# Direct visualization of lipid aggregates in native human bile by light- and cryo-transmission electron-microscopy

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## Abstract

The evolution of microstructures present in human gallbladder and hepatic bile was observed simultaneously by video-enhanced light microscopy (VELM) and transmission electron microscopy of vitrified specimens (cryo-TEM), as a function of time after withdrawal from patients. Fresh centrifuged gallbladder bile samples contained small (6 nm) spherical micelles in coexistence with vesicles (40 nm). Out of the seven bile samples investigated four contained, in addition, two types of elongated aggregates that have not been previously described. Uncentrifuged gallbladder bile also contained a mixture of ribbon- and plate-like crystals seen by VELM, but not by cryo-TEM. In aged (3–6-week-old) gallbladder bile samples VELM also revealed spiral and helical crystal structures. No such crystals were present in hepatic bile samples, although microcrystals, not observable by VELM were seen by cryo-TEM in addition to micelles and vesicles. The similarity of these observations to those observed in bile models lends strong support for the validity of the model systems. Furthermore, the presence of microcrystals in hepatic bile samples, apparently devoid of crystals by light microscopy, indicates that under certain conditions the common criterion of 'nucleation time' (NT), based on light microscopy, does not represent the real time of nucleation. In the human bile samples investigated in this study the dissociation between NT and the time of observation of microcrystals was seen in hepatic but not in gallbladder bile samples. Hence, crystal growth may be rate limiting only in dilute biles.

Key words: Cholesterol; Crystallization; Bile; Gallstone formation

## 1. Introduction

The mechanisms of cholesterol crystallization in bile are of great interest with respect to gallstone formation [1]. Supersaturation of bile is a prerequisite for crystal formation (and growth) but many other factors affecting the rate of processes involved in this frequent pathology have been shown to play a significant role [2,3]. Thus, promoters of crystallization commonly denoted nucleating agents [4] and inhibitors of this process (anti-nucleating agents [5]) have been investigated in great detail by several groups, both in bile and in lipid model systems. In spite of significantly advancing our knowledge on the relative importance of such factors, the detailed mechanisms that eventually lead to the formation of microscopically observable 'plate-like' cholesterol monohydrate crystals in bile are not well understood.

Specifically, the term 'nucleation time' (NT) is commonly used to describe that time at which cholesterol monohydrate crystals can be first observed upon incuba-

tion of an initially crystal-free bile sample [6]. In fact this NT is made up of the time it takes for cholesterol crystals to nucleate (in native bile probably by heterogeneous nucleation [7]) and subsequently to grow to become observable by light microscopy. NT represents the true nucleation time only if the crystallization process is highly cooperative. Under such conditions, once a critical cholesterol nucleus has formed the subsequent crystal growth will almost instantaneously produce crystals that are large enough to be seen by light microscopy [7]. Inhibition of crystal growth is not necessarily less feasible than inhibition of 'nucleation'. Under such circumstances, microcrystals can possibly be observed by electron microscopy much earlier than at NT.

In an effort to dissociate the actual nucleation from the growth of initially formed microcrystals, we have studied human bile samples, from both gallstone and non-gallstone patients, as a function of time, using simultaneously light microscopy and cryo-transmission electron microscopy (cryo-TEM).

