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Composites Comprising Cholesterol and Carboxymethyl Cellulose

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Abstract

Whereby cholesterol presents one of the major fatty substances in human body, carboxymethyl cellulose is a water-soluble derivative of cellulose, the most abundant dietary fiber. Whereas on one hand *in vivo* precipitation of cholesterol is the major cause of atherosclerosis, dietary fibers are on the other hand known for their ability to clean the fatty plaque deposited on intestinal pathways, and prevent its build-up in other critical areas within the organism. In this work, a method for the preparation of a composite material comprising cholesterol and carboxymethyl cellulose from 1-hexanol/water biphase mixtures is reported. Specificity of the interaction between the composite components in the given conditions of synthesis inhibits the tendency of solid cholesterol to adopt typical plate- or needle- shaped morphologies. Instead, control of the thixotropic behavior of the constituent polymer phase leads to the formation of bubbling, multi-layered cholesteric films. In view of the major illnesses that involve biological precipitation of cholesterol crystals, these findings may be considered as pointing towards the interactional specificity of potential chemotherapeutic and/or nutritional significance. Scanning electron microscopy, thermal and diffractometric analyses were performed as parts of the characterization of the prepared material.

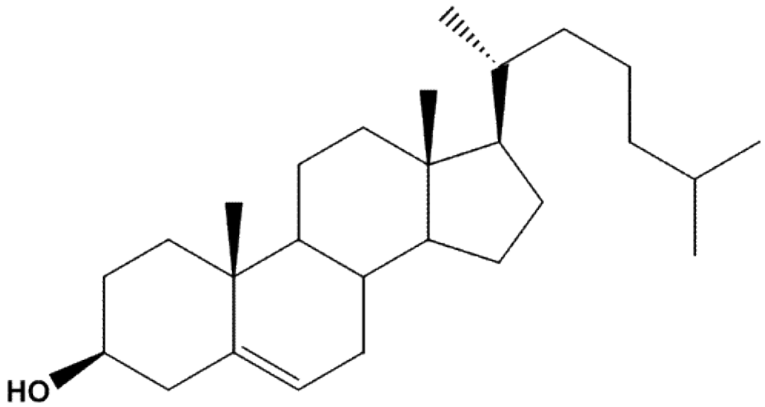
1. Introduction

Cholesterol is the most abundant steroid in mammalian species. It is also an essential biomolecule for the functioning of human body, where it is involved in maintaining the flexibility and proper transport balance of cellular membranes, and acts as a precursor for the synthesis of bile acids and steroid hormones. Its abundance in the body is governed by the levels of its dietary intake and internal production. On the other hand, its efficient transport and utilization throughout the body depends on the solubilizing action of lipid bilayers, micelles and vesicles in bile and lipoprotein complexes in blood. When an unbalanced state between these two effects occurs, the conditions for its precipitation *in vivo* become favored. Thus it forms gallstones and atherosclerotic deposits, and eventually endangers health of individual organisms. Understanding the physico-chemical pathways involved in crystallization and deposition of cholesterol, as has been the aim of this work, therefore presents one of the important contemporary challenges with far-stretching potential benefits.

It has been long known, for instance, that solvents affect morphology and structure of cholesterol crystals[1,2]. However, in all previous cases of re-crystallization in solution, either plate- or needle-shaped cholesterol crystals (respectively corresponding to monohydrate and anhydrate modifications[3], although this is a rule with exceptions[4]) were obtained. Spiral, helical and tubular morphologies of cholesterol crystals have been also observed, but only as transient microstructures that physiologically evolve into

thermodynamically stable cholesterol platelets[5,6]. Beside plates, needles, arcs and spirals as forms of cholesterol crystals[7,8], different cholesterol derivatives have recently been observed to self-assemble into tubular structures[9]. Nevertheless, the typical observations of platelet-shaped cholesterol crystals are consistent with the well-known crystallographic fact according to which the crystals of 3-hydroxy steroids and their hydrates show a tendency towards adopting double layer crystal structure with end-for-end arrangement of approximately parallel molecules[10]. Whereas cholesterol monolayers have never been observed yet[11], the double-layer ordering mechanism explains the formation of typical platelet morphologies during cholesterol precipitation. As a matter of fact, layered structural arrangement of cholesterol precipitates can be considered as a natural mechanism for maximization of cholesterol coagulates build-up in arteries, gallbladders and intestinal lumens without constraining the natural flow of life-sustaining fluids. On the other hand, relatively high free surface energy of plate-shaped crystallites (comparing to spherical particle shapes that correspond to minimal surface energy of multi-atomic or multi-molecular aggregates[12]) favors their adhesion on membranes and walls of the respective organs and tissues, decreasing their susceptibility towards efficient dissolution and removal by means of proper cleansing agents.

Discovery of the ways to achieve a disruption of the chemical mechanisms that lead to crystallization of cholesterol precipitates in layered forms would present a significant step in the development of chemotherapeutic and/or nutritional methods for fighting against cholesterol coagulates *in vivo*. The only reported case of the formation of spherical cholesterol particles so far involved the use of spray-freezing[13]. However, although such an approach is important in understanding the physical mechanisms of cholesterol dissolution and crystallization, it does not possess direct chemotherapeutical significance.

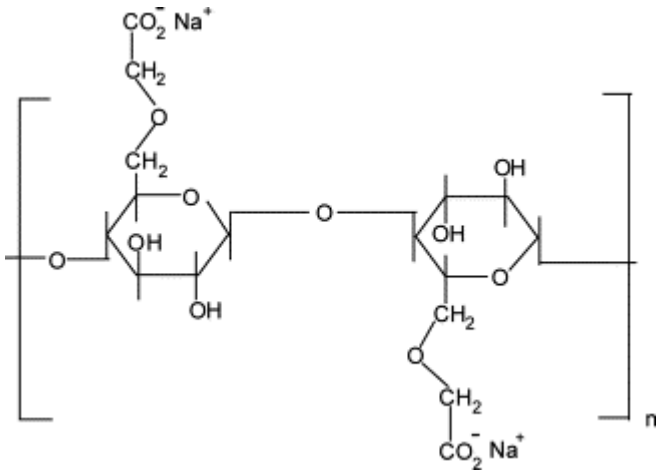


Chemical structure of a cholesterol molecule

Numerous investigations of the effects of various chemical environments on the crystallization and dissolution of cholesterol are reported in the literature. For example, the effects of solvent type[4], non-solvent phases[14], temperature[1], pH[15], electrolytes[16], the dynamics of solvent systems[17], magnetic field[18], mineral[19] and protein[20] substrates and co-existing phases[21,22] (such as hydroxyapatite deposits, often found interspersed within cholesterol layers in atherosclerotic plaques[23,24]), model bile composition[25-28], various medicinal plants[29] and synthetic biochemical compounds (including phospholipids[30], cholic acid[31] and other sterols[32]) on cholesterol morphology and crystallization

mechanisms were previously acknowledged. The majority of these studies suggested a significant sensitivity of cholesterol crystallization processes on the limiting conditions of imposed environments. Such an observation is mostly consistent with the biological nature and multi-functional character of cholesterol molecules. It has also provided a starting point for the actual inquiry into morphological dependencies of cholesterol crystallites upon variously set conditions of its re-crystallization.

