Palmitic acid sophorolipid biosurfactant: from self-assembled fibrillar network (SAFiN) to hydrogels with fast recovery

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Nanofibres are an interesting phase into which amphiphilic molecules can self-assemble. Described for a large number of synthetic lipids, they were seldom reported for natural lipids like microbial amphiphiles, known as biosurfactants. In this work, we show that the palmitic acid congener of sophorolipids (SLC16:0), one of the most studied families of biosurfactants, spontaneously forms a self-assembled fibre network (SAFiN) at pH below 6 through a pH jump process. pH-resolved in situ small-angle X-ray scattering (SAXS) shows a continuous micelle-to-fibre transition, characterized by an enhanced core–shell contrast between pH 9 and pH 7 and micellar fusion into a flat membrane. © 2021 The Author(s) Published by the Royal Society. All rights reserved.
between pH 7 and pH 6, approximately. Below pH 6, homogeneous, infinitely long nanofibres form by peeling off the membranes. Eventually, the nanofibre network spontaneously forms a thixotropic hydrogel with fast recovery rates after applying an oscillatory strain amplitude out of the linear viscoelastic regime: after being submitted to strain amplitudes during 5 min, the hydrogel recovers about 80% and 100% of its initial elastic modulus after, respectively, 20 s and 10 min. Finally, the strength of the hydrogel depends on the medium’s final pH, with an elastic modulus fivefold higher at pH 3 than at pH 6.

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1. Introduction

Stimuli-responsive peptides, proteins and lipids [1–4] attract a great deal of interest in the field of nanotechnology for their ability to self-assemble into two- and three-dimensional soft materials, which can in turn be employed in a growing number of high-tech applications [5], such as protective coating for cells [6], regenerative medicine [7], laboratory-on-a-membrane prototyping [8] or self-healing materials [9]. Lipids are particularly interesting compounds because they are ubiquitous natural molecules, which are still easy to synthesize and, despite their often simple structure, can self-assemble into a variety of soft architectures [10], possibly leading to complex isotropic (e.g. disordered entangled fibres) or anisotropic (e.g. lamellar) nano and meso-structures having interesting mechanical properties and potential applications [11–13].

Nanofibres, whether reported in the shape of nanotubes [14], twisted or helical ribbons [15], lipid peptides [19] and proteins [20]. Generally driven by both specific (H-bonding, π–π stacking) intermolecular forces and non-specific (steric hindrance, hydrophobic effect) interactions [12,13,21], sometimes driven by interactions with chiral counterions [22], self-assembled fibrillar networks (SAFiN) are often involved in the development of soft gelled materials [12,13,23–25], with applications as scaffolds for tissue engineering [26], wound healing [27] and cancer treatment [28].

Microbial glycolipids are a class of compounds generally addressed as microbial amphiphiles, or biosurfactants. They all have in common their microbial fermentation production source from vegetable oils and glucose [29,30]. Commonly considered as safe compounds from a cytotoxicity point of view [31,32], their high-end applicative potential is still to be unveiled and one of the main reasons is the poor knowledge of their phase behaviour in water under application-relevant conditions, that is at volume fractions below 10 wt%, at pH between 5 and 8 and ionic strength above the mM range. A set of recent studies has shown the ability of a wide range of microbial glycolipids to form micelles [33,34], ribbons [35], vesicles [36–38], sponge [39], lamellar [39,40] structures and even simple [41] or complex coacervates [42].

Fibrillation in the form of nanoribbons and nanohelices from microbial biosurfactants has been shown for celllobioselipids [36,43], symmetrical sophorolipids [44], amine-derivatives of sophorolipids [45] and, above all, the neutral form of stearic acid (C18:0) derivative of sophorolipids [35,46,47]. The latter shows fibrillation at both acidic and alkaline pH for, respectively, the –COOH-ending [35,46] and –NH2-ending [47] congeners. However, hydrogel formation from microbial biosurfactants in the absence of additives (e.g. gelators, polymers) was mentioned for celllobioselipids [48] and more deeply studied for C18:0 glucolipids (lamellar gel) [40,49], symmetrical C16:0 [44] and acidic C18:0 sophorolipids (fibrillar gels) [50]. In the latter cases, it was shown that the gel strength depends on either the temperature or pH variation rates, in agreement with previous studies on low-molecular weight gelators (LMWG), of which the gel strength was shown to be related to the content of spherulitic structures, which are in turn controlled by the supersaturation extent and, consequently, by kinetics [51].

In this work, we address the self-assembly properties of the recently discovered non-acetylated palmitic acid sophorolipids, SLC16:0, derived by fermentation of S. bombicola CYP1BMR in the
Figure 1. Acidic C16:0 sophorolipids are obtained by fermentation of S. bombicola CYP1BMR in the presence of palmitic acid and glucose. The compound is referred to as SLC16:0 throughout this work. (Online version in colour.)

presence of palmitic acid and glucose (figure 1) [52]. We employ pH-resolved in situ small-angle X-ray scattering (SAXS) to follow its self-assembly in water at room temperature. We find that nanofibres form at pH below 6 following a micelle-to-fibre phase transition. We then find that thixotropic hydrogels spontaneously form below pH 6, of which the strength depends on the concentration and final pH, similar to peptidic LMWG [53,54]. This behaviour is different from what was previously found for the deacetylated acidic C18:0 sophorolipid, for which fibrillation is a diffusion-limited process, with the gel strength depending on the pH change rate [50]. Finally, C16:0 sophorolipids hydrogels reach elastic moduli above 10 kPa for concentrations below 5 wt%, a range of values comparable to the best LMWG, such as FMOC derivatives [21,55]. Typically of thixotropic hydrogels, they also show fast recovery to about 80% of their mechanical strength within 20 s, and 100% after 10 min, after being destructured with large strain amplitude beyond the linear domain during 5 min.

