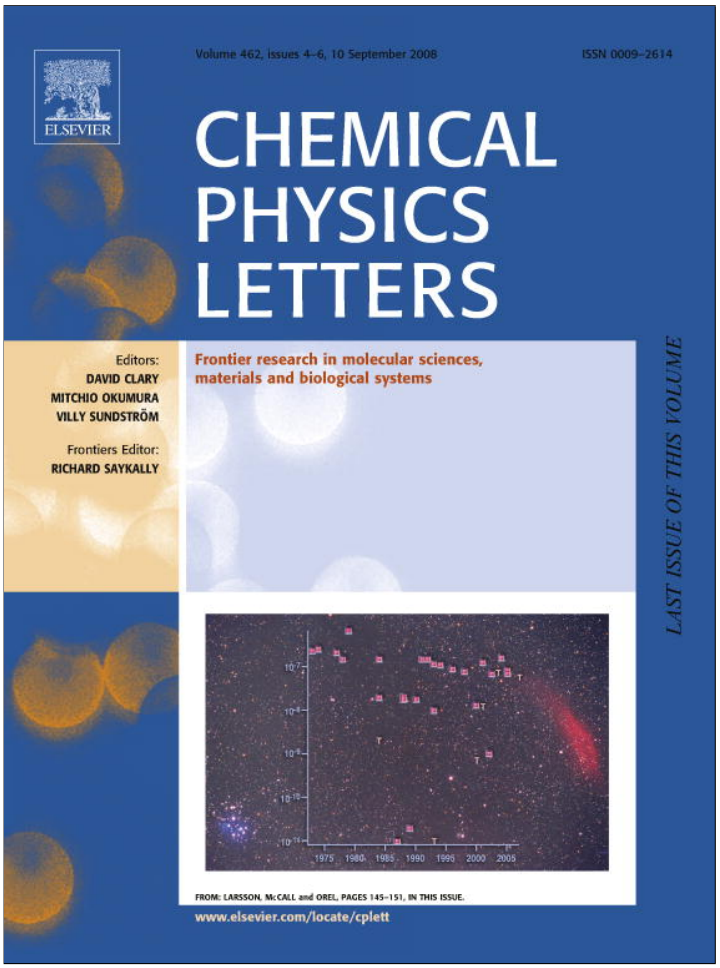

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# Effect of hydration on the structure of solid-supported Niosomal membranes investigated by in situ energy dispersive X-ray diffraction

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ABSTRACT

The supramolecular structure of Niosomal vesicles (Niosomes) made of a binary mixture of polysorbate 20 (Tween 20) and Cholesterol in aqueous solution was investigated by means of synchrotron small angle X-ray scattering (SAXS). Solid-supported Niosomal membranes at full hydration exhibit the same structural properties, as determined by in situ energy dispersive X-ray diffraction (EDXD), than their counterpart in solution. Both Niosomes and solid-supported Niosomal membranes are made of highly swollen bilayers rich in Tween 20 coexisting with Cholesterol crystallites. EDXD patterns from oriented samples suggest that at least some Cholesterol crystals are aligned along the normal to the solid support.


## 1. Introduction

Phospholipid (Liposomes) and non phospholipid (Niosomes) vesicles are extensively studied as drug delivery systems to modify pharmacokinetics of drugs and to improve their action in target cells [1,2].

The use of liposomes in the drug delivery field is widely reported in literature [3], but their clinical applications are still limited by their high cost and by chemical, physical and biological low stability [3]. For these reasons surfactant vesicles, showing higher chemical stability than liposomes, are currently emerging as a primary alternative to lipid-based vesicles. Among surfactants employed as drug carriers, polysorbate 20 (Tween 20) is widely used in the food and pharmaceutical industry, is not expensive and not toxic. When bearing poly(ethylene glycol) units, Tween 20 is also resistant to the reticuloendothelial systems capture and is most useful to produce stable drug delivery systems. It is a central point since long-circulating time will be a primary requirement to project highly efficient vectors and, in turn, to sharply increase their activity in treatment of various diseases [4].

