Journal of Crystal Growth 401 (2014) 242–247

Contents lists available at ScienceDirect

Journal of Crystal Growth

journal homepage: www.elsevier.com/locate/jcrysgro

Crystal growth of cholesterol in hydrogels and its characterization

J. Manuel Bravo-Arredondo [a], A. Moreno [b,*], M.E. Mendoza [a,**]

[a] Instituto de Física, Benemérita Universidad Autónoma de Puebla, Av San Claudio y 18 sur, 72570 Puebla, México
[b] Instituto de Química, Universidad Nacional Autónoma de México, Circuito Exterior C.U., México D.F. 04510, México

ARTICLE INFO

Available online 9 January 2014

Keywords:
A1. Bile stones
A1. Cholesterol crystals
A1. Crystal growth
A1. Gels
A1. Polymorphic transition

ABSTRACT

In this work, we report the crystallization of cholesterol in ethanol solution and in three different hydrogel media: tetramethyl orthosilane, sodium metasilicate, and poly(vinyl)alcohol, whose structures are similar to the gel-like polymer structure of mucin, which is found in the mucus present in bile stone formation. The monohydrated triclinic phase was identified in all the samples by means of X-ray powder diffraction. The characteristic polymorphic crystalline transition of the anhydrous cholesterol was detected by differential thermal analysis and modulated differential scanning calorimetry only in crystals grown in ethanol, sodium silicate, and tetramethyl orthosilane. Finally, hysteresis of the phase transition temperature was measured by modulated differential scanning calorimetry in crystals grown in ethanol. The biological implications of the crystallization of cholesterol for bile stones formation are discussed in the last part of this contribution.

1. Introduction

Nowadays, the challenge within the pathological aspects of biomineralization processes is to understand and to comprehend the physicochemical mechanisms of many of the diseases in which biominerals are involved. These mineralization processes are cardiovascular precipitation of hydroxyapatite [1,2], renal diseases [3], pancreatic diseases [4,5], and gallbladder stones [6,7]. For the particular case of *in vivo* crystallization, bilirubin and cholesterol stones in the gallbladder usually happen at very high supersaturation. They not only interact with some biological macromolecules in human beings, but also play an important role in modern diseases in industrialized countries [8,9]. Moreover, the molecule of cholesterol is also involved in several immunological processes as well as in a variety of pathologies in humans [10]. Crystallization of cholesterol is rather difficult due to its lack of solubility in aqueous solutions [11]. Some approaches, focused on new and original strategies, to obtain single crystals of cholesterol [12,13] have already been published.

From the structural point of view, cholesterol crystals have a monohydrate triclinic structure with a space group P1, having eight-unit-formula per cell, at room temperature with cell parameters $a=14.172(7)$ Å, $b=38.443(18)$ Å, $c=10.481(5)$ Å, $\alpha=93.88(4)^\circ$, $\beta=90.67(4)^\circ$ and $\gamma=117.81(4)^\circ$. However, cholesterol crystals have also a triclinic anhydrous phase, described also by the space group P1. There are sixteen-unit-formula per cell for this phase and its cell parameters obtained at 37 °C are $a=27.565(10)$ Å, $b=38.624(16)$ Å, $c=10.748(4)$ Å, $\alpha=93.49(3)^\circ$, $\beta=90.90(3)^\circ$ and $\gamma=117.15(3)^\circ$ [14].

The reported crystal habit for the monohydrate phase is the plate-like one, which is structurally generated by the assembly of filaments that developed gradually in needles, helical and tubular microstructures to yield finally the plates [15], whereas for the anhydrous phase a needle-like habit is commonly found [16].

It is known that cholesterol crystals undergo several phase transitions in the anhydrous phase such as the polymorphic transition at 39 °C and the crystalline to liquid phase transition at 151 °C. The monohydrate phase shows three reversible endothermic transitions: the first at 86 °C, the second at 123 °C, and the third at 157 °C [17]. The existence of these phase transitions, closely related to polymorphism in different pharmaceutical drugs, is a well-known process [18]. Also isosymmetric phase transitions associated with intergrowths have been demonstrated for some triclinic minerals [19].

