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Niki Baccile, Alexandre Poirier, Chloé Seyrig, Patrick Le Griel, Javier Perez, et al.. Chameleonic Amphiphile: the Unique Multiple Self-Assembly Properties of a Natural Glycolipid in Excess of Water. *Journal of Colloid and Interface Science*, 2023, 630 (404-415), 10.1016/j.jcis.2022.07.130 . hal-03576358v2

**HAL Id: hal-03576358**

**<https://hal.science/hal-03576358v2>**

Submitted on 12 Oct 2022

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# Chameleonic Amphiphile: the Unique Multiple Self-Assembly Properties of a Natural Glycolipid in Excess of Water

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## Keywords

Low-molecular weight amphiphiles; Self-assembly; Surfactants; Lipids; Gelators; Multiphase;

## Abstract

Chameleons are stunning reptiles which change colour according to the surrounding environment. In astrophysics, chameleons are particles whose mass varies in the surrounding matter. Here, we show the chameleonic self-assembly behavior of a low molecular weight (LMW) amphiphile, a broad class of molecules widely studied for several decades. Their ability to self-assemble in water make them both fascinating and useful compounds for a number of applications. Under thermodynamic conditions, their thermotropic and lyotropic phase behavior is generally predicted in relation to their molecular shape, as seen for classical head-tail molecules like surfactants or phospholipids. However, many exceptions do exist, either when amphiphiles have unconventional shapes, e.g., bolaform or gemini, or when they contain functional groups which undergo specific interactions such as H-bonding or  $\pi$ - $\pi$

stacking. In excess water, surfactants form micelles, phospholipids form vesicles or lamellar phases, and functional amphiphiles often form micelles or fibers. Here, we show the multiphase behavior, much richer and more unpredictable than what it is known for most amphiphiles, of a biobased glycolipid produced by the yeast *S. bombicola*  $\Delta$ ugtB1. In excess water and within a narrow pH range around neutrality, this compound assembles into micelles, uni- and multilamellar vesicles, lamellae and fibers, simply as a function of changing pH, temperature and counterions. This rich phase behavior is not only interesting in itself, it also generates a number of diverse biocompatible and biodegradable soft self-assembled materials like hydrogels, complex coacervates and drug carriers.

## Significance statement

How many different structures can a single amphiphilic molecule self-assemble into in water under diluted conditions in the vicinity of neutral pH, and at room temperature? A natural glycolipid obtained by microbial fermentation contains a glucose headgroup, a bolaform shape, a monounsaturated C18:1 aliphatic chain and a reactive carboxylic acid. Their synergy drives the self-assembly towards at least nine different crystalline or liquid crystalline structures under similar physicochemical conditions, depending on pH, type of counterion and direction of pH variation.

## Introduction

The variation of a given property in living or inert systems in response to the environment is a remarkable phenomenon in nature. Chameleons are mimetic reptiles which adapt themselves to their surrounding environment by changing color. In physics, chameleons refer to particles which change mass as a function of the amount of matter surrounding them: the closer they are to a planet or a star, the higher their mass.[1] In chemistry, the concept of the chameleonic behavior of both low molecular weight (LMW) compounds and polymers was proposed long ago[2,3] in the context of morphological variation or aggregation according to their surrounding conditions. However, the possibilities offered by the compounds in these early works (catenanes, polyisobutylene-based biopolymers) were limited to only a few states of assembly. More recently, an LMW peptide amphiphile was shown to assume four different states of aggregation (amyloid-like  $\beta$ -sheet,  $\beta$ -sheet, super-helix, random coil) according to the type of metal ion in solution.[4] Despite such richness, the

variation in terms of self-assembly of a single, given LMW amphiphile is still limited to a few morphologies of equivalent structure and curvature.

LMW amphiphiles are molecules having a size of roughly 1 kDa or less and composed of hydrophilic and hydrophobic moieties. Their intrinsic properties are determined by the chemical nature and relative size of each block, but they are also tuned by their interaction with external chemical and physicochemical stimuli, like pH, ions, light, etc. Amphiphiles with surface-active properties are referred to as surfactants,[5] while those with membrane-, or fiber-forming, properties are known as lipidic[6] compounds or LMW gelators.[7] From the chemical standpoint, and according to the packing parameter approach described by Israelachvili, surfactants have a large hydrophilic surface area compared to the volume of the aliphatic chain, while lipidic compounds have a small surface area compared to the volume of the hydrophobic moiety. Typical surfactants are aliphatic derivatives of sulfates or quaternary ammonium salts[5] while the most common lipidic compounds are phospholipids.[6] Fiber-forming amphiphiles are generally characterized by a hydrophobic moiety, often presenting aryl or cyclic groups, and/or by a functional hydrophilic moiety, offering multiple H-bonding interactions.[8–10] Typical compounds are derivatives of peptides, peptide amphiphiles or derivatives of steroids.[8–10] If given compounds can undergo transitions from one category to another, for example induced by ions[11,12] or pH,[7] compounds that display a phase behavior common to the three categories of amphiphiles are rare. For instance, specific peptide amphiphiles[4] can assemble into four structurally-related fibrous structures. If one includes additional constraints like the use of an aqueous medium, a narrow set of physicochemical conditions, high abundance of the amphiphile, low cost and enhanced biocompatibility, the list is most likely empty.

This communication shows that all of the above can be satisfied by a natural glycolipid, C18:1 glucolipid - G-C18:1 (Figure 1) - produced by an engineered yeast strain (*Starmerella bombicola* *AugtB1*).[13] Microbial glycolipid amphiphiles are LMW compounds widely known in the literature for their potential for replacing petrochemicals in all applications where synthetic surfactants are employed.[14–17] G-C18:1 shows a rich chameleonic behavior under multiple stimuli, each driving a well-defined phase under high dilution, up to 99% of water or even above. By combining both published and unreleased data, we show how, within a narrow range of pH around neutrality, G-C18:1 can spontaneously assemble into (Figure 1): micelles (1),[18,19] unilamellar vesicles (2),[18,19] a lamellar phase (3),[18–20] complex coacervates (4),[21,22] multilamellar-wall vesicles (MLWV) (5),[21,22] vesicular aggregates (6), [21] crystalline fibers (7),[23–26] cylindrical

and wormlike micelles (8),[18,19,24,26] and multilamellar vesicles (9).[20] The richness and complexity in terms of self-assembly are very specific to G-C18:1, even compared to other natural glycolipids, like sophorolipids and rhamnolipids.