The intracellular fate of particulate drug delivery systems is determined by the initial mode of cell internalization and the subsequent intracellular trafficking. The internalization pathway might be influenced by different parameters: particle dimensions [5] and vesicle surface characteristics [6]. For these reasons, the knowledge of vesicle supramolecolar structure plays a fundamental role in designing efficient carrier to optimize drug targeting.

In this Letter we investigated, for the first time, the supramolecular structure of Niosomal vesicles at the nanometer scale by means of synchrotron small angle X-ray scattering (SAXS). Niosomal vesicles are made of a binary mixture of Tween 20 and Cholesterol [7] (chemical structures reported in Fig. 1). Structural properties of Niosomal vesicles in bulk were compared to those of solid-supported Niosomal membranes fully hydrated from a vapor-saturated atmosphere as determined by in situ energy dispersive X-ray diffraction (EDXD).

For a long time there has been concern in studying aligned systems due to many reports of oriented membranes hydrated from water vapor exhibiting *d*-spacings much less than liposomes immersed in water [8,9]. Since the chemical potential of water vapor in equilibrium with bulk water is the same, a question arose in the scientific community: why should the *d*-spacings of aligned systems be shorter than those of liposomes in bulk? As a result, the so-called 'vapor pressure paradox' (VPP) appeared to be well established [10] and theories were proposed to explain it [11,12]. Recently, Katsaras [13,14] proved that VPP was simply an experimental artifact due to temperature gradients inside hydration chambers and designed a sample cell capable of RH = 1. Taking advantage of this, fully hydrated oriented systems (RH = 1) exhibited, for the first time, the same physical characteristics as liposomes in solution [13–15]. Even though vesicles dispersed in

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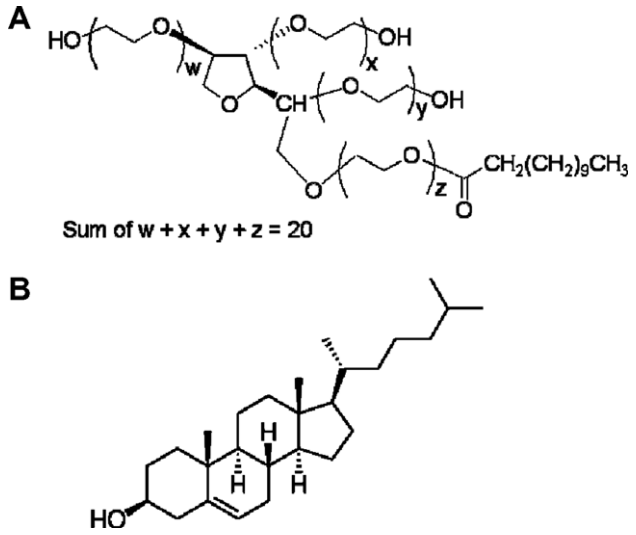


**Fig. 1.** Chemical structures of polysorbate 20 (A) and Cholesterol (B).

aqueous solutions remain a frequent measurement condition, solid-supported samples, allowing a precise discrimination between in-plane and out-of-plane structures, are currently emerging as a useful alternative to achieve detailed structural information.

Here we show that mixing Tween 20 and Cholesterol in equimolar ratio results in vesicles made of highly swollen membranes rich in Tween 20 coexisting with Cholesterol crystallites. The structure of solid-supported Niosomal membranes at full hydration was found to be the same as their counterpart in aqueous solution. Upon hydration, Niosomal membranes swell continuously and two different hydration regimes were identified. EDXD patterns from oriented samples suggest that, at least some Cholesterol crystals are aligned along the normal to the solid support.

