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Abstract

Uniform crystalline colloidal cholesterol particles of narrow size distribution were obtained by precipitation. The method consisted of adding a miscible non-solvent (water) into cholesterol solutions of different alcohols and acetone, without any additives. The properties of the resulting particles depended in a sensitive way on the concentration of all reactants, temperature, pH, ionic strength, and aging time. The major observed effects were due to the solubility of cholesterol, which was strongly affected by the solvent mixture and temperature. Precipitation in 1-propanol/water system yielded stable dispersions of well-defined particles, which were used to evaluate the effects of different experimental parameters on their properties. Aging of stable dispersions resulted in multi-layered aggregation of the primary platelets, the degree and rate of which process was strongly affected by temperature. Finally, it was shown that the colloidal cholesterol particles could be coated with homogeneous silica layers in order to alter their surface characteristics.

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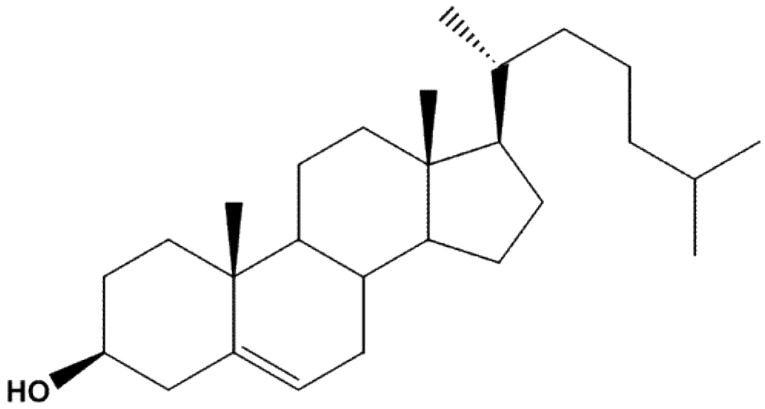
1. Introduction

This investigation serves a two-fold purpose. The first refers to some general aspects of uniform colloid dispersions, the importance of which is now widely recognized. However, the efforts have mostly been focused on inorganic systems. For example, in the comprehensive compendium on fine particles [1], out of thirty-seven chapters only one deals with dispersed organic matter, i.e. polymer latexes (which are not pure compounds, but different polymers). While the importance of inorganic systems cannot be denied, neglecting the preparation of dispersions of organic compounds is unjustified both for theoretical and practical reasons. The formation of inorganic particles by precipitation in different liquids mostly involves a number of solute complexes, which can be very sensitive to minor changes in experimental conditions. For this reason, it is sometimes difficult to reproduce or scale-up a given process on one hand and to elucidate the mechanism of the particle formation on the other [2]. In contrast, organic compounds of identical molecules represent considerably simpler systems from the chemical point of view, and making the precipitation process dependent only on physical conditions.

Among the first organic dispersions to be prepared as uniform particles are carotenoids [3,4], the optical properties of which strongly depend on particle size, so that they have found a major application as food colorants, such as for fruit juices. Drug molecules are of particular interest, not only because their molecular properties are known with great certainty, but also because there is now a growing evidence that the efficiency and even activity of these materials depend on the degree of dispersity and the structure when in solid state [5-7]. Several studies involving different drugs have been reported, which showed that dispersions of uniform particles in different modal sizes and shapes can be produced [8-15].

The second purpose of this work relates specifically to cholesterol. This steroid represents one of the essential biochemical compounds in the animal kingdom, where it is involved in numerous body-functioning tasks, ranging from the synthesis of bile acids and steroid hormones *in vivo* to maintaining a proper transport balance within cellular membranes [16]. However, due to inability to solubilize cholesterol from different sources by micellar, vesicular and bilayer cleansing agents in bile, as well as lipoprotein complexes in blood, this steroid may deposit on the vessel walls, causing problematic health issues that include gallstone formation, intestinal lumen deposits, and artheriosclerotic plaque.



Chemical structure of a cholesterol molecule

Numerous investigations of the influence of various chemical environments on crystallization and dissolution pathways of cholesterol molecules have been reported, including those on the effects of solvent type [17-19], non-solvent phases [20], temperature [17], pH [21], magnetic field [22], mineral substrates [23,24] and co-existing phases [25-28], model bile composition [29-31], various medicinal plants [32] and synthetic biochemical compounds, such as phospholipids [33], and other sterols [34]. These studies have indicated significant sensitivity of cholesterol crystallization process on many of these physical and chemical factors. In order to be able to control the deposition and dissolution of cholesterol, investigations of its crystallization in simple environments may useful in providing information critical for medicine and chemotherapeutics. Uniform colloidal cholesterol particles of various morphologies could also serve as excellent model systems for the evaluation of different factors on the properties and reactivity of this steroid. This study has demonstrated that such dispersions can, indeed, be

obtained by precipitation when an immiscible non-solvent (water) is introduced into cholesterol solutions in organic solvents in the absence of any additives. The most uniform such dispersion was then used to explore the effects of various experimental parameters, including solubility, reactant concentrations, pH, ionic strength and temperature on the morphological characteristics of the resulting particles, their stability in the dispersed form, and the ability to form multi-layered structures. While the developed precipitation method is capable of generating uniform platelet-type particles, it would be of special interest to establish conditions that would yield finely dispersed cholesterol in other shapes, especially spherical.

2. Experimental

2.1. Materials

The chemicals used were: cholesterol (99+%, Alfa Aesar), methanol, ethanol, 1-propanol, 2-propanol, acetone, aqueous sodium silicate solution (29.2 % amorphous silica and 9.1 % sodium oxide, Fisher) and Amberlite ion-exchange resin of medium porosity (Mallinckrodt), all of the highest purity available.

2.2. Methods

Two substantially different precipitation methods were employed for the preparation of colloidal cholesterol. The first procedure involved rapid addition of a miscible non-solvent (water) into a cholesterol solution of an organic solvent (different low-chain alcohols and acetone), to induce precipitation. The second method was based on the evaporation of cholesterol solutions in solvent/non-solvent mixtures in the vicinity of the corresponding supersaturation limit.

2.2.1. Precipitation

Numerous experiments were carried out to optimize conditions for producing well-defined cholesterol particles, which included the effects of solvent type, concentrations of the reactants, pH, ionic strength, temperature, and subsequent aging. Finally, a *standard precipitation procedure* (SPP) was developed with 1-propanol, and used as a reference system to observe any changes in the properties of the particles by altering the experimental parameters. Accordingly, in the SPP 20 mg of cholesterol were dissolved in 10 cm^3 of 1-propanol, to which were added 15 cm^3 of water to supersaturate the solution. After aging for 10 min, a few drops of the suspension were deposited onto a SEM sample holder, dried in air, and analyzed.

Throughout the entire procedure, the suspension was kept undisturbed, i.e. without any agitation. To study the effects of the pH or electrolytes or both, the required amount of additives was always introduced into the aqueous phase before

mixing with the cholesterol solution. In the case of acids and bases, all other conditions were the same as in the SPP case, while when salts were added, additional 5 cm^3 of the electrolyte solution were admixed in the system.

2.2.2. Evaporation

In the evaporation-based procedure, 20 mg of the cholesterol powder were dissolved in a solution mixture containing 10 cm^3 of 1-propanol and 10 cm^3 of distilled water. A few droplets of the so prepared solution were deposited on a watch glass, and dried in air.

