ENGINEERING OF BIOMATERIALS

INŻYNIERIA BIOMATERIAŁÓW

JOURNAL OF POLISH SOCIETY FOR BIOMATERIALS AND FACULTY OF MATERIALS SCIENCE AND CERAMICS AGH-UST
CZASOPISMO POLSKIEGO STOWARZYSZENIA BIOMATERIAŁÓW I WYDZIAŁU INŻYNIERII MATERIAŁOWEJ I CERAMIKI AGH

Number 146
Numer 146
Volume XXI
Rok XXI

JULY 2018
LIPIEC 2018

ISSN 1429-7248

PUBLISHER:
WYDAWCA:

Polish Society for Biomaterials in Krakow
Polskie Stowarzyszenie Biomateriałów w Krakowie

EDITORIAL COMMITTEE:
KOMITET REDAKCYJNY:

Editor-in-Chief
Redaktor naczelny
Jan Chłopek

Editor
Redaktor
Elżbieta Pamuła

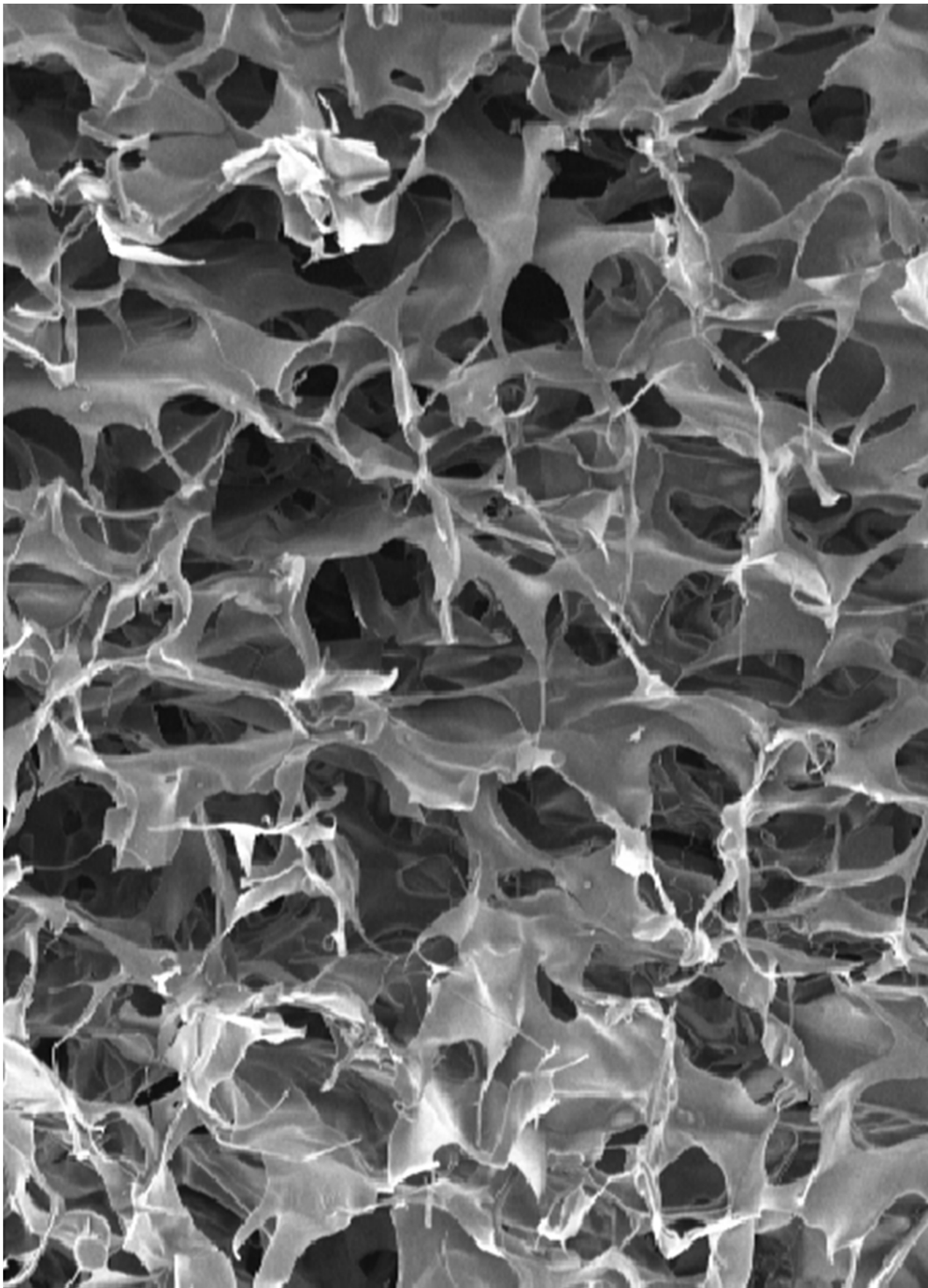
Secretary of editorial
Sekretarz redakcji
Design
Projekt
Katarzyna Trała

ADDRESS OF EDITORIAL OFFICE:
ADRES REDAKCJI:

AGH-UST
30/A3, Mickiewicz Av.
30-059 Krakow, Poland
Akademia Górniczo-Hutnicza
al. Mickiewicza 30/A-3
30-059 Kraków

Issue: 250 copies
Nakład: 250 egz.

Scientific Publishing House AKAPIT
Wydawnictwo Naukowe AKAPIT
e-mail: wn@akapit.krakow.pl



ENGINEERING OF BIOMATERIALS

EDITORIAL BOARD
KOMITET REDAKCYJNY

EDITOR-IN-CHIEF
Jan Chłopek - AGH University of Science and Technology, Krakow, Poland

EDITOR
Elżbieta Pamuła - AGH University of Science and Technology, Krakow, Poland

INTERNATIONAL EDITORIAL BOARD
MIĘDZYNARODOWY KOMITET REDAKCYJNY

Iulian Antoniac - University Politehnica of Bucharest, Romania
Lucie Bacakova - Academy of Science of the Czech Republic, Prague, Czech Republic
Romuald Będziński - University of Zielona Góra, Poland
Marta Błażewicz - AGH University of Science and Technology, Krakow, Poland
Stanisław Błażewicz - AGH University of Science and Technology, Krakow, Poland
Maria Borczuch-Łączka - AGH University of Science and Technology, Krakow, Poland
Wojciech Chrzanowski - University of Sydney, Australia
Jan Ryszard Dąbrowski - Białystok Technical University, Poland
Timothy Douglas - Lancaster University, United Kingdom
Christine Dupont-Gillain - Université Catholique de Louvain, Belgium
Matthias Epple - University of Duisburg-Essen, Germany
Robert Hurt - Brown University, Providence, USA
James Kirkpatrick - Johannes Gutenberg University, Mainz, Germany
Ireneusz Kotela - Central Clinical Hospital of the Ministry of the Interior and Administr. in Warsaw, Poland
Małgorzata Lewandowska-Szumieł - Medical University of Warsaw, Poland
Jan Marciniak - Silesian University of Technology, Zabrze, Poland
Ion N. Mihailescu - National Institute for Laser, Plasma and Radiation Physics, Bucharest, Romania
Sergey Mikhalovsky - University of Brighton, United Kingdom
Stanisław Mitura - Technical University of Liberec, Czech Republic
Piotr Niedzielski - Technical University of Lodz, Poland
Abhay Pandit - National University of Ireland, Galway, Ireland
Stanisław Pielka - Wrocław Medical University, Poland
Vehid Salih - UCL Eastman Dental Institute, London, United Kingdom
Jacek Składzień - Jagiellonian University, Collegium Medicum, Krakow, Poland
Andrei V. Stanishevsky - University of Alabama at Birmingham, USA
Anna Ślósarczyk - AGH University of Science and Technology, Krakow, Poland
Tadeusz Trzaska - University School of Physical Education, Poznań, Poland
Dimitris Tsipas - Aristotle University of Thessaloniki, Greece

ENGINEERING OF BIOMATERIALS

Wskazówki dla autorów

1. Prace do opublikowania w kwartalniku „Engineering of Biomaterials / Inżynieria Biomateriałów" przyjmowane będą wyłącznie w języku angielskim. Możliwe jest również dołączenie dodatkowo polskiej wersji językowej.
2. Wszystkie nadsyłane artykuły są recenzowane.
3. Materiały do druku prosimy przysyłać na adres e-mail: kabe@agh.edu.pl.
4. Struktura artykułu:
• TYTUŁ • Autorzy i instytucje • Streszczenie (200-250 słów) • Słowa kluczowe (4-6) • Wprowadzenie • Materiały i metody • Wyniki i dyskusja • Wnioski • Podziękowania • Piśmiennictwo
5. Autorzy przesyłają pełną wersję artykułu, łącznie z ilustracjami, tabelami, podpisami i literaturą w jednym pliku. Artykuł w tej formie przesyłany jest do recenzentów. Dodatkowo autorzy proszeni są o przesłanie materiałów ilustracyjnych (rysunki, schematy, fotografie, wykresy) w oddzielnych plikach (format np. .jpg, .gif., .tiff, .bmp). Rozdzielczość rysunków min. 300 dpi. Wszystkie rysunki i wykresy powinny być czarno-białe lub w odcieniach szarości i ponumerowane cyframi arabskimi. W tekście należy umieścić odnośniki do rysunków i tabel. W przypadku artykułów dwujęzycznych w tabelach i na wykresach należy umieścić opisy polskie i angielskie.
6. Na końcu artykułu należy podać wykaz piśmiennictwa w kolejności cytowania w tekście i kolejno ponumerowany.
7. Redakcja zastrzega sobie prawo wprowadzenia do opracowań autorskich zmian terminologicznych, poprawek redakcyjnych, stylistycznych, w celu dostosowania artykułu do norm przyjętych w naszym czasopiśmie. Zmiany i uzupełnienia merytoryczne będą dokonywane w uzgodnieniu z autorem.
8. Opinia lub uwagi recenzentów będą przekazywane Autorowi do ustosunkowania się. Nie dostarczenie poprawionego artykułu w terminie oznacza rezygnację Autora z publikacji pracy w naszym czasopiśmie.
9. Za publikację artykułów redakcja nie płaci honorarium autorskiego.
10. Adres redakcji:

Czasopismo
„Engineering of Biomaterials / Inżynieria Biomateriałów"
Akademia Górniczo-Hutnicza im. St. Staszica
Wydział Inżynierii Materiałowej i Ceramiki
al. Mickiewicza 30/A-3, 30-059 Kraków
tel. (48) 12 617 25 03, 12 617 25 61
tel./fax: (48) 12 617 45 41
e-mail: chlopek@agh.edu.pl, kabe@agh.edu.pl

Szczegółowe informacje dotyczące przygotowania manuskryptu oraz procedury recenzowania dostępne są na stronie internetowej czasopisma:
www.biomat.krakow.pl

Warunki prenumeraty

Zamówienie na prenumeratę prosimy przesyłać na adres:
mgr inż. Augustyn Powroźnik
apowroz@agh.edu.pl, tel/fax: (48) 12 617 45 41
Cena pojedynczego numeru wynosi 20 PLN
Konto: Polskie Stowarzyszenie Biomateriałów
30-059 Kraków, al. Mickiewicza 30/A-3
ING Bank Śląski S.A. O/Kraków
nr rachunku 63 1050 1445 1000 0012 0085 6001

Prenumerata obejmuje 4 numery regularne i nie obejmuje numeru specjalnego (materiały konferencyjne).

Instructions for authors

1. Papers for publication in quarterly journal „Engineering of Biomaterials / Inżynieria Biomateriałów" should be written in English.
2. All articles are reviewed.
3. Manuscripts should be submitted to editorial office by e-mail to kabe@agh.edu.pl.
4. A manuscript should be organized in the following order:
• TITLE • Authors and affiliations • Abstract (200-250 words) • Keywords (4-6) • Introduction • Materials and Methods • Results and Discussions • Conclusions • Acknowledgements • References
5. All illustrations, figures, tables, graphs etc. preferably in black and white or grey scale should be additionally sent as separate electronic files (format .jpg, .gif., .tiff, .bmp). High-resolution figures are required for publication, at least 300 dpi. All figures must be numbered in the order in which they appear in the paper and captioned below. They should be referenced in the text. The captions of all figures should be submitted on a separate sheet.
6. References should be listed at the end of the article. Number the references consecutively in the order in which they are first mentioned in the text.
7. The Editors reserve the right to improve manuscripts on grammar and style and to modify the manuscripts to fit in with the style of the journal. If extensive alterations are required, the manuscript will be returned to the authors for revision.
8. Opinion or notes of reviewers will be transferred to the author. If the corrected article will not be supplied on time, it means that the author has resigned from publication of work in our journal.
9. Editorial does not pay author honorarium for publication of article.
10. Address of editorial office:

Journal
„Engineering of Biomaterials / Inżynieria Biomateriałów"
AGH University of Science and Technology
Faculty of Materials Science and Ceramics
30/A-3, Mickiewicz Av., 30-059 Krakow, Poland
tel. (48) 12) 617 25 03, 12 617 25 61
tel./fax: (48) 12 617 45 41
e-mail: chlopek@agh.edu.pl, kabe@agh.edu.pl

Detailed information concerning manuscript preparation and review process are available at the journal's website:
www.biomat.krakow.pl

Subscription terms

Contact:
MSc Augustyn Powroźnik,
e-mail: apowroz@agh.edu.pl
Subscription rates:
Cost of one number: 20 PLN
Payment should be made to:
Polish Society for Biomaterials
30/A3, Mickiewicz Av.
30-059 Krakow, Poland
ING Bank Slaski S.A.
account no. 63 1050 1445 1000 0012 0085 6001

Subscription includes 4 issues and does not include special issue (conference materials).

ISBPPB 2018



4th International Conference on

Biomedical Polymers & Polymeric Biomaterials

15–18 July 2018, Kraków, POLAND

Organizers:

Polish Society for Biomaterials

Faculty of Materials Science and Ceramics, AGH-UST

International Society for Biomedical Polymers and Polymeric Biomaterials

www.isbppb2018.org

27th Biomaterials in Medicine and Veterinary Medicine Annual Conference

11 – 14 October 2018 Rytro, Poland

SAVE THE DATE

11-14 OCTOBER 2018

www.biomat.agh.edu.pl

REGISTER AND SUBMIT AN ABSTRACT



SPIS TREŚCI
CONTENTS

Wersja papierowa czasopisma „Engineering of Biomaterials / Inżynieria Biomateriałów" jest jego wersją pierwotną
Printed version of „Engineering of Biomaterials / Inżynieria Biomateriałów" is a primary version of the journal

FABRICATION AND PHYSICO-CHEMICAL PROPERTIES OF PECTIN/CHITOSAN SCAFFOLDS

Viktoryia Kulikouskaya*, Maryna Lazouskaya, Vladimir Agabekov

Institute of Chemistry of New Materials
of National Academy of Sciences of Belarus,
36 F. Skaryna str., Minsk, Belarus, 220141
*e-mail: kulikouskaya@gmail.com

Abstract

Scaffolds from chitosan and its combinations with other polymers are widely used for tissue engineering application. This is due to the favourable biological properties of chitosan such as antimicrobial activity, biocompatibility, biodegradability, cell adhesion and proliferation, etc. The aim of the study was the creation of 3D porous scaffolds based on pectin/chitosan polyelectrolyte complexes and study the influence of components ratio on their physico-chemical properties, as well as degradation behaviour in solutions modelling the media of the human body. Porous "sponge-like" polysaccharide films were produced using freeze-drying technique from gel-like pectin-chitosan polyelectrolyte complexes. The weight ratio of chitosan:pectin in complexes was varied in the range from 1:1 to 1:2. Obtained samples were characterized by infrared spectroscopy and scanning electron microscopy. It has been shown that pectin-chitosan films turned out to have sponge-like structure with highly interconnected pores with the size about 50-300 μm. It has been determined that all samples regardless of the chitosan:pectin ratio possess high swelling properties. The degradation profile of scaffolds in different media was studied. It has been determined that the largest weight loss is observed in water and reaches more than 80% after 1 day, while in NaCl and PBS solutions weight loss is approximately 50-60% after 25 days. For samples with different chitosan:pectin weight ratio, weight loss slightly rise with increasing amount of pectin. It has been shown that mesenchymal stem cells adhered to the surface of obtained pectin:chitosan porous scaffolds in viable state. Hence, it can be served as a potential material for tissue engineering applications.

Keywords: *chitosan, pectin, 3D scaffold, degradation*

[Engineering of Biomaterials 146 (2018) 2-7]

Introduction

The preparation of polyelectrolyte complexes (PECs) is an actual trend in modern material science due to their possible application in pharmaceutics, tissue engineering, food industry, for water purification, etc. [1-6]. Polyelectrolyte complexes possess unique properties, which are significantly different from those of the initial components. Polyelectrolyte complexes are products of intermolecular reactions of complementary polyelectrolytes and, depending on the preparation conditions, can be obtained in the form of nano- and microparticles, gels, films, membranes, porous structures and fibrous materials [2-7].

