Significance of Surface Structure on Orthopedic Materials

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The surface effect of nano- and microscale structures of some orthopedic materials, containing nano hydroxyapatite (HAP) and collagen type I (COL), on the interactions of osteoblasts with the surface of these materials is investigated and analyzed. The size and shape of inorganic particles are examined by TEM, SEM and AFM imaging. The surface morphology of collagen self-assemblies is also explored by AFM and SEM observations. Results show that the effect of the surface structure is significant on cell behaviour and can be correlated with cell constructs on these materials in cell culture.

Keywords: orthopedic materials, surface structure, osteoblasts, TEM, SEM, AFM

An increasing interest is documented for the uses of nanostructured surfaces to continually improve the cytocompatibility and osteointegration of orthopedic materials [1-8]. Generally, orthopedic nanobiomaterials are formed from constituent particles with sizes less than 100 nm in at least one dimension using nanotechnology for making surface structures for cellular engineering as well as for coatings of orthopedic implants. The request for nanostructured materials is currently especially high.

Although, the surface characterization of biomaterials has been extensively investigated [3], still a lack of reliable data about material cytocompatibility exists. Particularly, the cellular response to different surfaces with a defined morphology is not well known or it is inadequately understood, due to many variables that influence the cell interactions with surface structures.

The cytocompatibility of nanostructured materials is strongly influenced by its chemical composition and surface topography, which are important factors for cell and surface interactions. They can influence the behavior of osteoblasts (bone making cells), particularly cellular viability, adhesion, migration, differentiation and proliferation.

Collagen type I (COL), might be used for the enhancement of surface biocompatibility of nanoparticles of calcium phosphates, like hydroxyapatite (HAP), particles which are used for coatings on titanium implants [2]. Undoubtedly, COL became an interesting biocompound due to its auto-associative properties [9, 10]. On the other hand, COL is the major fibrillar protein in the extracellular matrix and is abundant in bone, cartilages, ligaments and tendons.

This work is carried out to determine the role of nanoscale structure and roughness of the surface of some nanostructured materials, made of nanoHAP and COL, on the interactions of osteoblasts with these materials, using scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atomic force microscopy (AFM). The effect of nano- and microscale structures on the interactions of osteoblasts with the surface of these materials is described and appears to be related to the COL cellular production.

Experimental part

Materials and methods

The starting compounds used include calcium nitrate, $Ca(NO_3)_2 \cdot 4H_2O$, calcium acetate $Ca(CH_3COO)_2 \cdot 2H_2O$, diammonium hydrogen phosphate, $(NH_4)_2HPO_4$, ammonium hydroxide (NH_4OH), sodium silicate in the molar ratio of $Na_2O:SiO_2$ of 1:3.2, nonylphenol, acetic acid (CH_3COOH) and tetraethyl ortosilicat (TEOS, 98 wt%, used as the silica source). They were acquired from Merck. Bovine Achilles tendon collagen, type 1 (COL), was purchased from Sigma. All compounds were used without further purification. Deionized ultrapure water was used in all experiments. Collagen was independently dispersed in deionized water at a pH of about 3, made with acetic acid (CH_3COOH).

Synthesis of nanoHAP

Nanostructured hydroxyapatite (nanoHAP) was prepared by the synthesis procedure. The $Ca(NO_3)_2 \cdot 4H_2O$ was completely dissolved in water at a concentration between 0.15 M and 0.24 M, in the presence of nonylphenol at *p*H 6.3. Separately, $(NH_4)_2HPO_4$ was dissolved in water at a concentration between 0.036 and 0.090 M, in the presence of nonylphenol and the pH was adjusted at about 11.5 with NH_4OH. The two solutions were mixed and the *p*H of the resulted colloidal dispersion was adjusted at a value between 9.5 and 11.5, with ammonium hydroxide. Then, the colloidal dispersion was sealed in a container and kept inside of a water bath for 48 h at 80 °C up to 85 °C for its maturation. During the maturation process, the dispersion was vigorously and continuously stirred, to allow the calcium phosphate to nucleate and grow properly to give nano hydroxyapatite (nanoHAP). In the presence of a surfactant, such as nonylphenol, the nucleation and the growth of HAP nuclei can be controlled. The resulted final suspension was filtered, and the precipitate was washed with deionized water until no NO_3^- ions were detected. Then, the precipitate was dried by lyophilization. After that, it was heated at about 650 °C, to obtain nano crystals of HAP. This optimized hydrothermal method resulted in a nanostructured HAP powder of controlled stoechiometry, high cristallinity, and nano sized particles (nanoHAP).