2.2.3. Coating

To coat cholesterol particles with silica, a modified procedure described earlier [10] was employed. A silicic acid solution was first obtained by mixing 1 cm^3 of a sodium silicate solution and 100 cm^3 of water containing 320 mg of Amberlite ion-exchange resin with the resulting pH = 10.5. After the resin had settled, the supernatant solution was removed and used in the coating experiment. Thirty cm^3 of a dispersion of cholesterol particles, prepared according to the SPP (section 2.2.1), were diluted with 10 cm^3 of this active silicic acid solution. The suspension was aged at atmospheric conditions without stirring for 7 days, and then analyzed.

2.3. Characterization

The cholesterol particles were examined with JEOL JSM-6300 Scanning and JEOL JSM-7400F Field Emission Scanning electron microscopes. Considerable effort was applied to establish the optimum conditions and prevent any distortion of the particles during the observation. Accordingly, the lower secondary emission detector, voltage of 1.0 kV and the working distance of 8.0 mm comprised the microscopic settings. Electrophoretic measurements were performed with the BIC ZetaPlus Analyzer, using dispersions of cholesterol over the pH range of 2 – 10. The pH was adjusted with HCl or KOH solutions. Crystallinity of the prepared powders was examined with a Bruker D8 Axis X-ray diffractometer. The particle size distribution was determined by laser diffraction, using a Malvern Hydro-Masterseizer 2000 instrument.

3. Results

3.1. Particles obtained by precipitation

3.1.1. Solvent effect

To investigate the influence of the nature of the solvent on the morphology of precipitated particles, four different alcohols (methanol, ethanol, 1-propanol, and 2-propanol) and acetone were employed. For comparison reasons, in all cases the SPP described in section 2.2.1 was used. SEM micrographs of cholesterol dispersions precipitated from these alcohol/water mixtures are shown in Fig.1, whereas that from the acetone/water mixture is displayed in Fig.2.

Particles obtained in solutions of methanol and ethanol appeared as irregular flakes. In contrast, cholesterol formed in solutions of 1-propanol consisted of well-defined, brick-shaped colloids with smooth surfaces and of narrow size distribution, while those from 2-propanol were thinner platelets, less uniform in size. The precipitates in acetone/water mixtures were irregular aggregates, rather than individual entities, giving the appearance of partly melted cholesterol. In all cases, turbidity appeared in each system on addition of water, and no settling was observed for extended periods of time (~ 2 days).

3.1.2. Precipitation of cholesterol from 1-propanol/water solutions

Since the best results in terms of cholesterol particles morphology and uniformity were achieved using 1-propanol as solvent, the effects of other experimental parameters on the properties of the resulting dispersions were examined with this solvent system, and compared with the product obtained by the SPP (Fig.1c).

3.1.2.1. Characterization of the "standard" sample

The particle size distribution of the "brick"-shaped particles obtained by the SPP exhibits two distinct peaks, one at 150 nm and the other at 1.2 μm (Fig.3a). The first peak corresponds to the width, and the second one to the length of cholesterol particles, which sizes are consistent with the results of electron microscopy measurements, as is evident from Fig.3b.

The XRD pattern of cholesterol powder prepared by the SPP is shown in Fig.4. Two most intensive diffraction peaks at $2\theta = 2.6^{o}$ and $2\theta = 5.2^{o}$ correspond to Bragg distances of d = 33.4 and 16.7 Å, derived from bilayer and monolayer reflections, respectively. Diffractometric measurements of particles obtained in other solvents did not exhibit significant changes in XRD patterns. A number of X-ray diffractograms of crystalline cholesterol have been reported in the literature [18,32,34,35], which were somewhat dependent on the method of the sample preparation, but none detected the two sharp peaks seen in Fig.4. The peaks between 2θ of 10^{o} and 20^{o} have been shown in a number of cases, and specifically in the sample prepared in methanol/water mixture [18], which identified that sample as the monohydrate modification. The IR spectra (not reproduced here) of the brick-like particles have the same features as those published elsewhere [32]. They corroborate the XRD data by confirming the presence of typical atomic groups of cholesterol molecules.

The dependence of the zeta potential of cholesterol particles on the pH of the dispersing medium is presented in Fig.5, which shows an isoelectric point (IEP) at pH ~ 1.8.

3.1.2.2. Reactant concentration effects

Table 1 summarizes experimental parameters in the study of the effects of the cholesterol concentration and of different 1-propanol/water volume ratios on the precipitated particles, while Fig.6 displays the corresponding SEM micrographs. Adding the same volume of water to alcohol solutions of different cholesterol concentrations yields essentially the same kind of particles, but of broader size distribution (Fig.6a,b) than obtained by the SPP. Changing the volume of added water had a profound effect on the nature of the particles, which was obviously due to the difference of solubility in the respective solvent/non-solvent mixtures. Thus, much larger and multi-layered particles are observed with less water (sample D, Fig.6c), while aggregates of significantly smaller individual entities appear when larger amount of water is added (sample E, Fig.6d) relative to the SPP case (sample B, Fig.1c).

3.1.2.3. Aging time and temperature effects

To follow the formation of particles prepared in accordance with the SPP, samples were taken out at different times after adding water. Fig.7a is the SEM of solids removed after only 10 seconds, while (b) is for the same system after 10 min of reaction, all at room temperature. Immediately after the addition of water, uniform thin platelets precipitate, which then grow to the final particles in a few minutes. The latter particles remain stable and do not change after aging for extended periods of time (~ 2 days), when aggregation effects take place. It is quite apparent that the initially generated platelets grow by diffusion of cholesterol molecules to form larger crystals.

Since the solubility of cholesterol increases strongly with temperature [19], the latter has a major effect on the precipitates in terms of their particles size, shape, and stability. This influence was explored in the present system by two modifications of the process. In the first case, the system was prepared according to the SPP, but the subsequent aging was carried out at different temperatures for various periods of time. In the second case, the precipitation was carried out at elevated temperatures, using preheated reactant solutions, and then the dispersions were aged at still higher temperatures. An essential experimental detail in the last cases was the need to immediately separate solids by filtration at the desired times to avoid any further reaction on subsequent cooling. A large number of variations in the conditions was investigated to evaluate these parameters, but only a few representative cases are exemplified here. Fig.8 shows particles prepared by the SPP (i.e. at room temperature), but these dispersion were aged at 34 ^{o}C for two

days (a) and at 37 ^{o}C for two hours (b). The shorter time in the latter case was chosen because the observed morphological changes take place more rapidly at higher temperatures. As a consequence of this treatment, the instability of primary particles increases, which is again more pronounced at higher temperature. Fig.8c and d refer to samples prepared at elevated temperatures, both during the precipitation and aging stages, as given in the legend. The most obvious effect is the dramatic increase in the multilayered aggregation.

3.1.2.5. pH and electrolyte effects

To study the effect of the pH on the precipitation of cholesterol particles, reactant solutions were modified by the addition of HNO_3 or KOH, using otherwise the SPP, at which the pH was 6.3. It should be noted that the cited operational pH values are simply instrument readings. Furthermore, it has been shown that pH values in pure water and alcohol-water mixtures do not differ significantly [36]. Electron micrographs in Fig.9 display the cholesterol particles obtained at pHs 1.3, 2.0 and 7.7. At the highest and the lowest pH value, the charged particles are rather well-defined and smooth, while at pH 1.8, which is close to the IEP, they appear eroded and aggregated.

