Cryo-TEM and AFM for the characterization of vesicle-like nanoparticle dispersions and self-assembled supramolecular fatty-acid-based structures: a few examples.

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1. Introduction

For some decades now, the development of advanced materials with novel properties has been a research field of growing interest. The production of stable systems in liquid and aqueous solutions can provide three-dimensional networks for the design of original structures with properties adapted to specific advanced applications.

Assemblies formed in an aqueous solution extracted from renewable resources are of a particular interest when they have competitive functional properties. Supramolecular assemblies formed with biomolecules from natural sources could constitute new ecological surfactants to replace petroleum-based products [1, 2]. Other recyclable polymeric micelle-based systems can also lead to the formation of mesoporous materials [3].

However, the design of new materials formed by assembling numerous organic compounds requires the parallel development of an ability to characterize their considerable complexity in detail. In particular, when studying systems formulated with lipids or biopolymers (proteins, polysaccharides), microscopy techniques are widely employed for this purpose and constitute a group of complementary tools that can be used separately or in correlation [4].

For all these techniques, the general principle is based on a close interaction between a probe and the atoms in the sample under investigation. This probe may be a light source, for optical and fluorescent microscopy techniques, a solid tip in atomic force microscopy (AFM), or an electron beam for electron microscopy, in the scanning or transmission modes. For each type of microscope, numerous analytical modes have been developed to supply complementary information on structure and chemical composition.

The diagram in Fig. 1 tries to summarise some of the electron microscopy techniques applicable at present to sorting information on morphology, size and structure from an organic sample. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) can be used to solve structures by high resolution imaging, electron diffraction (available for crystals or semi-crystals) or electron backscatter diffraction (EBSD). Chemical compositions can be determined by energy dispersive X-ray analysis (EDX) or cathodoluminescence coupled with SEM or TEM, or by electron energy-loss spectroscopy (EELS) coupled with TEM. Electron microscopes can operate under both ambient and cryogenic conditions and using different imaging modes: transmission imaging in SEM and, inversely, scanning mode in TEM, bright field or dark field imaging in TEM and secondary electron or backscattering imaging in SEM. A similar diagram could also be drawn to summarise the operability of AFM in numerous modes in terms of both sample characteristics and the data required.

Conventional TEM and SEM observations, combined with the use of staining, have often demonstrated their efficiency in characterizing the polyphasic composition of organic nanoparticles using different sample preparation and staining strategies [5, 6]. The use of electron microscopy in conventional or more advanced modes, such as the cryogenic mode, together with other microscopy techniques (e.g. AFM), or physicochemical techniques (e.g. light and neutron scattering) constitutes a powerful tool for the investigation of soft-matter-based assemblies at the nanometer scale [7-11].
When using TEM, it is important to consider several features of a sample before selecting the most appropriate technique for its preparation and observation. Sample thickness may be a crucial criterion. If the dimensions of the object under investigation are within the nanometer range (up to 100-200 nm depending on its density), direct observation is possible because such thickness remains compatible with transmission of the electron beam.

If the sample is thicker and contains some water (as is often the case for cells, tissues, colloids and gels), it is important to apply specific preparation protocols which include chemical or physical fixation, dehydration, resin inclusion and ultramicrotomy, so as to obtain ultrathin sections across the thick sample (thinner than 200 nm). When a sample is produced in a dehydrated state, it can be embedded directly in resin and then cut by ultramicrotomy. In this case, the presence of water in the structure or as the dispersion liquid (as with all colloids) is an essential criterion to be considered, as well as thickness.

The nature of a sample is also crucial to estimating the risk of e-beam damage, i.e. the electron radiation effect that may occur in the TEM chamber [12]. Electron scattering across the sample causes elastic or inelastic collisions. The kinetic energy absorbed by the sample may create local heating, leading to structural damage or loss of mass. These effects are more pronounced in the case of organic and hydrated samples [13].