To the best of our knowledge, we are not aware of other amphiphiles that display such varied multiphase behavior, merging the properties of at least three families of LMW amphiphiles with opposite tendencies of self-assembly: surfactants, lipids and gelators. G-C18:1 can act as a detergent but also as an encapsulating agent or gelator under nearly comparable conditions, thus being of potential interest to many areas of application. In terms of market perspectives,[27,28] developing a single, multi-property, biobased compound is also potentially cheaper than investing in a large panel of different molecules.[29] Natural microbial glycolipids are notoriously more expensive than petrochemical compounds, and concentrating the investment in a single molecule is certainly a highly attractive argument for the private sector, which has recently massively invested this domain.[30] Last but not least, G-C18:1 is obtained from genetically-modified strains[13] which could be a potential limitation for the market, as a large number of consumers reject the use of GMOs. However, G-C18:1 can also be efficiently produced by the enzymatic hydrolysis of sophorolipids,[31] one of the most common and commercially available bioamphiphiles, recently developed as a specialty chemical,[30] thus circumventing the use of GMOs.

## Materials

**Chemicals.** The glycolipid biosurfactant, glucolipid G-C18:1 (Figure 1), was purchased from Amphistar (Gent, Belgium) and produced by the Bio Base Europe Pilot Plant (Gent, Belgium), batch No. APS F06/F07, Inv96/98/99 and used as such. The monounsaturated glucolipid G-C18:1 ( $M_w = 460 \text{ g.mol}^{-1}$ ) contains a  $\beta$ -D-glucose unit covalently linked to oleic acid. The molecule is obtained by fermentation from the yeast *Starmerella bombicola*  $\Delta$ ugtB1 according to the protocol given before.[13] According to the specification sheet provided by the producer, the batch (99.4% dry matter) was composed of 99.5% G-C18:1, according to HPLC-ELSD chromatography data. NMR analysis of the same compound (different batch) was performed elsewhere.[18]

**Sample preparation.** For all the samples, G-C18:1 powder was dispersed in milli-Q water followed by vortexing and sonication. When needed, the pH was adjusted by a few  $\mu\text{L}$  of concentrated NaOH or HCl, generally prepared at 0.1 M, 0.5 M, 1 M or 5 M. pH was monitored with a standard KCl electrode. For each specific preparation protocol, please refer to Table S 1 in the Supporting Information.

## Results

G-C18:1 is a molecule that was first synthesized by enzymatic hydrolysis of sophorolipid biosurfactants produced by the yeast *S. bombicola*. [31] Rediscovered years later by the direct fermentation of a genetically-modified, glycosyl transferase-free strain of *S. bombicola*  $\Delta$ ugtB1, [13] the self-assembly properties in water of G-C18:1 have only been studied recently by us. [18,19] As with many other microbial biosurfactants, like sophorolipids, rhamnolipids or surfactin, pH has a strong impact on self-assembly, as reviewed recently. [29] However, more recent published and unpublished data show that other parameters, such as the nature of the counterion and the direction of pH variation, have a dramatic influence on phase behavior.

The known phase behavior in water of G-C18:1 under conditions of strong dilution (< 5 wt%) is reported in Figure 1, with most of the corresponding experimental fingerprints, namely SAXS and cryo-TEM, shown in Figure 2 and Figure 3, respectively. Below, we discuss each phase and the typical physicochemical conditions necessary to obtain them, separately.

### Surfactant-like behavior.

(*Spherical*) *micelles* (**1** in Figure 1). Micelles are self-assembled objects typically observed in surfactant solutions above their critical micelle concentration (cmc).[32] Above their cmc, they are generally spheroidal with aggregation numbers below 100, although they can grow into cylindrical and wormlike morphologies above a second cmc value,[33] generally reached by increasing concentration or by reducing the repulsive forces between headgroups.[34–36] Here, the case of spheroidal micelles is discussed. For most microbial bioamphiphiles, including G-C18:1, neutral-alkaline pH deprotonates the carboxylic acid, turning the molecule, *de facto*, into an anionic surfactant, which tends to form an aqueous solution of micelles.[29] Its diameter is roughly in the order of the length of the molecule itself.[29] This is expected,[37] as most of them have a bolaform shape. Micellar solutions of G-C18:1 (**1**, Figure 1) are clear and easy to obtain by simply increasing the pH above about 7.5 at concentrations below 5 wt%.[18,19] Their SAXS profile is characterized by a flat low- $q$  plateau and an oscillation of the form factor at high- $q$  (Figure 2, **1**) typical of spheroidal micelles. The amplitude of the oscillation may vary with pH, attributable to a local rearrangement of the sugar and carboxylate groups inside the micelle.[19] The typical microscopy image obtained by cryo-TEM (Figure 3, **1**) shows classical, poorly-contrasted, spheroidal objects.

*Complex coacervates* (**4** in Figure 1). The association between charged micelles and oppositely-charged polyelectrolytes has long been described in the literature as forming complex coacervates (CC).[38–40] CC are a unique state of soft matter which has been described to be responsible, for instance, for mussel adhesion and that have been developed as underwater adhesives.[41] The micellar solution of G-C18:1 at pH above neutrality contains mostly negatively-charged micelles whose charge is counterbalanced by  $\text{Na}^+$ . Adding a polycation, whether synthetic or biobased (e.g., chitosan, poly-L-lysine), to this system induces a strong polycation-G-C18:1 interaction, driven both by specific enthalpic and non-specific entropic contributions to the free energy.[21,22] The combination of cryo-TEM (**4** in Figure 3) and pH-resolved *in situ* SAXS (typical profile, **4**, in Figure 2) shows the systematic formation of more or less hydrated colloids of a hundred nm in size and which can be safely associated with complex coacervates, by comparison with the literature.[42] Similar results were also obtained with another glycolipid biosurfactant, sophorolipids, also in its negatively charged micellar phase domain.[43]

