COMPOSITES CONTAINING HYDROXYAPATITE AND POLYURETHANE IONOMERS AS BONE SUBSTITUTION MATERIALS

ROXANA MIOARA PITICESCU[a], LAURA MADALINA POPESCU[a,b*],
TINCA BURUIANA[b]
[a]*National R& D Institute for Non-Ferrous and Rare Metals, 102 Biruintei Bd.,
Pantelimon, 077145, Ilfov, Romania*
[b]*Romanian Academy – Institute of Macromolecular Chemistry "Petru Poni", 41A
Grigore Ghica Voda Alley, Iasi, 700487, Romania.*

Two types of polyurethane ionomers with different content of carboxyl groups have been prepared and further used for hydrothermal synthesis of hydroxyapatite – polyurethane composites. The polyurethane structures were verified by ^{1}H NMR and IR spectroscopy, respectively. Prior to composite preparation, polyurethane ionomers were subjected to hydrothermal treatment in order to verify the stability of polymeric structures in conditions of pressure (20 atm) and temperature (140^0C). This treatment didn't induced changes in the absorption band of FT-IR spectra, which was an evidence that polymeric structure remains the same. The interactions between hydroxyapatite and polyurethanes during hydrothermal synthesis were investigated using FT-IR spectroscopy, showing the presence of characteristic absorption bands of hydroxyapatite and transformation of carboxyl groups from polyurethane in carboxylate groups, thus confirming the formation of chemical bonds with hydroxyapatite. Compatibility of polyurethane ionomers and hydroxyapatite based composites with mesenchymal stem cells has been quantified using MTT assay. Preliminary results of the in vitro tests showed a good proliferation of mesenchymal stem cells on the surface of hydrothermally treated polymers and composites. Further studies will focus on biocompatible properties of these composites and their potential application as bone substitution materials.

1. Introduction

Tissue engineered constructs must exhibit tissue-like functional properties, including mechanical behavior compatible to the native tissues they are intended to replace. Polyurethanes consist of hard segments, composed of alternating diisocyanate and chain-extender molecules and amorphous soft, linear diol segments. Polyurethane foams have good
mechanical property degradation and biocompatibility characteristics [1, 2].

Ionomers with carboxylate and sulphonic groups have important applications in preparation of biomedical components, elements senstive to temperature variations, polyelectrolytes, etc [3-5]. Carboxylic groups have been introduced in the hard segments of polyurethane chain using a chain extender which contains ionic or ionogenic groups such as α,α-dimethylol propionic acid [6-13], 2-hydroxyethylmethacrylate [14, 15], 3, 4-dihydroxycinnamic acid [16], 4-hydroxycoumarin [17], dimethylol butanoic acid [18], phenolphtaleine [19, 20],

---

followed by neutralization with bases. Another approach to introduce carboxyl groups would be two-step derivatization of a neutral polyurethane [21].

Such polyurethanes functionalized with carboxylic groups (PU-COOH) could be used for the development of polymer/hydroxyapatite composites as bone tissue engineering scaffolds.

In fact, human bone tissue is a kind of composite composed of about 65 wt% nano-hydroxyapatite (HAp) crystals and collagen matrix. [22]. An ideal material for bone repairing must be biocompatible and bioactive, which is able to initiate osteogenesis. Moreover, its mechanical strength and composition should be similar to those of bone. In recent years, composite materials with high content of HAp nanoparticles have been studied, such as polyamide/HAp (PA/HAp) [23], polymethylmethacrylate/HAp (PMMA/HAp) [24-26], polylactide/HAp (PLA/HAp) [27-29] and polyethylene/HAp (PE/HAp) [30-32] etc., for biomedical applications [33]. Many researchers investigated polyurethane (PU) based composites with HAp as reinforcing filler ($\leq$ 10 wt%) prepared by in situ polymerization [33, 34]. Boissard et al [35] reported the synthesis and processing of nano-hydroxyapatite/poly(ester urethane) composite scaffolds using a salt-leaching-phase inverse process. Five different nano-hydroxyapatite weight fractions were added: 5, 10, 20, 30 and 50 wt.% of the poly(ester urethane).

To our knowledge, untill now, no work has been reported on template synthesis of composites based on hydroxyapatite as major phase (80 wt%) and polyurethane ionomers with carboxyl groups on its surface, excepting our previous papers [36, 37]. In this study, two types of polyurethane ionomers with different content of carboxyl groups have been prepared and further used for hydrothermal synthesis of HAp/PU-COOH composites.