Currently, tissue engineering is an actively developing direction and important therapeutic strategy for present and future medicine. According to this strategy, tissue engineering scaffolds are used for the formation of new viable tissue for a medical purpose. Tissue engineering scaffolds have to include cells, scaffolds and growth factors. Hence, one of the main directions in tissue engineering is designing a suitable scaffold. The scaffold should have the following properties: biocompatibility, biodegradability, non-toxicity, non-immunogenicity, desired mechanical strength. The morphology of scaffolds should be suitable for cell attachment and allow providing the possibility of transporting nutrients, signal molecules, metabolites. For this reason, highly porous films are very attractive materials for use as scaffolds. It should be noted that the rate of scaffolds biodegradability has to be corresponding to the one of new tissue formation. There are a lot of biodegradable polymers that can be used for scaffolds creation, but one of the promising types among them are biopolymers (polysaccharides, proteins, etc.) due to their biological and chemical similarities to natural tissues.

In recent years, scaffolds based on polyelectrolyte complexes of chitosan (CS) with various anionic natural polyelectrolytes, such as alginates, carrageenans, gelatin, have attracted increasing attention for tissue engineering application due to their porous structure and beneficial biological properties [4,8-10]. Chitosan is a linear polycationic polysaccharide composed of randomly distributed β-(1→4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It possesses such favorable biological properties as antimicrobial activity, biocompatibility, biodegradability, non-toxicity, cell adhesion and proliferation, as well as mucoadhesive behaviour. Combination of chitosan with other polymers allows to achieve improved physicochemical and permeability properties of material in use. For example, PECs on the basis of chitosan and pectin (Pect) have properties that overcome the limitations of individual polymers (stability, mechanical strength, sustained release of the entrapped substance, controlled degradation rate, etc.) [11,12]. Fabrication of highly porous scaffolds based on pectin/chitosan PECs are also gaining more attention due to anti-inflammatory activity of pectin, as well as simplicity of PECs formation from these two oppositely-charged polysaccharides. Pectin is a branched anionic heteropolysaccharide, which is rich in galacturonic acid. The residues of α-(1-4)-D-galacturonic acid partially etherified with methanol form the main chain of pectin.

The stability in solutions and the degradation rate of PECs strongly depends on pH, temperature, charge density and ionic strength, as well as other conditions [13]. Degradation of scaffolds is the key parameter for their application in tissue engineering, because scaffolds should degrade as new tissue formation takes place [14]. Therefore, it is important to establish the factors by which the biodegradation rate of scaffolds can be controlled. The key parameter for film based on PEC may be the ratio of components in it, especially if one of the component is water soluble. Thus, the aim of this work was the creation of 3D porous scaffolds based on pectin/chitosan polyelectrolyte complexes and study the influence of components ratio on their physico-chemical properties, as well as degradation behaviour in solutions modelling the media of the human body.

Materials and Methods

Low-molecular chitosan (M_v ~ $3.4 \cdot 10^5$, the content of NH_2 group = 74.8%) and citrus low methoxylated pectin (M_v ~ $1.4 \cdot 10^5$, the content of COOH group = 19.6%) used for this study were obtained commercially from Sigma-Aldrich.

Pectin/chitosan scaffolds in the form of porous "sponge-like" films were prepared by the freeze-drying of insoluble polyelectrolyte complexes formed by these two polysaccharides in an aqueous solution. For this, 5 mg/ml solution of chitosan in 1% acetic acid was added dropwise into 5 mg/ml water solution of pectin under vigorous shaking on the vortex to obtain a solution with a whole polysaccharide concentration 5 mg/ml (1.7-2.5 mg/ml chitosan, 2.5-3.3 mg/ml pectin). Mass proportions of the two polysaccharides (CS:Pect, w/w) was in the range from 1:1 to 1:2. The resulting gel-like complexes were left for 1 h to complete the formation of PECs, maintained at -20°C for 20 h and freeze-dried (Labconco FreeZone 1.0.) for 8 h.

The structure of pectin/chitosan PECs was studied by scanning electron microscopy (SEM, Jeol JCM-6000 Plus, Japan). Films were placed on a graphite tape, attached onto a metal support, and coated with platinum on Smart Coater (Neo Coater). SEM was also used for analysis the surface of pectin/chitosan films after culturing mesenchymal stem cells on them. The scaffolds, incubated with the cells (24 hours), were rinsed with PBS and fixed with glutaraldehyde (2.5% in PBS) for 10 min. Then films were dehydrated in graded ethanol of 70, 80 and 90% for 10 min each, frozen and freeze-dried. Finally, the obtained samples were coated with platinum (Smart Coater) and their morphology was investigated by SEM.

The infrared (IR) spectra of the initial polysaccharides and PECs were recorded on a Tensor 37 FTIR spectrometer ("Bruker", Germany) in the range of 4000-400 cm^{-1}. Samples for the study were prepared in tablets with KBr. To compare the intensity of the selected characteristic peaks of pectin and pectin/chitosan PEC, baseline correction of them was carried out before data analysis. Automatic baseline correction procedure was conducted using Bruker OPUS 6.5.97 Software.

The yield of pectin/chitosan films was calculated from the masses of chitosan and pectin used initially in preparation and the mass of the formed samples:

$$\text{Yield, \%} = \frac{m_{Pect} + m_{CS}}{m} \cdot 100\%$$

where m is the mass of pectin/chitosan film, m_{Pect} and m_{CS} are the respective initial masses of pectin and chitosan used for PEC film formation.

The swelling behavior of scaffolds was investigated at 37ºC by exposing them for 1 h to solutions with different pH and ionic strength: distilled water, 0.9% NaCl (pH 5.5, ionic strength 0.17 M), Dulbecco's phosphate buffered saline (PBS, 8 g/L NaCl, 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 1.15 Na_2HPO_4 g/l, pH 7.0-7.4, ionic strength 0.15 M). The wet weight of the scaffold was determined by first blotting the scaffold surface with filter paper, to remove the excess surface water, and then weighed immediately. The percentage of water uptake (W) by the scaffold was calculated from the equation [12]:

$$W, \% = \frac{m_t + m_0}{m_0} \cdot 100\%$$

where m_0 is the mass of the initial dry sample (mg) and m_t is the weight of the hydrated sample (mg).

Degradation behavior of scaffolds was investigated by immersion them into solutions with different pH and ionic strength (distilled water, 0.9% NaCl, PBS) with weight loss determination during 25 days. For this, samples of porous pectin/chitosan films were dipped into 10 ml of a model solution and incubated at 37°C (LOIP LB-140 water bath, Russia) for 25 days. The samples were removed within 1, 7, 14 and 25 days, carefully washed with distilled water to remove low molecular weight electrolytes, frozen, freeze-dried for 8 h and finally weighed. For each kind of scaffolds, ten samples were tested. Data were plotted as mean ± SD. Stability of scaffolds was estimated as weight loss (WL, %):

$$WL, \% = \frac{m_0 - m_1}{m_0} \cdot 100\%$$

where m_0 is the mass of initial dry sample (mg) and m_1 is the mass of sample after aging in the test medium and dried at time t (mg).

Rat mesenchymal stem cells (3rd passage) obtained from adipose tissue were used to assess the possibility of application the obtained porous pectin/chitosan films as scaffolds. The viability of cells taken off from the surface of scaffolds was evaluated using 0.4% trypan blue solution.

Results and Discussions

Chitosan (polycation) and pectin (polyanion) are electrostatically complementary to each other and form PECs by mixing their solutions due to electrostatic interactions between their ionized amino (NH_3^+) and carboxy groups (COO^-), respectively. The resulting PEC can be also stabilized by hydrophobic and van der Waals interactions, as well as hydrogen bonds between the different groups in polymer-polymer complexes. Chitosan and pectin are weak polybase and polyacid respectively, so the optimal pH range in the reaction mixture for the formation of PECs is between the values of their pKa when the macromolecules are charged. For pectin the value of pKa is ~ 2.9 [15], therefore, at a lower pH its carboxyl groups are unionized and the charge density of macromolecules is insufficient to form a complex. The amino groups of chitosan will be ionized at pH <6.5 (pKa = 6.5 [16]) and are able to interact electrostatically with COO- groups of pectin. Therefore, in this work the formation of complexes was carried out in acetic acid medium at pH ~ 4-5.

The formation of pectin/chitosan PECs was confirmed by IR spectroscopy. The IR spectra of pectin (FIG. 1) contain characteristic bands: the band identified at 1750 cm^{-1} corresponds to the stretching vibrations of the carbonyl group (vC=O) of the methyl ester ($COOCH_3$) or in the undissociated carboxyl group, and the one at 1627 cm^{-1} deals with the asymmetric stretching vibrations of the dissociated carboxyl (COO-) group [17-19]. In the spectrum of chitosan, there are overlapping bands at 1670 cm^{-1} and 1660 cm^{-1}, corresponding to stretching vibrations of the vC=O of the amid group of the acetylated units of chitosan (Amid I band) and deformation vibrations δN-H of the primary amine (Amid II band) [17,20]. The main changes in the IR spectra of pectin/chitosan PEC are in the region between 1800 and 1400 cm^{-1} and associated with the formation of intermolecular ionic salt bonds between chitosan and pectin (FIG. 1). Thus, a decrease in the intensity of the band at 1750 cm^{-1}, associated with the vC=O vibration of the non-ionized carboxyl group, is observed. Also, a hypsochromic shift to the short-wave region and intensification of the band at 1620 cm^{-1}, assigned to the stretching vibrations of the vC=O of the dissociated carboxyl (COO-) group, were determined.

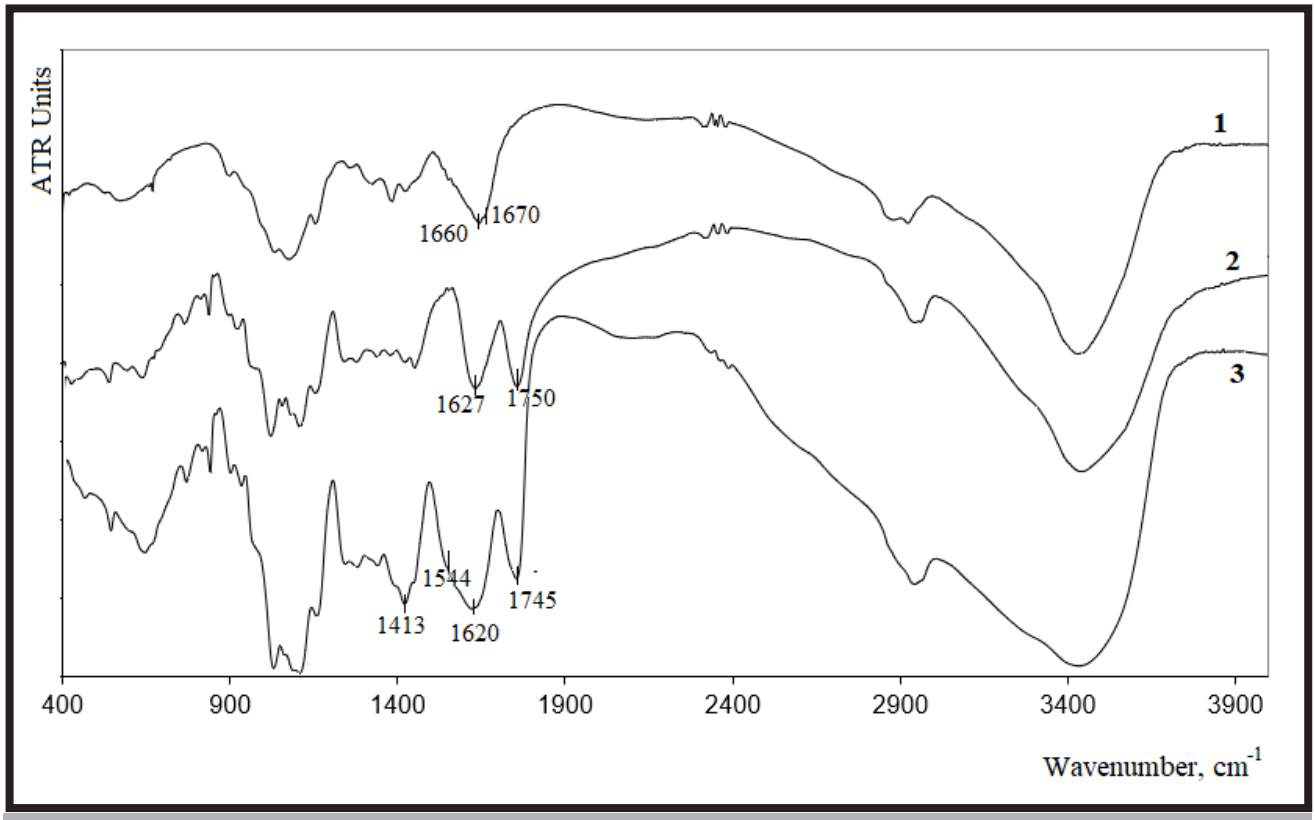


FIG. 1. IR spectra of chitosan (1), pectin (2) and polyelectrolyte complex pectin-chitosan (3).

TABLE 1. Yield of PECs and their swelling behaviour in dependence of chitosan:pectin weight ratio in scaffolds.

Weight ratio of CS:Pect in PEC	Yield, %	Water uptake, %		
		NaCl	H_2O	PBS
1:1	108.2 ± 5.4	3000 ± 450	2900 ± 300	2200 ± 400
1:1.5	117.6 ± 4.3	3530 ± 290	2240 ± 100	2470 ± 220
1:2	119.3 ± 3.6	3230 ± 230	2310 ± 210	2250 ± 300

In the IR spectrum of PEC also appears a band at 1413 cm^{-1}, corresponded to the symmetric stretching vibrations vC=O of the dissociated carboxyl (COO-) group [19], and a shoulder at 1544 cm^{-1}, which is responsible for the symmetric bending vibrations of the protonated NH_3^+ group [20].

In addition, IR spectra of pectin/chitosan PEC in comparison with the spectrum of individual polysaccharides shows an increase in the intensity of the absorption band in the region between 3500 and 2900 cm^{-1}, which is assigned to the superposition of stretching vibrations of NH groups of chitosan and free (3550-3500 cm^{-1}) hydroxyl groups. It should be noted that a wide intense band at the 3500-3200 cm^{-1} region also indicates the formation of intra- and intermolecular hydrogen bonds. The bands in the region of 950-1150 cm^{-1}, related to the pulsation vibrations of the pyranose ring skeleton, remain unchanged [20].

The yield of PEC films based on chitosan and pectin is more than 100% (TABLE 1). It increases with the raise of pectin content in the complexes. This can be caused by the retention of small amounts of water by the sample due to the hydrophilic nature of pectin. The amount of retained water, indeed, raises with increasing weight fraction of pectin in PEC.

Highly porous structure is favorable for tissue engineering as interconnected porous and cavity structure have to ensure a biological environment conducive to cell attachment and proliferation as well as tissue growth. Based on the observations of SEM micrographs, obtained pectin/chitosan polyelectrolyte complexes turned out to have sponge-like structure regardless of the polysaccharides ratio in the complex (FIG. 2). The scaffolds present an irregular, highly porous structural form (from sheet-like to fibrous-like structures). Moreover, samples possess highly interconnected pores with the size about 50-300 μm (FIG. 2). Increases in pectin content in the films leads to smaller pores formation (FIG. 2). The porous structure of obtained films is due to the formation of ice crystals during freezing process. Their removing by lyophilization causes pores formation. Apparently, an increase in the content of pectin during the PECs preparing leads to the formation of a denser network of intermolecular bonds between polysaccharide macromolecules resulting in smaller voids which are filled with the dispersion medium (water).

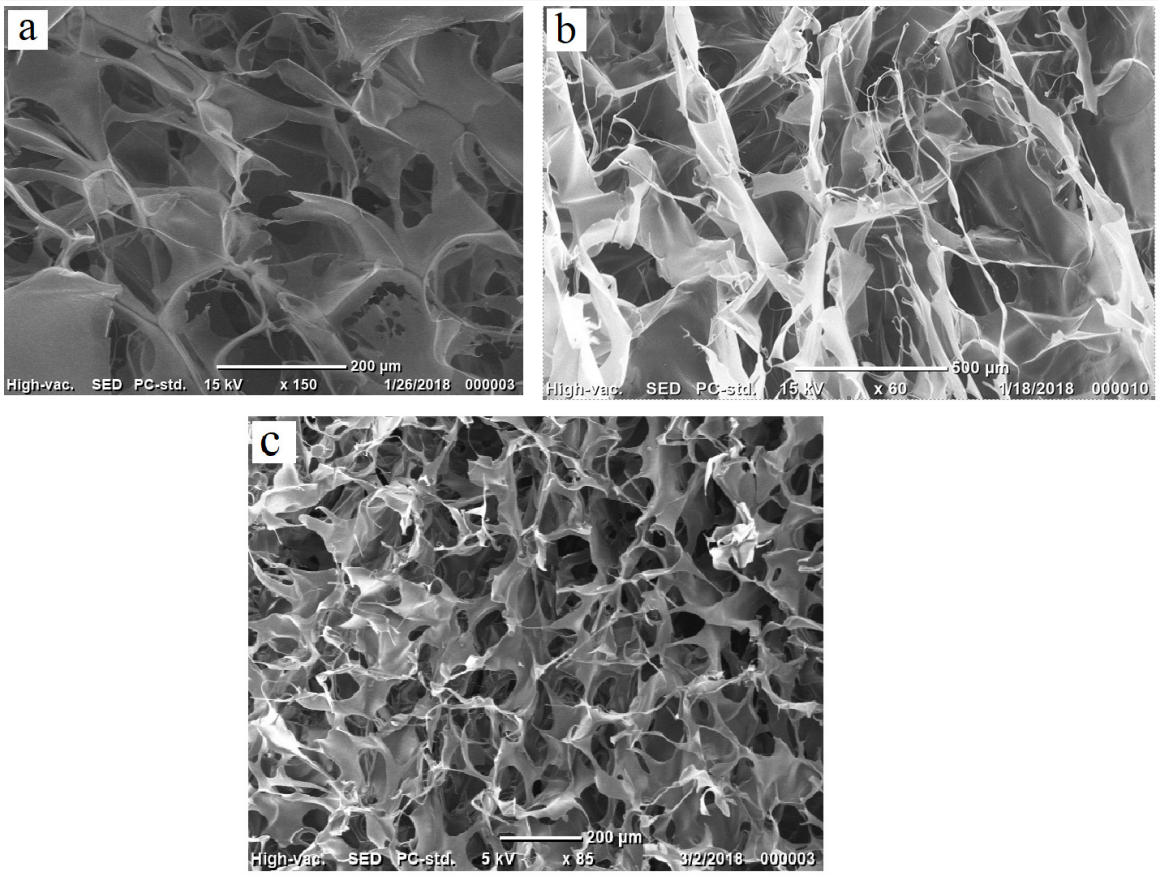


FIG. 2. SEM image of pectin/chitosan PECs. The weight ratio of CS:Pect are 1:1 (a), 1:1.5 (b) and 1:2 (c).