Dietary fibre is, on the other hand, well-known for its ability to lower blood cholesterol levels[33,34]. Soluble fibre may, for example, in the small intestine form a gel which binds excessive blood cholesterol delivered by the action of liver, and eventually expel it from the body. As an aqueous-soluble derivative of cellulose, an important cleansing agent against the intestinal plaque, carboxymethyl cellulose (CMC) was chosen as an additional component for the procedures of cholesterol precipitation, attempted at overcoming the inherent and seemingly irresistible tendency of cholesterol crystallites to adopt platelet morphologies. Beside its application as surfactant, thickener, water-binder, disintegrant, lubricant, polymer flocculant, phase and emulsion stabilizer, suspending agent and dirt-attractive, anti-redeposition agent in detergent chemistry, CMC is also used as a chelating agent in precipitation of diery lipoproteins and the corresponding fabrication of cholesterol-free food products[35]. Viscous CMC has even proven to be a more effective agent for decreasing plasma cholesterol levels through the digestive usage comparing to nonviscous cellulose[36,37]. Similar to cellulose itself, CMC was noticed to decrease liver cholesterol concentrations when introduced in the diet[38]. It has also been used as a hemostatic agent, promoting cellular adhesion and healing from injuries[39,40]. It has lately been used as an enteric polymer, coating pharmaceutical dosage forms[41] and magnetic nanoparticles[42], with a potential application in targeted drug-delivery products. CMC has recently been employed as a pore-sealing additive in cement chemistry, improving strength, fracture toughness, corrosion resistance and durability of the final composite products[43], as well as a biodegradable, low-cost and eco-friendly binding agent in graphitic anodes for lithium ion batteries[44]. It has also been used as an active ingredient in the production of artificial tears[45]. Being a mild acid, CMC may offer an aid in the suppression of electrostatic repulsion present at naturally slightly alkaline pH (~ 7.5) of cholesterol solutions, at which *in vivo* deposition of cholesterol usually proceeds[46]. Commercial CMC normally comprises a certain amount of sodium carboxymethyl groups which promote water solubility, and which contribute to an increase of the ionic strength of solution. In accordance with DLVO theory, this effect promotes the contraction of charged layers surrounding individual suspended particles, leading to additional suppression of electrostatic repulsion at large distances.



Molecular structure of sodium carboxymethyl cellulose

2. Experimental

2.1. Materials

The chemicals used in the presented procedures of synthesis were: cholesterol (99+%, *Alfa Aesar*), carboxymethyl cellulose (M_w = 250,000 g/mol, *Polysciences, Inc.*), ethanol (anhydrous, 99.5 %, *Pharmco*), and 1-hexanol (*J. T. Baker*).

2.2. Synthesis

Twelve milligrams of CMC were poured in 10 ml of 1-hexanol, with the subsequent addition of 7 ml of distilled water. Ultrasound treatment was used until complete dissolution of CMC particles in biphase aqueous/alcoholic liquid. Twenty milligrams of commercial cholesterol were then introduced in the previously prepared CMC solution comprising immiscible phases of organic solvent and water and further treated with ultrasound until complete dissolution of cholesterol particles. One milliliter of water and 13 ml of hexanol were subsequently introduced in the mixture, so that the hexanol-to-water volume ratio was increased twice, initiating dispersion of the solid phase throughout the water phase. A part of the liquid was taken through a pipette, poured on top of the sample carrier for SEM analysis, and left to dry in air overnight. An identical procedure in which 1-hexanol was substituted with ethanol, and with an additional rapid introduction of 5 ml of water to induce precipitation, was performed as a synthesis in a miscible water/organic solvent, providing an experimental comparison to the aforementioned synthesis in immiscible, biphase water/organic solvent medium.

2.3. Characterization

Morphology of the dried cholesterol samples was examined using scanning electron microscopy (SEM) and field-emission scanning electron microscopy (FE-SEM), whereby crystallinity of the prepared powders was investigated by means of X-ray diffractometry (XRD). Thermogravimetric analyses (TGA) and differential scanning

calorimetry (DSC) were performed in air, from 50 to 700 ^{o}C and from 25 to 250 ^{o}C, respectively, at the scanning rate of 5 ^{o}C/min.

3. Results and discussion

3.1. Morphology of the cholesterol/CMC composite

Complex morphology of cholesterol/CMC composite prepared as described in section 2.2 may be discerned from the following SEM images. Topographically smooth, extremely flat and transparent film, easily peeled off as a whole, is obtained after a sufficient aging/drying treatment (~ 7 days), as shown in Figs.1-2. However, shorter aging times and the deposition of smaller quantities of the sample (hence preventing the formation of continuous, millisized film structure by depositing the precipitate in form of micro-sized "islands") results in the formation of "sea shell"-shaped cholesterol/CMC aggregates, having few tens of microns in diameter each, presented in Fig.3. It is worth noting that cholesterol precipitates are typical of being composed of many different morphologies, even after many days of incubation[47].

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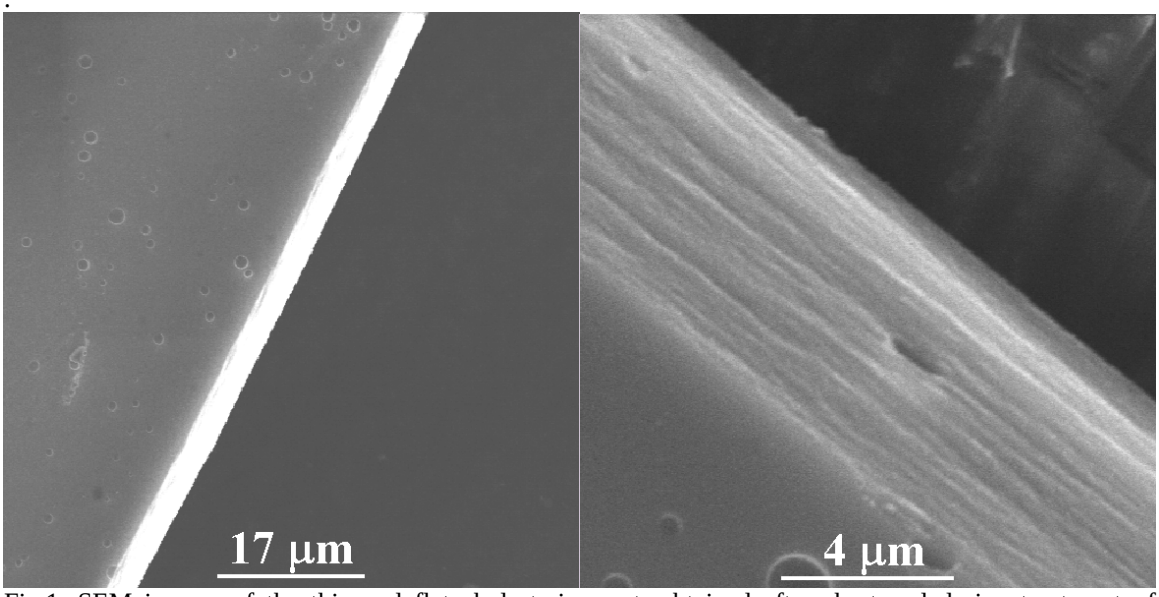

Fig.1. SEM images of the thin and flat cholesteric crust, obtained after shortened drying treatment of cholesterol/CMC composite.