It is a well-known fact that ionic strength plays an essential role in the formation and stability of colloidal dispersions. For this reason, the experiments were carried out, in which NaCl and $BaCl_2$ were introduced in the reaction mixture

as described in section 2.2.1. The range of electrolyte concentrations that could be used was limited by the fact that salts cause separation of phases in the 1-propanol/water mixture [37]. Since the systems were kept at pH > IEP, resulting in negatively charged cholesterol particles, Na^+ and Ba^{2+} acted as counterions. Fig.10a and b display the results obtained at two different NaCl concentrations, i.e. 0.1 and 0.4 mol/dm^3, which at the lower concentration show platelet-type particles of lesser uniformity than without the electrolyte, but greatly enhanced multilayered aggregation at the higher salt content. In view of the higher charge of Ba^{2+}, lower concentration of $BaCl_2$ was used at two different pH values, i.e. 6.3 and 10. Obviously, the more strongly charged particles at the higher pH are essentially single platelets, while those at the lower pH are quite unstable (Fig.10c, d).

3.2. Cholesterol obtained by evaporation of the solvent/non-solvent mixture

The solids obtained by evaporation of dissolved cholesterol in 1-propanol/water solutions by the procedure described in section 2.2.2 yielded, as a rule, thin layered deposits illustrated in Fig.11. The so prepared cholesterol “films” had the same structural characteristics as dispersed particles obtained by precipitation.

3.3. Silica-coated cholesterol particles

To prevent aggregation of cholesterol particles (when unstable) and to modify their surface characteristics, the samples prepared in 1-propanol/water solution were coated with silica as described in section 2.2.3. Fig.12a of such a dispersion shows only brick-like coated cholesterol. No separate silica particles are seen, which would appear as spheres. Furthermore, energy-dispersive spectrogram in Fig.12b shows a silicon peak in the sample of coated particles.

4. Discussion

This study has demonstrated that stable dispersions of colloidal cholesterol can be obtained by a simple process in solvent/non-solvent mixtures. The most uniform particles of the narrowest size distribution were observed when 1-propanol was employed as the solvent (Fig.1c). Whereas the propensity of cholesterol to crystallize in layered, biaxially-grown structures has been known [18,34,38], dispersions described here are the first to consist of well-defined and "monodisperse" particles of this compound.

The solubility of cholesterol in n-alkanols increases significantly with their chain length and temperature [19]. A larger number of nuclei formed at lower solubilities should account for the formation of flaky particles in methanol- and ethanol-water mixtures under otherwise the same conditions. The uniformity and modal size of particles in 1-propanol can be also explained by the solubility. Fig.7

shows that immediately upon adding water, the precipitated platelets are rather small and thin, in agreement with the bluish appearance of the dispersion due to the Rayleigh scattering. At the low critical supersaturation in this alcohol the initial platelets then grow by diffusion of cholesterol molecules to form the final brick-shaped particles. The difference in the particle size observed when 2-propanol is used instead of 1-propanol appears to be caused by the different molecular configuration of the two alcohols, which should affect the solubility of cholesterol. While no data on the latter for 2-propanol could not be found, one may assume that solubility should be lower, because of its shorter chain length as compared to 1-propanol. Note that in all described cases, the same SPP was used.

To preserve the uniformity of cholesterol crystals, the concentration of cholesterol reactant solutions needed to be kept within an optimal range. Experimentally, the concentration conditions were altered in two different ways, i.e. by changing the cholesterol content and keeping the solvent/non-solvent volume ratio the same, or by having the same initial cholesterol concentration, but changing the solvent composition. In the first case, as expected, at lower cholesterol concentration, fewer but larger particles (Fig.6a) are obtained, while at the higher concentrations, much larger number of smaller particles (Fig.6b) is formed. When the volume of water in the system is increased relative to that of alcohol, due to lesser solubility smaller particles in higher concentrations are precipitated (Fig.6d). In contrast, at lower water content, considerably larger aggregates of stacked, platelet-type subunits (Fig.6c) are produced. It appears that

in this case, constituent platelets are formed first, which then aggregate face-to-face.

The stability of cholesterol dispersions must be considered in connection with the presence of OH groups in the steroid molecules. Since the reaction medium contains water, this group will orient itself toward the surface of crystals, especially at their sides [26,39-41]. These surface sites play an important role in the multilayered aggregation of individual particles and in the overall stability of the entire dispersion. At very low pH of the medium (below the isoelectric point), the hydroxyl groups are protonated, imparting positive charge to the crystals, which accounts for their stability (Fig.9a). The exact nature of the negative charge on the particles above the IEP is not well understood, but it causes particles to remain well dispersed (Fig.9c). Finally, at the IEP, as expected, the dispersions were unstable (Fig.9b). The face-to-face layering should be caused by the location of the OH groups at the particle sides. Finally, these groups would be preferential sites for alcohol adsorption, thus affecting the polarity of the dispersed solids.

Carrying out the precipitation process in the presence of the added electrolyte had no major effect on the shape of the resulting particles, but greatly affected their ability to stack up. The observed behavior is typical of hydrophobic dispersions, which depends in a pronounced way on the charge of the counterion on one hand and the magnitude of the surface potential of the dispersed particles on the other. Thus, Na^+ initiates aggregation of the particles above a critical concentration (Fig.10a,b), typical for monovalent ions, while the double charged

Ba^{2+} destabilizes the system at considerably lower concentrations, unless particles become strongly charged at sufficiently high pH (Fig.10c,d), as predicted by the classical Schulze-Hardy rule.

The significant effect of higher temperature on precipitation of cholesterol in mixed solvents is essentially caused by the increased solubility. In addition, a polymorphic phase transition of cholesterol was reported over the range of 37 – 39 ^{o}C [38,42]. Thus, experimental conditions to produce well-defined particles are rather limited due to the competition effect caused by solvent composition and temperature; the higher amount of water in the solvent mixture lowers and temperature raises the solubility.

Of particular interest are temperature effects related to the precipitation process itself, and to the subsequent aging of the resulting dispersions. At the SPP conditions no precipitation takes place above 34 ^{o}C and, therefore, no experiments were performed above 31 ^{o}C, at which conditions brick-shaped particles are still obtained. The post-precipitation aging of the dispersions was greatly influenced by temperature, i.e. dispersions obtained by the SPP and left at room temperature remained essentially unchanged for days, but if kept at 34 ^{o}C enhanced multi-layered aggregation occurred after 2 days (Fig.8a). Further increase of the aging temperature to 37 ^{o}C shortened this aggregation process to only 2 hours (Fig.8b), which may be due to a combination of Ostwald ripening and the increased solubility of the original particles. In these conditions, the latter are negatively charged, yet their face-to-face layering still takes place. The aggregation of

primary platelets must be promoted by hydrogen bonding of –OH groups operating within the layers, and the van der Waals force between them.

It is worth noting that by changing the solvent mixture composition by evaporation produces rather flat, large cholesterol films, which differ morphologically from any products obtained by direct precipitation. This study has also demonstrated that cholesterol particles can be coated with an inorganic layer, in this case silica. The latter is again possible due to the charges and the hydroxyl groups on the particles enabling the highly hydrated silicate species to adsorb and polymerize on the particle surface to form a homogeneous silica layer.

Conclusion

This investigation describes the preparation of uniform crystalline colloidal cholesterol by a simple method without any additives. The so obtained dispersions have been used to explore the effects of various experimental conditions on the morphological properties and stability of the resulting particles, with special reference to the multi-layered aggregation of primary platelets. Furthermore, it is possible to provide a smooth inorganic coating on these particles to impart different properties in terms of charge, interactions, and the degree of hydration. The method employed coating with silica, but it could be readily extended to other inorganic layers.