## 2. Experimental section

### 2.1. Samples preparation

Unilamellar vesicles were obtained from a non-ionic surfactant/cholesterol aqueous dispersion by means of the 'film' method as previously reported [16]. For this purpose equimolar quantities of polysorbate 20 (Tween 20) and Cholesterol were dissolved in a $CHCl_3/CH_3OH$ (3:1 v/v) mixture in a round-bottomed flask. Tween 20 concentration was always remarkably above CMC. After evaporation of the solvents, the dried film was hydrated by addition of 5 ml of distilled water. The dispersion was vortexed for about 5 min and then sonicated for 20 min at 60 °C using a tapered microtip operating at 20 kHz at an amplitude of 16% (Vibracell-VCX 400-Sonics, USA).

### 2.2. Size and zeta potential measurements

Size measurements after film-hydration were carried out by means of dynamic light scattering. The vesicle dispersion was diluted about 100 times in the same buffer used for their preparation. Vesicle size distribution was measured on a Malvern Nano ZS90 (Malvern, UK) at 25 °C, with a scattering angle of 90.0°.

The same apparatus was used for the evaluation of zeta potential using a vesicle preparation appropriately diluted (1:10) in distilled water at 25 °C. The polidispersity index was directly calculated by the software of the apparatus and the values obtained are in agreement with mono disperse vesicular systems.

### 2.3. Synchrotron SAXS experiments

Small-angle X-ray scattering (SAXS) measurements were carried out at the high-brilliance beamline ID02 at the European Synchrotron Radiation Facility (Grenoble, France). The energy of the incident beam was 12.5 keV ($\lambda = 0.995$ Å), the beam size was 100 μm and the sample-to-detector distance was 1.2 m. The diffraction patterns were collected by a 2D CCD detector. We investigated the $q$ range from $q_{min} = 0.04$ Å$^{-1}$ to $q_{max} = 0.5$ Å$^{-1}$ with a resolution of $5 \times 10^{-4}$ Å$^{-1}$ (FWHM). The sample was held in a 1 mm-size glass capillary. Measurements were performed at 25 °C. To avoid radiation damage, a maximum exposure time of 3 s/frame was used for any given sample. Satisfactory statistics were attained by repeating several measurements on fresh samples. The collected 2D powder diffraction spectra were angularly integrated to get 1D intensity vs. $q$ patterns. The data have been normalized for primary beam intensity and detector efficiency, as well as the background has been subtracted.

### 2.4. In situ EDXD experiments

EDXD experiments were carried out by using an apparatus elsewhere described [17]. The X-ray source is a standard Seifert tube (W target) operating at 50 kV and 40 mA whose Bremsstrahlung radiation is used whereas the detecting system is composed of an EG&G liquid-nitrogen-cooled ultrapure Ge solid-state detector. The diffractometer, equipped with step motors and a collimating system, operates in vertical $\theta/\theta$ geometry and both the X-ray tube and the detector can rotate around their common center in which the lipid coated wafer is placed. A diffraction angle $\theta = 0.67°$ was fixed that allowed to cover simultaneously an overall region of the reciprocal space $0.05 < q < 0.5$ Å$^{-1}$ momentum $q$ ($q = \cos\ t \times\ E \times \sin\ \theta$; cos $t = 1.01354$ Å$^{-1}$ keV$^{-1}$). In contrast to traditional angular dispersive X-ray diffraction, energy dispersive X-ray diffraction (EDXD) permits the simultaneous acquisition of the diffraction pattern. This peculiar characteristic of the EDXD technique allows to perform kinetic studies. Biological samples are not damaged by EDXD experiments as elsewhere discussed [18].

A drop of 100 μl of the sample solution was carefully spread onto the freshly-cleaved surface of oriented silicon wafers. After drying under a vacuum over at least 12 h to remove any residual solvent, the samples was transferred to the hydration chamber allowing a precise control of relative humidity (RH) and temperature. The sample chamber, designed to overcome the experimental inadequacy that had previously led to the VPP, is elsewhere described [19]. The dry film was carefully hydrated by vapor. The hydration kinetics was followed in time due to the peculiar characteristics of EDXD. Each EDXD scan was collected at room temperature for $t = 1000$ s.