The development of cryogenic preparation methods adapted to hydrated samples of differing thickness (high pressure freezing, freeze fractures, gradual lowering of the inclusion temperature, cryosubstitution, cryoultramicrotomy) have considerably improved our ability to analyse objects with varied morphology, size and nature, while maintaining optimum conditions that will preserve the initial structure [14-18]. In particular, and although the vitrification of thin films was a forerunner of other cryo-methods, it remains remarkably useful when characterising numerous aqueous dispersions of nanometer-sized objects [19, 20].

The advantage of vitreous thin films is that they can be observed at the highest possible resolution using a TEM equipped with a field emission gun and operated with an acceleration voltage of 200 or 300 kV. These conditions normally enable determination of the macromolecular structure of protein complexes. However, vitreous films can also be observed using TEM operated at a lower tension acceleration (80 – 120 kV) and equipped with a thermionic filament (tungsten or LaB₆), typical of those available in microscopy facilities specialized in cell and tissue analyses. Resolution remains sufficient to characterise most of the nanoparticles targeted by current research projects in the soft-matter and biological fields.

Our aim here to demonstrate the considerable usefulness of cryo-TEM of vitreous thin films operated at 80 kV to clarifying the complex morphology of lipid-based nano-objects and supramolecular assemblies. We also discuss its complementarity with conventional negative staining TEM techniques and atomic force microscopy (AFM) in order to characterize vesicle-like nanoparticles and fatty acid-based supramolecular assemblies.

2. Experimental Part (general procedures) [22-24]

2.1 Sample preparation methods

Transmission electron microscopy (TEM).

One 50 μl drop of an aqueous dispersion specimen was first of all deposited on a carbon film-coated TEM copper grid (Quantifoil, Germany) and allowed to dry in air for a few seconds. The surface of the carbon film had previously been glow-discharged by exposure under plasma to render it hydrophilic. The sample was then negatively stained with uranyl
acetate (Merck, Germany). The sample-coated TEM grid was then placed successively on a drop of an aqueous solution of uranyl acetate (1% w/w) and a drop of distilled water. The grid was then air-dried before its insertion in the electron microscope.

**Cryo-Transmission electron microscopy (cryo-TEM) of vitreous thin films.**

Specimens for the cryo-TEM observation of vitreous thin films were prepared using a “cryoplunge” cryo-fixation device (Gatan, USA) which enables rapid immersion in a cryogenic fluid (liquid ethane or propane). A diagram summarising the principles of this method is shown in Fig. 2.

A typical support is a TEM copper grid (3 mm in diameter) recovered with a holey-type carbon film that contains regularly or randomly dispersed holes in which the aqueous dispersion is placed (Ted Pella Inc., USA). A glow-discharged treatment is applied to the carbon film to render it hydrophilic, before the deposition of a 10 μl microdrop of the dispersion that is reduced in thickness by blotting with Whatman paper (cf. Fig. 2a). The liquid film is placed in a humidity-controlled chamber (which maintains a relative humidity of 97-99%) to prevent its evaporation until the TEM grid (previously fixed on a holder under a pressure of 6-8 bars), is projected into a cold liquid (cf. Fig. 2b) to enable the most efficient absorption of sample heat. This liquid is often ethane or propane that has previously liquefied from gas by placing it in contact with the cold walls of a metal goblet plunged into liquid nitrogen. The direct use of liquid nitrogen is usually avoided because heat transfer is restricted by the existence of an intermediate thin layer of gas (the Leidenfrost effect). 5 ml of liquid ethane, maintained at -180°C, are sufficient for sample preparation. Below this temperature, liquid ethane solidifies (at around 182.5°C) and while above this temperature, water crystallizes (at around -135°C).

Finally, the objects of interest in the aqueous dispersion were embedded in the vitreous water film through the holes in the carbon film support (cf. Fig. 2c). The TEM grids were mounted on a Gatan 910 liquid nitrogen-cooled sample holder (Gatan, USA) equipped with a liquid nitrogen reservoir, and then transferred to the microscope using a CT-3500-cryotransfer system (Gatan, USA) designed to ensure that the frozen state of the sample is uninterrupted. Figure 2d shows a typical TEM image of partly-folded a vitreous aqueous film.