The goal of this paper is to study the formation of HAp based composites, containing two types of specially polyurethane ionomers, designed to replace and/or repair the damaged bone tissue.

2. Experimental

Materials

Poly(tetramethylene oxide) diol (PTMO; M_w=1000), isophorone diisocyanate (IPDI), butanediol, dimethylol propionic acid and tartaric acid were purchased from Sigma–Aldrich. Calcium nitrate $(Ca(NO3)_2 \cdot 4H_2O)$ and ammonium di-hydrogen phosphate $(NH_4H_2PO_4)$ of analytical grade were purchased from Merck. Ammonium hydroxide (NH_4OH) 25 % solution (pure chemical reagent) was purchased from Chimopar, Bucharest.

Synthesis of PU functionalized with carboxylic groups

PU-COOH1

First type of polyurethane ionomer was synthesized starting from poly(tetramethylene oxide) diol (M_w=1000), isophorone diisocyanate and butanediol. Carboxyl groups were introduced by the dimethylol propionic acid. The evolution of the reaction was monitored with FT-IR spectroscopy until the isocyanate and hydroxyl groups were reacted, as confirmed by the disappearance of their stretching bands located at 2260 and 3480 cm^{-1}, respectively. The resulting polyurethane was precipitated in methanol and dried for 48 h at 60 ^{o}C under reduced pressure. The structure of PU-COOH1 ionomer is given in figure 1.
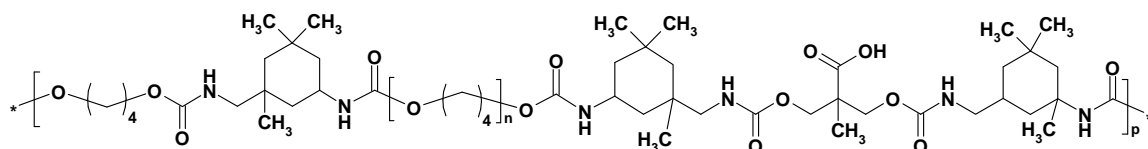

Fig. 1. Structure of polyurethane ionomer of type 1 (PU-COOH1) functionalized with carboxyl groups

PU-COOH2

Second type of polyurethane ionomer was synthesized starting from poly(tetramethylene oxide) diol (M_w=1000), isophorone diisocyanate and butanediol. Carboxyl groups were introduced by the tartaric acid. The evolution of the reaction was monitored with FT-IR spectroscopy until the

isocyanate and hydroxyl groups were reacted, as confirmed by the disappearance of their stretching bands located at 2260 and 3480 cm^{-1}, respectively. The resulting polyurethane was precipitated in methanol and dried for 48 h at 60 °C under reduced pressure. The structure of PU-COOH2 elastomer is given in figure 2.
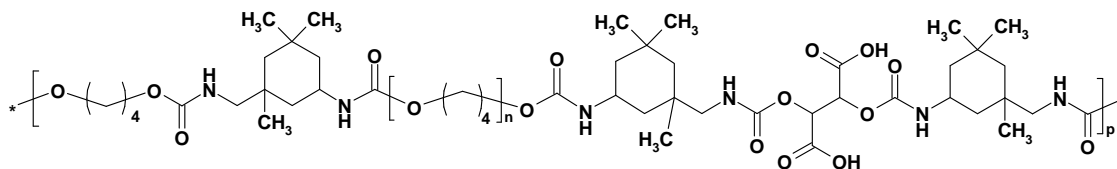

Fig. 2. Structure of polyurethane ionomer of type 2 (PU-COOH2)

Hydrothermal treatment

Polyurethane ionomers prepared as described above were dissolved in dimethylacetamide (DMAA, c = 5 %) and diluted in water (DMAA:H2O = 1: 4). The solution was magnetically stirred and then put into autoclave vessel for 2h of hydrothermal treatment at 140^{0}C and 20 atm. The resulted polymer solutions were dried by lyophilization in a freeze drier and further characterized using Fourier Transform infrared spectroscopy (FT-IR) and differential scanning calorimetry (DSC).