The results of these studies, reported in this communication, show that in some cases a wide variety of microstructures or aggregates occur in native bile at times
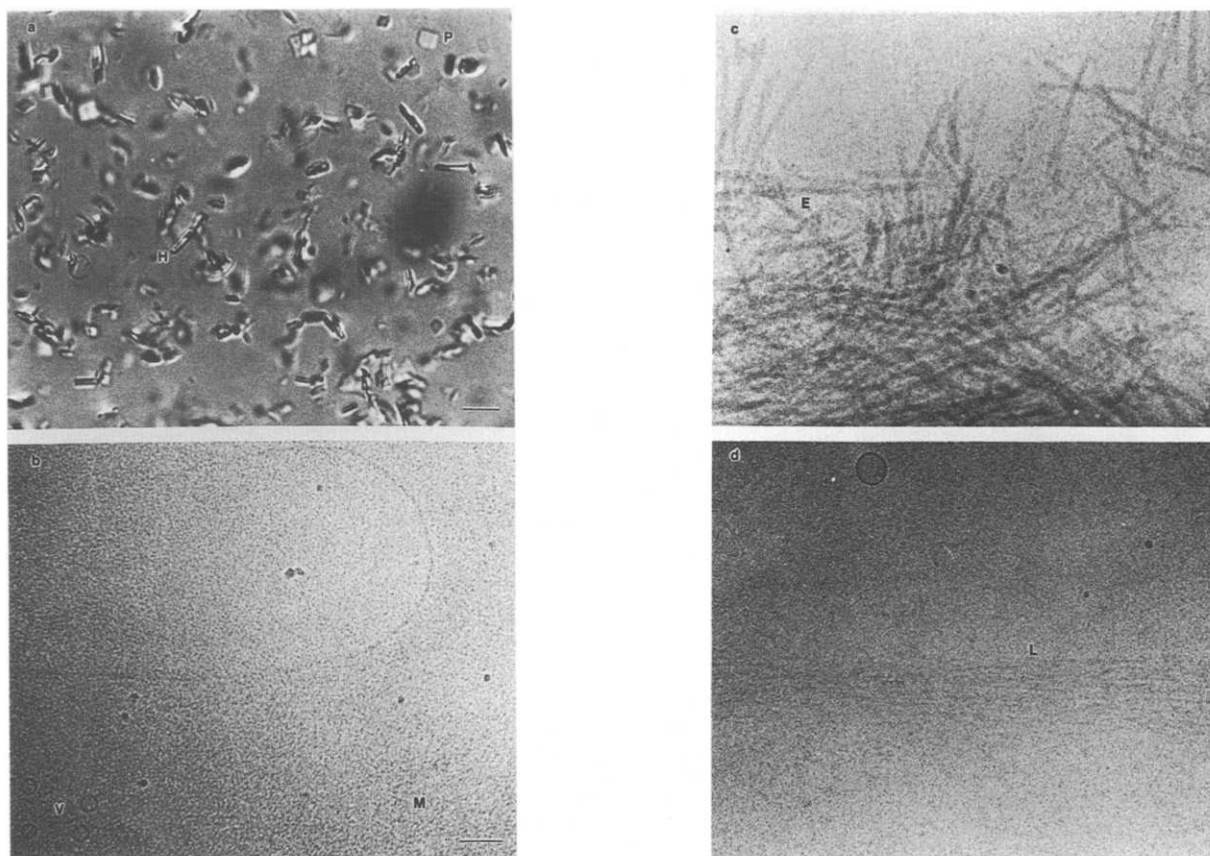
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Fig. 1. (a) Light-micrograph of a one-day-old gallbladder bile sample from a gallstone patient revealing plate-like crystals (P) and elongated (H) structures; (b–d) Cryo-TEM micrographs of a specimen vitrified from the same bile at the same time (b), and of another gallstone patient (c,d) within one day after bile collection. Note small spheroidal micelles (M), spherical vesicles (V), electron dense elongated structures (E), and very thin and long structures (L). Bar = 10 μm for light-micrograph and 100 nm for TEM micrographs.

when no 'typical' cholesterol crystals can be observed. Furthermore, the sequence of appearance of these aggregates prior to formation of microcrystals is very similar to that observed in previously studied bile models [8,9]. This similarity lends strong support to the previously proposed mechanism, that describes biliary cholesterol crystal formation as being a consequence of cholesterol reorganization within aggregates of cholesterol-rich licithin/cholesterol vesicles [9–12]. These findings may lead to a better understanding of the mechanisms of gallstone formation.

## 2. Materials and methods

### 2.1. Materials

Native biles of gallstone and non-gallstone patients were received from Beilinson (Petach Tikva) and Rambam (Haifa) Medical Centers and maintained at room teperature with no additional treatment. Gallbladder bile samples were aspirated by puncture prior to ligation or manipulation of the gallbladder. Hepatic bile was collected from an indwelling T-tube catheter after cholecystectomy. All samples were obtained following informed consent of the patients.