**Freeze fracture of gels.**

Small pieces of gels were cut and placed in specific supports for high-pressure freezing using a HPM100 device (Leica Microsystems, Wetzlar, Germany). The samples were then freeze-fractured using a Balzers BAF 400T (Balzers, Liechtenstein) at -150°C under 1.8 mBar of vacuum before sputtering the surfaces with platinum/carbon. Replicas were then obtained by washing the samples in water and organic solvents (ethanol, chloroform and methanol) and detected on TEM grids before TEM examination.

**Atomic force microscopy (AFM).**

Two types of support (glass or mica) were used, depending on the ability of the sample to attach itself to the surface. The mica sheets (10 mm x 10 mm, Agar) were freshly cleaved before use. The glass support (square slides of 22 mm x 22 mm, Menzel-Glaser) were washed under sonication in a Twin surfactant solution (0.02 %), rinsed with acetone and dried under an argon flux. A 10 μl droplet of the aqueous dispersion was then spread on the support and allowed to incubate for one minute before rinsing the surface with Millipore water to eliminate the unfixed fraction and then drying under argon.

### 2.2 Microscopy

**TEM and cryo-TEM.**

All samples were observed using a JEM 1230 cryo-microscope (JEOL, Japan) operated at 80 kV and equipped with a LaB₆ filament. For cryo-TEM experiments, the microscope was operated under low-dose conditions (<10 e⁻/Å²) while maintaining the sample at -178°C as previous studies had shown that these conditions are appropriate for the characterisation of nanometer-sized objects in vitreous thin films with an adequate contrast and signal/noise ratio. The micrographs were recorded on a Gatan 1.35k x 1.04k x 12 bit ES500W CCD camera. Energy electron loss spectroscopy and imaging (EELS/EFTEM) analysis was performed with a Gatan Imaging Filter (GIF 2001, Gatan, Sunnyvale CA) equipped with a 1k x 1k x 12 bit Multiscan CCD camera.

**AFM.**

AFM images were acquired either in air using a Park Scientific Instrument Autoprobe CP (Sunnyvale, CA) or in liquid using a Bruker Instruments Bioscope (Santa Barbara, CA). The AFM images were recorded in the non-contact mode using conventional pyramidal silicon nitride cantilevers obtained from Digital Instruments (Santa Barbara, CA). All non-contact mode images (both error-signal and height imaging modes) were acquired at the lowest possible stable scanning force (less than 10 nN) with a line scan frequency of 1 Hz.
**Image analysis.**

For TEM: ImageJ software (Research Services Branch NIMH & NINDS) was used to determine particle size distributions and to apply contrast enhancement to the cryo-TEM images [25]. For AFM: all AFM images were processed and analysed using the WSXM4.0 software program [26].

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**Fig. 2** Diagram of the different steps involved in preparing a vitreous thin film adapted for cryo-TEM observation. (a) A microdrop of a liquid dispersion is placed on a carbon holey-film recovered on the TEM grid. The grid is held in place with tweezers fixed on a holder connected to an air-pressure flow; (b) while applying an air-flow pressure of around 8 Bar, the grid is quickly plunged into liquid ethane cooled to −178°C using liquid nitrogen; (c) representation of the vitreous thin film retained in the holes of the holey-type carbon membrane laid on the TEM grid; (d)-(d’) Cryo-TEM images showing the vitreous thin film on the holey carbon membrane (d) and partly folded (d’).

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**3. Results and Discussion**

3.1 Cryo-Transmission electron microscopy of vitreous thin films

Cryo-electron microscopy includes a broad range of sample preparation and observation techniques which apply the benefits of low temperature in terms of keeping samples free of artefacts and damage. The main feature is the use of cryogenic conditions during one or more experimental steps. A cryo-observation can be achieved using a liquid nitrogen (or helium) cooling holder (TEM) or a Peltier cooling stage (SEM). Cryo-preparation is often used to harden a soft material (for example, an elastomer) or hydrated sample (cells, tissues) so that it can be cut in ultrathin slices (thinner than 100 nm) while ensure that the sample remains as native as possible. Cryo-preparation methods may differ in terms of their cooling rates and degree of vitrification free from ice crystals: cryo-plunge, metal-mirror, high pressure freezing, jet or spray freezing, cryofracture, freeze-drying, Tokoyasu cryosectioning, cryoultramicrotomy. Cryo-microscopy then refers to a preparation obtained under low temperature conditions (mainly at the temperature of liquid nitrogen) followed by observations in ambient conditions, or inversely, but is much more efficient when all preparation and observation steps are performed at a low temperature.