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Collagen aqueous dispersion

The collagen dispersion was prepared at *p*H 3, as elsewhere presented [9, 10]. Collagen fibrils were re-assembled from acidic aqueous solutions of COL in the absence and the presence of nanoHAP. The COL self-assemblies were engineered by deposition method (e.g., casting method) of COL aqueous dispersions on different solid supports, like glass.

Preparation of nanoHAP/COL thin layers on glass support (known as nanostructured fibrous scaffolds)

The nanoHAP powder is redispersed at a wanted concentration in deionized water and the resulted colloidal dispersion is further used both for TEM investigations and for the preparation of composites with collagen, as layered scaffolds. The mixed nanoHAP/COL layers were made of nanoHAP and collagen (COL) by self-assembling layer by layer method on solid substrates, such as flat glass plates.

More specific, the optically flat glass (solid support) was initially cleaned with sulphochromic acid and washed with deionized water. Then, it was treated with 5% HCl aqueous solution for 15 min, and after that, rinsed with deionized water for three times. Then, it was shortly immersed in 2% sodium silicate aqueous solution for ten times. Through this surface treatment, the hydrophilic glass surface was enriched in hydroxyl groups able to couple chemically with the deposited layers on the glass by adsorption from colloidal dispersions. Thus, the successive layers of nanoHAP and collagen were built from their respective colloidal solutions on glass support by vertical adsorption for 5 min, by washing and drying between the layer depositions.

After that, the inorganic thin layer was built by vertical adsorption (about 5 min) from the 6% nanoHAP aqueous dispersion (*p*H 7) on that hydrophilic glass support, activated with sodium silicate solution. The resulted nanoHAP layer was washed and dried and then, it was activated by treatment with sodium silicate aqueous solution for about 3 min. Subsequently, the inorganic layer was dried and COL layer was deposited by adsorption from collagen colloidal solution (*p*H 3) for about 5 min. After the drying process, the biocomposite material (HAP/COL layered scaffold) was washed with 1% acetic acid solution in order to remove the excess of Na^+ ions.

Synthesis of nanoHAP-COL composite cement

The composite cement has been prepared by precipitating nanoHAP and assembling COL fibrils simultaneously by the self-organization mechanism of hydroxyapatite and collagen. A solution of TEOS about 1 % in ethyl alcohol was independently prepared. The COL solution was mixed under continue stirring with 1 ml TEOS solution for 30 min. Then, collagen solution was mixed with nanoHAP aqueous dispersion under intense stirring for 1 hr at *p*H 7 adjusted using sodium hydroxide solution. The HAP to collagen weighted ratio was monitorred at 7: 3. After that, the obtained precipitate (HAP-COL cement) was kept under continuous stirring at room temperature for 24 h and then it was filtered and dried by lyophilization.

Human osteoblasts

The human osteoblasts have been obtained in vitro from adult mesenchymal stem cells derived from the human bone marrow of the iliac crest using monoclonal antibodies [11]. Osteoblasts were cultured on combined nanoHAP/COL layered scaffolds under the standard cell culture conditions for up to seven days. Osteoblasts were cultured in complex osteogenic medium (DMEM), supplemented with 15% fetal calf serum (FCS), 2 mM L-glutamine, 10 nM dexametazone, 1% non-essential amino acids, 50 μg ascorbic acid, 10 mM β-glycero-phosphate, μg insulin, 2 ng/mL transforming growth factor β1 (TGF-β1μg) and 3 ng/mL bone morphogenic protein 2 (BMP-2) (all purchased from Sigma-Aldrich).

For cell culture, nanostructured fibrous scaffolds made of nanoHAP/COL thin layers self-assembled on flat glass support were first sterilized under ultraviolet light for 4 h. These combined scaffolds were again sterilized with 70% ethanol for 1 h, and then washed for five times with phosphate buffered saline (PBS) for 30 min; finally, they were soaked in culture medium overnight. Osteoblasts were then seeded at a seeding density of 10^4 cells/cm^2 on the combined scaffolds. Cells viability and proliferation were monitored using MTT assay [11].

The obtained composites, nanoHAP and nanoHAP/COL cement, COL fibers prepared in the absence and the presence of nanoHAP deposited layers were characterized by imaging techniques. The samples were imaged by the scanning electron microscope (SEM), JEOL, JSM 5510 LV, using the secondary electron imaging technique (SEI) [12], the transmission electron microscope (TEM), JEOL-JEM 1010 [13] and atomic force microscope (AFM) JEOL 4210, operating in tapping mode [14-16].