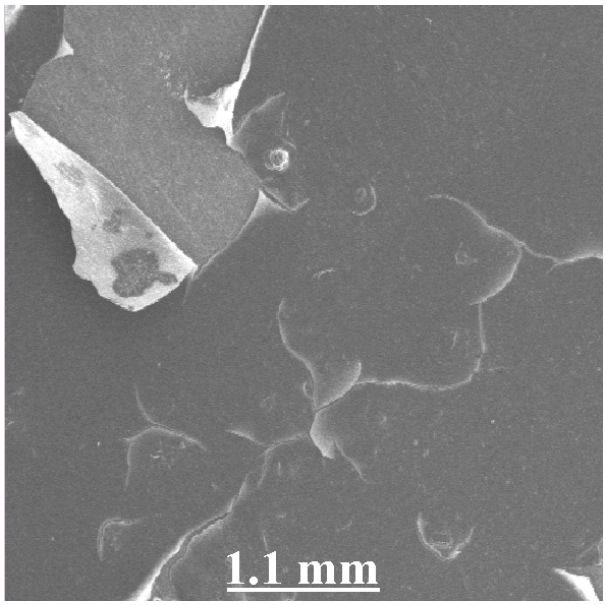

Fig.2. SEM image of the peeling cholesteric crust obtained after 6 days of aging of the cholesterol/CMC composite

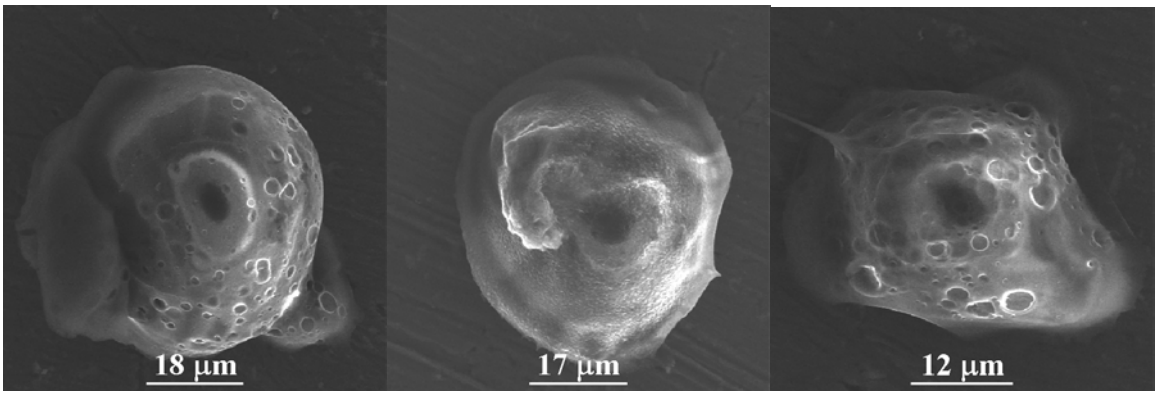

Fig.3. SEM images of cholesterol/CMC composite aggregates, observed immediately upon their precipitation.

The mechanism of the formation of “sea shell”-shaped cholesterol/CMC aggregates presumably comprises the precipitation of plate-shaped cholesterol deposits on the surfaces of CMC fibers or granules at first. The subsequent swelling of the polymer phase takes place, during which compositional and morphological integrity results, together with the formation of surface bubbles, pores, pits and holes. The later deswelling of CMC molecules (i.e. contraction of molecular aggregates of the polymer phase followed by the loss of absorbed water) from beneath the flat, multi-layered cholesterol deposits in the course of the drying treatment may initiate additional deepening of the holes in the already cracked cholesterol crystals. Pits on these structures obviously originate from the sudden drops in CMC viscosity during the ultrasound heating treatment, as thixotropic phenomena are involved in the process. Note that thixotropy can be defined as a property of some non-Newtonian pseudoplastic fluids to exhibit a time-dependent change in viscosity under the influence of shear. The thixotropic behavior of CMC is well-known, and regularly used in the food industry to improve the volume yield of the CMC-containing baked products by encouraging the bubble gas

formation. This process in the actual case results in the formation of erected structures (Fig.4) with either pronounced central holes (Figs.5,6) or smaller sideway pits, albeit corresponding to an overall equal amount of polymer bubbling. Partial transition of the thixotropic polymer phase into a gel-like form while keeping the suspension still at room temperature, i.e. without the influence of ultrasound agitation, might present the reason for the loss of "sea shell"-like morphological character with prolonged aging. After 6 days of aging, the dried composite takes the form of a wide and continuous leaf, as is shown in Fig.2. A layered substructure of the cholesterol/CMC crust, comprising bubbles as the artifacts of CMC swelling processes, is clearly seen from Fig.1. The way the process of bubbling underneath the precipitated cholesterol deposits proceeds over time may be seen from the sequence of SEM images in Figs.4-6.

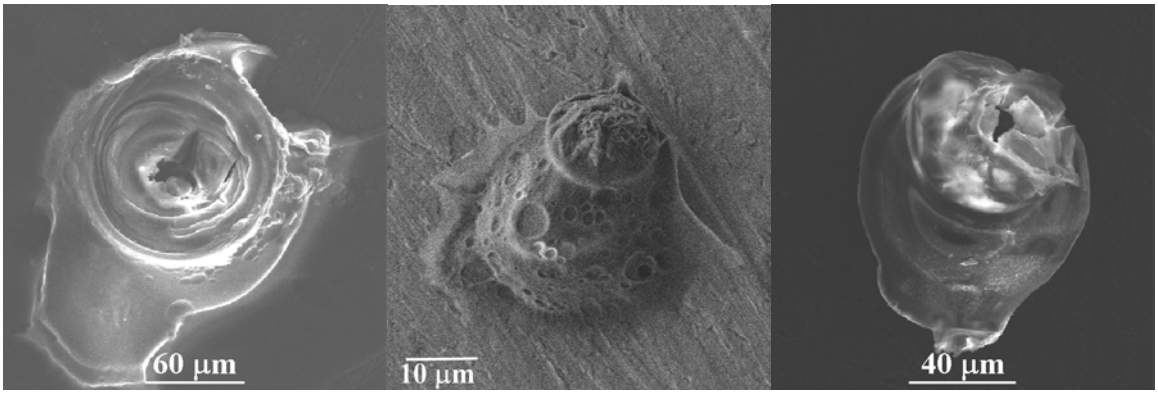

Fig.4. SEM images of cholesterol/CMC aggregate structures that correspond to the step wherein polymeric bubbling induces curving and erection of flat cholesterol deposits.

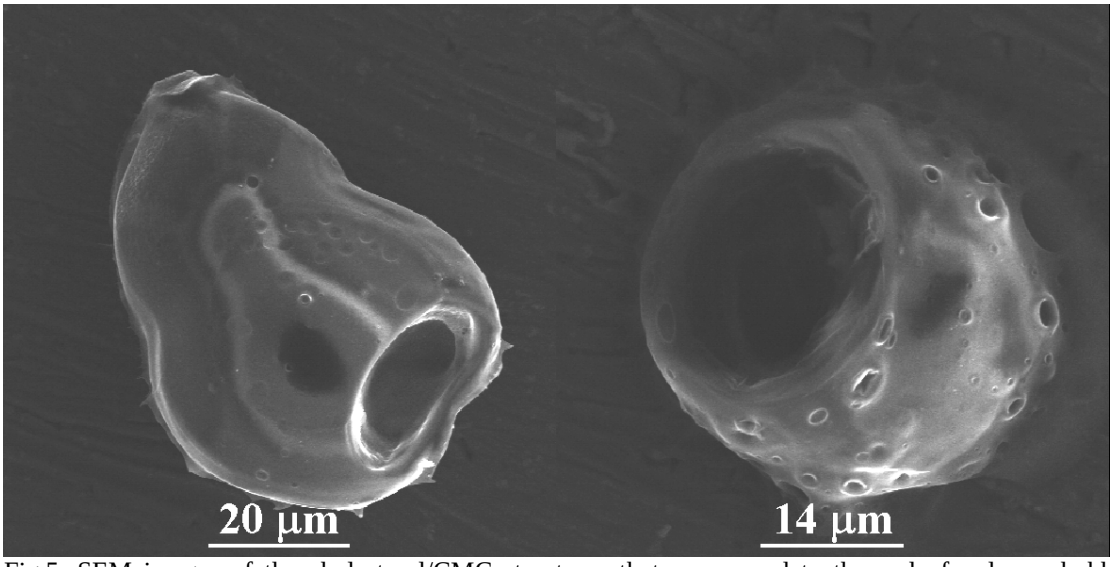

Fig.5. SEM images of the cholesterol/CMC structures that correspond to the end of polymer bubbling phase, when large central holes are formed.