2. Material and methods

(a) Chemicals

Palmitic acid sophorolipids SLC16:0 (Mw = 596.7 g.mol⁻¹) were produced at a production rate of 33 mg.l⁻¹.h⁻¹ in a bioreactor system using a strain of the yeast Starmerella bombicola, modified by the heterologous expression of the cytochrome P450 cyp1 gene of Ustilago maydis and feeding with palmitic acid [52]. The molecule was hydrolysed under alkaline conditions and only the non-acetylated acidic form of SLC16:0 was collected and used in this study. The detailed conditions of biosynthesis, purification as well as the full HPLC-ELSD, LC-MS and NMR characterization are provided in [52]. The typical ¹H NMR spectrum of the compound used in this work is reported in the electronic supplementary material, figure S1.

(b) General method to prepare SLC16:0 hydrogels

SLC16:0 is generally insoluble in water at room temperature. However, the solubility of its deionized form at basic pH is improved, as is classically observed for many glycolipid biosurfactants [36,37,56]. For this reason, SAFiN and hydrogels are prepared by a pH-jump process, from basic to acidic pH, following a procedure developed in previous studies [35,37]. The SLC16:0 solution (exact concentrations are given in the legends of figures) is adjusted to pH approximately 10 by adding 1–5 µl of NaOH 5 M. The solution becomes clear, indicating solubilization of the compound. pH is then reduced by adding 1–20 µl of HCl 0.5 M or 1 M (0.1 M can also be used for refinement). The exact amount of NaOH and HCl depends on the lipid concentration in the solution. More precise data are given for the in situ SAXS experiments and can be found in the electronic supplementary material, table S1, which also provides the typical dilution factors and NaCl concentrations after the pH jump for an SLC6:0 system at C = 0.5 wt% acidified with either a 0.1 M or 0.5 M HCl solution. Fibrillation and gelation occur below pH approximately 6.

Additional information on the analytical techniques (in situ SAXS, analysis of SAXS data, rheology, cryo-TEM) is given in the electronic supplementary material.
3. Results and discussion

(a) Self-assembly of SLC16:0 in water

The non-acetylated palmitic acid derivative of sophorolipids, SLC16:0, is a new molecule belonging to the broad family of biobased amphiphiles and its self-assembly properties in water are not known. Similarly to other sophorolipids, it contains a free-standing ionizable COOH group, of which the pKa is evaluated in this work at a pKa approximately 7.0 by acido-basic titration (electronic supplementary material, figure S2).

In this work, we study the aqueous phase behaviour of SLC16:0 at concentrations between 0.5 wt% and 5 wt% as well as its hydrogel-formation properties. The self-assembly is characterized by pH-resolved in situ SAXS (figures 2 and 3; electronic supplementary material, figure S3 and S4), and by cryogenic transmission electron microscopy (cryo-TEM, figure 4; electronic supplementary material, figure S5). The pH-resolved in situ SAXS experiment is designed according to previous studies: the sample solution (2 ml, 0.5 wt%) is pumped from the reaction beaker in a flow-through 1.5 mm quartz capillary by means of a peristaltic pump. pH is controlled by adding microlitre-amounts of a 0.5 M, or 0.1 M, HCl solution at a rate of 0.136 µl s⁻¹ (electronic supplementary material, table S1 for more information) by means of a computer-controlled push-syringe. pH is monitored and acquired using a computer-controlled pH metre. pH and SAXS acquisitions are synchronized (1 acquisition every 5 s) and triggered manually with an error of ±1 s [37].

The full SAXS profiles recorded from pH 9.6 to 2 are shown in figure 2a for an added 0.5 M HCl solution and in the electronic supplementary material, figure S4a for a 0.1 M HCl solution. The advantage of the former is the negligible dilution factor (1.6% against 17% for the 0.1 M HCl solution, electronic supplementary material, table S1 for more information), although the rate of pH change is much faster than the latter, as pH 2 is reached within 13 min against 50 min when the 0.1 M HCl solution is employed (electronic supplementary material, figure S4b). The rate of pH variation was shown to be important in the fibrillation process of the C18:0 sophorolipid congener, for which fast rates induce precipitation due to spherulite formation [50]. On the contrary, solutions of SLC16:0 fibres are always homogeneous and stable, independently of the molarity of the acid solution used and/or the rate of pH variation. This particular feature will be discussed later.
Figure 3. (a) Typical SAXS profiles extracted at pH greater than 7 (grey) and pH less than 5 (black) from the experiment in figure 2a. Continuous black and red lines indicate the q-domains of linear fits in log–log scale, while P1 and P2 schematically show the Lorentzian peaks used to fit the experimental diffraction peak above 0.1 Å\(^{-1}\). A specific analysis of the structural features of P1 and P2 is given in the electronic supplementary material (Page S15). (b) Contour plot profile of experiment in figure 2a centred around the diffraction peak and selected, corresponding two-dimensional SAXS images. (c) pH-resolved evolution of low-q and mid-q slopes (left ordinate) and peak position (right ordinate). (Online version in colour.)

Two selected scattering curves, at pH 9.38 and 6.54, are given in figure 2b, and they display a similar signal, over an analogous q-range, to that observed for the fully ionized form at basic pH of other microbial glycolipids, sophorolipids, glucolipids and cellobioselipids [33,37,59]: an intense scattering below \(q = 0.01\) Å\(^{-1}\) and a broad oscillation of low intensity above \(q = 0.02\) Å\(^{-1}\). In analogy with the scattering signal of other microbial glycolipids, we qualitatively attribute the low-q scattering to aggregated objects and the oscillation to micellar aggregates. A more detailed description will follow in the next paragraphs.