## 3. Results and discussion

The synchrotron SAXS scans of Niosomes in bulk is reported in Fig. 2, panel A. The diffraction pattern shows strong first- and second-order Bragg peaks (00l) of a lamellar structure with a periodicity $d = 2\pi/q_{001} = 33.9$ Å. The value of $q_{001}$ was determined by fitting a Lorentz function to the peak. Panel B of Fig. 2 shows the EDXD scan of solid-supported Niosomal membranes at full hydration (RH $\sim$ 1). Two diffraction peaks are observed corresponding to a characteristic spacing $d = 2\pi/q = 33.5 \pm 0.5$ Å. The varying background in the EDXD pattern is due to the scattering of excess water at the surfactant/air interface. This finding confirms that fully hydrated aligned systems adsorbed to the silicon substrate effectively exhibit the same structural properties as their counterpart in liquid water [14,15].

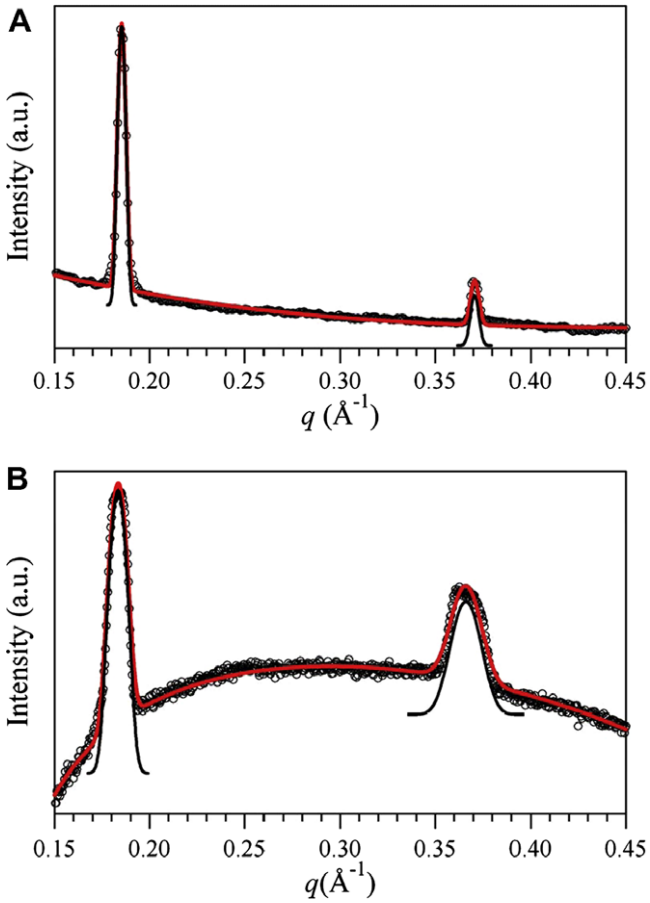


**Fig. 2.** Panel A: synchrotron SAXS pattern of Niosomal vesicles in aqueous solution. Panel B: EDXD pattern of solid-supported Niosomal membranes. Scattering in the region between Bragg peaks arises from water molecules. For clarity, fitting functions were multiplicated by a scale factor.

