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Original Article

ORTHOPEDIC SURGERY AND BONE REGENERATION. ADVANCED FUNCTIONAL BIOMATERIALS BASED ON NANOHYDROXYAPATITE AND COLLAGEN

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Abstract

The effect of composition and surface structure of novel biomaterials, containing nano hydroxyapatite (nanoHAP) 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 transmission electron microscopy (TEM), scanning electron microscopy (SEM) and atomic force microscopy (AFM) imaging. The surface morphology of collagen self-assemblies is explored by AFM and SEM observations. The osteoblasts are cultivated on five groups of scaffolds, namely scaffolds made of nanoHAP, COL layers, nanoHAP-COL cement, nanoHAP/COL combined layers as well as of nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL combined layers in cell culture. The cellular production of collagen, osteopontin and osteocalcin is visualized by fluorescence microscopy (FM) by using monoclonal antibodies and immunocytochemical methods. Results show that the effect of composition and surface structure is significant on cell behaviour and function and can be correlated with cell constructs on these scaffolds in cell culture. The data indicate that the scaffolds made of nanoHAP/COL combined layers and nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL combined layers have a significantly improved bioactivity compared with the other three groups, e.g. nanoHAP scaffolds, especially in promoting the formation of mineralized bone matrix. The nanoHAP/COL scaffolds and especially nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL scaffolds substantially enhance the osteoblasts function and can have a high potential clinical application in nanomedicine from orthopedic surgical treatment of nonunion fractures to bone cancer therapy.

Keywords*: orthopedic biomaterials, bone scaffolds, osteoblasts, nanotechnology*

Rezumat

În lucrarea de față este analizat efectul compoziției şi structurii de suprafață a noilor biomateriale ce conțin nano-hidroxiapatită (nano-HAP) şi colagen de tip I (COL), asupra interacțiunii osteoblaştilor cu suprafața acestor materiale. Forma şi dimensiunea particulelor anorganice sunt examinate cu ajutorul microscopiei electronice de transmisie (TEM), microscopiei electronice de scanare (SEM) şi microscopiei de forță atomică (AFM). Morfologia de suprafață a particulelor de colagen auto-asamblate este explorată prin observații de AFM şi SEM. Osteoblaştii sunt cultivați pe cinci grupe de scaffold-uri (matrițe), şi anume scaffold-uri de nano-HAP, straturi de colagen, ciment de nano-HAP şi colagen, straturi combinate de nano-HAP/COL, ca şi straturi combinate de nano-HAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL în culturi celulare. Prin microscopie de fluorescență (FM) este vizualizată producția celulară de colagen, osteopontină şi osteocalcină, cu ajutorul anticorpilor monoclonali şi a metodelor de imunocitochimie. Rezultatele arată că efectul compoziției şi structurii de suprafață este semnificativ pentru comportamentul şi funcția celulelor şi poate fi corelat cu structurile celulare pe aceste scaffold-uri, la nivelul culturilor celulare. Datele indică faptul că scaffold-urile formate din straturi combinate de nano-HAP/COL şi straturi combinate de nano-HAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL au o bioactivitate semnificativ îmbunătățită comparativ cu celelalte trei grupuri (spre exemplu scaffold-urile de nano-HAP), în special în ceea ce priveşte promovarea formării de matrice osoasă mineralizată. Scaffold-urile de nano-HAP/COL, şi în special cele de nano-HAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL, îmbunătățesc substanțial funcția osteoblaştilor şi pot avea un deosebit potențial pentru aplicații clinice in nanomedicină şi ortopedie, de la tratamentul chirurgical al pseudartrozelor, la tratamentul tumorilor osoase.

Cuvinte-cheie: *biomateriale ortopedice, osul scafoid, osteoblaşti, nanotehnologie*

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Introduction

Functional biomaterials (scaffolds) that can mediate bone tissue formation might have clinical applications in nanomedicine for the orthopedic surgical treatment of bone fractures and bone cancer.

Briefly, natural physiological bone is a multifunctional biomineralized system with an unique structure and specific properties [1- 4]. Healthy bone exhibits hydroxyapatite nanocrystals arranged with their c-axis regularly aligned with the long axis of the collagen fibrils, within the polymeric matrix of type I collagen [5, 6]. The mineralized collagen fibrils are associated as bundles and aligned along their long axis.

Ideally, the bone scaffolds should mimic the composition and the structure of natural bone. From a clinical perspective, the use of bone scaffolds made of collagen (COL) and nano hydroxyapatite (nanoHAP) is very attractive [7-9].

These scaffolds can provide a favorable matrix to osteoblasts adhesion and proliferation and subsequently promote the formation of new bone. In vivo, they can minimizes patient discomfort and risk of infection being constructed by mineralized collagen fibrils.

Bone scaffolds with surface structure and properties similar to natural bone would mediate the production of new bone at the interface between bone tissue and biomaterials, and consequently, improve orthopedic implant efficacy [10]. These materials are expected to give a mechanical support to the bone fracture until osteogenesis is induced. Also, they should degrade in a timely manner so that the new bone will fill the bone defect [11, 12]. The scaffolds might also serve as drug delivery vehicles for the local release of essential proteins, growth factors or various drugs to further increase the bone healing [13, 14].

