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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, chameleon are particles, of which the mass varies on the surrounding matter. Here, we show the chameleonic self-assembly behaviour of a low molecular weight (LMW) amphiphile, a broad class of molecules largely studied since 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 behaviour is generally predicted in relationship 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 π - π stacking. In excess of water, surfactants form micelles, phospholipids form vesicles or lamellar phases and functional amphiphiles often form

micelles or fibers. Here, we show the multiphase behaviour, much richer and unpredictable than what it is known for most amphiphiles, of a biobased glycolipid, produced by the yeast *S. bombicola* Δ ugtB1. In excess of water, and within a narrow pH range around neutrality, this compound assembles into micelles, uni- and multilamellar vesicles, lamellae, fibers, simply by playing between pH, temperature or counterion. This rich phase behaviour is not only interesting *per se*, but it also shows generates a number of diverse biocompatible and biodegradable soft self-assembled materials like hydrogels, complex coacervates or drug carriers.

Significance statement

How many different structures can a single amphiphilic molecule self-assemble into, in water under diluted conditions and in the vicinity of neutral pH and at room temperature? A natural glycolipid, composed of a glucose and a free fatty acid obtained by microbial fermentation, self-assembles into at least nine different crystalline, or liquid crystalline structures, under close physicochemical conditions, depending on pH, type of counterion and sense of pH variation.

Introduction

The variation of a given property in living or inert systems in response to the environment is a stunning phenomenon in Nature. Chameleons are mimetic reptiles, which adapt themselves to their surrounding environment by changing colours. 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 chameleonic behaviour of both LMW compounds and polymers has been proposed long ago^{2,3} within the context of a morphological or aggregation variation to the surrounding conditions. However, the possibilities offered by the compounds in these early works (catenanes, polyisobutylene-based biopolymers) were limited to few states of assembly. More recently, a LMW peptide amphiphile was shown to assume four different states of aggregation (amyloid-like β -sheet, β -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 few morphologies of equivalent structural and curvature.

LMW amphiphiles are molecules having a size roughly in the order of 1 kDa or less and composed of a 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 the 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 lipidic⁶ compounds or LMW gelators.⁷ From a chemical point of view, 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 salts⁵ 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 rare are those compounds, which display a phase behaviour common to the three categories of amphiphiles. For instance, peptide amphiphile in ⁴ assembles into four, structurally-related, fibrous structures. If one includes additional constraints like the use of an aqueous medium, 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 -, produced by an engineered yeast strain (*Starmerella bombicola* *ΔugtB1*).¹³ Microbial glycolipid amphiphiles are LMW compounds widely known in the literature for their potential in replacing petrochemicals in all applications where synthetic surfactants are employed.¹⁴⁻¹⁷ G-C18:1 shows a rich chameleonic behaviour 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} 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} multilamellar vesicles (9).²⁰ The richness and complexity in terms of self-assembly is 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, which can display such a wide multiphase behaviour as single molecules, merging the behaviour of at least three families of LMW amphiphiles with opposite tendencies of self-assembly: surfactants, lipids and gelators. From the properties point of view, 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 applicative domains. In terms of market perspectives,^{27,28} developing a single, multi-properties, biobased compound is also potentially cheaper than investing into 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 larger share of concerned consumers does not accept 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 specialty chemical by BASF,³⁰ thus circumventing the use of GMOs.

Materials and methods

Chemicals. Glycolipid biosurfactant, glucolipid G-C18:1, is purchased from the Bio Base Europe Pilot Plant, Gent, Belgium, lot N° APS F06/F07, Inv96/98/99 and used as such. The monounsaturated glucolipid G-C18:1 ($M_w = 460 \text{ g}\cdot\text{mol}^{-1}$) contains a β -D-glucose unit covalently linked to oleic acid. The molecule is obtained by fermentation from the yeast *Starmerella bombicola* *AugtB1* according the protocol given before.¹³

Sample preparation. For all samples, G-C18:1 powder is dispersed in milli-Q water followed by vortexing and sonication. When needed, the pH is adjusted by few μL of concentrated NaOH or HCl, generally prepared at 0.1 M, 0.5 M, 1 M or 5 M. pH is monitored with a standard KCl electrode. For each specific preparation protocol, please refer to the cited works according to Table 1.

Table 1 – Known structures, conditions and type of behavior of G-C18:1 in water (mQ-grade) at concentrations below 5 wt%. Unless otherwise state, temperature is always room temperature (T~ 23°C).

