Methylene Blue based Antiseptic Chitosan/hydroxyapatite Composite Materials

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Bone graft materials based on chitosan and hydroxyapatite were obtained. The materials were characterized by XRD, FTIR spectroscopy and microscopy and SEM microscopy. Based on XRD, pure HA has been obtained while FTIR analyses confirm the formation of the desired composite materials, and in the case of chitosan/hydroxyapatite/methylene blue composite materials, even the presence of methylene blue can be proved. Based on the FTIR microscopy it seems that methylene blue is preferentially distributed onto the hydroxyapatite. The antibacterial activity was tested against E. coli and prove that chitosan induce antibacterial effect while the addition of methylene blue further improve this activity. The precipitation conditions of hydroxyapatite were similar with those used for the preparation of collagen and hydroxyapatite composite materials. Consequently, the method can be further used in case of the synthesis of more complex antiseptic composite materials containing both collagen and chitosan, the antiseptic properties of the composite being tailored by the proper choosing of chitosan:methylene blue ratio.

Keywords: chitosan/hydroxyapatite composite material, antiseptic bone grafts, infrared microscopy

Bone grafts are extensively studied in the last decades because of the increasing need of bone grafts [1]. Ceramics and composite materials are by far, the most efficient bone grafts. Most synthetic bone grafts contain calcium phosphates (hydroxyapatite and tricalcium phosphates being the most used) and/or different natural (collagen, chitosan, cellulose) or synthetic polymers (polylactic acid, polyvinyl alcohol, polyethylene glycol, poly(ε-caprolactone), etc) [2-8].

Hydroxyapatite (HA) is the most important phase belonging to human hard tissues (both bone and teeth). This is why calcium phosphates and especially hydroxyapatite and its derivatives are extensively used for the preparation of ceramic and composite materials for bone tissue repairing and grafting. For this reason, gel, block or powder-like materials were obtained, tested and some of them clinically applied [9]. Although powder ceramics and composites as well as gels still remain the best choice for filling small irregular defects even if the risk of losing particles from the defective site is quite high. Based on extensive researches in the field of polymer/hydroxyapatite composite materials, new injectable support material with good biocompatibility, osteoinductivity, osteoconductivity and osteointegration were developed [10].

Hydroxyapatite demonstrates an exceptionally good osteoconductivity and osteointegration, but also a very low osteoinductivity, leading to low mechanical properties both in tissue culture or surgical operations, not only after the implantation procedure. Even so, long time resistance till fully degradation (more than one year) going even after the bone has been improved and regenerated into the pores, thus increasing the fracture risk, will be inhibiting the regeneration further on.

Chitosan is one of the most promising natural polymers for tissue engineering because it combines some very important properties such as good biocompatibility, intrinsic antibacterial activity, ability to bind growth factors and facile processing ways being a good candidate for cartilage, intervertebral disc, bone tissue engineering as well as delivery support of a wide number of drugs, including genes [11]. Chitosan based composite materials are of great importance for bone grafting, most usually in combination with calcium phosphates (and especially hydroxyapatite), collagen, alginate, hyaluronic acid, poly(methyl)methacrylate, or poly-L-lactic acid [12-14].

Experimental part

Materials and methods

The synthesized CS/HA/MB antiseptic composite materials were investigated by Fourier, transforming infrared spectroscopy and microscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) as well as by determining the *in vitro,* antimicrobial activity against *E. coli*.

Infrared spectroscopy (IR) measurements were performed on an iN10 MX mid infrared FTIR microscope operated in reflection mode. The spectra were recorded over the wavenumber range of 400–4000 cm^{-1} by co-adding 32 scans with a resolution of 4 cm^{-1}.

SEM images were recorded on a HITACHI S2600N instrument with an EDS probe. Before imaging, all samples were covered with a thin gold layer.

The antibacterial activity was evaluated against *Escherichia coli* (*E. coli* ATCC 8738) bacterium. It was cultured in a tube containing Luria Bertani (LB) medium at 37 ^{o}C (L.B. medium composition: peptone, 10g/L; yeast extract 5g/L, NaCl 5g/L) [15].

Sterile LBA (LB +20% agar) plates were prepared by pouring the sterilized media in sterile Petri dishes under aseptic conditions. The test organism (1 mL) was spread on agar plates and the samples were placed on the agar surface.

