Multifunctional Materials Based on Chitosan, Globular Protein and Gold Nanoparticles

Emil Indrea*, Simina Dreve*, Ioan Bratu*, Gheorghe Mihailescu*, Liliana Olenic*, Gheorghe Tomoaia**, Aurora Mocanu***, Ossi Horovitz*** and Maria Tomoaia-Cotisel***

**National Research and Development Institute of Isotopic and Molecular Technologies, P.O. Box 700, 400293 Cluj-Napoca, eindrea@s3.itim-cj.ro*

***"Iuliu Hatieganu" University of Medicine and Pharmacy, Department of Orthopedic Surgery, Traian Mosoiu Str., no. 47, 400132 Cluj-Napoca*

**** "Babes-Bolyai" University, Faculty of Chemistry and Chemical Engineering, Physical Chemistry Department, Kogalniceanu Str., no. 1, 400028 Cluj-Napoca,* mcotisel@yahoo.com

ABSTRACT

Our studies are focused on the structure of chitosan thin films and globular protein in both the absence and the presence of negatively charged gold nanoparticles. Deacetilated chitosan of high molecular weight and globular protein from aleurone cells of barley were used to prepare the thin organic films deposited on different solid substrates, i.e. glass, polystyrene, and quartz by various methods, such as, layer by layer, cast and spin coating methods. The protein used is a globular storage protein from aleurone cells of barley (*Hordeum vulgare* L.). In order to study the influence of protein (P) on gold (Au) and chitosan (CTS) films, the protein aqueous solution was prepared with a protein content of 2 mg/L in ultra pure water. Gold nanoparticles of 20 to 35 nm diameter range were prepared by reduction of gold complex salt with sodium citrate and then were embedded into chitosan and protein films. We present the effect of annealing on the nanostructured gold self-assembled nascent film, obtained at room temperature. The effect of protein adsorption on both gold nanostructured films, one obtained at room temperature and the other annealed at high temperature, was also studied.

The structure of the obtained functional nanocomposite films and of their surface at atomic and molecular level is explored by FTIR, UV-VIS spectroscopy, scanning probe microscopy (e.g. AFM), and X-ray diffraction. The structure and the morphology of nanocomposites were different than for the films cast from chitosan solution due to the presence of protein and gold nanoparticles. These functional materials might have industrial and medical applications, particularly in the development of new drug delivery systems with good sorption and biodegradability of the carrier.

Keywords: globular protein, chitosan, gold nanoparticles, nanocomposites, AFM, X-ray diffraction

1 MATERIALS AND METHODS

Synthesis of gold nanoparticles was based on the well documented Turkevitch process with the exception that $HAuCl_4$ has been replaced with $Na_3Au(SO_3)_2$ prepared in our laboratory [1]. In brief: 20 ml of 0.015 M of $Na_3Au(SO_3)_2$ was added to 500ml of double-distilled water and the solution is heated until boiling. Upon boiling, 20 ml 0.67 M of sodium citrate solution (Merck) is added to it. After two hours, the color changes from almost colorless solution of diluted gold salt to the typical deep red color of gold nanoparticles. At this point, the gold colloidal solution is cooled to room temperature.

The gold films were dried slowly at room temperature (sample 1). Then, one of these gold nascent films was annealed for two hours, at 200 ^{o}C. The resulting aged gold film is called sample 2. The nanostructure of both gold film types (samples 1 and 2) was investigated by AFM in the absence (Fig. 2) and in the presence of protein layers (Fig.3) adsorbed on the gold films. The thermal treatment has been applied only to nascent gold film without protein.

The storage protein extracted and purified from aleurone cells of barley (*Hordeum vulgare* L.) was provided by Prof. Yupsanis (Aristotle University, Thessaloniki, Greece, [2]). The protein was further purified by high pressure liquid chromatography and its purity was assessed by mass spectrometry. This protein contains 4 subunits of molecular weights about 20, 25, 40 and 50 kDa [2].