*: manuscript submitted but not peer-reviewed, yet, and available free of charge from a repository.

Structure	Conditions	Number	Reference	Behaviour
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Spheroidal micelles	pH > 7.5	1	18,19	Surfactant-like
Unilamellar vesicles	pH < 6.5	2	18,19	Lipid-like
Lamellar	pH < 4	3a	18–20	Lipid-like
Lamellar	pH > 4, T < 15°C	3b	18,19	Lipid-like
Complex coacervates	pH > 7.5, polycations (poly-L-lysine, chitosan...)	4	21,22	Surfactant-like
Multilamellar wall vesicles (MLWV)	pH < 7.5, pH reduced from complex coacervate phase	5	21,22	Lipid-like
Vesicle aggregates	pH < 6.5, polycations	6	21	Lipid-like
Crystalline fibers	pH > 7.5, Ca ²⁺ , Ag ⁺ , Mn ²⁺ , Cr ²⁺	7	23–26*	Gelator-like
Cylinders, wormlike micelles	pH > 7.5, Fe ²⁺ , Co ²⁺ , Al ³⁺ , Cu ²⁺	8a	24,26*	Surfactant-like
Cylinders, wormlike micelles	6.5 < pH < 7.5	8b	18,19	Surfactant-like
Multilamellar vesicles (MLV)	4 < pH < 6.5, basification from pH < 4	9	20	Lipid-like

Small angle X-ray scattering (SAXS). SAXS experiments have been performed at Diamond (UK), Soleil (France) and ESRF (France) synchrotron radiation facilities. Please, refer to the references in Table 1 for more experimental details of each system.

Cryogenic-transmission electron microscopy (Cryo-TEM). All cryo-TEM images are recorded on an FEI Tecnai 120 twin microscope operating at 120 kV with an Orius 1000 CCD numeric camera. The sample holder is a Gatan Cryo holder (Gatan 626DH, Gatan). Digital Micrograph software is used for image acquisition. Cryo-fixation is done with low dose on a homemade cryo-fixation device. The solutions are deposited on a glow-discharged holey carbon coated TEM nickel grid (Quantifoil R2/2, Germany). Excess solution is removed and the grid is immediately plunged into liquid ethane at -180°C before transferring them into liquid nitrogen. All grids are kept at liquid nitrogen temperature throughout all experimentation. Cryo-TEM images are treated and analyzed using Fiji software, available free of charge at the developer's web site.

Results

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

The known phase behaviour 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 to obtain them, separately. The reader should remark that this communication contains both published and unpublished data. However, at the time of writing, all unpublished data have been submitted for evaluation and are available free of charge in the form a full manuscripts from a public repository. Table 1 specifies which documents are peer-reviewed and which are available from a repository.

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 spherical 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 spherical 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 tend to form a aqueous solution of micelles.²⁹ Micelles are spheroidal in shape and of diameter 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 typical of spheroidal micelles, characterized by a flat low-*q* plateau and an oscillation of the form factor

at high- q (Figure 2, **1**). The amplitude of the oscillation may vary with pH, and this was attributed 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). Association between charged micelles and oppositely-charged polyelectrolyte complexes have 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, of mussel adhesion and that have been developed for underwater adhesives.⁴¹ The micellar solution of G-C18:1 at pH above neutrality contains mostly negatively-charged micelles, of which the charge is counterbalanced by Na^+ . Adding a polycation, may it be 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 hundred of nm in size and which can be safely associated to 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⁻ forms of G-C18:1. Wormlike micelles have also been observed upon addition of specific transition metal ions (Al^{3+} , Fe^{2+} , Co^{2+} , Cu^{2+}) to a micellar solution of G-C18:1 at 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 experimentally found for G-C18:1 (**8** in Figure 2), when pH is reduced and when 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.