Generally, scaffolds formed from hydroxyapatite and polymer components to support bone cells and tissue growth are of a great interest. In our previous studies, the utility of developing nanoHAP, modified nanoHAP with Si, Mg, and Zn, and scaffolds of these nano materials with collagen and/or chitosan was demonstrated [1, 7, 8] for osteoblasts culture and for the new bone formation.

It is likely that functionalization of nanoHAP with polymers, particularly with collagen, will lead to a better integration of inorganic nano structures into the composite scaffolds [1] and consequently, into the new bone production. However, there are still unanswered questions when it comes to reconstruction of bone, implant integration or rejection, acceleration of fractures consolidation, or healing of bone infections.

Thus, a continued research is needed to improve the cytocompatibility of biomaterials by using high nanotechnology. The advanced processed biomaterials might support the osteointegration, which will lead to a faster fracture healing, better implant function and tolerance without need for implant removal.

In the current study, an overview on advanced nanoHAP and COL scaffolds is presented. The main objective is to develop bone-like nano-hydroxyapatite / collagen scaffolds with a very porous structure, in which the bone forming cells (osteoblasts) can spread and divide, and finally produce physiological new bone.

Scaffolds and cytocompatibility

Osteoblasts grow differently on flat plastic surface (2D scaffold) than on the 3D scaffolds. In the situation of 2D scaffolds, cells can divide and spread laterally until they hit an obstacle, which can be the side of the plastic dish or another cell. Our preliminary studies demonstrated that the 3D scaffolds represent a more realistic way to grow cells in vitro [1] and consequently, they can be used in vivo. Definitely, the cell function is controlled by the composition and the structure of the 3D scaffolds, which provide physical, chemical and biological features for

cell-cell communication, migration paths, adhesion and proliferation. Thus, the interest to improve the cytocompatibility of scaffolds is very high.

Although, the surface characterization of biomaterials has been extensively investigated [15-21], 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 of scaffolds.

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

Generally, collagen type I (COL), is used for the enhancement of surface biocompatibility of hydroxyapatite nanoparticles. Undoubtedly, COL became an interesting biocompound due to its auto-associative properties [22, 23]. On the other hand, COL is the major fibrillar protein in the extracellular matrix and is abundant in bone, cartilages, ligaments and tendons.

Materials and Methods

Synthesis of nanoHAP

Nanostructured hydroxyapatite (nanoHAP) was prepared from starting compounds, including calcium nitrate, calcium acetate, diammonium hydrogen phosphate, ammonium hydroxide, sodium silicate, nonylphenol, acetic acid and tetraethyl ortosilicat, by co-precipitation method in aqueous medium. The pH of the obtained 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 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 nitrate 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. Our optimized hydrothermal method resulted in a nanostructured HAP powder of controlled stoechiometry, high cristallinity, with nano sized particles.

Nanostructured silicon, magnesium and zinc modified hydroxyapatite [nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)] was prepared by a chemical synthesis procedure similar with that presented above. The selected content in Si(0.2%),

Mg(0.6%) and Zn(0.2%) was introduced in the synthesis of the inorganic powder to better simulated the bone chemical composition. This complex nano powder, nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%), was firstly reported by us.

Collagen aqueous dispersion

Bovine Achilles tendon collagen, type 1 (COL), was independently dispersed in deionized water at a pH of about 3, made with acetic acid, as elsewhere presented [22, 23]. Collagen fibrils were reassembled 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 scaffolds

The nanoHAP powder or nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%) 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 scaffolds formed of nanoHAP or nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%) and for composites with collagen, as well as for (nanoHAP/COL or nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL) layered scaffolds. The mixed scaffolds were made of nanoHAP or modified nanoHAP with Si, Mg, and Zn, and collagen (COL) by self-assembling layer by layer method on solid substrates, such as flat glass plates.

Synthesis of nanoHAP-COL composite cement

The composite cement was prepared by precipitating nanoHAP and assembling COL fibrils simultaneously by the self-organization mechanism on hydroxyapatite particles at pH 7, adjusted using sodium hydroxide solution. The nanoHAP to collagen weighted ratio was monitored at 7:3. After that, the obtained precipitate (nanoHAP-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 and characterized by using monoclonal antibodies [24]. 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 Lglutamine, 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).

For cell culture, 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 controlled seeding density on these scaffolds.

Imaging techniques: SEM, TEM, AFM, FM

The obtained materials, nanoHAP and nanoHAP-COL cement, as well as COL fibers prepared in the absence and the presence of nanoHAP or nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL, 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) [25], the transmission electron microscope (TEM), JEOL-JEM 1010 [26] and atomic force microscope (AFM) JEOL 4210, operating in tapping mode [27, 28].

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 re-dispersed 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 trypsinization procedure. The dried cellular constructs were examined by AFM.

The activity of osteoblasts on scaffolds were visualized by using fluorescence microscopy (FM: optical microscopy, inverted microscope stage, ZeissAxio and Axiocam MRM camera) and fluorescent compounds, like FITC: fluorescein isothiocyanate, DAPI: 4,6 diamidino-2-phenylindole, and Texas red: sulforhodamine 101 acid chloride, which are used for staining cell specimens, in immunohistochemical applications.

