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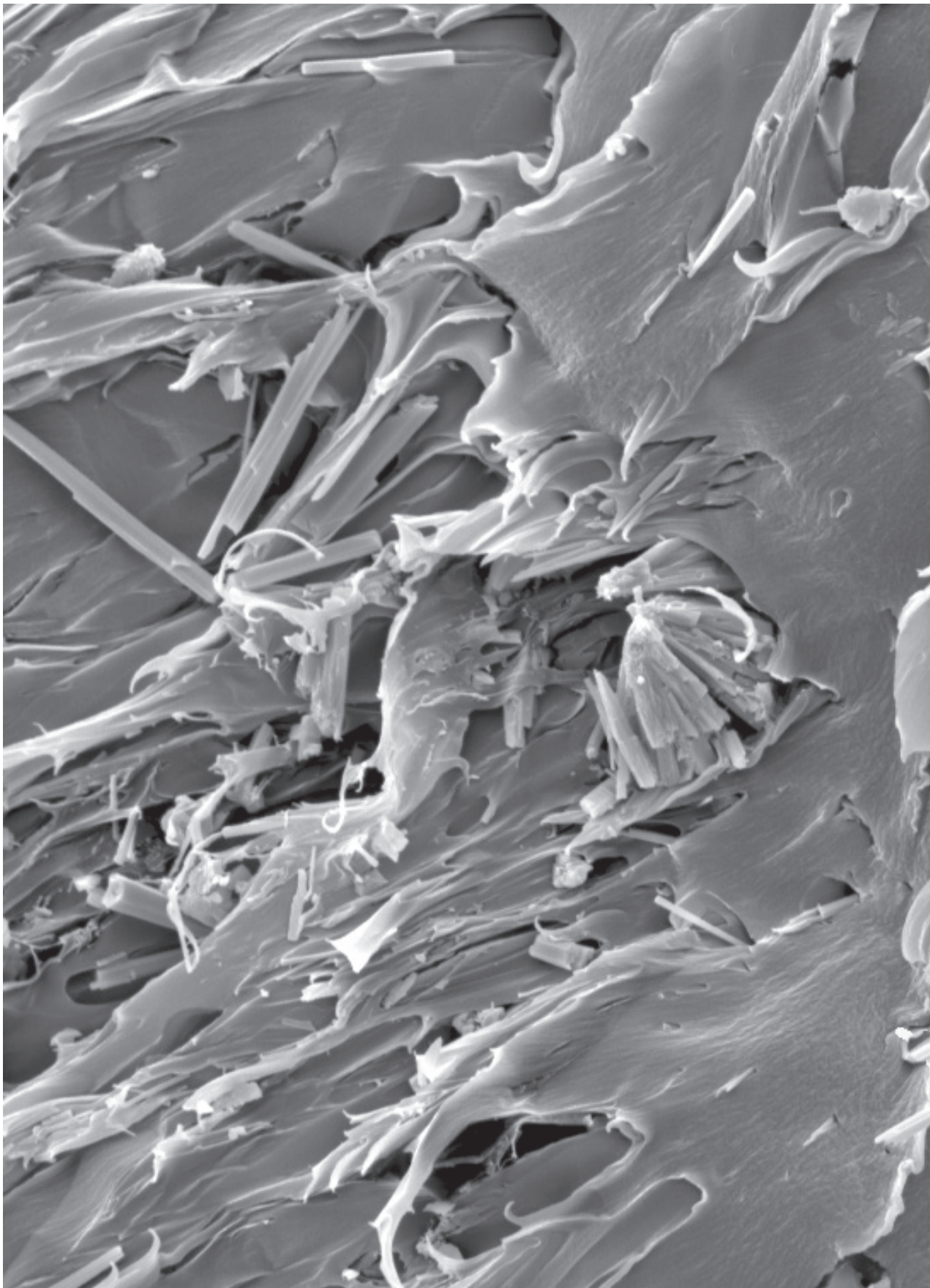

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ENGINEERING OF BIOMATERIALS

MODELING COMPUTATIONAL STUDIES OF MODIFIED DRUG MOLECULES BINDING TO THE LRRK2 PROTEIN IN THE TREATMENT OF PARKINSON'S DISEASE

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Abstract

Parkinson's disease is a neurodegenerative and progressive disease of the central nervous system. It affects more than 10 million patients worldwide and the symptoms allow for little to no control for movement. These symptoms appear because the chemical messenger dopamine is being made in small quantities from impaired cells. However, the disease often forms when there is a mutation in the LRRK2 gene, as the functions of the protein become abnormal. The IC50 value is essential information about molecules because it measures their effectiveness. The goal of this research was to design molecules with a lower IC50 value. This was first done by modeling molecules on the molecular modeling program, Gaussian 09. Modifications were made to molecules that were said to bind to the LRRK2 protein. Modifications ranged from adding a single atom or replacing atoms with groups. After running these molecules on the program, the total energy was found. Using the equation found from the correlation between 1/IC50 and the total energy, the IC50 value was predicted for each of the modified molecules. Many of the modified molecules portrayed a positive percent difference between the original IC50 value and the new one. This saves both time and money because the molecules with lower IC50 values can be made, preserving the resources. After creating the molecule with a low IC50 value, further experimental procedures can be taken; this is a large step in assisting researchers to reach a potential treatment because it is more efficient.

Keywords: *LRRK2 proteins, Parkinson, Computational Modeling, IC50, Density Functional Theory, DFT*

[Engineering of Biomaterials 145 (2018) 2-7]

Introduction

Parkinson's disease is a progressive, neurodegenerative disease that affects the central nervous system. A patient experiencing this disease suffers from a lack of movement and muscle control. More than 10 million people worldwide are affected by this and the United States receives about 60,000 cases per year [1]. Likewise, Parkinson's disease stands as the top 14th cause of death in the United States. Doctors are able to diagnose patients with Parkinson's if they are experiencing symptoms, such as bradykinesia, tremor, and rigidity. Although symptoms can be controlled using medication and surgical therapy, there is no standard treatment for Parkinson's disease [2].

The neurotransmitter, or chemical messenger, dopamine is responsible for controlling movement and emotions, including pleasure and pain. Those who are diagnosed with Parkinson's have impaired cells making dopamine. As this disease progresses, cells that are producing dopamine die and the brain eventually reaches a state in which no dopamine is being produced in a significant amount. This is the leading cause of abnormal movement as a symptom of Parkinson's disease [2].

The precise cause of Parkinson's is unknown, however research shows that it can be caused by genetic and environmental factors. It is known that the disease results from a loss of cells in the brain (substantia nigra). This area of the brain produces the neurotransmitter dopamine, which will transmit signals that allow for movement. A loss of this, as stated above, leads a patient to have no control over their movement. Some researchers have found that Parkinson's disease develops from a genetic mutation that is passed from generation to generation. It has been increasingly evident that the mutation occurs in the Leucine-Rich Repeat Kinase 2 (LRRK2) gene, causing it to produce an abnormal protein [3,4].

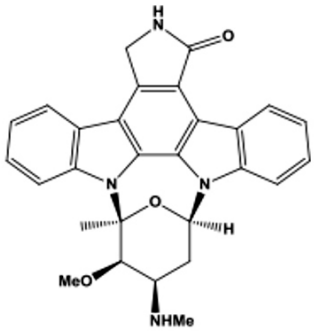
The LRRK2 gene is located on chromosome 12 and it gives proper instructions to make the LRRK2 protein as a kinase. It is made as a kinase to maintain a cell's ability to survive. However, mutations in this gene can make it hyperactive, causing cell death. A study illustrates that the LRRK2 gene is negatively affecting a set of proteins that originally function to traffic cargo. Thus, a mutation in this gene is shown to cause traffic jams in the cell [4].

Many organizations are working towards this specific issue from using drug therapy to using technology. They are focusing on the LRRK2 gene because it is responsible for a defect. The Michael J. Fox Foundation (MJFF) is taking a novel and therapeutic approach to develop a drug for the gene. They are studying the mutation and its surroundings and plan to take a further step by determining drugs that could bind to the active site of the protein [5][6]. Finding a balance that will lower the kinase activity allows the pharmaceutical industry to believe that this gene is drugable. The Dawson Duo, in a recent study, showed that regulating a low dose of anisomycin will help tremendously by blocking protein production. They further established that removing the phosphate group that attaches to the ribosomal s15 could prevent degeneration and brain cell death.

Several studies have shown that having a specific molecule bind to the LRRK2 gene may result in further progress towards creating a drug to bind to the protein in the treatment of Parkinson's disease. The plan of this research is to design new molecules that could potentially bind to the LRRK2 protein and to see how well this can be done by looking at the IC50 values [4]. The IC50 value is a measure of the effectiveness of a substance in inhibiting a specific biological or biochemical function or the concentration of a potential drug molecule with 50% inhibition; this allows the data to be quantified. The values will be found by using a correlation that was found in a previous study [4]. The new molecules designed can be more efficient by saving both time and money.

Original Molecules

A) Staurosporine



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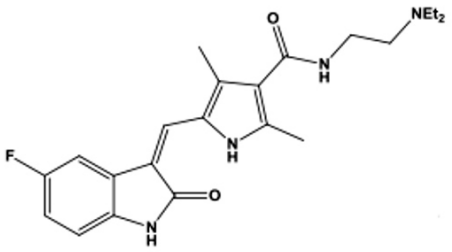
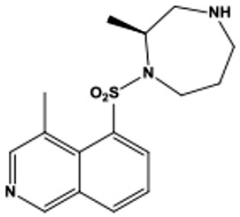
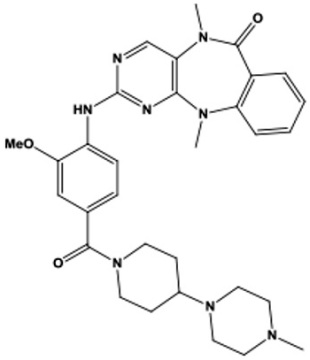


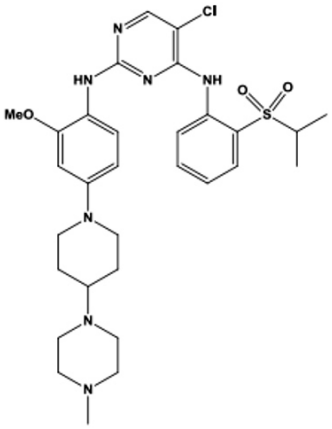
C) H1152



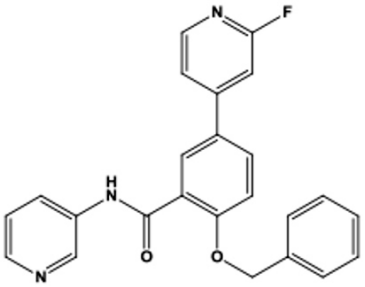
D) LRRK2-IN-1



E) TAE684



F) GSK2578215A



G) HG-10-102-01

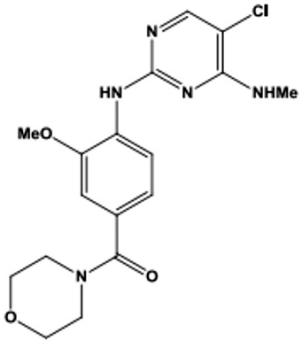


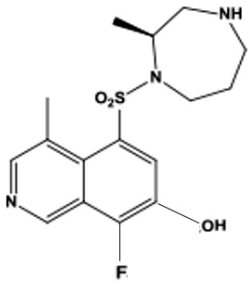
FIG. 1. These are all of the molecules that are modified and said to bind to the LRRK2 protein.

TABLE 1. The table displays all of the modifications made to specific molecules, as well as the IC50 values, the total energy, and the percent difference between the old IC50 value and the new one. The new IC50 values were found by using the strong correlation found in previous research. The top eight compounds that indicated a significant percent difference is highlighted by *.

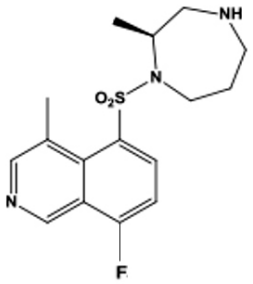
	Total Energy	1/IC50	IC50	Original IC50	Percent Difference
Staurosporine(1) - changed C to N	-1556.16	0.0338	29.57	40	29.98
* **Staurosporine(2)** - added =O	-1599.44	0.0380	26.30	40	41.32
* **Sunitinib(1)** - added a carboxyl	-1512.00	0.0295	33.86	79	79.99
* **Sunitinib(2)** - changed F to Cl	-1683.86	0.0462	21.64	79	113.99
* **H1152(1)** - added F	-1433.63	0.0219	45.61	244	137.01
* **H1152(2)** - added OH	-1409.61	0.0196	51.03	244	130.81
* **H1152(3)** - added F and OH	-1508.82	0.0292	34.22	244	150.80
LRRK2-IN-1(1) - added NH	-1923.65	0.0695	14.39	13	-10.16
LRRK2-IN-1(2) - added a carboxyl	-2056.80	0.0824	12.14	13	6.88
LRRK2-IN-1(3) - added OH^-	-1943.49	0.0714	14.00	13	-7.44
LRRK2-IN-1(4) - added F	-1967.48	0.0737	13.56	13	-4.23
* **LRRK2-IN-1(5)** - added Cl	-2327.88	0.1087	9.20	13	34.25
* **LRRK2-IN-1(6)** - added a carboxyl and Cl	-2516.36	0.1270	7.87	13	49.12
TAE684(1) - added methyl	-2674.10	0.1423	7.03	7.8	10.43
TAE684(2) - changed F to Cl	-2274.42	0.1035	9.66	7.8	-21.30
TAE684(3) - added a carboxyl	-2823.26	0.1568	6.38	7.8	20.06
TAE684(4) - added a carboxyl and methyl	-2862.57	0.1606	6.23	7.8	22.44
TAE684(5) - added OH	-2709.96	0.1458	6.86	7.8	12.84
TAE684(6) - added NH	-2690.11	0.1439	6.95	7.8	11.51
TAE684(7) - added OH, NH, carboxyl, and methyl	-2993.06	0.1733	5.77	7.8	29.90
GSK2578215A(1) - added a carboxyl	-1528.00	0.0311	32.17	10.9	-98.77
GSK2578215A(2) - added O_2S	-1848.64	0.0622	16.08	10.9	-38.38
GSK2578215A(3) - added NH	-1394.84	0.0182	55.06	10.9	-133.90
GSK2578215A(4) - added OH	-1414.69	0.0201	49.78	10.9	-128.15
GSK2578215A(5) - changed F to Cl	-1699.86	0.0478	20.94	10.9	-63.05
HG-10-102-01(1) - changed C to NH	-1635.02	0.0415	24.11	20	-18.65
HG-10-102-01(2) - added methyl	-1658.35	0.0437	22.86	20	-13.37
HG-10-102-01(3) - added carboxyl	-1807.54	0.0582	17.18	20	15.18
HG-10-102-01(4) - added OH	-1694.22	0.0472	21.18	20	-5.73
HG-10-102-01(5) - added NH	-1674.37	0.0453	22.08	20	-9.88