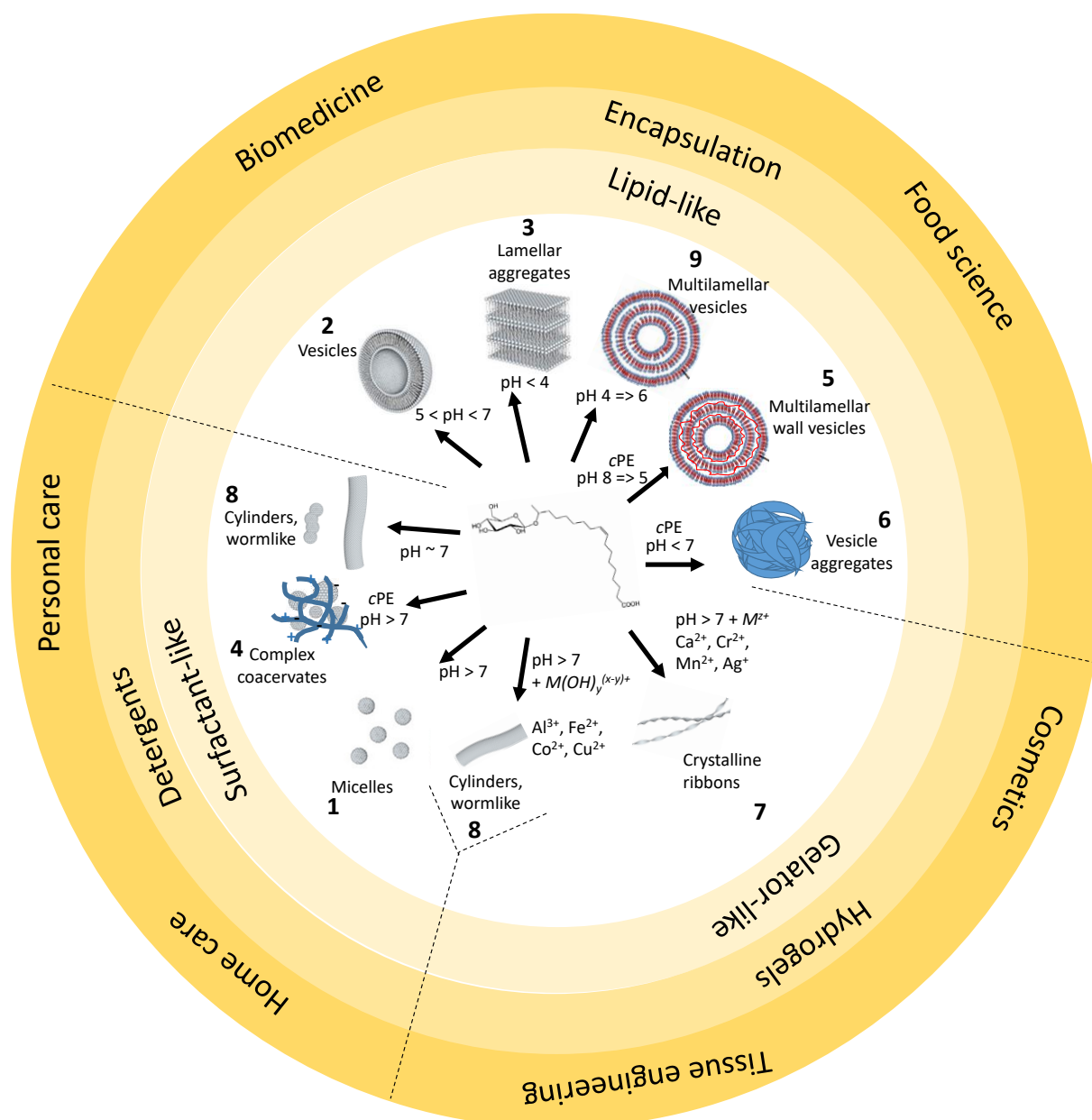


Figure 1 – Scheme summarizing the phase behavior of G-C18:1 ($C < 5$ wt% in water) with pH and salt addition. *cPE*: cationic polyelectrolytes. Phases: (1) micellar,^{18,19} (2) unilamellar vesicles,^{18,19} (3) lamellar phase,^{18–20} (4) complex coacervate,^{21,22} (5) multilamellar wall vesicles (MLWV),^{21,22} (6) aggregated vesicles,²¹ (7) fiber phase (under review)^{23–26} (8) micellar wormlike phase pH-^{18,19} and cation- (under review)^{24,26} dependent, (9) multilamellar vesicle (MLV) phase.²⁰