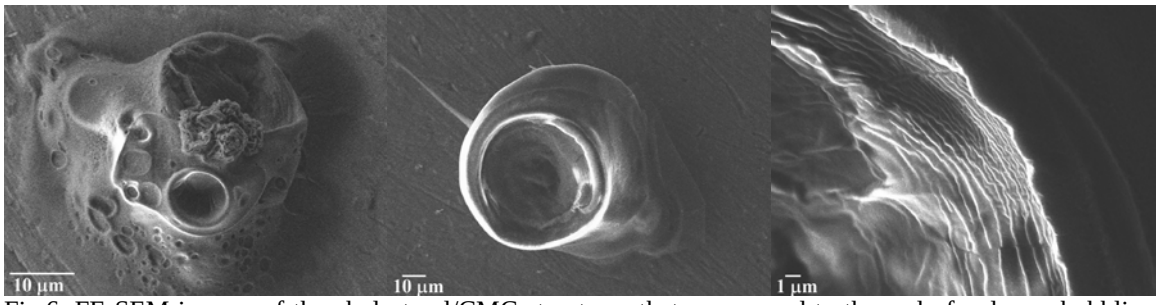

Fig.6. FE-SEM images of the cholesterol/CMC structures that correspond to the end of polymer bubbling phase, when large central holes in the structures are formed. The image on the right presents a magnified detail of the ear-like cholesterol/CMC aggregate in the middle image, showing dune-like structure of the inner "ear" surface, and thus representing an indication of the layered, cholesteric substructure thereof.

As a comparison, the synthesis in ethanol/water mixture yields narrowly dispersed and well-defined rectangular cholesterol crystallites with few microns in length, attached on the bubbling remains of the polymer phase, as can be seen from Fig.7. The shape of these particles is consistent with the way typical, monohydrate crystalline modification of cholesterol appears[3].

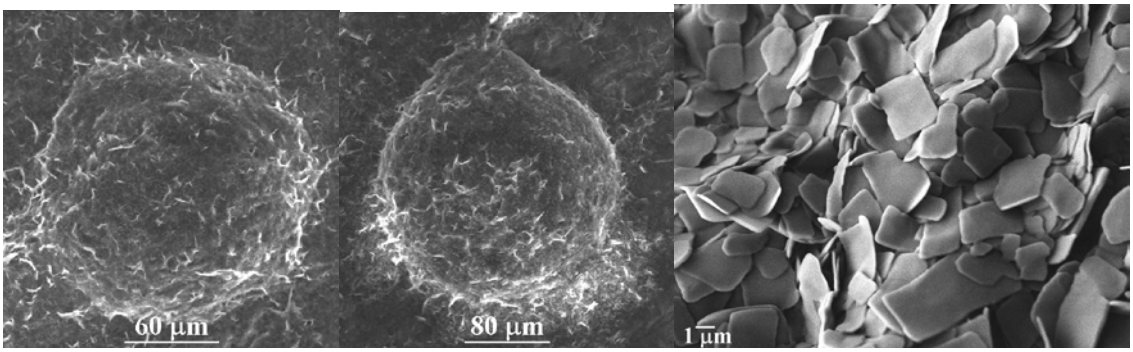

Fig.7. SEM and FE-SEM images of cholesterol/CMC aggregates prepared by abrupt supersaturation method in ethanol/water solutions (left and middle), together with their substructure composed of narrowly dispersed cholesterol platelets (right).

3.2. Diffractometric and thermal characterization of cholesterol/CMC composite

XRD patterns, TGA diagrams and DSC diagrams of the commercial, precursor cholesterol and CMC particles, and cholesterol/CMC composites prepared by employing precipitation in ethanol/water monophase and hexanol/water biphase media, are respectively presented in Figs.19-21. XRD pattern of commercial CMC provides two wide peaks at $2\theta \sim 20^{o}$ and 11^{o} (Fig.8b), that are typical of CMC[41,48] and wood pulp[49]. However, upon the incorporation of CMC in both of the investigated composite structures, amorphization of the polymer phase takes place. This is evidenced by the disappearances of the two typical diffraction lines in the XRD patterns that correspond to the composite structures. Decreased diffraction peak intensities and increased background noise present a sign that the crystalline structure of cholesterol has also been transformed to a less ordered state with its dissolution, re-crystallization and incorporation to composite structures with the co-existent polymer phase. However, whereas in the case of precipitation in monophase ethanol/water medium, intensities of reflections from all the

major crystalline faces are proportionally decreased, in case of the transparent film obtained from the biphase 1-hexanol/water mixture, the only two peaks, corresponding to interfacial, Bragg distances of d = 33.4 Å and d = 16.7 Å, are derived from cholesterol bilayer and monolayer reflections, respectively (Fig.8c). This indicates a higher level of structural synergy achieved between the components of the composite prepared in the biphase solvent/non-solvent mixture comparing to the one prepared in the monophase system. The same observation could have been previously noticed by the comparison of the corresponding morphological patterns. Whereas a powder is obtained after the final stage of drying in the former case, in the latter case a thin and highly elastic composite film is produced. More preferential orientations of individual molecules within the film structure may be responsible for the detection of only two peaks derived from cholesterol crystals. These are not the most intensive ones, although they correspond to typical bilayer and monolayer lattice distances (Fig.8c). Extraordinarily preserved short-range order (as shown by the appearance of relatively sharp diffraction peaks at lower 2θ angles) with the disappearance of long-range order in the case of cholesterol/CMC composite prepared in hexanol/water mixture (Fig.8c), suggests significant chemical and structural modifications of the typical molecular arrangement of solid cholesterol within the given composite, when compared to both commercial cholesterol (Fig.8a) and cholesterol/CMC composite prepared in ethanol/water medium (Fig.8d).

TGA diagrams for each of the analyzed samples show both cholesterol and CMC decomposition processes in the range of 200 – 300 ^{o}C. The existence of two weight loss peaks and the residual content equal to 17 % of the initial weight (Fig.9b) suggest that the decomposition of commercial CMC proceeds in at least three stages. The first peak, in the range of 200 – 240 ^{o}C, is due to decarboxylation[50] of CMC and the subsequent loss of CO_2. Whereas the total weight loss of commercial cholesterol and the composite prepared in ethanol/water mixture occurs at ~ 550 ^{o}C (Fig.9a,d), the decomposition process of both commercial CMC and the composite prepared in hexanol/water mixture (Fig.9b,c) is not finished after TGA heating to 700 ^{o}C (with ~ 20 % of the initial weight left in the pan), indicating more thermally stable structure of the latter two compounds. Non-existence of the interlinking effects between the two phases in case of the sample prepared in ethanol/water medium (Fig.7) is in contrast with the highly intertwined phases in case of the sample prepared in hexanol/water environment (Figs.1-6), and presents the reason for the observed differences in the TGA results. Pronounced interlinking effects in the latter case cause the transformation of a stepped degradation of pure CMC to a more continuous decomposition curve, which is consistent with a highly uniform final structure of the composite film. Due to highly ordered crystalline structure (as is reflected on the disappearance of CMC diffraction peaks on the composite diffractograms), and increased number of glycosidic linkages between monomer units, precursor CMC proves to be thermally more stable comparing to cholesterol/CMC composite prepared in ethanol/water mixture. However, activation of these bonds in the case of “sea shell”-shaped aggregates increases thermal stability of the CMC phase, previously decreased due to its dissolution in course of the synthesis. DSC curve of the precursor CMC also shows a thermally reversible exothermic peak at ~ 150 ^{o}C (Fig.10b), undetected on the corresponding diagrams of the composite structures (Fig.10c,d), and thus probably derived from depolymerisation reaction, that is due to the cleavage of glycosidic linkages. Disappearance of this transition on DSC curves of cholesterol/CMC composites suggests

a less ordered state of CMC molecules within the final composites, comparing to the initial CMC. The loss of the second and third stage of CMC decomposition process with the dissolution and precipitation of CMC phase, leading to its complete decomposition through the prolonged first stage (as shown on TGA) has already been noticed[42], similar as the mutual influence of decomposition ranges of individual components within grafted CMC copolymer composites[50].