Top Modified Molecules

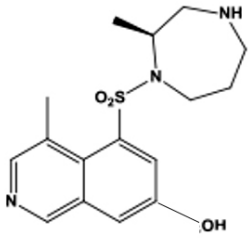
A) H1152(3)



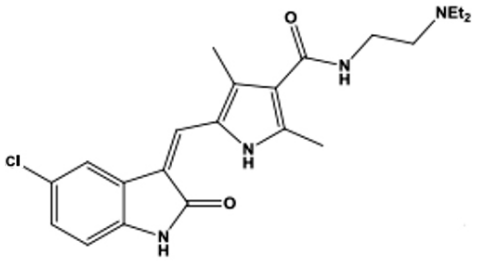
B) H1152(1)



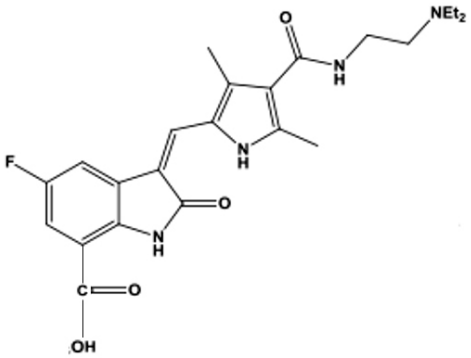
C) H1152(2)



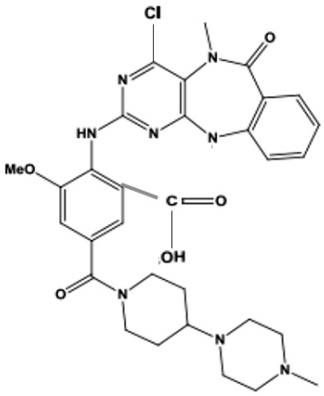
D) Sunitinib(2)



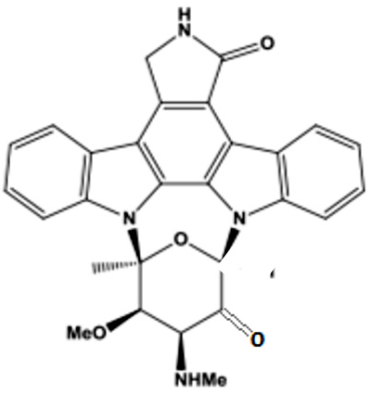
E) Sunitinib(1)



F) LRRK2-IN-1(6)



G) Staurosporine(2)



H) LRRK2-IN-1(5)

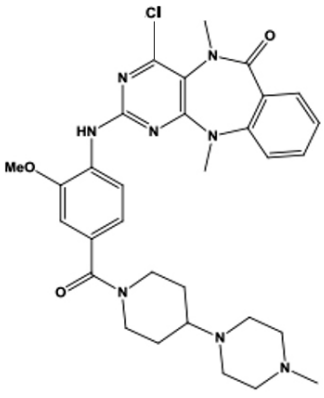


FIG. 2. These are the top eight modified molecules with the greatest percent difference between the original IC50 value and the new IC50 value (ordered with decreasing percent difference).

Materials and Methods

Designing and Modeling New Molecules

A strong correlation has been found in a previous study between the 1/IC50 of molecules that are said to bind to the LRRK2 protein and the total energy of those molecules [7]. This research aims to use this correlation to design new molecules with significantly improved IC50 values. The molecules that were used were modified in this research. Modifications consisted of adding a certain group or element, such as adding a methyl, carboxyl acid, OH, fluorine, chlorine, etc. In order to modify a compound, the original compound was opened in the Gaussian 09 program and then the corresponding group or element that needed to be added was performed. The proper bonds were made as well. After modifying the compound, the Gaussian 09 program was used under the DFT and 6-38G settings; the total energy of the modified compound was recorded. A total of 30 new molecules were designed.

Analysis - Determining the New IC50 Value

Using the total energy of each molecule in the previous step, a table was made. The table listed the name of the original compound, the total energy, the original IC50, the modified IC50 (which was calculated using the equation from the graph "1/IC50 vs. Total Energy"), and the percent difference. The equation that was used was y = -10304x - 1207.7, where y represents the total energy and x represents the 1/IC50 value. FIG. 1 represents the top eight compounds that illustrated the highest percent difference, which significantly lowered the IC50 value of the molecule before it was modified.

Results

In order to determine the IC50 value of the modified molecules, a correlation that was previously found (between 1/IC50 and total energy) was used [7]. The equation given from the line of best fit allowed the total energy to be plugged in and the IC50 value to be solved. The molecules that were modified were chosen because research has shown that they will bind to the active site of the LRRK2 protein [8]. The total energies of the new and modified molecules were found using the computation molecular modeling program, Gaussian 09. Each molecule was modeled and ran under the density functional theory (DFT) method of this program. This method was used because it examines the electronic structure of the molecule, allowing the total energy to be utilized. This research plans to take a further step in targeting the goal of binding a molecule/drug to the active site of the LRRK2 protein; thus, the strength of a binding affinity is essential information. This is because the binding affinity is the capability of ligands to form bonds with a reception. Because the total energy is an interactive force of attraction that can affect the strength, it can be related to the IC50 value. Additionally, the IC50 value is necessary information because it will tell the effectiveness of a substance; predicting the IC50 value before making a molecule is more efficient because only the better ones can be made, saving both time and money.

TABLE 1 summarizes the results of the modifications to the original structures, which include the percent difference is the difference between the original IC50 value and the new IC50 value. Most of the molecules showed a positive percent difference, whereas some had a negative percent difference. A positive percent difference means that the new IC50 value was better and a negative percent difference means that the new IC50 value was worse. The top 4 compounds had a percent difference in the range of 113-150% and they include the following: Sunitinib (Mod. 2), H1152 (Mod. 1), H1152 (Mod. 2), and H1152 (Mod. 3). This illustrates a significant change in the molecules IC50 value, displayed in FIG. 2 and TABLE 1. As a result, these molecules could be made with the modification because the IC50 value was significantly better. For example, after adding a fluorine atom and a hydroxyl group to the H1152 molecule, the percent difference was 150.80% because the IC50 value went from 244 to 34.22.

Some of the molecules that were modeled had a positive percent difference that was very low. This was because the original IC50 value was already significantly low. For example, when adding a hydroxyl group, a NH, a carboxyl, and a methyl to the TAE684 molecule, the IC50 value went from 7.8 to 5.77. This is important because a low original IC50 value decreased to an even lower number. Despite this, some modifications to molecules did not result in a positive percent difference at all, such as GSK2578215A(1) and parts of HG-10-102-01(5). If we are able to find molecules with more efficient mechanisms for treatment of the negative symptoms of Parkinson's disease, life for those afflicted with this disease could be vastly improved [10].

Discussion

A mutation in the LRRK2 protein causes a change in function, resulting in Parkinson's disease [9]. This research designed new molecules and found the new IC50 value that it had by using a correlation that was found in previous research between the total energy and the 1/IC50 value. Finding the new IC50 value as was done in this research can save time and money because resources don't have to be spent for every molecule made; rather only the ones with positive percent differences can be made to be tested.

In computational molecular modeling, finding a reference when creating new molecules is necessary and helpful. Consequently, one of the biggest applications of this project is the fact that it can be used as a reference for future procedures. This research designs new molecules that can bind to the active site of the LRRK2 protein. After running the modified molecules on the Gaussian 09 program, the total energy can be found. The total energy that was found gives the y-value of the equation that is used. After plugging in the y-value, the x-value can be found by solving for it. Because the definition of IC50 states the concentration of a potential drug molecule with 50% inhibition, the lower the value, the stronger the interaction. For the graph with the outcome of a strong correlation, the x-axis is "1/IC50." This means that the highest value of 1/IC50 produces the lower value of IC50. Therefore, the graph can be used to estimate an approximate IC50 value, increasing efficiency and saving time. If the IC50 value is good, then the experimental procedures can be taken in the future.

Each molecule that was modified had a different effect on the IC50 value. It is plausible that the sole reason for this depended on the effects of the new atoms or groups that were added or replaced. FIG. 1 illustrates the original molecules and FIG. 2 illustrates the top eight modified molecules when regarding the percent difference. Examples of modifications included adding fluorine or chlorine atoms or adding carboxyl or hydroxyl groups. Furthermore, the placement (ortho, meta or para) of each of these atoms and groups could create a larger effect. However, this is countered with the fact that modifications to some molecules always lead to a positive percent difference, such as shown in FIG. 2 with the H1152 molecule.

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BLENDS OF POLY(L-LACTIDE) AND POLY(L-LACTIDE-CO-TRIMETHYLENE CARBONATE) AS PROMISING MATERIALS FOR BONE AND CARTILAGE TISSUE ENGINEERING

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Abstract

The aim of this work was to evaluate the properties of polymer blends of poly(L-lactide) and poly(L-lactide--co-trimethylene carbonate) 15/85 and to select two compositions to be used to prepare the materials for bone and cartilage tissue engineering. PLLA and PLLATMC as the polymers with significantly different mechanical properties could be used to prepare a wide range of polymer blends. They were investigated by ultimate tensile strength and Young modulus measurements. Considering similar mechanical properties to these types of human tissues PLLA:PLLATMC 80:20 and 30:70 blends have been chosen as appropriate materials for bone and cartilage engineering, respectively. The materials were examined by thermal analysis (thermogravimetry and differential scanning calorimetry), ATR-FTIR spectroscopy and surface analysis (roughness and contact angle measurements). Miscibility of two polymers was discussed. High thermal stability of the blends allow to process them by fused deposition modelling which is one of the most promising methods for the manufacturing of computationally designed scaffolds. Based on the results of thermal analysis at least partial miscibility of PLLA and PLLATMC in the examined blends is indicated. Detailed interpretation of ATR-FTIR spectra let to distinguish the polymers despite their structural similarities. Surface properties of the materials depend on the preparation method and on their form.

Keywords: *poly(L-lactide); poly(L-lactide-co-trimethylene carbonate); polymer blends; mechanical properties; biomaterials*

[Engineering of Biomaterials 145 (2018) 8-15]

Introduction

Most of the materials for bone and cartilage regeneration are prepared on the base of natural and synthetic polymers [1]. Natural polymers like collagen [2-3], fibrin [4-5], chitosan [6-7], alginates [8-9] and hyaluronates [10-11] are used in the form of hydrogels as the carriers of growth factors and cells. The advantage of applying natural polymers is a simple homogeneous distribution of cells, medicines and other bioactive substances. Synthetic polymers including polylactide, polyglycolide and their copolymers [12-14] are applied in the form of fibres or three-dimensional structures as scaffolds for cell growth.

Preparation of a polymer blend as two- or multi-component physical mixture of homopolymers, copolymers or both of them is one of the basic method used for production of new materials. Polymers could be modified by addition of small amounts of other components to achieve desired properties, or they could be mixed in various ratios to produce blends with properties significantly different from these of the individual components [15-16]. Homogeneity of new materials is an important issue so polymer miscibility should be analyzed in detail [17-18]. Biodegradable polymer blends are often used to prepare materials for tissue regeneration with precisely adjusted properties [19].

The most commonly used polymers for bone and cartilage regeneration are polylactide blends [20] including blends with polycaprolactone [21-22], polyglicolic acid or poly(lactic-co-glycolic acid) [23-24], poly(ethylene glycol) [25-26], etc. Blends of polylactide and poly(trimethylene carbonate) [27-28] dedicated for biomedical applications [29-30] are only described by few authors. The aim of this research was to evaluate the properties of the new poly(L-lactide) and poly(L-lactide-co-trimethylene carbonate) blends with potential application in bone and cartilage tissue engineering.

Materials and Methods

Materials

Semi-crystalline poly(L-lactide) sold under the trade name BIOCOP® PLLA Sn free (PLLA) and amorphous poly(L-lactide-co-trimethylene carbonate) 15/85 sold under the trade name BIOCOP® PLLATMC 15/85 (PLLATMC) were purchased from BioMatPol, Poland. They were medical-grade polymers synthesized with non-toxic compounds. Chemical structure of the polymers is presented in FIG. 1. TABLE 1 summarizes their basic properties according to the manufacturer. Dichloromethane (≥99.5%) was purchased from Avantor Performance Materials, Poland.

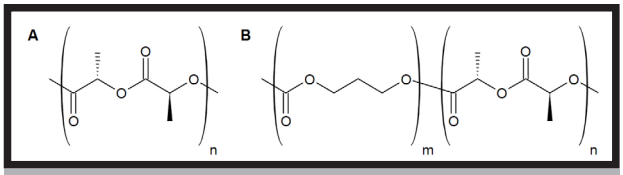

FIG. 1. Chemical structure of PLLA (A) and PLLATMC (B).

TABLE 1. Basic properties of PLLA and PLLATMC.

polymer	PLLA	PLLATMC
number average molar mass M_n [g/mol]	160000	60000
dispersion coefficient D	2.1	2.0

Poly(L-lactide) (PLLA) and poly(L-lactide-co-trimethylene carbonate) 15/85 (PLLATMC) blends were prepared by polymer solution casting. For this, an appropriate amount of each polymer for a total of 4.0 g was added to 40 mL of dichloromethane. The polymers were combined in the following ratios: 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100. The mixtures were stirred at room temperature until homogeneous solution was obtained. Then, resulting solutions were carefully poured on Petri dishes (10 cm in diameter) and left overnight to dry at room temperature. Prepared films were peeled off and two different sides, air-facing and plate-facing, were marked.

Methods

Ultimate tensile strength and Young's modulus were determined using Zwick 1435 (Zwick Roell, Germany) universal testing machine. 75 mm x 10 mm foils placed in the steel clamps with a distance of 50 mm were analyzed at an extension rate of 5 mm/s until the fracture occurred. Five measurements were conducted for each sample.

Thermogravimetry (TG/DTG) in the temperature range of 25 to 600°C were performed with Netzsch STA 449 F3 Jupiter (Netzsch, Germany). 10 mg samples were analyzed at a heating rate of 10°C/min in an inert atmosphere of 99.999% nitrogen with a flow rate of 40 mL/min. Differential scanning calorimetry (DSC) analysis in the temperature range of -50 to 200°C were conducted with Mettler Toledo DSC 1 (Mettler Toledo, Switzerland). 5 mg samples were analyzed at a heating rate of 10°C/min in an inert atmosphere of 99.999% nitrogen with a flow rate of 30 mL/min.

Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra were collected at room temperature in a range from 4000 to 400 cm^{-1} after 256 scans at 2 cm^{-1} resolution using Vertex V70 (Bruker, USA) with ZnSe crystal.

Two different sides of the films were examined and photographed under an Olympus BX41 (Olympus, Japan) light microscope at magnification of 100 x.