Lipid-like behaviour

(Unilamellar) vesicles (**2** in Figure 1). Unilamellar vesicles, or liposomes, are classically prepared with lipids and lipid mixtures.⁴⁸ They are generally promoted by the larger hydrophobic moiety in amphiphiles compared to the surface area of the hydrophilic headgroup, in agreement with the thermodynamic theory of self-assembly of amphiphiles.^{44,49} Surfactants generally do not form vesicles, unless the interactions among 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 the 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 mean of pH-resolved *in situ* SAXS.¹⁹ The typical profile corresponding to the vesicle phase is reported in Figure 2 (**2**) and it is characterized by a -2 low-q dependence of the intensity⁴⁷ and a broad oscillation of the form factor, both features typical for flat membrane with 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, typically composed of phospholipids and their mixtures. MLV are more interesting for encapsulation and delivery of actives than unilamellar vesicles because their multilamellar structure make 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, one can 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 precipitation of a lamellar aggregate, which, by increase of pH above 5, transform into MLV (cryo-TEM, **9**, Figure 3),²⁰ which are colloidally stable in water at pH in the vicinity, and slightly below, neutrality. Size can be controlled through the typical methods employed for phospholipid MLV, sonication and filtration, and that MLV from G-C18:1 can easily encapsulate and stabilize the dispersion of both stable (ferritin) and unstable (iron nanoparticles, hydrophobic phosphors) colloids.²⁰

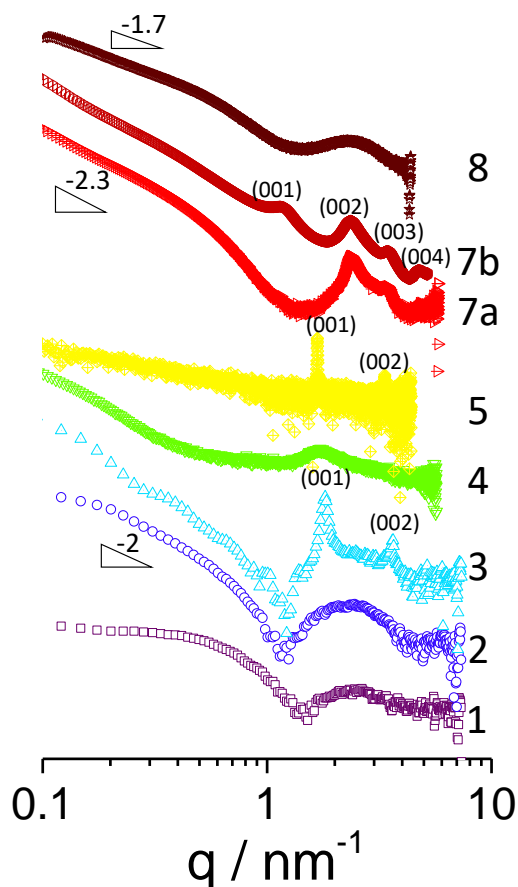


Figure 2 – SAXS profiles of various phases composed of G-C18:1 in water, room temperature, at concentration below 5 wt%: 1) micellar,^{18,19} (2) unilamellar vesicles,^{18,19} (3) lamellar phase,^{18–20} (4) complex coacervate,^{21,22} (5) multilamellar wall vesicles (MLWV),^{21,22} (7) fiber phase from (7a) Ca^{2+} and (7b) Ag^+ (under review).^{23–26} (8) micellar wormlike phase, cation-dependent (under review).^{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, practically quantitatively, stable MLWV colloids through a coacervate-to-MLWV phase transition in bulk water at concentrations below 5 wt% and by simple decrease of the solution pH 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 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, entirely composed of biocompatible molecules and their use in drug encapsulation and delivery to cancerous HeLa cells was recently demonstrated through a selective membrane fusion process.⁵³

(*Aggregated*) vesicles (**6** in Figure 1). Agglutination of vesicles is a typical phenomenon observed when vesicles interact with ions in water.⁵⁴ In the presence of polycations, 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. For this reason, 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 higher pH but at lower temperatures, below 15°C.¹⁸ Lamellar phase of G-C18:1 is generally characterized by the typical SAXS signature, composed of two sharp diffraction peaks in a 1:2 ratio (**3** in Figure 2). 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 lower curvature. If a thermodynamic understanding of the vesicle and lamellar phases could be attempted,^{44,49} one must keep in mind that G-C18:1 assumes an interdigitated arrangement inside the membrane, differently than 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

those glucose 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 not compatible, with the thermodynamic theory of self-assembly.^{32,44,49}