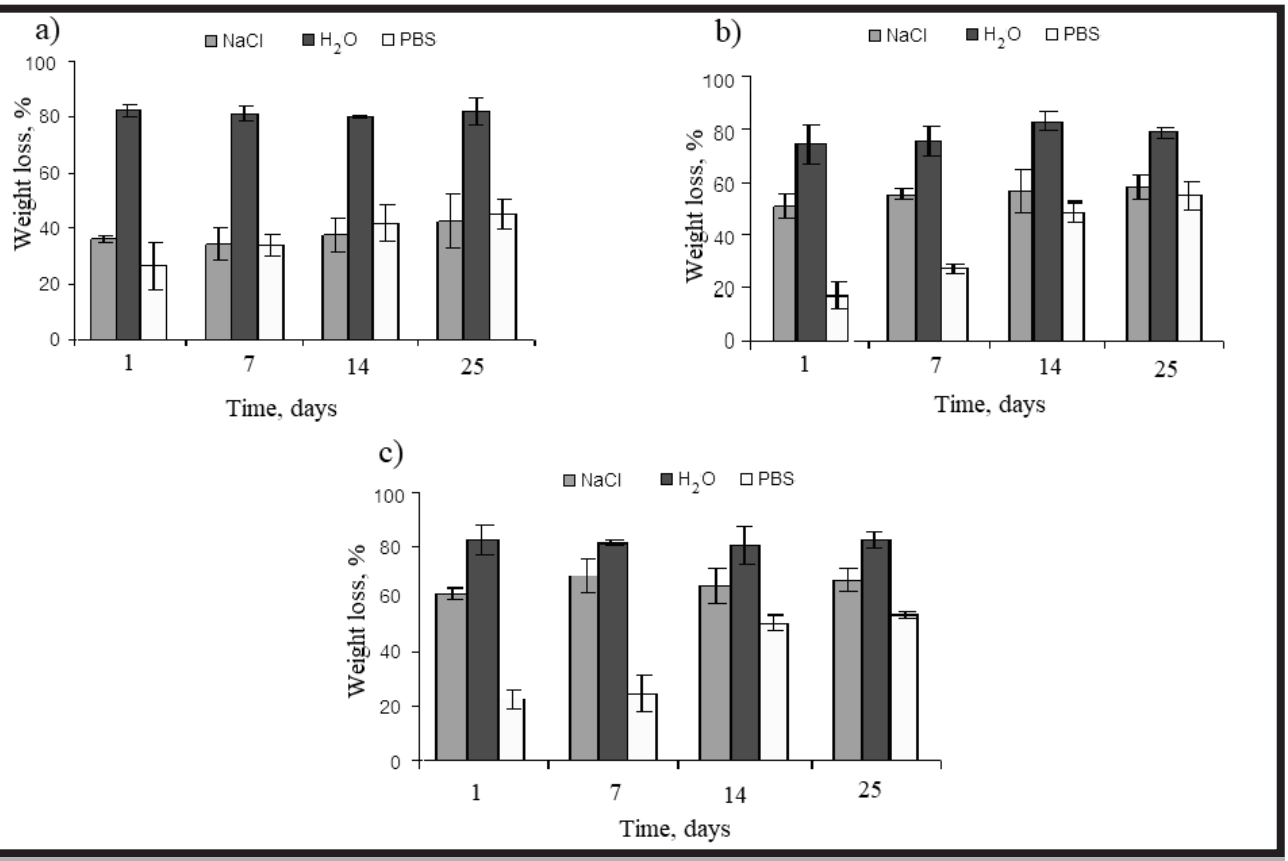


FIG. 3. Weight loss behaviour of the obtained scaffolds after submersion in NaCl, PBS and distilled water. The weight ratio of CS:Pect was 1:1 (a), 1:1.5 (b) and 1:2 (c).

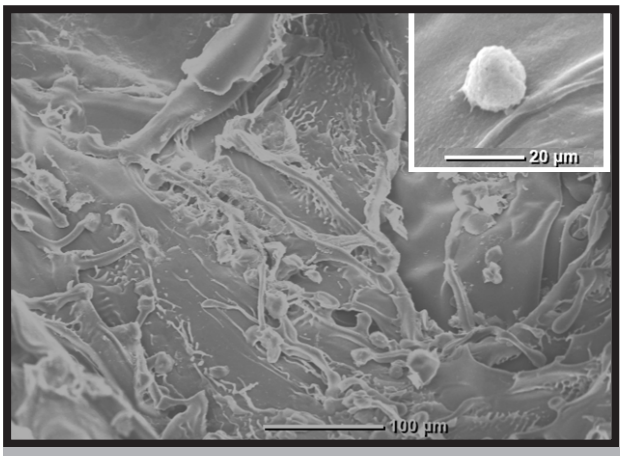


FIG. 4. SEM image of pectin/chitosan PEC with mesenchymal stem cells. The weight ratio of CS:Pect was 1:1.

Swelling behaviour and structural stability of scaffolds are critical for their practical using in tissue engineering. It is well known that natural polymers swell readily in biological fluids. Pectin/chitosan complexes are stabilized due to ionic interactions between positively charged chitosan and negatively charged pectin and exhibited ionic strength and pH-sensitive swelling (TABLE 1). So, the swelling behaviour of the scaffolds differed distinctly in used solutions (TABLE 1). The maximum swelling of films appeared for physiological solution (0.9% NaCl): water uptake is 3000-3530%. Decrease in solution ionic strength while preserving the pH value results in slightly reduction in water uptake (up to 2240-2900%). As swelling medium pH changes from 5.5 (NaCl) to 7.4 (PBS), the water uptake decreases in approximately 1.4 times (TABLE 1). From the data obtained by swelling tests for different scaffolds, it can be concluded that the addition of pectin does not influence on scaffolds' swellable and all samples characterized by high swelling behaviour.

The degradation profiles of the obtained pectin/chitosan scaffolds in different media are presented in FIG. 3. For all samples, the largest weight loss is observed in distilled water, reaches more than 80% after 1 day, and does not change after 25 days regardless of the type of used films. Obtained scaffolds are less degradable in 0.9% NaCl. For films with weight ratio of CS:Pect = 1:1, weight loss in NaCl solution was about 40% after 1 day and does not vary significantly after 25 days. Increase in pectin content in the PEC leads to decrease in sample stability in NaCl solution (FIG. 3). For example, weight loss of scaffold with weight ratio of CS:Pect = 1:2 reaches approximately 60% within 1 day storage in physiological solution. Another situation was observed for PBS: in this media the scaffolds decreases gradually with time. In PBS scaffolds showed reduction in weight 17-26% and 45-55% after 1 and 25 days, respectively (FIG. 3). Weight loss occurs mainly due to the break of ionic interactions, established between the chains of two polymers. The observed slowest destruction of scaffolds in PBS may be due to the interaction of chitosan chains with presented in the buffer hydrogen phosphate anions acting as additional physical crosslinkers that can lead to strengthening of the polymer matrix. So, it is known [21], that the addition of hydrogen phosphate anions to chitosan resulted in the gel formation at 37°C. The author [22] also shown, that chitosan fibers and films treatment with solutions containing phosphate ions resulted in samples' mechanical properties enhance. The obtained experimental data on the degradation of pectin/chitosan scaffolds are in good agreement with the literature: the authors [13] also showed that percentage of weight loss in the pectin/chitosan polyelectrolyte complex scaffolds with higher pectin content was slightly superior compared to the one with less it amounts. They suggested that the weight loss is mainly due to the loss of pectin chains rather than to an equal loss of both polysaccharides and with time the scaffolds become rich in chitosan [13].

The possibility of application the obtained porous pectin/chitosan complex films as scaffolds in tissue engineering constructions for cell culturing was investigated using mesenchymal stem cells as model culture. It has been shown that mesenchymal stem cells effectively adhered on the surface of films after 1 day of incubation. The data of scanning electron microscopy after 1 days of stem cells seeding on the surface of pectin/chitosan films indicated the presence both individual and clusters or agglomerates of cells (FIG. 4). It has also been determined that adhered stem cells are in predominantly viable state: the viability of cells was >90%. According to the obtained data, the adhesion of mesenchymal stem cells to the surface of pectin/chitosan porous films doesn't depend on the content of pectin and chitosan in the scaffold.

Conclusions

Highly porous "sponge-like" scaffolds were obtained based on pectin/chitosan interpolyelectrolyte complexes using freeze-drying technique. Formed pectin/chitosan films turned out to have sponge-like structure with highly interconnected pores with the size about 50-300 µm and theirs morphology depends on the weight ratio of CS:Pect in complexes. Increase in pectin content leads to the formation of a denser network of intermolecular bonds and results in smaller pores size. All obtained samples regardless of the chitosan:pectin ratio possess high swelling behaviour. The investigation of degradation profiles of pectin/chitosan scaffolds showed that the largest weight loss is observed in water and reaches more than 80% after 1 day, while in NaCl and PBS solutions weight loss is around 50-60% after 25 days. For samples with different CS:Pect weight ratio, weight loss slightly increase with improving amount of pectin. It has been shown that mesenchymal stem cells adhered to the surface of obtained pectin-chitosan porous scaffolds in viable state. Hence, it has been demonstrated that obtained pectin-chitosan scaffold have structure and possess properties which are favourable for their application in tissue engineering.

Acknowledgments

This research has been financial supported from the State Program of Scientific Research "Convergence-2020" (task 3.05).

The author sincerely wishes to express their gratitude to the staff of the Laboratory of Biochemical Methods of the Belarusian State Medical University, headed by Dr. Zh.A. Ibragimova, as well as to Dr. A. Zhura for their help in carrying out experiments on cell seeding.

References

[1] Philipp B., Dautzenberg H., Linow K-J., Kötz J., Dawydoff W.: Polyelectrolyte complexes - recent developments and open problems. Prog. Polym. Sci. 14 (1989) 91-172.
[2] Il'ina A.V., Varlamov V.P.: Chitosan-Based Polyelectrolyte Complexes: A Review. Applied Biochemistry and Microbiology 41 (2005) 9-12.
[3] Recillas M, Silva L.L., Peniche C., Goycoolea F.M., Rinaudo M., Román J.S., Argüelles-Monal W.M.: Thermo- and pH-responsive polyelectrolyte complex membranes from chitosan-g-N-isopropylacrylamide and pectin. Carbohydrate Polymers 86 (2011) 1336-1343.
[4] Inamdar N., Mourya V. K.: Chapter 15. Chitosan and anionic polymers – complex formation and application. Polysaccharides: development, properties and application (Edited by Tiwari A. New Delhi: Nova Science Publishers Inc.) (2010) 333-377.
[5] Krayukhina M.A., Samoilova N.A., Yamskov I.A.: Polyelectrolyte complexes of chitosan: formation, properties and applications. Russian Chemical Reviews 77 (2008) 799-815.
[6] Schatz C., Domard A., Viton C., Pichot,C., Delair Th.: Versatile and Efficient Formation of Colloids of Biopolymer-Based Polyelectrolyte Complexes. Biomacromolecules 5 (2004) 1882-1892.
[7] Shybaila T.N., Savitskaya T.A., Kislyakova T.A., Albulov A.I., Grinshpan D.D.: Chitosan-cellulose sulfate acetate complexation in acetic acid solutions. Colloid Journal 70 (2008) 661-665.
[8] Kim H.-J., Lee H.-C., Oh J.-S., Shin B.-A., Oh Ch.-S., Park R.-D., Yang K.-S., Cho Ch.-S.: Polyelectrolyte complex composed of chitosan and sodium alginate for wound dressing application. Journal of Biomaterials Science, Polymer Edition 10 (1999) 543-556.
[9]. Hamman J.H.: Chitosan Based Polyelectrolyte Complexes as Potential Carrier Materials in Drug Delivery Systems. Marine. Drugs. 8 (2010) 1305-1322.
[10] Martino A.D., Sittinger M., Risbud M.V.: Chitosan: A versatile biopolymer for orthopaedic tissue-engineering. Biomaterials 26 (2005) 5983-5990.
[11] Morris G.A., Kok M.S., Harding S.E., Aams G.G.: Polysaccharide drug delivery systems based on pectin and chitosan. Biotechnology and Genetic Engineering Reviews 27 (2010) 257-284.
[12] Bigucci F., Luppi B., Cerchiara T., Sorrenti M., Bettinetti G., Rodriguez L., Zecchi V.: Chitosan/pectin polyelectrolyte complexes: selection of suitible preparative conditions for colon-specific delivery of vancomycin. European Journal of Pharmaceutical Sciences 35 (2008) 435-441.
[13] Coimbra P., Ferreira P., Sousa H.C., Batista P., Rodrigues M.A., Correia I.J., Gil M.H.: Preparation and chemical and biological characterization of a pectin/chitosan polyelectrolyte complex scaffold for possible bone tissue engineering applications. International Journal of Biological Macromolecules 48 (2011) 112-118.
[14] Vacanti J. P, Langer R.: Tissue engineering. Science 260 (1993) 920-926.
[15] Ralet M-C., Dronnet V., Buchholt H.C., Thibault J.F.: Enzymatically and chemically de-esterified lime pectins: characterisation, polyelectrolyte behaviour and calcium binding properties. Carbohydrate Research 336 (2001) 117-125.
[16] Birch N.P., Schiffman J.D.: Characterization of Self-Assembled Polyelectrolyte Complex Nanoparticles Formed from Chitosan and Pectin. Langmuir 30 (2014) 3441-3447.
[17] Ghaffari A., Navaee K., Oskoui M., Bayati K., Rafiee-Tehrani M.: Preparation and characterization of free mixed-film of pectin/chitosan/Eudragit® RS intended for sigmoidal drug delivery. European Journal of Pharmaceutics and Biopharmaceutics 67 (2007) 175-186.
[18] Sinitsya A., Čopíková J., Prutyanov V., Skoblya S., Machovič V.: Amidation of highly methoxylated citrus pectin with primary amines. Carbohydr. Polym. 42 (2000) 359-368.
[19] Filippov M.P.: IR spectra of pectin films. J Appl Spectrosc. 17 (1972) 1052-1054.
[20] Lawrie G., Keen I., Drew B., Chandler-Temple A., Rintoul L., Fredericks P., Grøndahl L.: Interactions between Alginate and Chitosan Biopolymers Characterized Using FTIR and XPS. Biomacromolecules 8 (2007) 2533-2541.
[21] Nair L.S., Starnes,T., Ko J.-W. K., Laurencin C.T.: Development of Injectable Thermogelling Chitosan–Inorganic Phosphate Solutions for Biomedical Applications. Biomacromolecules 8 (2007) 3779-3785.
[22] Knaul J.Z., Hudson S.M., Creber K.A.M.: Improved Mechanical Properties of Chitosan Fibers. Journal of Applied Polymer Science 72 (1999) 1721-1732.

EVALUATION OF THE DIFFERENTIATION OF STRUCTURAL AND PHYSICO-CHEMICAL PROPERTIES OF ORTHODONTIC WIRES OF AISI 304 STAINLESS STEEL

Dominika Grygier[1], Victoria Hoppe[2], Anna Zięty[3], Małgorzata Rutkowska-Gorczyca[1]

[1] Wrocław University of Science and Technology, Department of Materials Science, Strength and Welding Technology, Poland
[2] Wrocław University of Science and Technology, Scientific Association of Material Science of the Nation doc. Rudolf Haimann, Poland
[3] Wrocław University of Science and Technology, Department of Mechanics, Materials Science and Engineering, Poland
*e-mail: dominika.grygier@pwr.wroc.pl

Abstract

Wires used for orthodontic arches play a very important role in the process of orthodontic treatment. In combination with the lock attached to the tooth, they move and align the teeth along the set trajectories. Wires of stainless steel are commonly used in orthodontics for several reasons: they are characterised by high resistance to corrosion, high strength and elasticity, formability and a possibility of obtaining defined properties through cold working and annealing during production process. The purpose of the research presented in the work is the analysis of differences of the selected structural properties in the context of corrosion resistance of the orthodontic wire material. The object of the research was edge arches of the 0.016"x0.022" size made of the stainless steel type AISI 304, provided by two different producers: G&H Orthodontics and Adenta. The research methodology involved analysis of chemical and phase composition of the tested alloy, microscopic tests with application of the light and electron microscopy methods, as well as electrochemical direct current measurements.