One time again, sample thickness is an essential criterion in TEM when selecting the appropriate cryo-TEM method. When the size of particles in the aqueous dispersion is compatible with transmission of the electron beam (which also depends on the acceleration voltage and the specific density of the object), it is possible to apply the cryo-TEM method directly by plunging it into a cold liquid (liquid ethane, propane) [27], as is also described in the experimental part of this paper.

Various artefacts may appear on a cryo-TEM image and they must be detected (cf. Fig. 3). A typical vitreous thin film should be flat and homogeneous across all the holes in the holey carbon film [28]. In some cases, film thickness may vary because of partial drying of the liquid water before freezing or to some liquid ethane remaining on the surface of the solid water film after freezing/removal from the liquid ethane containers (see Fig. 3a). To prevent this, any liquid ethane should be removed by carefully placing the frozen sample in contact with a filter paper maintained at liquid nitrogen temperature, close to the liquid ethane container.

Water (or a buffer) remains the fluid most frequently used for the vitrification process. Organic solvents may freeze under the combined effects of cold and pressure. Many non-polar solvents (pentane, cyclohexane, dioxane, etc.) are often soluble in liquid alkanes (ethane, propane) and may easily be damaged by the e-beam (cf. Fig. 3b). However, several organic solvents have been efficiently frozen in liquid nitrogen in their vitreous state, such as toluene [29, 30], hexane [31] or diethylphthalate [32].

Some restrictions may affect this technique when freezing large objects (in the micron range) and they may limit the vitrification of a regular film of water (Fig. 3.c); a thicker layer of water may also be retained around the object, resulting in a loss of resolution (Fig. 3.d). The vitreous thin films thus obtained must also be free of artefacts arising from...
from the condensation of ambient humidity or a partial recrystallization of amorphous water in the different cubic or hexagonal phases (see Fig. 3e-h).

3.2 Characterization of dispersions of monolayered or multilayered lipid-based self-assemblies

Cryo-TEM of vitreous thin films remains a method of choice for the characterisation of lipid-based self-assemblies such as vesicles [33], micelles [34] and liposomes [16]. Lipid nano-assemblies have long been developed as nanovectors because of their unique ability to encapsulate and deliver bioactive molecules for therapeutic applications [35].

Figure 4 shows some examples of typical structures that have been characterized using cryo-TEM operated at 80 kV. Under some conditions, fatty acids extracted from milk or egg yolk products spontaneously self-assemble as colloidal vesicles. Their relative instability limits any extensive study of size and morphology using microscopy. Few methods are able to retain the water content at the same time as an ability to image lipid bilayers with sufficient resolution to distinguish each layer. Cryo-TEM of vitreous thin films is a technique that can achieve detailed observations with a minimum of structural alterations.

Onion-like multi-lamellar vesicles (Fig. 4a) and hybrid nanostructures containing a dense lipid particle attached to an empty vesicle (Fig. 4b) have been prepared from egg yolk lipoproteins (LDL) as vitreous thin films and then investigated using cryo-TEM in an attempt to elucidate their structures [36]. Another example is the characterization of a bimodal dispersion of sub-micrometer sized vesicles and small uni-lamellar vesicles (SUV) of DOPC:DOPG:CHOL [37], which were prepared by vitrifying their corresponding aqueous dispersions (see Fig. 4c and 4d). In the case of SUV particles, cryo-TEM analysis revealed the self-assembly of a relatively homogeneous population of nano-sized vesicles with a diameter of around 16 nm (Fig. 4d).