The AFM investigations were executed using an AFM JEOL 4210 equipment operating in tapping (noted *ac*) mode on nanostructured ordered gold films adsorbed on hydrophobic glass plates. Standard cantilevers with non-contact conical shaped tips of silicon nitride, coated with aluminum, were used. The tip was on a cantilever with a resonant frequency in the range of 200 - 300 kHz and with a spring constant of 17.5 N/m. AFM observations were repeated on different areas from 30 x 30 μm^2 to 250 x 250 nm^2 of the same gold film. The images were obtained from at least ten macroscopically separated areas on each sample. All images were processed using the standard AFM procedures [3].

The X-ray diffraction patterns were obtained by means of a standard D8 Advance Bruker X-Ray Diffractometer, working at 45 kV and 30mA. The Cu K_α radiation, Ni filtered, was collimated with Soller slits. The data of the Au (1 1 1) profile were collected in a step-scanning mode with $\Delta 2\theta = 0.02^0$ steps. Pure silicon powder standard sample was used to correct the data for instrumental broadening. The UV-VIS investigations were performed with a ABL&Jasco V 500 spectrophotometer and FTIR spectrophotometry was performed with the ABL&Jasco 6100.

2 RESULTS AND DISCUSSION

Multifunctional materials based on chitosan, globular protein and gold nanoparticles were obtained as follows. First, the 1g/L of chitosan (600 kDa, Sigma-Aldrich) dissolved in 1% aqueous acetic acid solution was mixed with nanogold solution and separately mixed with protein solution (in 1:1 volume ratio for the two solutions). Secondly, the chitosan acidic solution was mixed in a 1:1:1 volume ratio with the nanogold and protein solutions. The mixed colloidal solutions were stirred overnight and filtered. Then, each of them was individually poured in a Petri dish and allowed to dry slowly.

The resulting nanocomposites are almost flat and transparent mixed films made from chitosan and gold nanoparticles (sample CTS + Au visualized in Fig. 1), chitosan and protein (CTS + P), and chitosan, gold nanoparticles and protein (CTS + Au + P). These films have controllable thickness, depending on the volume of the mixed solution poured on the Petri dish surface.

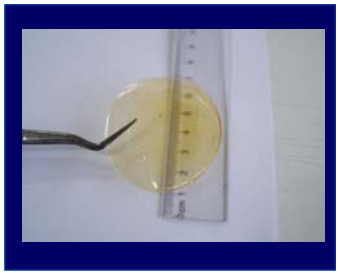


Fig.1. The composite film made of chitosan and gold nanoparticles (sample CTS + Au)

The nanostructured gold films coated by protein biomolecules were prepared by placing several drops (2.5 mL) of protein aqueous dispersion (protein content of 2 mg/L) on about 1 cm^2 of the gold film surface. These protein coated gold films were dried slowly at room temperature.

2.1 Atomic Force Microscopy (AFM)

Fig. 2 displays the top view of the nanostructured nascent gold film (sample 2) prepared by self-assembled gold nanoparticles on hydrophobic glass, at room temperature (20°C), and aged two hours at 200°C. From Fig. 2 one can clearly observe that the gold nanoparticles are packed in distorted pentagons or hexagons when aging the nascent gold film at high temperature (Figs. 2a and 2b).

Therefore, we are lad to conclude that in the initial stage of gold nascent film formation, the gold nanoparticles are aggregated and rather deformed by surface forces that are not isotropic for the surface particles. However, the gold particles retain their rounded shape at the side exposed to air.

