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Biocompatible structures based on hybrid organic-inorganic nanocrystalline materials

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Finding new biomaterials for regenerative medicine is a major challenge to researchers working in the fields of materials science and health. Some of the most significant advances in biomaterials over the last 20 years have been referred to hybrid organic-inorganic compounds. Here we study the influence of organic synthetic polymers on biocompatible properties of hybrid nanostructured materials prepared by hydrothermal synthesis. The bonding between organic phase and inorganic phase, represented by hydroxyapatite or other calcium phosphates, takes place in situ during hydrothermal treatment. Maleic anhydride copolymers form hydrogen bonds with P=O group of inorganic phase. The bonding between organic polymers and hydroxyapatite, as well as chemical composition, cristallinity and microstructure were revealed by spectral methods such as Fourier -Transformed infrared (FT-IR) , structural characterization by X ray diffraction analysis (XRD), and scanning electron microscopy (SEM). The proliferation of human fibroblasts on the nanocomposite surface performed by in vitro tests demonstrated the biocompatible properties of this hybrid nanostructured material.

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Keywords: Polymer/ceramic nanocomposites, Biocompatible structures, In vitro tests

1. Introduction

Nanoscience and nanotechnology only became possible in the 1980s with the first tools to measure and make nanostructures. In describing nanostructures it is necessary to differentiate between the numbers of dimensions of the nanoscale. Nanostructures could be classified in three main groups: one dimension on the nanoscale (for e.g. nanostructured surfaces with a thickness of the surface of about 0.1-100 nm), two dimensions on the nanoscale (for e.g. nanotubes with a diameter of about 0.1-100 nm) and three dimensions on the nanoscale (for e.g. spherical nanoparticles, the particle size being between 0.1-100 nm in each spatial dimension). The unique properties of nanomaterials have been exploited by biological and medical research communities for various applications (e.g. contrast agents, cell imaging and therapeutics for treating cancer, implants etc). A series of terms such as bionanotechnology, nanotechnology and nanomedicine are used to describe the new trends in medicine field. Nanomaterials can be functionalised to enhance their compatibility with biological molecules or structures. The size of nanomaterials is similar to that of most biological molecules and structures. Therefore, nanomaterials can be useful for both in vivo and in vitro biomedical research and applications. The main requirements which must be fulfilled by all biomaterials (ceramics, metals and their alloys, polymers, composites) are corrosion resistance, biocompatibility, bioadhesion (bone in growth), biofunctionality, adequate mechanical properties, processability and availability. A corrosion resistant material may not necessarily be biocompatible and contrarily a more biocompatible material may be less corrosion resistant [2]. New types of materials were developed in other areas of chemical engineering and materials science [3]: starburst polymers, PEO based biomaterials, genetically engineered biomaterials, electrically conducting polymers, memory metals and materials, new degradable materials, adhesive materials, bioactive materials, piezoelectric materials. These new materials ofers properties to be developed by the scientists for new potential medical devices or other biomaterials based systems. The performance of a ceramic material is described by a series of factors: the structure of the materials on different levels including atomic, electronic, grain boundary micro and macrostructure, chemical composition, the nature and distribution of the impurities, crystal structure, grain size, defects [2]. Many biomaterial investigators agree that long term performance is often limited by poor control over the material-tissue response [2,4].

Composites of polymers and ceramics were developed with the aim to increase mechanical scaffold stability and to improve tissue reactions. The composite systems combining the advantages of polymers and ceramic seem to be a promising choice in particular for bone tissue engineering [5]. Biodegradable polymers can undergo a passive hydrolysis process or an enzymatic one. For example, in scaffolds materials it is very important to achieve a surface erosion of the polymer instead of usually polymer bulk erosion. In this way the toxic effect (for e.g. local acidity) will be minimised. Enzymatic degradation may lead to individual variation because enzyme levels may vary between people. In addition the cellular response around a polymer implant may change over time [4]. All individuals have excess water and as a consequence

polymer hydrolysis could be considered a mechanism with a high degree of reproducibility. Polymer behaviour is dependent firstly on the monomers type (hydrophobic or hydrophilic). The chain of the most popular monomers contains carbon, oxygen, nitrogen, some hydrolytically susceptible groups (ester, urethane, ether linkages, and hydroxyl groups).