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Table 1
Concentration conditions in precipitation of cholesterol in 1-propanol/water mixtures

Sample and Figure	Grams of cholesterol in 10 cm^3 of 1-propanol	Volume of added water [cm^3]	Final concentration of cholesterol [mol/dm^3]
Sample A (Fig.3a)	$5 \cdot 10^{-3}$	15	$5.2 \cdot 10^{-5}$
Sample B (SPP, Fig.1c)	$2 \cdot 10^{-2}$	15	$2.0 \cdot 10^{-3}$
Sample C (Fig.3b)	$8 \cdot 10^{-2}$	15	$8.3 \cdot 10^{-3}$
Sample D (Fig.3c)	$2 \cdot 10^{-2}$	12	$2.3 \cdot 10^{-3}$
Sample E (Fig.3d)	$2 \cdot 10^{-2}$	30	$1.3 \cdot 10^{-3}$

Figure Captions:

Fig.1. Scanning electron micrographs (SEM) of particles precipitated from cholesterol solutions in (a) methanol; (b) ethanol; (c) 1-propanol and (d) 2-propanol, by addition of water using the *standard precipitation procedure* (SPP, section 2.2.1).

Fig.2. SEM of cholesterol particles precipitated as in Fig.1, using acetone as solvent.

Fig.3. Particle size distribution obtained by laser diffraction analysis (a) and a SEM image (b) of cholesterol particles prepared by the SPP.

Fig.4. X-ray diffractogram of the cholesterol powder prepared according to the SPP.

Fig.5. Zeta potential as a function of the pH of aqueous dispersions of cholesterol particles prepared according to the SPP.

Fig.6. SEM of cholesterol particles produced at different cholesterol concentrations (a,b) and at different solvent/non-solvent volume ratios (c,d), under conditions given in Table 1.

Fig.7. SEM of cholesterol particles prepared with 1-propanol as solvent using the SPP, sampled after 10 s (a) and 10 min (b) of reaction time.

Fig.8. SEM of cholesterol particles prepared using reactant concentrations as in the SPP, but different temperature and aging conditions: (a) and (b) precipitated at room temperature and the resulting dispersions aged for 2 days at 34 ^{o}C (a) and for 2 hours at 37 ^{o}C (b). Samples (c) and (d) precipitated at 31 ^{o}C and aged at 37 ^{o}C for 10 minutes (c) and for 5 hours (d).

Fig.9. SEM of cholesterol particles precipitated according to the SPP, but at different pH values; (a) pH = 1.3, (b) pH = 2 (isoelectric point) and (c) pH = 7.7.

Fig.10. SEM of cholesterol particles precipitated in the presence of (a) 0.1 mol/dm^3 of NaCl at pH = 6.3, (b) 0.4 mol/dm^3 of NaCl at pH = 6.3, (c) 0.05 mol/dm^3 of $BaCl_2$ at pH = 6.3, and (d) 0.05 mol/dm^3 of $BaCl_2$ at pH = 10, in accordance with the procedure described in section 2.2.1.

Fig.11. SEM of cholesterol films obtained by the solvent-evaporation method described in section 2.2.2.

Fig.12. SEM of cholesterol particles prepared according to the SPP and coated with silica as described in section 2.2.3 (a), and the corresponding energy-dispersive spectrogram (b).