Whereas the anhydrous modification of cholesterol during heating typically exhibits endothermic, crystalline polymorphic transition at 37 ^{o}C, the monohydrate form shows broad endotherm at ~ 80 ^{o}C, resulting from the loss of water molecules. The sample prepared in hexanol/water mixture is the only one showing an endothermic DSC peak at ~ 80 ^{o}C that results from the loss of entrapped water (Fig.10c). Endothermic phase transition at 37 ^{o}C, detected only in case of the commercial cholesterol (Fig.10a), indicates an anhydrous nature of the starting compound. However, it is known that prolonged drying even at room temperature transforms cholesterol crystals, independently on their precipitation environment, into their anhydrous form. The reversible endothermic peak at ~ 150 ^{o}C (Fig.10a,d), not followed by the corresponding transition on TGA curve, presents the melting point of cholesterol. It is particularly interesting that whereas the melting point of cholesterol is detected on the DSC diagram of cholesterol/CMC composite prepared in ethanol/water mixtures (Fig.10d), it is absent in case of the sample prepared in hexanol/water mixture (Fig.10c). More uniformly intertwined composite structure with a higher degree of chemical interlinks might present the reason for the shift of the melting point of the latter composite structure towards higher temperatures.

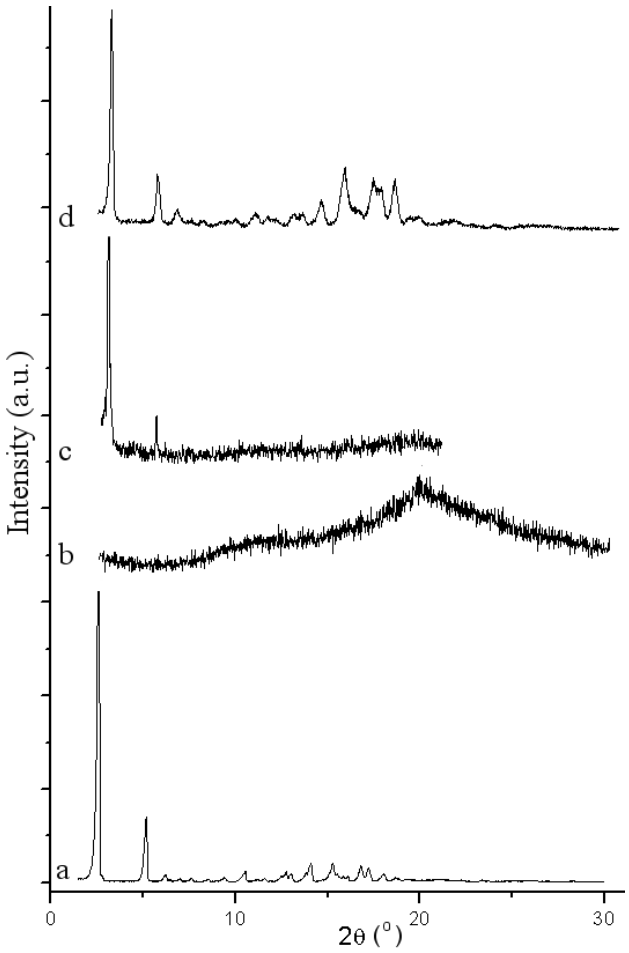

Fig. 8. Normalized XRD patterns of: a) commercial cholesterol; b) commercial CMC; c) cholesterol/CMC composite prepared in hexanol/water mixture; d) cholesterol/CMC composite prepared in ethanol/water mixture.

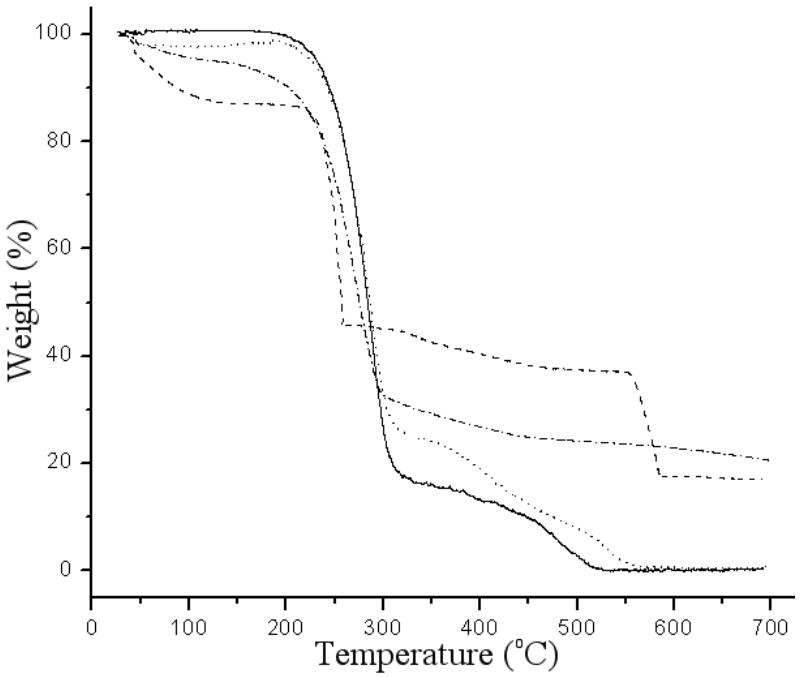

Fig.9. TGA curves of commercial cholesterol (——), commercial CMC (------), cholesterol/CMC composite prepared in hexanol/water mixture (– · – · –), and cholesterol/CMC composite prepared in ethanol/water mixture (·····).

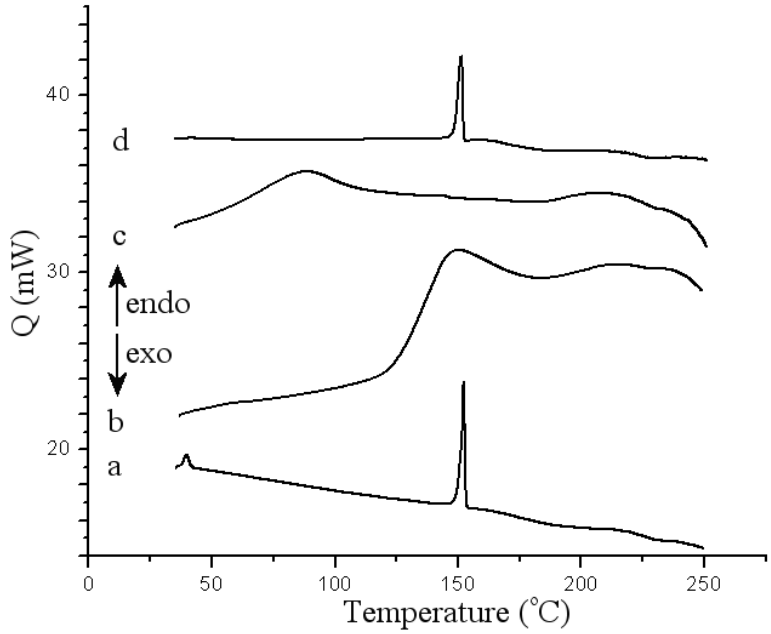

Fig.10. DSC curves of: a) commercial cholesterol; b) commercial CMC; c) cholesterol/CMC composite prepared in hexanol/water mixture; d) cholesterol/CMC composite prepared in ethanol/water mixture, drawn on a relative heat flux scale.