The pH-driven experiment in figure 2a shows that a phase transition occurs between pH 6.5 and pH 5.5, as indicated by the evolution in the scattering signal in the mid and low-q portion and by the appearance of a broad correlation peak at \(q \approx 0.2\) Å\(^{-1}\) (figures 2a and 3a,b). Below pH 5.5, the signal has evolved into a strong, better defined, scattering contribution at low q and a well-defined diffraction peak at \(q \approx 0.2\) Å\(^{-1}\).

The qualitative evolution of the SAXS profiles over the basic-to-acidic pH range has a similar behaviour to the previously studied microbial glycolipids [33,37,59,60], and it identifies a complex behaviour where at least two phases coexist at the same time. For this reason, the analysis of the SAXS data is not straightforward, and it was approached by two methods: a model-independent slope and peak analyses, presented in figure 3, but also a model-dependent analysis of the form factor at q greater than approximately 0.03 Å\(^{-1}\), presented in the electronic supplementary material, figure S3. The scattering data in the basic-to-neutral pH region hardly show a clear-cut Guinier plateau at low-q, thus preventing a clear-cut attribution of the origin of the low-q
Figure 4. (a–e) Cryo-TEM images recorded on an SLC16:0 sample at $C = 0.25$ wt% and pH 3. Sample is diluted 10 times from a hydrogel prepared at $C = 2.5$ wt% using the pH jump approach (pH 10 → pH 3). Images have been analysed with Fiji software [58]. (Online version in colour.)
scattering. For this reason, we prefer to present the model-independent analysis in the main text and the model-dependent approach in the electronic supplementary material.

In the model-independent analysis, the value of the slope is related to q-dependence of the scattered intensity in the log(I)-log(q) representation of SAXS data. It can be characteristic of well-defined, although simple, morphologies (e.g. spheres, flat sheets, cylinders, sharp interfaces) or more complex fractal systems. This approach does not need any preliminary hypothesis on the morphology, an obvious advantage, although the final interpretation needs complementary data. The model-dependent analysis requires a hypothesis on the morphology, and it eventually provides quantitative pieces of information on the structure (e.g. core–shell structure, size, thickness, density…). For the latter, we base our hypotheses on the data collected on similar systems and on the fact that the low-q scattering could be associated with aggregated objects of ill-defined nature (aggregation of micelles or other morphologies, like platelets, as found elsewhere).

The region below q less than 0.03 Å\(^{-1}\) is informative on the type of morphology at typical scales larger than about 200 Å. The q dependence of the intensity in the log–log scale, referred to as the slope, provides the fractal dimension, Df, a model-independent parameter which is either related to a specific morphology (e.g. –4 for spheres or sharp interfaces, –1 for cylinders, rods and elongated objects, –2 for lamellar and flat objects) or describing the presence of bulk, or surface, fractal objects for intermediate values. In the present work, the SAXS data in figure 3c show two distinct scattering regimes at q less than 0.1 Å\(^{-1}\); for this reason, we analyse the values of the slope in two regions, below q = 0.03 Å\(^{-1}\) (low-q regime) and between 0.03 < q/Å\(^{-1}\) < 0.1 (mid-q regime, figure 3a) for the entire pH range. The values of the slopes against pH for these regimes are reported in figure 3c.

The slope in the low-q region (black circles) is practically contained between –2 and –2.5 throughout the entire pH range. The non-integer values indicate the presence of fractal objects but its proximity to –2 strongly suggests that the morphology of single objects is planar. The slope in the mid-q region (red circles) evolves, on the contrary, between close to –1 (pH 9 to 7) and –3.5 (pH 3). At pH greater than 7, one can reasonably make two hypotheses: coexistence between the micellar phase and a second phase, presumably composed of platelets or bilayer fragments, as it was found for other acidic sophorolipids and glucolipids, or aggregation of the micelles at scale larger than about 600 Å.

Within this framework, one can push further the analysis of the broad oscillation above about 0.1 Å\(^{-1}\) and use a model-dependent approach to estimate the size and structure of the micellar objects. This is shown in the electronic supplementary material, figure S3 and a detailed discussion can also be found. As a short summary, micelles could be described as ellipsoidal objects with an equatorial radius of 13 ± 1.3 Å and a polar dimension of 49 Å ± 4.9 Å above pH 7. Below pH 6, that is upon increasing content of the COOH form of SLC16:0, the equatorial and polar radii, respectively, increase at 26 ± 2.6 Å and 72 ± 7.2 Å (electronic supplementary material, figure S3b). In the present model, we assume a core electron density, constituted by the aliphatic part of SLC16:0, and shell density, constituted by sophorose, the COOH group and water. Considering the coexistence of COOH and COO\(^-\), of which the ratios vary with pH, we suppose that the electron density distribution of the shell is not homogeneous. To rationalize such heterogeneity, we employ a model form factor where the shell thickness is not homogeneous. This model is certainly an approximation, but it is the only one which helps evaluate at best the fluctuation in electron density around the hydrophilic shell associated with the coexistence of the carboxic and carboxylate forms of SLC16:0.

When pH is between 5 and 6, the mid-q slope reaches the value of –2, being the same range as the low-q slope. This behaviour strongly suggests a continuous morphological change from ellipsoidal to flat objects. When the pH falls below 5.5, the cross-section varies by no more than 10 Å, as indicated by the contained evolution of the minimum of the form factor from 0.2 Å\(^{-1}\) to 0.15 Å\(^{-1}\). The cross-section is most likely flat, as indicated by the slope around –2 at low-q but the length increases monotonously much above 150 nm, beyond the detection window of the present SAXS configuration. This is indicated by the slope settled around –3.5 (interface) in the mid-q
and by the lack of a plateau at low-q. The evolution of the SAXS signal below 5.5 is then typical of either anisotropic fibrillation or formation of flat lamellae.