Hydrothermal synthesis of HAp/PU-COOH composites

Organic-inorganic composites with hydroxyapatite 80 wt% were obtained by hydrothermal process. Polyurethane prepared as described above was dissolved in 5% (w/v) N,N-dimethylacetamide solvent. Purity grade hydroxylapatite precursors, namely calcium nitrate tetrahydrate and ammonium dihydrogen phosphate were solubilized in water under a strict pH control, adding ammonium hydroxide as a mineralizing agent. The aqueous suspension thus obtained was further mixed with the polymer solution under vigorous stirring, followed by a hydrothermal reaction in a Cortest autoclave, USA. Reactions were conducted in a closed system at 120 ^{0}C and 20 atm, leading to nanostructured composite powders. The detailed hydrothermal synthesis process is described in our previous papers [36, 37].

Characterizations

The polyurethane structures were verified by ^{1}H NMR and IR spectroscopy with a Bruker 400-MHz spectrometer and a Specord M80 spectrophotometer, respectively.

FTIR spectra were recorded in the transmission mode on an ABB MB 3000 spectrometer from 4000 to 550 cm^{-1}, averaging 64 scans at a resolution of 4 cm^{-1}. The solid samples were carefully mixed with KBr powder for 30 mins. A homogenous mixture of about 1 % sample in KBr powder was obtained. Quantitative Fourier-transformed infrared spectra were collected using the PIKE Technologies EasiDiff diffuse reflectance accessory. The Horizon[MB] software was used for automatic data processing. For the analysis of samples an electronic scanning microscope (SEM) Vega -TESCAN LSH microscope was used, at an acceleration voltage of 30 kV. DSC measurements were performed with a Netzsch DSC 200 F3 Maia thermal analyzer. The instrument was calibrated using a sapphire standard. The samples were weighed and encapsulated in aluminium pans. An empty aluminium pan served as the reference. After baseline acquisition, the samples were heated from 20 to 300 ^{0}C at a rate of 15 ^{0}C min^{-1}.

Cell culture and proliferation using MSCs

Mesenchymal stem cells (MSC) were isolated from mouse bone marrow as previously described [38]. Cells were seeded at a density of 5000 cells/cm^{2} on polyurethane and composite thin films deposited on glass coverslips. Substrates used for the deposition were chemically etched with hydrofluoric acid to improve roughness and the adherence of the bioactive layer. Viability and cell proliferation tests were performed after 5 days using MTT assay.

3. Results and discussion

Polyurethane synthesis

The structure and purity of the polyurethane ionomers were verified by FT-IR and ^{1}H – NMR.

Figure 3 displays the ^{1}H NMR spectrum of the carboxylic acid polyurethane PU-COOH1 in deuterated dimethyl sulfoxide (DMSO-d$_6$).
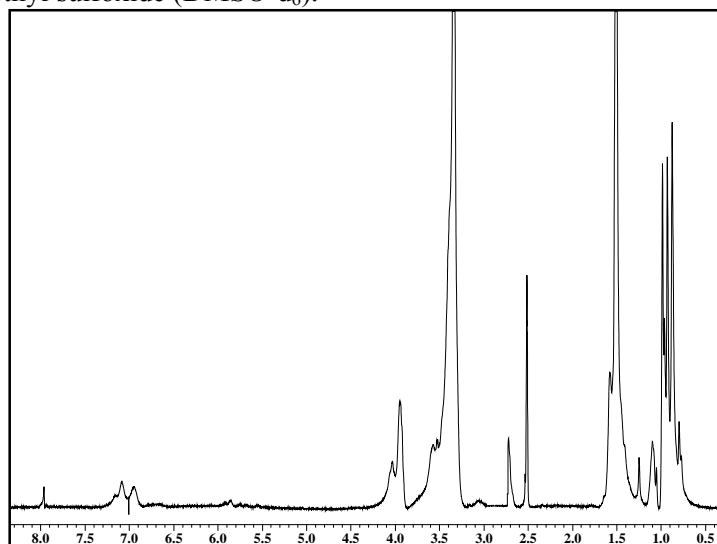

Fig. 3. ^{1}H NMR spectrum of PU-COOH1 in DMSO-d$_6$

In NMR spectrum of polyurethane ionomer PU-COOH1 the signals corresponding to the urethane protons appear in the interval of 7,1-6,9 ppm; at 3,9 ppm is observed the signal given by methylene protons situated near ester-urethane group -CH2-OCONH-, while at 3.33 ppm methylene proton signal O-CH2-(CH2)2-CH2-O in PTMO and butanediol can be observed.