Table 1. Some examples of polymer/ceramic composites fabrication [5].

Technique	Advantages	Drawbacks
Solvent casting and particle leaching (dissolution of a polymer in an organic solvent, mixing with ceramic particles and casting the solution into a 3D mould)	Ease of fabrication without a specialised equipment	- Shape limitation - Possible retention of the toxic solvent - Possible denaturation of the proteins due to the used organic solvent
Solvent aggregation method (polymer microspheres are first formed and then mixed with solvent, salt or sugar particles, ceramic granules)	3D structure with controlled porosity	- Shape limitation - Possible retention of the toxic solvent -Possible denaturation of the proteins due to the used organic solvent
Solid freeform fabrication technique (SFFT)	- Fully interconnected porous networks - Shape, pore architecture and porosity control	- Applied only for composites containing calcium phosphates as bioactive phase
Microsphere sintering (microspheres of bioceramic and polymer are first obtained by an emulsion /evaporation technique followed by sintering)	- Well integrated interconnected porous structures	
Slurry dipping and electrophoretic deposition (EPD) for coatings (foams, fibrous bodies, meshes)	- EPD could be an efficient solution to incorporate nanoparticles in porous structures	- EPD determines the seal of the interconnected pores -Thermal treatment is necessary after depositions by these two techniques

The specific monomers should be selected based on toxicological tests. Addition of bioactive phases to a bioresorbable polymer allows the rapid alkali protons exchange in water and as a consequence they can also alter the polymer degradation.

The bioactive phase type and the bonding nature between bioactive phase and polymer are essential in composites [6, 7]. The increasing of the bonding strength at the interface is a challenge and up to now this aspect was neglected in most studies.

Functionalised nanoparticles with a large specific surface area could be a solution to increase the interfacial bonding strength and as a consequence the overall mechanical properties of the composite could be effectively enhanced [5].

There are a good number of in vitro and in vivo studies for biodegradable polymers and bioactive ceramics alone, but in vitro studies for polymer /ceramic composites have just started [5].

Different techniques were used to synthesis polymer/ceramic composites [5]. In Table 1 are summarised some examples and their drawbacks and advantages. Taking into account all the literature data presented above, in this paper an innovative technology (hydrothermal procedure) is proposed to synthesis in situ at low temperatures and high pressures hydroxyl apatite/polymer nanocomposites with a strength bond between these two phases. Some preliminary in vitro tests were performed on the synthesised materials.

2. Experimental

Soluble salts of calcium and ammonium phosphate aqueous solutions were used as starting materials for hybrid nanocomposite synthesis. These solutions were mixed with an appropriate amount of mineralising reagent (ammonia solution) and synthetic polymer (maleic acid copolymer with vinyl acetate, in form of sodium salt). The weight ratio between hydroxyl apatite and polymer of 4:1 was selected based on the composition of dentin, enamel, cortical bone, trabeculae bone summarised in Table 2 (according to data from [2]).

Table 2. Basic constituents of human bone and dentition.

Human component	Water [weight %]	Organic [weight %]	Mineral [weight %]
Cortical bone	12	28.1	59.9
Trabeculae bone	20	26	54
Dentin	10	20	70
Enamel	3	1	96

The hybrid nanocomposite powders were hydrothermally synthesized in a CORTEST (USA) autoclave at 100 ^{o}C, for 3 hours at a pressure of 2 MPa. The precipitates thus obtained were filtered, washed and oven dried at 100 ^{o}C for several hours. The synthesis procedure is described elsewhere [8]. The hydroxyl-apatite-polymer hybrid nanocomposite was compositionally characterized using classical methods. Chemical volumetric and gravimetric methods as well as spectral methods (inductively coupled plasma - ICP, direct coupled plasma - DCP, and atomic absorption spectrometry - AAS) were used for quantitative determination of the main elements with a ± 2% standard