Both sides of the films were characterized by surface roughness and contact angle analysis. Surface roughness measurements were conducted with Hommel Tester T1000 (Jenoptik, Germany). Water contact angle measurements were performed with Kruss DSA10 MK2 drop shape analyzer (Kruss, Germany) using goniometer method and UHQ water (0.23 ± 0.05 µl volume of the drop). Each parameter was calculated as an average of ten measurements.

Results and Discussions

Poly(L-lactide) is a relatively hard and brittle material whereas poly(L-lactide-co-trimethylene carbonate) is an elastomer. Mixing them can lead to blends with different mechanical properties. Whole range of the compositions has been investigated to obtain blends with mechanical properties similar to bone or cartilage tissue. Young's modulus and tensile strength of the blends are presented in FIG. 2, and compared with bone and cartilage tissue properties in TABLE 2. As composition of 80 wt.% of PLLA and 20 wt.% of PLLATMC showed Young's modulus similar to bone tissue, and composition of 30 wt.% of PLLA and 70 wt.% of PLLATMC was similar to cartilage tissue, further research was performed using these two compositions. It should be pointed out that both of them withstand higher loads than the discussed natural tissues.

Mechanical properties measurements

Dependence of ultimate tensile strength and Young's modulus on blends composition are presented in FIG. 2. These parameters were significantly different for PLLA and PLLATMC, so a vast range of the materials with intermediate properties as blends of these polymers could be prepared. PLLA was a semi-crystalline rigid and brittle polymer. Measured ultimate tensile strength was 47.12 ± 0.98 MPa and Young's modulus was 2485 ± 153 MPa. PLLATMC was an amorphous ductile polymer. Ultimate tensile strength was 0.31 ± 0.02 MPa and Young's modulus was 0.64 ± 0.14 MPa, so it was two and four order of magnitude lower, respectively, in comparison with PLLA.

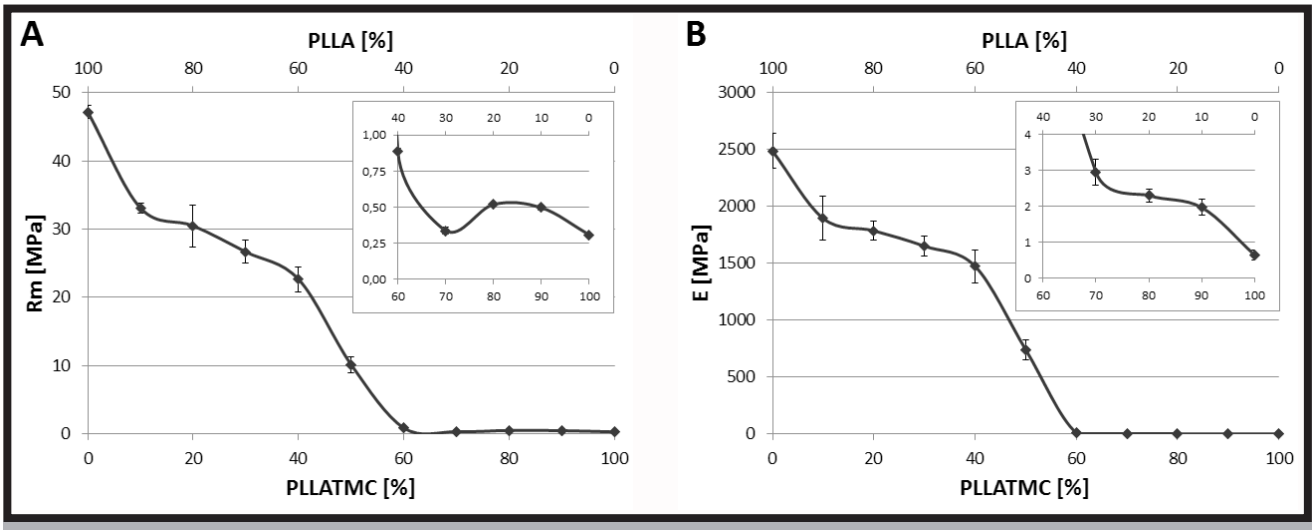

FIG. 2. Dependence of ultimate tensile strength Rm (graph A) and Young's modulus E (graph B) on blend composition.

TABLE 2. Mechanical properties of the selected blends of PLLA/PLLATMC and human bone and cartilage.

	PLLA:PLLATMC 80:20	Bone (wet) [Yamada]	PLLA:PLLATMC 30:70	Cartilage [Yamada]
Tensile strength [MPa]	30.49 ± 3.06	14.0	0.34 ± 0.02	0.29
Young's modulus [MPa]	1786 ± 81	1830	2.95 ± 0.36	2.5

PLLA and PLLATMC blends with a various ratios of both components were analyzed to select two compositions with mechanical properties appropriate to prepare materials for bone and cartilage tissue engineering. Two ranges of the mechanical properties could be seen in FIG. 2. In the first one (PLLA:PLLATMC from 90:10 to 60:40) ultimate tensile strength and Young's modulus were similar to PLLA and changed linearly with blend composition according to the following equations (where X is the content of PLLATMC [%] and R is the correlation coefficient):

$$Rm = -0.3508 \cdot X + 36.997 \quad (R^2 = 0.9910)$$

$$E = -13.969 \cdot X + 2050 \quad (R^2 = 0.9876)$$

They could be used to define a composition and to prepare a material with desired properties. In the second range (PLLA:PLLATMC from 40:60 to 10:90) both parameters were similar to PLLATMC but, contrary to the first one, a linear changes were not observed.

Mechanical properties of the human tissues were described in detail by Yamada (1970) [31]. In that review an average ultimate tensile strength for bone (wet) and cartilage were specified as 14.0 MPa and 0.29 MPa, respectively, and an average Young's modulus as 1830 MPa and 2.5 MPa, respectively. Two compositions of polymer blends, namely PLLA:PLLATMC 80:20 and 30:70, have been chosen as blends appropriate to prepare materials for bone and for cartilage tissue engineering, respectively. For 80:20 blend Young's modulus was similar to human bone tissue and ultimate tensile strength was almost two times higher whereas for 30:70 blend both parameters were slightly higher in comparison to cartilage tissue. The results are summarized in TABLE 2.

Thermal analysis

Thermal properties of the investigated blends are important from the point of view of their processing, filament fabrication, and 3D printing. TG and DTG curves of polymers (PLLA and PLLATMC) and polymer blends (PLLA:PLLATMC 80:20 and 30:70) are given in FIG. 3 and FIG. 4, respectively. Typical effects of decomposition of both polymers were observed. At measurement conditions degradation process started about 260°C and ended about 360°C. Based on the shape of curves parameters (onset, midpoint, endset, mass change) were determined and collected in TABLE 3. High thermal stability of both components as well as their blends in the temperature range of 20 to 260°C allows for their processing at about 200°C (fused deposition modelling).

DSC curves of PLLA and PLLATMC are shown in FIG. 5. PLLA as a semi-crystalline polymer had a melting peak with maximum at 177°C and enthalpy change of -42.82 J/g. According to Pyda et al. (2004) [32] enthalpy of crystallization for 100% crystalline PLLA is 91±3 J/g, and based on this value a degree of crystallinity for the sample was estimated as 47%. Glass transition at 46°C was also observed. For PLLATMC as an amorphous polymer the only observable effect was glass transition at -7°C. DSC curves of the blends presented in FIG. 6 revealed three effects. The first glass transition (at -4°C and at -11°C for 80:20 and 30:70 blends, respectively) can be associated with a presence of PLLATMC. The second glass transition (at 49°C both) and melting peak (at 180°C and at 177°C, respectively) were related to the presence of PLLA. All of observed thermal effects are described in TABLE 4.

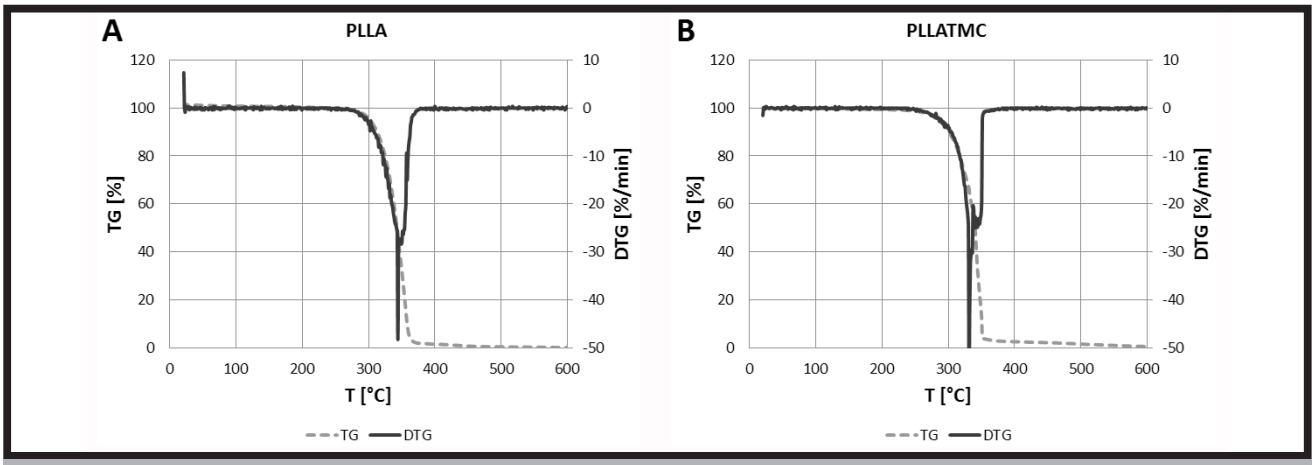

FIG. 3. TG and DTG curves of PLLA (graph A) and PLLATMC (graph B).

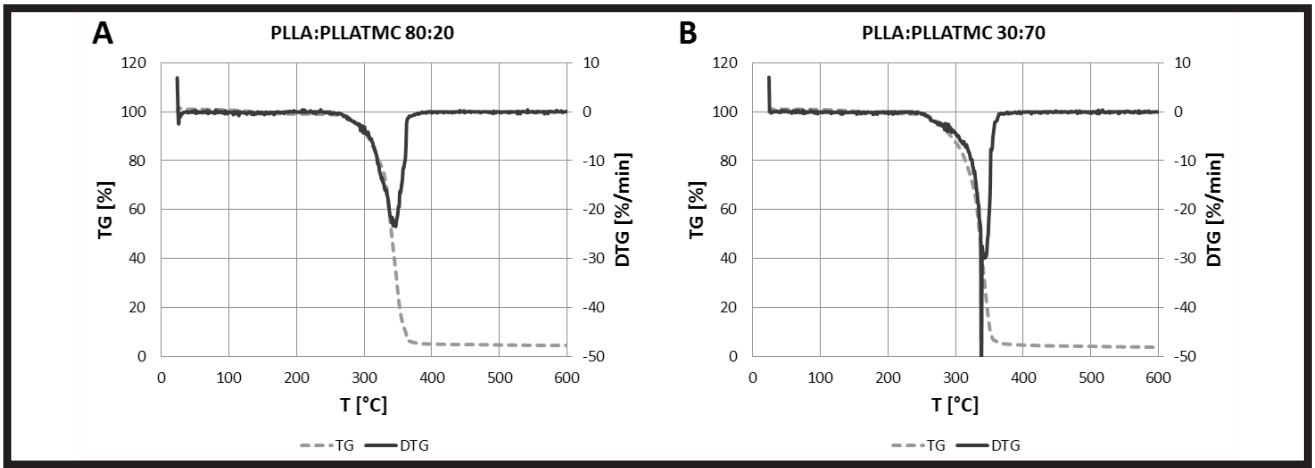

FIG. 4. TG and DTG curves of PLLA:PLLATMC 80:20 (graph A) and PLLA:PLLATMC 30:70 (graph B).

TABLE 3. Results of TG/DTG measurements for decomposition of the samples.

PLLA:PLLATMC	100:0	80:20	30:70	0:100
onset [°C]	323	320	318	327
midpoint [°C]	342	339	335	340
endset [°C]	362	358	352	352
mass change [%]	-98.2	-94.7	-94.8	-97.0

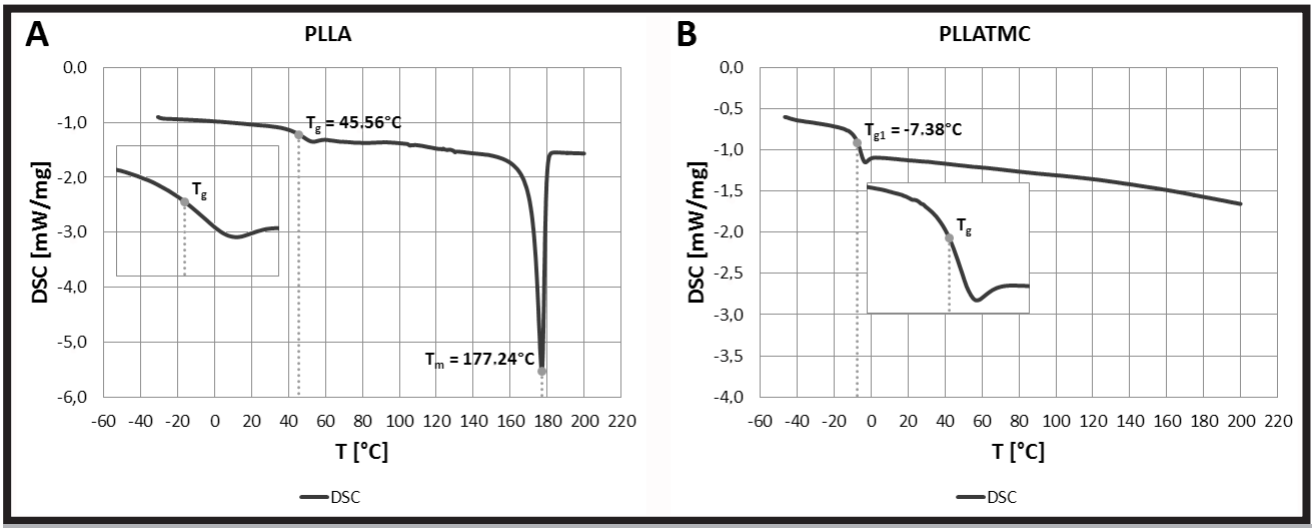

FIG. 5. DSC curves of PLLA (graph A) and PLLATMC (graph B) in the temperature range of -50 to 200°C.