Freeze fracturing is often suggested as an alternative method for the preparation of vitreous thin films in order to study vesicles and larger objects with different morphologies and sizes [38-41]. This technique is an efficient way to generate images of viscous systems from which vitreous thin films cannot be prepared as the method is restricted to fluid systems. As an illustration, Figures 4e and 4f show images of self-assembled vesicles of fatty acid salts which form viscous gels at room temperature, obtained after the freeze fracturing of high pressure-frozen gel pieces. To enhance the visualization of vesicles, a platinum/carbon shadow has been applied. A recent study of the self-assembling structures produced between fatty acids of with different chain lengths and lysine or guanidine salts demonstrated the possibility of producing new vesicles of varying diameters [42-43]. The existence of vesicles has also been proved in gels formulated using various backbone length fatty acids in the presence of guanidinium counter-ions (respectively,
C_{16}-palmitic acid salts of guanidinium and C_{18}-stearic acid salts of tetramethylguanidinium, as has the influence of the type of counter-ion on vesicle size [44].

Figure 4 shows cryo-TEM observations of a vitreous thin film with negative staining TEM, using small multi-lamellar phospholipid-based vesicles (Fig. 5a-b) [45, 46], and of a highly complex morphology based on multilayered structures generated by the self-assembly of myristic acid in the presence of organosilane counter-ions (Fig. 5c-e) [47].

The negative staining technique is often applied to the study of numerous biological systems because of its rapid application [48]. Standard negative stains are aqueous solutions of uranyl salts, ammonium molybdate and phosphotungstic acid [49]. Before applying a negative stain, its pH value must be determined and then adjusted if necessary adjusted to the correct value as this will mainly influence charged lipids (for example, the pH of 1% phosphotungstic acid solution is close to 7.2 whereas that of 1% uranyl acetate solution is around 4.6). However, numerous artefacts may arise from either the evaporation of water that damages hydrated samples or different interactions that can occur between the lipid structures and the stain [50]. In general, the type of lipid (with a saturated or unsaturated backbone, neutral or with a cationic or anionic charge) influences the resistance of the structure and the degree of damage that may occur under drying. Lipid type is also crucial to the efficiency of staining.

By comparing the Cryo-TEM images in Fig. 5a of L-alpha-phosphatidylcholine and L-alpha-phosphatidylserine (PC/PS) onion-like vesicles (prepared according to reference [46]) with the corresponding negatively-stained TEM image obtained using uranyl acetate (Fig. 5b), it is possible to measure the deformation of native morphology induced by the drying step in the staining protocol, while cryo-TEM enables preservation of the expected spherical-shaped morphology. It should also be noted that the electron dense atoms in the negative stain highlight the lipid bilayers surrounding the internal aqueous space, whereas the lipid phase appears with darker contrast when compared to the weaker contrast of the surrounding amorphous solid water.

Another example that demonstrates the advantages of cryo-TEM over negative staining when trying to characterize complex vesicular-shaped particles is given in Fig. 5c-d, which shows cryo-TEM images of myristate salts of aminopropyltriethoxysilane (APTES) self-assembled hydrated structures. Such particles may play an unique role as precursors for novel templates of mesoporous silica networks or as low-cost biosurfactants to produce thermodynamically stable assemblies [47]. Self-assembly processes and the resulting particle morphology, as well as surfactant properties, can be monitored by experimental parameters such as pH value and the nature of the counter-ion.
The detailed characterization of dispersions resulting from an interaction between a fatty acid and counter-ion functional groups is determinant to understanding its functional properties. The cryo-TEM images (Fig. 5c-d) clearly show the presence of particles with a “pine-cone”-like morphology, with a high degree of symmetry of the lipid membranes that appear to be arranged along a vertical axis.