The research presented in the work has shown significant differences in structural and physical-chemical properties of the orthodontic wires made of AISI 304 type stainless steel. Despite the fact, that the tested arches were manufactured of the theoretically the same materials, but by different producers, they significantly differ in chemical composition, metallurgical purity, phase composition and corrosion resistance. In addition, it is worth noticing that the tested materials, in terms of structure, do not meet the normative requirements obligatory for biomaterials.

Keywords: *stainless steel, orthodontics wire, microstructure*

[Engineering of Biomaterials 146 (2018) 8-13]

Introduction

Orthodontics is one of the fields of dentistry, dealing with the diagnosis, prevention and correction of malocclusion [1-3]. Orthodontic treatment is primarily aimed at correcting the appearance of dentition, facial features, but also restoring those activities that were incorrectly performed due to incorrect dentition, e.g. chewing food or correct pronunciation. Orthodontic tooth displacement results mainly from the application of force to the teeth [2]. The forces generated by orthodontic appliances are selected and activated by the orthodontist. Teeth, as well as supporting structures, react to exerted force through various biological responses and lead to tooth displacement in the supporting bone. Proper application of the principles of biomechanics leads to an increase in the efficiency of treatment through the improvement of planning and carrying out orthodontic care.

One of the popular methods of treating malocclusion is the orthodontic fixed braces. The main elements of the apparatus are wires, called arches and fastening elements [3,4]. Fixing elements include rings, ligatures and various types of locks, fixed directly to the tooth surface. However, the most important element of the apparatus is the arch. The role of the orthodontic arch in the treatment process is both as a spring and as a conductor. The force that is required in the process of deflecting the wire to the slit of the lock, causes a certain amount of work, called the activation energy that causes the tooth to move [3,5]. Wires used for orthodontic arches have many applications in the orthodontic treatment process. In conjunction with the lock attached to the tooth, they are to move and align the dentition along the set trajectories. Wires with larger cross-sections serve as retainers, which are supposed to prevent the return of teeth to the original arrangement in the arch [4]. Materials commonly used for orthodontic wires are austenitic stainless steel of the AISI 302 and AISI 304 grades, nickel-titanium alloys called Nitinol, beta-titanium and cobalt chrome alloys [5-7]. Each of these types of orthodontic wires, due to its specific, desirable properties, is used in various stages of orthodontic treatment. Stainless steel wires are widely used in orthodontics for several reasons: they are characterized by high corrosion resistance, high strength and elasticity, formability and the ability to achieve specific properties by cold working and annealing during the production process, as well as low manufacturing costs. These materials also meet the condition of biocompatibility in tissues and fluids of the stomatognathic system, have the required high corrosion resistance, permanent aesthetic features and specific organoleptic properties [5,8-10]. AISI 302 stainless steel is austenitic steel, containing from 17% to 19% chromium, 8% to 10% nickel and about 0.15% carbon, is non-magnetic, extremely durable and plastic. It is one of the most popular chromium-nickel and stainless steels. Cold working dramatically increases its hardness, and the range of applications ranges from stamping, broaching and forming wire to moulding in various types of washers, springs and plates. AISI 304 stainless steel is a non-magnetic alloy containing from 18% to 20% chromium, from 8% to 12% nickel and a maximum of 0.08% carbon content. The steel of this grade has less carbon, which minimizes the precipitation of carbide precipitates. It is widely used in the production of equipment in the mining, chemical, cryogenic, food, dairy and pharmaceutical industries. High corrosion resistance in acidic environments makes 304 stainless steel ideal for medical applications. Stainless steel orthodontic wires began to be used already in the 1920s. The wire manufacturing processes improved its properties and enabled the production of various wire shapes, which in time convinced sceptical orthodontists.

A few years later Begg started using stainless steel round wires, and in the early 1940s, he started working with Wilcox, to produce a different type of stainless steel wire: the Australian stainless steel. Stainless steel has not been completely accepted in the physicians' environment, to the point where Archie Brusse (founder of Rocky Mountain Metal Products) presented a few decades later in 1933 at the American Society of Orthodontics, complete clinical treatment on the first system made of stainless steel, which resulted in the fact that by 1950, the 300 series was used in most orthodontic materials [11-13].

Materials and Methods

Purpose of the research presented in the work is an analysis of differentiation of the selected structural properties in the context of corrosion resistance of the material of the orthodontic wire. The object of the research was edge arches of the 0.016"x0.022" size made of the stainless steel type AISI 304, provided by two different producers: G&H Orthodontics and Adenta.

The X-ray microanalysis of the tested orthodontic wires was performed with the use of the SEM-EDS method. The quantitative and qualitative analysis was performed with the use of Zeiss EVO Ma25 scanning electron microscope equipped with the EDS Quantax probe from Bruker. The measurements were conducted with the accelerating voltage of 20 kV at the areas of 110 μm x 150 μm, at the counting time 500 s. The percentage of carbon and sulphur in the steels was determined additionally using the Leco CS-444 carbon and sulphur analyser, which uses the HF-400 induction furnace and measures carbon and sulphur by infrared absorption. Phase analysis studies were performed using an Ultima IV Rigaku X-ray diffractometer, in the range of angles 2θ from 40 to 90 with a step of 0.05. The resulting diffractograms were analysed using FindIt software using the Inorganic Crystal Structure Database Fiz Karlsruhe. The obtained results were presented using the OriginLab software. The assessment of the degree of contamination of steel with non-metallic inclusions was made in accordance with the ISO 4967: 2013 standard, comparing the recorded metallographic specimens of wires in the non-etched condition, with a magnification of 100x with the standards included in the standard. The microstructure observations were conducted using the NIKON ECLIPSE MA200 light microscope with the use of NIS Elements BR software, image recording was performed using the CCD Nikon DS.-Fi1 camera at magnifications: 100x, 200x and 500x. The microscopic tests were performed in the etched and non-etched states. As the etching agent, the 10% oxalic acid was used. The samples were electrolytically etched for 90 s with the 5.4 V voltage and 1.4 A current.

Determining the average number of carbide grains in the microstructure was performed with the application of quantitative metallography and the Jeffries's method. The Jeffries's method enables determining the average number of grains or precipitations at 1 mm^2 of the surface. A circle with a diameter of 79.8 mm and a surface of A = 0.5 mm^2 should be applied to the photo taken of the microstructure. Next, the number of grains or precipitations lying completely inside the NW circle and the number of grains or precipitates cut by the N_i circle is calculated, where after the total number of grains or NT precipitations on the surface of circle A is calculated:

$$N_T = N_w + kN_i \quad (1)$$

where:
k – usually assumed as equal to 0.5.

The corrosion tests were performed with the use of the three-electrode system – the working electrode (WE), created by a fragment of wire applied for orthodontic wire of the AISI 304 steel, calomel reference electrode (RE) and the platinum counter electrode (CE). For the measurements, the ATLAS 1131 potentiostat from EU&IA was applied. The corrosion resistance of the materials applied for orthodontic wires was evaluated at the base of the polarisation curves run performed with the potentiodynamic method in the Ringer solution – NaCl + KCl + $CaCl_2 \cdot 6H_2O$ – (8.6 mg + 0.3 mg + 0.33 mg)/ml, at the room temperature. The polarisation curves were recorded for the rate of potential change equal to 1 mV/s.

Results and Discussions

The tests of the chemical composition of the orthodontic wires were performed with the use of the SEM-EDS technique (TABLE 1). The content of phosphorus, sulphur, chromium and nickel tested in the orthodontic arches of the AISI 304 steel does not exceed the values specified in the standard. The content of chromium and nickel oscillates within the limits of minimum values given by the standard. Due to the fact, that nitrogen and carbon are light elements and are not easily detected in the EDS method, it was decided to determine the carbon content by another method. The studies have shown that for light elements such as sulphur and carbon much better method of quantitative analysis of the elements is the method of combustion with the use of the infrared detector. The content of carbon in the tested arches slightly exceeds the permissible content given by the standard, however, this is the value in the 3rd decimal place, which can be considered as an admissible measurement error (TABLE 2). Orthodontic wires have an insignificant amount of sulphur in its chemical composition.

TABLE 1. Comparison of alloy element contents in the tested orthodontic wires with reference to the standard AISI 304 [14].

	AISI 304		G&H Orthodontics	Adenta
	wt.%	max	wt.%	wt.%
Si	1.00		0.83	1.10
Mn	2.00		2.10	2.16
P	0.045		0.000	0.000
S	0.030		0.030	0.010
N	0.11		0.00	0.01
Cr	17.0	19.5	17.85	17.86
Ni	8.00	10.50	8.64	8.56

TABLE 2. Percentage contents of carbon and sulphur in the tested samples in reference to the AISI 304 standard [14].

AISI 304		G&H Orthodontics		Adenta	
chemical element	max wt.%	[g]	wt.%	[g]	wt.%
C	0.07	0.2752	0.07631	0.2946	0.07641
S	0.03		0.00061		0.00195

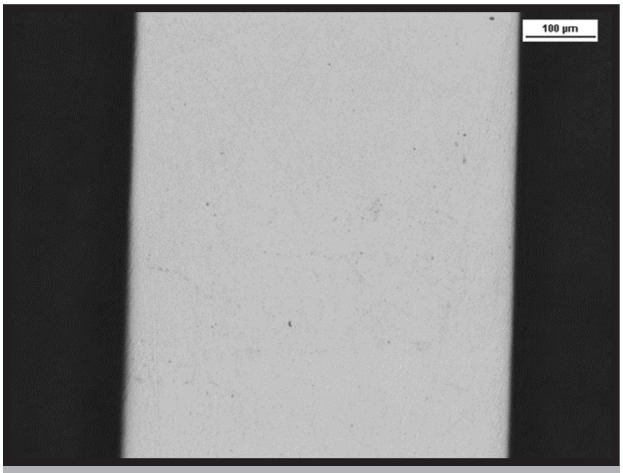


FIG. 1. Metallographic section of G&H Orthodontics orthodontic wire. Non-Etched state. LM

FIG. 2. Metallographic section of Adenta orthodontic wire. Non-Etched state. LM

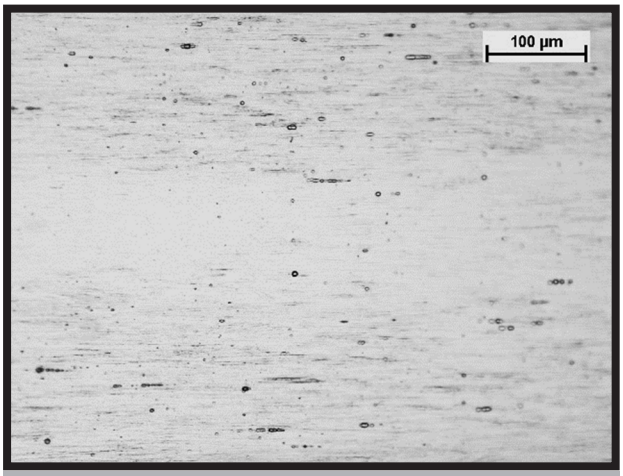


FIG. 3. The microstructure of the G&H Orthodontics orthodontic wire. Visible fibrous texture and precipitations of the $Cr_{23}C_6$ carbide arranged in bands. Etched state. LM

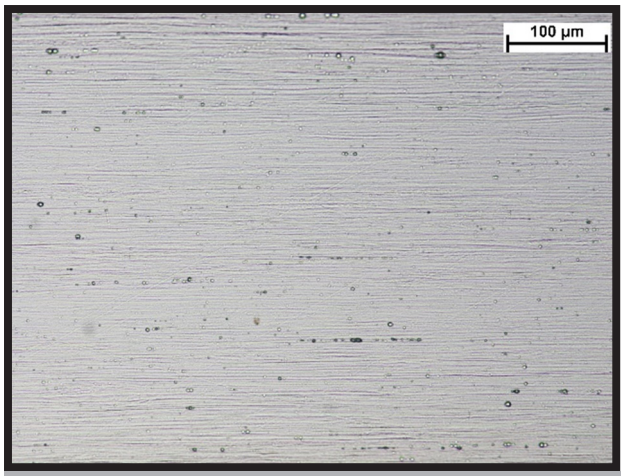


FIG. 4. The microstructure of the Adenta orthodontic wire. Visible strongly crushed structure with precipitations of the $Cr_{23}C_6$ carbide. Etched state. LM

Microscopic studies of the tested orthodontic arches material in the non-etched state have shown the appearance of non-metallic inclusions in the form of oxides in the quantity not exceeding the standard number 2 according to the PN-64/H-04510 standard (FIGs. 1 and 2). Such large number of the non-metallic inclusions is not allowed in materials for medical applications. Moreover, the type of inclusions, their shape, quantity and the way of their deployment can have a huge impact on the anisotropy of the mechanical properties of the material [8,10]. Mechanical properties of the orthodontic wires are of great importance because they are the main factor determining the effectiveness of treatment [2,10].

Microscopic studies in the etched state have shown the presence of strongly deformed austenite structure, being the result of the arches forming process by cold drawing (FIGs. 3 and 4). The size and deployment of grains in the microstructure cannot be determined. In the microstructure of the tested wires, which is confirmed by subsequent studies, also the chromium carbide of the $Cr_{23}C_6$ type is found and noticeable in the form of spheroidal, arranged in bands precipitations between grains of the deformed austenite.

In order to determine the quantitative content of chromium carbide present in the tested material, the quantitative metallography method with the use of Jeffries method was applied (TABLE 3). The smallest number of the $Cr_{23}C_6$ type carbide is in the wire used for the Adenta orthodontic wire, amounting to some 1330 grains/1 mm^2. The G&H Orthodontics orthodontic wire has by 200 precipitations of the carbide more at the same surface.

TABLE 3. Quantitative metallography of the $Cr_{23}C_6$ type chromium carbide content with the use of Jeffries method.

Sample	N_i	N_w	N_T	k	N_A
G&H Orthodontics	5	47	49	0.5	1560
Adenta	4	40	42	0.5	1332

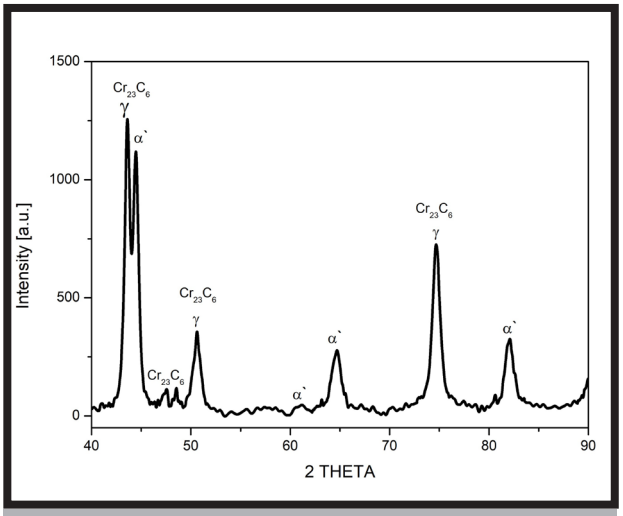


FIG. 5. X-ray radiation spectrum with marked reflexes coming from individual phases contained in the tested alloy of the G&H Orthodontics arch.

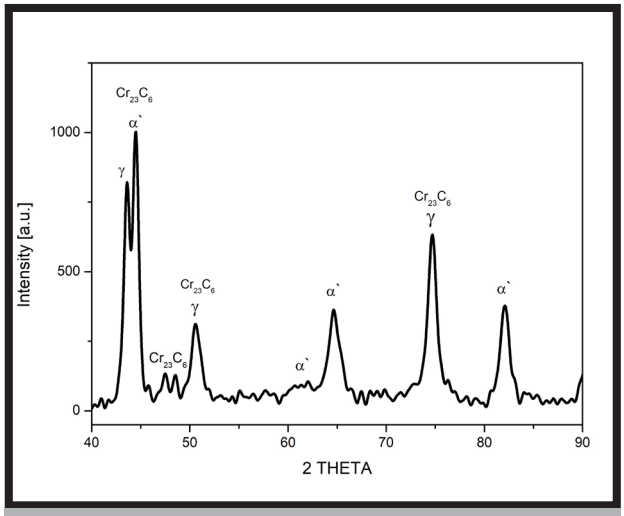


FIG. 6. X-ray radiation spectrum with marked reflexes coming from individual phases contained in the tested alloy of the Adenta arch.

The phase composition analysis studies using the XRD method have shown that materials of the orthodontic arches have a three-phase structure. The reflexes corresponding to the phase γ, α` and $Cr_{23}C_6$ have been identified (FIGs. 5 and 6). The primary phase of the type AISI 304 alloys is austenite (γ). As a result of the cold plastic working from the γ phase, the martensite induced by crushing is created, the so-called strain-induced martensite (α`). The $Cr_{23}C_6$ carbide is created as a result of annealing of the austenitic steel after cold plastic working at temperatures above 400°C.