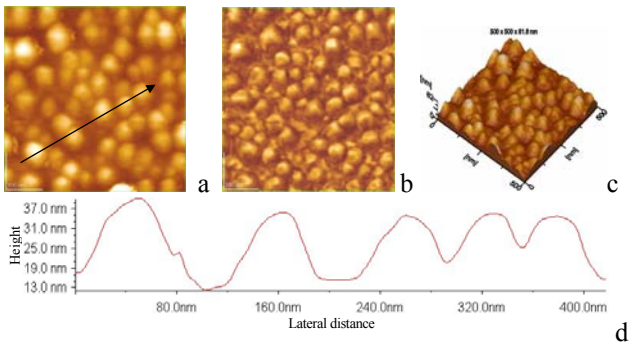


Fig. 2. 2D-topographic (panel a) and phase (b) AFM images of nanostructured gold nascent film after heating two hours at 200°C and, then, slowly cooled at room temperature (sample 2); scanned area 500 x 500 nm^2; panel c gives 3D view of AFM image from panel a; panel d represents the cross section profile along the arrow in (a).

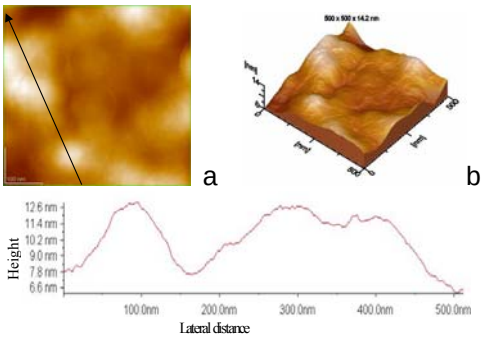


Fig. 3. 2D-topographic (panel a) AFM image of sample 2 in the presence of high protein concentration (a thicker adsorbed protein layer); panel b gives 3D-view of AFM image (a); cross section profile (c) along the arrow in (a); scanned area 500 x 500 nm^2.

The surface of thicker protein adsorbed layer is shown in Fig.3. In all cases, protein was found to be irreversibly adsorbed on gold and to form a stable protein adsorbed layer decreasing the roughness of the gold surface (Fig. 3). The protein adsorption might also occur by its spreading on the gold surface followed by protein conformational changes, probably, in the protein secondary structure.

In addition, there is a loss of contrast in the AFM images of the samples with high protein concentration adsorbed on gold film surfaces (Fig. 3). This loss of contrast corresponds to the diminished film surface roughness and to the fading of boundaries among adjacent

gold nanoparticles with protein molecules. The surface structure of these gold films coated with thicker protein adsorbed layers has nearly disappeared, especially for the heated gold film (Fig. 3). Occasionally, a few rounded shapes can be observed as in Figs. 3 (a and b) and they probably correspond to initial gold nanoparticles coated by protein or to new aggregates formed by deposited protein molecules on the gold film surface. The resulting nanoparticles show a mean particle size of about 80 nm for both films, as observed in Fig. 3c.

2.2 X-ray Diffraction (XRD)

The microstructural information obtained by single X-ray profile Fourier analysis of gold nanoparticles were the effective crystallite mean size, $D_{eff}(nm)$, the root mean square (rms) of the microstrains averaged along the [*hkl*] direction, $<\varepsilon^2>^{1/2}_{hkl}$, and the α staking fault probability [4].

Fig.4 shows the X-ray diffraction pattern for chitosan and Au + chitosan biocomposite.

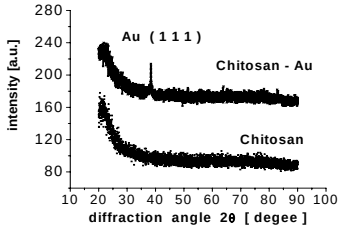


Fig.4. X-ray diffraction pattern for chitosan and Au + chitosan biocomposite (CTS+Au) : Au G.S. O_H^5 FM3M ; a = 4.0786 Å – File ASTM 4-784) , Cu K_α.radiation .