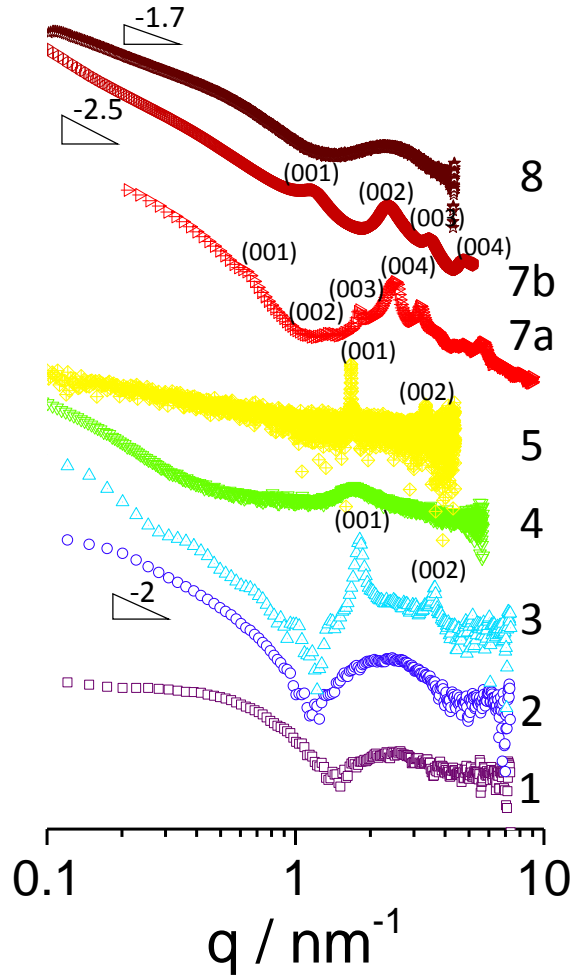
(Cylindrical/wormlike) micelles (**8** in Figure 1). Cylindrical or wormlike micelles are generally observed for surfactant solutions above a second cmc, or following a sphere-to-rod transition when repulsion between headgroups is screened, for example, by adding a cation.[34–36] This experimental fact is in agreement with the thermodynamic description of micellar self-assembly developed by Tanford and Israelachvili *et al.*[44,45] Sphere-to-rod (or wormlike) transitions for G-C18:1 are stimulated in two different ways. Cylinders and worms have been observed in a narrow pH region, between 7.5 and 6.2, when reducing pH from alkaline to acidic.[18,19] As found elsewhere,[46] worms appear to be a transitory phase between micelles (high pH) and vesicles (low pH) and are driven by a given ratio of the COOH and COO<sup>-</sup> forms of G-C18:1. Wormlike micelles have also been observed upon the addition of specific transition metal ions (Al<sup>3+</sup>, Fe<sup>2+</sup>, Co<sup>2+</sup>, Cu<sup>2+</sup>) to a micellar solution of G-C18:1 at a pH above 7.5.[24,26] This behavior is not surprising as metal-to-cylinder/wormlike transitions triggered by salts have also been long described for solutions of anionic surfactants. The typical SAXS profile of wormlike solutions show a dependency of the scattered intensity against the wavevector  $q$  (log-log scale) in the order of -1 (cylinders) to -1.6 (worms).[47] This is found experimentally for G-C18:1 (**8** in Figure 2), when pH is reduced and salts are added to the micellar solution. The typical cryo-TEM fingerprints for both systems are shown in Figure 3 and published elsewhere. They show either individual (**8b**, Figure 3)[18,19] or aggregated (**8a**, Figure 3)[24,26] cylinders, respectively for pH- and metal-stimulated transitions. Cryo-TEM data are complementary and agree with SAXS experiments.





headgroups are screened, by salt for instance.[50] G-C18:1 forms unilamellar vesicles (**2** in Figure 1 and cryo-TEM in Figure 3) by simple dispersion and sonication of G-C18:1 powder in water at acidic pH, slightly below neutrality, or by reducing pH from alkaline to acidic,[18,19] the latter promoting a micelle-to-vesicle transition, passing through a wormlike phase, in agreement with previous studies.[46] The micelle-to-vesicle transition of G-C18:1 has been studied in detail before by pH-resolved *in situ* SAXS.[19] The typical profile corresponding to the vesicle phase is reported in Figure 2 (**2**) and characterized by a -2 low-q dependence of intensity[47] and a broad oscillation of the form factor, both features typical for a flat membrane with a core-shell interface.[19]

(*Multilamellar*) vesicles (**9** in Figure 1). Multilamellar vesicles (MLV) are important soft colloids classically obtained by the hydration process of adsorbed multilamellar phases, and typically composed of phospholipids and their mixtures. MLV are more interesting for the encapsulation and delivery of active molecules than unilamellar vesicles because their multilamellar structure makes them more stable *in vitro* and *in vivo*. An analogous, but still conceptually different mechanism has been found to prepare MLV with G-C18:1. Instead of drying a thin multilamellar layer onto a round-bottomed flask, it is possible to work directly in bulk water at the desired concentration of G-C18:1 below 5 wt%. The process is quite simple: pH is reduced below 4 under stirring, so to induce the precipitation of a lamellar aggregate which, by increasing pH above 5, transforms into MLV (cryo-TEM, **9**, Figure 3),[20] that is colloidally stable in water at pH close to and slightly below neutrality. Size can be controlled using typical methods employed for phospholipid MLV, sonication and filtration, while MLV from G-C18:1 can easily encapsulate and stabilize the dispersion of both stable (ferritin) and unstable (iron nanoparticles, hydrophobic phosphors) colloids.[20]



**Figure 2 – SAXS profiles of various phases composed of G-C18:1 in water, room temperature, at concentration below 5 wt%: 1) micellar,[18,19] (2) unilamellar vesicles,[18,19] (3) lamellar phase,[18–20] (4) complex coacervate,[21,22] (5) multilamellar wall vesicles (MLWV),[21,22] (7) fiber phase from (7a)  $\text{Ca}^{2+}$  and (7b)  $\text{Ag}^+$ .[23–26] (8) micellar wormlike phase, cation-dependent.[24,26]**

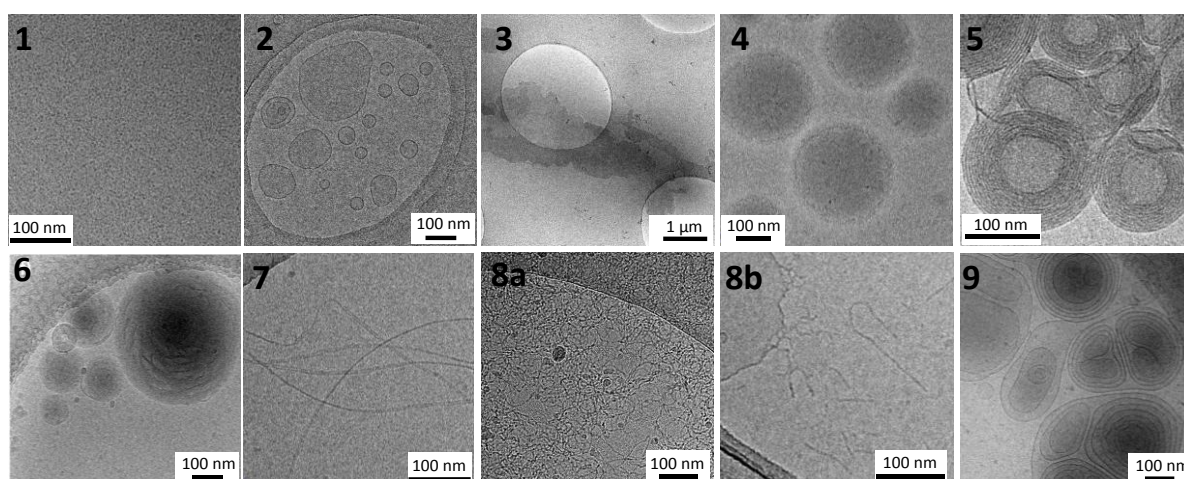
(*Multilamellar wall*) vesicles (**5** in Figure 1). Multilamellar-wall vesicles (MLWV) are conceptually similar to MLV, but they present a major difference, the lumen is void and the wall is composed of a dense multilamellar phase. MLWV are known in the literature as lipoplexes,[51,52] when the amphiphile is constituted by either a positively-charged lipid or surfactant interacting with a negatively-charged DNA strand.[51,52] Lipoplexes are commonly developed for gene transfection applications. G-C18:1 is able to form stable MLWV colloids through a coacervate-to-MLWV phase transition in bulk water at concentrations below 5 wt% and by simply decreasing the pH of the solution from about 8 to

