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Soft chemical methods integration with micro fabrication in developing new scaffolds for tissue engineering

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The aim of the work is to develop new scaffolds for tissue engineering. This supposes to integrate soft chemical methods (hydrothermal and electrochemical methods) for preparing multifunctional biomaterials based on hydroxyl apatite and functionalized polyurethanes. The innovative part is represented by the following aspects: in situ synthesis under hydrothermal conditions at low temperatures and high pressures of new nanostructured hybrid materials with a strong chemical/physical bonding between the components; applying the cyclic voltametry to monitor the electrical response of the nanostructured hybrid thin films deposited by spin-coating on gold-plated silicon wafers used as substrate. To accomplish these studies, cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were performed. Depending on the structural and compositional factors of the nanostructured hybrid thin films, the electrical response could be useful in monitoring by electrical stimulation of the cell response. SEM and HRTEM methods have been used to characterize the morphology of nanostructured hybrid powders. Biocompatibility studies by in-vitro tests of different immobilized biomolecules are under working.

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1. Introduction

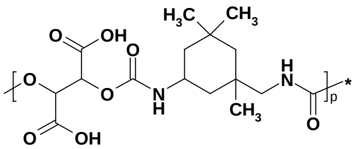
The main factor that distinguishes between a biomaterial and other materials is its ability to exist in contact with the human tissue without causing any harm to the body [1]. Biomaterials are used in very diverse fields, such as tissue engineering, invasive sensors, drug delivery and gene transfection systems. One goal of the tissue engineering is to replace compromised tissue functions. Classic biomaterials (metals, ceramics, and polymer composites) were used for teeth, hips, knees, heart valves and intervertebral discs implants, but they provided limited ability to repair and regenerate the tissue [2]. As a consequence new materials with enhanced functionality were developed by modifying the existing materials and synthesise new ones [2, 3]. Nanostructured inorganic-organic composites combining the toughness of the polymer phase and the strength of the inorganic one seem to be a very promising solution for bioactive materials with enhanced mechanical and biocompatible properties [3]. Among the polymers intensively exploited in biomedical applications the most popular are segmented polyurethanes, due to their generally favorable physical and mechanical properties, together with fairly good biocompatibility and antithrombogenicity characteristics, as results of a difference in their chemical structure [4]. Moreover, polyurethanes have received recent attention for achieving degradable polymers because of their great potential in tailoring polymeric structure to realize mechanical properties and biodegradability suitable for tissue engineering applications [5]. One modern approach to this problem is to use polyurethanes for nanostructured composite development containing various nanofillers including hydroxyapatite for applications requiring high strength, durability and not only [6, 7, 8] where is very important to understand the specific interaction of these polymers with inorganic species (for instance, modified silicate), since interface between organic-inorganic components can be crucial, in the case of implanted cardiac assist devices. Like the present situation, modification of polymer chemistry, particularly that of the hard segment, seems to be also a possibility. Micro and nanotechnology application in the biomedical field has been increasingly used to study bio/nonbio interactions. Initially the scientists focused their work to develop miniaturized diagnostic tools, but in the last years they concentrated the efforts to develop new therapeutic applications [9]. Microfabricated substrates present some advantages over traditional biomaterials, namely: ability to control the surface architecture, topography and size (from nanometer to microns size), control of surface chemistry [9].

The aim of the work is to develop new scaffolds for tissue engineering. This supposes to integrate soft chemical methods (hydrothermal and electrochemical methods) with micro-nanofabrication for preparing multifunctional biomaterials based on hydroxyl-apatite and functionalized polyurethanes.

2. Experimental

The basic idea of this work was to explore the special nanostructure (hydroxyapatite, HAp) developed by hydrothermal procedure to be used as inorganic phase in the in situ preparation of hybrid polyurethane nanocomposites containing a relatively small amount (up to 20%) of ionic polyurethane capable to realize specific interactions between them.

For that reason, a new hard polymer (100% hard segment) was first synthesized by reacting isophorone diisocyanate and tartaric acid in stoichiometric proportions to yield carboxylic hard polyurethane (PU-COOH: Mw = 9200; Carboxylic groups: 537 mechiv. ionic groups/100 g polymer) (Scheme 1).



Scheme 1. Structure of the hard polyurethane with carboxylic groups

Hybrid organic-inorganic structures were synthesized in situ through a hydrothermal procedure under high pressure conditions and low temperatures starting from carboxylic hard polyurethane dispersed in a mixture of water and DMAc and inorganic salt precursors of hydroxyl apatite. Nanostructured organic-inorganic thin films were further synthesized by spin coating deposition on gold coated Si substrate.