SEM, TEM and AFM samples

SEM samples have been prepared by deposition of each powder in thin films on carbon coated SEM grids or on an adhesive metallic support. Before SEM imaging, samples were gold coated for a better conductivity in the AGAR Auto Sputter Coater. The thin gold coating (thickness 10 nm) was sputtered in three sputtering cycles taking about 10 s each. These metallized samples were examined by SEM at different magnifications.

TEM samples were prepared using the standard protocol by the adsorption on TEM grids of various materials, which are redispersed at a wanted concentration in deionized water forming the aqueous colloidal dispersions.

AFM samples were prepared by spreading out of each composite powder in thin layers on a double adhesive band. Then, each thin film composite sample was independently affixed to the AFM sample support for AFM observation. Both COL layers and nanoHAP/COL layered biocomposite scaffolds are made as self-assembled deposited layers on flat glass support and used as manufactured for AFM investigation.

The structure and morphology of cellular constructs, made due to the interaction of osteoblasts with newly created layered scaffolds, were investigated by AFM imaging. After three days in cell culture, the fibrous combined scaffolds were washed with PBS to remove non adherent cells. Afterwards, the adherent osteoblasts were removed by tripsinization procedure [11]. The dried cellular constructs were examined by AFM.

Results and discussions

The surface morphology of nanoHAP powder and nanoHAP-COL composite cement is given in figures 1-4. The surface structure of COL self-assemblies is given in figures 5-7, both in the absence (figs 5 and 6) and the presence of nanoHAP (fig. 7). The collagen network produced by adhered cells on scaffolds made of nanoHAP-COL self assembled layers is given in figure 8.

TEM images, given in figure 1, confirm that powders of nanoHAP (fig. 1a) and nanoHAP-COL composite cement (Fig. 1b) present a rod like structure and acicular particles are smaller than 100 nm long.

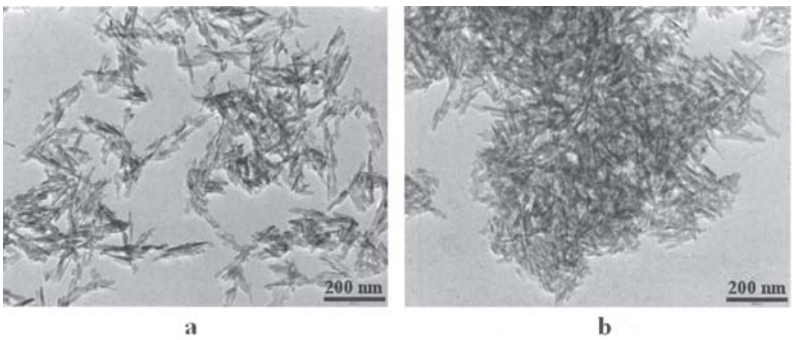

Fig. 1. TEM images of nanoHAP powder (a) after thermal treatment at 650°C and of nanoHAP-COL cement (7:3, weight ratio) (b); bars in the images correspond to 200 nm

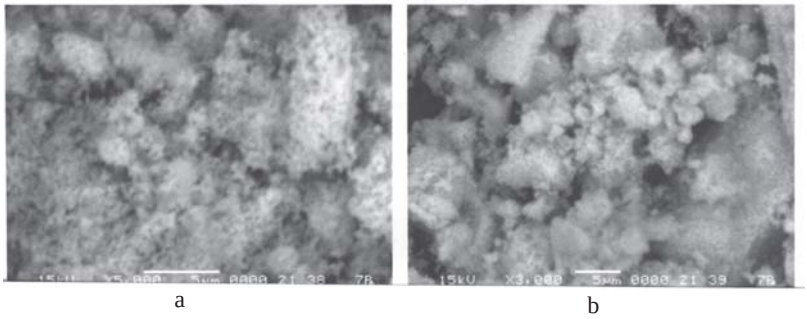

Fig. 2. SEM micrographs of nanoHAP powder (a) and of nanoHAP-COL cement (7:3, weight ratio) (b); bars correspond to 5µm.