### 2.2. Video enhanced light-microscopy (VELM)

Bile specimens were observed at 25°C by an Olympus BH-2 light microscope at the same time of cryo-TEM specimen vitrification.

Samples (200 μl) were put on a micro concavity slide (Clay–Adams) and sealed with a cover glass to avoid evaporation. The light microscope operated in the differential interference contrast (DIC) mode [13], and was connected to a Cue-4 (Galai) image analysis system for contrast enhancement. Images from the monitor screen were recorded on a Kodak TMX film and developed with 1:4 diluted T-MAX developer (Eastman Kodak Company, Rochester, NY).

### 2.3. Cryo-transmission electron microscopy (cryo-TEM)

Vitrified specimens for transmission electron microscopy were prepared in a controlled environment vitrification system (CEVS), as previously described [14]. In short, a bile sample (1 ml) was equilibrated in the CEVS to 23°C at 100% relative humidity. A drop (5 μl) was applied onto a holey carbon film, supported on an electron microscopy copper grid, held by the CEVS tweezers. The sample was blotted and immediately plunged into liquid ethane at its freezing point (90 K). The vitrified sample was then stored under liquid nitrogen (77 K). Specimens were loaded into a cooling-holder (model 626, Gata Inc., Warrendale, PA) and transferred to the electron microscope (JEOL JEM 2000FX). Specimens were equilibrated in the microscope at about 100 K. The TEM was operated at 100 kV accelerating voltage, in a low-dose mode to minimize electron beam radiation damage. Images were recorded at a nominal underfocus of 4 μm on Kodak SO-163 film and developed in full-strength D-19 developer (Eastman Kodak Company, Rochester, NY) for maximum electron speed.

## 3. Results

The microstructures formed during cholesterol crys-

tallization in native bile were studied using both VELM and cryo-TEM. Gallbladder and hepatic bile samples of gallstone and non-gallstone patients were examined as a function of time during ex-vivo incubation. Fresh samples (age less than one day) were vitrified as cryo-TEM specimens and observed by light microscopy at the same time. Some of these samples were further examined as a function of time. Samples aged from two to six weeks were observed only by light-microscopy.

### 3.1 Gallbladder bile

Light-microscopy of a fresh, non-centrifuged gallbladder bile sample from a gallstone patient revealed the coexistence of plate-like (P) and apparently hollow elongated (H) structures (Fig. 1a). None of these large structures were observed in the cryo-TEM micrographs (for reasons discussed below). Instead, cryo-TEM (Fig. 1b) revealed the coexistence of vesicular particles of a diameter of approximately 40 nm (V), and predominant small spheroidal particles of about 6 nm diameter (M). These particles resemble in structure and size vesicles (V) and micelles (M) that have been previously observed in a model system composed of egg-phosphatidylcholine/sodium-cholate [15].

Gallbladder biles, obtained from four other patients, were centrifuged (4,000 × g, 10 min, 23°C) prior to specimen preparation and similar aggregates (V,M) were observed by cryo-TEM. In some of these samples additional aggregates appeared (Fig. 1c,d). Of note are (i) electron dense, elongated structures (E) of 300–450 nm length and 30 nm width, and (ii) lighter contrast, thin structures (L) exceeding 2 μm in length.

Fig. 2 presents light-micrographs of gallbladder bile samples from three gallstone patients, as observed after three-to-six weeks of incubation at room temperature. These micrographs revealed a wide range of coexisting structures with helical, spiral, needle-like and plate-like morphologies. Noticeably, there were marked differences between the structures observed in the various bile samples: in one patient helical structures predominated (Fig. 2a,b), whereas in other samples most particulate material was either needle- or plate-like (Fig. 2c,d). Similar microstructures have been found in bile models made of bile-salts, phosphatidylcholine and cholesterol, and shown to represent cholesterol crystals [8,9]. While this polymorphism can not be used for evaluation of the mechanism of gallstone formation, the mere observation of similar habits in bile models and native bile lends further support to the validity of studies performed in model systems.