Antibacterial activity was determined by measuring the sizes of inhibition zone (IZ, mm) as clear, distinct zones of

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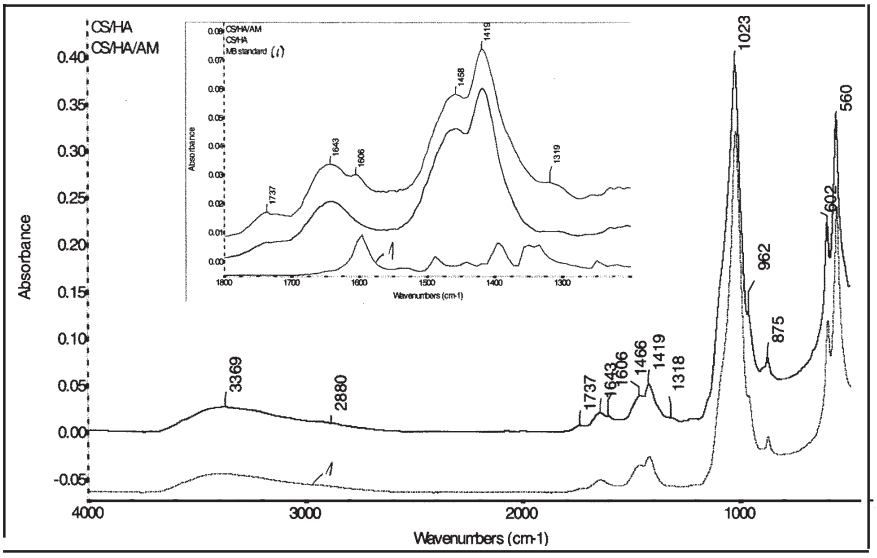

Fig. 1. FTIR spectra of CS/HA and CS/HA-MB; insert magnified spectra between 1200 and 1800cm^{-1}

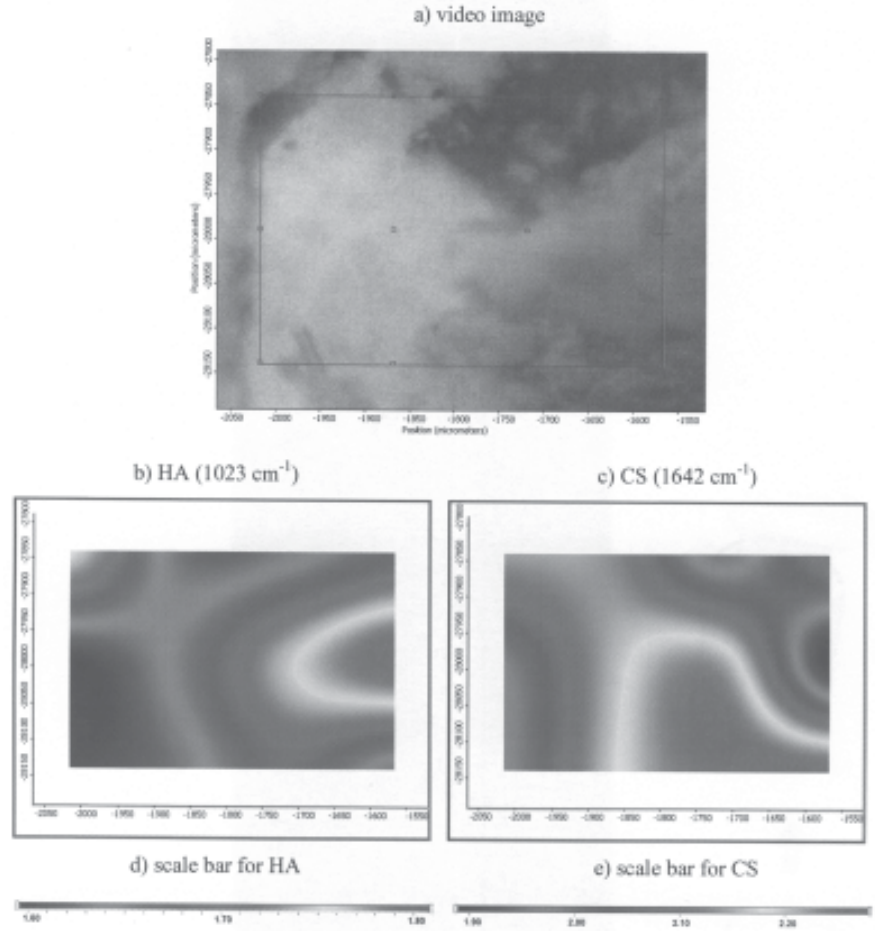

Fig. 2. FTIR maps recorded on CS/HA/MB: a) video image 350x500 m^2; b) FTIR image recorded at 1023cm^{-1}; c) FTIR image recorded at 1642 cm^{-1}; d,e) scale bar for b) and c)

inhibition surrounding the samples, and values < 8 mm were considered as not active against microorganisms.

All of the experiments were performed in triplicate. The results are reported as the average of three experiments and are presented as mean ± standard deviation (SD). Standard deviation was calculated as the square root of variance using STDEV function in *Excel* 2010.

Results and discussions

The obtained samples were analyzed by FTIR in order to identify the components and their distribution. Based on the FTIR spectra (Figure 1), we analysed the main bands of HA (560, 602, 1023 cm^{-1}) [16], CS (1642, 1418 and 1021 cm^{-1}) [17] and MB (~1599, 1395, 1350, 1337 cm^{-1}) [18]. Unfortunately, the absorption bands of methylene blue cannot be visualized without peaks deconvolution.