During plastic straining of metals and alloys with the A1 lattice (regular wall centred) a microstructure is created, dependent mainly on such parameters as chemical composition, strain temperature and thermodynamic phase stability. The phase change taking place during plastic straining with the course γ→ε→α' or γ→α' causes strong material hardening, which can be verified by previously performed hardness tests. The bibliography gives that the AISI 304 steel in the plastically undeformed state has the structure of equiaxed grains of the γ phase, characteristic, and instead, after deformation of the steel with the crush of about 50% – the structure of elongated austenite grains with the α' martensite phase. The formation of the α' phase causes the fragmentation of the structure and, as a consequence, its strengthening [15,16]. While analysing the X-ray diffractograms, the intensity of reflexes coming from martensite and comparing the images of microstructure in the etched states it is assumed that the material crush significantly exceeded 80%, and the dominating phase in the studied materials is martensite - α'.

The higher values of the reflex coming from martensite indicate for the higher degree of the material crush [15]. The higher contents of the ferromagnetic phase α' will adversely affect the system, where orthodontic wires will stay possibly causing magnetothropism of blood components and form the corrosion centres. It is believed, that the carbide of the $Cr_{23}C_6$ type was created as a result of the metallurgy process and was not dissolved in the matrix, so that its small amounts remained in the orthodontic wire material, and the temperature of the annealing process after cold working exceeded 500°C, which causes precipitation of chromium carbide along the grain boundaries with simultaneous depletion of chromium within the grain [17].

Corrosion of orthodontic apparatus in the oral environment has for some time been a common topic among clinicians. These concerns focus on two main issues: whether the corrosion products, if they are manufactured, are absorbed by the body and cause local or systemic effects, and what are the effects of corrosion on the physical and clinical operation of orthodontic appliances. Due to the fact that the three-phase structure observed in the tested arches is particularly unfavourable because of the corrosion resistance, and the presence of the $Cr_{23}C_6$ carbide favours the intercrystalline corrosion, the electrochemical constant current measurements were performed and the potentiodynamic polarisation curves were determined.

At the base of the obtained study results it has been noticed that the orthodontic wires of stainless steel in the environment of the Ringer solution are not characterised by ability to passivation over the whole sample surface – no flattened current characteristics was observed at the graphs in the anodic range, which is confirmed by low abilities of the AISI 304 material in the above conditions to creating the stable passive layer (FIGs. 7 and 8). It has been observed that during exposal of the G&H Orthodontics orthodontic wire to the activity of the Ringer solution, the created passive layer is unstable, which increases the risk of ions flows favouring the development of the pitting corrosion. In the case of the Adenta orthodontic wire, the course of the curve indicates for typical pitting corrosion appearing at the material surface. The material of the arch No. 2 is characterised with the higher value of the corrosion potential (E_{corr} = -0.075 V) than the arch No. 1 (E_{corr} = -0.126 V), which indicates for better corrosion resistance (TABLE 4).

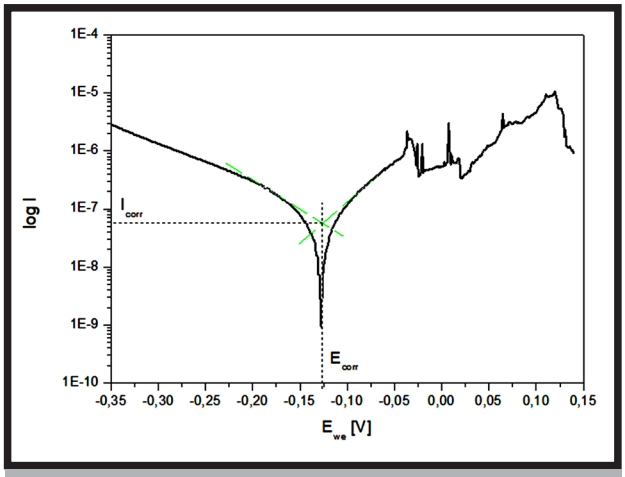


FIG. 7. The potentiodynamic polarization curve of the wire applied to the G&H Orthodontics orthodontic wire - the Ringer solution.

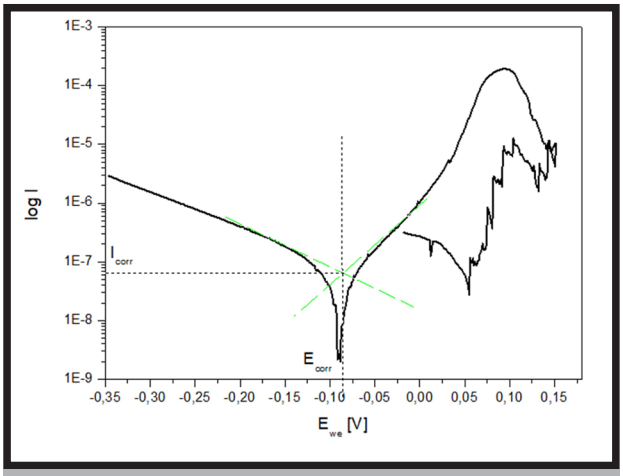


FIG. 8. The potentiodynamic polarization curve of the wire applied to the Adenta orthodontic wire - the Ringer solution.

TABLE 4. Summary of results of the polarization curve analysis (the Ringer solution).

Sample	E_0 (V)	I_{corr} (A/cm^2)	E_{corr} (V)
G&H Orthodontics	-0.117	$5.54 \cdot 10^{-8}$	-0.126
Adenta	-0.099	$7.05 \cdot 10^{-8}$	-0.075

Conclusions

The chemical composition tests have shown that the orthodontic wires from various producers differ in contents of individual alloy elements, as well as they exceed the contents of permissible manganese and sulphur values allowed by the standard. However, it could be stated, that the information provided by the producers that the wires are made of stainless steel of the AISI 304 type are confirmed.

Evaluation of the metallurgical purity degree has shown considerable diversity in terms of deployment and appearance frequency of non-metallic inclusions in the orthodontic wires from different producers. The research has shown that presence of the non-metallic inclusions in the tested materials is equal to the standard No. 2, according to the ISO standard, which is unacceptable for materials applied in the living body.

Microstructure of the tested wires applied for orthodontic arches have shown appearance of strongly deformed structure of austenite (the fibrous texture along the drawing direction). Between strongly deformed grains of austenite the clear initial etchings around other microstructures have been observed. The XRD analyses have shown occurrence of austenite in the microstructure of martensite induced by α' draft, as well as chromium carbide of the M23C6 type. The three-phase structure is particularly unfavourable due to the corrosion resistance, as presence of the $M_{23}C_6$ carbide in the tested material will favour the intercrystalline corrosion. Instead, presence of martensite, the ferromagnetic phase will adversely affect the body the orthodontic wires will be in, possibly causing magnetotropism of blood components and additionally be a source of corrosion.

The corrosion of orthodontic wires is closely related to the acidic environment of the mouth and the presence of fluoride ions, prophylactic agents and mouthwash solutions. Due to the thermic, microbiological and enzymatic properties aspects, the mouth environment is favourable in terms of biodegradation of metal and their alloys aspect, resulting in releasing metallic ions in the mouth. Along with the release of ions from metals or alloys the corrosion of orthodontic wires may lead to increase in surface roughness and their weakening, which can seriously impact the material strength, leading to mechanical damage or even fracture of the orthodontic materials. Based on the obtained test results of the orthodontic wires of the same geometry coming from two different producers it has been observed that in the environment of the Ringer solution they do not show the ability to passivate. At the polarization curves in the anodic area only the clear dissolution area is noticable. At the surface of the orthodontic wires also the phenomena of pitting corrosion take place, which is confirmed by the course the anodic curves. Moreover, it has been found that content of non-metallic inclusions and carbides occurring in the material microstructure definitely lowers corrosion resistance of the material.

The research presented in the work have shown significant differences in structural and physical-chemical properties of the orthodontic wires of the AISI 304 type stainless steel. Despite the fact, that the tested arches were manufactured of the theoretically the same materials, but by different producers, they significantly differ with chemical composition, metallurgical purity, phase composition and corrosion resistance. In addition, it is worth noticing that the tested materials, in terms of structure, do not meet the normative requirements obligatory for biomaterials.

Acknowledgments

Work financed within the statutory activity of the Faculty of Mechanical Engineering Wroclaw University of Science and Technology.

References

[1] Karłowska I.: Zarys współczesnej ortodoncji, Podręcznik dla studentów i lekarzy dentystów, Wydawnictwo Lekarskie PZWL, Warszawa, 2001
[2] Nanda R., Mielnik-Błaszczyk M., Chang F.H.: Biomechanika i estetyka w ortodoncji, Wydawnictwo Czelej, Lublin, 2009
[3] Isaacson K.G., Williams J.K.: Wprowadzenie do aparatów stałych, Wydawnictwo Kwintesencja, Warszawa, 1994
[4] Kahl-Nieke B., Masztalerz A.: Wprowadzenie do ortodoncji, Urban&Partner, Wrocław, 1999
[5] Craig R.G., i inni: Materiały stomatologiczne, Elsevier Urban&Partner, Wrocław 2008
[6] Combe E.C., Purzyński M., Wstęp do materiałoznawstwa stomatologicznego, Wydawnictwo Medyczne Sanmedica, Warszawa, 1997
[7] Karłowska I. i inni, Zarys współczesnej ortodoncji: Podręcznik dla studentów i lekarzy dentystów, Wydawnictwo Lekarskie PZWL, Warszawa 2016
[8] Marciniak J.: Biomateriały, Wydawnictwo Politechniki Śląskiej, Gliwice, 2002
[9] Nałęcz M. (red.): Biocybernetyka i inżynieria biomedyczna, tom 4 Biomateriały, Akademicka Oficyna Wydawnicza Exit, Warszawa, 2003.
[10] Łaskawiec J., Michalik R.: Zagadnienia teoretyczne i aplikacyjne w implantach, Wydawnictwo Politechniki Śląskiej, Gliwice, 2002
[11] S. Arango Santander, C.M. Luna Ossa, Rev. Nac. de Odont. 11 (2015)
[12] Robert P. Kusy: A review of contemporary archwires: Their properties and characteristics. The Angle Orthodontist: 67(3) (1997) 197-207.
[13] Nikolai R.J.: Orthodontic wire: a continuing evolution, Seminars in orthodontics 3(3) (1997) 157-165 .
[14] American Society for Testing and Materials, Standard Specification for Deformed and Plain Stainless Steel Wire and Welded Wire for Concrete Reinforcement 77.140.20, ASTM International, West Conshohocken 2001,
[15] Ozgowicz W.S.: Effect of deformation-induced martensite on the microstructure, mechanical properties and corrosion resistance of X5CrNi18-8 stainless steel. Archives of Materials Science and Engineering 43(1) (2010) 42-53.
[16] Ozgowicz W., Kalinowska-Ozgowicz E., Kurc A.: Influence of plastic deformation on structure and mechanical properties of stainless steel type X5CrNi18-10. Archives of Materials Science and Engineering 32(1) (2008) 37-40.
[17] Mohd Warikh, Abd Rashid, Miron Gakim, Zulkifli Mohd Rosli, Mohd Asyadi Azam: Formation of Cr23C6 during the sensitization of AISI 304 stainless steel and its effect to pitting corrosion. International Journal of Electrochemical Science 7 (2012) 9465-9477.

THE INFLUENCE OF COLLAGEN FROM VARIOUS SOURCES ON SKIN PARAMETERS

Weronika Prus, Justyna Kozlowska*

Nicolaus Copernicus University in Torun,
Faculty of Chemistry, Department of Chemistry
of Biomaterials and Cosmetics,
Gagarina 7, 87-100 Torun, Poland
*e-mail: justynak@chem.umk.pl

Abstract

Collagen is the main component of connective tissue – it represents 30% of total proteins in the animal body. This protein occurs in a wide range of tissues, e.g. in bone, skin, tendon, ligaments and cornea. It provides structural integrity, strength, resistance to tensile stress and elasticity. Due to its excellent biocompatibility and controlled biodegradability, collagen has found diverse application in the biomedical field such as wound dressing, drug carrier and tissue engineering. However, concerns about contamination of mammalian collagen have stimulated the search of another source of this biopolymer. Fish wastes are thought to be an attractive and safe new source of collagen. Fish and mammalian collagen differ in physical and chemical properties.

The aim of this work was to examine the influence of collagen extracted from different sources (rat tail tendons, fish scales of northern pike (Esox lucius) and fish skin of Brama australis) on skin parameters such as hydration, colour, pH and skin's barrier quality. The measurements had been taken on the skin surface before as well as after application of the collagen solutions.

The most harmful effect on skin parameters was observed after application of rat tail collagen solution. Collagen extracted from scales of Esox lucius showed the most favourable effect on the skin parameters.

The source of collagen has a significant influence on its effectiveness. The greatest virtues for human body were observed in the case of fish collagen extracted from Esox lucius scales.

Keywords: *fish collagen, Brama australis, Esox lucius, rat tail tendons, skin parameters*

[Engineering of Biomaterials 146 (2018) 14-17]

Introduction

Collagen, as the main component in the extracellular matrix and connective tissue, it is the most abundant protein in mammals – it constitutes nearly 30% of total proteins in the animal body [1,2]. During the last years, several different collagenous proteins have been isolated and characterized, which lead to expanding the collagen family to at least 29 types of collagen. They are marked as types I - XXIX [3,4]. The various types of collagen differ in amino acid sequence, structure and function.

Collagenous proteins are characterized by a triple-helix structure in which three left-handed polypeptide chains are supercoiled into a right-handed triple helix [5,6]. Each polypeptide chain consists of about 1000 amino acids and they are formed of a repeating triplet: Gly-X-Y, where Gly is glycine, X is generally proline and Y is hydroxyproline [4]. This sequence stabilizes the triple helix and is responsible for its specific shape. Proline and hydroxyproline are heterocyclic amino acids containing a nitrogen atom, which causes a significant effect on the structure of the triple helix. Collagen helix also contains polar amino acids, such as lysine, arginine, glutamic acid and aspartic acid [7]. The collagen comprises non-helical fragments, i.e. telopeptides, which are located at the ends of the collagen molecule or are embedded in the superhelix [8].

This biopolymer is the main structural protein responsible for the structural integrity of the connective tissue of many multicellular organisms. A wide range of tissues from tendons and ligaments to skin, cornea, bone and dentin have divergent mechanical requirements. Some of them need to be elastic and other require to be stiff and tough. Therefore, collagen as a building material shows a broad versatility - it provides mechanical stability, toughness, tensile strength and elasticity [9,10]. The skin contains mainly type I, III and V collagen, which determine the tension, elasticity, durability and hydration of skin [11].

Type I collagen is distributed in bone, skin, tendon, ligaments, cornea and other organs [1]. For that reason, the most common raw materials for collagen extraction are skin, bones, tendons and cartilage [12]. Due to the potential risk for viral and prion contamination of collagen derived from animal tissues, non-mammalian sources of this protein are sought. Collagen extracted from fish wastes presents an attractive new source of collagen as it is a by-product of food production. Mammalian and fish collagens differ in thermal stability and the amino acid composition – fish collagen is characterised by a lower proline and hydroxyproline content and a lower denaturation temperature [13-15].

The paper focuses on comparing the influence of collagen extracted from different species (rat tail tendons, fish scales of northern pike (*Esox lucius)* and skin of *Brama australis*) on skin parameters such as hydration, colour, pH and skin's barrier quality.

Materials and Methods

Collagen from rat tail tendon [16] as well as collagens from fish tissues – scales of *Esox lucius* [15] and skin of *Brama australis* [17] were prepared in our laboratory.

Afterwards, 0.1% solutions of each collagen were prepared and applied on the forearm skin of voluntarily probants. Then, the evaluation of skin condition after the application of collagen solutions was made, including hydration, pH, colour and skin's barrier quality. The measurements were conducted with the participation of five probants (women, aged 22 years).

The hydration level of the skin surface (*stratum corneum*) was determined using Corneometer CM 825 (Courage+Khazaka, Germany). Skin's pH was tested using pH-meter (Elmetron, Poland), skin's barrier quality (TEWL - Transepidermal Water Loss) was examined using Tewameter TM 300 (Courage+Khazaka, Germany) and skin colour was measured by Skin-Colorimeter CL 400 (Courage+Khazaka, Germany).

The measurements had been taken on the skin surface in three places before application and after 10, 20, 30, 60, 120 and 180 min from application of the collagens solutions. The results of these measurements were averaged and the standard deviation was calculated. All measurements were performed in the laboratory in controlled temperature and humidity conditions (20-22°C, relative humidity 40-60%).

Results and Discussion

The results of evaluation of skin condition after the application of collagen solutions are shown in FIGs. 1-4.

The application of obtained collagen from rat tail tendons and fish tissues (marine *Brama australis* and fresh water *Esox lucius*) had initially deteriorated the skin's barrier quality manifesting itself as the increase in TEWL (FIG. 1). The highest TEWL value was observed after application of collagen from rat tail tendons. The level of TEWL had returned to the initial level 120 min after application of collagen solutions from rat tail tendons and *Esox lucius* scales and after 180 min in the case of collagen from the skin of *Brama australis*. The solution of collagen extracted from the scales of *Esox lucius* improved the skin's barrier quality – 180 min after application of this solution the level of TEWL decreased below the preliminary level.

Slight redness of skin appeared after the application of collagen solutions (FIG. 2). Collagen from rat tail tendons made the skin the more red and irritated. After 120 min from the application of collagen solution from *Esox lucius* scales the skin colour solely had returned to the initial level.

The application of collagens solution had improved the hydration of the outer skin layers (FIG. 3). After 30 min the level of hydration of the skin surface decreased, however, within three hours of the study it remained at a higher level than the initial one regardless of the source of collagen. The solution of collagen from *Esox lucius* scales have the best long-term moisturizing properties. The increase in TEWL could have an indirect impact on corneometric measurements.