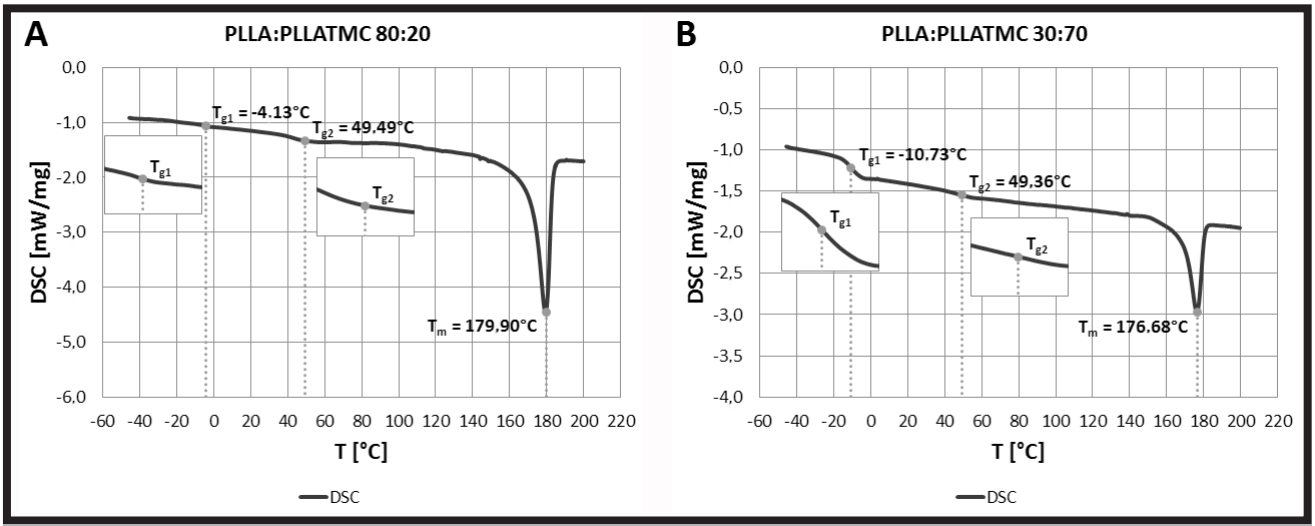

FIG. 6. DSC curves of PLLA:PLLATMC 80:20 blend (graph A) and PLLA:PLLATMC 30:70 blend (graph B) in the temperature range of -50 to 200°C.

TABLE 4. Results of DSC measurements of the samples.

PLLA:PLLATMC		100:0	80:20	30:70	0:100
glass transition I	T [°C]	-	-4.13	-10.73	-7.38
	ΔCp [J/(g·K)]	-	0.030	0.430	0.531
glass transition II	T [°C]	45.56	49.49	49.36	-
	ΔCp [J/(g·K)]	0.297	0.210	0.150	-
melting	T [°C]	177.24	179.90	176.68	-
	ΔH [J/g]	-42.82	-43.28	-15.14	-

Glass transition temperature is a very useful factor to assess miscibility of polymers. Full miscibility of the components of polymer blend is recognized by a single glass transition temperature. It can be calculated according to the Fox equation where T_{g1} and T_{g2} are glass transition temperatures of pure polymers, and w_1 and w_2 are weight fractions of the components in the blend:

$$\frac{1}{T} = \frac{W_1}{T_{g1}} + \frac{W_2}{T_{g2}}$$

In the case of full miscibility of PLLA and PLLATMC single glass transition temperatures at 33.35°C and 6.56°C should be observed for 80:20 and 30:70 blends, respectively. Detection of two values of glass transition temperature for polymer blends can be an evidence for phase separation of the components. However, the T_g values observed for the blends are different from these obtained for pure polymers. They also vary depending on weight ratio of the components. Glass transition assigned to PLLA was 49°C for both blends, while pure PLLA exhibited glass transition at 45°C. Higher T_g in the blends suggests that PLLA in its amorphous phase is packed more tightly than in pure polymer and needs more energy to increase the interchain distances. Glass transition of PLLATMC in 80:20 and 30:70 blends was observed at -4°C and -10°C, respectively, comparing to -7°C in pure polymer. It shows that PLLATMC as elastic copolymer is much more sensitive to the addition of rigid PLLA. It seems that relatively small amounts of PLLA (in our studies 30%) in the blend help the PLLATMC to achieve the elastic state, while excess of PLLA makes it more difficult. Most probably the distribution of polymer molecules and the interchain interactions play a significant role in glass transition. In the case of our studied system both components possess lactide units in their molecules that should induce better miscibility. However, high content of PLLA causes stronger interactions with PLLATMC molecules hindering their movements. This is why the T_g of PLLATMC in 80:20 blend is higher than in pure polymer. On the other hand, low content of PLLA interacting with lactide units of PLLATMC helps in creation of crystalline rigid segments in elastomer and microphase separation. No crystalline phase (melting) is observed in pure PLLATMC, however it is expected in thermoplastic copolymers like the investigated one.

Glass transition width as a difference between endset and onset temperatures is the other parameter used to evaluate the miscibility of polymer blend components. It is described to be about 6°C for pure homopolymers, about 10°C for fully miscible polymers and over 32°C for immiscible polymers. Width of glass transition assigned to PLLA was 8°C for pure, 13°C for 80:20 blend and 15°C for 30:70 blend and assigned to PLLATMC was 4.5°C, 2.5°C and 10°C, respectively. These values could indicate at least a partial miscibility of PLLA and PLLATMC in 80:20 and 30:70 blends, which is in agreement with the discussion on T_g changes.

ATR-FTIR analysis

ATR-FTIR spectra of the samples are presented in FIG. 7 and detailed interpretation is summarized in TABLE 5. The chemical structure of PLLATMC as a 15/85 copolymer consists of carbonate moieties but contains also some amount of the same units as PLLA. Comparing the spectrum of PLLATMC to the spectrum of PLLA the most recognizable are peaks attributed with $-CH_2-$ rocking in an aliphatic chain of carbonate moiety at 925 cm^{-1} and 787 cm^{-1}. The most intense peaks in each spectrum were attributed to C=O stretching (at about 1735-1750 cm^{-1}), C-O asymmetric stretching (1230-1265 cm^{-1} and 1180-1195 cm^{-1}) and C-O-C symmetric stretching (1075 cm^{-1} and 1025-1040 cm^{-1}). Apart of the peak at about 1075 cm^{-1} characteristic absorption varied with the change of PLLA:PLLATMC ratio. For example for C=O stretching it decreased from 1747 cm^{-1} to 1736 cm^{-1} for 100:0 and 0:100 samples, respectively.

Surface characterization

Microscopic images of two different sides of the films are given in FIG. 8. Using polymer solution casting air-facing and plate-facing surfaces are formed as a result of solvent evaporation. Diverse morphology between them was clearly visible in the pictures. The most characteristic was air-facing side of 30:70 sample with noticeable inhomogeneity.

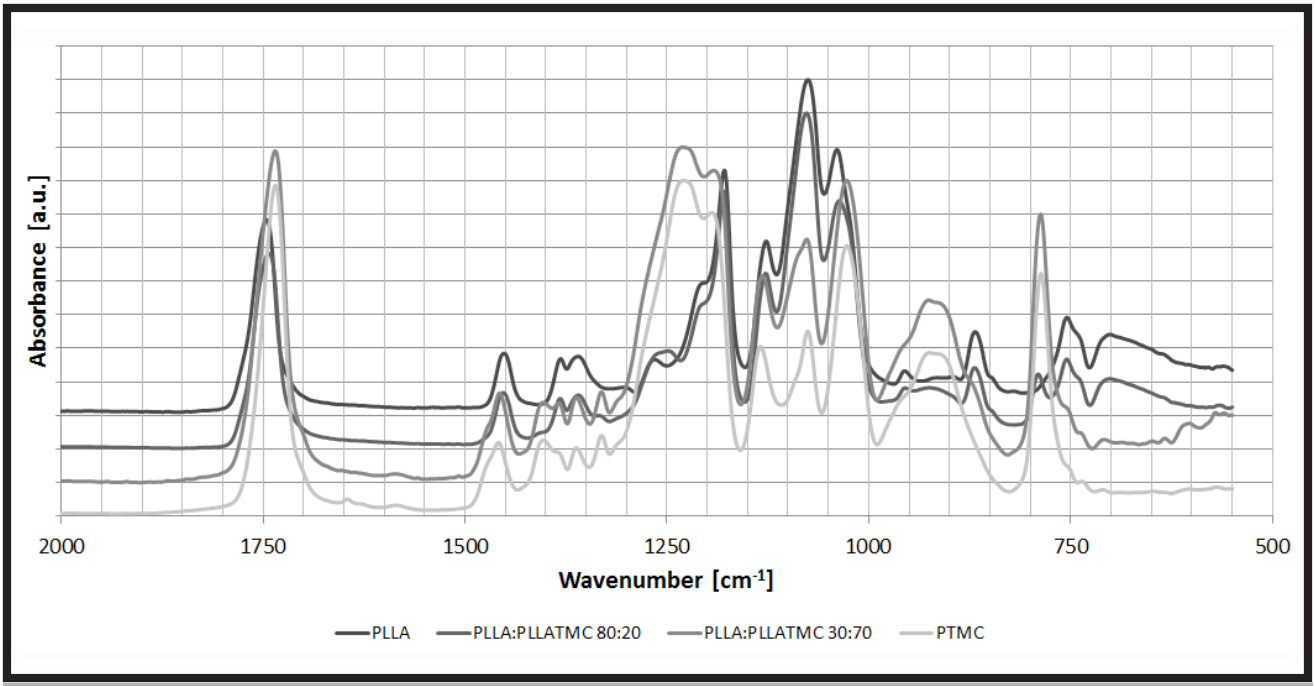

FIG. 7. ATR-FTIR spectra of PLLA, PLLATMC and PLLA:PLLATMC blends (80:20 and 30:70).

TABLE 5. Detailed interpretation of ATR-FTIR spectra of the samples (abbreviations: vs - very strong, s - strong, m - medium, w - weak, sh - shoulder, nv - not visible).

Type of vibration	Characteristic absorption [cm^{-1}] and intensity			
	PLLA	PLLA:PLLTMC		PLLATMC
		80:20	30:70	
C-H symmetric stretching (O-CHR- moiety)	2997 w	2997 w	2974 w	2974 w
C-H symmetric stretching (C-CH_3 moiety)	2943 w	2943 w	nv	2921 w
C=O stretching	1747 s	1744 s	1736 vs	1736 vs
CH_3 scissoring	1453 m	1453 m	1456 m	1458 m
CH_3 rocking	1382 m	1382 m	1403 m	1403 m
CH_3 rocking	1361 m	1359 m	1362 m	1362 m
-CH_2- rocking	nv	nv	1330 m	1330 m
C-O asymmetric stretching (O-C=O moiety)	1265 m	1249 m	1230 vs	1230 vs
C-O asymmetric stretching (O-CH_2- moiety)	1179 s	1179 s	1193 s sh	1194 s sh
C-C stretching	1127 s sh	1128 m	1132 m	1134 m
C-O-C symmetric stretching	1075 vs	1077 vs	1076 s	1075 m
C-O-C symmetric stretching	1039 s sh	1037 s sh	1028 s	1028 s
-CH_2- rocking (O-CH_2- moiety)	nv	nv	926 m	925 m
C-O stretching (O-CH(CH_3) moiety)	868 m	869 m	nv	nv
-CH_2- rocking (-$(CH_2)_n$- moiety)	nv	790 m	787 s	787 s
-CH_2- rocking (-$(CH_2)_n$- moiety)	754 m	754 m	nv	nv

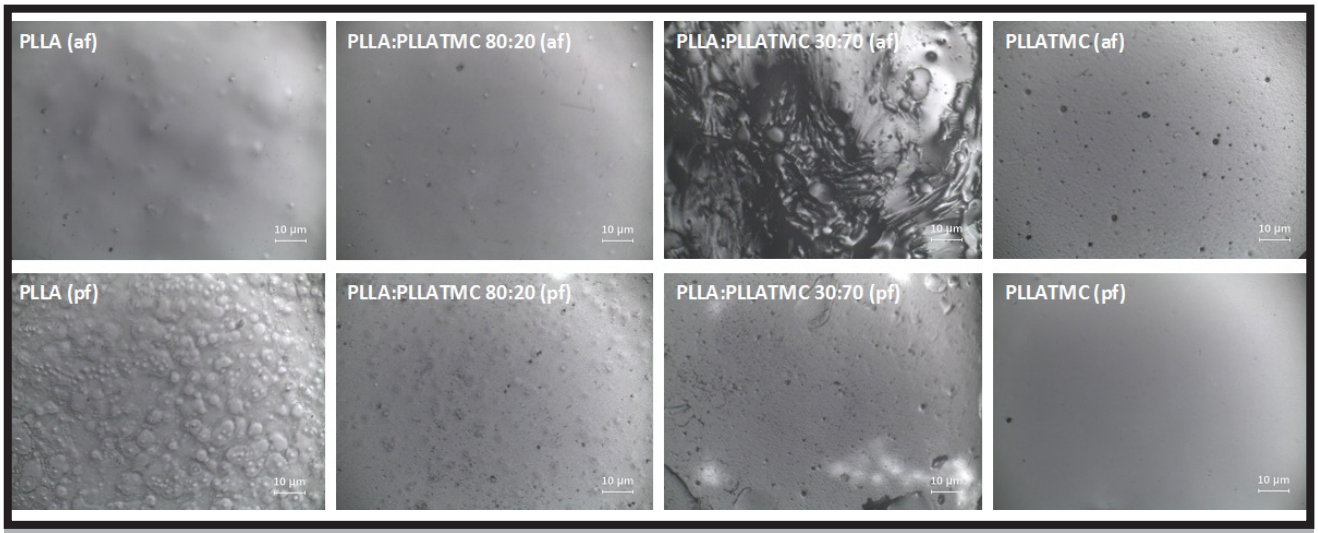

FIG. 8. Microscopic images of the air-facing (af) and plate-facing (pf) sides of the films.

TABLE 6. Roughness parameters (Ra - arithmetical mean height, Rt - total height of profile, Rz - maximum height of profile) of air-facing and plate-facing sides of the films.

PLLA:PLLATMC		100:0	80:20	30:70	0:100
Ra [μm]	air-facing	0.72 ± 0.10	0.36 ± 0.06	11.31 ± 1.41	1.36 ± 0.27
	plate-facing	0.55 ± 0.05	0.28 ± 0.07	10.52 ± 1.05	1.64 ± 0.36
Rt [μm]	air-facing	6.22 ± 1.23	2.34 ± 0.50	95.88 ± 12.54	15.31 ± 2.75
	plate-facing	4.31 ± 0.70	2.41 ± 0.54	87.67 ± 9.33	17.79 ± 3.19
Rz [μm]	air-facing	3.91 ± 0.65	1.69 ± 0.31	62.02 ± 5.85	7.85 ± 1.26
	plate-facing	3.25 ± 0.29	1.66 ± 0.23	55.31 ± 5.22	10.24 ± 1.54

TABLE 7. Water contact angle of air-facing and plate-facing sides of the films.