6-7.[21,22] The use of complex coacervates composed of G-C18:1 micelles and an oppositely-charged polyelectrolyte, described above, is crucial to obtain MLWV. This is attributed to two factors, the proximity between G-C18:1 and the polyelectrolyte combined with an internal phase transition of G-C18:1, from high (micelles) to low (vesicles) curvature.[22] The MLWV of G-C18:1 can be observed by cryo-TEM (**5** in Figure 3), which agrees well with pH-resolved *in situ* SAXS experiments,[22] of which the typical fingerprint of MLWV is shown in Figure 2 (**5**). MLWV colloids from G-C18:1 are stable in time, composed entirely of biocompatible molecules, and their use in drug encapsulation and delivery to cancerous HeLa cells was recently demonstrated.[53]

(*Aggregated*) *vesicles* (**6** in Figure 1). The agglutination of vesicles is a typical phenomenon observed when vesicles interact with ions in water.[54] In the presence of polycations, the vesicles of G-C18:1 agglutinate into “cauliflower-like” structures, observed by cryo-TEM (**6** in Figure 3).[21] This phenomenon is reported here as being anecdotic and mainly opposed to the assembly into MLWV (see above), also observed when G-C18:1 interacts with polycations under the same conditions, except that MLWV are only obtained when G-C18:1 undergoes a micelle-to-vesicle transition inside a complex coacervate.

*Lamellar phase* (**3** in Figure 1). Lamellar phases are very common in the lyotropic behavior of surfactants and lipids.[55] They generally reflect the tendency of the amphiphile to occupy a large apolar volume compared to the surface area of the hydrophilic group. Consequently, they are generally observed for double-tailed amphiphiles or for surfactant-cosurfactant mixtures or, in some rare cases, surfactants with salts.[56] G-C18:1 forms a lamellar phase at room temperature only when the pH is reduced below 4, that is, when the fatty acid tail is entirely protonated.[18–20] Lamellae have been observed (**3** in cryo-TEM, Figure 3) at a higher pH but at lower temperatures, below 15°C.[18] The lamellar phase of G-C18:1 is characterized by the typical SAXS signature. Profile **3** in Figure 2, extracted from a pH-resolved *in situ* experiment,[19] shows two sharp diffraction peaks in a 1:2 ratio, expected for lamellar phases, and a low- $q$  slope of -3.6, a value close to -4 and typical for interfaces. The SAXS profile reflects the solid precipitate nature of the lamellar form of G-C18:1. Modulations of intensity below  $1\text{ nm}^{-1}$  can also be observed and attributed to possible residues of multilamellar vesicles in solution, seen in the pH region close to the vesicle-to-lamellar transition.[18] Lamellar precipitates of G-C18:1 below pH 4 have also been reported elsewhere.[22] pH-resolved *in situ* SAXS has shown how the lamellar phase for G-C18:1 is

obtained from the unilamellar vesicle phase,[19] directly in bulk water and at the same concentration, thus showing how the neutral glucose headgroup is not bulky enough to increase its surface area compared to the tail, when the latter is entirely protonated. The presence of spurious amounts of negative charges at pH above 4 is enough to introduce local repulsion and stabilize a higher curvature. When attempting to understand the thermodynamic behavior of the vesicle and lamellar phases,[44,49] it must be borne in mind that G-C18:1 assumes an interdigitated arrangement inside the membrane, different from what it is classically found in phospholipid-based membranes, notoriously composed of bilayers. Interdigitation, driven by the bolaform structure of G-C18:1, could induce additional H-bonding interactions between these glucose units and COOH close in space. Although not demonstrated, this mechanism could be plausible, thus making the specific case of G-C18:1 more complex and incompatible with the thermodynamic theory of self-assembly.[32,44,49]



**Figure 3 – Typical cryo-TEM images of various phases composed of G-C18:1 in water, room temperature, at concentration below 5 wt%:** (1) micellar,[18,19] (2) unilamellar vesicles,[18,19] (3) lamellar phase,[18–20] (4) complex coacervate,[21,22] (5) multilamellar wall vesicles (MLWV),[21,22] (6) aggregated vesicles,[21] (7) fiber phase from Ag<sup>+</sup> [23–26] (8) micellar wormlike phase with (8a) Fe<sup>2+</sup> [24,26] or (8b) at pH ~7,[18,19] (9) multilamellar vesicle (MLV) phase.[20]

### **Gelator-like behavior**

*Fiber phase* (7 in Figure 1). Self-assembled fiber networks (SAFiN)[57–59] are commonly found for amphiphiles with a highly marked hydrophobic character, often, although not necessarily, combined with a multifunctional headgroup like a peptide derivative.[8–10] Due to their fibrillar structure, most of these systems spontaneously form hydro- or organogels at low concentrations in water.[7] This behavior is generally not always predictable on the sole basis of the molecular structure, and it is not considered in the thermodynamic treatment of

amphiphiles in water.[32,44,45,49] Fibrillation can be induced[60] by temperature, pH[61] or salts[62] but in general the molecules, which display a SAFiN behavior, may show a tendency to form either micelles or vesicles, but not the three at the same time. Classical anionic or cationic surfactants do not show a tendency to fibrillate, not even with salts (refer to Table S 1 in ref. [24] for a broader literature survey), unless surfactants with atypical shapes (e.g., gemini) are mixed with uncommon chiral counterions.[63]

G-C18:1 forms crystalline fibers from its charged micellar phase above pH 7.5 at room temperature in water, by simple addition of a source of specific cations, namely  $\text{Ca}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Mn}^{2+}$ ,  $\text{Cr}^{2+}$ . [23–26] These also induce prompt hydrogel formation with remarkable stability against temperature, shear strain and both combined.[25] The fibers are observed by cryo-TEM (**7** in Figure 3), while the SAXS fingerprint of the corresponding hydrogels contain a series of diffraction peaks at mid- $q$  and high- $q$  (**7** in Figure 2). In the case of silver (**7b**), the peaks are systematically broad and in a 1:2:3:4 ratio, while in the case of calcium (**7a**) the peaks are also found in a 1:2:3... ratio, but with a more complex distribution of their width. The dependency of the intensity with the scattering vector follows a power law contained between -2 and -3. Typical values of the slope in the *low-q* range, -1, -2 or -4, are generally associated with well-defined cylindrical, flat morphologies or a sharp interface, respectively.[47] Non-integer values are on the contrary associated with mass (1 – 3) or surface (3 – 4) fractals,[64,65] where a value of -4 commonly identifies a smooth interface. Taken together, the combination of cryo-TEM and SAXS show a mass fractal network in which fibers organize themselves in a rare side-by-side arrangement, forming raft-like structures. The periodicity in the side-by-side arrangement is responsible for the lamellar signal in SAXS. This curious structure is only found for specific proteins like silk fibroin or actin and it is not known for any diluted, unsheared, bulk solution of SAFiN. Despite these similarities, the calcium and silver fibrous systems have locally different structures. More details on the mechanisms of formation and fiber structure of **7a** and **7b** are described in detail elsewhere.[23,26]

Interestingly, many other cations drive the formation of a wormlike morphology (see *Cylindrical/wormlike micelles* paragraph, above). The tendency to fibrillation has been attributed to a ligand-metal complex between the metal center and the  $\text{COO}^-$  group of G-C18:1,[23] whereas the speciation diagrams of these cations indicate that they exist in a free ion form in the pH range of the experiment[24] while most other cations exist as a complex mixture of hydroxylated species.