Results and discussions

The surface structure 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 (*figure* 5 and 6) and the presence of nanoHAP (*figure* 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 (*figure* 1a) and nanoHAP-COL composite cement (*figure* 1b) present a rod like structure and acicular particles are smaller than 100 nm long.

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

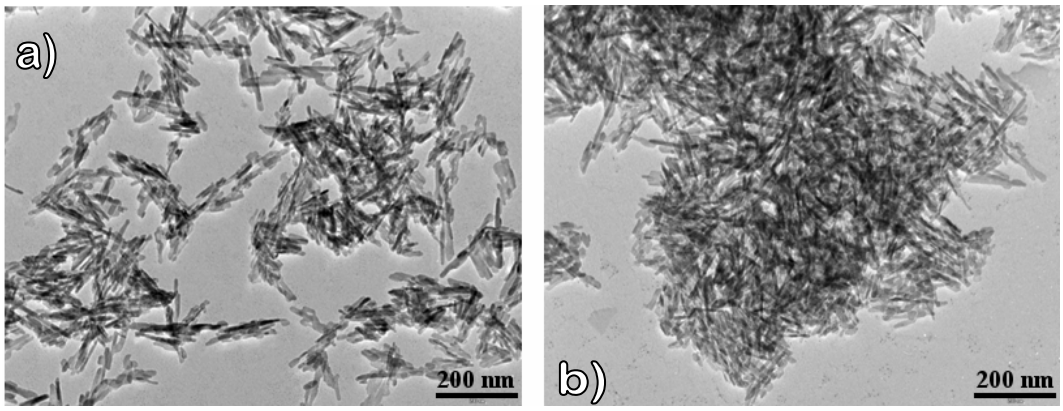

Figure 1: TEM images of nanoHAP powder (a) after thermal treatment at 650°C and of nanoHAP-COL cement (7:3, weight ratio) (b); bars in the images correspond to 200 nm

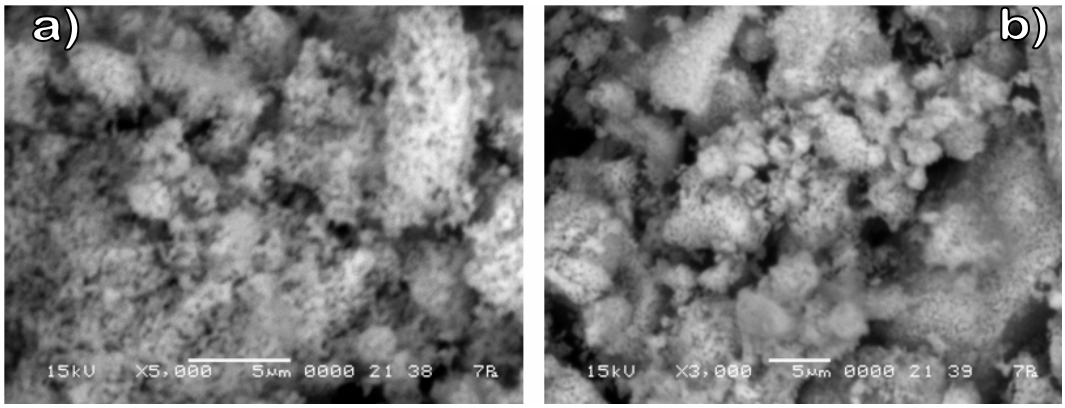

Figure 2: SEM micrographs of nanoHAP powder (a) and of nanoHAP-COL cement (7:3, weight ratio) (b); bars correspond to 5µm.

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

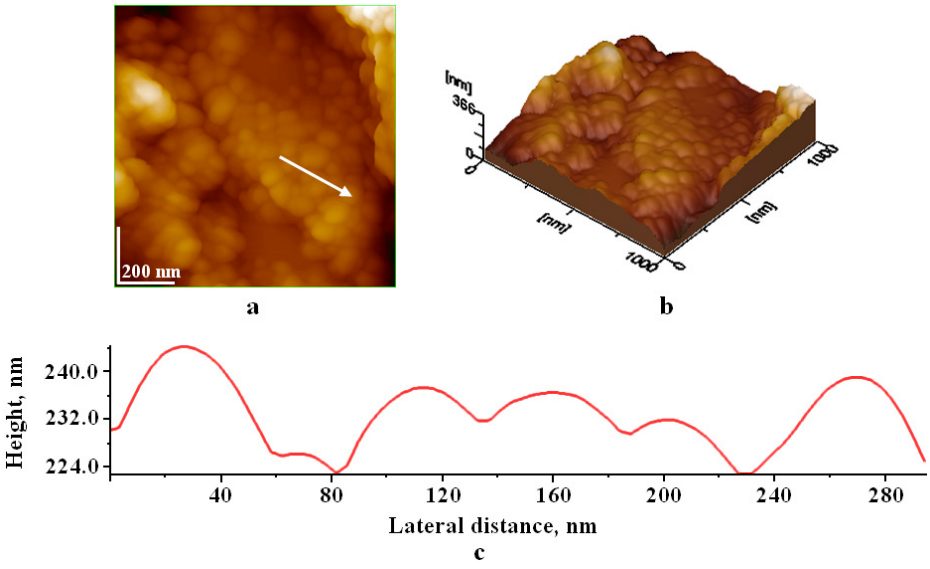

Figure 3: AFM images of nanoHAP powder; a) 2D topography image; b) 3D view of image; c) cross section profile along the arrow in panel a. Scanned area 1µm x 1 µm. Surface roughness, given by RMS, 43 nm (± 6 nm) on scanned area. Standard deviation is denoted in parentheses.