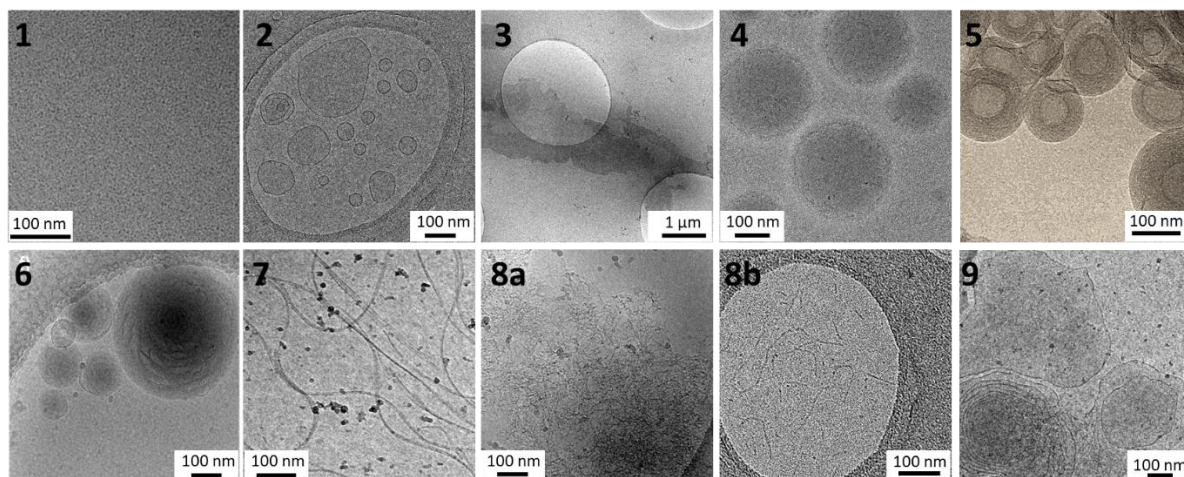


Figure 3 – Typical cryo-TEM images of various phases composed of G-C18:1 in water, room temperature, at concentration below 5 wt%: (1) micellar,^{18,19} (2) unilamellar vesicles,^{18,19} (3) lamellar phase,^{18–20} (4) complex coacervate,^{21,22} (5) multilamellar wall vesicles (MLWV),^{21,22} (6) aggregated vesicles,²¹ (7) fiber phase from Ag⁺ (under review)^{23–26} (8) micellar wormlike phase with (8a) Fe²⁺ (under review)^{24,26} or (8b) at pH ~7,^{18,19} (9) multilamellar vesicle (MLV) phase.²⁰

Gelator-like behaviour

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 to a multifunctional headgroup, like a peptide derivative.^{8–10} Due to their fibrillar structure, most of these systems spontaneously form hydro- or organo-gels 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 induced⁶⁰ by temperature, pH⁶¹ or salts⁶² 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 Ca²⁺, Ag⁺,

Mn^{2+} , 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) and they display a characteristic SAXS fingerprint with a -2 dependency of the intensity at low-q, typical for flat structures, including tape or ribbon fibers, and a series of diffraction peaks at mid-q and high-q (7 in Figure 2). In some cases, the peak follow a 1:2:3... ratio, demonstrating a rare lamellar, raft-like, arrangement of the fibers.²³ 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. Interestingly, many other cations drive the formation of a wormlike morphology (see above). The tendency to fibrillation has then been attributed to a ligand-metal complex between the metal center and the 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.

If full understanding is still out of reach, metal-induced fibrillation is very specific to G-C18:1, when compared to other glycolipid bioamphiphiles: addition of Ca^{2+} ions to other biosurfactants having similar structures, namely C18:1 sophorolipids⁶⁴ and rhamnolipids,⁶⁵ 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, has been observed for other biosurfactants, but rather having a saturated fatty acid and only in their acidic form at pH below neutrality (refer to Ref. ²⁹ for a comprehensive discussion on the fibrillation of biosurfactants).

General comments.

G-C18:1 is a polyvalent molecule, of which the self-assembly behavior cannot be directly described by the classical thermodynamic description of amphiphiles.^{32,44,45,49} Its bolaform morphology makes it similar to bolaamphiphiles, a class of molecules, which is notoriously known to have a stunning phase behavior, including micellization, vesiculation and fibrillation.⁶⁶ However, to the best of our knowledge, these cannot be obtained by the same compound and in such a narrow range of pH and high dilution regime. Under diluted conditions, classical charged, neutral or zwitterionic surfactants generally express a standard micellization behavior, where micelles are spheroids with aggregation number below 100.⁵ Transition to wormlike occurs with increasing concentration or by adding salt,^{34-36,67,68} which screens repulsive interactions between polar headgroups. Transition towards low curvature morphologies can also be induced by adding either cosurfactants or salts,^{50,56} but fibrillation is