This hypothesis is strongly supported by the anisotropic scattering signal in the two-dimensional SAXS images recorded below pH 6.25 (figure 3b). These are typical of anisotropic structures aligned in the flow direction within the capillary and orthogonal to the direction of the incident beam. The flat anisotropic structures are also characterized by a broad diffraction peak at $q = \text{approximately } 0.20 \, \text{Å}^{-1}$, indicating the formation of a crystalline order. The peak can actually be deconvoluted into two Lorentzian contributions (figure 3a,c), P1, evolving from 0.19 Å$^{-1}$ at pH 5.5 to 0.2 Å$^{-1}$ at pH 3, and P2, evolving from 0.25 Å$^{-1}$ to 0.24 Å$^{-1}$ in the same pH range. A more detailed analysis of the evolution of the structural features (position, full width at half maximum and intensity) of P1 and P2 is given in the electronic supplementary material (Page S15). All in all, as discussed later, the peak centred at about 0.19 Å$^{-1}$ seems to be the most important in the final material, irrespective of the synthesis conditions.

A broad diffraction peak at a comparable q-value is commonly observed for other biosurfactants’ systems. It has been attributed to the typical inter-lipid distance laying in the plane of twisted nanofibres [35] but it could also correspond to the intermembrane repeating distance in liquid crystalline lamellar phases stabilized by repulsive electrostatic interactions [40,64]. We anticipate that the second hypothesis is ruled out both by cryo-TEM arguments and by the insensitivity of the peak position to increasing ionic strength. In conclusion, despite such modest variations, we do not observe any other specific differences (form factor, position of minima, low-q or mid-q slopes) between a faster or slower acidification rate. We then conclude that this parameter has a negligible influence on the structure of SLC16:0 structures within the length scale explored by SAXS.

The nature of the self-assembled structures at acidic pH has been studied by complementary cryo-TEM experiments, presented in figure 4, and electronic supplementary material, figure S5, and recorded at pH 3 and at the concentration of 0.25 wt%, so to avoid overload of the TEM support grid. At low magnification (figure 4a; electronic supplementary material, figure S5a), the sample is massively constituted by ‘infinitely’ long fibres, organized in bundles and often aligned in a given direction, thus explaining the anisotropic signal found in SAXS experiments (figure 3b). A closer look (figure 4b) shows the presence of flat structures, with an average diameter of 8.6 ± 0.9 nm, that is about 10% polydispersity, measured over about 50 different fibres. Interestingly, tilting of the TEM sample holder (+33°, +35° and +40° tested) shows a homogeneous electron density across the fibres’ section, as indicated by the arrows 1 through 3 at 0° (figure 4c) and at +33° (figure 4d). Estimation of the cross-section on a tilted sample confirms an average diameter of 8 nm. This curious result could suggest that the fibres are actually cylinders, or nanotubes. However, these morphologies would provide a −1, or close to −1, slope in the low-q portion of SAXS experiments at pH less than 6 and this is in contradiction with all our SAXS data, showing an approximate −2 slope, instead (figure 3c), and typical for flat structures [35,61,65,66]. The structures in figure 4a–c could then be compatible with a nanobelt, also displaying a −2 slope in SAXS [19], but a difference in the diameter of the cross-section would be expected upon tilting the sample holder [19]. This is not the case here (figure 4c,d). Flat ribbons then constitute the only plausible structure. This hypothesis would agree with the self-assembled morphology of the analogue stearic C18:0 sophorolipid [35], although the typical twisted ribbon structure is not frankly visible in our TEM images. The possible combination of long pitch values, thin cross-section (less than 10 nm), poor contrast and limited resolution of our TEM camera could probably explain such a discrepancy.

Interestingly, cryo-TEM also reveals the presence of large flat crystals, of which the extremities disassemble into fibres. This is illustrated in the close-up of figure 4e and in electronic supplementary material, figure S5b–e. The Fourier transform (panel 2-FT) of region 2 and plot profile 1 of figure 4e show a highly crystalline region with the repeating distance of 2.63 nm. Plot profiles 2 through 4 in the electronic supplementary material, figure S5c–e show additional crystalline regions of a typical interplanar distance of 3.33 nm, 2.75 nm and 2.67 nm. These values are in agreement with positions of P1 (0.19–0.20 Å$^{-1}$ ≡ 3.31–3.14 nm) and P2
Both SAXS and cryo-TEM then suggest the simultaneous existence of several structural polymorphs in terms of lipid organization within the fibres, as explained below.

The d-spacing values found here are all typical for lipid nanoribbons, nanobelts and nanotubes [16,19,67], although the size of the molecule and its packing are of major importance [67–70]. Masuda and co-workers have described in detail the correlation between the position of the strongest reflection in lipid nanotubes formed by unsymmetrical bolaamphiphiles and the type of polymorph and polytype in relation to the estimated length of the molecule in an all-trans configuration [67]. In the case of multiple peaks in a narrow q-range around \( q = 0.2 \text{ Å}^{-1} \), the coexistence of more than one polymorph and polytype is then not to be excluded.

The calculated length of the SLC16:0 is approximately 31 Å, where about 21 Å are related to palmitic acid, the length of which is estimated with the Tanford formula (1.54 + 1.265*\( n \), \( n \) = number of CH\(_2\) groups) [71] and 10 Å is a typical size for a disaccharide [72]. If this calculation shows that the d-spacing is in the order of the molecular length, the coexistence of two main distances centred around 32 Å and 26 Å suggests the presence of both untilted and tilted polymorphs [16,67].