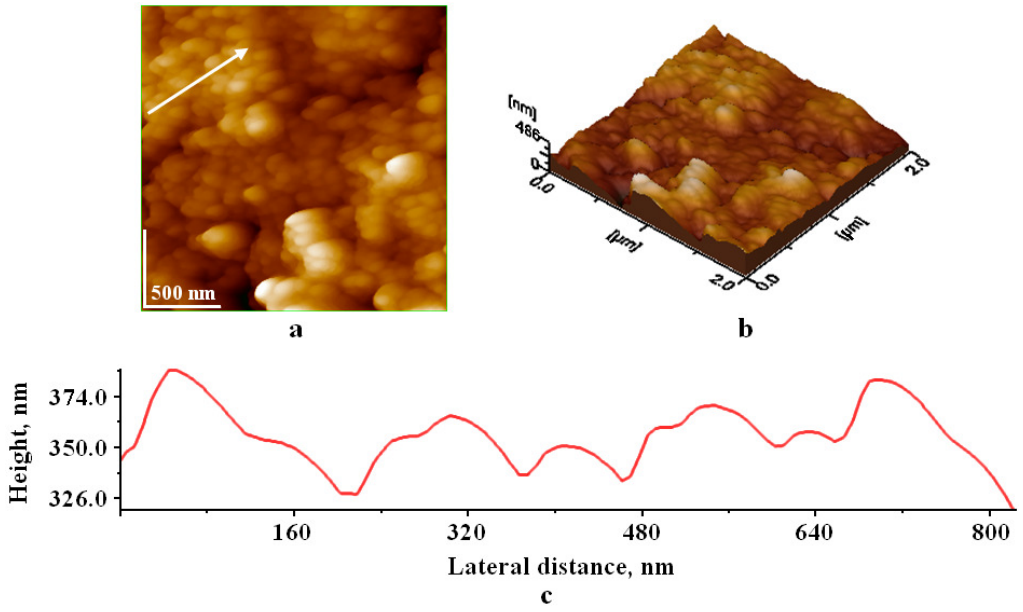

Figure 4: AFM images of nanoHAP-COL cement powder: a) 2D-topography image; b) 3D view of image; c) cross section profile along the arrow in panel a. Scanned area 2 µm x 2 µm. RMS 64 nm (± 8 nm). Symbols as in figure 3.

Furthermore, a study was carried out to determine the nanoscale structure of collagen fibers self assembled on glass support from colloidal solutions using AFM (*figure* 5) and SEM (*figure* 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 (*figure* 6). Furthermore, the nodule structure of collagen fiber is shown by AFM imaging (*figure* 5) and indicates a different mass density across the COL fiber. It also exhibits a nanoscale banding pattern with a periodicity of about 67 ± 4 nm (*figure* 5f) on the long axis of COL fiber in substantial agreement with collagen organization [22, 23] at interfaces.

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 (*figures* no 7a and 7d) in good agreement with some reported data on related systems [29]. 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 (*figure* no 7e).

Moreover, *figure* no 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 a surface (external) mineralization process of collagen, the mixed nanoHAP/ COL fibers are formed. The mineralized COL fibers become thicker and wider than the COL fibers obtained in the absence of inorganic particles (*figure* 5). Undoubtedly, a stabilizing effect of collagen fibers might appear through the interaction of COL with nanoHAP particles due to electrostatic forces, van der Waals forces and hydrogen bonds.

