Maciej Boguń[1], Ewa Stodolak[2], Elżbieta Menaszek[2,3], Anna Ścisłowska-Czarnecka[4]

[1)]**Technical University of Lodz, Department of Man-Made Fibres,**
ul. Żeromskiego 116, 90-924 Łódź, Poland,
E-mail: maciek.bogun@wp.pl

[2)]**AGH University of Science and Technology, Faculty of Materials Science and Ceramics, Department of Biomaterials,**
al. Mickiewicza 30, 30-059 Kraków, Poland

[3)]**CMUJ - Jagiellonian University Medical Collage, Faculty of Pharmacy, Department of Cytology,**
ul. Medyczna 9, 30-688 Kraków, Poland

[4)]**University School of Physical Education, Faculty of Rehabilitation, Physiotherapy Department,**
Al. Jana Pawla II 78, 31-571 Krakow, Poland

Composites Based on Poly-ε-Caprolactone and Calcium Alginate Fibres Containing Ceramic Nanoadditives for Use in Regenerative Medicine

Abstract
The aim of the present work was to develop new composite materials based on two biocompatible polymers (sodium alginate and polycaprolactone) intended for use in the treatment of bone tissue defects. Tests carried out to obtain polymer-fibre composites using two resorbable polymers demonstrated the possibility of attaining composites with mechanical properties that are suitable from the point of view of their applications. Young's modulus values for the composite systems analysed (254-389 MPa) are higher than for an unmodified PCL sheet. Irrespective of the fibrous phase used, the PCL matrix demonstrates stability in in vitro conditions. The constant pH values and small changes in the ionic conductance of the water indicate that these materials undergo gradual but slow degradation.

Key words: *alginate fibres, composite, polycaprolactone, biological properties.*

Introduction

Regenerative medicine is one of the fastest developing branches of medicine at the present time, serving to support the reconstruction of damaged tissues resulting from various types of illnesses or genetic defects. An important role in this branch of medicine is played by tissue engineering, which makes use of materials engineering, broadly defined. The current development of materials engineering provides scientists with ever greater possibilities of creating new implant materials, for example for use in the reconstruction of bone tissue. The setting up of interdisciplinary teams to work on obtaining new materials makes it possible to take full and simultaneous advantage of the potential of the developing technologies. New implant materials fulfilling the criteria of regenerative medicine for the reconstruction of bone tissue should have the following characteristics among others

- a specified time of degradation enabling the overgrowing of the implant with regenerating tissue;
- a supporting action (based on physicochemical and bioactive properties) on the bone cells so as to increase their rate of multiplication;
- an appropriate microstructure and porosity.

One of the directions being taken to obtain new solutions for medicine is the development of new implant materials using biodegradable and biocompatible polymers such as co-polymers of lactic acid, polycaprolactone, cellulose, chitin, and alginate [1 – 5]. At the same time, the rapid development of nanotechnology which occurred at the end of the 20th century opened up new prospects for modelling the properties of developed materials, especially their bioactivity. An implant with suitable bioactivity can be given features which are responsible for good chemical activity, and this leads to the possibility of creating a bond between natural tissue and the implant.

The aim of the present work was to develop new composite materials based on two biocompatible polymers (sodium alginate and polycaprolactone) intended for use in the treatment of bone tissue defects. These materials will be characterised by a random dispersion of fibres in the polymer matrix, i.e. they will be composites of a MD type. The paper presents the results of testing the biocompatibility of the polymer-fibre composites used. Cell tests are of great importance in predicting the effectiveness of the action of a new type of polymer-fibre composite produced with alginate fibres containing ceramic nanoadditives. It should be noted, however, that with new types of implant materials, a reliable assessment of the effectiveness of their action is provided by *in vivo* studies, which are outside the scope of this work.

These composites will serve to accelerate the treatment of patients. At the same time, bringing implant materials of this type onto the market can be expected to reduce patient hospitalisation costs, since the use of implant materials based on bioresorbable and biodegradable polymers eliminates the need for a repeat operation, which is necessary, for example, in the case of metal implants. Also the use of bioresorbable and biodegradable implants makes it possible to limit the use of autogenic and allogenic transplants, which carry an ever greater risk of post-operative complications of various kinds.