The samples were characterized as follows:

a) hard carboxylic polyurethane structure was determined by ^{1}HNMR, FTIR spectroscopy, decomposition temperature (t = 150 ^{o}C) was evaluated from thermal analysis and morphology by atomic force microscopy (AFM) analysis;

b) nanostructured hybrids powders were compositionally characterized by atomic absorption spectroscopy (AAS-Zenith 700 apparatus) and inductively coupled plasma spectroscopy (ICP-Spectroflame). The particle size distribution was analyzed with a NANOSIZER ZS90, Malvern granulometer endowed with auto titration unit. The formation of bonding between organic and inorganic phase was evaluated by differential scanning calorimetric analysis (DSC F3 Maya Netzsch calorimeter);

c) nanostructured hybrid powders and gold coated silicon substrates microstructure and morphology were determined by scanning electron microscopy (QUANTA INSPECT F microscope), endowed with field emission gun –FEG (resolution 1.2 nm) and EDAX spectrometer (resolution at MnK 130 eV):

d) hybrid thin layers deposited on gold coated substrate were characterised by Fourier transform infrared spectroscopy (FT-IR-ABB MB 3000 spectrometer endowed with HATR component and Horizon MB FT-IR software) to reveal the bonding between inorganic and organic phase. Si/SiO$_2$/Au electrodes modified with hydroxyl apatite-polyurethane hybrid (HAp-PUCOOH) in neutral (KCl) solutions, in the absence of electroactive species were preliminary characterized by electrochemical impedance spectroscopy (EIS) to monitor the electrical response of the hybrid layers and to estimate to what extent the electrical response could be useful in monitoring the cell response by electrical stimulation. 0.1M KCl (Aldrich 99.99%) supporting electrolyte was used. Electrochemical impedance experiments were performed with an Autolab 302N potentiostat/galvanostat equipped with the FRA2 impedance module. All measurements were carried out in a conventional one-compartment three-electrode cell, equipped with two graphite counter electrode and a silver-silver chloride (Ag/AgCl) reference electrode. The Si/SiO$_2$/Au and Si/SiO$_2$/Au /HAp-PU modified electrodes were used as working electrodes. The exposed surface area of the working electrode used in electrochemical experiments, was 0.38 cm^2. Electrochemical impedance spectra were recorded at open circuit potentials in aqueous solution containing 0.1M KCl as the supporting electrolyte. All spectra reported here were measured at the open circuit potential. The tested frequency range was from 0.01Hz to 100 kHz. The sinusoidal potential amplitude was 10 mV. All electrochemical measurements were performed at room temperature. The experimental data were fitted to the equivalent electrical circuit by a complex non-linear least squares procedure using the ZView- Scribner Associated Inc., software.

3. Results and discussion

3.1 Hard polymer characterization

FTIR analysis performed on hard carboxylic polyurethanes revealed the characteristic peaks of νNH and CO vibrations from urethane structure at 3390 and 1740 cm^{-1} respectively, secondary amides (CONHR) vibrations at 1560 cm^{-1} , amides I vibrations at 1625 cm^{-1} and stretching vibrations of C-H bonding at 2955 cm^{-1}. In table 1 the NMR signals that confirm the structure of anionic polyurethane are presented.

Table 1. RMN spectra results for the hard carboxylic polyurethane

δ ppm	Assignation
7.95	carboxylic protons
7.4-7.3	urethane protons
3.9-4.2	C**H** near carboxilic groups
3.66	Methylenic protons near ester-urethane group
0.7-1.6	Aliphatic protons from remnant isophorone

AFM micrographs of the hard carboxylic polyurethane are presented in Fig. 1. It can be observed the crystalline structure of this type of polyurethane formed only from hard segments of crystalline domains is around 15 nm.. The maximum height