Methylene protons -CH2-CH2- from PTMO and butanediol appear at 1,5 ppm, whereas the protons from the remaining isophoron give a multiple signal between 1,1 and 0,8 ppm. Abundance and the integral ratio of the of the protons described above confirms the proposed structure for the synthesized polyurethane ionomers of type 1 (PU-COOH1).

Structure of polyurethane elastomer PU-COOH2 was verified using the same spectral methods. NMR spectrum of polymer PU-COOH2 is shown in Figure 4, where one can identify aliphatic protons from the remaining isophoron in the region 1,07-0,77 ppm, methylene protons -O-CH$_2$-C**H**$_2$-C**H**$_2$-CH$_2$-O- (1,5 ppm), aliphatic protons -O-C**H**$_2$-(CH$_2$)$_2$-C**H**$_2$-O- (3.42 ppm). At 3,9 ppm is observed the signal given by methylene protons situated near ester-urethane group -CH2-OCONH-, while urethane protons appear between 6,9 and 7,1 ppm.
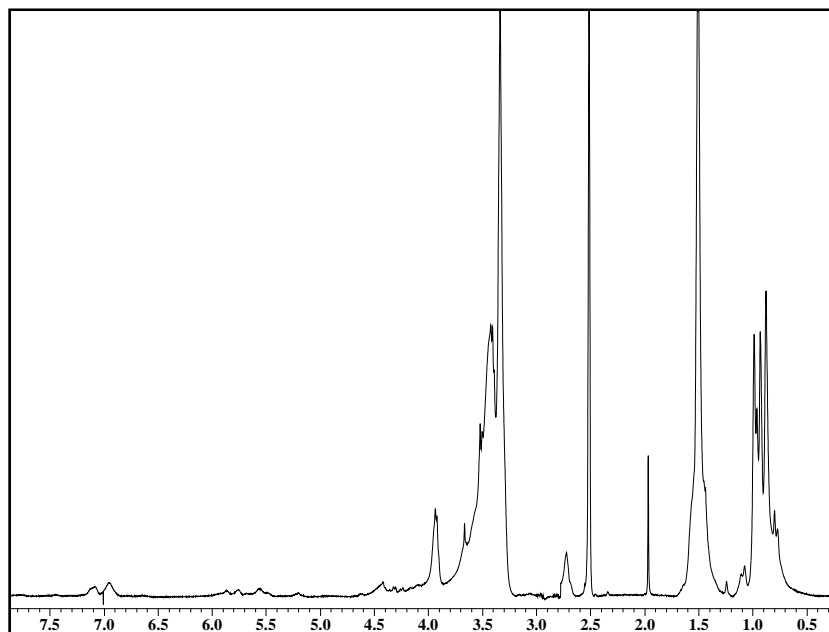
Fig. 4. NMR spectrum of polyurethane PU-COOH2 in DMSO-d$_6$

Hydrothermal treatment of polyurethanes

As obtained carboxylic polyurethanes were further used for in situ hydrothermal synthesis of HAp/PU-COOH composites. Before this, polyurethane ionomers were subjected to hydrothermal treatment in order to verify the stability of polymeric structures in conditions of pressure (20 atm) and temperature (140^0C). This treatment doesn't induce changes in the absorption band of FT-IR spectra, which is an evidence that polymeric structure remains the same. Figure 5 presents FT-IR spectrum of polyurethane PU-COOH1 before and after hydrothermal treatment at 140^0C and 20 atm.
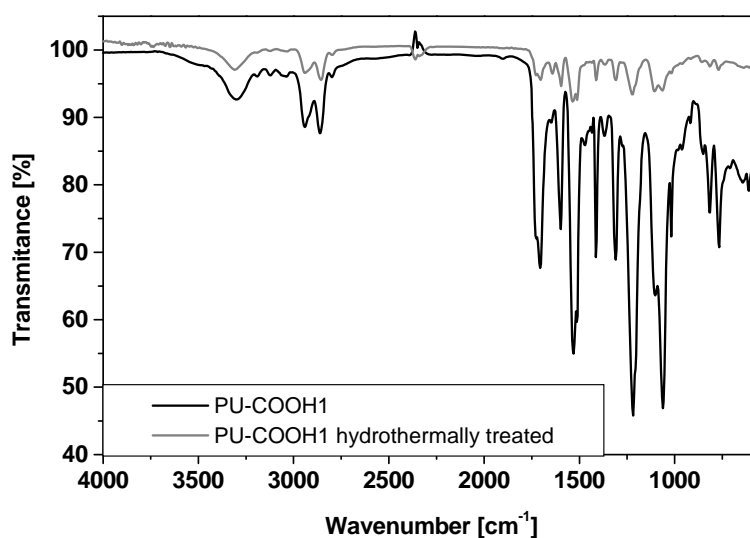

Fig. 5. FT-IR spectra of polyurethane PU-COOH1 before (black) and after hydrothermal treatment ($140\,^oC$, 2 h, 20 atm) (gray).