Alginate is currently one of the most commonly used polymers in medicine [6]. This polymer is a linear polysaccharide built of residues of β-D-mannuronic acid (M) and α-L-guluronic acid (G) [7]. It has many features that are favourable for medical application, such as the absence of toxicity, antibacterial properties, and a controlled biodegradation time. It is currently used in tissue engineering for the treatment and regeneration of skin, cartilaginous tissue, bone tissue, the liver, and myocardial tissue [8 - 12]. In the present work it was used to obtain calcium alginate fibres whose fibre-forming material contained bioactive nanoadditives, such as hydroxyapatite and bioglass. This creates the possibility of giving one of the components of the composite (the alginate fibres) additional osteoconductive or osteoinductive properties, brought about by the nanoadditive introduced into the material of the fibres.

Polycaprolactone (PCL) belongs to the class of biodegradable aliphatic polyesters. It is a linear, semi-crystalline, hydrophobic polyester. Thanks to its good solubility, its low melting point (around 60 °C), glass transition temperature

(–60 °C) and its exceptional ability to mix with other polymers, it has motivated numerous studies relating to possible applications in medicine. It is used, among other things, as surgical thread, in systems of controlled drug release, in dentistry and in blood-vessel surgery [13 - 17].

Therefore polymer-fibre composites based on poly-ε-caprolactone (PCL) and nanocomposite calcium alginate fibres are an interesting biomaterial in view of the possibility of designing their material characteristics depending on the nanoadditive used. When the fibrous phase contains bioactive ceramic nanoadditives, a strong osteoinductive potential can be expected.

Description of the materials used

Spinning solutions were made using sodium alginate from FMC Biopolymer (Norway) under the trade name Protanal LF 10/60LS, with an intrinsic viscosity $\eta = 3.16$ dL/g. The polydispersity index Mw/Mn determined by gel chromatography (SEC/GPC) was 6.8. The majority of the polymer was composed of blocks formed from mannuronic acid (up to 65%), with the rest being residues of guluronic acid.

The nanoadditives introduced into the spinning solution were:

- hydroxyapatite (HAp) - $Ca_{10}(PO_4)_6(OH)_2$ obtained at the AGH Uniwersity of Science and Technology in Kraków, according to a patent [18];
- bioglass obtained at AGH Kraków with the following oxide composition: CaO 16%mol, SiO_2 80%mol, P_2O_5 4%mol.

The biocomposite matrix consisted of a resorbable polymer from the aliphatic polyester group: poly-ε-caprolactone (PCL) from Sigma-Aldrich. The average polydispersity index of this polymer, determined as a Mw/Mn ratio, was 4.8.

Fibre formation and obtaining of composites

Alginate fibres were formed wet from the solution, using distilled water as a solvent. The process of the solidification of the calcium alginate fibres took place in baths containing a 3% aqueous solution of $CaCl_2$ with the addition of a small quantity of hydrochloric acid (0.03% HCl), at a temperature of 40 °C. Stretching of the fibres took place in two stages: The first stage of the stretching process was carried out in a plastification bath, with the same composition as the solidification bath, and at a temperature of 67 °C. The second stage of the stretching process took place in an environment of overheated steam at a temperature of 135 – 140 °C. Following the process of stretching and rinsing, the fibres were dried at a temperature of 250 °C in isometric conditions. A description of the fibre forming process and the effect of basic process parameters on the properties of the fibres can be found in our earlier papers [19 – 21].

Sheets of hybrid polymer-fibre composites were obtained by the tape casting method at the Biomaterials Department of AGH Kraków. The fibrous component was broken down into short fibres approximately 20 – 30 mm in length. The polymer and alginate fibres were then subjected to a process of homogenisation in methylene chloride (a solvent of poly-ε-caprolactone), and the resulting solution was poured onto plates in order to evaporate the solvent.

Testing of composites

Mechanical testing of the polymer-fibre composite sheets was carried out on a Zwick 1435 universal mechanical testing machine. Samples in the form of strips were subjected to a stretching test in the following conditions: stretching speed 40 mm/min, working base 40 mm, and measured range of Young's modulus 5 - 15 MPa.

Physicochemical testing of the surface of the composite sheets was based on the following determinations: roughness profile, surface wettability, and the microstructure of the composite materials obtained.

The surface roughness of the composite sheets was tested using a T-500 surface tester from Hommelwerke (Germany). The results of the measurements consisted of the basic roughness parameters R_a, R_t, and R_z.