Although full understanding is still out of reach, metal-induced fibrillation is very specific to G-C18:1 when compared to other glycolipid bioamphiphiles: the addition of  $\text{Ca}^{2+}$  ions to other biosurfactants having similar structures, namely C18:1 sophorolipids[66] and rhamnolipids,[67] does not produce any remarkable effects besides charge screening of the corresponding micellar phase. On the other hand, salt-free fibrillation, and in some cases gelation, have been observed for other biosurfactants, but rather with a saturated fatty acid and only in their acidic forms at pH below neutrality (refer to Ref. [29] for a comprehensive discussion on the fibrillation of biosurfactants).

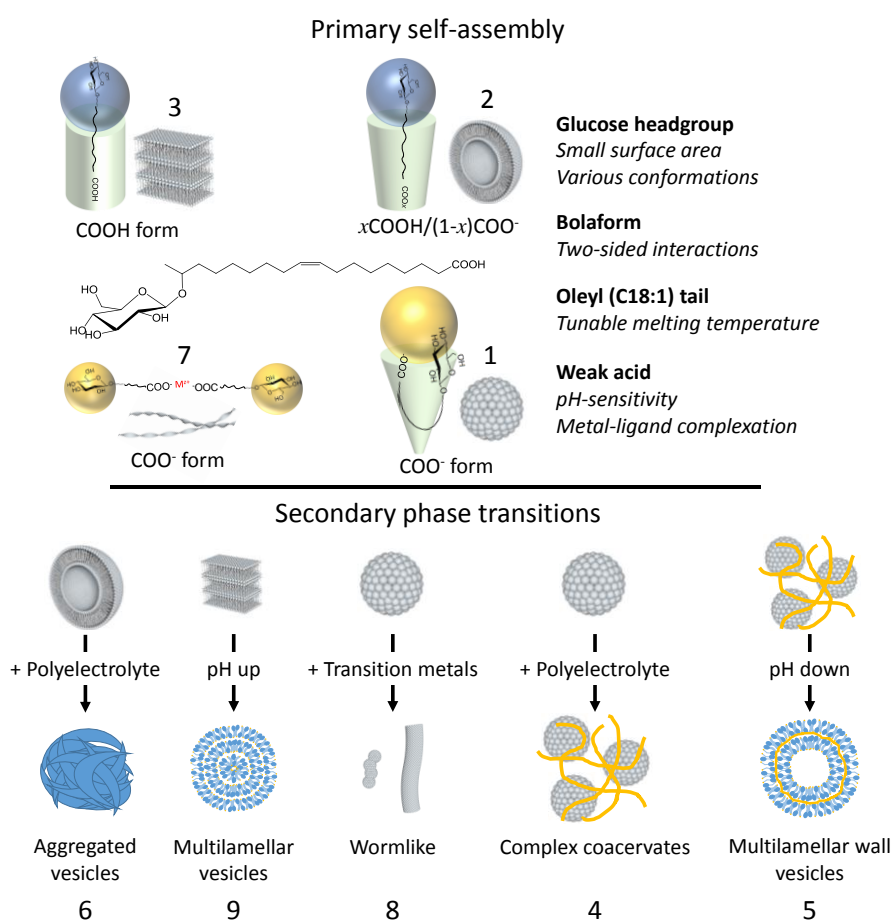
### **Structure-properties relationship of G-C18:1 self-assembly and mechanistic insights.**

G-C18:1 is a polyvalent molecule whose self-assembly behavior can obviously not be directly explained by the classical thermodynamic description of amphiphiles.[32,44,45,49] Its bolaform morphology makes it similar to bolaamphiphiles, well-known for having remarkable phase behavior, including micellization, vesiculation and fibrillation.[68] However, to the best of our knowledge, these were not immediately associated with a single compound. On the other hand, under diluted conditions, classical surfactants (charged, neutral or zwitterionic) generally express a standard micellization behavior, where micelles are spheroids with an aggregation number below 100.[5] Transition to a wormlike form occurs with increasing concentration or by adding salt,[34–36,69,70] which screens repulsive interactions between polar headgroups. Transition towards low curvature morphologies can also be induced, for instance by adding either cosurfactants or salts,[50,56] but fibrillation is generally not observed unless specific conditions are met, such as the combination of exotic gemini cationic surfactants associated with chiral organic counterions.[71] Lipids and LMW gelators also have their own specific behavior, which can include a micelle-to-vesicle (lipids)[46] or micelle-to-fiber (LMW gelators)[11] transition, but not the three at the same time for the same amphiphile.

Although much less is known compared to classical amphiphiles, the phase behavior of bioamphiphiles under diluted conditions displays a certain degree of richness, as reviewed recently.[29] However, the behavior of G-C18:1 seems to be quite unique, even compared to a number of sister molecules. Rhamnolipids and surfactin can form micelles or vesicles at alkaline and acidic pH,[29] respectively, but they do not fibrillate, even in the presence of divalent salts.[67,72] Monounsaturated C18:1 sophorolipids generally form micelles in a broad range of pH [29] and the effect of mono and divalent salts is limited to charge screening.[66] Other forms of saturated derivatives of sophorolipids and cellobioselipids, or

even various forms of bolaform sphorolipids,[73,74] tend to form micelles ( $\text{pH} > 7$ ) and fibers ( $\text{pH} < 7$ ), but not vesicles.[29] If the number of unsaturation of sphorolipids increases, the neutral form tends to form vesicles, but not fibers.[75] Why does G-C18:1 display such a unique behavior, compared to both synthetic and biobased amphiphiles?

Figure 4 shows the four main primary self-assembled structures of G-C18:1 in relation to the key structural features of the molecule, namely the type of headgroup, the bolaform morphology, the oleyl C18:1 backbone and the carboxylic end-group. Their simultaneous coexistence within a single molecule is responsible for its unique properties, as explained in detail below. Figure 4 also illustrates the main secondary phase transitions resulting from various phases and driven by specific conditions of pH variation or type of counterion. Taken altogether, they show that G-C18:1 can be found in nine different phases (Figure 1).