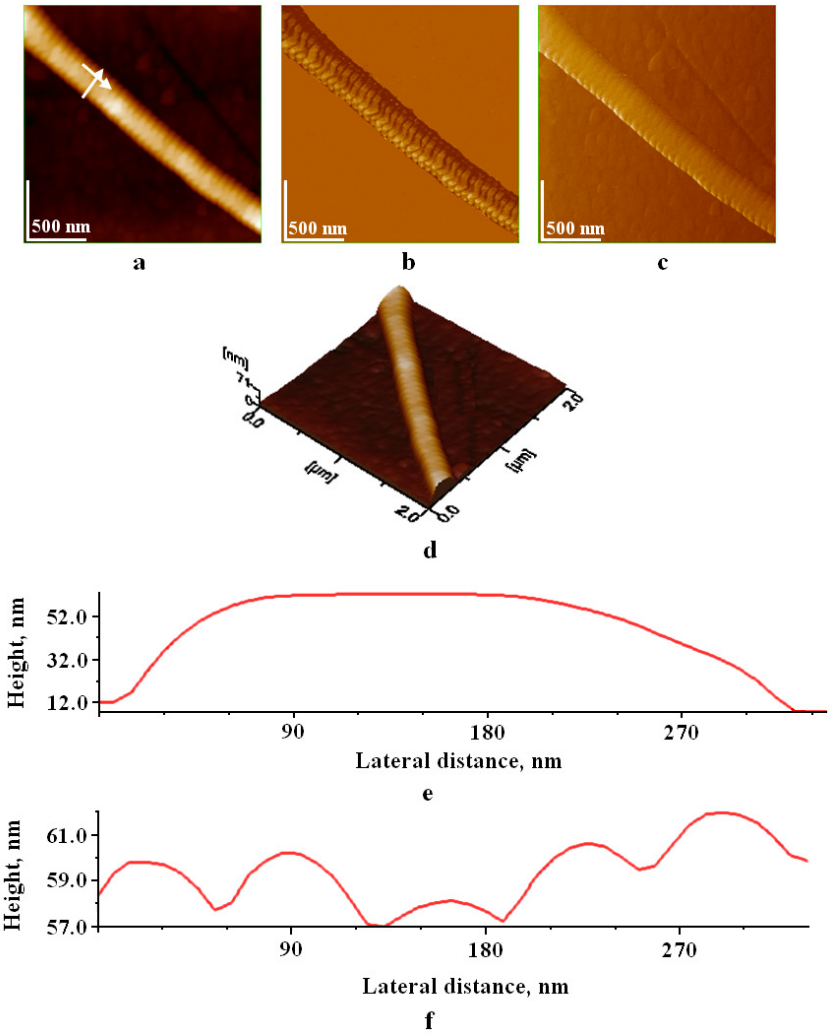

Figure 5: Collagen fiber self assembled on glass surface from acidic dispersion of collagen.

a) 2D-topography;

b) phase image;

c) amplitude image;

d) 3D-view of panel a; 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).

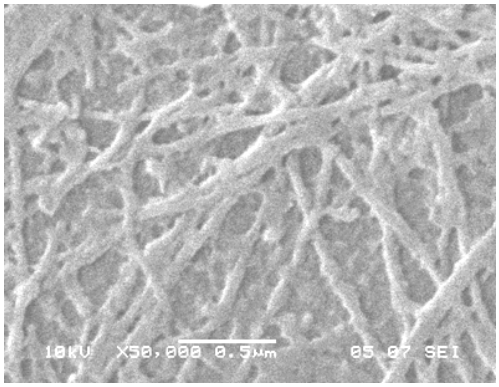

Figure 6: SEM image of collagen fibers self-assembled on carbon coated grid. Scale bar of 0.5 µm.

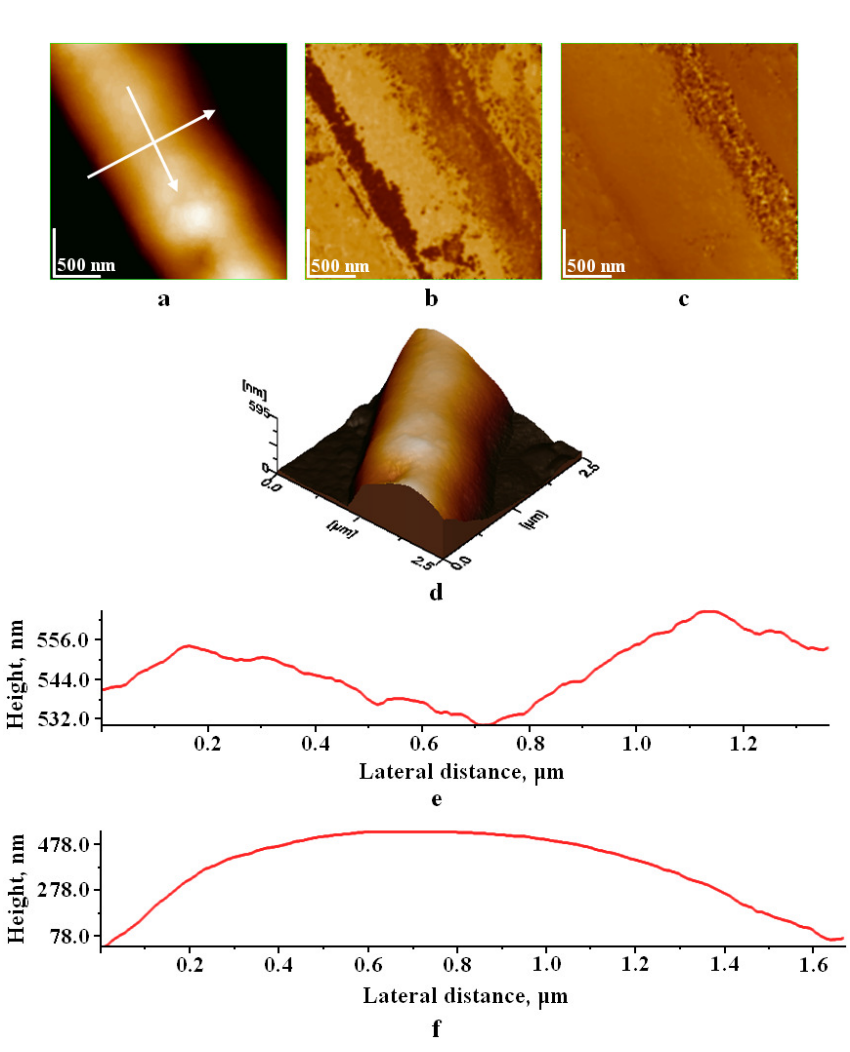

Figure 7:

Collagen fiber mineralized with inorganic phase (nanoHAP/COL layers deposited on glass surface).
a) 2D-topography;
b) phase image;
c) amplitude image;
d) 3D-view of panel a; 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).

