sup> with permission from The Royal Society of Chemistry). c) Comparison of frequency-dependent  $G'$ ,  $G''$  between fibrillar and lamellar hydrogels respectively obtained from C18:0 SLs and GLs at acidic pH.<sup>172,190,207</sup> d) Comparison of the axial compression applied to solid foams prepared from fibrillar and lamellar hydrogels using the ice-templating process (c) and d) are reproduced from Ref. <sup>207</sup> with permission from The Royal Society of Chemistry).**

In conclusion, the purity, homogeneity and mixture of congeners is a limit for the understanding of the properties of biosurfactants in solution. Similarly, their double hydrophilic nature and responsivity to pH, temperature or ionic strength have a strong impact on their properties. Surfactant-like or phospholipid-like behaviours can be observed for their ionic and neutral forms, respectively. Many aspects remain to be understood both for their properties in solution (CMC, for instance) but, and above all, for their self-assembly and phase behaviour. When these aspects will be understood and controlled, the panorama of possible applications will certainly explode in fields other than the actual ones.

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