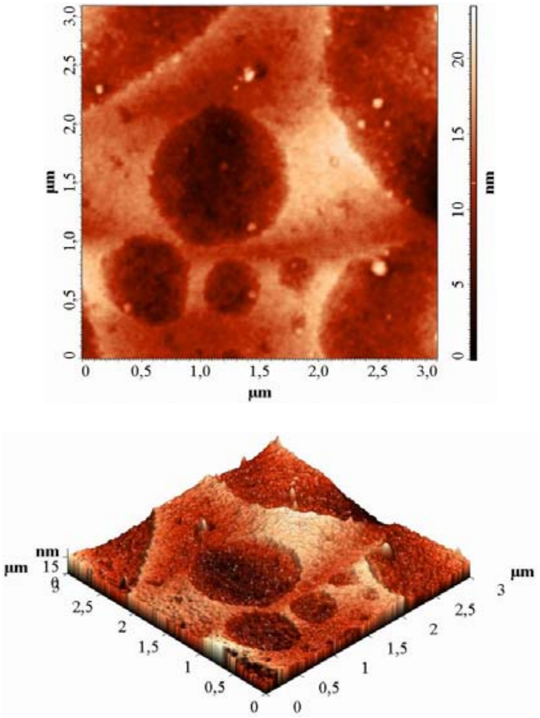


Fig. 1. 2D and 3D (3x3 μm^2) AFM images of hard carboxylic polyurethane.

3.2 Nanostructured hybrid powders characterization

In Fig. 2 the particle size distribution of hybrid powders (chemical composition: Ca=37.50%; P=16.28%; C=6.21%) is presented. It can be observed that the average particle size of hybrid powders synthesized in situ in hydrothermal conditions is around 80.03 nm.

Fig. 3 reveals the thermal behavior of nanostructured hard carboxylic polyurethane –hydroxylapatite hybrid synthesized in situ under hydrothermal conditions comparing to hard carboxylic polyurethane. In table 2 the activation energy and some considerations for a possible reaction mechanism are synthetically presented. It can be observed that in the case of polyurethane there are three transition domains, while for hybrid nanopowders only one transition domain can be identified. A possible explanation could be the formation of a strong bonding between inorganic and organic phases during hydrothermal treatment that sterically hindered the microstructure rearrangements.

In Fig. 4 a detail of the morphologic aspect of nanostructured hybrid powder is presented. The powder is formed from nanometric hydroxyl apatite particles with acicular shape (width is about 5-7 nm). In general the distribution of polymeric material in inorganic matrix is uniform; at higher magnifications a foam structure is visible.

Table 2. Results summary regarding the thermal behavior of hybrid powders and polyurethane

Sample	Peaks [^{0}C]	Activation energy [J/g]	Thermal effect	Considerations
Hard carboxylic polyurethane	107.7	109.1	endo	Transition domains: microstructure rearrangements, or/ and morphological modifications
	175.6	4.139	endo	
	190.9	8.821	endo	
	238.6	37.31	endo	Polyurethane thermal decomposition
Hybrid nanopowders	104.6	107.8	endo	Morphological modifications
	322.8	9.161	endo	Polyurethane thermal decomposition
	482.8	11.8	endo	

3.3 Gold coated silicon substrate and thin hybrid layers characterization

In the micrograph from figure 5 the gold layer deposited on the silicon wafer surface is revealed. It can be observed the nanometric structure of fine gold grains depositions with a polyhedral morphology and relative uniform sizes. The gold layer is homogeneous on the silicon wafer surface.

FT-IR analysis performed on thin nanostructured hard carboxylic polyurethane – hydroxyl apatite hybrid layer deposited on gold coated silicon wafers is presented in figure 6. In table 3 the main characteristic peaks and their assignation is synthetically presented. The vibration νC=O at 1658 cm^{-1} (amide I band) corresponding to CONHR group from polymer structure has a very low intensity in the FT-IR spectra of the hybrid thin layer. Probably a part of the carbonyl groups are sterically hindered due to the interaction with hydroxyl apatite.

3.4 Si/SiO$_2$/Au electrodes modified with hydroxyl apatite-polyurethane hybrid characterization by EIS

Complex plane plots obtained on several electrodes in 0.1 M KCl solution are displayed in figure 7. The main feature in the complex plane plots is the appearance of a depressed semicircle at low frequencies with the diameter strongly dependent on the nature of the electrode, followed by a straight line at higher frequencies related to a diffusion process. The spectra were approximated using a simple model widely used in electrochemical studies: a solution resistance Rs, in series with a parallel combination of the double layer capacitance C_{dl}, the polarization resistance R_P and the Warburg impedance Z_W. The Warburg impedance represents the resistance to mass transport and can be approximated as a parallel combination of C_P and R_P.

Fig. 2. Particle size distribution of nanostructured hard carboxylic polyurethane –hydroxylapatite. hybrid powder.