The behaviour of osteoblasts, cultured for three days on four groups of scaffolds, made of nanoHAP powder, nanoHAP-COL cement, pure collagen layers and combined nanoHAP/COL layers deposited on glass support, was evaluated by SEM and AFM imaging. For this purpose, the cells were removed from scaffolds by trypsinization procedure. 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* no 8.

Results suggest that the cell collagen production is substantially improved on combined fibrous scaffolds (*figure* 8), made of nanoHAP/COL combined 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 (*figure* 7) is substantially increased than for nanoHAP-COL cement (*figures* no 1b, 2b and *figure* 4), or on pure COL layers (*figure* 5 and *figure* 6), or on nanoHAP powder (*figure* 1a, 2a and *figure* 3). 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 RMS surface roughness 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, *figure* 7) > nanoHAP-COL cement (64 ± 8 nm, *figure* 4) > nanoHAP (43 ± 6 nm, *figure* 3) > pure COL layers (18 ± 3 nm, *figure* 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 the following, a representative AFM image is shown in *figure* no 8, in which a collagen fiber (*figure* 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 (*figure* 8e) are observed on the surface of combined fibrous nanoHAP/COL scaffold, after three days in cell culture.

The COL fibers reveal highly ordered arrangements of rather thick mineralized collagen fibers (width about 480 ± 33 nm, *figure* 8f). They are preferentially arranged parallel to each other, in a particular plan (*figures* 8b and c) closely packed and better arranged than those observed for the pure COL fibers (*figure* 6). As pointed in *figure* 8, COL fibers (fiber width of about 480 nm) are

wider than the pure COL fiber (about 270 nm, *figure* 5) but thinner than artificially mineralized COL fiber (1400 nm, *figure* 7). The surface roughness (4 ± 1 nm, *figure* 8a) of collagen network is very low leading to a rather compact structure. The difference in the periodic pattern (*figures* 5f, 7e and 8e) shows clearly the influence of the inorganic particles incorporated inside the collagen fiber structure. The inorganic particles are not individually seen because they are incorporated intrafibrilary within COL matrix. They might enhance the mechanical stability and the resistance of collagen fibers through their binding process and molecular interactions with collagen molecules.

The COL fibers shown in *figure* 8 are arranged almost parallel or in different directions as mainly illustrated in *figures* no 8b and 8c. AFM images given in *figure* no 8 are similar to the AFM images observed on human trabecular bone [6].

By comparing the AFM images, given in *figures* 5 and 8, an important characteristic of type I collagen is demonstrated, namely that collagen exists as a structure with nanoscale morphologies (e.g. nanoscale banding patterns on the long axis of the collagen fiber) both in self-assembled layers at interfaces (*figure* 5) and in the collagen network (*figure* 8) produced by osteoblasts on scaffolds. This fundamental characteristic of type I collagen is for the first time reported and provides an understanding of the nano structure of collagen involved in the formation of new bone, particularly in the mineralization process of this essential component in bones. A distribution of similar nanoscale morphologies was also recently found in bones and teeth regardless of the anatomical location, the presence of mineral or the mechanical function of that tissue [2].

Recently, we have further studied the osteoblasts activity by the cell production of collagen, osteopontin and osteocalcin by immunocytochemical staining technique for osteoblasts cultivated on five groups of scaffolds, namely scaffolds made of nanoHAP, COL layers, nanoHAP-COL cement, nanoHAP/COL combined layers, and nanoHAP-Si(0.2%)-Mg(0.6%)-Zn(0.2%)/COL combined layers.

As an example, the collagen production of osteoblasts is evidenced in *figure* 9 by fluorescence microscopy. A rather strong production of collagen by osteoblasts on nanoHAP-Si-Mg-Zn/COL scaffolds at 3 days in cell culture is clearly observed, which was greater than that observed when osteoblasts were cultured on nanoHAP scaffolds, on nanoHAP-COL cement scaffolds or on COL layers scaffolds. However, the type I collagen production was rather similar for the nanoHAP/COL combined layers compared to nanoHAP-Si-Mg-Zn/COL combined layers, but still in a slightly low quantity for the nanoHAP/COL scaffolds than for nanoHAP-Si-Mg-Zn/COL scaffolds combined layers.