- l_r – length of the section (elementary section) for which the roughness parameters are defined;
- R_a in μm – arithmetic mean of the deviation of the profile. R_a relates to the whole of the elementary section Ir. The representativeness of R_a is very limited. Single summits have a minimal effect on the value of parameter R_a;
- R_t in μm – maximum height between the highest peak and lowest trough. This parameter gives the vertical distance between the highest and lowest points of the filtered roughness signal within the section of measurement (computation);
- Rz in μm – height of the roughness profile based on 10 points. This parameter gives the principal level of absolute values of the five highest peaks and five lowest troughs within the section of measurement (computation).

The contact angle of all samples was determined by direct measurements. The liquid used was ultra-pure (UHQ) water. Measurements were performed using DSA (Drop Shape Analysis) apparatus. The results for the contact angle were the averages of 15 measurements, at a significance level of $\alpha = 0.05$.

Surface microstructure testing was based on observations of the microstructure of the composite materials, made using a JMS-5400 scanning electron microscope (SEM) from JEOL (Japan). Prior to testing, the samples were covered in a conducting carbon layer.

Biological properties of the composite sheets, as well as their degree of degradation, were measured during incubation in distilled water. The samples were kept in a heat chamber at 37 °C for a period of 16 weeks. Their degradation was monitored through changes in the ionic conductance and pH of the immersion fluid (water) in which the composite materials were kept.

Cell culture. Cells were grown in 75 ml plastic bottles (Nunclon, Denmark) in a MEM nutrient medium (PAA, Austria), with the addition of 10% foetal calf serum (PAA, Austria) and a 5% solution of antibiotic penicillin (10 UI/ml) and streptomycin (10 mg/ml) (Sigma, Germany), in a 5% CO_2 atmosphere and at a temperature of 37 °C. A suspension of cells was obtained by adding 5% trypsin EDTA (PAA, Austria). After rinsing and centrifuging, the cells were brought to a concentration of 3×10^4 cells/ml, and then 1 ml of the cell suspension was placed in the wells of a 24-well plate (Nunclon, Denmark) containing sterile discs of the materials tested. Positive control was the polystyrene bottom of the wells of the plate (TCPS). The incubation of MG-63 cells in the presence of PCL discs containing nanocomposite alginate fibres was carried out for 3 and

7 days in an incubator in an atmosphere of 5% CO_2 at 37 °C.

Cell numbers were determined based on the supernatant taken from above the cells, rinsed with PBS and covered with a 5% solution of trypsin EDTA. After 10 minutes the cells that had been separated from the medium were counted in a Bürker chamber.

Cell adhesivity was tested using the crystal violet absorption test. The cells adhering to the medium were set in 2% paraformaldehyde for one hour and then dyed with crystal violet (CV; 0.5% in 20% methanol) for 5 minutes. After this time the discs with adhering cells were rinsed in running water and transferred to a new 24-well plate, and after drying out, the absorbed dye was extracted by adding 1 ml of 100% methanol to each well. Next the optical density (OD) was measured for a wavelength of 570 nm, using an Expert Plus spectrophotometer (Asys Hitach, Austria).

Discussion of results

The introduction of short fibres (20 – 30 mm) into the poly-ε-caprolactone (PCL) polymer matrix in a quantity of 2% by weight to the mass of the polymer matrix leads to a reduction in the elongation strength of the hybrid composite materials. However, the purpose of introducing alginate fibres in this quantity was not to improve the mechanical properties, but merely to obtain a composite with a bioactive component (nanocomposite alginate fibres) – our preliminary tests showed that a content of fibres in the matrix in a quantity of 2% by weight is below the critical value given by the law of mixtures. As the elongation strength falls, the deformability of the composite materials is observed to decrease; they have much lower values than the reference material, in this case the PCL sheet. The opposite is observed with Young's modulus. The value of this parameter in the case of polymer-fibre composites is higher than for the PCL sheet; the highest value of the indicator is found for composites containing calcium alginate fibres without a nanoadditive or containing the nanoadditive HAp. Analysis of mechanical properties showed that the introduction of fibres into the polymer matrix causes a reduction in those properties. Nonetheless these composites still meet the basic requirements for materials intended for use in the regeneration of bone tissue.