**Figure 4 – Structure-properties relationship for the primary self-assembly of G-C18:1 in water and illustration of the secondary phase transitions.**

*Glucose headgroup.* Glucose plays a double complementary role. First of all, it defines the surface area at the hydrophilic-hydrophobic interface of G-C18:1, thus contributing to the molecular shape as described by the packing parameter theory.[45] Secondly, like all linear



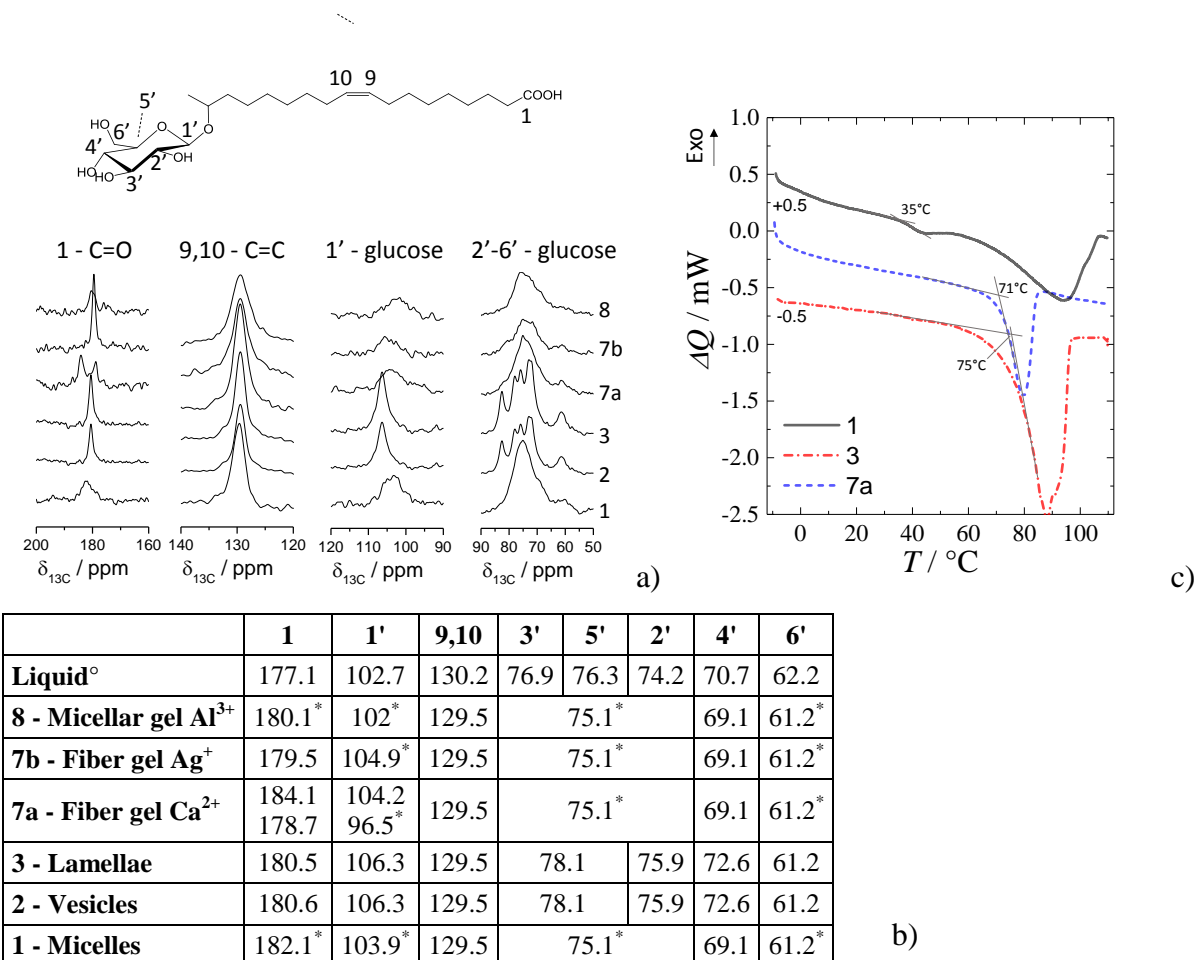
and cyclic carbohydrates, glucose has a complex energetic landscape, where many conformations are possible.[76] The interplay of these two aspects widen the range of possibilities for the self-assembly properties of G-C18:1. The area per molecule in single glucose lipids is in the region of 40-50 Å<sup>2</sup>, [77,78] and comparable values, between 35 and 47 Å<sup>2</sup>, are measured for G-C18:1.[31] These values are also comparable with the typical surface area of phospholipids (50-60 Å<sup>2</sup>).[45] G-C18:1 has an 18-carbon aliphatic chain, which is also comparable with the hydrophobic moiety of most phospholipids. According to the packing parameter theory,[45] as with phospholipids, G-C18:1 can be expected to aggregate into low-curvature morphologies, like lamellae (3) or vesicles (2), found experimentally at acidic pH. The important role of the single glucose unit in determining nearly flat membranes is demonstrated by the otherwise highly curved micellar shape observed for the di-glucose derivative (sophorolipid) of G-C18:1[19,79] and characterized by a larger headgroup, with a surface area above 70 Å<sup>2</sup>. [80]

At the same time, glucose has multiple energetically-stable conformations,[76] which can potentially adapt to more than one configuration of G-C18:1 with a potentially self-assembled structure. The direct role of carbohydrate conformations in glycolipids has been shown in the variety of crystal structures reported for alkyl gluconamides, each associated with a given conformation of the gluconamide group.[81] Similar effects were even reported for bolaform sophorolipids.[82] The most appropriate way to correlate structure and carbohydrate conformation is by <sup>13</sup>C cross polarization magic angle spinning (CP-MAS) solid-state nuclear magnetic resonance (ssNMR),[81,83,84] which was employed in this work to correlate glucose conformation and phase.

Figure 5a shows the most relevant peaks corresponding to the <sup>13</sup>C CP-MAS ssNMR spectra of freeze-dried G-C18:1 samples prepared from micelles (1), vesicles (2), lamellar precipitate (3), fibrillar (7) and micellar gel (8) solutions (full spectra and attributions are given in Figure S 1 and Table S 2). The peak of the C=C group in the oleyl moiety has a chemical shift at 129.5 ppm, the same across all samples, in good agreement with its solution resonance (130.2 ppm, Figure 5b). On the contrary, the anomeric (1') and 2'-6' positions in glucose differ strongly across samples. Membranes (2,3), whether in vesicle (2) or precipitated lamellar crystalline (3) phase, show well-defined peaks with reasonable resolution and equal chemical shift, in good agreement (although shifted) with the solution resonance. The actual peak shift of the glycosyl moiety in glycolipids between the solid and liquid state is expected, as previously shown for alkyl gluconamides[81] and bola sophorolipids[82]. On the other hand, the 2'-6' peak is broad, centered on 75.1 ppm and

essentially unresolved in both micellar and fibrous morphologies (**1,7,8**). The 1' peak is also broad, but characterized by chemical shift variations between 1 and 2 ppm between the micellar and fibrous samples.

ssNMR shows that the sugar conformation in **2** and **3** is not only the same but also well-defined, while all the other structures are characterized by a broad distribution of glucose conformations. Considering the strong morphological differences between micelles and fibers, this is unexpected and shows that the glucose conformation is most likely not involved in the micelle-to-fiber transition.