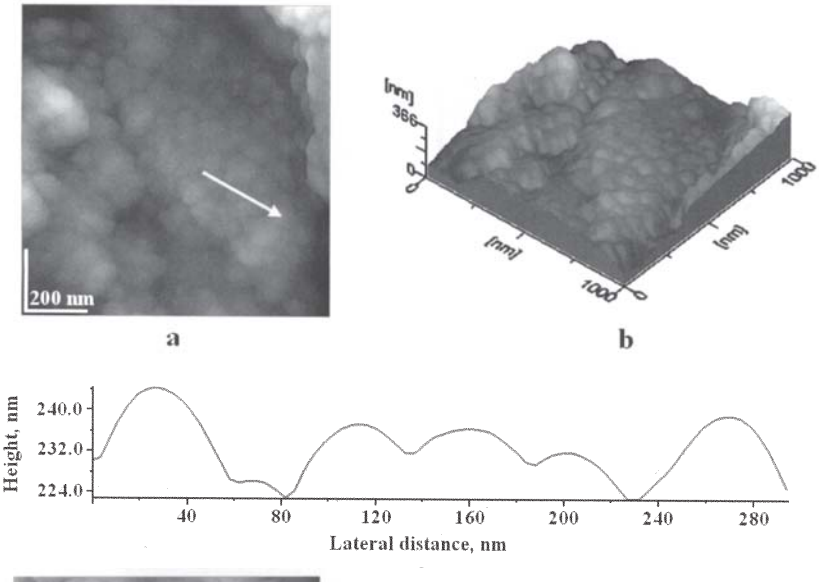

Fig. 3. AFM images of nanoHAP powder; a) 2D-topography image; b) 3D view of image a (height 366 nm); c) cross section profile along the arrow in panel a. Scanned area 1µm x 1 µm. Surface roughness, given by RMS, 43 nm (± 6 nm) on scanned area. Standard deviation is denoted in parentheses

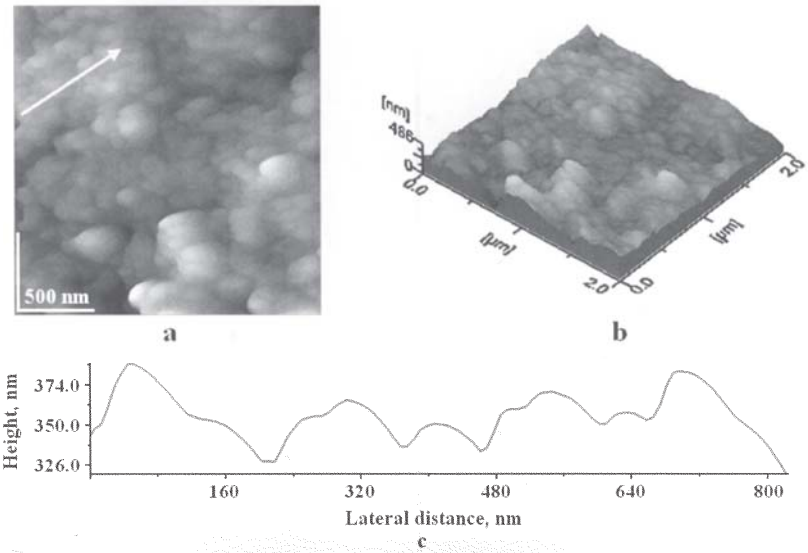

Fig. 4. AFM images of nanoHAP-COL cement powder; a) 2D-topography image; b) 3D view of image a (height 486 nm); c) cross section profile along the arrow in panel a. Scanned area 2 µm x 2 µm. RMS 64 nm (± 8 nm). Symbols as in figure 3.

Figure 2 shows SEM images for nanoHAP powder (fig. 2a) and for nanoHAP-COL composite cement (fig. 2b) and a different morphological aspect is observed than in TEM images (fig. 1). However, both materials present a similar porous structure made of almost spherical particles. They are further investigated by AFM (figs. 3 and 4).

The average diameter of about 40 nm (± 3 nm) is determined for nanoHAP (fig. 3) and about 75 nm (± 7 nm) is estimated for nanoHAP-COL cement (fig. 4). Evidently, depending on the preparation process the size and the shape of particles can be modified and controlled. The nanoparticles within the nanoHAP-COL cement are