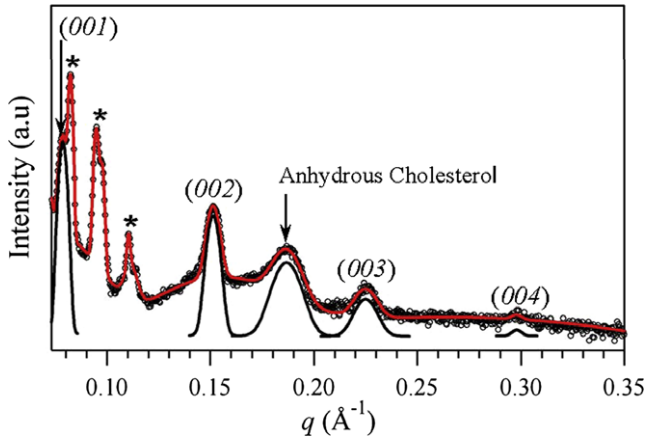


**Fig. 3.** EDXD pattern of solid-supported Niosomal membranes hydrated from a vapor-saturated atmosphere (RH = 0.98). Solid red line is the best fit to the experimental data (open circles), while black full lines show the Gaussian fitting functions (for better visualization fitting functions were multiplicated by a scale factor). Symbols (∗) indicate the fluorescence lines from the X-ray source (W anode). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The lamellar structure as found here is called phase T (thin lamellar phase) in the subsequent text. About its arrangement, some hypotheses can be made. Given the molecular structure of Tween 20 (Fig. 1), the observation of an extremely small lamellar periodicity could be due to the formation of interdigitated multibilayers with a high degree of interpenetration of alkyl chains [20–22]. Alternatively, the Bragg peak at may be due to the formation of Cholesterol crystallites. Cholesterol is well known to form precipitates in lipid bilayers by exceeding its solubility [23] and experimental reports on the Cholesterol crystallite formation in Cholesterol–phospholipids binary mixtures are abundant in the literature [24–26].

In situ EDXD experiments allowed us to clarify the exact nature of the T phase. Hydration kinetics of solid-supported Niosomal membranes was followed in time due to the distinctive characteristics of EDXD [18] over the hydration range of 0.45–1 RH. For $0.45 < RH < 0.97$ (data not reported) we did not observe any appreciable structural change. For RH $\sim$ 0.97, the EDXD pattern (not reported) closely resembled that collected at full hydration with changes in the peak line shapes probably given by different lattice disorders associated with a different content of interlamellar water molecules [27,28].

For RH $\sim$ 0.985 the EDXD pattern started to exhibit four more sharp Bragg peaks (Fig. 3). They were indexed as the (001) reflections of a lamellar phase with $d = 2\pi/q_{001} = 78$ Å and indicated a high degree of translational order along the normal to Niosomal membranes. Thus, two lamellar phases started to coexist at RH $\sim$ 0.985, i.e. the T phase ($d \sim 34$ Å) and the 'swollen lamellar phase' ($d \sim 78$ Å). While the former phase was observed in the case of unoriented (i.e. liposomes in bulk, Fig. 2, panel A) as well as of oriented systems (Fig. 2, panel B), the latter was only detected when aligned membranes were employed. Hydration EDXD experiments allowed us to clarify the exact nature of these two phases.

In Fig. 4, representative EDXD patterns of Niosome membranes at increasing RH are reported. Upon hydration, the larger lamellar phase did spontaneously swell as shown by the continuous shift of Bragg maxima towards lower $q$-values, while the T phase did not.

Upon progressive hydration, two regimes were identified. In the first stage, water adsorption increased the long-range order along the normal to the Niosomal membranes (Fig. 4, grey panel) as revealed by the intensities of the diffraction peaks that increased with RH. This means that more layers contribute coherently to Bragg diffraction which leads to the notion of a better packing along the normal to the solid support [27,28]. In the second step, (Fig. 4, white panel), the resulting scattering distribution showed simultaneous loss in intensity and broadening of Bragg peaks. Both these effects are due to 'second-order disorder' [29] promoted by free water molecules that penetrate the multilayer system and produce fluctuations of the bilayers around well defined mean layer positions (affecting intensity of Bragg peaks) as well as small variations in the bilayer separations (affecting widths of Bragg peaks).

In particular, the full width at half maximum of Bragg peaks increase with increasing RH confirming that the inter-membrane coherence progressively extends less far [29]. Thus, first adsorbed water molecules ($RH < 0.988$) could progressively modulate the molecular arrangement of surfactants into a more ordered and stable structure [27,28], while further hydration ($RH > 0.988$) produced increased structural disorder which is consistent with the penetration of free water molecules between the inter-bilayer region [27,28].