deviation. A diffraction study for hybrid nanocomposites phase analysis was carried out on a DRON 2 diffractometer, linked to a computer for automatic data processing. CuK_α radiation was used in the range of Bragg angles between 5 and 40. The CuK_β radiation was removed using a carbon monochromator placed between the source and the sample. Spectral methods like Fourier transformed infrared spectroscopy (FT-IR) (JASCO FT/IR-620 spectrometer) and UV-VIS-NIR spectroscopy (JASCO V570 spectrometer) were used to study the nature of the bonding between organic and inorganic phases. The microstructure was investigated by scanning electron microscopy (LEO 1530 with Gemini column type). Some of the synthesised samples are presented synthetically in the Table 3.

Table 3. Hydrothermal synthesised nanocomposite hybrids.

No	Sample type	Sample name	Synthesis parameter	Polymer composition
1	Hybrid nanocomposite	P1HPA 11	150^0C /3h	AM:AV=1:1
2		P1HPA 12	150^0C /3h	AM:AV= 1:1.15
3		P1HPA 13	150^0C /3h	AM:AV= 1:1.2
4		P1HPA 14	150^0C /3h	AM:AV=1: 1
5		P1HPA 15	150^0C /3h	AM:AV= 1:1.15
6		P1HPA 16	150^0C /3h	AM:AV= 1:0.92

Preliminary in vitro tests were performed using human skin fibroblasts grown on different nanocomposites samples synthesised in situ in hydrothermal conditions. Cell suspension in RPMI culture medium was layered on the hybrid surface or hydroxyl apatite synthesised in hydrothermal conditions and incubated 24 hours at 37 ^{o}C. A control sample was represented by cells grown on cover glass support. After incubation the samples were washed with a phosphate buffer solution and then incubated with NBD C6 ceramid for 30 minutes at 40 ^{o}C. NBD C6 ceramid is incorporated in biotic cells and it is used as marker for the Golgi complex (a cell organelle positioned in the nucleus proximity and involved in protein maturation and secretion in proteins age). The adhered cells to the hybrid nanocomposite substrates, to hydroxyl apatite synthesised in hydrothermal conditions and control glass substrates, respectively, were observed by fluorescence microscopy and the results are presented in section 3.

3. Results

Fig. 1 represents the X-ray diffraction pattern for one of the nanocomposites hybrid samples synthesised in situ in hydrothermal conditions.

Figs. 2 a and 2 b represent scanning electron micrographs for one of the nanocomposite samples synthesised in situ in hydrothermal conditions.

Figures 3a, 3b and 3c represent FT - IR spectra for samples P1HPA 12, P1HPA 13 and P1HPA 14 by comparing to the FT-IR spectra of organic phase used as raw material for the in situ hydrothermal nanocomposite synthesis.

Figs. 4 and 5 present the fibroblasts grown on hybrid nanocomposite, hydrothermal synthesised hydroxyl apatite without polymer and control glass sample and visualised by fluorescence microscopy.

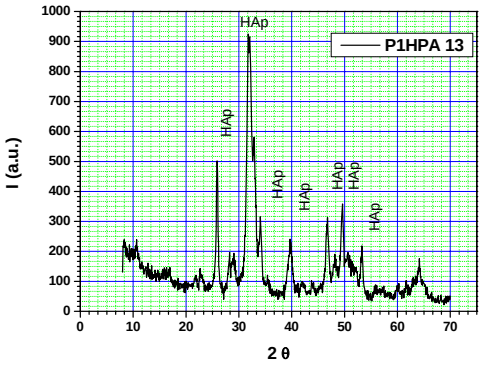
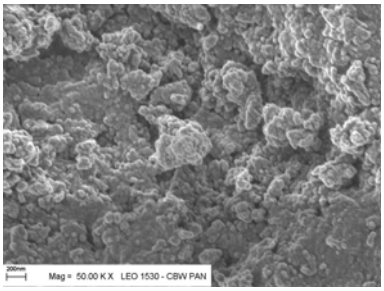
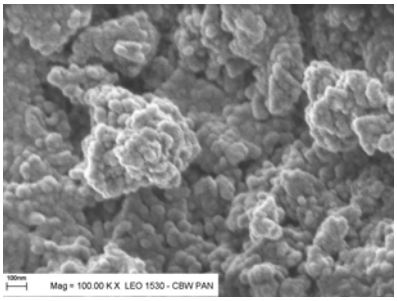


Fig. 1. X-ray diffraction patterns for hybrid nanocomposites synthesized in hydrothermal conditions



a



b

Fig. 2. Scanning electron micrograph for sample P1HPA 12.