generally not observed, unless specific conditions are met, such as the combination of a gemini cationic surfactants with chiral organic counterions.⁶⁹ Lipids and LMW gelators have also their own specific behavior, which can possibly 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 recently reviewed.²⁹ However, the behaviour 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.^{65,70} Monounsaturated C18:1 sphorolipids generally form micelles in a broad pH range²⁹ and the effect of mono and divalent salts is limited to charge screening.⁶⁴ Other forms of saturated derivatives of sphorolipids and cellobioselipids, or even various forms of bolaform sphorolipids,^{71,72} tend to form micelles (pH > 7) and fibers (pH < 7), but not vesicles.²⁹ If the number of unsaturation of sphorolipids increases, the neutral form tends to form vesicles, but not fibers.⁷³

The self-assembly behavior of G-C18:1 is strongly affected by pH. At the extremes, upon complete deprotonation or protonation, G-C18:1 behaves as surfactant (micellar phase) or a lipid (lamellar phase).^{18,19} In between the two, pH determines the relative content of the COOH and COO⁻ forms, making *de facto* this system behave as a mixture of a surfactant and a lipid, thus allowing a tunable curvature controlled by pH.¹⁹ For this reason, one can observe cylinders, worms and vesicles in a continuous fashion.¹⁹ This process is fully reversible, although a new phase appears when the vesicle phase is approached from the lamellar phase. In this case, disruption of the lamellar precipitate in bulk water drives the formation of a MLV phase,²⁰ in analogy with MLV prepared by hydration from adsorbed multilamellar layers of phospholipids, except that this process occurs in bulk water for G-C18:1. This allows the possible encapsulation of both stable and unstable colloids in solution.²⁰

The effect of counterions on the phase behavior of G-C18:1 is 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 bilayers⁷⁴ and fibrillation in LMW gelators.^{75,76} The effect of cations on biosurfactants has been less studied from a phase behavior point of view. Micellar solutions of sphorolipids

seem to be poorly affected by mono and divalent cations,⁶⁴ just as rhamnolipids,⁶⁵ and surfactin, except, in the latter, for Ca^{2+} and Ba^{2+} , which do provoke a structural change, although not well identified so far.⁷⁰

The nature of the counterion, combined to the phase behavior of G-C18:1, drives the self-assembly. At neutral acidic pH, in the micellar phase, mono and divalent cations induce precipitation of vesicles,²⁶ most likely in a lamellar phase, while polycations drive agglutination of the vesicles into a less-defined “cauliflower-like” aggregate.²¹ Adding cations in the micellar phase spans from major to no impact at all: 1) Na^+ ions simply screen the repulsive electrostatic interactions between micelles;²⁴ 2) most transition metal cations drive a sphere-to-wormlike transition, with possible gelification of the aqueous phase;²⁴ 3) specific mono and divalent cations drive fibrillation, with possible formation of SAFiN hydrogels (metallogels);^{24,25} 4) polycations, synthetic or biobased, drive the micellar aggregation into complex coacervates;^{21,22} 5) polycations, combined with a micelle-to-vesicle transition, drive the formation of multilamellar wall vesicles from complex coacervates;^{21,22} 6) cations existing as free ions in solution (M^{z+}) seem to drive complexation and fibrillation, while cations existing as a mixture of complex species, $\text{M}_x(\text{OH})_y^{(xz-y)+}$ (with z being the valence),⁷⁷ seem to induce charge screening and drive the formation of wormlike micelles.²⁴

Conclusions

This work presents the wide range of phases obtained under comparable physicochemical conditions and low concentration (< 5 wt%) found for a new biobased molecule obtained by fermentation of the yeast *S. bombicola* ΔugtB1 . 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 Ag^+ , Ca^{2+} , Mn^{2+}), wormlike micelles (pH > 7, with Fe^{2+} , Cu^{2+} , 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 behaviour in diluted 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. The lack of chemical modification and its biological origin make this compound extremely interesting for a broad range of applications, from detergency to biomedicine or cosmetics, as shown by its ability to form strong hydrogels (fiber phase) or encapsulate drugs

(multilamellar wall phase). This work illustrates how complex physicochemical functionality can be found in natural molecules, making them not only interesting compounds to push the boundaries of understanding of amphiphiles further,⁷⁸ but also a source for matter with low environmental and ecological impact for many applications

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Competing interests

Authors declare no competing interests

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Data sharing plan

Raw data corresponding to each Figure will be shared on the Zenodo repository after acceptance of the manuscript.

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