PLLA:PLLATMC		100:0	80:20	30:70	0:100
contact angle [°]	air-facing	82.7 ± 1.4	81.5 ± 0.5	75.4 ± 2.0	103.9 ± 1.4
	plate-facing	73.3 ± 1.8	74.8 ± 1.1	74.7 ± 1.4	104.1 ± 1.7

The results of surface roughness and water contact angle analysis are shown in TABLE 6 and TABLE 7, respectively. Roughness parameters of air-facing side were higher than plate-facing side for films of PLLA and two PLLA:PLLATMC blends in contrast to PLLATMC. The differences between both sides were a result of the gradient formed by solvent evaporation during preparation of the films. For 30:70 PLLA:PLLATMC film roughness parameters were one order of magnitude higher compared to the others. This could be a result of the lower miscibility of both components in this polymer blend. For PLLA and 80:20 sample water contact angle of air-facing sides was higher than these of plate-facing sides, while for PLLATMC and 30:70 sample it was similar. The measurements were affected by differences in surface roughness of the samples, and contact angles were the lowest for 30:70 sample with the highest roughness.

Conclusions

Biodegradable polymers like poly(L-lactide) and poly(L-lactide-co-trimethylene carbonate) 15/85 with significantly different mechanical properties are an appropriate materials to prepare a wide range of polymer blends for various applications. 80:20 and 30:70 weight ratios of PLLA to PLLATMC were chosen to develop materials for bone and cartilage tissue engineering, respectively. Importantly, a linear relationship between mechanical properties (ultimate tensile strength, Young's modulus) and composition for PLLA:PLLATMC ratios from 90:10 to 60:40 was observed. Thermal processing of the materials is possible in the temperature range from above 180°C when they melt up to 260°C when decomposition starts. The changes in glass transition temperatures of blends comparing to pure polymers suggest partial miscibility of the components. PLLA and PLLATMC in spite of structural similarity are discriminated by ATR-FTIR spectroscopy so the method could be used to investigate polymer blends. Depending on the preparation method surface properties of the materials (roughness and water contact angle) are different.

Fused deposition modelling (FDM) as one of the most promising method is proposed to be used to produce bone and cartilage scaffolds with a structure precisely described by a computer-designed model. Developed PLLA:PLLATMC 80:20 and 30:70 blends after processing to the form of a filament could be used in FDM 3D printers.

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IMPACT OF SINTERING TEMPERATURE OF HYDROXYAPATITE ON BIOLOGICAL AND PHYSICOCHEMICAL PROPERTIES OF ALGINATE/HA BIOMATERIALS FOR REGENERATIVE MEDICINE APPLICATIONS

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Abstract

Synthetic hydroxyapatite (HA) has gained considerable attention in regenerative medicine over recent decades. It is widely used as a bone filler and constituent of various biomaterials. HA possesses high biocompatibility, osteoconductivity, bioactivity, and bioresorbability. There are many different synthesis methods for HA described in the available literature. It is worth noticing that even slight changes in pH, reaction conditions or chemical composition during synthesis, can influence biological, physicochemical, and mechanical properties of resultant HA.

The aim of this study was to evaluate the impact of sintering temperature of hydroxyapatite on biological and physicochemical properties of biomaterial made of alginate and hydroxyapatite granules. Alginate/HA material was produced using HA sintered at temperature of 800°C and HA sintered at temperature of 1150°C. Microstructure of the fabricated biomaterials was visualized by SEM. Osteoblast growth on the composites was assessed using human foetal osteoblast cell line. Moreover, ion reactivity, plasma/serum protein adsorption ability as well as water/NaCl uptake capability of the biomaterials were compared.

Obtained results demonstrated that although both biomaterials had the same chemical composition, composite comprising hydroxyapatite sintered at temperature of 1150°C had smoother surface, revealed lower ion reactivity, was more favourable to osteoblast growth, and adsorbed lower amount of fibrinogen (which is known to promote biomaterial-induced inflammatory response), compared to the material made of hydroxyapatite sintered at temperature of 800°C. Thus, the type of bioceramics used for the production of biomaterials should be tailored to their specific applications – bone fillers for primarily in vivo implantation or in vitro cell-seeded scaffolds.

Keywords: *ion reactivity, bone scaffolds, alginate, water uptake*

[Engineering of Biomaterials 145 (2018) 16-19]

Introduction

Over recent decades, synthetic hydroxyapatite (HA) has gained considerable attention as an implantable material which is widely used in regenerative medicine as a bone filler and component of various biomaterials. HA is characterized by high biocompatibility, bioactivity, osteoconductivity, and bioresorbability [1,2]. A great variety of synthesis methods for HA have been published, e.g. dry methods, wet methods (i.e. co-precipitation, hydrolysis, emulsion, sol-gel), or microwave-assisted method [3]. It is important to note that slight changes in pH, chemical composition or reaction conditions during synthesis, can affect biological, physicochemical, and mechanical properties of resultant HA [2-4].

According to the available literature, an increase in the grain size, density and crystallite size is observed with increasing sintering temperature of bioceramics [1,2,4]. Moreover, ceramics sintered at high temperature (> 900°C) reveals low ion reactivity, poor bioactivity [5,6], and does not show cytotoxic effects against cells *in vitro* [5]. Whereas bioceramics sintered at low temperature (< 900°C) shows high ion reactivity [7], and thus may critically decrease Ca^{2+} and HPO_4^{2-} concentrations in the culture medium, reducing cell viability *in vitro* [4-6].

The goal of this study was to determine the effect of sintering temperature of HA on biological and physicochemical properties of a biomaterial made of alginate and hydroxyapatite granules. For this purpose, biomaterials with the same chemical composition (alginate and hydroxyapatite) were fabricated using HA sintered at low and high temperature. Then, liquid uptake behaviour, ion reactivity, plasma/serum protein adsorption ability as well as osteoblast growth on the biomaterials were compared.

Materials and Methods

Alginate/hydroxyapatite composites were produced by mixing 1.5 wt.% sodium alginate in distilled water (Sigma-Aldrich Chemicals) with 60 wt.% HA granules followed by material gelation in 0.2 M $CaCl_2$ (Avantor Performance Materials). Hydroxyapatite granules were fabricated according to the procedure described somewhere else [8-10] using sintering temperature of 800°C (alg/HA_L) and 1150°C (alg/HA_H).

Surface of the biomaterials was visualized using scanning electron microscopy (Nova NanoSem 200). Cell culture test was performed using human foetal osteoblast cell line (hFOB 1.19, ATCC) cultured in DMEM/Ham F12 medium without phenol red, and supplemented with 10% foetal bovine serum (Pan-Biotech). Before cell culture experiment, sterile biomaterials (cut into discs approximately 1 mm thick and 8 mm in diameter) were placed in 48-well plate and preincubated for 24 h in a complete culture medium at 34°C. Osteoblasts were seeded directly on the materials at a concentration of $1x10^5$ cells/sample and cultured for 24 h at 34°C in a 5% CO_2 humidified atmosphere. Cell growth on the biomaterials was assessed by fluorescent staining of nuclei with DAPI (Sigma-Aldrich Chemical) and F-actin filaments with AlexaFluor635phalloidin (Invitrogen) as described earlier [6]. Stained cells were observed under confocal laser scanning microscope (Olympus Fluoview equipped with FV1000).

Ion reactivity test was performed by placing the biomaterials in DMEM/Ham F12 medium for 15 days at 37°C as described earlier [6]. The culture medium was changed every 2-3 days to simulate procedure during long-term *in vitro* culture of the cells. The concentration of Ca^{2+} and HPO_4^{2-} ions in medium after incubation with biomaterials as well as in fresh culture medium (control) was determined by colorimetric method using commercially available kits for the detection of calcium and phosphate ions (BioMaxima).

The protein adsorption test was carried out according to the procedure described by Kar et al. [11]. Briefly, biomaterials were incubated in 250 µl of 25% human plasma and 25% human serum solutions (obtained from Blood Bank in Lublin) prepared in phosphate buffered saline (PBS, Sigma-Aldrich Chemical) for 3 h at 37°C on a shaker (125 rpm). The total amount of proteins in prepared plasma/serum solutions was determined before and after incubation with biomaterials using commercially available kit for the determination of total proteins in human serum/plasma (BioMaxima). The amount of proteins adsorbed to the biomaterials (P_a) was calculated using the formula: $P_a = P_0 - P_s$, where P_0 is the amount of proteins in the initial solution before incubation with the material (µg) and P_s is the amount of proteins left in the solution after incubation with the material (µg). The results were expressed as the amount of adsorbed protein (µg) per cm^2 of the material. The relative amount of fibrinogen adsorbed to the biomaterial surface (Fib) was determined using the formula $Fib = P_p - S_p$, where P_p is the quantity of adsorbed plasma proteins per material (µg/cm^2), S_p is the quantity of adsorbed serum proteins per material (µg/cm^2) [12].

Liquid uptake/retention ability of the biomaterials was assessed according to the procedure described by Venkatesan et al. [14] via immersing the samples in normal saline solution (0.9% NaCl) and deionised water at room temperature. At specific time intervals, the biomaterials were removed from the solutions in which samples were soaked and were weighed on an analytical balance. The test was carried out for 100 min. Liquid uptake behaviour of the biomaterials was expressed as the percentage of the weight increase (W_i) with time. W_i was calculated using the formula: $W_i = (W_t - W_0)/W_0 \times 100$ (W_0 – weight of dry material at time 0, W_t – weight of material at time t) [14,15]. Completely soaked samples were subjected to the determination of rehydration index (R_i), which was calculated using the formula: $R_i = (m_s - m_d)/(m_0 - m_d)$, where m_s is the weight of the completely soaked material, m_0 is the weight of the material in a wet state just after fabrication, and m_d is the weight of the dried material [16].

Obtained results were expressed as mean values ± standard deviation (SD). Statistical analysis of the results was performed using GraphPad Prism 5, Version 5.03 Software. The unpaired t-test was applied to assess statistical differences between tested group and control group. Statistical significance was considered at a probability of $P < 0.05$.

Results and Discussions

SEM images demonstrated that biomaterial comprising sintered at low temperature HA had noticeably more rough surface compared to the alg/HA_H material (FIG. 1a, b). It was probably due to high microporosity of HA granules, which is typical of sintered at low temperature bioceramics [4]. Although rough surfaces are considered to be more favourable to osteoblast adhesion [17], more cells were observed on the alg/HA_H biomaterial compared to the alg/HA_L (FIG. 1c, d). It was most likely the result of high ion reactivity of sintered at low temperature HA, which changed ion concentrations in the culture medium reducing cell viability [4-6].

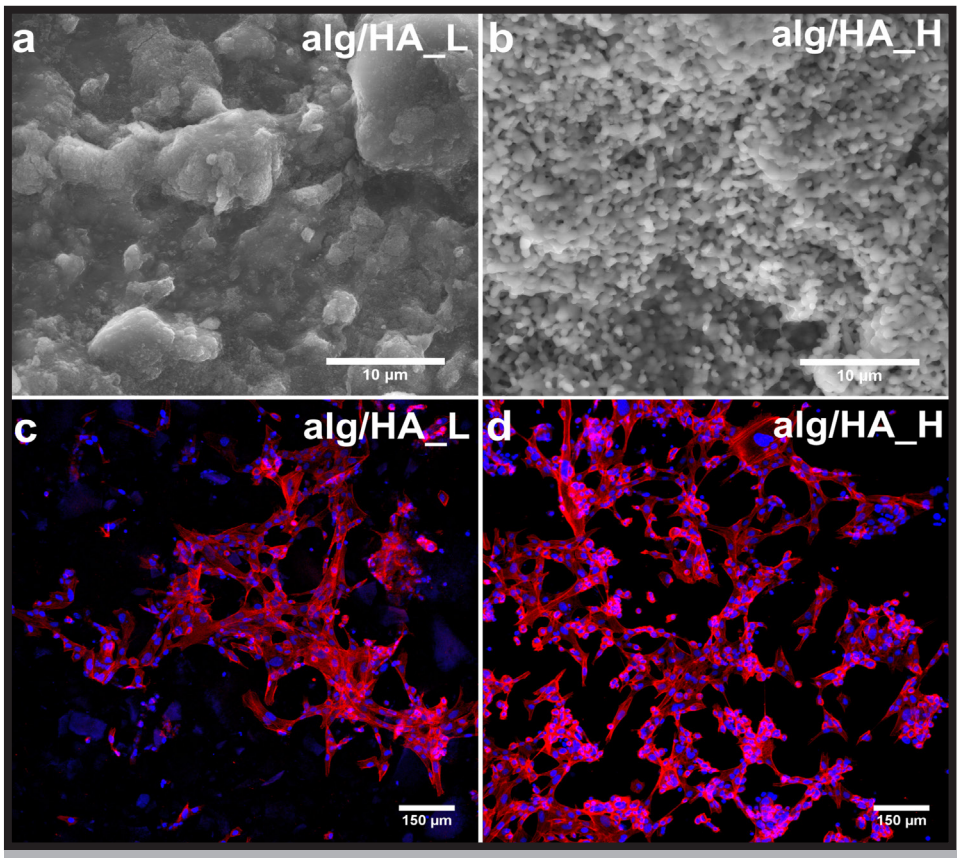

FIG. 1. SEM images (a, b) of the surfaces of biomaterials; confocal microscope images (c, d) presenting osteoblast growth on the biomaterials (red fluorescence – cell cytoskeleton, blue fluorescence – cell nuclei).

This assumption was confirmed by ion reactivity test, which showed that alg/HA_L biomaterial caused higher uptake of Ca^{2+} and HPO_4^{2-} ions than alg/HA_H throughout the duration of the experiment, compared to the control fresh medium (FIG. 2).

According to the available literature, decreased concentration in Ca^{2+} and HPO_4^{2-} ions in the culture medium may be the result of either ionic substitution [18] or the formation of a layer of apatite on the surface of biomaterials [19]. Importantly, critically low concentrations of Ca^{2+} and HPO_4^{2-} ions in culture medium are lethal to the cells *in vitro* [4-6].

It is well known that biomaterials with rough surfaces reveal ability to adsorb serum/plasma proteins inducing cell adhesion [17]. In our study, it was observed that both materials adsorbed great amount of plasma proteins. Nevertheless, the alg/HA_H composite adsorbed over 2-fold more serum proteins compared to the alg/HA_L, indicating that the latter had higher specificity towards fibrinogen binding (TABLE 1). It should be noted that adsorption of fibrinogen to the implants is considered as a key factor responsible for post-implantation biomaterial-induced inflammatory response [12,13].