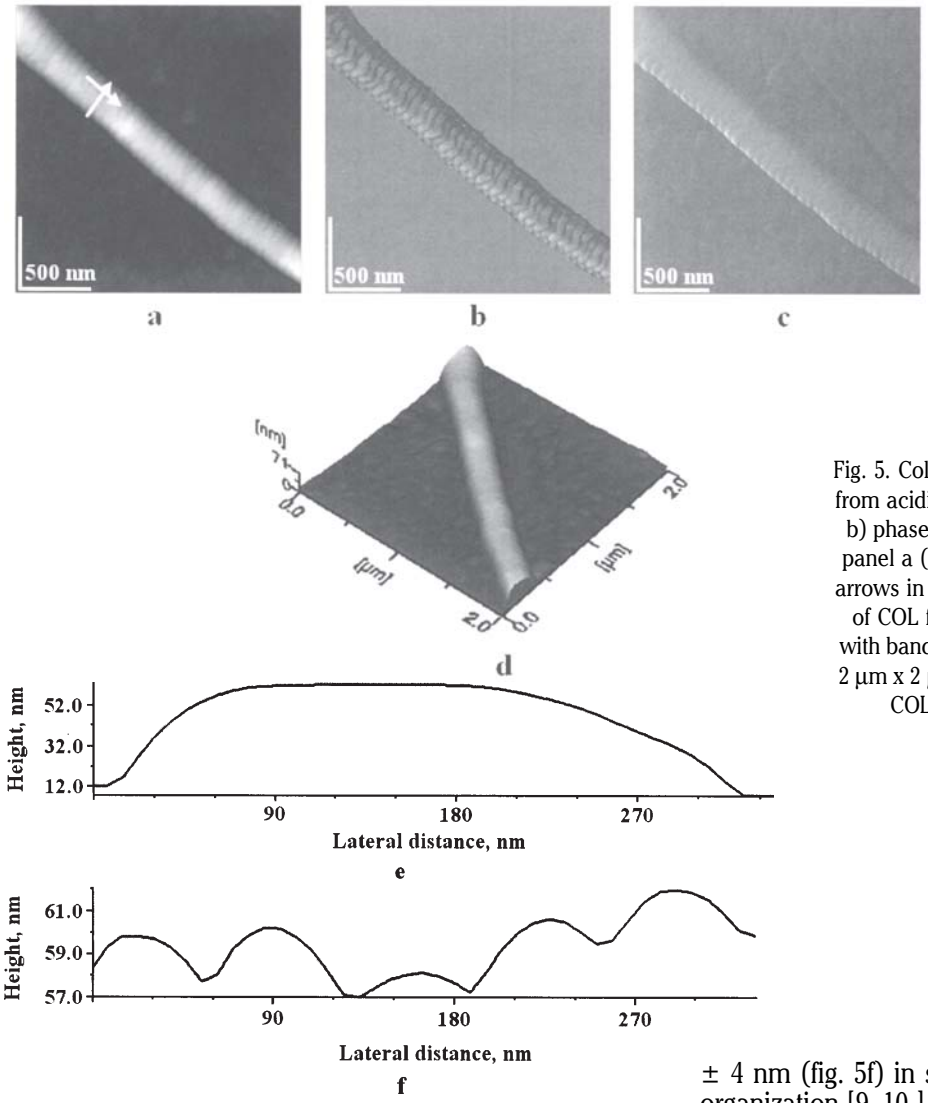

Fig. 5. Collagen fiber self assembled on glass surface from acidic dispersion of collagen. a) 2D-topography; b) phase image; c) amplitude image; d) 3D-view of panel a (height 71 nm); cross section profiles along arrows in panel a: perpendicular (e) on the long axis of COL fiber and (f) on the long axis of COL fiber with banding pattern 67 nm (± 4 nm); scanned area: 2 µm x 2 µm; RMS 18 nm (± 3 nm) on scanned area. COL fiber width about 270 nm (± 19 nm).

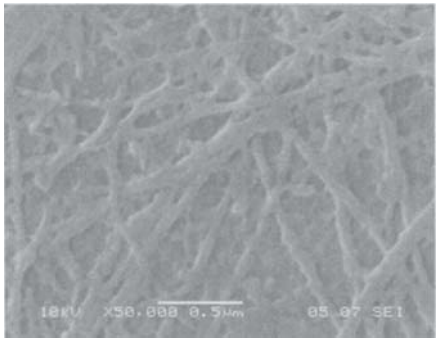

Fig. 6. SEM image of collagen fibers self-assembled on carbon coated grid. Scale bar of 0.5 µm.

bigger than in nanoHAP powder due to the COL layer which is coating the nanoHAP particles. Correspondingly, the surface roughness (given as root mean square, RMS) of about 43 ± 6 nm of nanoHAP layer is smaller than for nanoHAP-COL cement layer (64 ± 8 nm).

Further, a study was carried out to determine the nanoscale structure of collagen fibers self assembled on glass support from colloidal solutions using AFM (fig. 5) and SEM (fig. 6) observations. It is to be noted that the COL fibers artificially created are randomly oriented on glass support as also evidenced by SEM image (fig. 6).

Furthermore, the nodule structure of collagen fiber is shown by AFM imaging (fig. 5) and indicates a different mass density across the COL fiber. It also exhibits a transverse banding pattern with a periodicity of about 67 ± 4 nm (fig. 5f) in substantial agreement with collagen organization [9, 10].