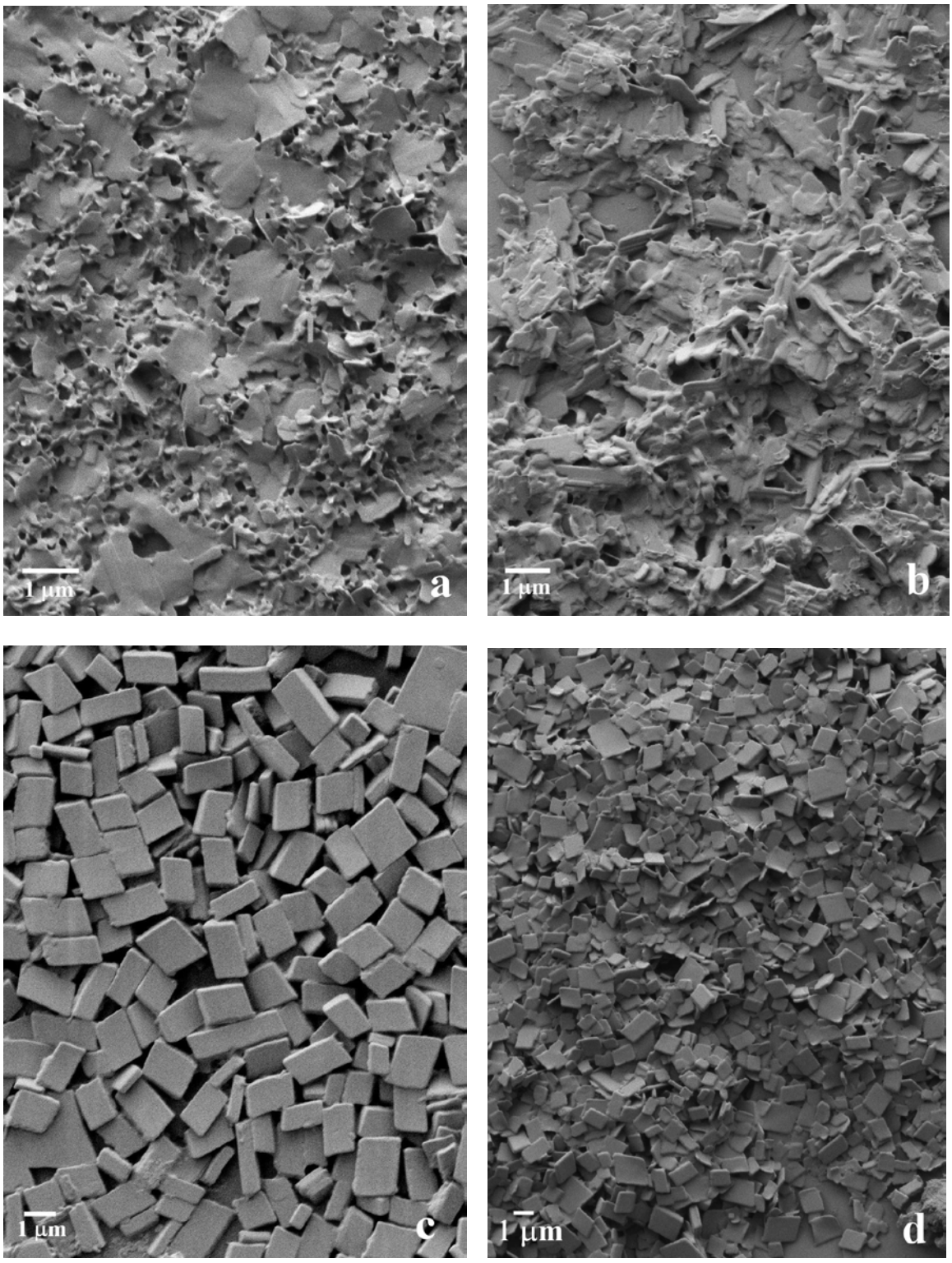

Fig.1

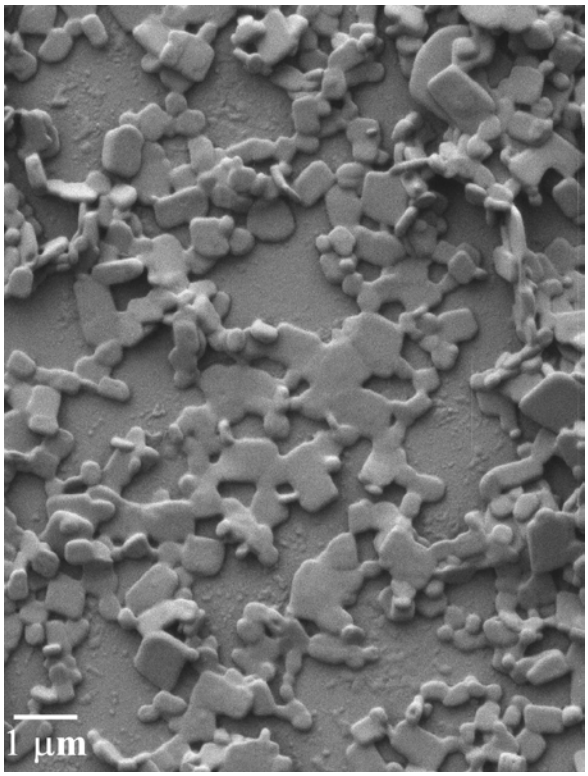

Fig.2

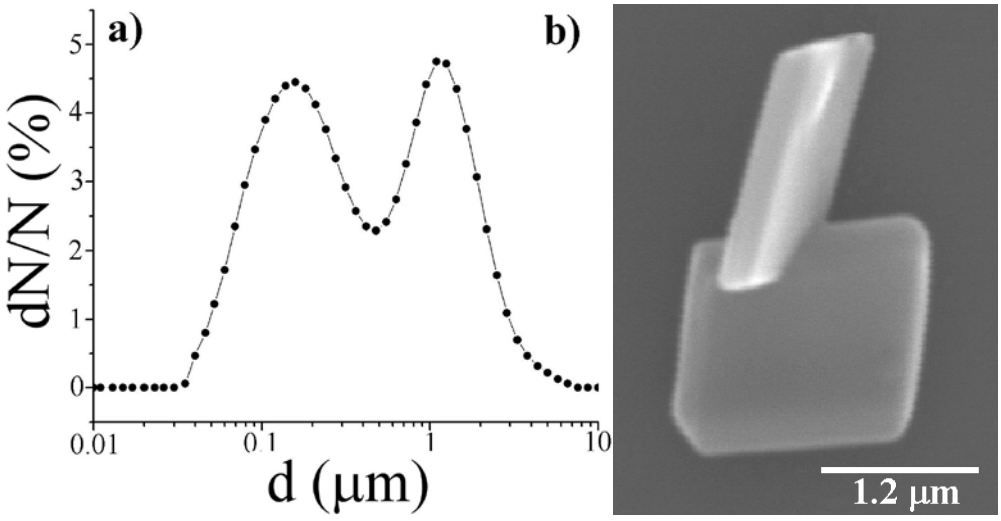

Fig.3