Fig. 5 shows the Bragg $d$-spacing of both the phases as a function of RH. Interestingly, the lamellar $d$-spacing of the larger phase increases monotonously as a function of increasing RH without reaching any finite swelling limit. Such a swelling behaviour is typical of charged membranes and has been reported several times in literature. The obvious force responsible for indefinite swelling is the electrostatic repulsion [30,31].

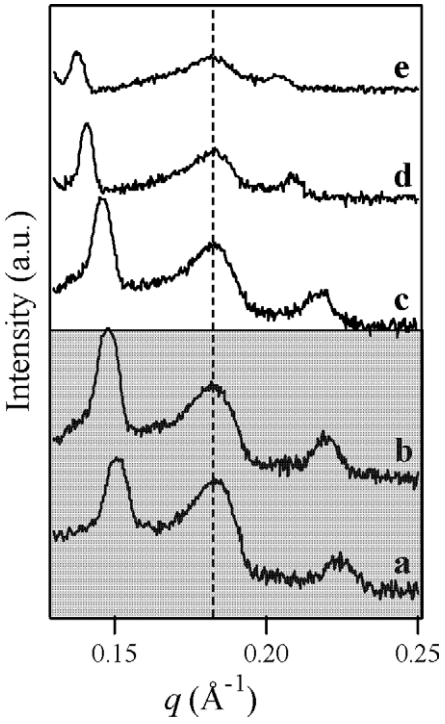


**Fig. 4.** Representative EDXD patterns of solid-supported Niosomal membranes as a function of increasing RH (a: RH = 0.98; b: RH = 0.985; c: RH = 0.988; d: RH = 0.99; e: RH = 0.995). Dashed line indicates the position of the first-order Bragg peak of Cholestreol crystallites. To clarity, the diffraction range was restricted to $q = 0.25$ Å$^{-1}$.

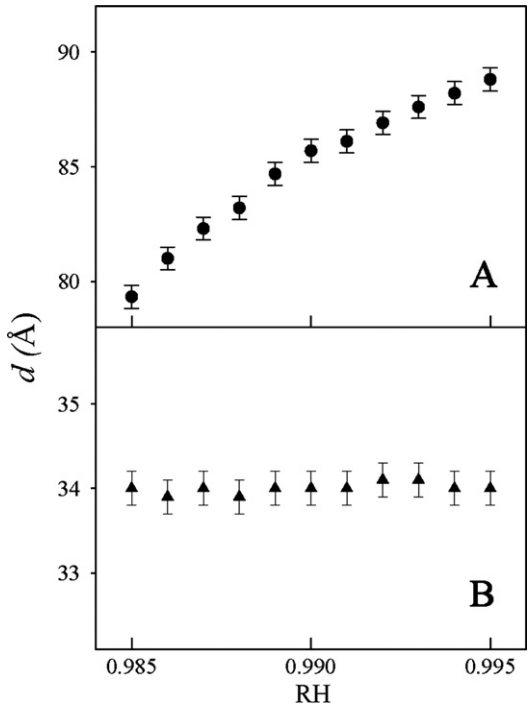


**Fig. 5.** Lamellar *d*-spacings of Niosomal membranes and Cholesterol pseudo-bilayers as a function of increasing RH.

Size and zeta potential analyses confirm that in a water solution, vesicle suspension possesses a negative charge of −28 mV and a mean diameter of 380 nm with a polidispersity index (PI) of +0.57. This PI value can be ascribed to a non homogeneous distribution of vesicle dimensions.

The negative zeta potential value is the result of the orientation of the Cholesterol and Tween 20 oxydrile groups oriented vs. the aqueous medium and the subsequent re-distribution of the ion charges in water. The fact that droplets stabilized by non-ionic surfactants can have a negative charge is well established in the literature, where it has been attributed to the ability of oil–water interfaces to preferentially adsorb OH species from water [32,33].