Characterization of HAp/PU-COOH composites

FT-IR spectroscopy

FT-IR spectra of composite hybrids HAp/PU-COOH1 and HAp/PU-COOH2 (figure 6) containing 20% anionic polyurethane PU-COOH1 or PU-COOH2, show the presence of characteristic absorption bands of hydroxyapatite namely, OH band at 3572 cm^{-1}, and $(PO_4)^{3-}$ bands at 1091-1031 si 633-563 cm^{-1}. Another absorption band appears at 3435 cm^{-1} being attributed to N-H urethane group; absorption bands between 2925-2855 cm^{-1} are assigned to C-H vibrations, while carbonyl groups which are usually found at 1735 cm^{-1} in polymer spectrum are shifted to 1616 cm^{-1}, due to transformation of carboxyl groups from polyurethane in carboxylate groups, thus confirming the interactions with hydroxyapatite.
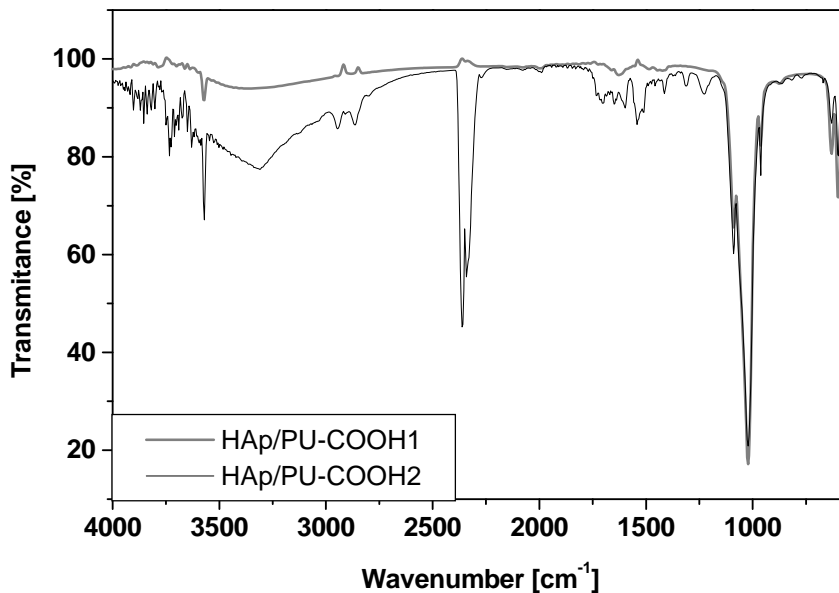

Fig. 6. FT-IR spectra of two selected composites: HAp/PU-COOH1 and HAp/PU-COOH2

Thermal characterization (DSC measurements) DSC thermographs of two representative samples are displayed in figure 7.
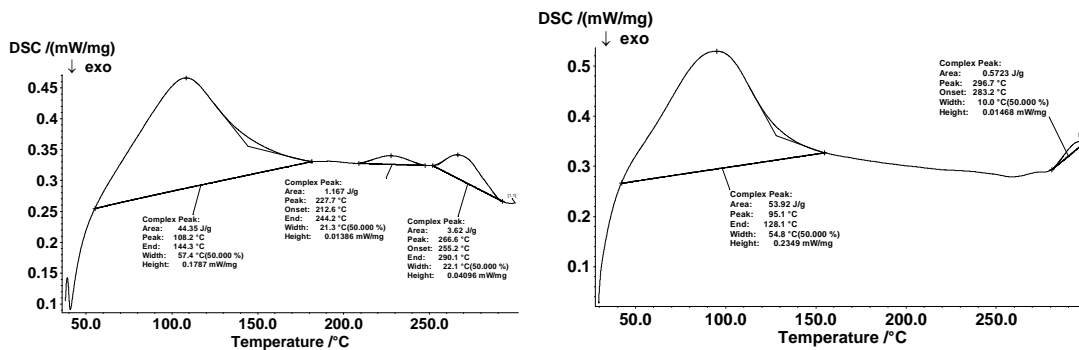