3.3. Discussion of the physico-chemical features of the cholesterol/CMC composite and modifications of the basic procedure of synthesis

Cholesterol crystallites precipitated in aqueous environments have been shown to possess plate faces terminated with 3-hydroxyl groups, whereby the ones precipitated in anhydrous alcoholic solutions are terminated with alkyl groups[51]. The rates and the mechanisms of dissolution of cholesterol deposits are also shown to be dependent on local surface topographies[11]. Such an inherent structural flexibility of cholesterol deposits is crucial in explaining stability and consistency in appearances of cholesterol crystals in both aqueous and organic solutions. Due to both hydrophobic and hydrophilic functionalities of cholesterol crystal surfaces, any changes at the crystal-solution interfaces during a cholesterol precipitation result in a re-balance of the crystal growth, albeit always in the direction of formation of typical, biaxially-grown morphologies. However, CMC and cholesterol have been observed to accumulate at oil-water and water-air interfaces, respectively. In that sense, note that the dispersed precipitate in the experiment described in section 2.2 forms a white curtain that extends only through the upper part of the bottom, aqueous phase. In addition to thickening or gelling the water phase, CMC molecules are known to form interfacial films, typically characterized by their strength and stability[52]. On the other hand, cholesterol as an amphiphilic molecule is known to regulate the fluidity and order-disorder behavior of the lipid part of cell membranes. It is also efficient in suppressing the swelling of water layers in certain systems through broadening or eliminating gel to liquid-crystalline phase transitions[53]. As a matter of fact, attracted by polar/non-polar interface, cholesterol particles were typically observed as segregating from simple suspensions through both sedimentation and creaming mechanisms. Planar biomolecules with sufficiently extended π-systems, that cholesterol belongs to, have found particularly wide use in interfacial self-assembly procedures. Namely, they tend to bond to surfaces in a flat-lying geometry (typical of cholesterol deposits), which allows functional groups at the molecular periphery to approach each other easily and to engage in non-covalent interactions[54]. Specific interfacial interactions that may direct the growth of cholesterol deposits may thus possess a significant and highly influential role. The use of the biphase solvent/non-solvent system in which an oil-water interfacial film acts as a self-organizing pattern for the growth of peculiar composite morphologies might, therefore, present an essential factor in favor of avoiding the crystallization of plate-shaped cholesterol crystals.

By performing the precipitation of CMC particles only (with excluding cholesterol from the procedure described in section 2.2), it was verified that the composite morphology is the product of specific interaction between cholesterol and CMC phases, and does not appear when precipitations of the separate phases are carried out. The microstructures obtained in this case are presented in Fig.11a, whereby an SEM image of the commercial CMC particles is displayed in Fig.11b. Higher transparency of the aqueous phase in the experiments that excluded the presence of cholesterol presents another indication that “sea shell”-like structures arise out of the cholesterol-CMC interaction. However, a partial swelling of the CMC phase, required for the formation of “sea shell”-shaped composites in the presence of cholesterol phase, can be witnessed by comparing Fig.11a and Fig.11b. Slight changes in the solvent/non-solvent ratio can also cause significant discrepancies in terms of the final particle morphologies. As an example, when the applied 1-hexanol/water volume ratio was 0.50 instead of 2.87 as in the experiment described in section 2.2, the microstructures presented in Fig.12 were obtained.

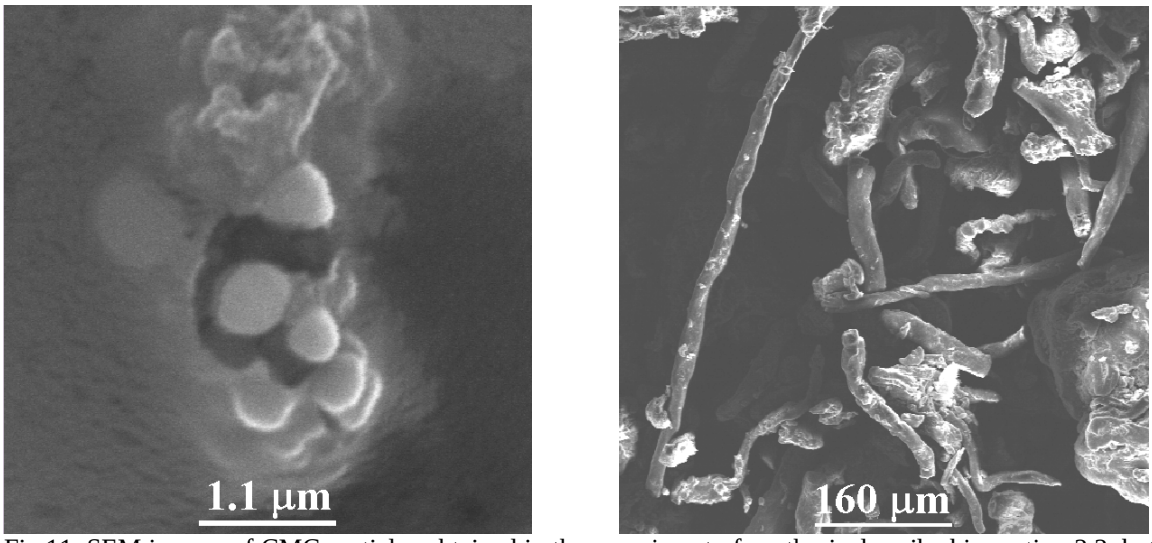

Fig.11. SEM images of CMC particles obtained in the experiment of synthesis described in section 2.2, but with excluding the presence of cholesterol (left), and of commercial CMC (right).

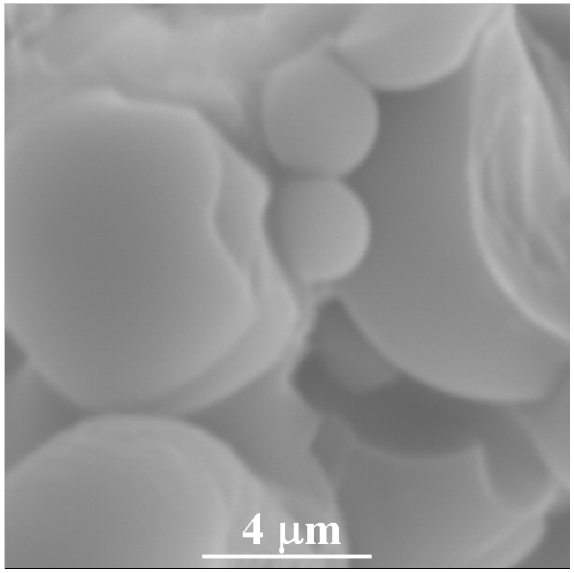

Fig.12. Cholesterol/CMC microstructure obtained when a different hexanol-to-water ratio was used in qualitatively identical procedure to the one described in section 2.2.

It is also important to note that the procedure of preparation of cholesterol/CMC composites within the 1-hexanol/water mixture has not shown to be simply scalable. Namely, when the synthesis yield of the procedure described in section 2.2 was increased by the factor of ten, a transparent solution was obtained prior to the addition of cholesterol molecules in the former case (in 50 ml beaker), whereby an opaque white color comprised both organic and aqueous phase in the latter case (in 500 ml beaker). The differences in the surface area of contact of the beaker with the vibrating bed of the ultrasound bath (*Fisher Scientific FS110*), i.e. available ultrasound energy per particle, presumably presented a practical obstacle in scaling-up the described procedure. Significant morphological sensitivity of the reaction products, with an emphasis on the vessel volume effect, has frequently been observed in the syntheses of inorganic

particles[55]. Considering the organic nature of the applied compounds, a similar or even a more pronounced sensitivity on seemingly negligible modifications of the experimental procedure can be expected. The 'butterfly effect' in the form of an extreme sensitivity towards the initial conditions of reaction has already been witnessed in precipitations from salt bile systems, involved in gallstone formation[56].