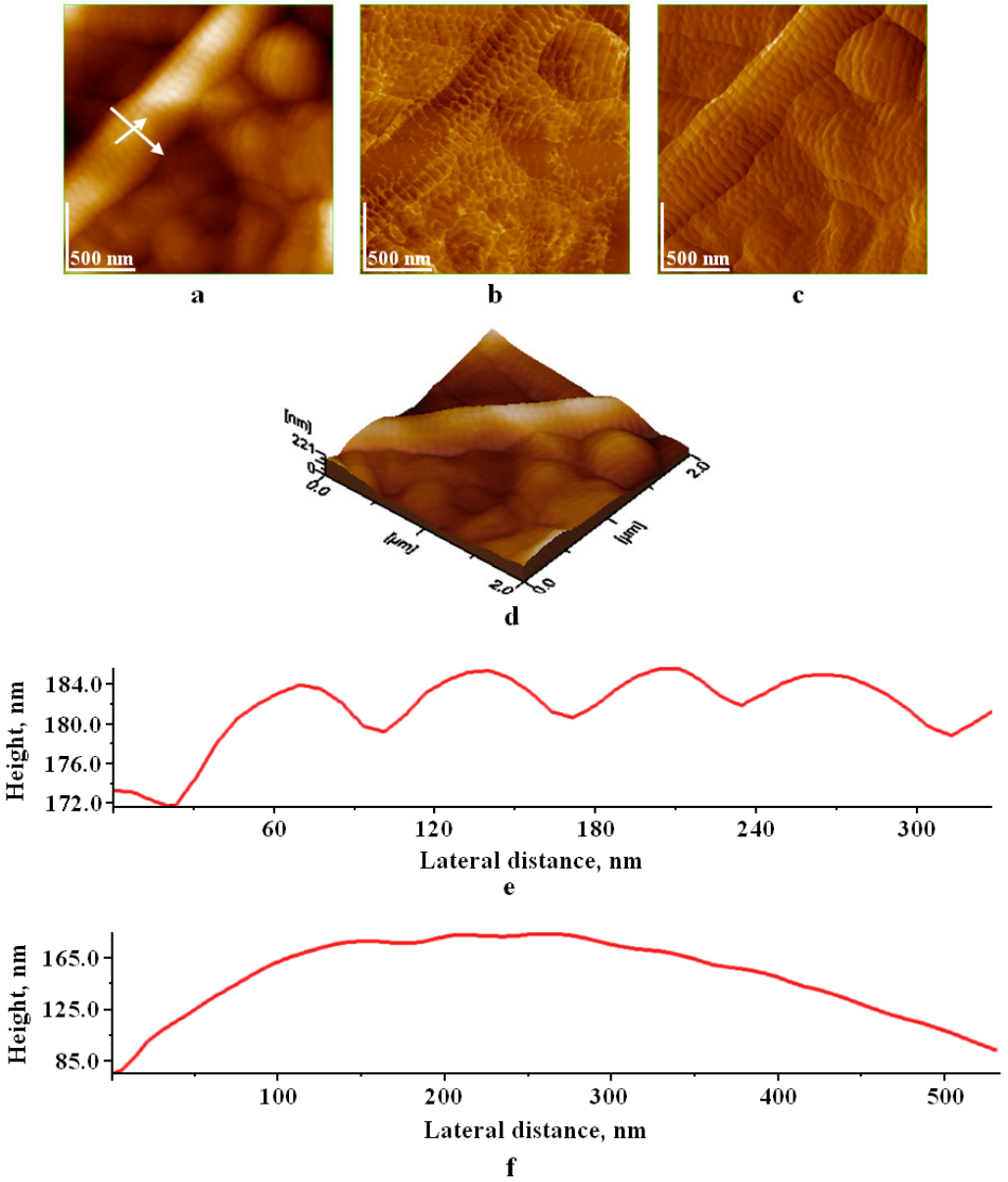

Figure 8: Collagen fibers were produced by osteoblasts on the scaffold surface, made of nanoHAP and COL combined layers (see, HAP/COL layers in absence of cells in fig. no 7). Osteoblasts were grown on the scaffold for three days and then, cells were removed by trypsinization procedure. a) 2D topography; b) phase image; c) amplitude image; d) 3Dtopography; 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.

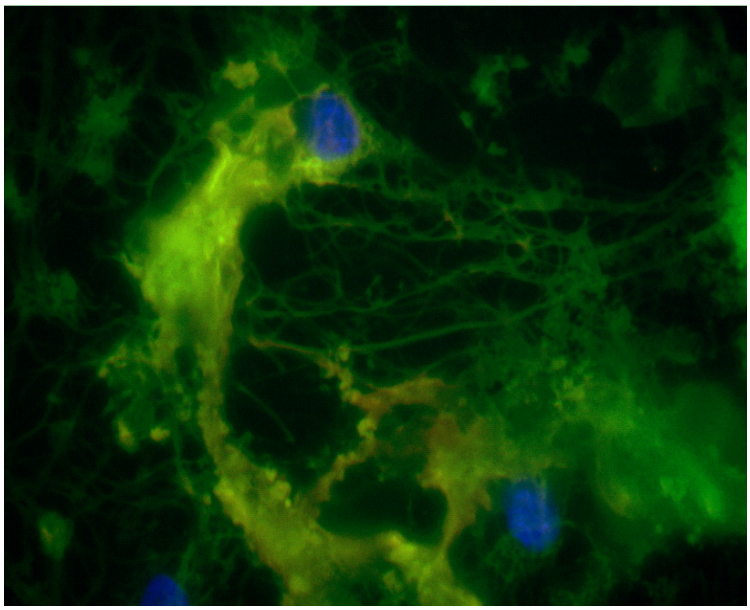

Figure 9: Osteoblasts cultured for three days on nanoHAP-Si(0.2%)-Mg(0.6%)-Zn (0.2%)/COL scaffolds; cellular collagen production visualized by fluorescence microscopy using anti collagen antibody conjugated-FITC (green, for collagen fibers network); FITC: fluorescein isothiocyanate; DAPI: 4,6 diamidino-2-phenylindole (blue, DAPI staining for cellular nuclei); magnification x 400.