The surface morphology of self-assembled collagen in the presence of inorganic phase is revealed in figure 7. The analysis of AFM images shows that the COL fiber can be identified as a number of rather short cylindrically shaped fibrils arranged within that COL fiber (figs. 7a and 7d) in good agreement with some reported data on related systems [17]. During the COL adsorption on nanoHAP layer, previously deposited on glass support, a structuration process appears and collagen fiber is assembled and simultaneously mineralized with nanoHAP particles at the interface with nanoHAP layer. As illustrated in figures 7a-d, nanoparticles of nanoHAP are imbedded within the COL fiber and the fine banding pattern is not possible to be identified (fig. 7e).

Alternatively, figure 7a shows an internal mineralization of COL fiber and the banding pattern is not any longer observed. In addition, figures 7b and 7c reveal the presence of nanoparticles of nanoHAP attached to the surface of COL fiber. As a consequence of an internal and external mineralization process of collagen, the mixed nanoHAP/COL fibers are formed. A stabilizing effect of collagen fibers might appear through the interaction of COL with nanoHAP particles. Accordingly, the fibrous combined scaffold is made from nanoHAP/COL layers by using layer by layer technique. AFM images (fig. 7) indicate that the collagen fiber is mineralized in the presence of nanoHAP within the nanoHAP/COL layered scaffolds. The adsorption of COL on nanoHAP surface, previously activated with silicon (see, experimental section), is probably due to electrostatic forces, van der Waals forces and hydrogen bonds.

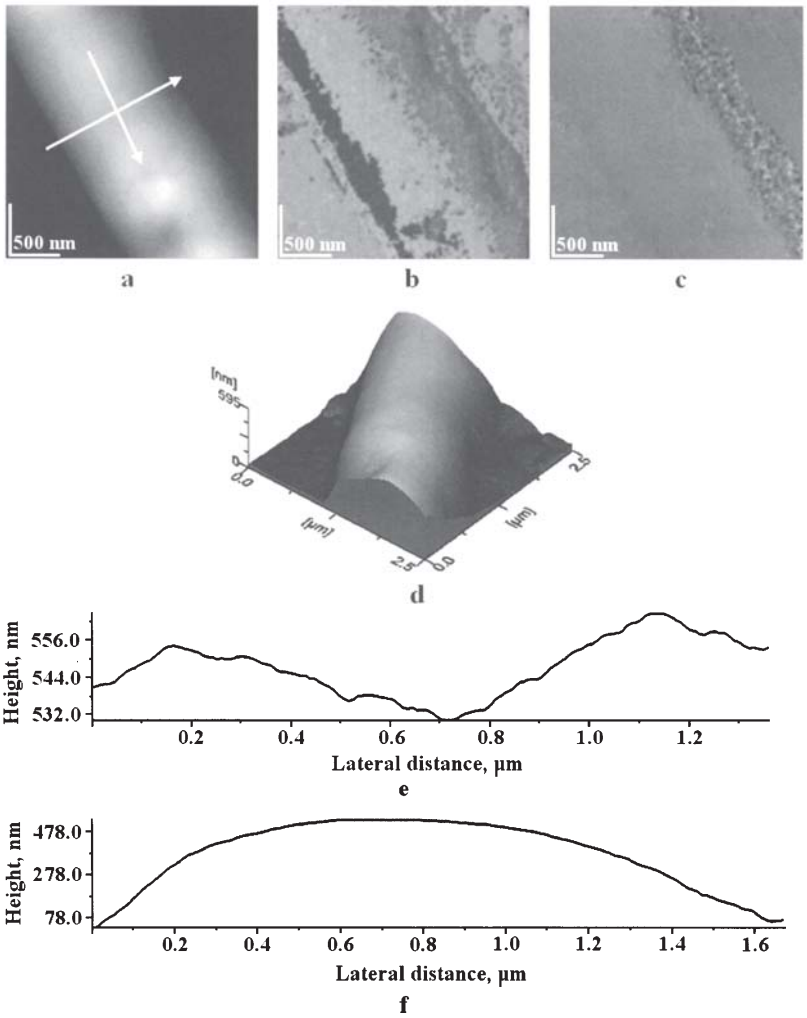

Fig. 7. Collagen fiber mineralized with inorganic phase (HAP/COL layers deposited on glass surface). a) 2D-topography; b) phase image; c) amplitude image; d) 3D-view of panel a (height 595 nm); cross section profiles along the arrows situated in panel a, on the long axis of fiber (e) or perpendicular (f) on it; scanned area 2.5 µm x 2.5 µm; RMS 207 nm (± 16 nm) on scanned area. COL fiber width about 1400 nm (± 110 nm)

Further, SEM and AFM imaging has been used to evaluate the behaviour of osteoblasts, cultured for three days on scaffolds, made of nanoHAP powder, nanoHAP-COL cement, pure collagen layers and combined nanoHAP/COL layers deposited on glass support. Then, the cells were removed by trypsinization procedure [11]. After that, the surface of these scaffolds was deeply analyzed by AFM and SEM investigations. In this respect, a representative example of AFM images on cellular constructs is shown in figure 8.