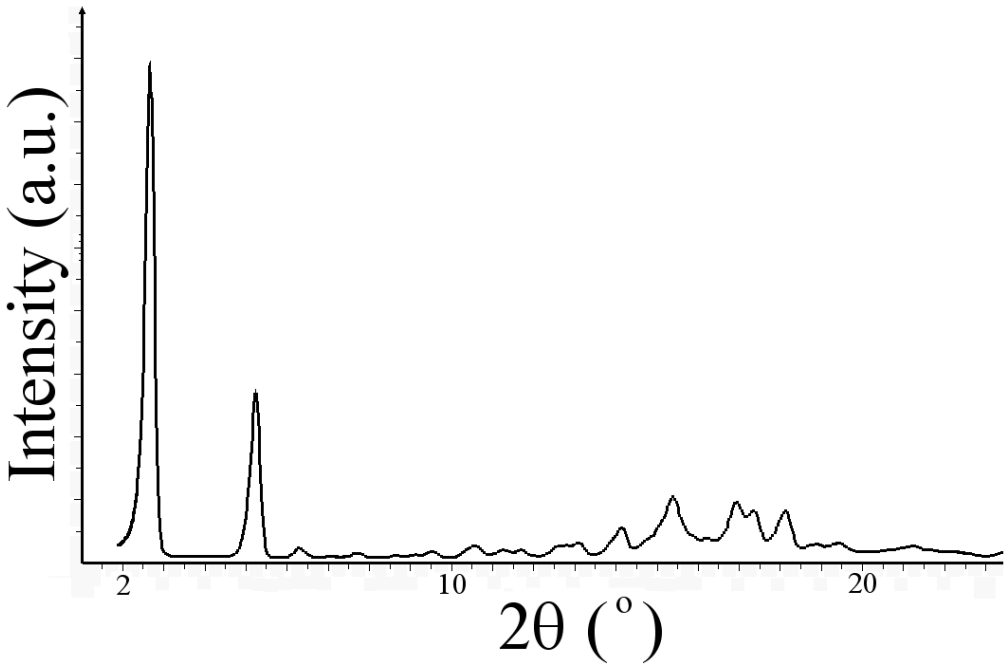

Fig.4

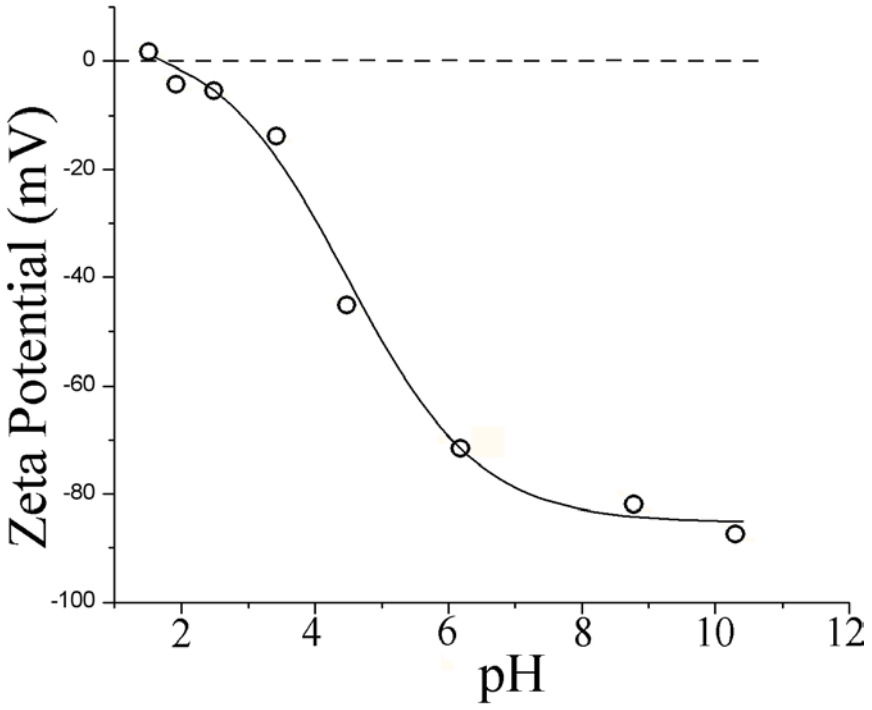

Fig.5

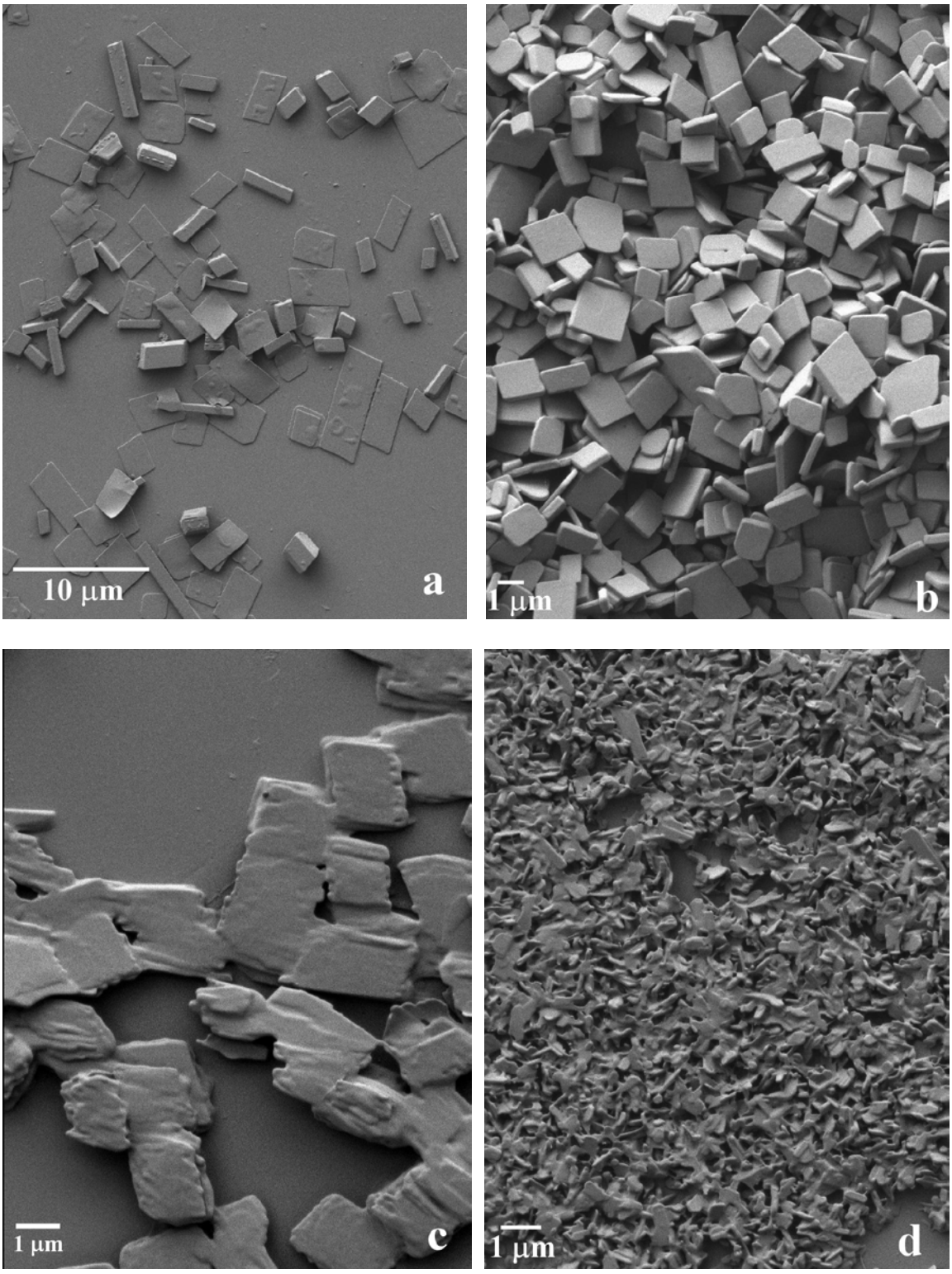

Fig.6