Table 1. *Mechanical properties and surface topography of the PCL composites.*

Symbol of sample	Strength tensile, MPa	Deformability, %	Module Young, MPa	Contact angle theta, °	Surface topography, μm		
					R_a	R_t	R_z
PCL	18.85	555.04	198.39	75.7 ± 2.54	1.72	18.86	12.51
PCL+Alg Ca	15.69	72.28	389.40	77.0 ± 1.66	1.27	15.78	9.32
PCL+Alg CaHAp	10.24	20.42	306.96	73.7 ± 2.14	2.59	25.40	15.99
PCL+Alg CaBG	11.13	31.13	254.03	76.8 ± 1.42	1.17	13.08	9.02

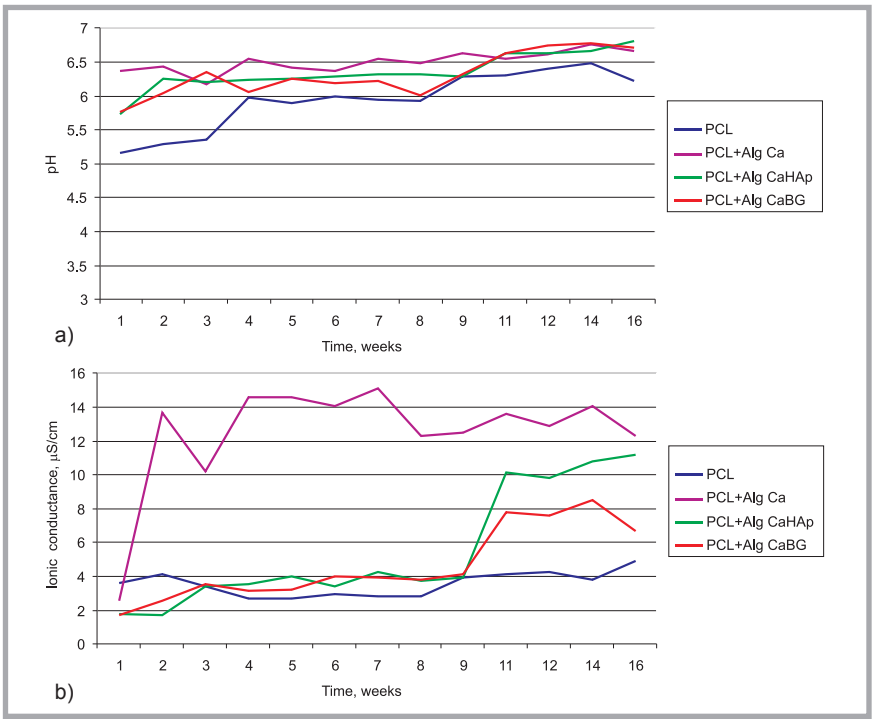

Figure 1. *Changes in the pH (a) and ionic conductanve (b) of the immersion medium (water) during degradation testing (37 °C for 4 months) for PCL composites.*

Among the most important tests serving to give a preliminary determination of the body's reaction to the material analysed are physicochemical tests. The introduction of a fibrous phase into the polymer matrix causes only slight changes in the hydrophilicity of the surface of the composites, which is shown by the similar values of the contact angle θ, which takes values in the range 73 – 77°. However, changes in the surface topography of the composites analysed (***Table 1***) show that the introduction of a fibrous phase into the polymer matrix affects the surface roughness indicators of the materials. The highest roughness profile values R_a and R_Z are recorded for composites with alginate fibres containing HAp, and the lowest for composites containing alginate fibres with bioglass as a nanoadditive. A similar relationship is found for the vertical roughness R_t: composites with a fibrous phase of alginate fibres containing nanoadditive HAp have the highest value of that parameter, compared with poly-ε-caprolactone sheet.