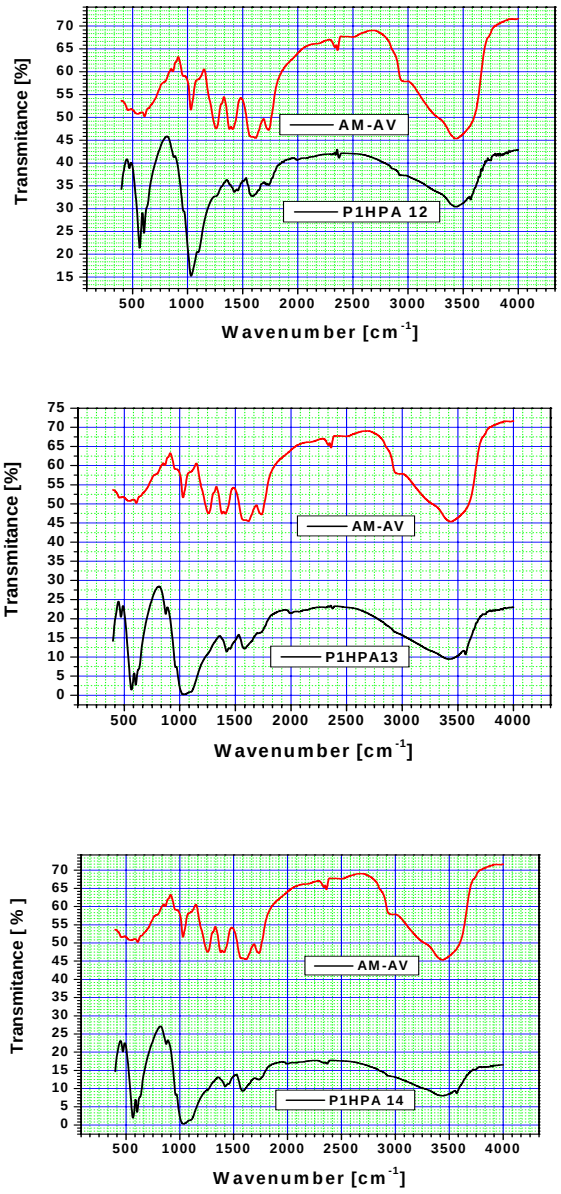


Fig. 3. FT-IR spectra for different hybrid nanocomposites samples synthesized in situ in hydrothermal conditions. a. FT-IR spectra for sample P1HPA 12, b. FT-IR spectra for sample P1HPA 13, c. FT-IR spectra for sample P1HPA 14.