Application of rat collagen solution had increased the skin pH, while a solution of fish scale collagen had slightly decreased the pH of skin (FIG. 4).

The most harmful effect on skin parameters was observed after application of rat tail collagen solution. Application of rat collagen irritated the skin and deteriorated the skin's barrier quality the most. While collagen extracted from scales of *Esox lucius* showed the most favourable effect on the skin parameters. Collagen from *Esox lucius* improved the skin's barrier quality and have the best long-term moisturizing properties.

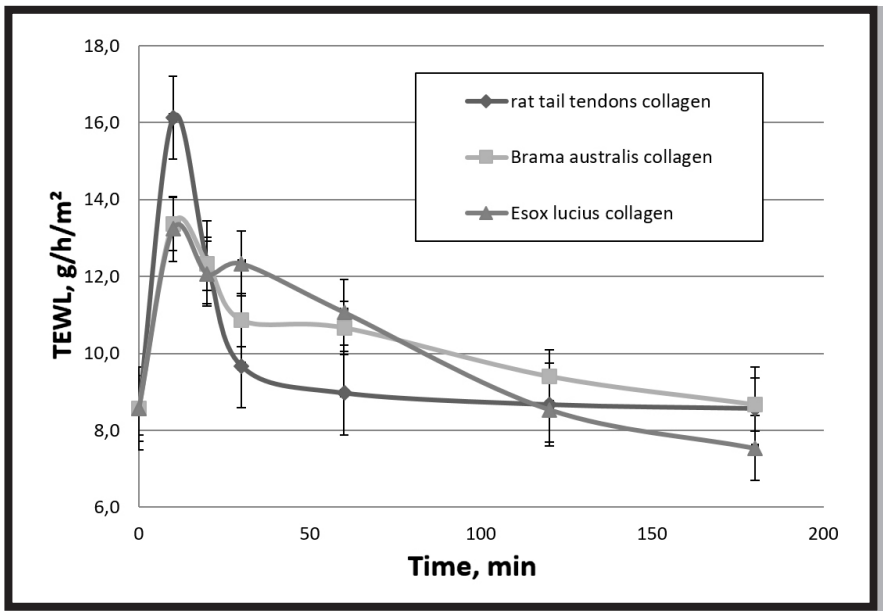


FIG. 1. The graph of dependence of skin's barrier quality (manifesting as Transepidermal water loss – TEWL) from time after application of collagen from different sources – rat tail tendons, scales of *Esox lucius* and skin of *Brama australis.*

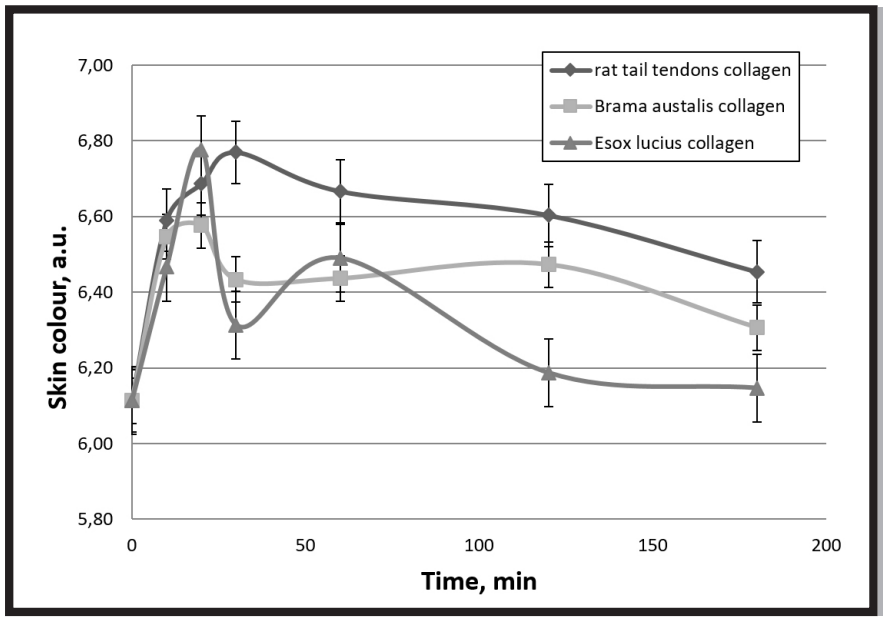


FIG. 2. The graph of dependence of skin colour from time after application of collagen from different sources – rat tail tendons, scales of *Esox lucius* and skin of *Brama australis.*

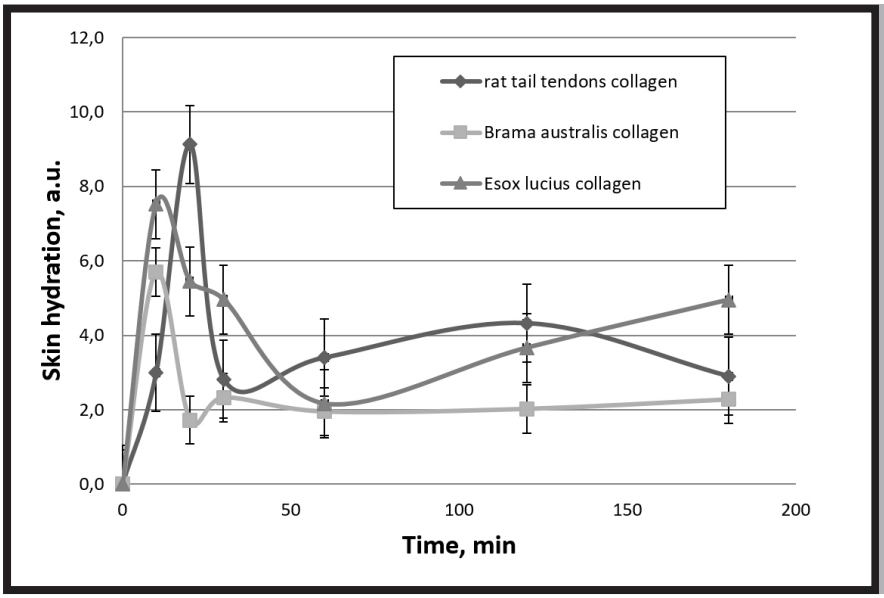


FIG. 3. The graph of dependence of skin hydration from time after application of collagen from different sources – rat tail tendons, scales of *Esox lucius* and skin of *Brama australis.*

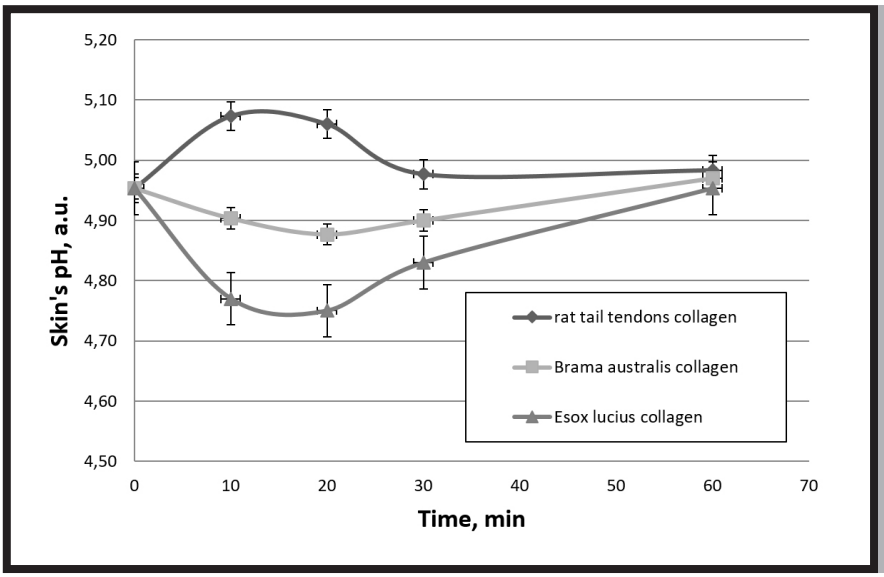


FIG. 4. The graph of dependence of skin's pH from time after application of collagen from different sources – rat tail tendons, scales of *Esox lucius* and skin of *Brama australis.*

Conclusions

The source of collagen has a significant influence on its effectiveness. The greatest virtues for human body were observed in the case of fish collagen extracted from *Esox lucius* scales.

Collagen, due to its biocompatibility, biodegradability and non-toxicity, is widely used for cosmetic, pharmaceutical and biomedical applications. Fish collagen may be a good base for the production of collagen matrices for the skin applications (e.g. for wound dressings) because it exhibits a positive active effect on the skin. Moreover, other active substances can be incorporated into these matrices. For that reasons, fish collagen may be an attractive alternative to mammalian collagen for biomaterials production.

Acknowledgments

Financial support from National Science Centre (NCN, Poland) Grant no. UMO-2016/21/D/ST8/01705 is gratefully acknowledged.

References

[1] Cameron G.J., Alberts I.L., Laing J.H., Wess T.J.: Structure of type I and type III heterotypic collagen fibrils: an X-ray diffraction study. Journal of Structural Biology 137 (2002) 15-22.

[2] Gelse K., Pöschl E., Aigner T.: Collagens - structure, function, and biosynthesis. Advanced Drug Delivery Reviews 55 (2003) 1531-1546.

[3] Veeruraj A., Arumugam M., Ajithkumar T., Balasubramanian T.: Isolation and characterization of collagen from the outer skin of squid (Doryteuthis singhalensis). Food Hydrocolloids 43 (2015) 708-716.

[4] Peng Y.Y., Stoichevska V., Vashi A., Howell L., Fehr F., Dumsday G.J., Ramshaw J.A.M.: Non-animal collagens as new options for cosmetic formulation. International Journal of Cosmetic Science 37 (2015) 636-641.

[5] Hoppe H.J., Barlow P.N., Reid K.B.: A parallel three stranded α-helical bundle at the nucleation site of collagen triple-helix formation. FEBS Letters 344 (1994) 191-195.

[6] Brodsky B., Ramshaw J.A.: The collagen triple-helix structure. Matrix Biology 15 (1997) 545-554.

[7] Howland M.R., Corr L.T., Young S.M., Jones V., Jim S., Van Der Merwe N.J., Evershed R.P.: Expression of the dietary isotope signal in the compound-specific δ13C values of pig bone lipids and amino acids. International Journal of Osteoarchaeology 13 (2003) 54-65.

[8] Hulmes D.J.: Building collagen molecules, fibrils, and suprafibrillar structures. Journal of Structural Biology 137 (2002) 2-10.

[9] Fratzl P.: Cellulose and collagen: from fibres to tissues. Current Opinion in Colloid & Interface Science 8 (2003) 32-39.

[10] P. Fratzl, Collagen: structure and mechanics, an introduction. In *Collagen* (pp. 1-13). Springer, Boston, MA, 2008.

[11] Cheng W., Yan-hua R., Fang-gang N., Guo-an Z.: The content and ratio of type I and III collagen in skin differ with age and injury. African Journal of Biotechnology 10 (2011) 2524-2529.

[12] Gómez-Guillén M.C., Giménez B., López-Caballero M.A., Montero M.P.: Functional and bioactive properties of collagen and gelatin from alternative sources: A review. Food Hydrocolloids 25 (2011) 1813-1827.

[13] Pati F., Adhikari B., Dhara S.: Isolation and characterization of fish scale collagen of higher thermal stability. Bioresource Technology 101 (2010) 3737-3742.

[14] Giraud-Guille M.M., Besseau L., Chopin C., Durand P., Herbage D.: Structural aspects of fish skin collagen which forms ordered arrays via liquid crystalline states. Biomaterials 21 (2000) 899-906.

[15] Kozlowska J., Sionkowska A., Skopinska-Wisniewska J., Piechowicz K.: Northern pike (Esox lucius) collagen: Extraction, characterization and potential application. International Journal of Biological Macromolecules 81 (2015) 220-227.

[16] Kozlowska J., Sionkowska A., Osyczka A.M., Dubiel M.: Stabilizing effect of carbodiimide and dehydrothermal treatment crosslinking on the properties of collagen/hydroxyapatite scaffolds. Polymer International 66 (2017) 1164-1172.

[17] Sionkowska A., Kozłowska J., Skorupska M., Michalska M.: Isolation and characterization of collagen from the skin of Brama australis. International Journal of Biological Macromolecules 80 (2015) 605-609.

INVESTIGATION OF THE INITIAL DEGRADATION STAGE AND TENSILE STRENGTH OF POLYLACTIDE AND ITS COMPOSITES WITH EGGSHELLS

Piotr Szatkowski[1]*, Katarzyna Greń[1], Michał Kisilewicz[2]

[1] Department of Biomaterials and Composites, Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow, Poland
[2] The Polytechnic Institute, Faculty of Materials Engineering, State Higher Vocational School in Tarnow, Poland
*e-mail: pszatko@agh.edu.pl

Abstract

The degradation rate of the composite polylactide (PLA) – eggshell powder (ESP), depends on many factors. The main are porosity, particle distribution, the weight load of ESP and chain length, the microstructure of bio-based and biodegradable PLA polymer. Bio-additives introduced to polymer matrix may have an impact on mechanical properties of the composite. Natural bone structure inspires to design mimicry materials. Materials which combine inorganic and organic components are of highly complex structure.

The paper focuses on the investigation of tensile strength and the initial stage of degradation of PLA and its composites with ESP in two media, i.e. H_2O and PBS. ESP was obtained by grinding, stirring with mixture 14.5 wt.% NaOH and 85.5 wt.% ethanol, and drying. Samples containing: 0, 10 and 20 wt.% of ESP were prepared. Dumbbell-samples were made by injection molding, and their mechanical properties were measured. Tensile results suggest that Young's modulus increased and tensile strength decreased as the amount of ESP is increased. Conductivity and pH of incubation media differed according to the material composition. SEM-EDS observations of PLA-ESP composites after fracture test were performed, and showed good adhesion between ESP and the polymer matrix. Influence of the incubation in H_2O and PBS on PLA and PLA--ESP composite surface degradation was checked. The PLA-ESP composites are characterized by a combination of unique properties, which may be suitable to use them as an eco-friendly packaging or medical material.

Keywords: *polylactide, eggshells, bio-composite, degradation, tensile strength*

[Engineering of Biomaterials 146 (2018) 18-23]

Introduction

Composite materials are made from the combination of two or more components, which allows achieving a new functional material. At the moment researchers focus on investigating new bio-composites for different purposes. Characteristics of the final material depends on many factors as size and amount of additive, its orientation, and adhesion to the matrix [1,2]. As an example: bone is strong and elastic. This duality is due to the complex structure of inorganic and organic phases, mainly collagen and hydroxyapatite [3-6]. Of course, there are many various methods for modifying polymers with particle reinforcement [1]. For example, PLA is a very popular biodegradable bio-based polymer, which can be used as an alternative to other non-biodegradable synthetic polymers [7]. PLA polymer may have a semi-crystalline or amorphous structure, which assures the possibility of physical properties modification. Many medical devices are made from PLA because of its unique properties [8]. PLA is not osteoconductive and has lower elastic modulus than bone. One of the methods of increasing the modulus of elasticity may be the manufacturing of a composite containing inorganic particles e.g. ESP. ESP addition to PLA matrix may bring its mechanical properties closer to those of bone, which can be very useful for medical application. ESP is also a cheap and easily available source of calcium. This type of material can avoid the need for a second surgery as well [2]. Kasuga et al. described the influence of calcium carbonate ($CaCO_3$) particles on PLA properties [9]. They showed that elastic modulus depends on the amount of additive. Young's modulus increased significantly to 4-6 GPa, i.e. comparable to that of natural bone by addition of 30 wt.% of $CaCO_3$ [9]. According to Ramakrishna et al., Young's modulus of cortical bone in the longitudinal direction is 17.7 GPa, and that of cancellous bone is 0.4 GPa. The tensile strength is approximately 133 MPa and 7.4 MPa for cortical and cancellous bone, respectively [5]. Some additives can also modify PLA degradation rate, which can differ depending on the size, shape, isomer ratio and temperature. Half-life time for PLA varies from 6 months to 2 years [10].

According to the previous studies, the amount of thrown chicken eggshells is significant, especially in the countries where egg production is well developed; in some articles, eggshell waste is described as an environmental problem [6,11,12]. What makes ESP an interesting additive for polymer composites is its chemical composition and availability [1,11]. ESP consist of $CaCO_3$ set on collagen and glycosaminoglycans films [6]. In various articles, some combinations of ESP with different polymers were described. Shuhadah and Supri investigated composites with low-density polyethylene (LDPE) and according to their study, the tensile strength of LDPE-ESP composites decreased and Young's modulus increased with increasing amount of ESP [1]. An interesting effect was observed in Asha and Sekhar work, where tensile strength of polyamide (PA) was higher or constant as the amount of ESP increased, and it depended on the type of PA matrix [12]. Petit et al. focused on testing degradation of polycaprolactone-ESP (PCL-ESP) composites; it was noted that soil pH, heat and an aerobic environment accelerate the degradation process, while an anaerobic and moisture-saturated environment delays this process, as compared with the composting system used as control [11].

TABLE 1. Literature review of types of ESP surface treatment processes and impact of ESP content on composite mechanical properties.