Fig. 7. DSC thermographs of : a) HAp/PU-COOH1; b) HAp/PU-COOH2

Both types of composites are thermally stable, as well as the initial polyurethanes PU-COOH1 and PU-COOH2. First endothermic peak at 95 and 108 ^{0}C, respectively is atributed to water evaporation. It can be observed that composites containing PU-COOH1 polymer are thermally stable until 300 ^{0}C comparing to HAP/PU-COOH2 composites which presents small endothermic peaks around 227 ^{0}C and 266 ^{0}C probably due to some morphological transformations or molecular rearrangements.

Morphological characterization (SEM analysis)

SEM images of composites based on hydroxyapatite and carboxylic polyurethane, obtained in hydrothermal conditions (Fig. 8) are distinguished by the appearance of irregular structures without a specific morphology, formed by the agglomeration of small crystallites, with size ranging from 50 to 5 μm. Overall, polymer distribution in inorganic matrix is uniform; spongy nano-sized structures can be observed at higher magnifications.
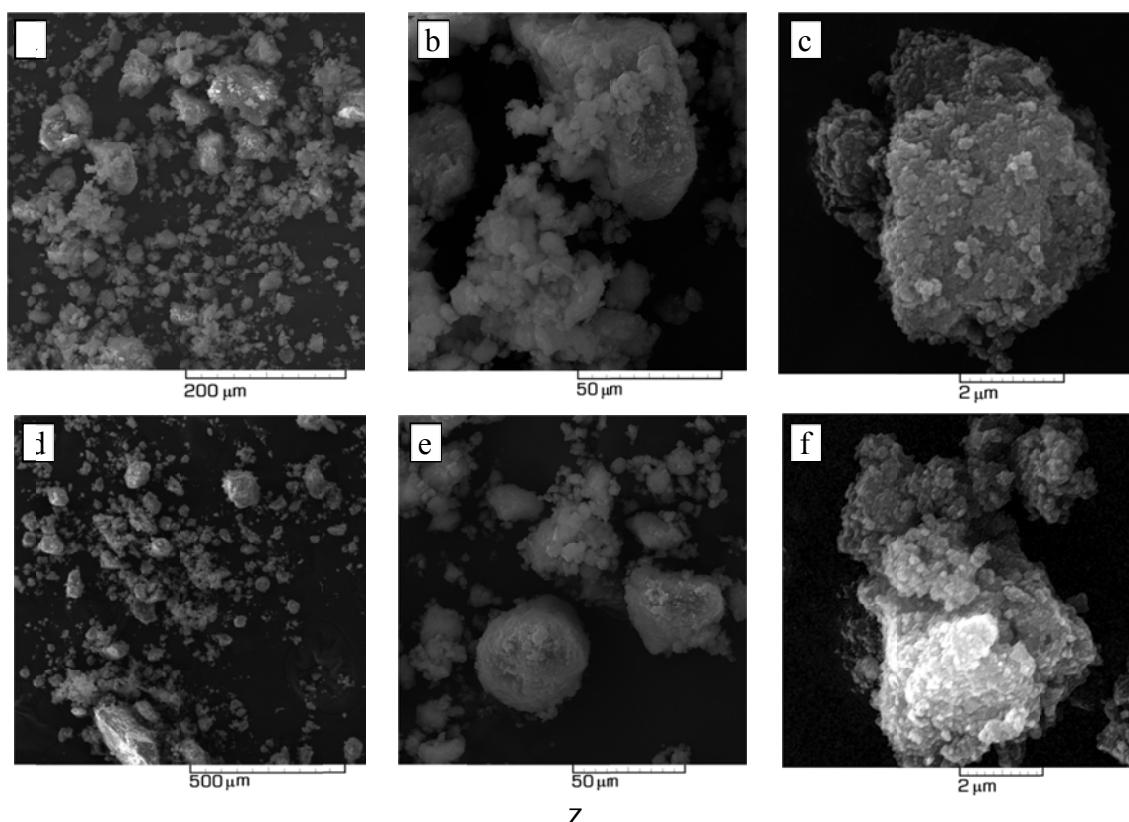