The mechanism of formation of SLC16:0 fibres undergoes a continuous micelle-to-fibre transition, characterized by a morphological evolution of the micelles. When the pH decreases, the hydrophilic shell becomes more homogeneous in the equatorial direction, most likely due to the increasing amount of carboxylic acids. Micelles then fuse together both in the equatorial and polar directions, probably through H-bonding interactions, with a morphological evolution from spheroids to flat objects, which crystallize below the pKa of SL16:0. Crystallization seems to occur on large scale lengths, of a few hundred nanometers, although such structures eventually peel into highly homogeneous fibres, of possible ribbon morphology, of about 8 nm in cross-section with 10% polydispersity. A similar peeling mechanism was reported before for peptide and lipid nanobelts as a function of concentration [19]. If peeling is possible and actually observed by cryo-TEM, it is at the moment unclear whether or not individual fibres can also grow directly from the micelles. This hypothesis cannot be excluded and it is probably concomitant with a peeling mechanism.

Interestingly, the self-assembly of SLC16:0 strongly differs from that of SLC18:0, which undergoes an abrupt micelle-to-ribbon transition with no apparent morphological evolution based on in situ SAXS [37]. Fibrillation was then explained by a classical nucleation and growth mechanism where the micellar phase is seen as a reservoir of matter only, from which molecules diffuse to the nuclei. On the contrary, SLC16:0 micelles seem to behave as growth sites for the nanofibres, as reported for amyloid fibrillation [73]. A possible explanation of such discrepancy will be given in the discussion section.

(b) Hydrogel from SLC16:0 SAFiN

From a minimal concentration of 0.5 wt%, the SLC16:0 solutions spontaneously form a gel when pH is lowered below approximately 6. To confirm that concentration does not modify the fibrillar structure, we perform complementary SAXS on the SLC16:0 hydrogels. The typical SAXS profiles recorded at higher concentrations (up to 2.5 wt%), given in figure 5a, show similar features to the ones recorded at a lower concentration (figure 3a): a \(-2\) slope at \( q < 0.03 \text{ Å}^{-1} \) is associated with a main diffraction peak at \( q = 0.194 \text{ Å}^{-1} \). For these reasons, the concentrations explored do not have any impact on the nature of the self-assembled fibrillar structures. Interestingly, the main diffraction peak for concentrated gels settles around 0.19 Å\(^{-1}\). First of all, this feature tells us that this specific peak position, corresponding to P1 in figure 3, is eventually favoured over P2. Second, it also indicates that its corresponding repeating distance of 32.4 Å, hence the untilted polymorph, compared to the expected length of SLC16:0, is the preferred arrangement of SLC16:0 molecules within the fibre. Interestingly, the peak at double its value, \( q = 0.388 \text{ Å}^{-1} \), suggests a lamellar order of SLC16:0 within the ribbon plane.
Salt (NaCl) is generally generated during the pH jump approach. In the case of SAFiN obtained from sophorolids, NaCl was shown to influence the homogeneity of the fibres’ cross-section [46]. In the case of lamellar liquid crystalline systems, including hydrogels, where electrostatic repulsions prevent the lamellar structures from collapsing, salt is responsible for screening the long-range electrostatic forces. This is experimentally followed by a shift of the lamellar peak towards higher q-values [40,74]. In the present work, the amount of salt generated during the pH jump process for a 0.5 wt% SLC16:0 solution is evaluated to 7.9 mM and 17.2 mM when the 0.5 M and 0.1 M HCl solutions are respectively employed (electronic supplementary material, table S1). These values are low and, considering the similarities among the structural parameters evaluated with the pH-dependent in situ SAXS experiments, one can safely state that NaCl does not have a major impact. However, to dissipate any doubt, we have performed additional SAXS experiments on hydrogel samples to which NaCl is deliberately added. Data presented in the electronic supplementary material, figure S6 show a substantial similarity among all scattering profiles in the entire q-range explored, thus confirming that NaCl does not have any unexpected structural effects on the SLC16:0 fibres up to 250 mM NaCl. It is also interesting to note that the diffraction peak at $q = 0.19 \, \text{Å}^{-1}$ is insensitive to ionic strength, which excludes inter-fibres repulsive electrostatic interactions and confirms the attribution of this peak to an intra-fibre long-range order.

The viscoelastic properties presented below are studied at $T = 20°C$ on a series of samples freshly prepared in water using the pH jump approach. Each sample has been systematically vortexed to remove its shear history, before loading it in a plate-plate geometry. The frequency-sweep experiments recorded in the linear viscoelastic regime (LVR) are performed after 30 min from loading. Frequency-sweep experiments are followed by strain sweep (electronic supplementary material, figure S7) and step-strain experiments (figure 6; electronic supplementary material, figure S8). The latter is employed to evaluate the recovery potential of SLC16:0 hydrogels after applying shear stress with an amplitude out of the LVR. Studying the rheological behaviour of the gels after 30 min from loading has the advantage of limiting the dehydration, although its main drawback consists of the fact that the mechanical properties could still evolve in time. If our choice may impact the absolute values of $G'$, it does not influence the general trends and relative comparison among the samples at a given time.