Reference	Material	Parameters changes	Surface compatibilization method
Shuhadah, Supri [1]	LDPE-ESP	↑ %ESP ↓ tensile strength ↑ %ESP ↑ Young's modulus	- wash - dry - grind to obtain 63 μm particles - dry in oven at 80ºC - mix with 10% NaOH, decant - dry - stir with mixture of 6% of isopthalic acid and ethanol - dry in 80ºC to constant weight
Timob [6]	Epoxy-ESP (nano particles)	2-4% nano-ESP ↑ bending modulus & strength 5/10% nano-ESP ↓ bending modulus & strength	- boil in 100ºC for 6 h - blend - wash with H_2O and then ethanol - dry at room temperature - mill - wash, stir etc. with absolute ethanol several times - vacuum dry at room temperature for 24 h
M.G. Petit, Z. Correa, M.A. Sabino [11]	PCL-ESP		- wash - dry - grind in a cryogenic mill (<190ºC)
A. Asha, V.C. Sekhar [12]	PA-ESP	ESP influence the tensile strength depending on the type of polyamide	- wash - dry - grind to a powder to obtain 100 μm particles - mix with NaOCl solution, stir for 30 min, decant, wash with distilled water - stir with 6% isophtalic acid in ethanol for an hour - dry in 140ºC to obtain constant weight

An important aspect is converting eggshells into particles, which can be added as an additive to the polymer matrix. Unmodified eggshells surface is hydrophilic [1,6]. Available articles mention various methods of eggshells powder surface compatibilization to the polymer matrix, which are described in TABLE 1. For example, treatment eggshells with NaOH solution leads to deproteinization of the material. Application of an isopthalic acid allowed the surface treatment of ESP, which was also compared by Shuhadah and Supri with unmodified eggshells [1]. Mechanical properties of polypropylene-ESP were investigated by Dhaliwal et al. They concluded that eggshells modification with isophthalic acid improved interfacial bonding [13].

This paper is focused on the investigation of tensile strength and the initial stage of PLA and PLA-ESP composite degradation in two media. This experiment allows considering the bio-based composite as an eco-friendly packaging or medical application material [6,7,12].

Materials and Methods

The tested bio-composite was made from PLA matrix (Ingeo, NatureWorks grade 3251D) and 0, 10, 20 wt.% ESP addition. ESP was prepared from hens organic eggshell.

The procedure of ESP preparation was started by washing hens eggshell in distilled water. Next stage was drying in Memmert drier at 100 ± 5ºC for 30 ± 1 min. Pulverisation was prepared in an electric grinder for 30 ± 1 min to obtain uniform particle distribution. Finally, ESP was sieved with sieves. Surface treatment was achieved in 14.5 ± 0.1% NaOH solution, and magnetic stirring for 30 ± 1 min was used for better removal of organic remnants. Then ESP was washed with distilled water and dried at 100 ± 5ºC for 30 ± 1 min. The last step of surface compatibilization was stirring for 30 ± 1 min in pure methanol. Cleaning was achieved by decanting and washing ESP with distilled water. Finally, drying was accomplished in the Memmert drier at 100 ± 5ºC for 60 ± 1 min.

PLA was dried in a Memmert drier at 50 ± 5ºC to achieve a constant weight. Three material composition samples were prepared using Zamak Mercator injection machine: PLA with 0, 10 ± 1, 20 ± 2 wt.% ESP. Samples mass was 4.4 ± 0.1 g. Settings of the injection process are listed in TABLE 2.

TABLE 2. Injection moulding settings of PLA and PLA-ESP composites.

Parameter	Value	Deviation	Unit
Injection time	10	0.1	[s]
Injection force	10	0.1	[kN]
Mould temperature	25	1	[ºC]
Injection moulding temperature mass	220	1	[ºC]

Tensile test

Dumbbell-samples were tested on universal testing machine Zwick 1435. The tensile test was performed at a speed of 2 mm/min, at room temperature (sample dimension 40 mm x 4 mm x 4 mm). Force-displacement data were registered, and tensile strength and Young's modulus were calculated. Samples were tested after injection and after one-week degradation in distilled water and PBS, and the average results and standard deviation were reported.

Degradation process

Degradation of samples was tested in two media: distilled water and phosphate-buffered saline (PBS). PBS was prepared by using 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 0.133 g $CaCl_2 \cdot 2H_2O$, 0.10 g $MgCl_2 \cdot 6H_2O$, mixing with distilled water to obtain a solution of 800 ml, and then a pH value in the range 7.2-7.4 was achieved by adding HCl and water.

The pH and conductivity values were measured for two suspension sets. Suspensions were made with dry to wet ratio 1:10. The sets were as follows:

- PLA + H_2O
- ESP + H_2O
- 90 wt.% PLA + 10 wt.% ESP + H_2O
- 80 wt.% PLA + 20 wt.% ESP + H_2O
- PLA + PBS
- 90 wt.% PLA + 10 wt.% ESP + PBS
- 80 wt.% PLA + 10 wt.% ESP + PBS

The first set was stored in a dryer at 37ºC. The second set was stored at 20ºC in distilled water, which mass was later measured, in a dried state and soaked, after different periods of time. This procedure allowed to measure solvent absorption and degree of degradation.

Conductivity, pH and mass changes

Solution conductivity was measured with ELMETRON microcomputer conductivity meter CC-315. Solution pH was measured with ELMETRON pH-meter CP-411. Measurements were made every 5 days. Mass of samples was measured with RADWAG PS 360/C/2 with an accuracy of 0.0001 g. Samples were weighed soaked for 10 and 22 days, then they were dried at 37ºC and again weighed.

Optical Microscope

Samples and eggshells were observed under a digital microscope Keyence VHX-900X with magnification 200x, which allowed evaluating surface topography.

Scanning Electron Microscope

The surface of samples before and after 3 weeks degradation was observed using NOVA NANO SEM 200 microscope with EDS. The fracture surface of the specimens was also investigated. Eggshells were also tested to determine their composition.

Results and Discussions

Injection process

After the injection process, it was observed that obtained dumbbell-shaped specimens had non-homogenous ESP distribution.

High transparency of PLA samples contained 0 wt.% ESP suggest the high volume of an amorphous phase [14]. Samples with: 10 wt.% ESP showed an injection line, for 20 wt.% ESP the homogeneity of material increased (FIG. 1).

Tensile test

Non-degraded samples with different ESP content after the tensile test are shown in FIG. 1. During the tensile test force-displacement characteristic curves were registered (FIG. 2). Their appearance indicates that samples behaved as a brittle material. Young's modulus measured from the characteristic curves increased with the increasing amount of ESP (FIG. 3). After one week degradation the parameter was increased for pure PLA, and for the composites, the differences were within the error limits (less than 5% of change).

As Young's modulus increased with the amount of ESP, the tensile strength decreased (FIG. 4). 1-week-degradation of the samples almost did not influence the tensile strength.

Visual appearance observation during degradation

The visual appearance of the samples during degradation in distilled water and PBS were observed under an optical microscope. Visible changes in the appearance of the samples did not occur, only light solution turbidity. The solution which contained a modified PLA-ESP composite was characterized by sulphur like smell, which may be connected to the presence of sulphur in eggshell.

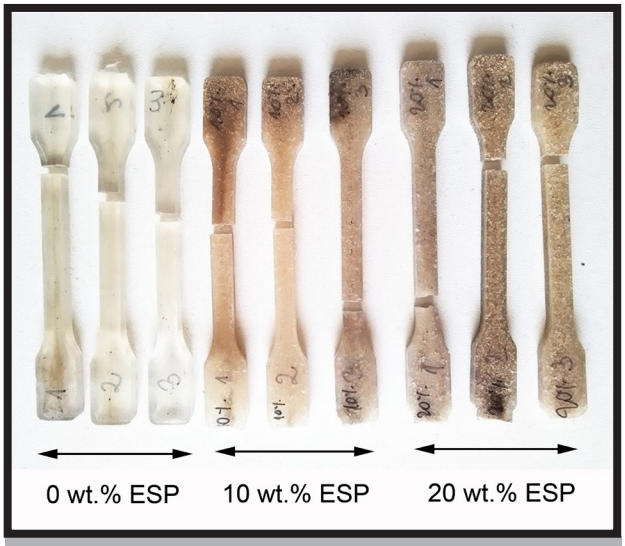


FIG. 1. Non-degraded samples after tensile test.

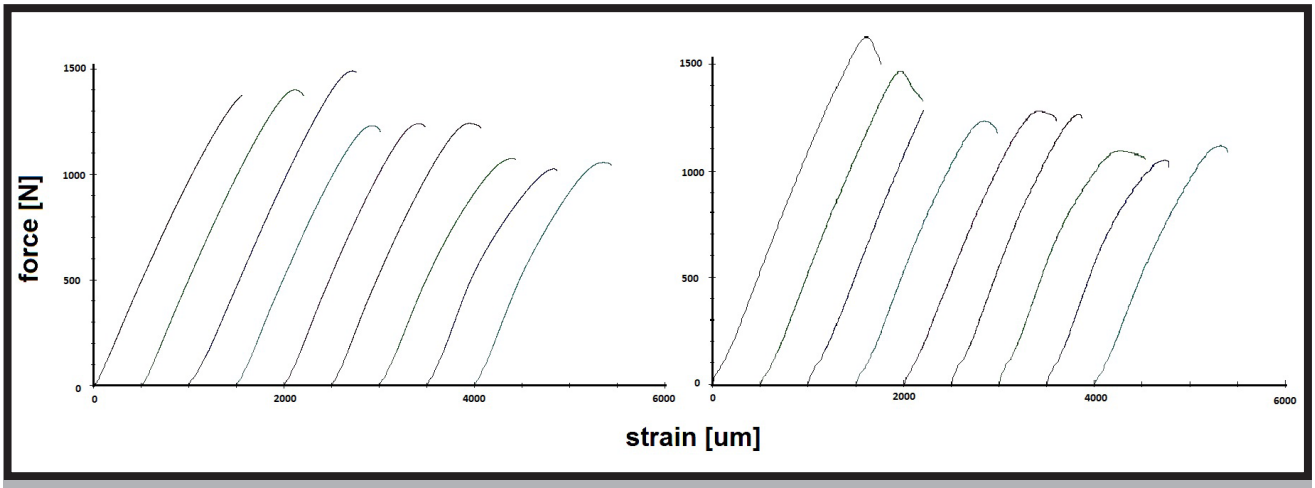


FIG. 2. Force-displacement characteristic curves for non-degraded (left) and after 1-week-degradation (right) samples. Example of samples type with 10% ESP additive.

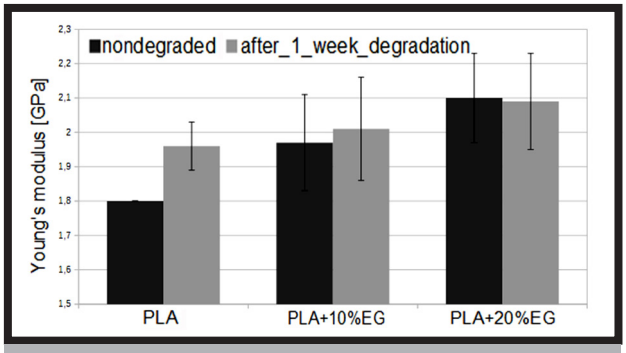


FIG. 3. Young's modulus of samples: non-degraded and after 1-week-degradation.

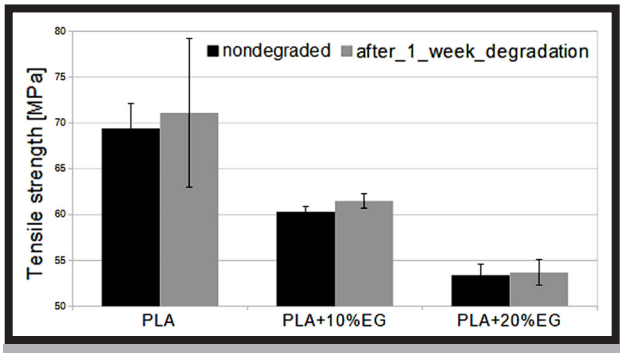


FIG. 4. Tensile strength of samples: non-degraded and after 1-week-degradation.

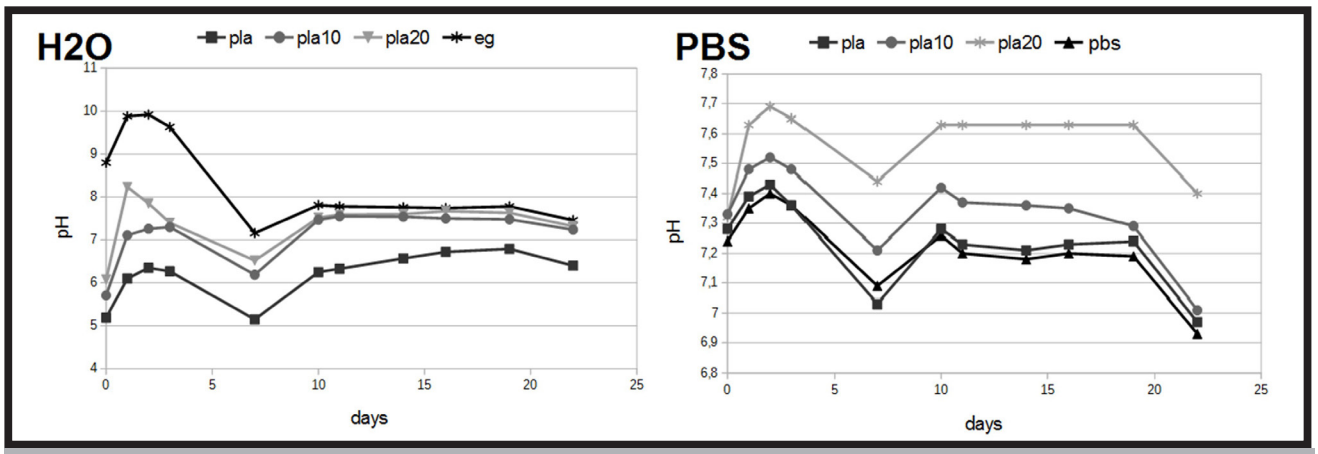


FIG. 5. Changes of pH for samples immersed in water (left) and PBS solution (right).

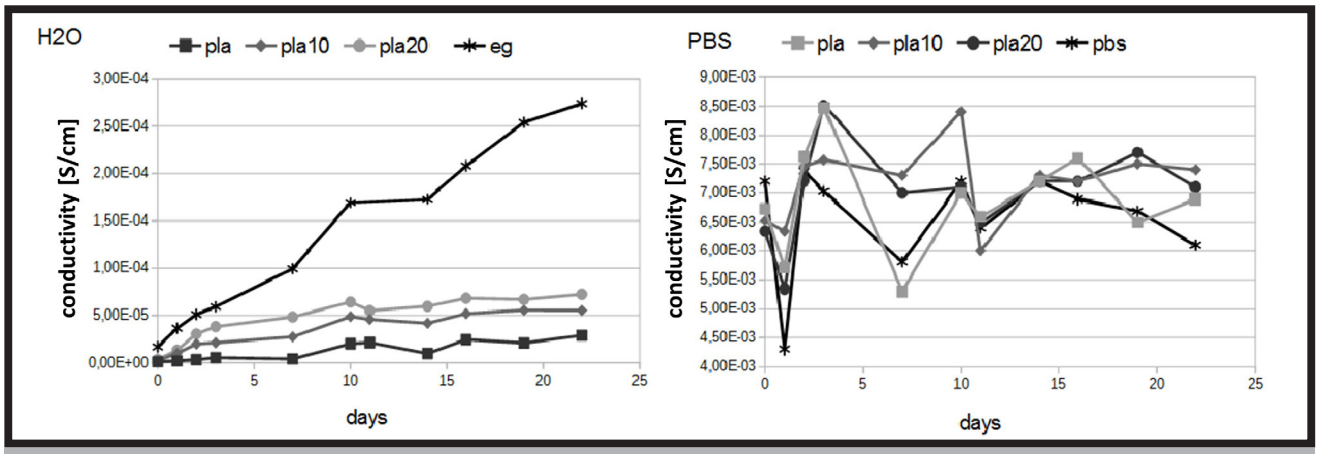


FIG. 6. Conductivity changes during degradation in water (left) and PBS (right).

pH

Up to 10 days pH of solutions was changing dynamically and then after 10 days of degradation it became constant in water (FIG. 5). Samples immersed in PBS showed decreasing pH with the time. These phenomena may be due to many possible factors, including the presence of bacteria. The highest pH value was observed for ESP solution and it can be explained with the chemical structure of ESP – which is mainly built from calcium carbonate. This also justified the fact that pH of composites increased with the increase in ESP amount. A pure solution of PLA+H_2O was acidic because of its degradation mechanism. It undergoes PLA hydrolysis, which produces lactic acid.

Conductivity

The conductivity of samples in distilled water at the beginning of the degradation was 0.5 [μS/cm] (FIG. 6). The conductivity value increased with time, intensively in the case of ESP. As ESP amount in the composite raised, the composite degradation accelerated, because conductivity was higher than in the sample with less ESP amount. It also means that the degradation time of PLA can be shortened by adding ESP. The conductivity of PBS solutions was not stable.

Mass changes

Mass change during the degradation process was investigated. The mass change during the 3 weeks was ≤ 0.0001 g.

Absorption of the solutions was measured and the results are shown in FIG. 7. Its value may be the evidence of open porosity in the composites what can facilitate the absorption of the liquids. Polylactide is hygroscopic, which is higher if it is in an amorphous state [2,15]. The value of absorptivity at the level of several percents confirms the fact. Then the value decreased and it was similar for all samples after three weeks of degradation.