### 3.2 Hepatic bile

Hepatic bile is more supersaturated with cholesterol, but less concentrated than gallbladder bile. Hence, nucleation may be more rapid but crystal growth is likely to be slower than in gallbladder bile. We therefore found
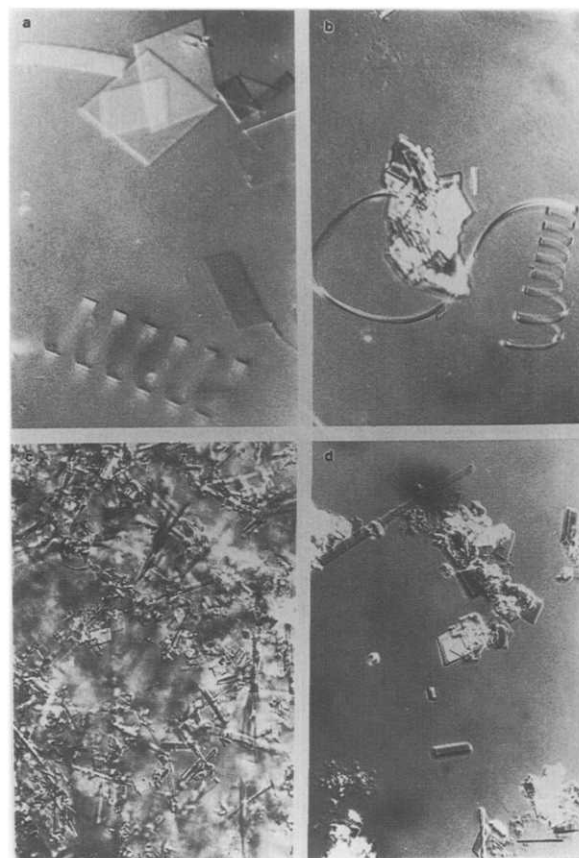


Fig. 2. Light-micrographs demonstrating polymorphism in aged gallbladder bile samples: (a,b) six weeks after aspiration from one gallstone patient; (c,d) six and three weeks after aspiration, from two other gallstone patients. Bar = 20 μm (a,b,d), and 40 μm (c).

it interesting to study continuously the structures present in hepatic bile as a function of time after bile collection. As expected, no evidence for crystal formation was revealed by light microscopy over a period of three weeks. However, cryo-TEM of a sample vitrified within one day after collection revealed the coexistence of several structures (Fig. 3a): spheroidal micelles (M), vesicles (V) and elongated structures (E). The vesicles observed differed in size and shape from those observed in gallbladder bile (Fig. 1b). The diameters of the vesicles were 100–250 nm. Some of these vesicles were non-spherical. Furthermore, in some of the vesicles, part of the bilayer was missing, indicating a process of vesicle opening. The elongated structures (E), resembling those seen in gallbladder biles, are possibly microcrystals that coexist with the vesicles and micelles.

The same type of structures were seen in specimens prepared two days later (three days after collection). In addition, cryo-TEM revealed a new type of structure, cylindrical tubes (165 nm in diameter) (Fig. 3b,c, T). Similar structures were observed by cryo-TEM in a bile model system [8], known to produce filamentous cholesterol crystals (unpublished results). These findings are
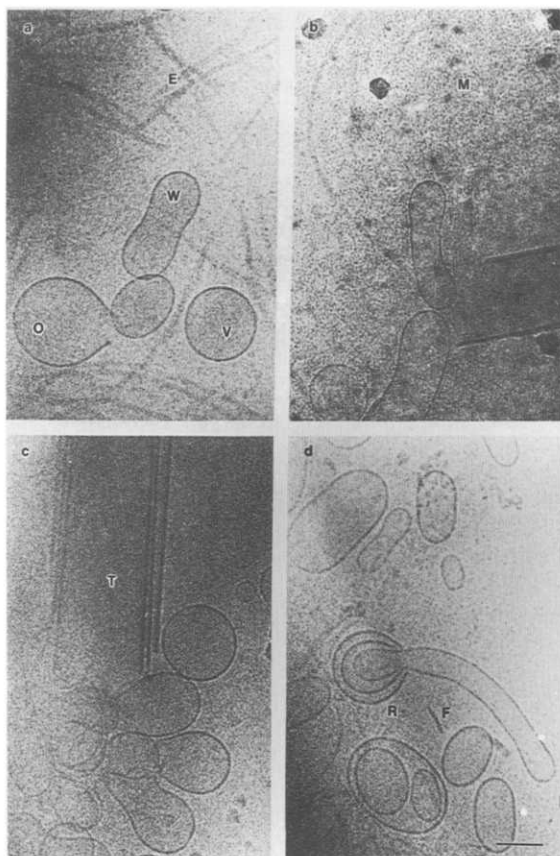
Fig. 3. Cryo-TEM micrographs of hepatic bile samples vitrified (a) one day, (b) three days, (c) six days, and (d) nine days after bile collection. Note spherical (V) and non-spherical (W) vesicles, some of which are open (O) or contain more than one bilayer (R). In addition, edge-on projections of lamellar fragments (F), small spheroidal micelles (M), electron dense elongated structures (E), and tubes (T) can be seen. Bar = 100 nm.