Anyway, by careful analysis of the EDXD patterns of Fig. 4, the hydration phenomenon appears to be quite attractive. Indeed, while the larger phase does swell monotonously in an ordinary way, the hydration behaviour of the T phase was peculiar and, to some extent, contradictory. Even though there is certainly a consensus in interpreting the decrease of the lamellar signal as due to water adsorption, there could remain controversy in justifying the observation that *d* remains constant as a function of increasing RH (Fig. 5). All these experimental observations let us conclude that the diffraction at 34 Å arises from Cholesterol crystallites embedded within (in contact with) oriented Niosomal membranes.

To the best of our knowledge, formation of Cholesterol crystallites in Tween 20/Cholesterol mixture has not been reported so far. Presence of Cholesterol crystallites separated from Niosomal membranes rich in Tween 20 molecules explains well why the observed lamellar phases exhibit largely different lamellar *d*-spacing and very distinct hydration behaviour (Fig. 5). Indeed, Tween 20 molecules have a large polar head-group region that can bind water molecules responsible for the large swelling of Niosomal membranes.

Several reports [24–26] suggest that, depending on the nature of the phospholipid and the history of the sample, the Cholesterol crystallites can be in the form of Cholesterol monohydrate, anhydrous Cholesterol or a mixture of both forms. Both the anhydrous and monohydrate forms of crystalline Cholesterol contain a pseudo bilayer structure with repeating distance ~34 Å. However, since the position of the diffraction peak is the same for both anhydrous and monohydrate crystals (010) reflection for anhydrous crystals and (001) for the monohydrate [24] its emergence alone cannot identify the crystallite form. Both anhydrous and monohydrate forms of crystalline Cholesterol have specific diffraction peaks in the wide angle region. The dominant peaks are found at 5.75, 5.02 and 4.91 Å for anhydrous Cholesterol and at 5.8, 4.7 and 3.8 Å for Cholesterol monohydrate. Further EDXD experiments carried out at $\theta = 3°$ ($0.3 < q < 2$ Å$^{-1}$, data not reported) allowed us the phase identification of anhydrous cholesterol. A necessary prerequisite for detection of Cholesterol crystallites by the appearance of the 34 Å reflection is a sufficiently large number of pseudo bilayers in the crystal [24]. There is evidence that crystalline Cholesterol associated with single lamellae or single shell vesicles does not satisfy this criterion, while Cholesterol crystallites associated with multilamellar vesicles are usually detectable [24]. In a recent study [34], Cholesterol crystallites formed in unilamellar vesicles of DMPS/Cholesterol were not detectable by XRD, but if the same mixture is not sonicated, thereby retaining multilamellar vesicles, the strong 34-Å reflection from Cholesterol crystallites was detected. As a consequence, the authors suggested that at least some of the Cholesterol crystals are embedded in the plane of the bilayer. Such expectation is seemingly confirmed by EDXD patterns reported in Fig. 4 showing broadening of diffraction peaks of Cholesterol crystallites with increasing RH. Since repeat spacing of Cholesterol pseudo-bilayers is definitely insensitive to progressive hydration (Fig. 5), loss in intensity of Bragg peaks can not be explained in terms of water molecules penetrating within the pseudo-bilayer structure, but probably reflects the hydration-dependent long range order of Niosomal membranes.

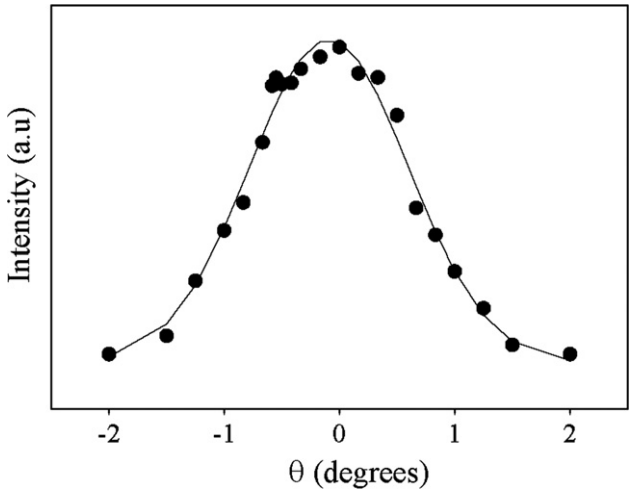


**Fig. 6.** Rocking curve taken at the second-order Bragg peak of Cholesterol crystallites (RH = 0.985) proving the low mosaicity of the sample (full width at half maximum ~1°).