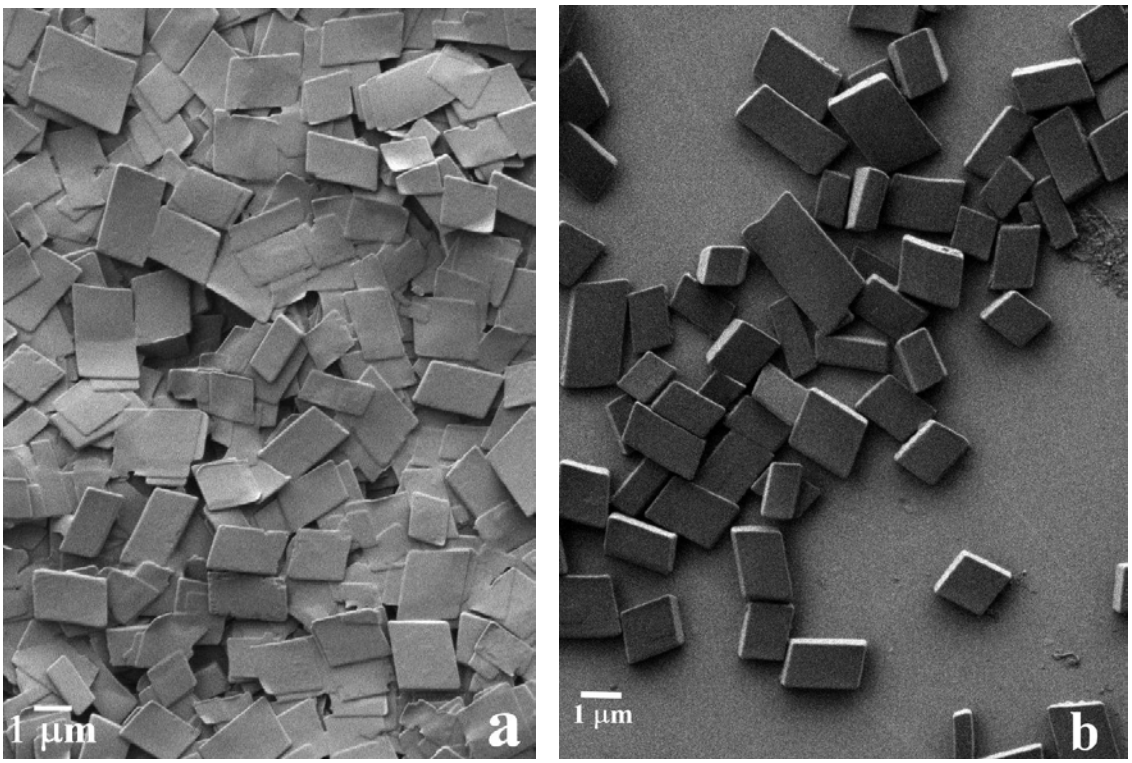

Fig.7

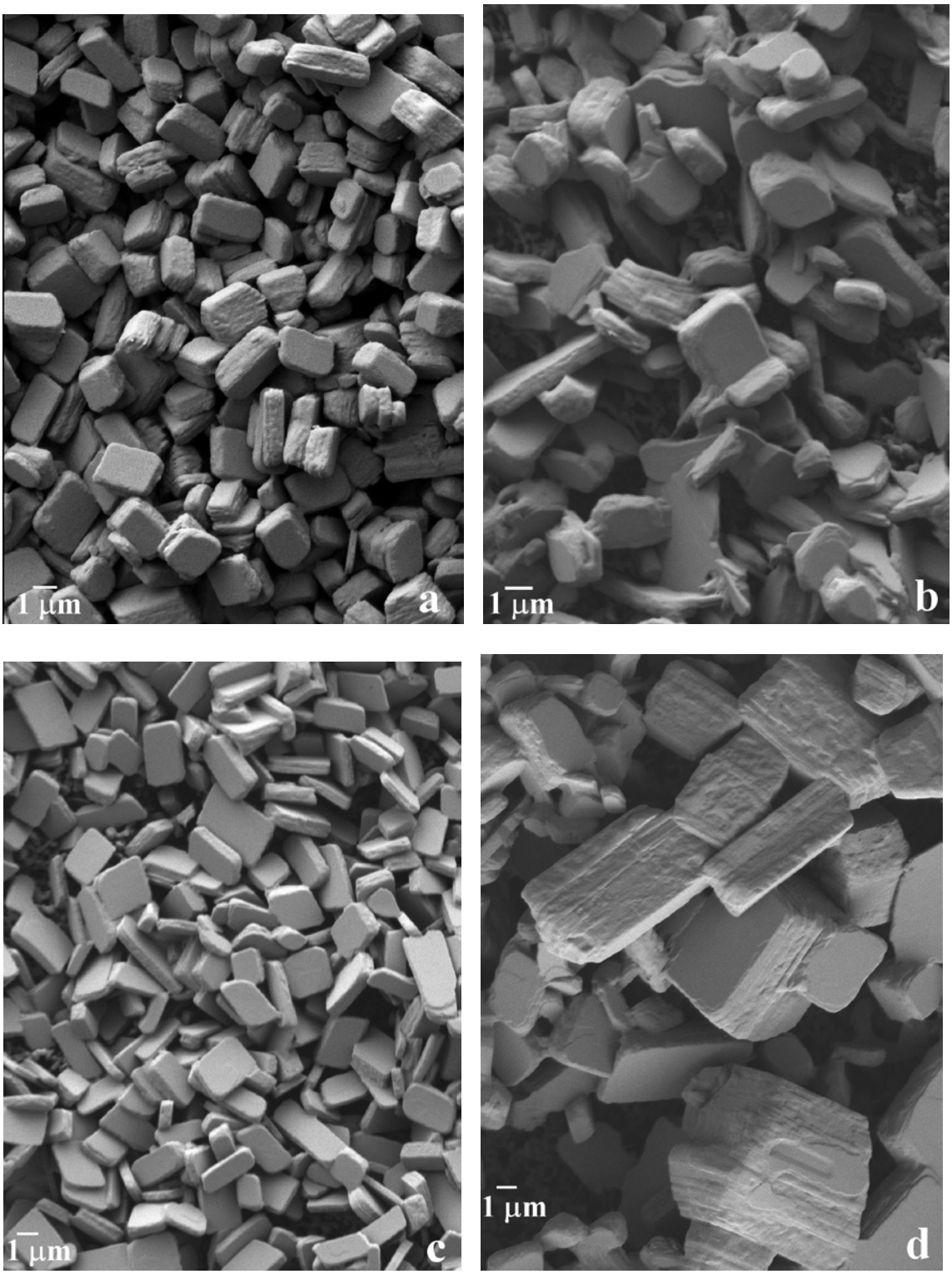

Fig.8

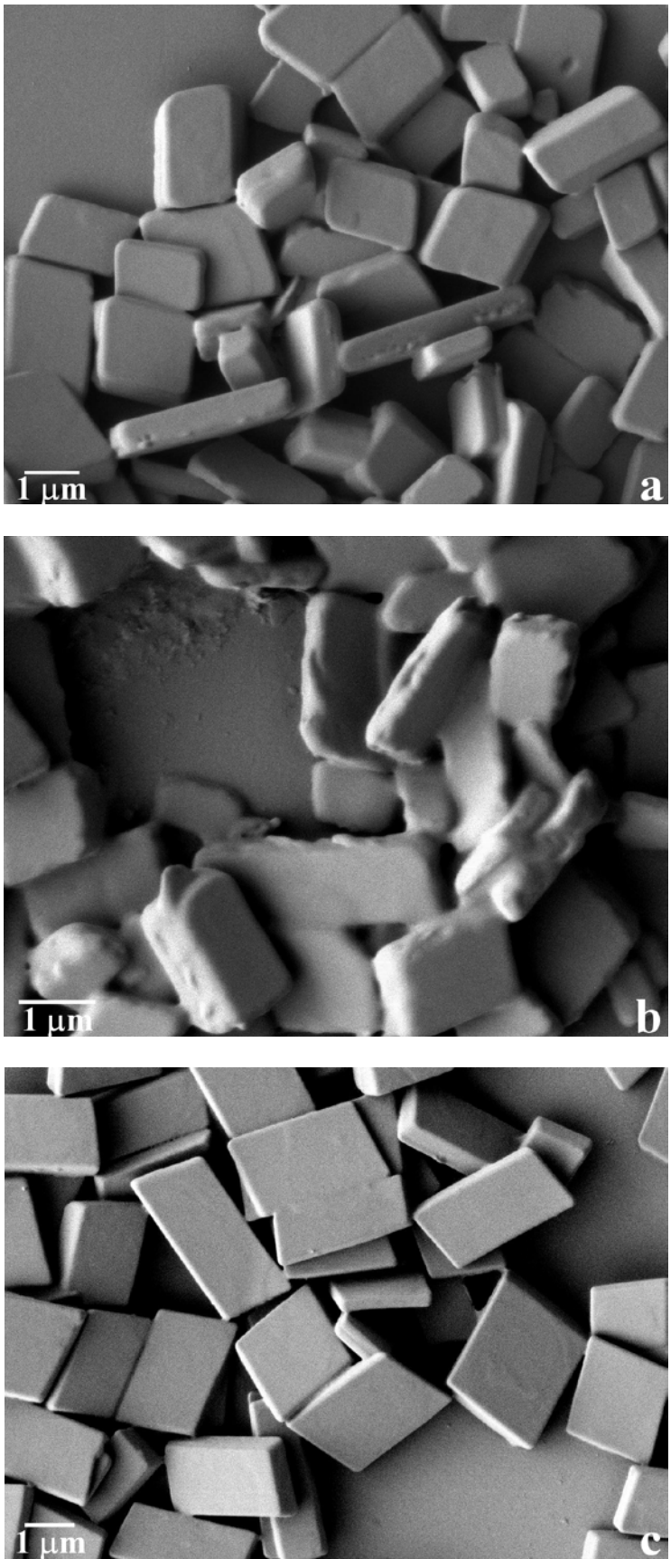

Fig.9