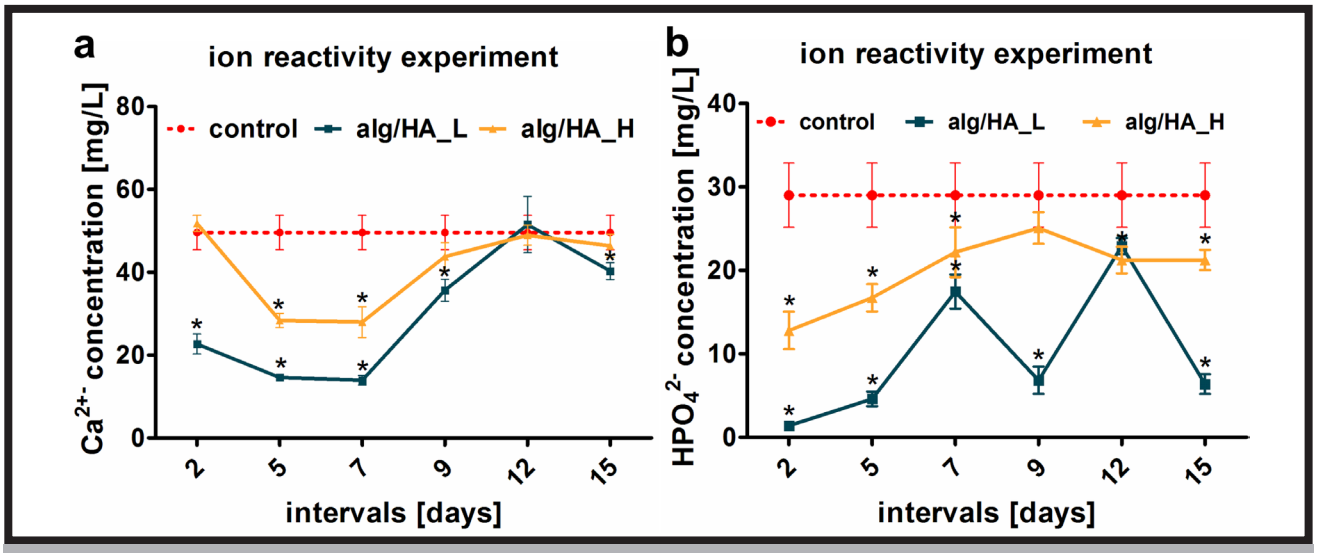

FIG. 2. Ion reactivity experiment with respect to Ca^{2+} (a) and HPO_4^{2-} (b) ions; *statistically significant results compared to the control fresh medium indicated as dotted line ($p < 0.05$, n = 4, unpaired t-test).

TABLE 1. Amount of adsorbed plasma and serum proteins to the biomaterials (mean ± SD, n = 3).

Biomaterial	Plasma proteins [μg/cm²] ± SD	Serum proteins [μg/cm²] ± SD	Relative fibrinogen [μg/cm²] ± SD
alg/HA_L	468.8 ± 94.7	134.1 ± 1.3	334.8 ± 94.7
alg/HA_H	529.3 ± 1.7	330.1 ± 66.0	199.8 ± 64.3

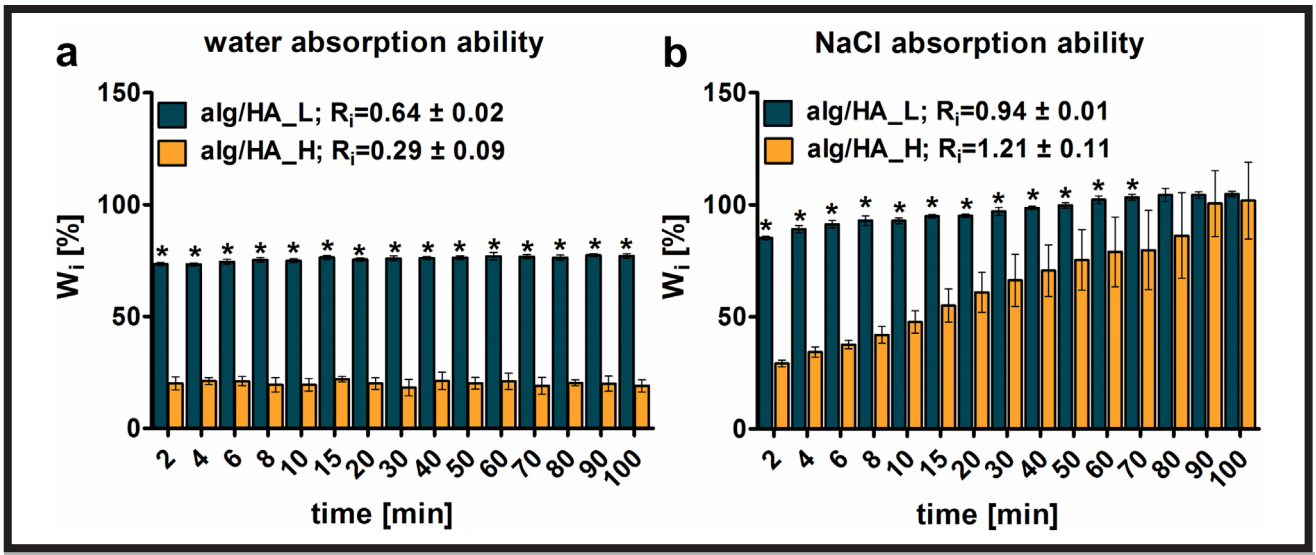

FIG. 3. Water (a) and 0.9% NaCl (b) absorption ability of biomaterials expressed as a percentage of weight increase (W_i) with time; *statistically significant results compared to the alg/HA_H ($p < 0,05$, n = 4, unpaired t-test); R_i – rehydration index.

Water/NaCl uptake test demonstrated that already after 2 min of soaking in deionised water, the alg/HA_L (W_i = 73.45%) and alg/HA_H (W_i = 20.11%) reached absorption equilibrium (FIG. 3a). Interestingly, biomaterials showed completely different behaviour in 0.9% NaCl since they required 70-90 minutes of soaking to reach absorption equilibrium (W_i = 100% for both composites) (FIG. 3b). Importantly, alg/HA_L was much more absorbent than alg/HA_H biomaterial regardless of the soaking solution due to greater microporosity of the sintered at low temperature HA [4]. This property is very important when material is designed to be a carrier of active substances like antibiotics or growth factors [14]. The R_i values determined for materials soaked in water were less than 1, but for biomaterials soaked in 0.9% NaCl were near 1 for alg/HA_L or higher than 1 for alg/HA_H. Similar phenomenon was observed by Vreeker et al. who demonstrated that rehydration of alginate gel highly depends on the ionic strength of rehydration fluid [16]. It should be noted that $R_i < 1$ indicates that completely soaked material sample contains less liquid than biomaterial just after fabrication process.

Conclusions

Based on obtained results it may be concluded that although biomaterials had the same chemical composition (alginate and HA) and were produced by exactly the same method, they contained different type of bioceramics (sintered at low and high temperature) which had great impact on their biological and physicochemical properties. Thus, the type of bioceramics used for the production of biomaterials should be tailored to their specific applications. Biomaterials for *in vivo* applications may contain highly reactive bioceramics, which show high microporosity, bioactivity, and bioresorbability [4]. Nevertheless, biomaterials designed for *in vitro* applications should be made of sintered at high temperature bioceramics which do not critically change ionic composition of culture medium and do not reduce cell viability.

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POLEPSZENIE WŁAŚCIWOŚCI KOMPOZYTÓW KRÓTKIE WŁÓKNA Ca-P/POLILAKTYD POPRZEZ MODYFIKACJĘ POWIERZCHNI WŁÓKIEN KWASEM LAURYLOWYM

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Streszczenie

Kompozyty polimerowo-ceramiczne są powszechnie stosowane w medycynie regeneracyjnej, zwłaszcza w ortopedii, gdyż istnieje wiele metod modyfikowania ich właściwości w zależności od przewidywanego zastosowania. Jedną z nich jest wypełnianie matrycy polimerowej wypełniaczami, które nadają kompozytom pożądane właściwości mechaniczne. Bardzo ważny jest wówczas dobór rodzaju i ilości wypełniacza, a także środka poprawiającego adhezję pomiędzy wypełniaczem a polimerem. Wypełniaczem poprawiającym wytrzymałość mechaniczną mogą być krótkie włókna (whiskersy) apatytowe (Ca-P). Jako środki proadhezyjne do materiałów implantacyjnych dobiera się natomiast związki nietoksyczne, wzmacniające połączenie polimer-wypełniacz. Przy wyborze bierze się również pod uwagę inne cechy, korzystne dla kompozytu, np. właściwości przeciwbakteryjne.

W pracy przedstawiono wyniki badań dotyczące wpływu modyfikacji powierzchni włókien Ca-P kwasem laurylowym (LA) na właściwości kompozytów, uzyskanych metodą prasowania na gorąco. Kwas laurylowy zastosowano w celu poprawy chemicznej kompatybilności pomiędzy włóknami a matrycą polimerową, a wytypowano go ze względu na jego nietoksyczność i właściwości przeciwbakteryjne. W celu przygotowania kompozytów zmieszano polilaktyd PLA - RESOMER® LR 706 S (Evonik) i zsyntezowane trójfazowe włókna Ca-P (niezmodyfikowane i zmodyfikowane LA). Właściwości otrzymanych kompozytów określano poprzez pomiar wytrzymałości na rozciąganie oraz pomiar kąta zwilżania. Analizowano również obrazy mikroskopowe przełamów.

Uzyskane wyniki badań wykazują, że modyfikacja powierzchni włókien Ca-P kwasem laurylowym pozwala na uzyskanie lepszego połączenia międzyfazowego pomiędzy tymi włóknami a matrycą z polilaktydu. W rezultacie daje to lepszą wytrzymałość na rozciąganie niż w przypadku kompozytów z udziałem włókien niezmodyfikowanych. Istotne jest również, że zastosowanie kwasu laurylowego jako modyfikatora powierzchni włókien pozwala uzyskać kompozyty o zwiększonej hydrofilowości, co ma znaczenie dla przyłączania i wzrostu komórek.

Słowa kluczowe: *kompozyty, włókna fosforanowo-wapniowe, kwas laurylowy, modyfikacja powierzchni*

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IMPROVEMENT OF SHORT Ca-P WHISKERS/POLYLACTIDE COMPOSITES BY SURFACE MODIFICATION WITH LAURIC ACID

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Abstract

Polymer-ceramic composites are widely used in regenerative medicine, especially in orthopedics, as there are many methods of modifying their properties depending on the intended use. One of them is filling the polymer matrix with fillers, giving the composite adequate mechanical strength. It is very important then to choose the type and amount of the filler, as well as the agent improving the adhesion between filler and polymer. Short apatite whiskers (Ca-P) are interesting fillers improving mechanical strength. As the proadhesive agents for implant materials, non-toxic compounds which strengthen the polymer-filler connection are chosen. Other features that are beneficial to the composite, e.g. antibacterial properties, are also contemplated in the selection.

The paper presents results of research on the effect of Ca-P whiskers surface modification with lauric acid (LA) on the properties of composites obtained by hot pressing. Lauric acid was used to improve the chemical compatibility between whiskers and polymer matrix, and it was selected due to antibacterial properties and non-toxicity. The polylactide PLA - RESOMER® LR 706 S (Evonik) and the synthesized triphasic Ca-P whiskers (unmodified and LA modified) were mixed to prepare the composites. The properties of the obtained composites were determined by measuring the tensile strength and the contact angle. Microscopic images of fractures were also analyzed.

The obtained tests results show that modification of the Ca-P whiskers surface with lauric acid allows to obtaining a better interfacial connection between these whiskers and the polylactide matrix. It results in better tensile strength than in the case of composites with unmodified whiskers. It is also important that the use of lauric acid as a surface modifier of the whiskers allows to obtain composites with increased hydrophilicity, which is important for the attachment and growth of cells.

Keywords: *composites, calcium-phosphate whiskers, lauric acid, surface modification*

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Wprowadzenie

Poli-L-laktyd (PLLA) to biodegradowalny polimer szeroko stosowany w medycynie, zwłaszcza w chirurgii ortopedycznej (płytki, wkręty, gwoździe) [1], a także w inżynierii tkankowej (rusztowaniach do wzrostu komórek) [2]. Niestety PLLA ulega degradacji do kwaśnych monomerów, które mogą powodować reakcje zapalne i alergiczne [3]. Ponadto właściwości mechaniczne PLLA są często niewystarczające dla niektórych, bardziej wymagających zastosowań. Wiadomo również, że PLLA jest hydrofobowy, co utrudnia wysiewanie, przyłączanie, proliferację i różnicowanie komórek.

W celu poprawy właściwości mechanicznych i zapewnienia lepszego środowiska do przyłączania i proliferacji komórek, do kompozytów PLLA często dodawane są jako wypełniacze fosforany wapnia (Ca-P). Kompozyty takie charakteryzują się zwykle lepszą osteokondukcyjnością niż czysty PLLA [4]. Materiały na bazie Ca-P są biokompatybilne i bioaktywne, a ich zasadowy charakter neutralizuje kwaśne produkty degradacji polimerów [5-7]. Jako obiecujące materiały do wzmocnienia kompozytów polimerowych brane są często pod uwagę włókna hydroksyapatytowe (HA) [8] i lepiej wchłanialne dwu- lub trójfazowe mieszaniny fosforanów wapnia [9].

Aby zapewnić maksymalne wzmocnienie kompozytów składających się z hydrofobowej matrycy polimerowej i hydrofilowych wypełniaczy apatytowych, powierzchnię tych wypełniaczy często poddaje się modyfikacji. Pozwala to na zwiększenie adhezji powierzchniowej i chemicznej kompatybilności pomiędzy wypełniaczem i matrycą, co z kolei pozwala uzyskać lepsze właściwości mechaniczne kompozytu. Wzmocnienie wzajemnego oddziaływania pomiędzy wypełniaczem a matrycą polimerową jest bardzo ważne przy stosowaniu kompozytów do implantacji. Warstwa międzyfazowa jest bowiem pierwszą warstwą ulegającą degradacji, co powoduje łatwe odłączenie wypełniacza od matrycy, a tym samym zmniejszenie wytrzymałości mechanicznej implantu [10].

Do modyfikacji powierzchni wypełniaczy nieorganicznych stosuje się różne związki, takie jak silanowe czynniki sprzęgające [8,11-13], izocyjaniany [14], polikwasy [15] i inne polimery [16]. Niektóre z nich jednak nie mogą być stosowane do materiałów dla zastosowań medycznych ze względu na ostrą toksyczność przy wysokim stężeniu [11]. Biorąc pod uwagę powyższe dane, wciąż poszukuje się nowych, nietoksycznych związków, które mogłyby działać jako promotory adhezji i wzmacniać połączenie polimer-wypełniacz w kompozytach. Dodatkową zaletą materiałów w zastosowaniach implantacyjnych byłyby również właściwości przeciwbakteryjne, które zapobiegałyby szkodliwym skutkom ubocznym, w tym zapaleniom bakteryjnym.

Jako modyfikator powierzchni można rozpatrywać łatwo dostępny, naturalny nasycony kwas tłuszczowy występujący w orzechach kokosowych, jakim jest kwas laurylowy (LA). LA występuje również w mleku matki, gdzie jest bardzo ważnym elementem budowania odporności rozwijającego się organizmu [17]. Jest uznawany za nietoksyczny i bezpieczny zarówno w zastosowaniu wewnętrznym, jak i zewnętrznym. LA wykazuje ograniczoną cytotoksyczność w zastosowaniach biomedycznych *in vitro* i *in vivo* [18,19]. Zgodnie z wcześniejszymi badaniami kwas laurylowy wykazuje aktywność przeciw szkodliwym bakteriom, dlatego jest stosowany w kosmetykach i dermatologii. Może on skutecznie hamować adhezję bakterii przeciwko P. acnes, S. aureus i S. epidermidis [20].