Results suggest that the cells adhesion and growth as well as the cell collagen production are substantially improved on combined fibrous scaffolds (fig. 8), made of nanoHAP/COL layers. The morphology of combined fibrous scaffold in absence of cells is shown in figure 7. A thoughtful analysis of AFM images shows that the cytocompatibility of combined fibrous scaffolds is substantially increased than for nanoHAp-COL cement (fig. 1b and fig. 2b), or on pure COL layers (fig. 5 and fig. 6), or on nanoHAP powder (fig. 1a and fig. 2a). Evidently, the addition of COL in these materials appears to enhance the osteoblasts activity leading to the improved collagen production at the early stage of bone development, in bone healing and remodeling.

The surface roughness, as root mean square (RMS), of these materials was also evaluated by AFM imaging. The RMS values are decreasing in the following order, combined fibrous nanoHAP/COL material (207 ± 16 nm, fig. 7) > nanoHAP-COL cement (64 ± 8 nm, fig. 4) > nanoHAP (43 ± 6 nm, fig. 3) > pure COL layers (18 ± 3 nm, fig. 5). The cell behavior was evaluated on these materials of different surface roughness. The increasing surface roughness appears to enhance the cell activity and the combined fibrous nanoHAP/COL material is superior to the nanoHAP-COL cement in cell culture. Thus, the surface roughness is clearly an important parameter governing the overall cell behaviour at cells and material interface, besides chemical composition and nanostructures at the material surface.

In this regard, a representative AFM image is shown in figure 8, in which a collagen fiber (fig. 8a) is observed on the top of other COL fibers as indicated in figures 8b-d and it appears to have a strong structural relationship to the underlying collagen fibers. In fact, many COL fibers with evident periodicity along their longitudinal axes, about 74 ± 5 nm (fig. 8e) are observed on the surface of combined fibrous scaffold, after three days in cell culture. The structure and organization of these COL fibers reveal highly ordered arrangements of rather thick mineralized collagen fibers (width about 480 ± 33 nm, fig. 8f). They are preferentially arranged parallel to each other, in a particular plan (figs. 8b and c) closely packed and better arranged than those observed for the pure COL fibers (fig. 6). As pointed in figure 8d, COL fibers (221 nm) are thicker than the pure COL fiber (about 71 nm, fig. 5d) but thinner than artificially mineralized COL fiber (height about 595 nm, fig. 7d). The surface roughness (4 ± 1 nm, fig. 8a) of collagen network is very low leading to a rather compact structure.

The difference in the periodic pattern (figs. 5f, 7e and 8e) shows clearly the influence of the inorganic particles incorporated inside the collagen fiber structure. These inorganic particles are not individually seen because they are incorporated intrafibrilary. They might enhance the mechanical stability and the resistance of collagen fibers through their binding process and molecular interactions with collagen molecules.