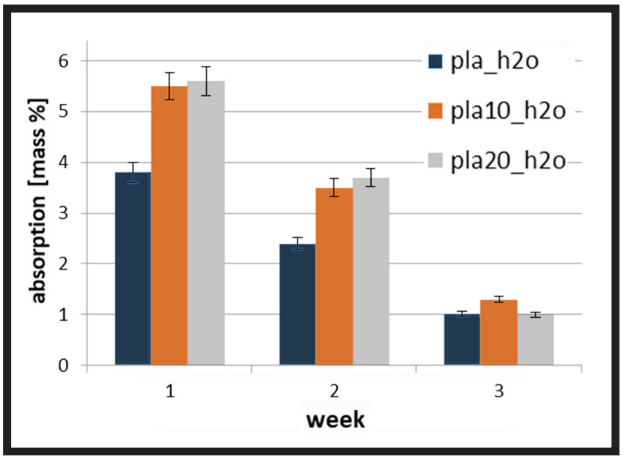


FIG. 7. Absorption of water vs time.

Scanning Electron Microscope

The SEM with EDS of non-degraded ESP surface and the ESP after 24 days of degradation in distilled water was investigated. EDS analysis showed that non-degraded ESP contains mainly: calcium, carbon, oxygen, and trace amounts of magnesium, sulphur and phosphorus.

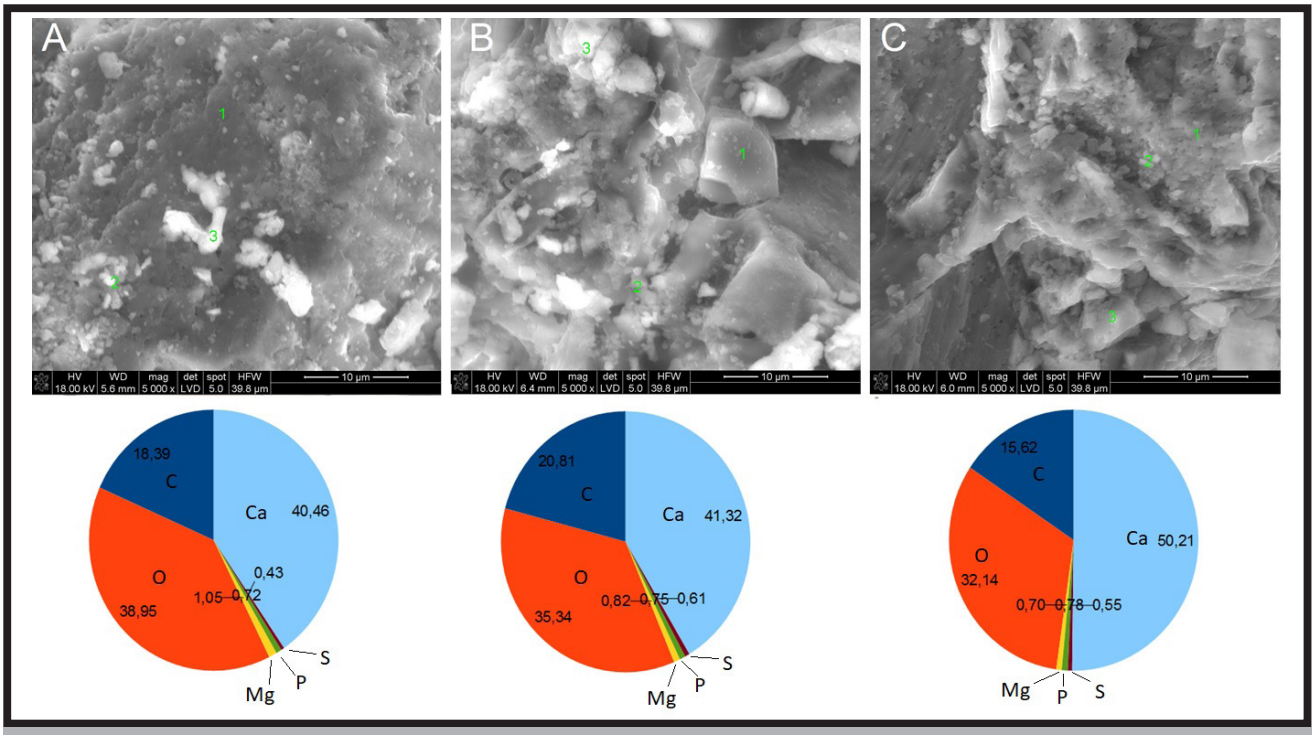


FIG. 8. SEM images of ESP and average chemical composition: A – unmodified, B – modified , C – after 24-days degradation in distilled water.

EDS analysis of the ESP showed during degradation that calcium amount increased (FIG. 8) suggesting that the organic part is removed and dissolution of calcium carbonate was occurred.

SEM surface images of the PLA and PLA-20 wt.% ESP samples (FIG. 9) have not shown visible degradation. The SEM surface image of PLA-10 wt.% ESP has shown a visible degradation of the PLA matrix. White spots on the surface are representing ESP, which was also investigated with EDS.

Fracture surfaces investigation under SEM after tensile strength measurement showed that ESP particles are strongly bond to the PLA matrix (FIG. 10).

Conclusions

1. The ESP can be a source of easily available $CaCO_3$.
2. The ESP modified PLA composite is chemically stable; in the long-term it does not cause significant acidification of the aqueous environment consisting of water and PBS.
3. Along with immersion time of PLA / ESP composites in water, salts from ESP are released to the solution.
4. The addition of ESP increased the hygroscopic properties of the composite.
5. As the ESP content in the composite increases Young's modulus of the resulting composite increases.
6. Together with the increase in the ESP content in the composite material, the strength of the composite material decreases.

Acknowledgments

This research was financed by the Dean grant No 11.11.160.182 of Faculty of Materials Science and Ceramics, AGH University of Science and Technology, Krakow, Poland.

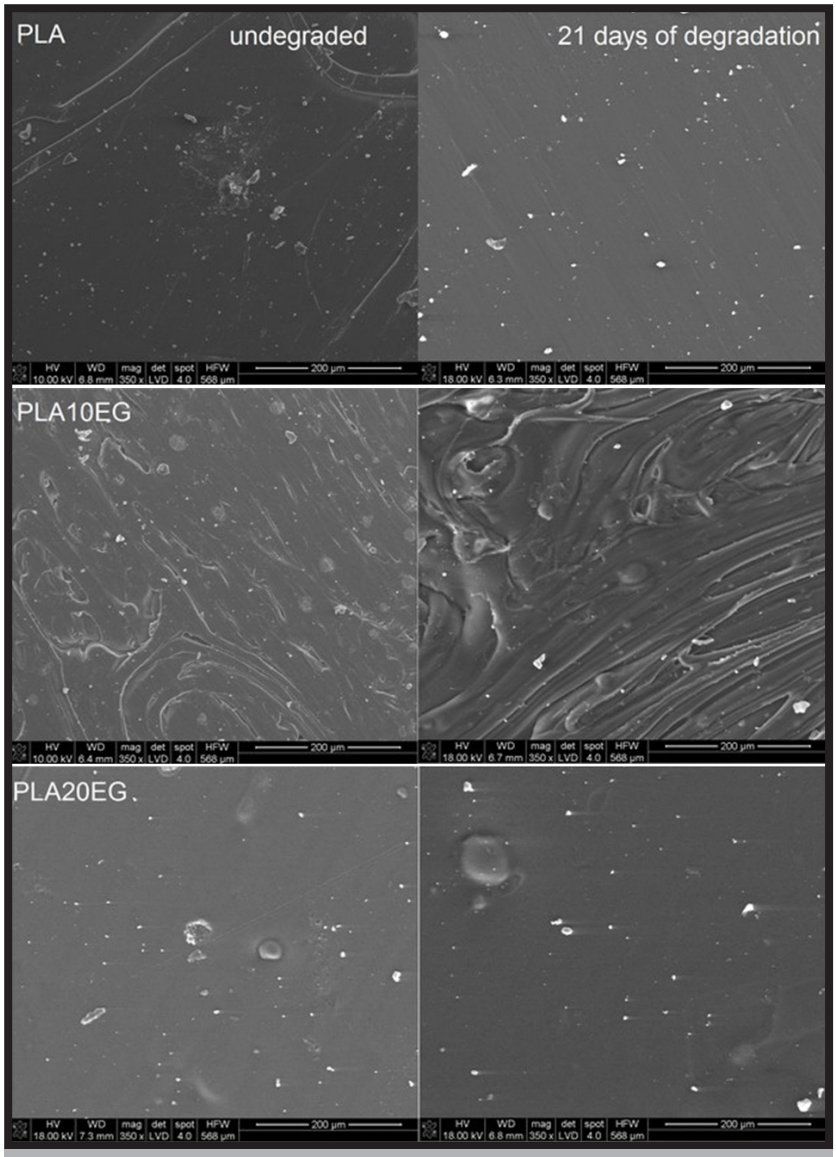


FIG. 9. SEM images of PLA-ESP samples surface before (left) and after 21-days degradation (right).

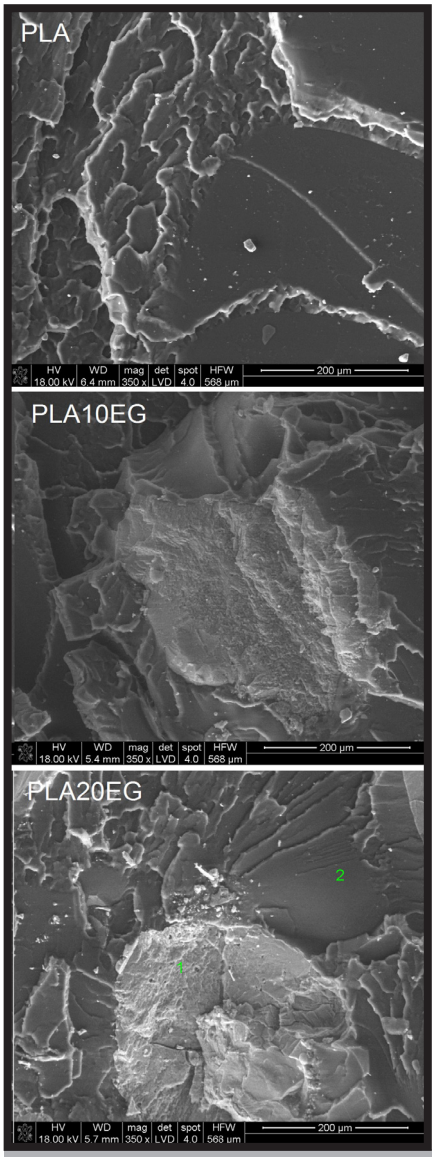


FIG. 10. SEM images of fracture surfaces of the samples after tensile test.

References

[1] S. Shuhadah, A.G. Supri: LDPE-Isophthalic Acid-Modified Egg Shell Powder Composites (LDPE/ESP$_i$). Journal of Physical Science 20 (2009) 87-98.
[2] J. Chłopek, A. Morawska-Chochół, B. Szaraniec: The influence of the environment on the degradation of polylactides and their composites. Journal of Achievements in Materials and Manufacturing Engineering 43 (2010) 72-79.
[3] A. D. P. Bankoff: Human Misculoskeletal Biomechanics, ed. dr. Tarun Goswami, 2012, www.intechopen.com, 07.06.0217
[4] T. Velnar, G. Bunc, L. Gradisnik: Fractures and Biomechanical Characteristics of the Bone. Surgical Science 6 (2015) 255-263.
[5] S. Ramakrishna, J. Mayer, E. Wintermantel et al.: Biomedical applications of polymer-composite materials: a review. Composite Science and Technology 61 (2001) 1189-1224.
[6] B.J. Tiimob, S. Jeelani, V.K. Rangari: Eggshell reinforced biocomposite – An advanced “green” alternative structural material. Journal of Applied Polymer Science 133 (2016) 431214.
[7] Y. Rudeekit, J. Numnoi, M. Tajan et al.: Determining Biodegradability of Polylactic Acid under Different Environments. Journal of Metals, Materials and Minerals 18 (2008) 83-87.
[8] K. Hamad, M. Kaseem, H.W. Yang et al.: Properties and medical applications of polylactide acid: a review. Express Polymer Letters 9 (2015) 435-455.
[9] T. Kasuga, H. Maeda, K. Kato et al.; Preparation of poly(lactic acid) composites containing calcium carbonate (vaterite). Biomaterials 24 (2003) 3247-3253.
[10] L. Xiao, B. Wang, G. Yang, M. Gauthier: Poly(Lactic Acid)--Based Biomaterials: Synthesis, Modification and applications, www.intechopen.com, 22.06.2017
[11] M.G. Petit, Z. Correa, M.A. Sabino: Degradation of a Polycaprolactone/Eggshell Biocomposite in a Bioreactor. Journal of Polymers and the Environment 23 (2015) 11-20.
[12] A. Asha, V.C. Sekhar: Investigation on the Mechanical Properties of Egg Shell Powder Reinforced Polymeric Composites. International Journal of Engineering Research&Technology 3 (2014) 288-291.
[13] J.S. Dhaliwal, G.S. Kapur, Shashikant: Chicken eggshell as biofiller in polypropylene composites. 4spepro.org, 26.04.2017
[14] Y. Srithep, D. Pholharn, L.-S. Turng, O. Veang-in: Injection moulding and characterization of polylactide stereo complex. Polymer Degradation and Stability 120 (2015) 290-299.
[15] M. Żenkiewicz, J. Richert: Synteza, właściwości i zastosowanie polilaktydu. Przetwórstwo Tworzyw 5 (2009) 192-199.

STUDIA PODYPLOMOWE
Biomateriały – Materiały dla Medycyny
2018/2019

Organizator: Akademia Górniczo-Hutnicza im. Stanisława Staszica w Krakowie Wydział Inżynierii Materiałowej i Ceramiki Katedra Biomateriałów i Kompozytów **Kierownik:** prof. dr hab. inż. Elżbieta Pamuła **Sekretarz:** dr inż. Małgorzata Krok-Borkowicz	**Adres:** 30-059 Kraków, Al. Mickiewicza 30 Pawilon A3, p. 208, 210 lub 501 tel. 12 617 44 48, 12 617 23 38, fax. 12 617 33 71 email: epamula@agh.edu.pl; krok@agh.edu.pl http://www.agh.edu.pl/ksztalcenie/oferta-ksztalcenia/studia-podyplomowe/biomaterialy-materialy-dla-medycyny/

Charakterystyka:
Tematyka prezentowana w trakcie zajęć obejmuje przegląd wszystkich grup materiałów dla zastosowań medycznych: metalicznych, ceramicznych, polimerowych, węglowych i kompozytowych. Słuchacze zapoznają się z metodami projektowania i wytwarzania biomateriałów a następnie możliwościami analizy ich właściwości mechanicznych, właściwości fizykochemicznych (laboratoria z metod badań: elektronowa mikroskopia skaningowa, mikroskopia sił atomowych, spektroskopia w podczerwieni, badania energii powierzchniowej i zwilżalności) i właściwości biologicznych (badania: *in vitro* i *in vivo*). Omawiane są regulacje prawne i aspekty etyczne związane z badaniami na zwierzętach i badaniami klinicznymi (norma EU ISO 10993). Słuchacze zapoznają się z najnowszymi osiągnięciami w zakresie nowoczesnych nośników leków, medycyny regeneracyjnej i inżynierii tkankowej.

Sylwetka absolwenta:
Studia adresowane są do absolwentów uczelni technicznych (inżynieria materiałowa, technologia chemiczna), przyrodniczych (chemia, biologia, biotechnologia) a także medycznych, stomatologicznych, farmaceutycznych i weterynaryjnych, pragnących zdobyć, poszerzyć i ugruntować wiedzę z zakresu inżynierii biomateriałów i nowoczesnych materiałów dla medycyny. Słuchacze zdobywają i/lub pogłębiają wiedzę z zakresu inżynierii biomateriałów. Po zakończeniu studiów wykazują się znajomością budowy, właściwości i sposobu otrzymywania materiałów przeznaczonych dla medycyny. Potrafią analizować wyniki badań i przekładać je na zachowanie się biomateriału w warunkach żywego organizmu. Ponadto słuchacze wprowadzani są w zagadnienia dotyczące wymagań normowych, etycznych i prawnych niezbędnych do wprowadzenia nowego materiału na rynek. Ukończenie studiów pozwala na nabycie umiejętności przygotowywania wniosków do Komisji Etycznych i doboru metod badawczych w zakresie analizy biozgodności materiałów.

Zasady naboru:
Termin zgłoszeń: od 20.09.2018 do 20.10.2018 (liczba miejsc ograniczona - decyduje kolejność zgłoszeń)
Wymagane dokumenty: dyplom ukończenia szkoły wyższej
Osoby przyjmujące zgłoszenia:
prof. dr hab. inż. Elżbieta Pamuła (pawilon A3, p. 208, tel. 12 617 44 48, e-mail: epamula@agh.edu.pl)
dr inż. Małgorzata Krok-Borkowicz (pawilon A3, p. 210, tel. 12 617 23 38, e-mail: krok@agh.edu.pl)

Czas trwania: 2 semestry (od XI 2018 r. do VI 2019 r.) 8 zjazdów (soboty-niedziele) 1 raz w miesiącu	**Opłaty:** 2 600 zł