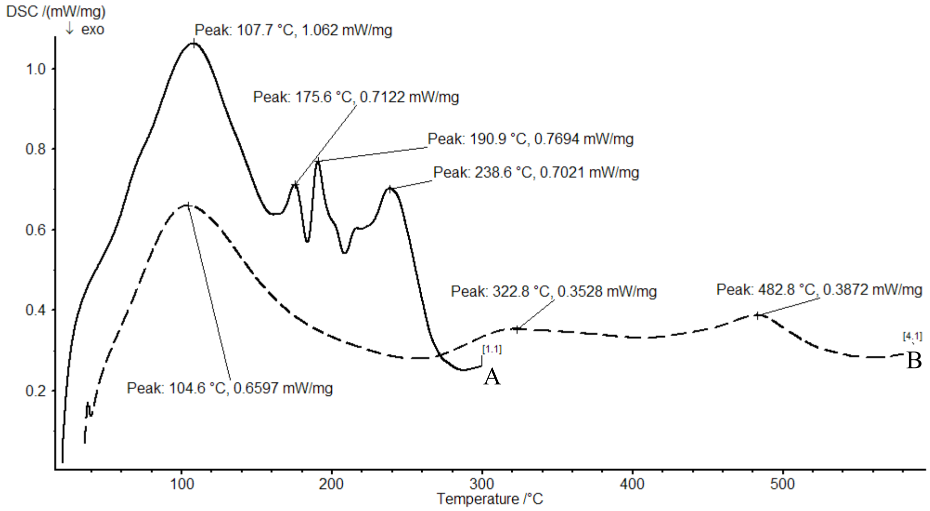


Fig. 3. DSC analysis: A. hard carboxylic polymer; B. nanostructured hard carboxylic polyurethane –hydroxylapatite hybrid.

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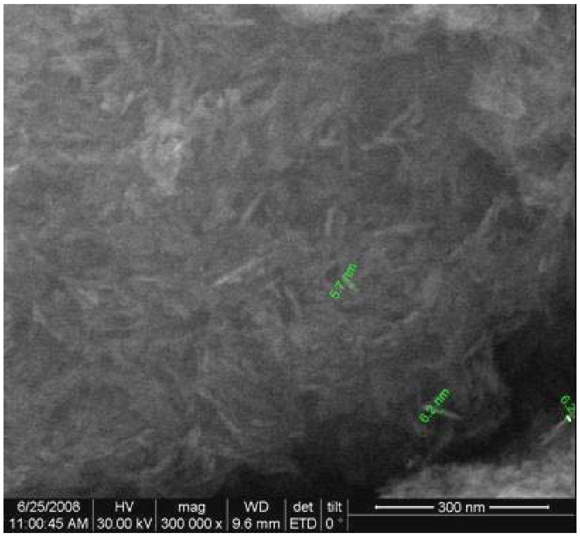

Fig. 4. Secondary electron image of nanostructured hard carboxylic polyurethane –hydroxylapatite hybrid