Based on the above presented spectra FTIR maps were obtained at selected wavelength. For monitoring, several bands were selected: 1023cm^{-1} (correspond to the main absorption band of HA), 1642cm^{-1} (correspond to the main absorption band of CS) and 1606cm^{-1} (correspond to the main absorption band of MB). Based on the video image (fig. 2a) and the two FTIR maps (fig. 2b and c) it can be assumed that CS/HA/MB sample is well heterogeneous; different repartition of HA and chitosan being visible. Unfortunately, due to the low content of MB, its monitoring in FTIR is not possible. Furthermore, it can easily be observed that the blue color (based on video image) is much more associated with the synthesized HA than with chitosan. This result was also confirmed based on UV-Vis measurements. For this, 1g of HA and CS were dispersed in 5mL methylene blue 0.1% and maintained for 1h. Based

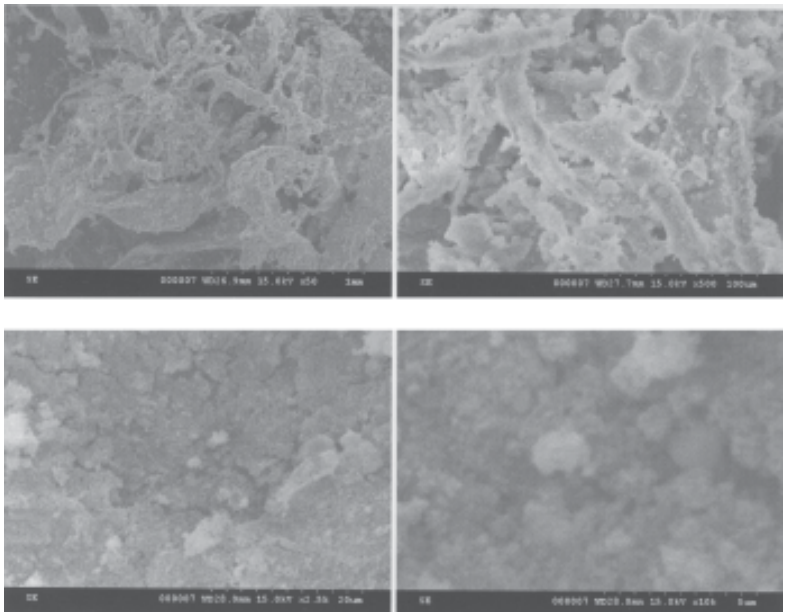

Fig. 3. SEM images of CS/HA

on the intensities of the methylene blue solutions recorded at 663nm (initial as well as the two solutions resulted after methylene blue retention on HA and CS), the affinity of HA seems to be two times higher than the affinity of CS against methylene blue.

SEM images were recorded on both materials (CS/HA and CS/HA/MB) between 50 and 10.000x magnification. As expected, there is no difference between the morphology of the two samples. At low magnifying, the well mineralized chitosan fibres are visible. When increasing magnifications, the HA agglomerates can be analysed, most probably the individual HA particles being in the nanometric region. Based on these high magnified images, the hydroxyapatite is homogenously distributed onto the chitosan fibres.

The antibacterial activity against *E. Coli* was determined for both CS/HA and CS/HA/MB. It can be observed that methylene blue improve the antibacterial activity against *E. coli* with ~16.5%. The results are indicated in table 1.

Table 1
ANTIMICROBIAL ACTIVITY OF CS/HA AND CS/HA/MB AGAINST *E. COLI*

No.	Sample	Antimicrobial activity (mm)
1	CS/HA	16.03±2.63
2	CS/HA/MB	18.67±1.53

Conclusions

Two new antiseptic materials based on chitosan and hydroxyapatite were obtained. The composite materials were obtained by precipitation. Even at low content of MB, its presence can be identified in FTIR spectrum, the FTIR microscopy allowing proving that MB is preferentially associated with hydroxyapatite. This heterogeneous distribution of MB between HA and CS can be explained, based on the higher affinity of methylene blue to hydroxyapatite than to chitosan. The obtained materials were tested from the point of view of antibacterial activity against *E. coli*, the obtained results being promising. Further works will be undertaken in order to obtain more complex composite materials based on collagen, chitosan and hydroxyapatite with tailored antimicrobial activity induced by proper CS:MB ratio.

Acknowledgements: This paper is supported by the Sectorial Operational Programme Human Resources Development, financed from the European Social Fund, and by the Romanian Government under the contract number POSDRU/86/1.2/S/58146 (MASTERMAT)" and 'Novel nanostructured prosthetic tubular devices with antibacterial and antibiofilm properties induced by physicochemical and morphological changes' PN-II-PT-PCCA-2011-3.2-0284, funded by the National University Research Council in Romania.

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Manuscript received: 18.06.2013