In this work, we present the results of the crystallization of cholesterol in three different types of hydrogels: tetramethyl orthosilane, sodium metasilicate, and poly(vinyl)alcohol, whose structures are similar to the gel-like polymer structure of mucin, which is a glycoprotein commonly observed in bile. Mucin is found in the mucus present in bile stone formation in humans. Crystallization of cholesterol was also done in ethanol solution as a control experiment. In addition, we present the results of cholesterol characterization by X-ray powder diffraction, polarized light microscopy, differential thermal analysis, and modulated differential scanning calorimetry. In the final part, the biological

* Corresponding author. Tel.: +52 55 56224467; fax: +52 55 56162217.
** Corresponding author. Tel.: +52 222 229 56 10; fax: +52 222 30 229 56 11.
E-mail addresses: carcamo@unam.mx, abel.moreno@mac.com (A. Moreno), emendoza@ifuap.buap.mx (M.E. Mendoza).

0022-0248/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jcrysgro.2013.12.030

implications of the crystallization of cholesterol in bile stones formation are discussed.

2. Materials and methods

2.1. Cholesterol crystal growth

Cholesterol crystals were obtained in ethanol by dissolving 0.125 g of cholesterol powder (Sigma-Aldrich, 98%) in 10 mL ethanol (Sigma-Aldrich. 99.8%). Afterwards, the solution was allowed to evaporate for 2 weeks at a constant temperature of 4 °C. The crystal growth in gels was performed at a constant temperature (18 °C) in three different types of gels: 1) tetramethyl orthosilane (usually named TMOS, Fluka, USA, 99%), 2) sodium metasilicate (SS, Sigma, USA, 97%), and 3) poly(vinyl)alcohol (PVA, Sigma, USA, 99%). A gel of tetramethyl-orthosilane (10% [v/v]) was prepared by using the hydrolysis and polycondensation methods [20]. After completion of the gelling process, 5% (w/v) cholesterol/ethanol solution was poured onto this gel in order to incorporate cholesterol in gel's network. For gels of sodium metasilicate, the neutralization method consisted of a solution of acetic acid (1 M) titrated with sodium metasilicate of 1.06 g/mL density value, to reach a pH of 7.0 [21]. A 12% (w/v) poly(vinyl)alcohol (PVA) concentration was prepared by the stirring/heating/freezing method. This PVA gel is usually prepared as agarose gels at 90 °C by the thawing–cooling down method [22,23]. Once the temperature is reduced, ranging from 5 °C to 10 °C, the PVA polymerization process starts, and the gel is formed. In this process, we first replaced water from the hydrogel and, then, we poured ethanol onto the gel medium and allowed it to diffuse for a week. After this time, ethanol was carefully decanted and replaced with 5 mL of an ethanol solution of 0.5% (w/v) cholesterol, and was allowed to rest for another week, at the end of which, we added pure water onto the gel to crystallize cholesterol. This procedure was repeated for each of the three different types of gels. In all of these gels, crystals were first observed at the end of the second week. Afterwards, the cholesterol crystals were carefully harvested from each medium and stored in sealed vials at 4 °C. Before characterizing these crystals by different techniques, they were dried at room temperature (26 °C) for at least 20 h.