Figure 5b shows the typical frequency-sweep experiments recorded in the LVR ($\gamma = 0.1\%$; electronic supplementary material, figure S7) for a set of samples at 2.5 wt% and pH between 3 and 6, while figure 5c reports the concentration-dependent $G'$ values recorded at pH 5. $G'$ and $G''$ curves are parallel with $G' > G''$ in the entire frequency range, thus demonstrating the

![Figure 5](https://example.com/figure5.png)

**Figure 5.** SAXS experiments recorded on SLC16:0 hydrogels at $C = 1\%$ and 2.5 wt%, both at pH 5. (a) Frequency-sweep experiments recorded in the linear viscoelastic regime (LVR) ($\gamma = 0.1\%$, $T = 20°C$) for a series of SLC16:0 samples prepared at $C = 2.5\%$ and various pH. (c) Concentration dependency of $G'$ measured in the LVR after 30 min from sample loading ($\omega = 6.28 \, \text{rad} \, \text{s}^{-1}$, $\gamma = 0.1\%$, $T = 20°C$). (Online version in colour.)
presence of a gel for all pH. The sample at a concentration as low as 0.5 wt% has an elastic modulus, $G'$, of about 45 Pa, thus showing that SLC16:0 forms hydrogels at concentrations even below 1 wt%. The elastic modulus scales linearly with concentration (figure 5c), with a slope of $3.14 \pm 0.03$. This value lies between typical exponents found in weakly aggregating polymer colloids (3.7 and 4.5) [75] and entangled polymer, biopolymer and fibrillary hydrogels (approx. 2.3) [44,50,53,76–80]. Such a discrepancy was observed before for similar molecules, and it is correlated to the gel equilibration time [44]. The storage modulus measured at concentrations between 2.5 wt% and 5 wt%, and pH 5 varies between 4 kPa and 40 kPa, a range which is consistent with other SAFiN hydrogels [54,80,81], including sophorolipids [50].

pH has a crucial effect on the elastic properties (figure 5b). In the vicinity of the micellar-to-fibre phase transition at pH 6, the elastic modulus at 2.5 wt% is in the order of 1 kPa, while it increases at 7 kPa at pH 3 for the same concentration. As far as the ionic strength is concerned, tested between 0 mM and 250 mM, we did not observe any impact on the SLC16:0 mechanical properties. The strong impact of pH is most likely related to the massive fibrillation region below the pKa. Closer to the pKa, the content of carboxylate molecules and the volume fraction of micelles is still too high to obtain the strongest hydrogels. However, upon pH decrease, the content of carboxylic molecules is maximized, as well as the volume fraction of fibres. Such a strong connection between final pH and mechanical properties in SAFiN constituted of LMWG is not uncommon and it was described before for FMOC peptides [53,54]. However, this mechanism strongly differs from the one found for C18:0 sophorolipids, for which the final pH has no impact on the mechanical properties, which are instead controlled by the pH change rate [50]. Such difference will be commented on further.

After application of an oscillatory strain with amplitude out of the LVR, SLC16:0 hydrogels show a time-dependent recovery of their elastic modulus, typical of thixotropic gels. The recovery properties of the gels are tested against oscillatory strain amplitude applied outside the LVR ($\gamma = 100\%$). Over 120 min, SLC16:0 hydrogels undergo a series of harsh step-strain cycles, of which six consecutive ones were designed with $\gamma = 100\%$ for 5 min followed by 10 min recovery in the LVR. Figure 6a; electronic supplementary material, figure S8 show a series of step-strain

Figure 6. Study of the recovery properties of SLC16:0 hydrogels ($C = 2.5 \text{ wt} \%, \text{pH} 5, \omega = 6.28 \text{ rad s}^{-1}$) after a series of imposed oscillatory shear strain with amplitude out of the linear viscoelastic domain. (a) Evolution of $G'$ (full grey circles) and $G''$ (empty red circles) in a typical step-strain experiment performed with an initial shear strain (logarithmic evolution, $4 \times 10^{-3} < \gamma$ less than 100%) followed by a recovery of 10 min at $\gamma = 0.1\%$. Six cycles of step-strain experiments then follow: the first cycle consists of applying a strain of $\gamma = 100\%$ for 30 s followed by a recovery of 10 min; cycles from 2 to 6 consist of applying a strain of $\gamma = 100\%$ for 5 min followed by a recovery of 10 min. (b) Specific highlight between the second and third cycle showing the region concerned with the recovery time of 20 s. (Online version in colour.)
experiments performed on SLC16:0 hydrogels at pH 3, 5 and 6 and C = 2.5 wt%. The restructuring process, which lasts over several minutes, is quantified by comparing the % of $G'$ recovery after 20 s and 5 min at the $N^{th}$ plateau with respect to the $N - 1$ plateau and with respect to the zero-plateau ($N = 0$). This is illustrated in figure 6a,b for the system at pH 5 while the % of recovery is given in figure 7 for all pH values.

Before beginning the first cycle, the value of $G'$ at plateau at pH 5 is at about 6900 Pa and $G' >> G''$ (more than one decade). A strain amplitude at $\gamma = 100\%$ is progressively applied, during which $G'' >> G'$, confirming the complete destructuring of the gel. When the strain is set again in the LVR, $G' >> G''$ within 5 s, with a recovery of 65% after 20 s and 83% after 10 min. Figure 6a also shows that longer (5 min) and repeated (six times) destructuring actions do not have a significant negative impact on the hydrogel’s mechanical properties. This is highlighted in figure 6b for the second step-strain action and on the evolution of the % of recovery averaged over six cycles, shown in figure 7. Whichever the pH in the sample, the recovery with respect to the initial, $N = 0$, plateau (red columns) settles between 60% and 70% after 20 s and between 80% and 90% after 10 min. The value found above 100% at pH 6 after 10 min is probably due to the continuous evolution of this gel. Figure 7 also shows that the recovery after 20 s with respect to the $N - 1$ plateau (grey columns) reaches an average of 78% at pH 3 and pH 5 and 62% at pH 6. After 10 min, the recovery is 100% for all pH values.