Introduction

Poly-L-lactide (PLLA) is a biodegradable polymer widely used in medicine, especially for orthopedic surgery (plates, screws, nails) [1] as well as scaffolds for tissue engineering [2]. Unfortunately, PLLA degrades to acidic monomers which may cause inflammatory and allergic reactions [3]. Furthermore, mechanical properties of PLLA are often insufficient for some applications. It's also known that PLLA is hydrophobic, what impedes cell seeding, attachment, proliferation and differentiation.

In order to improve mechanical properties and to provide a better environment for cell attachment and proliferation, calcium-phosphates (Ca-P) are often added into PLLA composites as fillers. Such composites usually have better osteoconductivity than pure PLLA [4]. Ca-P based materials are biocompatible, bioactive and their basic nature neutralizes the acidic degradation products from the polymers [5-7]. Hydroxyapatite (HA) whiskers [8], and more resorbable bi- or triphasic calcium phosphate mixtures [9] are considered as promising reinforcement for polymeric composites.

To ensure maximum reinforcement of composites consisting of hydrophobic polymer matrix and hydrophilic apatite fillers, these fillers are often subjected to surface modification. It allows to increase the surface adhesion and chemical compatibility between the filler and the matrix, which in turn allows to obtain better mechanical properties of the composite. Enhancement of the interaction between filler and polymer matrix is very important when using composites for implantation. Interfacial layer is the first to undergo degradation, resulting in easy detachment of the filler from the matrix and thus a decrease in the mechanical strength of the implant [10].

Various compounds are used for the surface modification of inorganic fillers, such as silane coupling reagents [8,11-13], organic isocyanates [14], polyacids [15] and polymers [16]. Some of them, however, cannot be used as modifying agents for medical applications due to their acute toxicity at high concentration [11]. Considering the above data, new compounds with no toxicity that could act as adhesion promoters and strengthen the polymer-filler connection in the composites are still being sought. An additional advantage of such compounds in implantation applications could be also their antibacterial properties, what prevent deleterious side effects, including bacteria-induced inflammation.

Lauric acid (LA) as an easily available, natural saturated fatty acid found in coconuts could be considered as surface modifier. LA is also found in breast milk, where it is a very important element in building the immunity of the developing organism [17]. It is recognized as non-toxic and safe in both internal and external use. LA shows limited cytotoxicity for biomedical applications in *vitro* and *in vivo* [18,19]. According to previous studies, lauric acid has activity against harmful bacteria, therefore it is used in cosmetics and dermatology. LA could efficiently inhibit bacteria adhesion against P. acnes, S. aureus and S. epidermidis [20]. It is likely that LA kills gram-positive bacteria by separating their inner and outer membranes, resulting in the cytoplasmic disorganization of the bacterium [20]. LA inhibits also the growth of gram-negative bacteria, especially Helicobacter [21]. LA limits bacterial growth already at very low concentrations (ppm) [22,23], and is also biodegradable.

LA zabija bakterie gram-dodatnie prawdopodobnie poprzez oddzielenie ich błony wewnętrznej i zewnętrznej, co prowadzi do cytoplazmatycznej dezorganizacji bakterii [20]. LA hamuje również wzrost bakterii gram-ujemnych, zwłaszcza Helicobacter [21]. LA ogranicza rozwój bakterii już przy bardzo niskich stężeniach (ppm) [22,23]. Ważną cechą w użyciu LA w kompozytach biodegradowalnych jest to, że sam ulega biodegradacji.

W niniejszej pracy, w celu przygotowania nowych biodegradowalnych kompozytów o ulepszonych właściwościach mechanicznych, zmieszano polilaktyd i trójfazowe włókna fosforanowo-wapniowe. Aby poprawić chemiczną kompatybilność pomiędzy włóknami a matrycą polimerową, powierzchnię tych włókien zmodyfikowano kwasem laurylowym (LA).

Materiały i metody

Materiały

Materiały wyjściowe obejmowały: dedykowany do celów medycznych (inżynieria tkankowa) polilaktyd PLA - RESOMER® LR 706 S (Evonik) będący kopolimerem poli(L-laktydu) i poli(D,L-laktydu) 70:30, kwas laurylowy (LA) o czystości 98% (Sigma-Aldrich), 99,8% etanol (Avantor), 99,5% dichlorometan (Avantor) stosowany jako rozpuszczalnik do polilaktydu oraz włókna fosforanowo-wapniowe (Ca-P) o składzie 72% hydroksyapatytu, 15% whitlockitu i 12% pirofosforanu wapnia, które zostały zsyntetyzowane w prostej metodzie, opisanej szczegółowo w naszej poprzedniej pracy [24].

Modyfikacja powierzchni włókien Ca-P z użyciem kwasu laurylowego

Włókna dyspergowano ultradźwiękowo przez 20 min w wodzie dejonizowanej z wytworzeniem zawiesiny. Do zawiesiny włókien wlano kwas laurylowy rozpuszczony w etanolu w temperaturze 60°C i zmieszano. Roztwór mieszano przez 1 godzinę, a następnie rozpuszczalnik odsączono. Włókna przemyto kilkakrotnie etanolem i wysuszono.

Przygotowanie kompozytów włókna Ca-P /polilaktyd

Przez rozpuszczanie polilaktydu w dichlorometanie przez 24 godziny przygotowano 10% roztwór polimeru. Następnie do roztworów dodano różne ilości włókien Ca-P (zmodyfikowane LA lub niezmodyfikowane) w celu otrzymania zawiesin o ustalonej proporcji włókien do polimeru. Zawiesiny wymieszano w celu homogenizacji, odlano do form, a następnie odparowano rozpuszczalnik. Przygotowano kompozyty o zawartości włókien Ca-P równej 10% wag., 20% wag. lub 30% wag. Następnie kompozyty w formach prasowano na gorąco w 195°C przez 1 min, stosując ciśnienie 0,7 MPa i przygotowano próbki do badań rozciągania i kąta zwilżania.

Charakterystyka i badania właściwości mechanicznych

Za pomocą STEM zbadano morfologię włókien przed i po modyfikacji kwasem laurylowym. Metoda SEM została wykorzystana do badań morfologii powierzchni kompozytów i obserwacji próbek zerwanych podczas rozciągania. Obserwacje mikroskopowe przeprowadzono przy użyciu elektronowego mikroskopu skaningowego z emisją polową (Nova NanoSEM 200, FEI). Obrazowanie mikrostruktury próbki przeprowadzono w warunkach wysokiej próżni za pomocą detektora ETD przy napięciu przyspieszającym 10 kV.

In this research polylactide and triphasic calcium-phosphate whiskers were mixed to prepare new biodegradable composites with improved mechanical properties. In order to improve surface chemical compatibility between whiskers and the polymer matrix, the whiskers surface was modified with lauric acid (LA).

Materials and Methods

Materials

The starting materials included: dedicated for medical purposes (tissue engineering) polylactide PLA - RESOMER® LR 706 S (Evonik) characterized as poly(L-lactide-co-D,L-lactide) 70:30, lauric acid (LA) with 98% purity (Sigma-Aldrich), 99.8% ethanol (Avantor), 99.5% dichloromethane (Avantor) used as the solvent for polylactide, calcium-phosphate (Ca-P) whiskers with composition of 72% hydroxyapatite, 15% whitlockite and 12% calcium pyrophosphate, that were synthesized by one-pot single method described in detail in our previous study [24].

Surface modification of Ca-P whiskers with lauric acid

The whiskers were dispersed ultrasonically for 20 min in deionized water to form a suspension. Lauric acid was dissolved in ethanol at 60°C, poured into the whiskers suspension and mixed. The solution was stirred for 1 h and then the solvent was filtered out. The whiskers were washed several times with ethanol and dried.

Preparation of Ca-P whiskers/polylactide composites

A 10% solution of polylactide was prepared by dissolving the polymer in dichloromethane for 24 h. Then various amounts of Ca-P whiskers (LA modified or unmodified) were added to obtained suspensions with fixed proportions of whiskers to polymer. The suspensions were mixed to homogenize, casted into molds and then the solvent was evaporated. Composites with 10 wt.%, 20 wt.% or 30 wt.% of Ca-P whiskers were prepared. Then the composites in the molds were hot-pressed at 195°C for 1 min under the pressure of 0.7 MPa. Samples for tensile tests and contact angle tests were prepared.

Characterization and mechanical properties tests

The morphology of the whiskers before and after modification by lauric acid was investigated by STEM. The SEM method was used for composites surface morphology investigations and observation of side view of tensile samples fractures. Microscopic observations were performed using a field emission scanning electron microscope (Nova NanoSEM 200, FEI). Imaging of sample microstructure was performed in high vacuum conditions using ETD detector at 10 kV accelerating voltage. Before the study, the samples were covered with conductive material (10 nm gold film) using a sputter coater, Leica EM SCD500. STEM detector was installed in SEM. In STEM observations, the whiskers were placed on a copper mesh and observed at 25 kV accelerating voltage.

Functional groups of samples were identified by Fourier transform infrared spectroscopy (FTIR). Measurements of absorbance were made using a TENSOR 27 (BRUKER) equipped with a DLaTGS detector. Analysis was performed in the wavelength range of 400 cm^{-1} to 4000 cm^{-1}. Samples were prepared as pressed tablet shape KBr molds.

The specific surface area for samples was measured by BET method using nitrogen adsorption at -195.8°C with Geminii VII instrument, Micromeritics. Before the measurements were taken, the samples were degassed for 1 h at 120°C.

Przed badaniem próbki pokryto materiałem przewodzącym (złota powłoka o grubości 10 nm) za pomocą napylarki Leica EM SCD500. Detektor STEM został zainstalowany w SEM. W obserwacjach STEM włókna umieszczono na miedzianej siatce i obserwowano przy napięciu przyspieszającym 25 kV.

Grupy funkcyjne próbek identyfikowano za pomocą spektroskopii w podczerwieni z transformacją Fouriera (FTIR). Pomiarów absorbancji dokonywano za pomocą TENSOR 27 (BRUKER) wyposażonego w detektor DLaTGS. Analizę przeprowadzono w zakresie długości fal od 400 cm^{-1} do 4000 cm^{-1}. Próbki przygotowano w postaci sprasowanych pastylek z KBr.

Powierzchnię właściwą dla próbek mierzono metodą BET z zastosowaniem adsorpcji azotu przy -195,8°C z użyciem aparatu Geminii VII, Micromeritics. Przed pomiarem próbki zostały odgazowane przez 1 godzinę w temperaturze 120°C.

Kąt zwilżania kompozytów wyznaczano metodą osadzonej kropli. W skrócie, 5 µl kroplę dejonizowanej wody osadzano za pomocą mikrostrzykawki na płaskiej powierzchni próbki, utrzymując tą samą minimalną wysokość igły nad badaną powierzchnią i kierunek ścinania jej wierzchołka. Po 5 sekundach od momentu nałożenia kropli na powierzchnię próbki wykonywano jej zdjęcie aparatem Canon EOS 450D. Oznaczenia kąta zwilżania dokonywano przez poddanie zdjęć kropli analizie za pomocą programu ZWCAD 2017, prowadząc styczną do konturu kropli w miejscu kontaktu z powierzchnią próbki. Każdą końcową wartość kąta zwilżania uzyskano przez uśrednienie sześciu wartości mierzonych w różnych punktach.

Właściwości mechaniczne kompozytów oceniano za pomocą prób rozciągania. Badania przeprowadzono na maszynie wytrzymałościowej Tinius Olsen w temperaturze pokojowej z prędkością rozciągania 1 mm/min. Rozmiar próbek rozciągania wynosił 20 mm długości, 3 mm szerokości i 2 mm grubości.

Wyniki badań kątów zwilżania i wytrzymałości na rozciąganie przedstawiono na wykresach zależności od rodzaju kompozytów z różną zawartością włókien Ca-P. Wyniki te przedstawiają wartości średnie ± SD (odchylenie standardowe). Dla uzyskanych wyników kątów zwilżania i wytrzymałości na rozciąganie przeprowadzono analizę statystyczną. Statystycznie istotne różnice (wartość p) dla dwóch niezależnych grup próbek oceniano za pomocą testu t-Studenta.

Wyniki i dyskusja

Stosując wyżej opisaną procedurę modyfikacji, otrzymano włókna Ca-P modyfikowane LA. Na RYS. 1 przedstawione są widma FTIR kwasu laurylowego i włókien Ca-P przed i po modyfikacji. Na RYS. 1a pasma absorpcji przy liczbie falowej 565, 609, 1033, 1093 cm^{-1} przypisane do drgań zginających i rozciągających grup fosforanowych PO_4^{3-} [25], pasma przy 729 i 1215 cm^{-1} przypisane grupom $P_2O_7^{4-}$ w pirofosforanie wapnia [26,27] i dwa pasma absorpcji przy 945 i 973 cm^{-1} przypisane do whitlockitu [25], potwierdzają skład trójfazowych włókien Ca-P. RYS. 1c przedstawia charakterystyczne pasma kwasu laurylowego. Pasma absorpcji przy 2850 i 2919 cm^{-1} odpowiadają odpowiednio pikom symetrycznych i asymetrycznych drgań rozciągających grup $-CH_2-$, pasmo absorpcji przy 2960 cm^{-1} odpowiada pikowi asymetrycznych drgań rozciągających grupy $-CH_3$, a pasmo absorpcji przy 1703 cm^{-1} odpowiada pikowi drgań rozciągających grupy karbonylowej w kwasie karboksylowym. Obecność tych charakterystycznych pasm kwasu laurylowego na widmie FTIR modyfikowanych włókien Ca-P potwierdza modyfikację ich powierzchni (RYS. 1b).

The water contact angle of the composites was measured according to the sessile-drop method. Briefly, 5 µl of deionized water droplet was deposited by microsyringe on a flat sample surface while maintaining the same minimum height of the needle over the surface being tested and the direction of shearing of its tip. The photo of the drop was made with the Canon EOS 450D camera after 5 seconds from the moment it was deposited on the surface of the sample. Measurement of the contact angle was made by subjecting photos of drops to the analysis using the ZWCAD 2017 program by leading a tangent to the outline of the drop at the point of contact with the surface of the sample. Each final water contact angle value was obtained by averaging six water contact angle values of different spots.

Mechanical properties of the composites were evaluated by tensile tests. The tests were performed on a Tinius Olsen testing machine at room temperature with loading speed of 1 mm/min. Size of the tensile samples was 20 mm in gauge, 3 mm in width and 2 mm in thickness.