Another example, it is given for the expression of two cell markers, like osteopontin and osteocalcin, which was also evidenced by immunocytochemical staining technique for osteoblasts cultivated at 3 days on nanoHAP-Si-Mg-Zn/COL scaffolds (*figure* 10). Both protein markers are evidenced showing that the nanoHAP-Si-Mg-Zn/COL scaffolds can facilitate the differentiation of osteoblasts.

It is to be noted that osteocalcin is also a specific marker for mineralized bone matrix formation. From *figure* 10B, it is observed that the osteocalcin production is induced at day 3, which is significantly earlier versus on day 21 when osteoblasts were cultured on nanoHAP scaffolds and on day 7 for nanoHAP/COL layered scaffolds or COL layers scaffolds. Therefore, the early osteocalcin production detected when cells were cultured on composite nanoHAP-Si-Mg-Zn/COL combined layers scaffolds may indicate that these scaffolds were superior to the other four groups of scaffolds in facilitating the mineralization process.

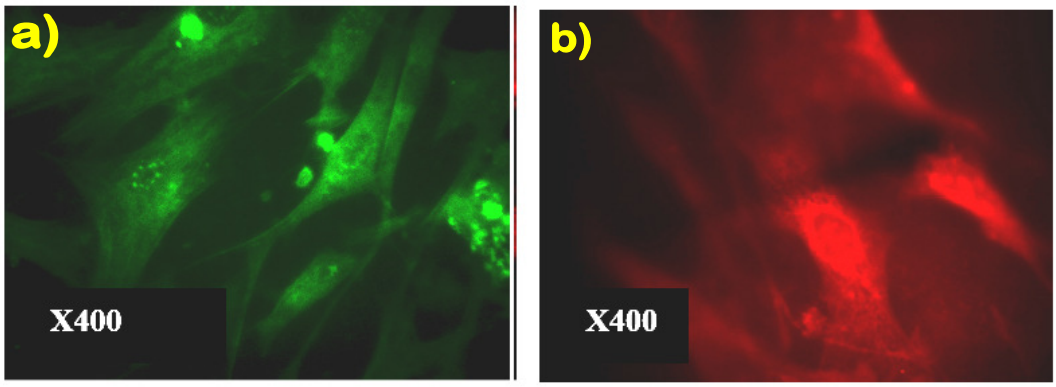

Figure 10: Osteoblasts cultured for three days on nanoHAP-Si(0.2%)-Mg(0.6%)-Zn (0.2%)/COL scaffolds (as in figure 9); imaged by fluorescence microscopy; osteopontin (*A*, visualized in green with specific antibody conjugated FITC) and osteocalcin (*B*, stained in red with characteristic antibody conjugated with Texas red); magnification x 400.

It is to be also mentioned that all five groups of investigated scaffolds, nanoHAP, COL layers, nanoHAP-COL cement, nanoHAP/COL combined layers or nanoHAP-Si-Mg-Zn/COL combined layers are biocompatible for bone regeneration and bone tissue engineering. However, these results have demonstrated the high potential for composite scaffolds, made of nanoHAP/COL combined layers or of nanoHAP-Si-Mg-Zn/COL combined layers, to be used in bone tissue engineering and orthopedic treatment of bone fractures and other bone diseases.

These findings support the idea that the general collagen structure developed by osteoblasts on combined fibrous scaffolds, engineered from nanoparticles of hydroxyapatite and collagen combined layers, is similar with the structure observed in bones [2, 6]. 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 COL fibers was also observed for the human trabecular bone [6].

Apparently, the collagen fibers might have direct relation to mechanical properties and consequently, to the quality of bone. As demonstrated above, the combined fibrous biomaterials (scaffolds made from nanoHAP/COL combined layers as well as from nanoHAP-Si-Mg-Zn/COL combined layers) can stimulate the cell collagen production (*figures* 8 and 9) and osteopontin and osteocalcin expression (*figure* 10) and consequently, it might improved the new bone formation both in vitro and in vivo.

Conclusions

The surface features observed by AFM, TEM and SEM on several biomaterials developed in this work (like, nanoHAP, nanoHAP-COL cement, pure collagen layers, fibrous nanoHAP/COL combined layers and fibrous nanoHAP-Si-Mg-Zn /COL combined layers) provide a new evidence supporting the concept of the significance of surface effect (composition and nano structures) on the cytocompatibility of biomaterials and on their uses for bone tissue engineering and bone regeneration.

Results showed a rather good adhesion of osteoblasts on the surface of all five different biocomposite scaffolds. However, it clearly appears that the most efficient scaffolds developed here to stimulate the cell growth are the nanoHAP/COL layered scaffolds and the nanoHAP-Si-Mg-Zn /COL layered scaffolds.

The imaging data have demonstrated that these combined scaffolds can stimulate the cell production of collagen, osteopontin and osteocalcin in vitro, and consequently, they 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 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.

A future challenge will be to build the 3D porous bioactive scaffolds with cells already inside the scaffolds and manufacture them by using advanced nanotechnology and bone tissue engineering approaches. Undoubtedly, the 3D scaffolds with incorporated cells could be used in clinical trials, and after words, be available in the market for orthopedic surgery and enhanced bone regeneration purposes.

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