consistent with a two-dimensional projection of a tube-like crystalline structure, close to the resolution limit of light microscopy.

Six days after collection, cryo-TEM of hepatic bile also revealed concentric tubes together with closed and open vesicles (Fig. 3c).

Nine days after collection, micrographs showed large vesicles (Fig. 3d), some made of more than one bilayer (R) and some non-spherical. In addition edge-on projections of lamellar fragments were observed (F).

## 4. Discussion

In this study VELM and cryo-TEM were used to study native human bile samples in an effort to reveal the structures present at early stages of cholesterol crystallization in bile. Cryo-TEM provides direct images of microstructures in the 4–250 nm size range [16]. Larger structures do not appear due to the blotting process during specimen preparation. The light microscope, operat-

ing in a differential interference contrast mode (DIC) [13] is limited to a size range of 100 nm to 500 $\mu$m, but allows visualization of the bulk and appearance of larger structures. Thus, combination of these two methods provides a comprehensive non-perturbing tool for structural studies within the whole size range of 4 nm to 500 $\mu$m [16].

In this study, micellar aggregates have been visualized in human bile (both gallbladder and hepatic) for the first time. Furthermore, the presence of vesicles previously observed after negative staining [17] or freeze-fracture fixation [18] has been confirmed by this less perturbing technique [19]. In addition, the morphology of vesicles could be seen in greater detail. For the first time, non-spherical as well as open vesicles were revealed in native human bile. Such structures have been previously described in model system studies of vesicle solubilization [15] and fusion [20]. The presence of these aggregates in native bile demonstrates the dynamic nature of the metastable aggregative forms in bile. Whether these structures bear any relation to some recently described lamellar structures in bile [21], remains to be studied. In addition, cryo-TEM revealed microstructures: tubes, lamellar fragments, and possibly early forms of crystallites that have not been previously described in bile (see Fig. 1). Further studies are required to fully characterize and evaluate the significance of these new structures. Nevertheless, the mere visualization by cryo-TEM of electron-dense, probably microcrystalline structures at a stage when no crystalline structures can be seen by light microscopy (Fig. 3), indicates that cholesterol crystallization in bile is not necessarily a cooperative process. Hence, the real nucleation time may be significantly shorter than the nucleation time based on light microscopic observation.

Ex-vivo incubation of bile is the best available method of distinguishing between lithogenic and non-lithogenic native biles [6]. Both gallbladder and hepatic biles from gallstone patients have been shown to exhibit rapid ex-vivo cholesterol nucleation and crystallization [22]. Hence, investigating ex-vivo biliary crystal formation is clearly of clinical significance. Moreover, since gallbladder hypomotility and bile stasis have been implicated to be pivotal in gallstone pathogenesis [23,24], a prolonged time-frame of events (days to weeks) has to be considered. All these aspects underscore the potential clinical relevance of the findings reported in the present study.

In conclusion, cryo-TEM adds a new dimension to bile and gallstone research in addition to the previously employed techniques, such as QELS [25], X-ray diffraction [8], ESR [26], and NMR [27]. The combination of cryo-TEM and light microscopy leads to a more complete characterization of bile samples in general, and specifically in relation to cholesterol crystallization. This may have an important impact on future research of bile, and contribute to a better understanding of gallstone formation in man.

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