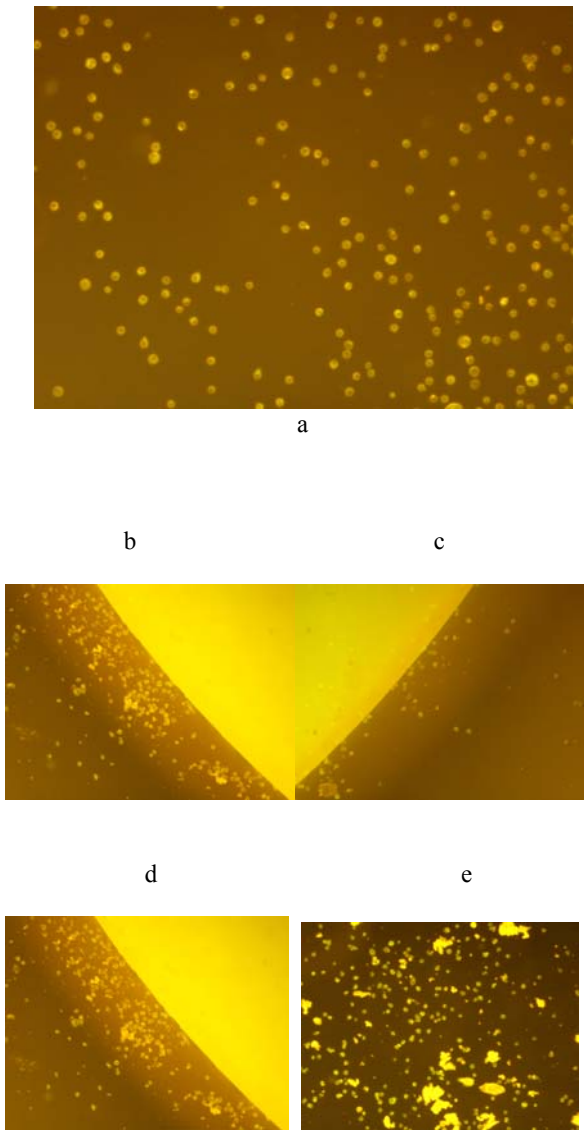
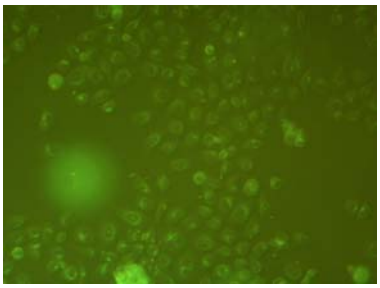
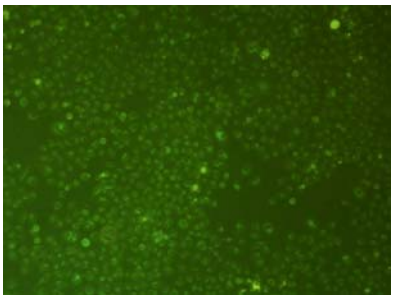


Fig. 4. Human skin fibroblasts grown on nanocomposite sample P1HPA 14: a- cells grown on cover glass(control); b,c,d – cells released from P1HPA 14 following 5 min incubation with trypsin e- trypsinised cells from P1HPA 14 in solution.



a



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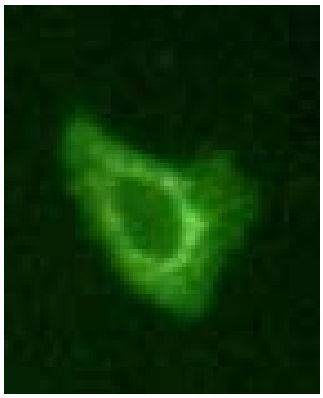
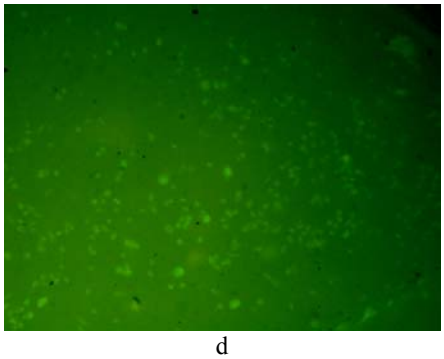


c



d

Fig. 5. Human skin fibroblasts grown on hydroxylapatite sample without polymer synthesised in situ in hydrothermal conditions and visualised by fluorescence microscopy: a, b – control sample representing cells grown on glass support (20x); c – cells grown on hydroxylapatite substrate (20x), d – Golgi marker localized in a typical position around the nuclei (40x).

4. Discussion

X ray diffraction spectra from Fig. 1 reveal a polycrystalline material with hydroxylapatite characteristic peaks. Polymer has an amorphous structure and can not be identified by XRD analysis.

Fig. 2 reveal the microstructure of a representative hybrid nanocomposite sample. Aggregates formed from nanosized particles (about 100 nm), giving the appearance of a homogeneous particulate phase can be observed. The micrograph illustrates the "cauliflower" like morphology of hydroxylapatite.