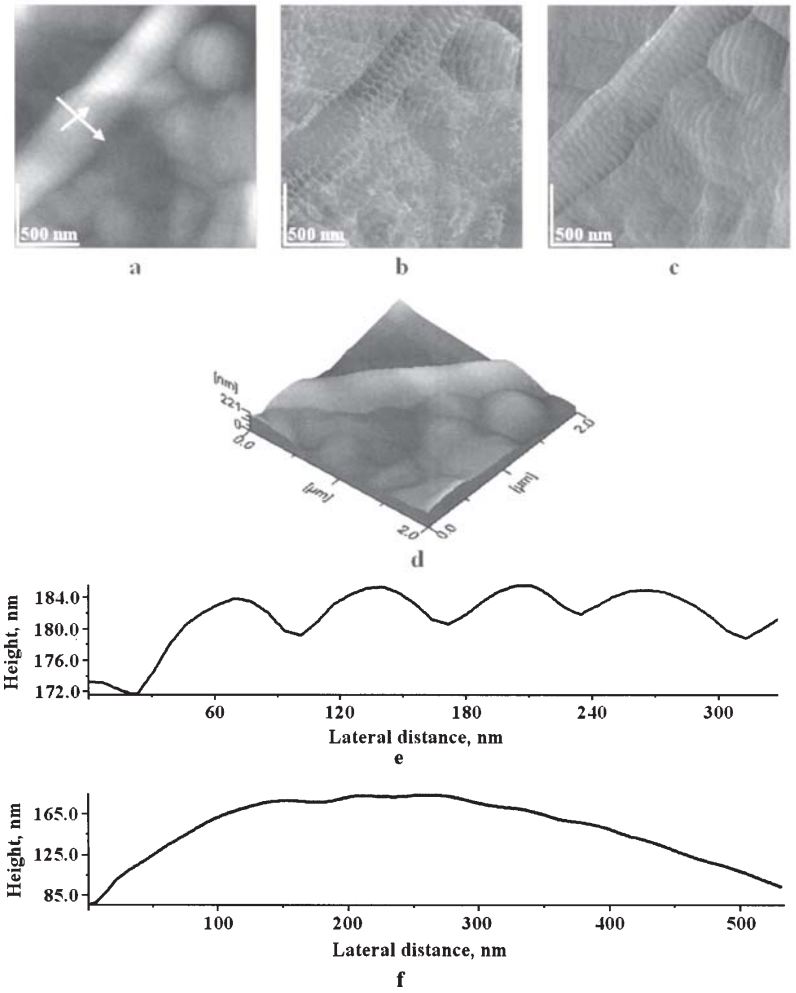

Fig. 8. Collagen fibers were produced by osteoblasts on the scaffold surface, made of nanoHAP and COL combined layers (see, HAP/COL layers in absence of cells in fig. 7). Osteoblasts were grown on the scaffold for three days and then, cells were removed by trypsinization procedure. a) 2D topography; b) phase image; c) amplitude image; d) 3D-topography (height 221 nm); cross section profiles along the arrows situated in panel a, on the long axis of fiber (e), banding pattern 74 nm (± 5 nm) or perpendicular (f) on it, COL fiber width about 480 nm (± 33 nm); scanned area 2 µm x 2 µm; RMS 4 nm (± 1 nm) on scanned area.

The fibers shown in figure 8 are very close and are arranged almost parallel or in different directions as mainly illustrated in figures 8b and 8c. In 2D- (fig. 8a) and 3D- (fig. 8d) topographies is not easy to distinguish individual COL fibers because the borders between fibers are somehow hidden, the fibers being at different levels in comparison with the outmost layer visualized by AFM images. However, the AFM images given in figure 8 are similar to the AFM images observed on the surface of a human trabecular bone [18].

These findings support the idea that the general structure developed by osteoblasts on combined fibrous scaffolds, engineered from nanoparticles of hydroxyapatite and collagen, is similar with the structure observed in human bone [18]. The size range of collagen fibers is roughly the same in this work as observed and reported by others [18]. Nevertheless, a clear evidence is also found in figure 8c of some individual collagen fibers that are running almost perpendicular to the main direction of the fibers from the underlying layer. This cross linking of fibers was also observed for the trabecular bone [18]. Apparently, the collagen fibers might have direct relation to mechanical properties and consequently, to the quality of bone. As demonstrated above, the combined fibrous material (scaffold from nanoHAP/COL layers) can stimulate the cell collagen production (fig. 8) and consequently, it might improved the new bone formation both in vitro and in vivo, in substantial agreement with reported data on related biomimetic films [19].

Conclusions

The summary of the surface structural features observed by AFM, TEM and SEM on several orthopedic materials developed in this work (like, nanoHAP, nanoHAP-COL cement, pure collagen layers and combined fibrous nanoHAP/COL layers) provides new evidence supporting the concept of the significance of surface effect (composition and nano structures) on the cyto-compatibility of materials and on their uses for bone tissue regeneration.

The obtained results showed a rather good adhesion of osteoblasts on the surface of all four different biocomposite scaffolds. However, it clearly appears that the most efficient scaffold developed here to stimulate the cell growth and the collagen cell production is the fibrous combined nanoHAP/COL layered scaffold.

Thus, the combined fibrous material (scaffold), built up from nanoHAP/COL layers on glass, has demonstrated that both the internal and external mineralization of COL fibers can stimulate the COL cell production in vitro, and finally might improve the development of a mineralized collagen network created by the cells, in vitro, with an important impact on new bone formation in vivo.

These results serve to fill a gap in knowledge between cell behaviour and the surface characteristics of these orthopedic materials, which can be used as scaffolds in cell culture, as bone cements, or as coating layers of bone implants to increase the osteointegration in vivo.

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