2.2. Cholesterol characterization

For X-ray powder diffraction experiments an Empyrean diffractometer system (PANalytical, Netherlands) was used, with Cu Kα radiation, a double monochromator, a Soller slit = 0.04, and an XCelerator detector. Step size = 0.004° in 2θ; X-ray datasets were collected at room temperature. Differential thermal analysis (DTA) and thermogravimetric analysis (TGA) were done simultaneously (SDT 2960, TA Instruments, USA), ranging from 30 °C to 500 °C, at a scan rate of 5 °C min^{-1} (an inert atmosphere of N_2 was used with flux of 100 cm^3 min^{-1}). Cholesterol crystals were placed in an alumina pan, using alumina powder as reference. Modulated differential scanning calorimetry spectra (MDSC) were obtained at temperature ranging from 25 °C to 90 °C (MDSC 2020TA Instruments, USA); each sample was introduced, this time, in an aluminum pan, using an empty pan as reference. The experiments were done in an atmosphere of N_2 with a flux of 100 cm^3 min^{-1}, with an underlying heating rate of 2 °C min^{-1}, modulation cycle time of 30 s, and a peak modulation temperature amplitude of 0.159 °C. Crystal habit of specimens was observed using a stereomicroscope (Discovery V12, Zeiss, Germany), zoom objective lens, and ocular lens 10 ×. Changes of birefringence associated with the phase transition were studied by thermomicroscopy (Universal polarized light microscope, Zeiss, Germany), composed of a heating stage (Leitz, Germany) in transmission mode, and crossed analyzer–polarizer lens, objective lens 16 × and ocular lens 10 ×.

3. Results and discussion

3.1. Cholesterol growth (in ethanol and hydrogels) and crystal habits

Transparent plate-like crystals were grown in all cases. Crystals were selected and harvested after 2 weeks of growing (Fig. 1). As previously mentioned in Introduction, they showed the typical crystal habit reported for monohydrated cholesterol phase.

The mean size of cholesterol crystals grown in ethanol was in the order of millimeters, whereas for crystals grown in sodium silicate (SS), poly(vinyl)alcohol (PVA), and TMOS gels this value was in the order of microns (Table 1). Generally speaking, crystals grown in gels are bigger in size compared to those grown in

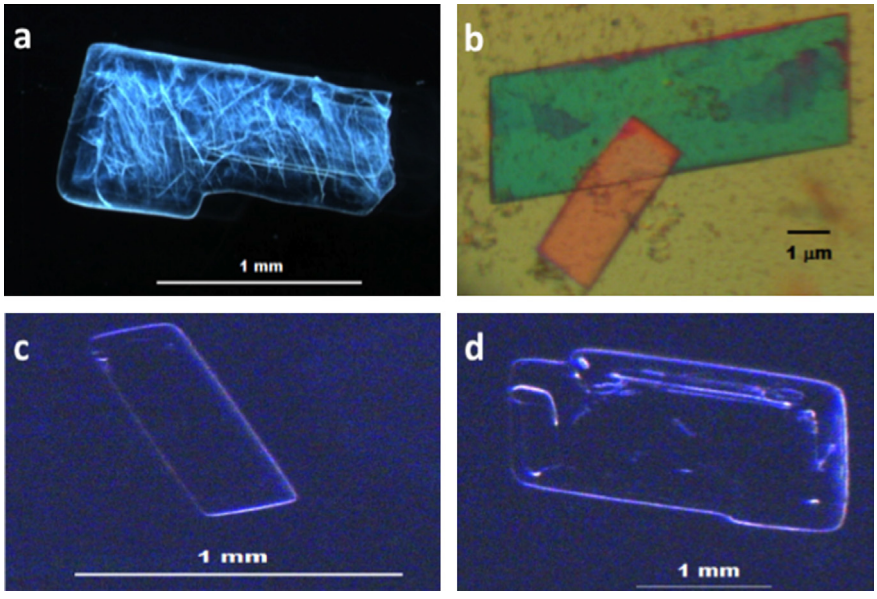

Fig. 1. Cholesterol crystals grown after 2 weeks in (a) ethanol, and three different hydrogels: (b) tetramethyl orthosilane (TMOS), (c) sodium silicate (SS), and (d) poly(vinyl) alcohol (PVA). All the crystals were transparent and colorless; the color in photographs is due to the piece of paper placed under the crystal to obtain a better contrast.