We stress, therefore, that at least some Cholesterol crystallites are in contact with Niosomal membranes. Among the advantages of using solid-supported samples is the possibility of investigating orientation with respect to the normal of the substrate. In general, the sample orientation can be monitored by observing the mosaic spread of the lamellar repeat peaks along the $q_z$ axis. Rocking curves provide a quantitative way to obtain mosaic spread. Fig. 6 shows the rocking curve taken at the second-order Bragg peak of Cholesterol crystallites (RH = 0.98) with mosaicity being above 1° (full width at half maximum). The rocking curve indicates that Cholesterol crystallites are poorly oriented along the normal to the solid support.

At RH = 0.995 the quasi-Bragg peaks of the lamellar phase rich in Tween 20 are still observable but weak and very broad. Low intensity and large peak width, which are characteristic of a system with a short scattering correlation length, are an indication that the bilayers are weakly bound. Further, the large lamellar periodicity $d$ of 88 Å shows that Tween 20 bilayers are in a highly swollen state.

For RH > 0.995, the Bragg peaks of Tween 20 disappear and the system exhibits pure diffuse scattering due to the bilayer form factor plus the diffraction peaks arising from Cholesterol crystallites. The diffraction pattern is practically indistinguishable from that reported in Fig. 2 (panel B) collected at RH = 1. This indicates that the positional correlations between Tween 20 membranes are completely lost [27]. This finding suggests that Niosomes in bulk are made of highly swollen membranes rich in Tween 20 (probably not observed in Fig. 2A due to the extremely large $d$-spacing and to the low positional correlation between membranes [27–29]) plus Cholesterol crystallites in contact with membranes or free in solution. Therefore, we underline that performing high-resolution synchrotron SAXS experiments on surfactant/Cholesterol mixtures with infinite swelling behaviour such that typical of charged cellular lipids could lead to misunderstanding when positional correlation between highly swollen membranes is almost completely lost [35]. In that case, swelling experiments performed by in situ EDXD technique on solid-supported samples are a powerful tool for investigating the structural properties of surfactant/Cholesterol mixtures.

Solid-supported samples are artificial systems, which confines the order, but are interesting to study the interaction between molecular components since one can tune by humidity the state of hydration (surfactant/water content) and therefore change the interaction. This is quite difficult in solution where different hydration levels are usually produced using the technique of applying osmotic pressure by adding the immiscible polymer polyvinylpyrrolidone (PVP) to bulk water. By varying concentration of PVP the osmotic pressure can be changed and RH can be finally calculated [36].

Nevertheless, experimental uncertainties can be large essentially due to the difficulty of preparing small samples with precise polymer concentrations [37]. We therefore claim the relevance of in situ EDXD because a single sample is used and the RH is not calculated but measured directly.

## 4. Conclusions

In conclusion we have shown that: (i) mixing Tween 20 and Cholesterol in equimolar ratio results in spontaneous formation of multilamellar vesicles rich in Tween 20 molecules coexisting with Cholesterol crystallites; (ii) EDXD experiments performed on oriented samples suggest that Cholesterol crystallites are in contact with Niosomal membranes and that a fraction of crystals are aligned along the normal to the solid support; (iii) the supramolecular structure of solid-supported Niosomal membranes hydrated from water vapor is essentially the same as their counterpart in bulk; (iv) Niosomal membranes swell largely with RH due to the large headgroup region of Tween 20 molecules that can bind several water molecules at the membrane surface; (v) in situ EDXD technique on solid-supported samples is a powerful tool for investigating the structural properties of surfactant/Cholesterol mixtures especially when (large water/surfactant volume fraction) positional correlation between highly swollen membranes is missing and does not produce any appreciable (weak or off-scale) X-ray scattering distribution.

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