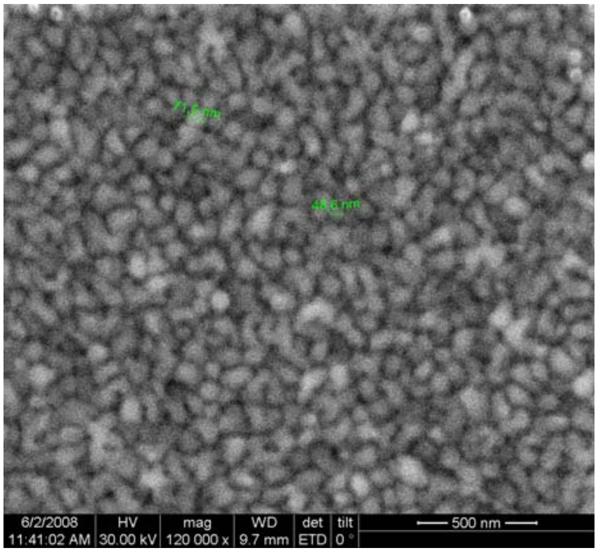

Fig. 5. Scanning electron microscopy analysis for gold coated silicon substrate

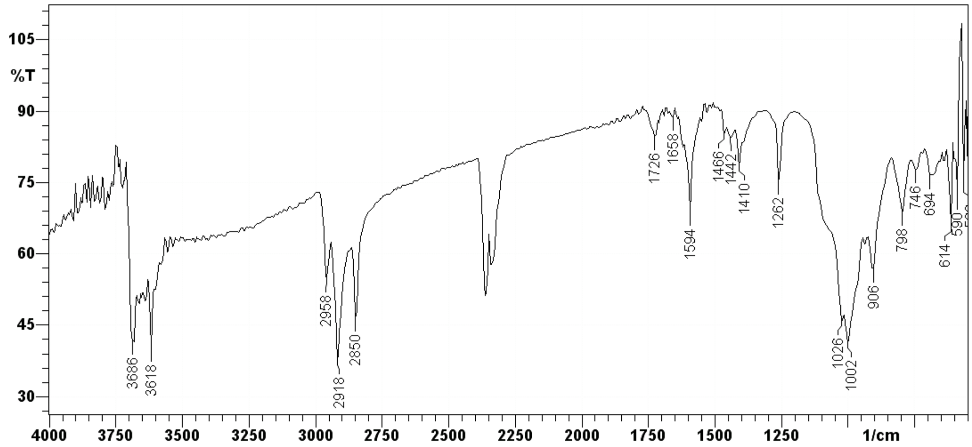

Fig. 6. FT-IR spectra of thin nanostructured hybrid layer.

Table 3. FT-IR main characteristic peaks and their assignation.

Peak[cm^{-1}]	Assignation
3686	νOH unassociated
3350	νOH (intermolecular hydrogen bonding)
2958	νCH3 asymmetric from polymer structure
2850	νCH3 symmetric from polymer structure
1726	νC=O from urethane structure
1594	δNH and νC-N(secondary amide band)-group CONHR from polymer structure
1026	symmetric (ν_1) and asymmetric (ν_3) P-O stretching vibrations of phosphate group
1002	
906	

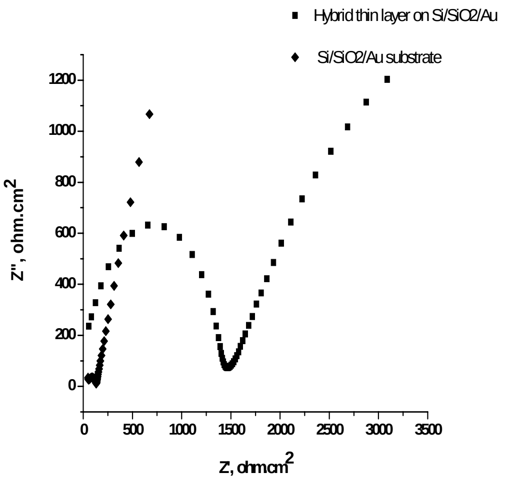

Fig. 7. Nyquist plots for the studied electrodes in KCl 0.1M at OCP potential.

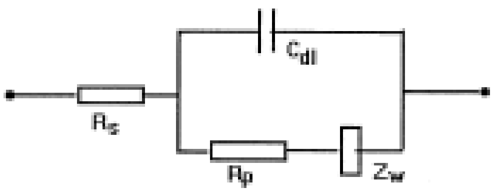

Fig. 8. Schematic representation of the equivalent circuit.

Table 4. Values of the circuit elements for the electrodes

Electrode	C_{dl}, F cm^{-2}	R_P, Ωcm^2	W1-R, Ω cm^{-2} s^{-1}	W1-T	W1-P, (α)
Si/SiO2/Au	6.94 E-08	54.67	371.4	1.72	0.42
Hybrid thin layer on Si/SiO2/Au substrate	8.05 E-09	1333	3750	7.06	0.42

4. Conclusions

Nanostructures of hybrid polyurethane composites containing a relatively small amount (up to 20%) of ionic polyurethane able to realize specific interactions between organic phase and hydroxyl apatite were prepared in situ under hydrothermal conditions at low temperatures and high pressures. Hybrid polyurethane composites with average particle size around 80.03 nm, acicular shape and uniform distribution of the polymer in the inorganic matrix were obtained. Both FT-IR and DSC analysis revealed the bonding formation between the hard carboxylic polyurethane and hydroxyl apatite during hydrothermal treatment. Thin hybrid layers deposited by spin coating on Si/SiO$_2$/Au substrates were preliminary characterized by EIS. These tests revealed that the presence of hybrid layer HAp-PUCOOH on the electrode surface changes the polarization resistance value and the thickness of the deposited layer influences the polarization resistance. Si/SiO$_2$/Au electrodes modified with hydroxyl apatite-polyurethane hybrid (HAp-PUCOOH) could be used to monitor the cell response by electrical stimulation. In vitro preliminary toxicity tests demonstrated the compatibility of the materials with the cells. Further works are necessary to better integrate the micro nanotechnology with material technology. In vitro biocompatibility tests are in progress.

Acknowledgements

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