Spherical particles of swelled CMC (composed of aggregated smaller spheres) and highly hydrated cholesterol layers (consisting of a layered substructure) comprising the sample prepared by the attempt to scale-up the yield of the final product through expanding the vessel volume are presented in Fig.13. This sample was observed immediately after the thin composite layer (while still in a slightly gelled state) had formed on top of the SEM sample carrier. However, slow deswelling of the polymer phase upon a prolonged drying gives rise to numerous hollow spheres on top of the layered cholesterol precipitate. Extremely long times required for the drying process of the cholesterol precipitates from hexanol/water mixtures to come to completion (cca. 1 week in case of the smaller and 2 weeks in case of the larger batch, when dried in vacuum) were an indicator of a slow deswelling of the composite structure. Cracks and bubbles were consequently showing up long after the last visible traces of the drying process had disappeared. The slow rate of deswelling of cholesterol/CMC gel into the final, thin and transparent crust, initiates the formation of an intrinsic bubbling structure, intruded with occasional cracks of the supporting cholesterol layers. Lighter circular regions noticeable at lower magnifications during SEM observations (Fig.14) thus belong to expanded and burst, extremely large (50 μm – 1 mm in diameter) cholesteric bubbles. On the other side, a larger number of smaller (~ 5 – 50 μm) hollow cholesterol spheres, comprising the darker areas in Fig.14 is obvious from Fig.15. The spheres are formed by thixotropic swelling and subsequent deswelling of the top layers of multilayered cholesterol deposits. They easily burst, either spontaneously (Figs.16-17) or by the influence of SEM electron beam, as can be seen from Fig.18. The exceptional surface sensitivity during microscopical imaging can be explained by the fact that weak molecular bonds figure between individual cholesterol molecules in their solid state. Namely, whereby hydrogen bonds form two-dimensional bonded network on the bilayer plane of cholesterol crystals, individual molecular bilayers measuring 3.4 nm in height are stacked through van der Waals forces along the crystallographic c axis[51]. Typical sharp and peeling edges of the burst hollow spheres, shown in Figs.18-19, indicate their cholesteric composition. A fine control over the intensity of thermal agitation and the corresponding thixotropic phenomena of the constitutive polymer phase may, therefore, yield a wide variety of reproducible morphological features of structurally entwined cholesterol/CMC deposits.

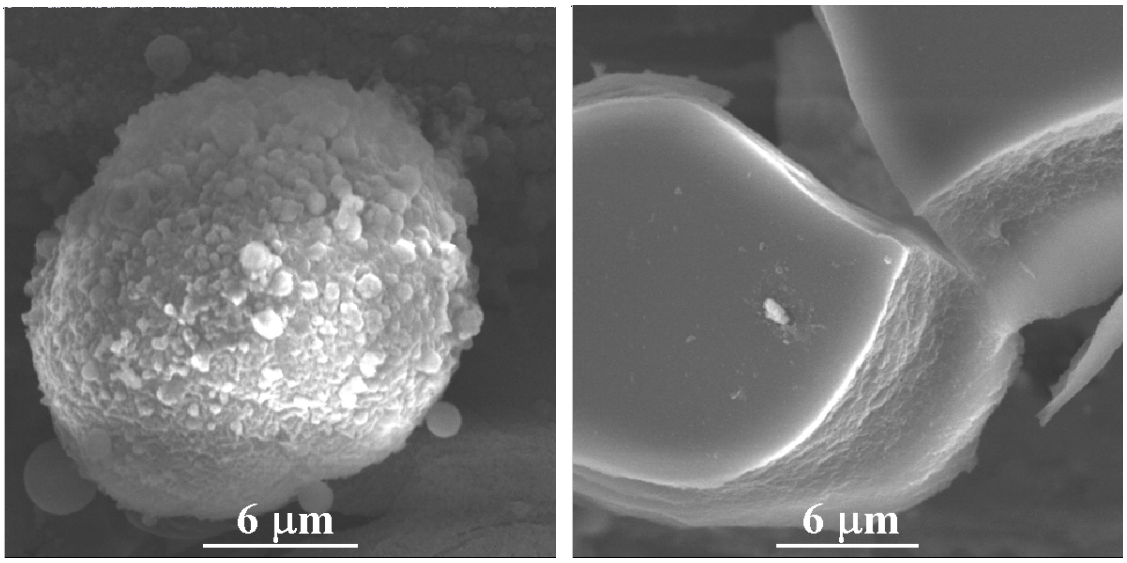

Fig.13. Spherical CMC particles, consisting of aggregated smaller spheres (left) and cholesterol cake-like precipitate, consisting of layered substructure (right), comprising the gelled sample prepared via scaling-up the procedure of preparation in biphase hexanol/water mixture.

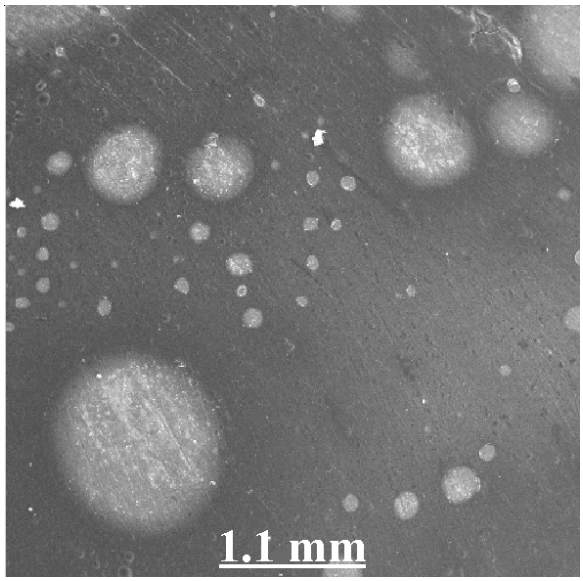

Fig.14. Cholesterol layers, precipitated via scaling-up the procedure of preparation in biphase hexanol/water mixture, intruded with hollow spherical regions.

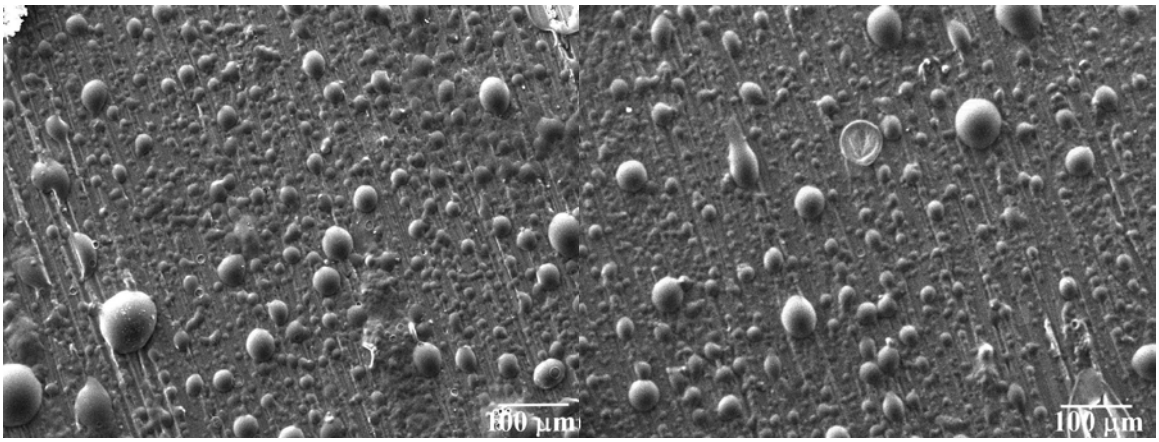

Fig.15. The darker, cholesterol layered areas from Fig.13, comprising hollow cholesterol spheres.