Typical FT-IR spectra of hybrid nanocomposites synthesised in situ in hydrothermal conditions are shown in Figs. 3a, 3b and 3c. The spectra contain the absorption bands characteristic for hydroxyl apatite. The large peak at 3442 cm^{-1} is due to the stretching of H-O (hydrogen intermolecular bond). At 1030 cm^{-1} can be observed a band which can be attributed to P-O stretching. Other two hydroxyl apatite characteristic peaks at 1092 cm^{-1} and 1048 cm^{-1} attributed to phosphate groups vibrations are missing in hybrid nanocomposite spectra. It can be assumed that some vibration modes of phosphate groups are sterically hindered by their participating in the bonding process with the copolymer. Two hydroxyl apatite characteristic peaks at 562 cm^{-1} and 601 cm^{-1} corresponding to P=O, P-O bonding can be identified. In hybrid nanocomposite the band at 633 cm^{-1} corresponding to hydroxyl vibration from hydroxyl apatite doesn't appear. This indicates the participation of H-O- unit in the bonding process with the copolymer. The spectra show also the copolymer characteristic peaks. Most significant are the aliphatic polyester band at 1735 cm^{-1} due to –C=O stretching, 1592 cm^{-1} attributed to the asymmetric stretching of carboxylate anion, 1422 cm^{-1} due to the symmetric stretching of carboxylate groups from the maleic moieties [9,10]. In the spectra of nanocomposites the bands corresponding to the acetate groups are considerably diminished, probably due to a partial hydrolysis of them during the hydrothermal synthesis, resulting in the increase of hydroxyl groups available for interaction with inorganic partner.

Preliminary in vitro tests performed on hybrid nanocomposites samples revealed their biocompatible potential.

Fig. 4a shows the cells grown on glass control sample with the Golgi marker in a typical perinuclear position. Due to an intense non-specific fluorescence of the hybrid nanocomposite the cells could not be directly visualised on this biomaterial as in the control sample. In order to demonstrate the presence of the cells on the biomaterial surface, we incubated the probe with a trypsin/ethylenediamino-tetracetic acid solution currently used to detach the adhered cells on different substrates. Following a 5 min incubation at 37 ^{o}C with trypsin the adhered cells were detached from the biomaterial and visualised in solution. The detached cells can be observed at the edge of the substrate shown in Fig. 4b, c, d whereas trypsinised cells are presented in Fig. 4 e. The cells

cultivated on substrates of nanostructured hydroxyl apatite synthesised in hydrothermal conditions were analysed by comparing to those cultivated on glass control sample (Fig. 5). The hydroxyl apatite substrates are not autofluorescent and allowed the direct cell visualisation by fluorescence microscopy. The cells adhere to the hydroxyl apatite substrates and are metabolically active for 48 hours. Interestingly, alteration of cell morphology is also be observed for fibroblasts grown on hydroxyl apatite substrates and this may represent a consequence of interaction with the substrate. The normal perinuclear position of Golgi marker suggests that there are not major alterations in cell functions. Unfortunately we can not estimate the effect of the substrate on cell morphology in case of nanohybrids, due to their autofluorescence which makes impossible their examination directly on the surface of the biomaterial. However, the cells grown on both hydroxyl apatite and nanocomposite are viable and proliferate within 24 hours as control cells.

5. Conclusions

Hybrid organic-inorganic nanocomposites were synthesised *in situ* in hydrothermal conditions at low temperatures and high pressures. FT-IR analysis revealed the formation of strongbond between organic phase (sodium maleate copolymer with vinyl acetate) and inorganic phase (hydroxyl apatite) with participation of phosphate and hydroxyl groups from hydroxyl apatite. Nanosized particles of about 100 nm were obtained. Both hydroxyl apatite alone and hybrid nanocomposites synthesised in situ in hydrothermal conditions are biocompatible, but they induce morphological modifications whereof it is not possible yet to define their repercussions on the cellular metabolism. Tests of other cellular markers and cells type are required.

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