Fig. 8. SEM images of composites based on hydroxyapatite and polyurethane ionomers (80/20) synhesized at 120 ^{o}C, 2 h and 20 atm: (a), (b), (c) – HAp/PU-COOH1; (d), (e), (f) – HAp/PU-COOH2

Cellular proliferation

It is known that bone marrow mesenchymal stem cells (MSCs) can differentiate into a various type cells. It has been identified that MSCs are an attractive cell source for various tissue engineering and play a central role in the repair and regeneration of mesenchymal tissue. Expansion of MSCs in vitro is prerequisite for their applications in tissue engineering [39]. In this study, we evaluated the effect of hydrothermally treated polyurethanes and HAp-based composites on growth and proliferation of MSCs by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphe-nyltetra-zolium bromide (MTT) assay. Cellular proliferation is presented in Fig. 9
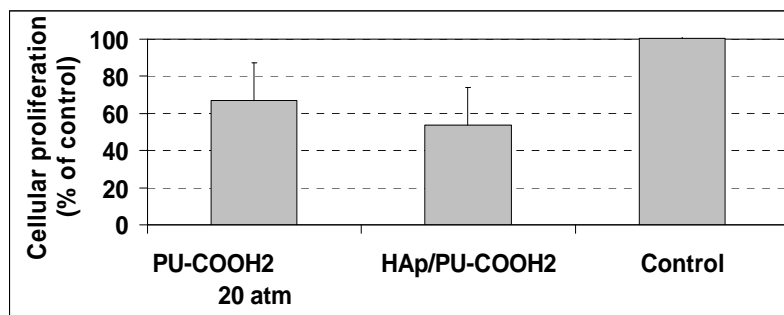
Fig. 9. MSC grown on PU-COOH2 treated at 20 atm and HAP-PUCOOH2 composites

Viability values of the cells cultured on the samples surface are statistically significant compared with control (glass coverslip) but there is no significant difference between the samples, regarding proliferation behavior of MSCs onto their surface. Mesenchymal stem cells showed a good proliferation on the surface of hydrothermally treated polyurethane (67%) and hydroxyapatite based hybrids (54%).

4. Conclusions

Hydroxypatite-polyurethane iononomers with 80 wt % HAp were prepared using hydrothermal method at 120^0C and 20 atm. Thermal stability of both polyurethanes before and after hydrothermal treatment was verified using DSC analysis prior to the synthesis of composites. Using this approach, we have demonstrated that polyurethane ionomers with carboxyl groups doesn't undergo any change during hydrothermal treatment. The interactions between hydroxyapatite and polyurethanes have been studied using FT-IR spectroscopy and the dissapearance of the vibration band assigned to free carbonyl group suggest that –C=O group is involved in the formation of new bonds with hydroxyapatite. SEM images underlined the homogenous distribution of polymer inside inorganic matrix and revealed the existence of spongy nano-sized structures which agglomerates in structures with 50 - 5 μm. Preliminary in vitro test using MTT assay showed a good proliferation of mesenchymal stem cells on the surface of hydrothermally treated polymers and composites, which represents a first step for further studies on biocompatible properties of these composites and their potential application as bone substitution or replacement materials.

References

[1] M. Sokolsky-Papkov, K. Agashi, A. Olaye, K. Shakesheff, A.J. Domb, Advanced Drug Delivery Reviews **59**, 187 (2007).
[2] J. Guan, J.J. Stankus, W.R. Wagner, Biomaterials **26**, 3961 (2005).
[3] H. B. Lin, W. Sun, D. F. Mosher, C. Garciaecheverria, K. Schaufelberger, P. I. Lelkes, S. L. Cooper, J. Biomed. Mater. Res. **28**, 329 (1994).
[4] D. H. Lee, J. W. Kim, K. D. Suh, J. Appl. Polym. Sci. **72**, 1305 (1999).
[5] H. Chen, D. Chen, Q. Fan, X. Yu, J. Appl. Polym. Sci. **76**, 2049 (2000).
[6] D. H. Lee, J. W. Kim, K. D. Suh, Colloid Polym. Sci. **277**, 265 (1999).
[7] Y. Luo, X. Xu, Eur. Polym. J. **34**, 1899 (1998).
[8] S. Y. Lee, J. S. Lee, B. K. Kim, Polym. Int. **42**, 67 (1997).

[9] D. J. Hourston, G. Williams, R. Satguru, J. D. Padget, D. Pears, J. Appl. Polym. Sci. **67**, 1437 (1998).