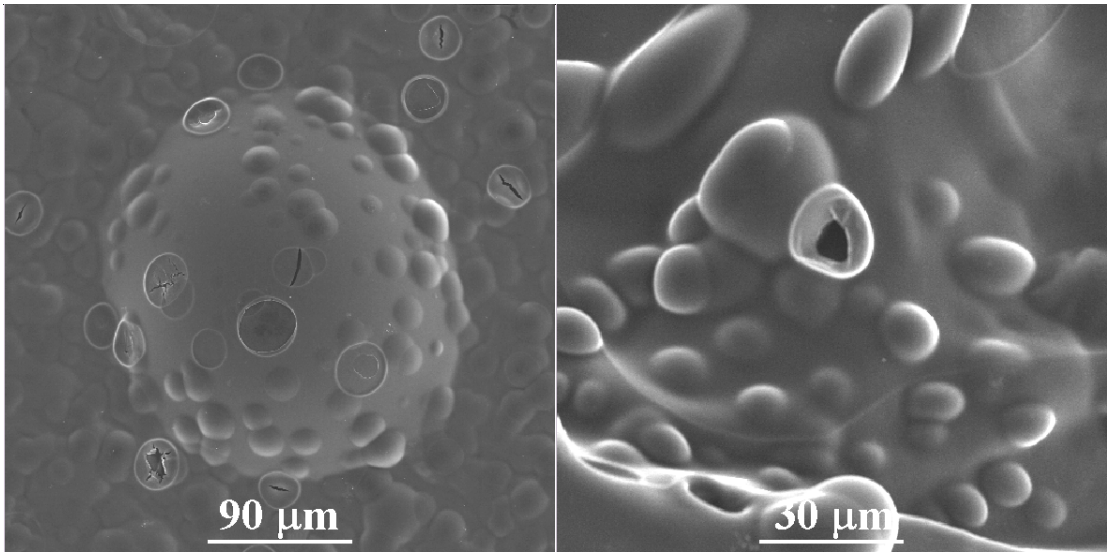

Fig.16. Bubbling structure of cholesteric deposits precipitated via scaling-up the procedure of preparation in biphase hexanol/water mixture.

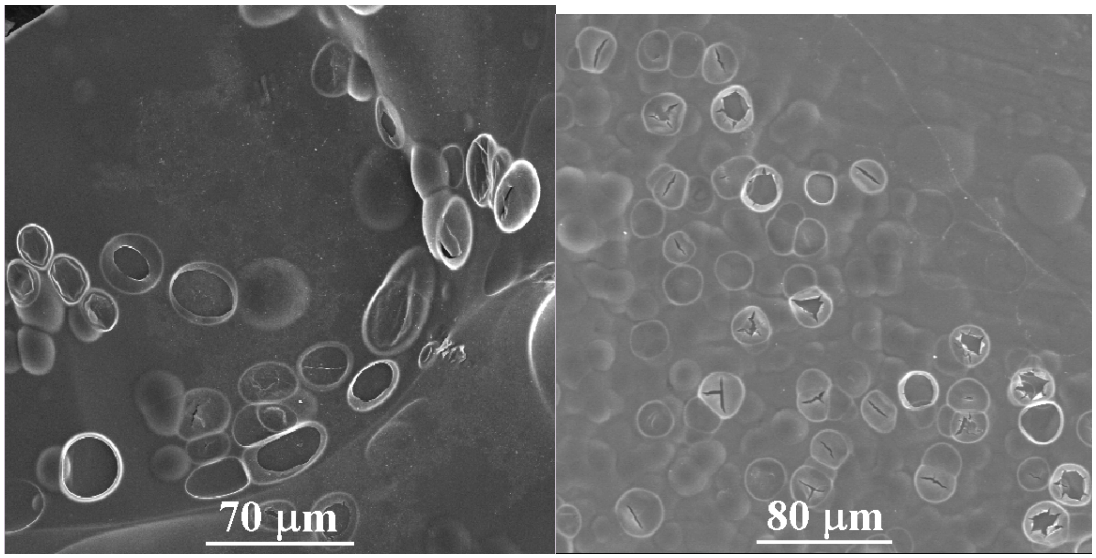

Fig.17. Burst cholesteric bubbles comprising the top layers of cholesteric deposits precipitated via scaling-up the procedure of preparation in biphase hexanol/water mixture.

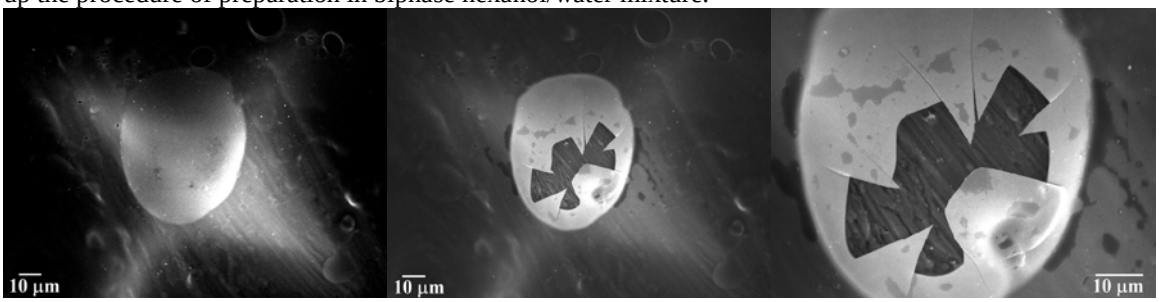

Fig.18. Hollow cholesteric spheres bursting under the influence of SEM electron beam.

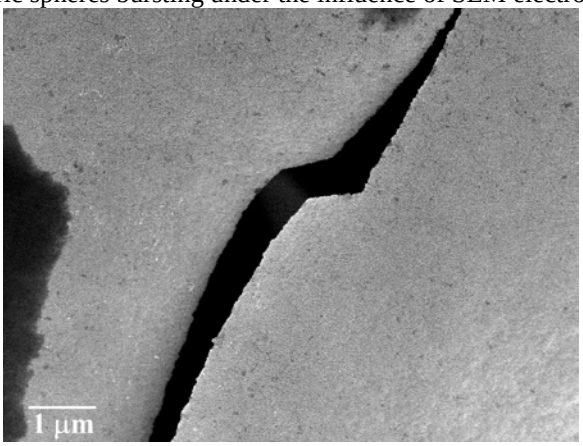

Fig.19. A segment of a burst hollow cholesterol sphere of the sample prepared via scaling-up the procedure of preparation in biphase hexanol/water mixture, showing a magnified crevice with sharp, arrow-like contours, typical of cracked cholesterol layers.

Conclusion

As a conclusion, a water-soluble cellulose derivative in form of CMC proves partly successful in subduing the inherent tendencies of cholesterol molecules towards imposing multi-layered structures in solid state. Numerous preceding investigations aimed at the production of untypical cholesterol morphologies. The applications of various solvents, additives, surface active agents, biochemical environments and physical modifications of experimental setups are notified, but in each reported case the typical, uniaxially or biaxially-grown morphologies of cholesterol aggregates have resulted. However, CMC as a semi-synthetic and biodegradable derivative of cellulose, the principal constituent of a variety of natural fibers[57], herein appears as a promising agent for modifying the surface properties of cholesterol precipitates. It may be, further on, used as a key additive for obtaining miscellaneous novel cholesterol morphologies. The application of cellulose derivatives in fight against cholesterol deposits *in vivo* proves as a reasonable choice after considering the results of the morphological investigation of cholesterol precipitation processes presented hereby. However, it is worth noting that this study of the co-precipitation of cholesterol provides insight into the investigated processes placed in a far simpler context than the one existing in a living organism. Just like about any similar study done *in vitro*, it can, therefore, provide only indications into how the interactions between these components may occur on a biological plane.

Morphological variety of the obtained CMC/cholesterol composite structures is consistent with the expected versatility of interactions between cholesterol, one of the major fatty substances in mammalian species, and CMC, a derivative of cellulose, the major naturally occurring dietary fiber. Such a diversity of the potential products of interaction between cellulose derivatives and cholesterol under different precipitation and environmental conditions, as shown in this work and as expected to occur in the intricate domain of biological phenomena, respectively, opens the door for preparation of numerous novel attractive cholesterol morphologies, significant for both fundamental understanding of life and humane beneficial reasons.

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