**Figure 5 – a)** Solid-state  $^{13}\text{C}$  CP MAS NMR spectra recorded on freeze-dried samples prepared from solutions of (1) micelles ( $t_c = 1$  ms), (2) vesicles ( $t_c = 3$  ms), (3) lamellar precipitate ( $t_c = 3$  ms), (7a)  $\text{Ca}^{2+}$  fiber gel ( $t_c = 3$  ms), (7b)  $\text{Ag}^+$  fiber gel ( $t_c = 1$  ms), (8)  $\text{Al}^{3+}$  micellar gel ( $t_c = 1$  ms).  $t_c$  stands for the contact time related to the magnetization transfer from  $^1\text{H}$  to  $^{13}\text{C}$  during cross polarization. **b)** Selection of the  $^{13}\text{C}$  chemical shift values related to various G-C18:1 phases. <sup>o</sup>: The  $^{13}\text{C}$  values recorded in the liquid state (solvent:  $\text{CDCl}_3/\text{CD}_3\text{OD}$  : 7/3) are reported from ref. [31]. \*: broad peak with no resolution of individual components. The list of full spectra and chemical shift is given in Supporting Information. **c)** Dynamic scanning calorimetry (DSC) of freeze-dried (1) micelles, (3) lamellar precipitate and (7a)  $\text{Ca}^{2+}$  fiber gel.

*C18:1 fatty tail.* Monounsaturations in fatty acids generally reduce their melting temperature, which for oleic acid is 13°C, in comparison to 69°C for stearic acid. Similar differences are observed for microbial glycolipids. The stearic and oleic derivative of sophorose are insoluble and soluble, respectively, at room temperature. The former assembles into crystalline ribbons[19,85] while the latter into micelles.[19,79] G-C18:0, the stearic derivative of G-C18:1, assembles into flat lamellae across a wide pH range, while the vesicle phase appears at higher temperature.[18,19,86] The C18:1 tail is also more flexible, for instance compared to aryl groups, like fluorine among others, where fluorine-derivatives (e.g., fluorenyl-9-methoxycarbonyl) are classically synthesized to enhance fibrillation.[7] The oleyl C18:1 moiety, due to its “kinked” structure, is more adaptable than saturated aliphatic chains to pack in both liquid crystalline and crystalline environments.[87] Despite the fact that the *cis*-olefin C=C group can potentially assume more than one conformation, as reported for various phases of oleic acid in the vicinity of 0°C,[87] its invariant <sup>13</sup>C chemical shift (Figure 5a,b) across all G-C18:1 structures seems to corroborate the hypothesis concerning its many degrees of freedom. This is illustrated in the melting profiles of three different phases of G-C18:1 probed by dynamic scanning calorimetry (DSC, Figure 5c).

The freeze-dried micellar phase (**1**) shows a broad, weak, pre-melting temperature at 35 °C followed by a much broader, poorly-defined, melting event starting at between 50°C and 60°C. These temperatures are higher than in oleic acid and illustrate the contribution of the glucose group to the physical state (solid at room temperature) of the sample. The lamellar precipitate (**3**) and the fiber phase (**7a**, from Ca<sup>2+</sup>) display sharper peaks at 75°C and 71°C, respectively. On the contrary, the lamellar precipitate (**3**) and the fiber phase (**7a**, from Ca<sup>2+</sup>) are characterized by a well-defined transition at higher temperature, as expected for more ordered systems. These reflect the important conformational changes in the oleyl backbone.

The combination of DSC and ssNMR data shows that the micelle-to-membrane transition is driven by the complete conformational rearrangement of G-C18:1, including the C=O, the oleyl and the glucose moieties at the same time. On the contrary, the micelle-to-fiber transition is driven by a combined effect involving the C=O (metal-ligand complexation, shift in the <sup>13</sup>C NMR spectra, discussed below) and the oleyl backbone (oleyl packing,[87] seen by the high temperature shift of the melting temperature, Figure 5c). The glucose headgroup plays a negligible role, as shown by the corresponding invariant <sup>13</sup>C signal compared to the micellar phase (Figure 5a).

*Bolaform shape.* Bolaform amphiphiles have been studied for decades due to their unique properties in water [68,88,89] and the two-sided amphiphilicity of G-C18:1 is a great advantage with respect to single head amphiphiles, with the bolaform shape of G-C18:1 driving the fiber (7). The COO<sup>-</sup> form of the carbonyl group strongly interacts with metal ions, most likely forming G-C18:1 dimers, in agreement with what has been reported for similar systems.[90,91] Meanwhile, the hydrophilic glucose group greatly improves solubilization in water, preventing the dimers from precipitating as a lamellar crystal, as classically found for metal salts of carboxylic acids.[90] According to ssNMR (Figure 5a,b), the 2'-5' and 6' signals of glucose are broad and centered at 75.1 and 61.2 ppm, respectively, thus indicating that the conformation of glucose is poorly defined for both micellar (1,8) and fibrillar (7) structures. The similar NMR response confirms that the role of glucose in the self-assembly of fiber (7) and micellar (8) gels is most likely not structural, different from what it is found for the membranes (2,3). This is schematized using different colors representing glucose in Figure 4. On the other side of G-C18:1, the C=O signal undergoes considerable shifts, discussed in detail in the next paragraph. The different roles shown by ssNMR played by glucose and COO<sup>-</sup> simultaneously in the micelle and fiber phases demonstrates the importance of the bolaform shape.

*COOH group.* Carboxylic acid is responsible for the pH-dependent properties of G-C18:1. This is unique compared to common head-tail amphiphiles and most bolaamphiphiles. Although fatty acids and bile salts also display pH-responsivity, the bolaform structure, double hydrophilic nature of G-C18:1 increase its stability in water over a much broader pH range than simple pH-responsive head-tail molecules, thus contributing to a richer phase behavior. Besides the extreme COOH and COO<sup>-</sup> forms below and above pH 4 and 8, respectively, mixtures of COOH and COO<sup>-</sup> characterize the broad intermediate range between pH 4 and 8. Increasing the content of negatively-charged COO<sup>-</sup> introduces intramolecular electrostatic repulsive interactions that have a critical impact on morphology, varying from flat to slightly curved (vesicles) to strongly curved (micelles). The mechanism of the transition is continuous and studied by pH-resolved *in situ* SAXS using different counterions.[19,22] This is in agreement with the packing parameter theory, where repulsive electrostatic interactions are of critical importance in the effective surface area of the hydrophilic headgroup,[34,45,92] as illustrated schematically by the use of different conical shapes in Figure 4. In other words, the amount of carboxylates tunes the curvature between the micellar and vesicular morphologies.