The stability of the polymer-fibre materials modified with ceramic nanoadditives was tested according to the recommendations of the ISO 10993-5 standard, which concerns degradation testing in an in vitro environment. These tests involved the incubation of the composites in distilled water, maintaining a 1% concentration of the extract relative to the incubated material. Degradation was carried out at a temperature of 37 °C for a period of 15 weeks. Materials with a 2% addition of a fibrous phase are stable in the given conditions of the pH of the immersion medium, and remain in the neutral range throughout the time of the experiment (***Figure 1.a***). The simultaneous observation of ions migrating to the solution – based on the change in conductance – shows that these materials are stable up to approximately the ninth month of incubation (over 2 months), after

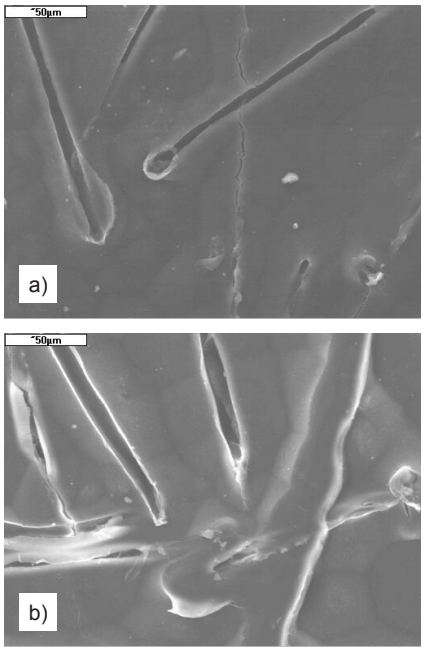

Figure 2. *Surface morphology of composites: a) PCL+Alg CaBG and b) PCL+AlgCaHAp, after 7 days' incubation in SBF.*

which time the processes of hydrolytic degradation take place, causing a jump in the ionic conductance of the water (***Figure 1.b***). The material which appears to be the least stable is the composite with alginate fibres without a nanoadditive (showing an increase in conductance after just two weeks of incubation).

The research included SEM observation of the surfaces of composites incubated in artificial blood plasma (SBF) over a period of 7 days. The microscope observations did not indicate the formation of typical apatite structures, which is probably due to the nature of the material, which undergoes faster degradation at places where fibres are exposed (***Figure 2***), and these in turn are of the nature of hydrocolloids, on which the crystallisation of apatite is difficult to achieve.

Cell testing of the polymer-fibre composites using lines of MG-63 osteoblast-like cells showed that the numbers of culture cells on the test media differed significantly between the composites depending on the type of fibrous component (***Figure 3***).

On the third day of cultivation, only on the composite made with alginate fibres containing nanohydroxyapatite (2% AH) is the number of cells higher than on the TCPS control (polystyrene culture discs). However, on the seventh day the number of cells on the composites produced is lower than on the control sample; the largest differences are found in the case of composites containing fibres without a nanoadditive and with bioglass (2% AC and 2% AB).

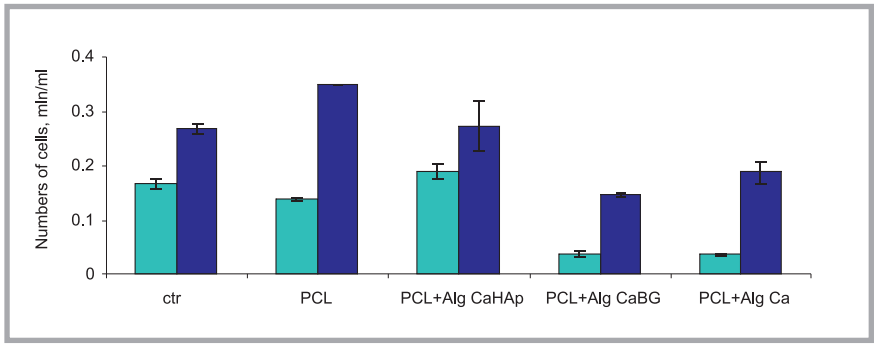

Figure 3. *Numbers of MG-63 osteoblast cells on the 3rd (■) and 7th (■) days of culture growing on a control surface of TCPS (ctr) and PCL and on the surfaces of PCL composite discs with the addition of 2% alginate fibres.*