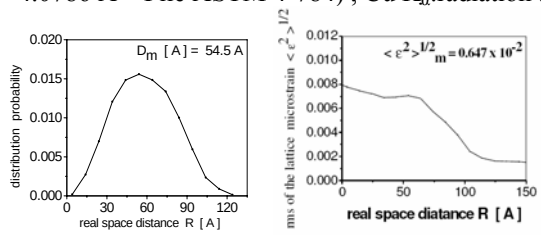
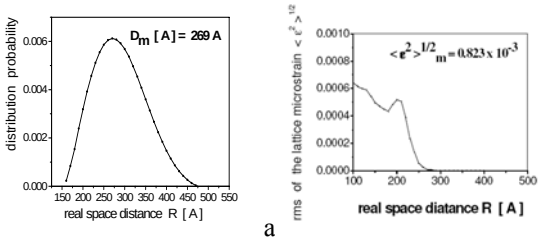


Fig.5. Effective crystallite size distribution and root mean square (rms) of the lattice microstrain $<\varepsilon^2>^{1/2}$ (L) distribution along the Au [111] crystallographic direction for Au thin layer (sample 2).



a b

Fig. 6. Effective crystallite size distribution and root mean square (rms) of the lattice microstrain $<\varepsilon^2>^{1/2}$ (L) distribution along the Au [111] crystallographic direction for Au + protein biocomposite (sample 2+ P).

The single (111) Au and (200) Au X-ray diffraction profiles were analyzed in order to determine the microstructural parameters of Au nanoclusters [5].

The Warren-Averbach X-ray profile Fourier analysis of the (111) Au peak profile was processed by a XRLINE [5] computer program. The crystallite size distribution function was determined from the second derivative of the strain-corrected Fourier coefficients [6].

Figs. 5 and 6 show the microstructural information obtained by single X-ray profile Fourier analysis of gold nanoparticles from sample 2 and sample 2 + P, respectively. Table 1 summarizes the microstructural parameters of gold nanoparticles from the gold film (sample 2), gold film with protein (sample 2 + P) as well as from chitosan with gold nanoparticles (CTS + Au biocomposite).

Samples	$D_{eff}(nm)$	$<\varepsilon^2>^{1/2}_{hkl} \times 10^3$	α
CTS+Au	27	1.45	0.008
sample 2	5.5	6.47	0.017
sample 2 + P	27	0.82	0.002

Table 1: Microstructural parameters of gold nanoparticles For abbreviations see the text

The X-ray diffraction pattern of Au + chitosan biocomposite (CTS+Au) contains a (111) reflection, indicating a nanocystalline system of the Au type structure (Fig.4). The Au chitosan biocomposite has been confirmed to be Au with f.c.c. structure with the lattice constant (a_0 = 4.0786 Å). The microstructural parameters of nanostructured gold film after heating two hours at 200 ^oC (sample 2) showed an average size of gold nanocrystals from about 5.5 nm (Fig. 5) to 8 nm (for gold nanocrystals within sample 2, after three months). In the presence of high protein concentration, the gold nanocrystals are more aggregated in the Au + protein biocomposite film (sample 2 + P) than in pure gold films (sample 2) in a similar way as found in the case of chitosan + gold nanoparticles (sample CTS + Au). The average size (about 27 nm) for these films is also indicated in Table 1.

The lattice microstrain $<\varepsilon^2>^{1/2}$ distribution along the Au [111] crystallographic direction for Au + protein biocomposite (sample 2 + P) shows an increases value in the intercrystallite zones that suggests a gold nanoparticles - protein interaction (Fig.6.).

2.3 FTIR and UV-VIS spectrophotometry

Fig.7. shows the FTIR spectra for chitosan films (CTS), chitosan films with gold nanoparticles (CTS+Au), chitosan and globular protein (CTS+P) and the spectra of chitosan with gold and protein (CTS+Au+P).

Chitosan (CTS) films with either gold nanoparticles (CTS + Au) or globular protein (CTS + P) and the films containing their three-component combination (CTS + Au + P) exhibit almost the same absorption bands, but, analyzing the details little differences still can be observed.