The role of carboxylates is however much broader, as they can be neutralized by single and polymeric cations but also undergo specific metal-ligand interactions with multivalent cations. In the former case, charge screening leads to either drive high-to-low curvature morphological transitions, e.g. sphere to worms, as described in the literature for single ions,[36] or complex coacervation, as reported for polyelectrolytes.[93] Both are listed as secondary phase transitions in Figure 4 and their corresponding mechanisms have been studied by *in situ* SAXS and reported elsewhere.[22,26]

On the other hand, specific metal-ligand interactions, common for carboxylates,[94] can drive more complex phenomena, like dimerization.[90] The important role of the COO<sup>-</sup> group is nicely shown by its chemical shift variation in <sup>13</sup>C ssNMR experiments (Figure 5b). The chemical shift of carboxylates is generally shifted downfield (larger values) with respect to carboxylic acids,[95] thereby explaining the high value (182.1 ppm) for the micelles (**1**) prepared at pH 8, while the pH of all other systems is generally equal to or lower than 7. Meanwhile, the splitting of the peak for the fibrillar Ca<sup>2+</sup> gel (**7a**) illustrates the more complex role of the C=O group in G-C18:1. The two peaks could either indicate the coexistence of COOH (178.7 ppm) and COO<sup>-</sup> (184.1 ppm) forms, but also the presence of two COO<sup>-</sup> sites, in agreement with the structural and mechanistic data of this fibrous system.[23,26] The variability in terms of peak width across samples is further proof that the COOH group plays a critical role in driving self-assembly. The membrane (**2,3**) and fiber (**7**) phases show a sharp C=O peak, illustrating its well-defined conformation, while the micellar phases (**1,8**) display a broad peak, more characteristic of a chemical shift distribution, showing a less defined structural role.

The effect of counterions on the phase behavior of G-C18:1 is also extremely important. Counterions are known to have an impact on the self-assembly of surfactants, lipids and gelators, although the possibilities are somewhat restricted for each category. For instance, cations can drive the formation of cylinders, worms or even vesicles in anionic surfactant systems, but not fibers (refer to Table S 1 in Ref. [24]). Ions may induce the formation of rafts in lipid bilayers[96] and fibrillation in LMW gelators.[4,91] The effect of cations on biosurfactants has been studied less from the standpoint of phase behavior. Micellar solutions of sophorolipids seem to be poorly affected by mono and divalent cations,[66] like rhamnolipids.[67] According to X-ray and neutron scattering observations, cations mainly screen repulsive electrostatic interactions in their micellar phase. Similar results were reported for surfactin, except for Ca<sup>2+</sup> and Ba<sup>2+</sup>, which drive under explored

structural changes, as shown by the modification of the scattering signal compared to the control.[72]

Counterions can either drive primary self-assembly (Figure 4), as in the case of the fiber phase (7) with free ions in solution ( $M^{z+}$ ), or they can impact secondary self-assembly (Figure 4). Polycations, synthetic or biobased, drive the agglutination of the vesicles into a less-defined “cauliflower-like” aggregate (6). Added to a micellar phase, they drive complex coacervation (4), whereas when added to a micelle-to-vesicle transition, they drive the formation of multilamellar wall vesicles (5). On the other hand, complex species of transition metal ions,  $M_x(OH)_y^{(xz-y)+}$  (with  $z$  being the valence),[97] drive sphere-to-wormlike transitions (8), with the possible gelification of the aqueous phase,[24] via a charge-screening mechanism.

## Conclusions

This work presented the wide range of phases obtained under comparable physicochemical conditions and low concentration (< 5 wt%) found for a new biobased molecule obtained by the fermentation of the yeast *S. bombicola*  $\Delta$ ugtB1. In water and at pH in the vicinity of 7, G-C18:1 assembles into micelles (pH > 7), unilamellar vesicles (pH < 7), multilamellar vesicles (pH < 4, followed by increase to pH < 7), lamellae (pH < 4 or pH ~7 at  $T < 15^\circ\text{C}$ ), fibers (pH > 7, with  $\text{Ag}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$ ), and wormlike micelles (pH > 7, with  $\text{Fe}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ni}^{2+}$ ). When a polycation is used instead of single ions, G-C18:1 forms complex coacervates (pH > 7), aggregated vesicles (pH < 7) and even multilamellar wall vesicles, analogues of lipoplexes (pH > 7, followed by pH < 7).

Such a broad phase behavior diluted in water (below 5 wt% and as low as 0.5 wt%) is uncommon for many classes of amphiphiles and practically unknown for a single compound. This is explained by the unique structural features of G-C18:1 which, combined together, are responsible for its remarkable phase behavior. The glucose headgroup is neutral and provides a surface area of about  $50 \text{ \AA}^2$ , explaining its packing into nearly flat membranes (vesicle, lamellae). At the same time, glucose is a cyclic sugar, known to assume more than one conformation. Solid-state NMR experiments show that the glucose headgroup in G-C18:1 can assume at least two conformations, one in the membrane and the other in the micellar and fibrillar phases. The bolaform structure is suitable for asymmetric interactions, for instance metal complexation by the carbonyl and hydration of the sugar end, thus favoring water-dispersible fibers. The C18:1 backbone is more versatile than a fully saturated aliphatic chain,

as it can assume both defective and well-ordered packing, with a wide range of melting temperatures. This was experimentally verified by DSC for the micellar, lamellar and fiber phases, with melting temperatures spanning between 40°C and 75°C. Finally, the ionization of the COOH group into carboxylates introduced electrostatic repulsive forces, strongly contributing to stabilizing the conical morphology of G-C18:1, thus driving the membrane-to-sphere transition. At the same time, negatively charged carboxylates could be neutralized by single (mono or divalent) or polymeric cations but also complexed by metals, with a critical impact on the final phase.

Last but not least, the lack of chemical modification and the biological origin of G-C18:1 make this compound extremely interesting for a broad range of applications, from detergency to biomedicines and cosmetics, as shown by its ability to form strong hydrogels (fiber phase) and encapsulate drugs (multilamellar wall phase). This work illustrated how complex physicochemical functionality can be found in natural molecules, making them not only interesting compounds to extend the boundaries of understanding amphiphiles,[98] but also a source for materials with low environmental and ecological impact for many applications

### **Acknowledgements**

Authors gratefully acknowledge the French ANR, Project No. SELFAMPHI - 19-CE43-0012-01 and Sorbonne Université (contract No. 3083/2018) for their financial support. We are most grateful to Amélie Leforestier and Jeril Degrouard (LPS, Saclay University) and Labex PALM (ANR-10-LABX-0039-PALM) for the cryo-TEM experiment in (Figure 3-8a) on the 200 kV Jeol instrument and to METSA (METSA Federation, FR3507 CNRS) for its financial support.

### **Competing interests**

Authors declare no competing interests.

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## Funding

French ANR, Project N° SELFAMPHI - 19-CE43-0012-01.

Sorbonne University, Ecole Doctorale, ED297 Physique and Chimie des Matériaux

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