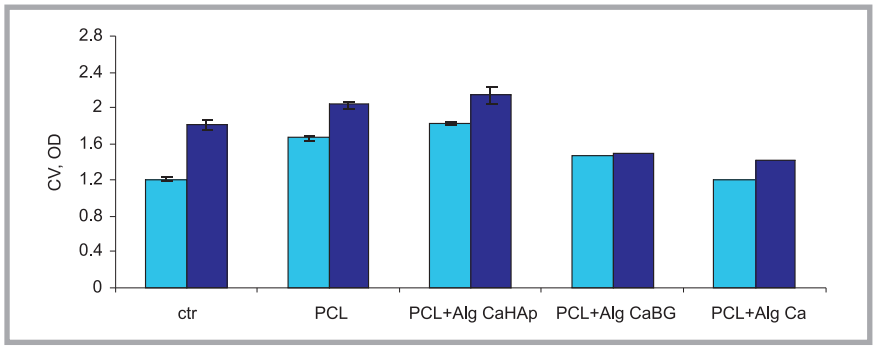

Figure 4. *Adhesion to the medium by MG-63 osteoblast cells on the 3rd (■) and 7th (■) days of cultivation on TCPS and PCL control surfaces and on surfaces of PCL discs containing alginate fibres (OD = optical density measured at wavelength 570 nm).*

Cell adhesion to the composite surface is important in view of the numerous phenomena taking place in a living organism, such as the immunological response, healing processes and the integration of tissue with the biomaterials. The contact, adhesion and spreading of cells are involved in the first phase of interaction, occurring at the point of contact between cells and the biomaterial. The proper course of these processes influences, in turn, the proliferation and differentiation of cells on the biomaterial surface. Materials designed for the regeneration of bone tissue ought to favour the adhesion and proliferation of osteoblasts. An orthopaedic or dental implant is effective if total integration is achieved between its surface and the bone tissue. The CV test used to evaluate the adhesion of cells also makes it possible to draw conclusions about their proliferation – here the test result may also be affected by the number of cells on the biomaterial surface. The results obtained from the CV test show an improvement in the cells' adhesivity on most of the composites, compared with the TCPS control sample, after three days. The lower values of this indicator obtained in the case of composites containing alginate fibres without a nanoadditive (2% AC) or with bioglass (2% AB) are primarily due to the smaller number of cells growing on their surface, as was concluded previously (***Figure 3***). On the other hand the adhesion of cells on composites made from fibres with the HAp nanoadditive is the highest out of the composite materials analysed (***Figure 4***). The lower cell adhesion values for composites containing alginate fibres without a nanoadditive and with bioglass may also be affected by the exposure of that component on the surface of the composite – alginate fibres partially gelify, thus making it harder for cells to adhere to the composite surface.

Summary

The tests carried out to obtain polymer-fibre composites using two resorbable polymers demonstrated the possibility of obtaining composites with mechanical properties that are suitable from the point of view of their applications. Young's modulus values for the composite systems analysed are higher than for unmodified

PCL sheet. Irrespective of the fibrous phase used, the PCL matrix demonstrates stability in in vitro conditions. The constant pH values and small changes in ionic conductance of the water indicate that these materials undergo gradual but slow degradation.

The most favourable of the materials analysed seem to be composites in which the modifying phase consists of fibres containing nanohydroxyapatite. These display suitable material characteristics, primarily roughness, wettability, as well as small changes in the pH of the immersion medium. These features may directly bring about the faster adhesion and proliferation of bone cells and stimulate a process of faster fixation of the implant material in a living body. In the cell testing, the system PCL-Alg CaHAp gave the most favourable results for cell adhesion among the composite materials analysed.

It is concluded that the polymer-fibre composites obtained may be used as membranes characterised by an active surface and faster degradation time of one of the phases, enabling the isolation of bone defects while at the same time allowing the material to become overgrown with tissue, in *in vivo* conditions.

Acknowledgment

This work was financed by the Ministry of Science and Higher Education in the years 2008–2010 as research project 3809/B/T02/2008/35.