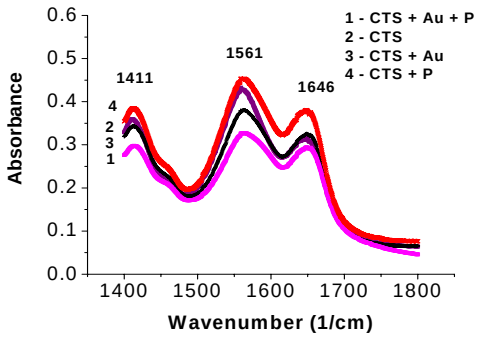
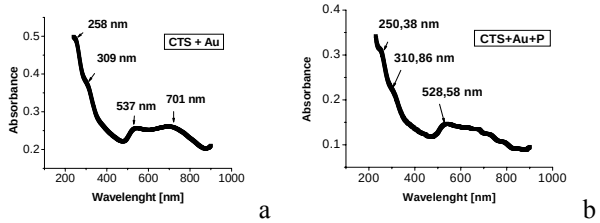


Fig.7. FTIR spectra for chitosan films (CTS), chitosan films with gold nanoparticles (CTS+Au), chitosan and globular protein (CTS+P) and the spectra of chitosan with gold and protein (CTS+Au+P).

The intensity modifications of bands centered at 1646 and 1561 cm^{-1}, for the three-component (CTS + Au + P) biocomposite against the intensities at the same wave numbers (Fig. 7) for the other investigated films, are suggesting the protein effect on the internal organization within this nanocomposite film.



a b

Fig.8. UV-VIS spectra of chitosan (CTS) film, with gold nanoparticles (panel a, CTS + Au) and with gold nanoparticles and globular protein (b, CTS + Au + P).

The UV-VIS spectra (Fig. 8), region 250 nm – 310 nm presents two maximum intensities corresponding to the chitosan film. A maximum intensity at 537 nm is present for gold nanoparticles (Fig. 8a) and its shift to 528.58 nm is shown in the presence of the protein (Fig. 8b). Also, a maximum at 701 nm is observed (Fig. 8a) suggesting the existence of quasi-ordered chitosan-gold aggregates. The interference fringes (Fig. 8b), appearing on chitosan with gold and protein film for wavelength bigger than 600 nm, are clearly suggesting an internal organization of protein secondary (β-sheet and α-helix) structure [7] in substantial agreement with FTIR observations (Fig. 7).

3 CONCLUSIONS

The AFM images of nanostructured gold nascent film obtained at room temperature and of gold film after heating two hours at 200°C showed a subtle increase in the average size of the gold particles from 40 nm (gold nascent film) to 50 nm (aged film at high temperature). The gold nanoparticles are aggregated in the nascent film, forming ordered rows of chain-like assembled nanoparticles. By aging the nascent gold film at high temperature, the gold nanoparticles are packed in distorted pentagons or hexagons on the film surface.

The AFM observations also allowed for a topographical examination of the nanostructured gold films coated with protein layers at two different protein concentrations. The analysis of AFM images confirmed that the protein forms a stable layer following the morphology of gold nanostructured films. In the presence of high protein concentration (a thicker adsorbed protein layer) gold nanoparticles are aggregated in the Au + protein biocomposite film. The lattice microstrain distribution along the Au [111] crystallographic direction for Au + protein biocomposite (sample 2 + P) suggests a gold nanoparticles - protein interaction.

The binding of protein to gold film surfaces leads to the stabilization of nanostructured gold films, forming crosslinks among gold nanoparticles. Electrostatic interactions between protein biomolecules (positively charged N-terminal amino acids of the protein, e.g. arginine repeated for several times in the protein N-terminal) and gold nanoparticles (negatively charged) appear to be very important in the chosen working conditions.

The X-ray diffraction pattern of Au + chitosan biocomposite (CTS+Au) contains a (111) reflection, indicating a nanocrystalline system of the Au with f.c.c. type structure. The UV-VIS and IR investigations are suggesting the self-organization of the protein in the chitosan thin films.

The findings make these materials based on chitosan, globular protein and gold nanoparticles well suitable for industrial and biomedical applications.

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