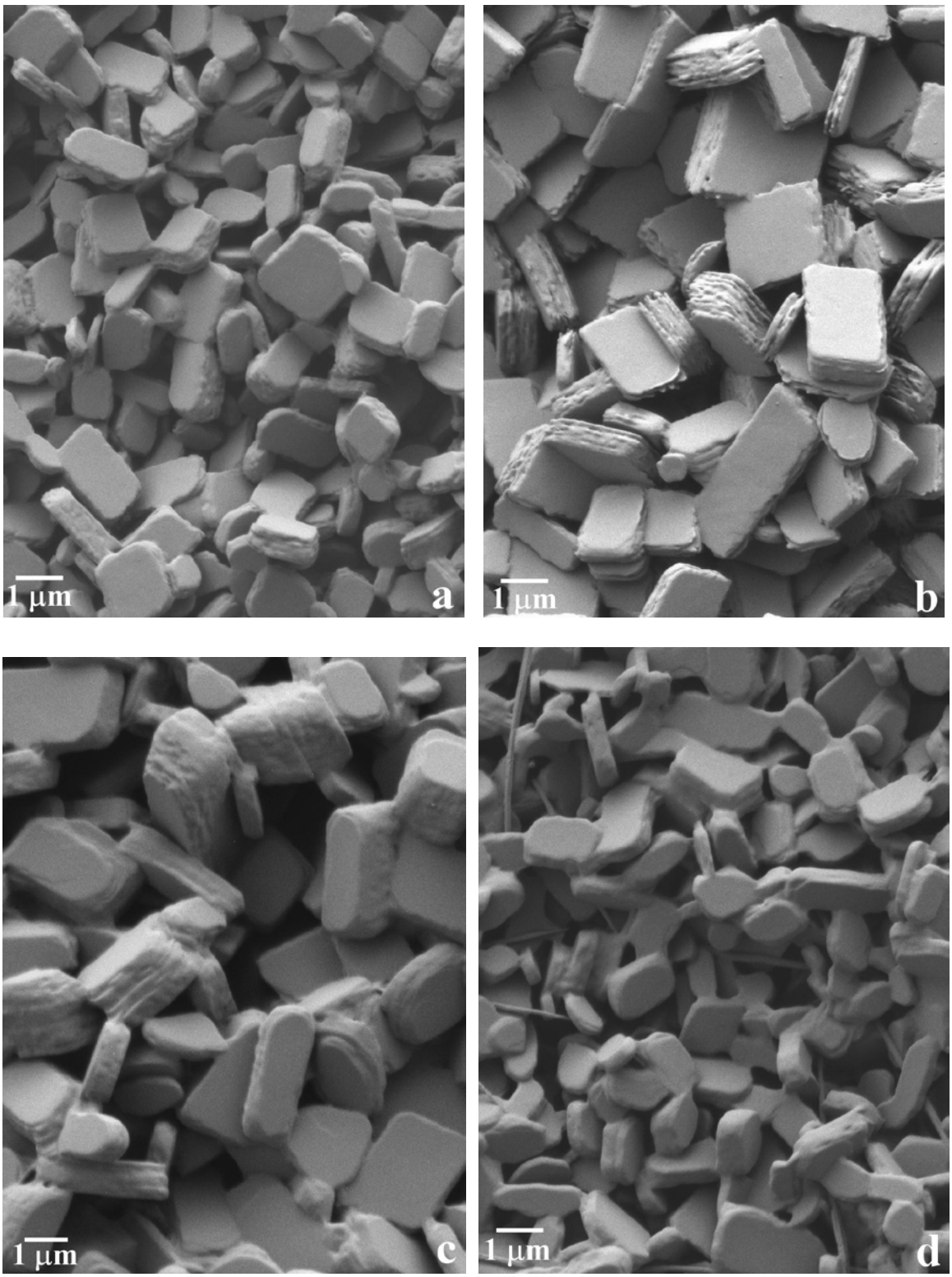

Fig.10

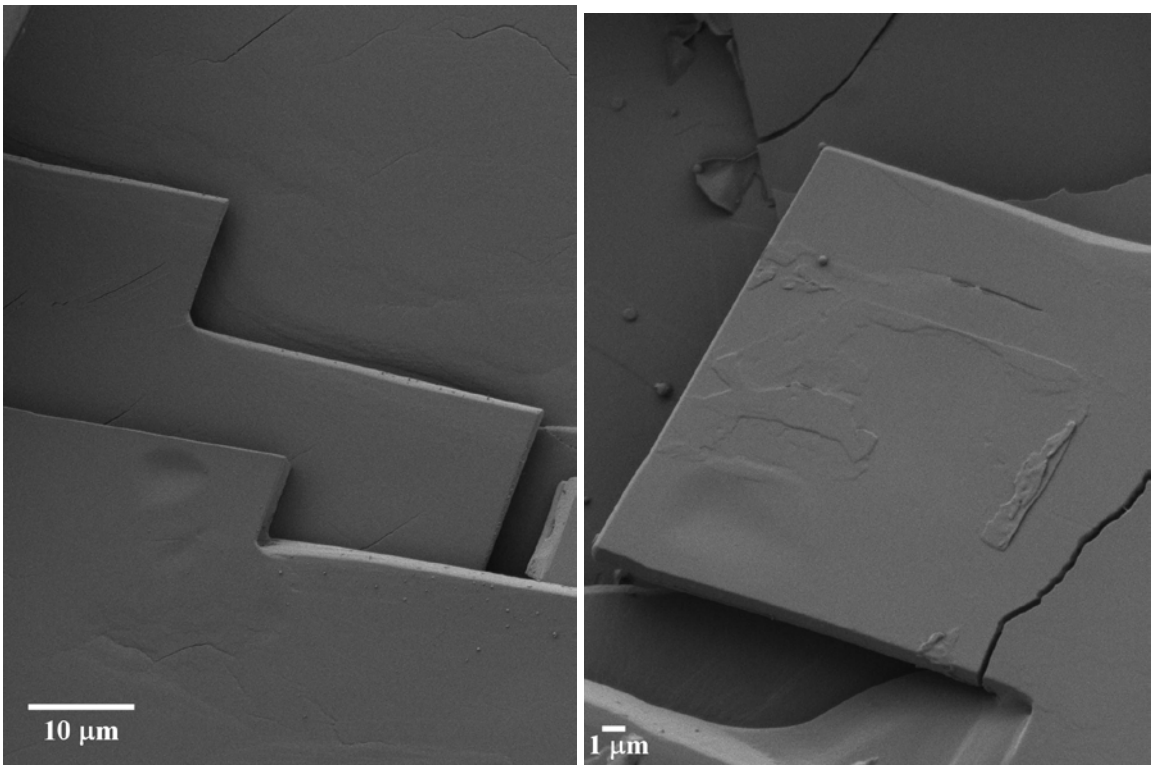

Fig.11

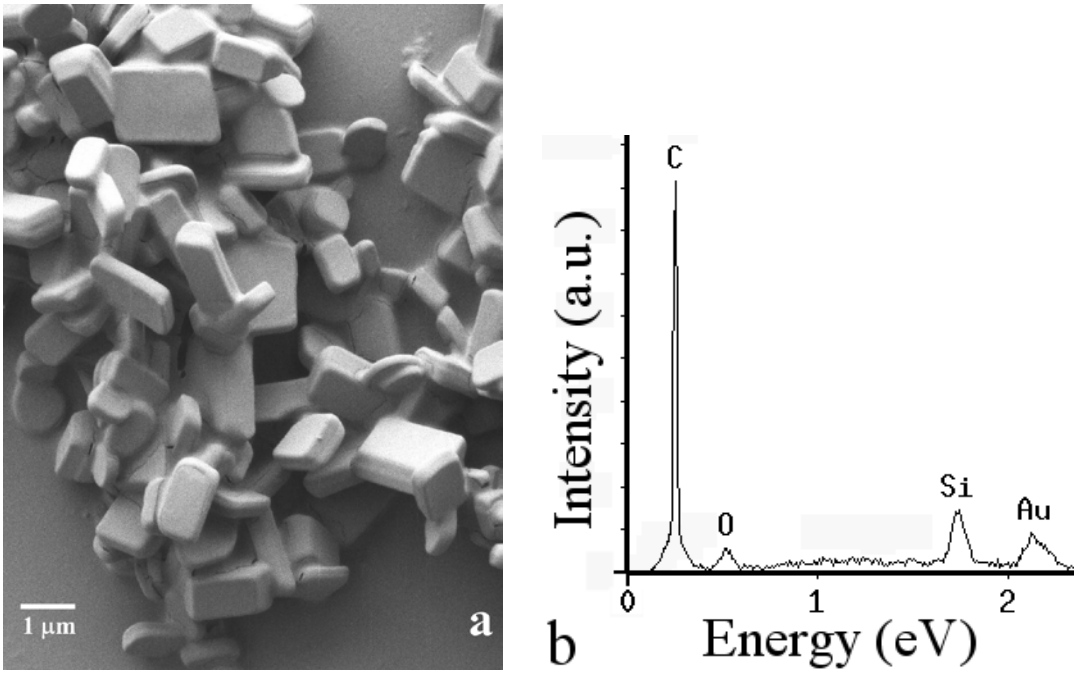

Fig.12