References

1. *Müller F. A., Müller L., Hofmann I., Wenzel M. M., Staudenmaier R.; Cellulose-based scaffold materials for cartilage tissue engineering, Biomaterials Vol. 27, 2006, pp. 3955-3963.*
2. *Boucard N., Viton Ch., Agay D., Mari E., Roger T., Chancerelle Y., Domard A.; The use of physical hydrogels of chitosan for skin regeneration following third-degree burns, Biomaterials Vol. 28, 2007, pp. 3478-3488.*
3. *Pamuła E.; Biomateriały dla inżynierii tkankowej. Badania nad kształtowaniem struktury i właściwości biologicznych poliestrów alifatycznych, Prace monograficzne AGH Kraków, Wydział Inżynierii Materiałowej i Ceramiki, Kraków, Vol. 1, 2008.*
4. *Shikinami Y., Matsusue Y., Nakamura T.; The complete process of bioresorption and bone replacement using devices made of forged composites of raw hydroxyapatite particles/poly l-lactide (F-u-HA/PLLA), Biomaterials Vol. 26, 2005, pp. 5542-5551.*
5. *Polyak B., Geresh S., Marks R. S.; Synthesis and Characterization of a Biotin-Alginate Conjugate and Its Application in Biosensor Construction, Biomacromolecules Vol. 5, 2004, pp. 389-396.*
6. *Abu-Rabeah K., Polyak B., Ionescu R. E., Cosnier S., Marks R. S.; Synthesis and Characterization of a Pyrrole-Alginate Conjugate and Its Application in a Biosensor Construction, Biomacromolecules Vol. 6, 2005, pp. 3313-3318.*
7. *Martinsen A., Skjåk-Bræk G., Smidsrød O., Zanetti F., Paoletti S.; Comparison of different methods for determination of molecular weight and molecular weight distribution of alginates, Carbohydr. Polym. Vol. 15, 1991, pp. 171-193.*
8. *Hench L. L., Jones J. R.; Biomaterials, artificial organs and tissue engineering, Woodhead Publishing Limited, Cambridge, 2005.*
9. *Hashimoto T., SuzukiY., Tanihara M., Kakimaru Y., Suzuki K.; Development of alginate wound dressings linked with hybrid peptides derived from laminin and elastin, Biomaterials Vol. 25, 2004, pp. 1407-1414.*
10. *Mikołajczyk T., Boguń B., Rabiej S., Król P. "Zinc Alginate Fibres Containing Nanosilica" Fibres & Textiles in Eastern Europe Vol. 18, No. 6(83), 2010, pp. 39-44.*
11. *Chung T. W., Yang J., Akaike T., Cho K. Y., Nah J. W., Kim S. II, Cho Ch. S.; Preparation of alginate/galactosylated chitosan scaffold for hepatocyte attachment, Biomaterials Vol. 23, 2002, pp. 2827-2834.*
12. *Boguń M., Mikołajczyk T.; "Sorption and Tensile Strength Properties of Selected Fibres of Cupric Alginate" Fibres&Textiles in Eastern Europe Vol. 16, No. 4(69), 2008, pp. 39-42.*
13. *Woodruff M. A., Werner-Hutmacher D.; The return of a forgotten polymer—Polycaprolactone in the 21st century, Prog Polym Sci, 2010.*
14. *http://www.osteopore.com.sg/index.htm*
15. *Klopp L. S., Simon B. J., Bush J. M., Enns R. M., Turner A. S.; Comparison of a caprolactone/lactide film (Mesofol) to two polylactide film products as a barrier to postoperative peridural adhesion in an ovine dorsal laminectomy model, Spine, Vol. 33, 2008, pp. 1518-26.*
16. *Ying W., Hua W., Xiaoying C., Siqin D.; Compressive mechanical properties and biodegradability of porous poly(caprolactone)/chitosan scaffolds. Polymer Degradation and Stability, Vol. 93, 2008, pp. 1736-1741.*
17. *Bertleff M., Meek M. F., Nicolai J. P. A.; A prospective clinical evaluation of biodegradable neurolac nerve guides for sensory nerve repair in the hand, J Hand Surg Am, Vol. 30A, 2005, pp. 513–8.*
18. *Haberko K., Haberko M., Pyda W., Pędzich Z., Chłopek J., Mozgawa W., Bućko M., Sawicki B.; Patent nr PL 202549 B1, 2009.*
19. *Boguń M., Mikołajczyk T., Rabiej S.; Effect of formation conditions on the structure and properties of nanocomposite alginate fibers, J. App. Polym. Sci. Vol. 114, 2009, pp. 70-82.*
20. *Boguń M., Rabiej S.; The influence of fiber formation an the structure and properties of nanocomposite alginate fibers containing tricalcium phosphate or montmorillonite, Polym. Compos. Vol. 31, 2010, pp. 1321-1331.*
21. *Boguń M., "Nanocomposite Calcium Alginate Fibres containing SiO_2 and Bioglass" Fibres & Textiles in Eastern Europe Vol. 18, No. 4(81), 2010, pp. 11-19.*

Received 04.10.2010 Reviewed 14.09.2011