[10] N. H. Park, K. D. Suh, J. Appl. Polym. Sci. **71**, 1597 (1999).

[11] M. E. Song, J. Y. Kim, K. D. Suh, J. Appl. Polym. Sci. **62**, 1775 (1996).

[12] C. Z. Yang, T. G. Grasel, J. L. Bell, R. A. Register, S. L. Cooper, J. Polym. Sci.: Part B: Polym. Phys. Ed. **29**, 581 (1991).

[13] J. Y. Kim, Y. S. Chon, D. J. Yoo, K. D. Suh, J. Polym. Sci.: Part B: Polym. Phys. Ed. **38**, 2081 (2000).

[14] J. Y. Kim, C. H. Kim, D. J. Yoo, K. D. Suh, J. Polym. Sci.: Part B: Polym. Phys Ed. **38**, 1903 (2000)

[15] G. N. Mahesh, P. Barm, G. Radhakrishnan, J. Appl. Polym. Sci. **65**, 2105 (1997).

[16] C. L. Wang, Y. M. Kuo, D. Y. Chao, Polym. Adv. Technol. **11**, 127 (2000).

[17] S. Y. Lee, J. S. Lee, B. K. Kim, Polym. Int. **42**, 67 (1997).

[18] D. J. Hourston, G. Williams, R. Satguru, J. D. Padget, D. Pears, J. Appl. Polym. Sci. **74**, 556 (1999).

[19] S. Ramesh, G. Radhakrishnan, J. Appl. Polym. Sci. **60**, 2273 (1996).

[20] S. Ding R. Register, C. Yang, S. L. Cooper, Polymer **30**, 1204 (1989).

[21] T.-C. Wen, Y.-J. Wang, Ind. Eng. Chem. Res. **38**, 1415 (1999).

[22] N. Watabe, Calcif Tissue Int **29**, 163 (1965).

[23] H. Wang, Y. Li, Y. Zuo, J. Li, S. Ma, L. Cheng, Biomaterials **28**, 3338 (2007).

[24] T.Y. Liu, S.Y. Chen, D.M. Liu, J Biomed Mater Res **71B**, 116 (2004).

[25] W.R. Walsh, M.J. Svehla, J. Russell, M. Saito, T. Nakashima, R.M. Gillies, W. Bruce, R. Hori, Biomaterials **25,** 4929 (2004).

[26] A.M. Moursi, A.V. Winnard, P.L. Winnard, J.J. Lannutti, R.R. Seghi, Biomaterials **23**, 133 (2002).

[27] Y. Shikinami, M. Okuno, Biomaterials **20**, 859 (1999).

[28] N. Ignjatovic, E. Suijovrujic, J. Budinski-Simendic, I. Krakovsky, D. Uskokovic, J Biomed Mater Res B: Appl Biomater **71**, 284 (2004).

[29] G. Wei, P.X. Ma Biomaterials **25**, 4749 (2004).

[30] L. Di Silvio, M.J. Dalby, W. Bonfield, Biomaterials **23**, 101 (2002).

[31] L. Fang, Y. Leng, P. Gao, Biomaterials **27**, 3701 (2006).

[32] Y. Zhang, K.E. Tanner, N. Gurav, L. Di Silvio, J. Biomed Mater Res **81**(A), 409 (2007).

[33] C-X Zhao, W-D Zhang, European Polymer Journal **44**, 1988 (2008).

[34] A.S. Khan, Z. Ahmed, M.J. Edirisinghe, F.S.L. Wong, I.U. Rehman, Acta Biomaterialia **4**, 1275 (2008).

[35] C.I.R. Boissard, P.-E. Bourban, A.E. Tami, M. Alini, D. Eglin, Acta Biomaterialia **5**, 3316 (2009).

[36] R.M. Piticescu, T. Buruiana, N. Plesu, E. Vasile, C. Moldovan , B. Fartat, C. Rusti, Optoelectron. Adv. Mater. - Rapid Comm. **4**, 401 (2010).

[37] L.M. Popescu, R.M. Piticescu, T. Buruiana, E. Vasile, R. Trusca, V. Badilita, Z. Naturforsch. B: J. Chem. Sci., **66**, 36 (2011).

[38] A.-M. Rosca, A. Burlacu, Stem Cells Dev 2010 Nov 10. [Epub ahead of print]

[39] G. Song, Y.Ju , H. Soyama, Mat Sci Eng C, vol **28**, 1467 (2008).