When preparing a vitreous thin film, the particles must be rapidly embedded in solid water, while maintaining all existing orientations in the initial aqueous dispersion. Fig. 5c’ presents two typical particles, with top view images (considering a principal vertical axis fitted to the longer axis of the particles) or lateral view images. The higher magnification image in Fig. 5d shows the symmetrical organization of membranes around a vertical axis. The corresponding negatively stained TEM image in Fig. 5e shows that some structural deformation has occurred under drying, resulting in a loss of evidence for a symmetrical arrangement of the lipid layers. Cryo-TEM operated at 80 kV is therefore well adapted to characterizing the complex morphology of sub-micrometer sized hydrated particles, in which the water content participates in structural integrity.

In recent years, atomic force microscopy (AFM) operated in liquid mode has appeared to be a promising and complementary method to achieve the cryo-TEM of vitreous thin films when evaluating both the 3D morphology and physicochemical properties (elasticity and adhesion) of lipid nanoparticle dispersions [51-53]. The principal objective of sample preparation for AFM studies is to determine the adsorption and fixation of nanostructures on solid surfaces while preventing any particle spreading and morphological deformation.

AFM was used as a complement to electron microscopy to identify the hybrid silica/lipid structures obtained by lowering the pH of the vesicle solutions of myristate APTES salts described above (see Fig. 5). Under highly acidic conditions (pH 2), no precipitate formed although it could logically be expected for fatty acids, but a bluish, stable solution was produced [47]. Figure 6 summarises the microscopy findings on the supramolecular structures corresponding to myristate APTES salts at pH2. Conventional TEM revealed the formation of flat, truncated lozenges of a few microns in size (see Fig. 6a-a’). The size range of the particles in the vitreous thin film was not considered adequate for further observation by cryo-TEM. The application of electron energy loss spectroscopy (EELS) and energy-filtered imaging (EF-TEM) was helpful to perform a chemical analysis and identify unambiguously the formation of hybrid silica/fatty acid composite particles (see Fig. 6b-b’). From the Si-L elemental maps of the particles, silica appeared as a fine textured network distributed throughout these lozenge-shaped fatty acid particles.

However, AFM was necessary to confirm the formation of a Si/lipid hybrid structure and to provide the heights of flat particles, which ranged from 100 to 250 nm (see Fig. 6c-d). AFM showed that the silica network appeared either to be out of the fatty acid zone with different morphologies (see Fig. 6c’, 6e, 6f) or completely embedded in the lipid (see Fig. 6d). The superimposition of lipid layers, each with a thickness of around 1.5 nm was visualized under a higher magnification the AFM images, which indicated that the fatty acid was crystallized (see Fig. 6g-g’).

3.3 Complementarity of AFM, TEM and cryo-TEM for the multiscale characterisation of the supramolecular self-assembly of 12-hydroxystearate salts

Structures with markedly variable morphologies are generated by systems based on fatty acids with different chain lengths and functional groups. Self-assembling supramolecular edifices based on membranes or vesicles are well known and have been studied intensively since the 1970s [54, 55]. The analogy which can be made between these structures
formed in vitro and biological membranes, in terms of their structural and functional roles, justifies the considerable value of constructing biomimetic systems based on saturated and unsaturated lipids [56, 57].

Although fatty acids are mainly used for the industrial manufacture of soaps, their capacity for dispersion in water opens new perspectives for the production of green detergents as an alternative to petroleum-based products [2]. The investigation of innovative systems based on hydroxyl fatty acids or ammonium salts has recently demonstrated that it is possible to generate systems with differing structural and physicochemical properties [58].

Similarly, because of their remarkable stability, hydroxyl fatty acid-based nanosized vesicles have also been used as templates for the synthesis of metallic and semiconductor nanoparticles in the form of stable aqueous dispersions [59, 60], or self-assemblies in nanoscopic optical fibres [61, 62].

The morphology of lipid-based systems, which typically ranges from the unilamellar phase to liquid crystals and vesicles or membranes, needs to be characterized with a high degree of precision that avoids any damage to the structure. As seen above, cryo-TEM of vitreous thin films is the appropriate method when the size range fits with the technique limitations [63]. When a supramolecular system with a larger size range is formed, the cryo-TEM of vitreous thin films encounters certain limitations to its efficient application (see Fig. 3c). As described, the freeze fracture technique can overcome this size limitation, but account must be taken of the fact that the fracturing plane occurs at random across the structures, which may complicate interpretation. In this case, the use of AFM can complement that of cryo-TEM.