4. Discussion

The combination of pH-resolved in situ SAXS and ex situ cryo-TEM shows that a micelle-to-fibre transition occurs in the vicinity of the pKa of SLC16:0. Fitting of the SAXS profiles using a core–shell ellipsoid of revolution form factor suggests that the equatorial shell region of the micelles becomes more and more occupied by the COOH groups when pH approaches 7, below which a morphological change occurs from spheroidal to flat objects. Redistribution of the COOH groups in the equatorial region could enhance lateral H-bonding interactions between adjacent micelles and drive the formation of flat fibres, which eventually grow in the polar, longitudinal, direction. Growth in the equatorial, lateral, direction is not excluded, although, in this case, individual fibres eventually peel off, as shown by cryo-TEM. Although the crystalline packing of SLC16:0 within the fibres can adopt several polymorphs, it seems that the untilted arrangement, with a diffraction peak at about 0.19 Å$^{-1}$, constitutes the equilibrium distance at the end of the fibrillation.
process. However, neither the exact polymorph (symmetrical or unsymmetrical) nor the exact polytype (head-to-head, head-to-tail) in terms of the respective bolaamphiphile arrangement within the fibres, as intended by Masuda et al. [67], is known with exactitude at the moment. Enhanced fibrillation below pH 6 is responsible for the spontaneous formation of hydrogels with very good mechanical properties, displaying elastic moduli above 10 kPa. The most probable micelle-to-fibre-to hydrogel mechanism is summarized in figure 8.

The fibre phase found in the SLC16:0 system at pH less than 6 seems to be at equilibrium at room temperature. The fibres’ cross-section diameter is highly homogeneous (10% polydispersity), compared to the corresponding SLC18:0 nanoribbons. The latter, which differ only in two methylene groups, fibrillate below pH 7.4, but the distribution of the fibres’ diameters was shown to vary between 8–10 nm and up to 20–30 nm [35]. Salt was shown to play an important role in the size dispersion; the lower the salt content, the narrower the size distribution [46]. In the meantime, salt was also observed to favour spherulite formation over homogeneous fibres and precipitation over gel formation for the same C18:0 sophorolipid molecule. Overall, the hydrogels’ mechanical properties of C18:0 sophorolipids were strongly affected by the rate of pH variation [50]. The strong sensitivity of the mechanical properties of SLC18:0 fibrillar hydrogels to pH change rate and ionic strength are most likely correlated to its abrupt micelle-to-fibre transition. pH-resolved in situ SAXS experiments have shown that micelles do not change their morphology into fibres upon increasing the COOH content at lower pH, but it was supposed that fibres nucleate in solution when an excess of carboxylic acid C18:0 sophorolipids are formed, whereas micelles only constitute a reservoir of matter. The fibrillation (nucleation and growth) process is then controlled by diffusion of the acidic C18:0 sophorolipids from the micellar environment to the fibres [37,50]. Diffusion-controlled fibrillation is well known for temperature-stimulated LMWG, for which it was shown that the rate of temperature variation controls the molecular solubility equilibrium. Fast temperature (or pH, in the case of SLC18:0) variation favours supersaturation of the insoluble component, thus driving spherulite formation and, eventually, poor mechanical properties of the hydrogel. On the contrary, slow temperature (or pH) variation favours equilibrium between soluble and insoluble species, thus minimizing the mismatch nucleation energy and, consequently, spherulite content. In the latter case, more homogeneous fibres and stronger gels are eventually formed [51,82].
The fibrillation mechanism seems to be completely different in the case of SLC16:0, which shows a continuous morphological evolution between micelles and fibre upon acidification of the medium (figures 2 and 3). In this case, fibrillation probably does not follow the nucleation and growth process controlled by the diffusion of the molecular SLC16:0 species in solution. In this case, hydrogel stability and strength do not depend on the rate at which fibrillation occurs, but rather on the overall volume fraction of the fibres in solution. If one assumes that the fibres are most likely composed of the carboxylic acid derivative of SLC16:0 (highly possible but not demonstrated at present), their volume fraction only depends on the final pH. This is experimentally verified through pH-dependent rheology experiments (figure 5b). These properties are consistent with what is classically reported for FMOC-based peptides [53,54].

Why do SLC16:0 and SLC18:0 sophorolipids, which only differ in two methylene groups, undergo a similar micelle-to-fibre transition with decreasing pH, but result in hydrogels with different sensitivity to pH? The answer could be found in the different melting-crystallization profiles between the C18:0 and the C16:0 derivatives of sophorolipids (electronic supplementary material, figure S9a). The former shows a glass transition temperature, T_g, at about 56°C and two well-defined first-order transitions at 77°C upon heating (fusion) and cooling (crystallization). This value is not much different than the melting temperature of stearic acid (69°C). On the other side, SLC16:0 shows no first-order transition in the 10°C to 110°C temperature range but only an ill-defined second-order transition between 85°C and 100°C (electronic supplementary material, figure S9b). Considering the fact that the melting temperature of palmitic acid is 63°C, it is highly unlikely to expect first-order transitions (fusion/crystallization) of SLC16:0 above 110°C. This suggests that acidic SLC16:0 molecules are in a more fluid state at room temperature, a fact which could explain the continuous micelle-to-fibre transition rather than abrupt crystallization.