The multiscale characterization of a supramolecular self-assembly of a 12-hydroxystearic acid salt of hexanolamine is given in Fig. 7 as an example of complementarity of AFM with electron microscopy.

Hollow tubes, more than 10 μm long and with outer diameters of 400 to 600 nm form spontaneously during the cooling of an isotropic solution of the hydroxyl fatty acid neutralized with ethanolamine [64, 65]. Whereas the TEM images of unstained samples provide valuable information on the tubular structure (see Fig. 7a), AFM observations of the surface of the tubes show a coiled ribbon-like structure with a helical pitch angle of about 45° (see Fig. 7b). It should be noted that the negatively stained TEM images confirm the structure described by AFM (not shown).

Similar racemic mixtures of 12-hydroxystearic acid and hexanolamine are able to form twisted ribbons following the aging of the tubular self-assemblies described above. In this case, a translucent gel is slowly formed and corresponds to the transition from tubes to a distinct supramolecular assembly. During this transition step, unstable hybrid supramolecular structures, which result from the unfolding of tubes into twisted ribbons, are formed (see Fig. 7c-d). The final transition step is represented by a homogeneous dispersion of monodispersed twisted ribbons with a width of 100 nm and a pitch period of 400 nm (see Fig. 7e-h) [66].

The AFM images in Fig. 7c-d-d’ clearly show the link between the smallest twin ribbons and the larger micron-sized tubes from which they are produced after aging. In this context, AFM was the correct method to provide high resolution views of both the larger and smaller objects. This was due in part to the simpler sample preparation for AFM (with no limitation in sample thickness) compared with that required for cryo-TEM. However, the resolution of cryo-TEM on twisted ribbons preserved in vitreous ice was the best to reveal the ribbons as being multi-lamellar and with a large water layer between the lipid bilayers, which was confirmed by neutron scattering data (see Fig. 7e-c’-c’’).
Negatively stained TEM, and AFM observations in air, as shown in Fig. 7f and Fig. 7g, respectively, also produced clear images of twists in the ribbons, although the pitch period was markedly reduced, as could be expected following evaporation of the water content. It should be noted that uranyl acetate negative staining revealed that the ribbons were composed of both right- and left-hand twists, as illustrated in the false-coloured image in the inset (Fig. 7f). The results of AFM carried out in water using a liquid cell, and which prevented any dehydration of the structures, had the advantage of determining both the right value for the pitch period and the orientation of twisting (see Fig. 7h).

These observations were obtained under a multiscale approach and contributed to our understanding of the self-assembly mechanism of hydroxy-derivatized fatty acids, and of the effects of temperature and aging on the transformation of such supramolecular structures to others with singular surfactant properties. The combination of AFM and electron microscopy is of great value to the study of fatty acid-based systems where the structure needs to be explained from the scales of the nanometer to the micrometer.

Using both AFM and MET also enabled clarification of the 3D anastomose-like structure resulting from the self-assembly of myristate guanidinium salts [42].

4. Conclusion

In this chapter, we have described several examples of techniques which enable a clearer understanding of the structure of complex fatty acid-based nanoparticle dispersions. Cryo-TEM of vitreous thin films operated at 80 kV has been shown to constitute a valuable tool to elucidate the complex morphology of highly hydrated supramolecular systems produced from the self-assembly of fatty acid salts. To overcome some of the limitations of cryo-MET, such as particle size and thickness, the complementary use of AFM can help to specify the multiscale organization of pure lipid or hybrid silica/lipid systems, from the nanometer to the micron size range. The emergence of AFM liquid and advanced force spectroscopy modes may be of a great interest for studying lipid-based supramolecular assemblies, particularly since it is now possible to correlate these modes with direct fluorescence and Raman spectroscopy measurements. It thus constitutes a promising innovation to supplement the cryo-TEM investigation of lipid-based systems in order to generate data on their multiscale structure.

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References