SLC16:0 forms fibrillar hydrogels, as observed for other synthetic [25,83] and microbial [44,48,50] glycolipids. SLC16:0 hydrogels have very interesting rheological properties, which can probably be explained by the good homogeneity of the fibrillar network. They can reach elastic moduli above 10 kPa below 3% and have thixotropic properties with fast recovery. For the latter, they show an average of 60% and 80% recovery, respectively, after 20 s and 5 min, of their initial storage modulus, while they show a 80% and 100% recovery, respectively, after 20 s and 5 min, of their storage modulus immediately preceding the applied stress. Thixotropic hydrogels with fast and complete recovery of the viscoelastic properties are interesting systems, especially for biomedical applications [27]. Specific peptide amphiphile hydrogels in the presence of HCl were submitted to a similar step-strain cycle as done in this work (5 min at 100% strain) and have shown recovery of 90% of the initial elastic modulus within 10 min [80], while the dipeptide 2NapFF was shown to recover 100% of its initial modulus after the first shear deformation and an average of 58% after five cycles [53]. In this regard, peptide amphiphiles are considered as one of the most performing LMWG gelators in the literature, both in terms of the absolute value of the elastic moduli and recovery after shear. The performances of SLC16:0 hydrogels can certainly be compared to the ones of this class of molecules [84–86].

If homogeneous fibrillation, absence of spherulites and low polydispersity of the fibres’ cross-section can explain the hydrogel’s properties, some open questions still remain. In particular, it could be interesting to know whether or not the fibre morphology (twisted, helical, tubular, belt-like...) has any impact on the macroscopic rheological properties. The extensive amount of work published in the field of physical self-assembled gels broadly addresses the structure as ‘fibrillar’, often disregarding their morphology or the relationship between the cross-section size distribution and elastic moduli. This is understandable because low-molecular weight gelators rarely self-assemble into a homogeneous well-defined structure with monodisperse diameter and length, but they are rather characterized by a complex network of poorly defined fibres with tip and/or side branching, greatly affecting the mechanical properties [51,81,87]. In addition, the mechanisms of gel destructuring and recovery upon application of a mechanical stress are far from being trivial and may actually depend on the fibre aggregation state but also on fibres’ breaking events. For instance, common sense suggests that the application of shear stress to a SAFiN gel results in fibre alignment, similarly to what we observe in this work during the
pH-resolved *in situ* study (figure 3b). However, Pochan et al. have actually shown by means of rheo-SANS experiments not a fibrillar alignment but rather the formation of fractured fibrillar domains allowing the gel to flow, although it was not clear whether or not the initial structure of the gel was constituted by a spherulitic, or fibrous, network. This is an important detail, which has a tremendous importance in the macroscopic behaviour of the gel under shear flow and recovery [84].

Unfortunately, fibrillar gels with very interesting recovery rates [84–86] are only partially characterized from a morphological perspective. Techniques like TEM or SEM are commonly used to characterize the fibrillar morphology. Still, these have several drawbacks: both of them require the drying of the sample and its study under vacuum, conditions which may strongly affect the real structure in solution; SEM only provides a surface survey but not an internal insight of the fibres. In addition, standard SEM rarely have the required resolution to probe local structures in the sub-10 nm range. Finally, in the absence of complementary techniques like cryo-TEM, even small-angle neutron or X-ray scattering do not provide a complete structural resolution, because fibrillar systems generally show common features characterized by a –2 slope in the low-q regime and a structure peak above 0.1 Å⁻¹.

### 5. Conclusion

This work shows that palmitic acid C16:0 sophorolipids spontaneously self-assemble into micelles at pH above 7 and into fibres at pH below 6. pH-resolved *in situ* SAXS experiments show a structural continuity from the micelles to the fibre morphology, differently than what is found for the congener C18:0 sophorolipids, which otherwise displays an abrupt transition between similar structures. The transition occurs below the pKa (7) and it seems to be driven by a redistribution of the carboxylic acids groups of SLC16:0 in the equatorial hydrophilic shell region of the micelles. The lack of first-order, melting or crystallization, transition below 10°C and 110°C on the solid powder supports the softer nature of SLC16:0 structures at room temperature and the possible absence of a nucleation and growth fibrillation mechanism when lowering pH, contrary to what was found with the C18:0 sophorolipid congener. The lack of spherulitic structures in cryo-TEM also supports this hypothesis. The advantage of the structural continuity between micelles and fibres is put in evidence by the homogeneity in terms of cross-sections of the fibres, which have an average diameter of 8.6 ± 0.9 nm and infinite length.

These structural features directly impact the elastic properties of the material at concentrations above 0.5 wt%, when fibrillar hydrogels form spontaneously below pH 6. The elastic modulus reaches values as high as $G' = 40 \text{kPa}$ at 5 wt% at pH 5, with final pH having a substantial impact: at 2.5 wt%, the elastic modulus increases by a factor of 5 from pH 6 to pH 3. This behaviour is in agreement with FMOC peptides-based self-assembled hydrogels and in disagreement with C18:0 sophorolipid fibrillar hydrogels. The origin of such discrepancy is attributed to the different fibrillation mechanism. Finally, SLC16:0 hydrogels show thixotropic properties with fast recovery after being submitted to large oscillatory strain amplitudes for as long as 5 min. It is shown that, after removing the mechanical constraint, 80% of their elastic modulus is recovered after 20 s and 100% after 10 min, when compared to the $G'$ values immediately preceding the constraint.

In summary, considering the ease of their synthesis procedure, strength, stability and fast recovery, SLC16:0 hydrogels have high potential for the development of sophorolipid-based soft fluids and materials.

**Data accessibility.** This manuscript contains new data. The standard in the field of soft matter/self-assembly is to present processed data. We present them both in the main text and in electronic supplementary material. For review purposes, the raw data can be accessed via the following link: https://doi.org/10.5281/zenodo.4323309.

**Authors’ contributions.** N.B. designed and performed the experiments, analysed the data and wrote the manuscript. G.B.M performed part of the rheology experiments. P.L.G. performed the cryo-TEM observations. N.C. and J.P. assisted in SAX experiments. M.D.G, S.R and W.S. produced the SLC16:0 compound.

**Competing interests.** We declare we have no competing interests.
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