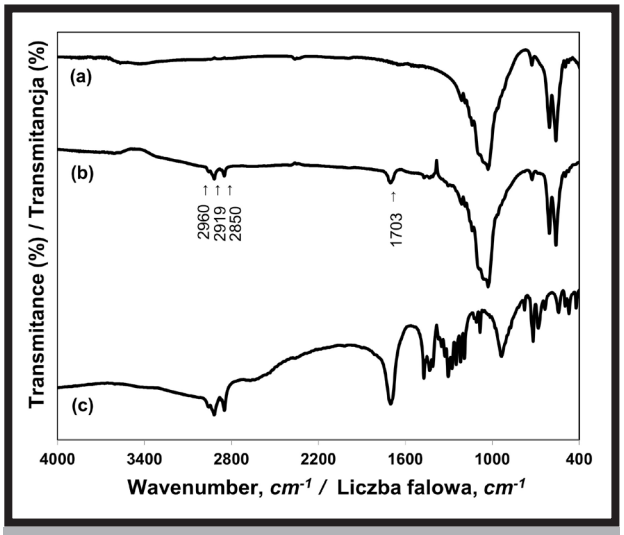
The results of water contact angles and tensile strengths measurements are presented on diagrams of dependence on the type of composites with different content of Ca-P whiskers. These results represent mean values ± SD (standard deviation). Statistical analysis for the obtained results of water contact angle and tensile strength was performed. Statistically significant differences (p-value) for two independent groups of samples were evaluated by t-Student test.

Results and Discussions

Using the above described procedure of modification, the LA modified Ca-P whiskers were obtained. The FTIR spectra of lauric acid, and Ca-P whiskers before and after modification are presented in FIG. 1. In FIG. 1a the absorption bands at 565, 609, 1033, 1093 cm^{-1} attributed to the bending and stretching modes of the phosphate PO_4^{3-} groups [25], the bands at 729 and 1215 cm^{-1} attributed to $P_2O_7^{4-}$ groups in calcium pyrophosphate [26,27] and two absorption bands at 945 and 973 cm^{-1} attributed to whitlockite [25] confirm the composition of triphasic Ca-P whiskers. FIG. 1c presents characteristic bands of lauric acid. The absorption bands at 2850 and 2919 cm^{-1} correspond to the symmetric and asymmetric stretching vibration peaks of $-CH_2-$ groups respectively, the absorption band at 2960 cm^{-1} corresponds to the anti-symmetric stretching vibration peak of $-CH_3$ group and the absorption band at 1703 cm^{-1} corresponds to the stretching vibration peak of the carbonyl group in carboxylic acid. The presence of these characteristic bands of lauric acid in the FTIR spectrum of modified whiskers confirms their surface modification (FIG. 1b).

The surface modification was also confirmed by observations on STEM micrographs (FIG. 2). It was noted that the boundaries of Ca-P whiskers before modification were clear and the surfaces were smooth. After modification, the surfaces of whiskers were rough and boundaries were unclear which is probably related to the presence of lauric acid on the surface of the whiskers.

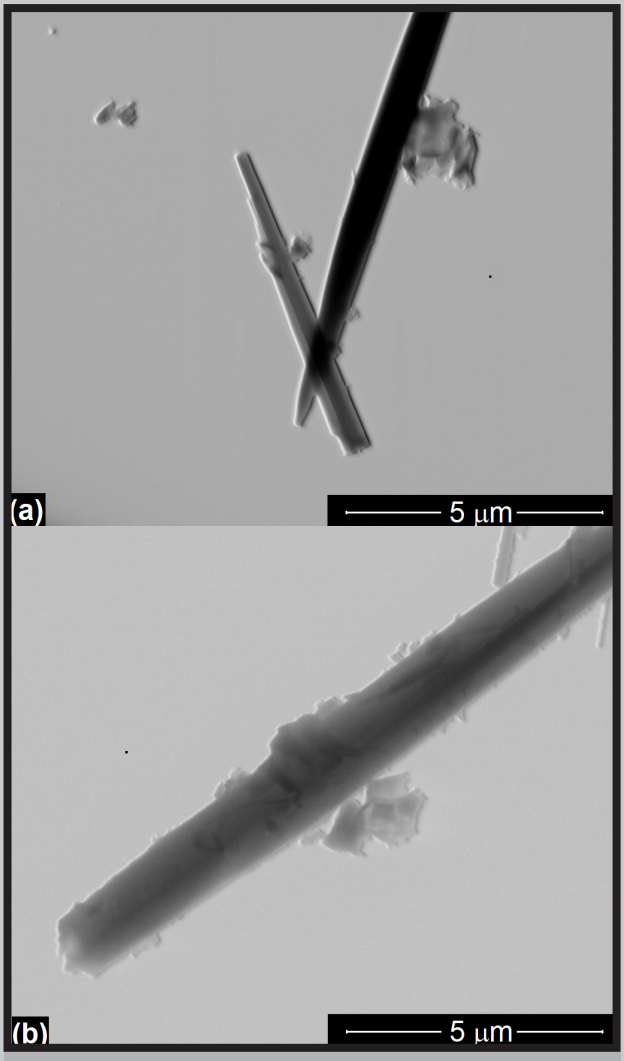
The formation of a rough and uneven LA layer on the whiskers surface resulted in a greater development of their surface. BET tests showed that specific surface area S_{BET} of LA modified whiskers was greater than the specific surface area of unmodified whiskers by nearly 9.5% (TABLE 1). On the basis of the obtained results, it was expected that the specific surface area growth of the whiskers would to a certain extent affect the strength of the interfacial connection between these whiskers and the polymer matrix in composites.



RYS. 1. Widma spektroskopii w podczerwieni z transformacją Fouriera dla: a) otrzymanych włókien Ca-P, b) włókien Ca-P zmodyfikowanych kwasem laurylowym, c) kwasu laurylowego.
FIG. 1. Fourier-transform infrared spectroscopy spectrum of: a) Ca-P whiskers as received, b) Ca-P whiskers modified with lauric acid, c) lauric acid.

Modyfikację powierzchni potwierdzono również obserwacjami STEM (RYS. 2). Zauważono, że granice włókien Ca-P przed modyfikacją były wyraźne, a powierzchnie gładkie. Po modyfikacji, powierzchnie były szorstkie, a granice niewyraźne, co prawdopodobnie jest związane z obecnością kwasu laurylowego na powierzchni włókien.

Utworzenie szorstkiej i nierównej warstwy LA na powierzchni włókien spowodowało zwiększenie stopnia jej rozwinięcia. Badania BET wykazały, że powierzchnia właściwa S_{BET} zmodyfikowanych włókien była większa niż powierzchnia właściwa włókien niezmodyfikowanych o prawie 9,5% (TABELA 1). Na podstawie uzyskanych wyników oczekiwano, iż wzrost powierzchni właściwej włókien będzie w pewnym stopniu wpływać na wytrzymałość połączenia międzyfazowego pomiędzy tymi włóknami a matrycą polimerową w kompozytach.



RYS. 2. Obrazy STEM: a) otrzymanych włókien Ca-P, b) włókien Ca-P modyfikowanych kwasem laurylowym.
FIG. 2. STEM images of: a) Ca-P whiskers as received, b) Ca-P whiskers modified with lauric acid.

TABELA 1. Wartości powierzchni właściwej S_{BET} włókien Ca-P niezmodyfikowanych i zmodyfikowanych kwasem laurylowym.
TABLE 1. Values of specific surface area S_{BET} of Ca-P whiskers as received and Ca-P whiskers modified with lauric acid.

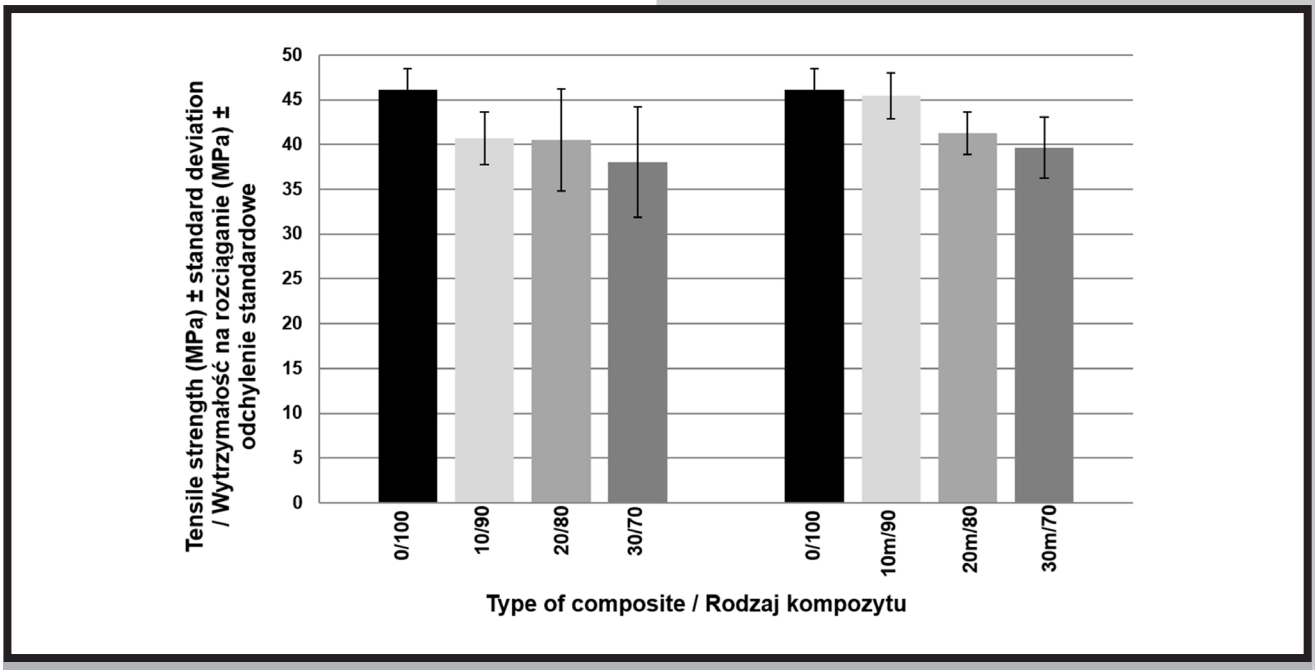
Próbka Sample	S_{BET} [m^2/g]	Współczynnik korelacji Correlation coefficient
Włókna Ca-P niezmodyfikowane Ca-P whiskers as received	3.7 ± 0.01	0.9999718
Włókna Ca-P zmodyfikowane LA Ca-P whiskers modified with LA	4.0 ± 0.03	0.9998477

Kompozyty otrzymano ze zmodyfikowanych i niezmodyfikowanych krótkich włókien Ca-P oraz polilaktydu, zmieszanych w różnych proporcjach wagowych (TABELA 2). Uzyskano próbki do badań wytrzymałości na rozciąganie i określono wytrzymałość na rozciąganie otrzymanych kompozytów. Wyniki wytrzymałości na rozciąganie przedstawiające wartości średnie ± odchylenie standardowe pokazano na RYS. 3. Kompozyty włókna Ca-P/polilaktyd wykazują mniejszą wytrzymałość na rozciąganie niż czysty polimer, co jest zgodne z doniesieniami innych autorów [28].

The composites were obtained on the base of modified and unmodified short Ca-P whiskers and polylactide mixed in various weight ratios (TABLE 2). Tensile samples were obtained and tensile strength of the obtained composites was determined. The results of tensile strengths presented mean values ± SD (standard deviation) are showed in FIG. 3. Ca-P whiskers/polylactide composites show lower tensile strength than pure polymer, what is consistent with reports by other authors [28].

TABELA 2. Skład procentowy kompozytów sporządzonych z polilaktydu i włókien Ca-P (niemodyfikowanych i modyfikowanych).
TABLE 2. Percent composition of composites made of polylactide and Ca-P whiskers (as received and modified).

Kompozyt Composite	Zawartość / Content [%]		
	polilaktyd polylactide	niezmodyfikowane włókna Ca-P Ca-P whiskers as received	zmodyfikowane włókna Ca-P modified Ca-P whiskers
0/100	100	-	-
10/90	90	10	-
20/80	80	20	-
30/70	70	30	-
10m/90	90	-	10
20m/80	80	-	20
30m/70	70	-	30



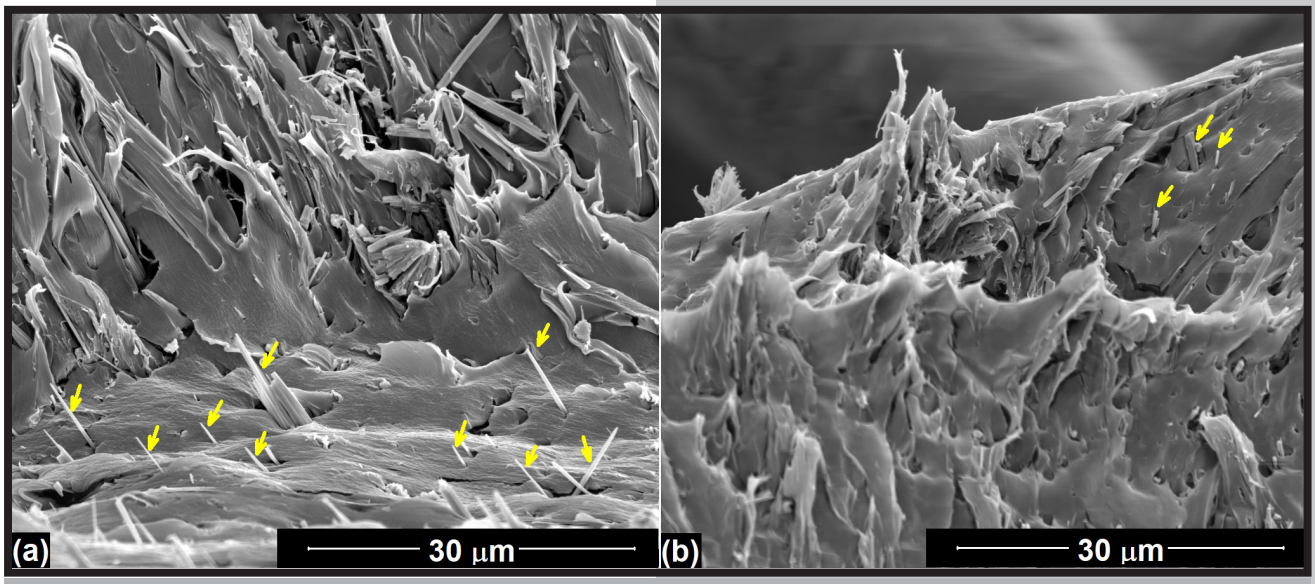
RYS. 3. Wytrzymałość na rozciąganie w zależności od rodzaju kompozytu.
FIG. 3. Tensile strength depending on the type of composite.

Badania wytrzymałości na rozciąganie tych kompozytów pokazują, że wraz ze wzrostem ilości włókien wytrzymałość na rozciąganie maleje. Jednakże kompozyty oparte na zmodyfikowanych włóknach wykazują nieco większą wytrzymałość na rozciąganie niż kompozyty z włóknami niezmodyfikowanymi (RYS. 3). Pomiędzy wartościami wytrzymałości na rozciąganie dla kompozytów zawierających niezmodyfikowane włókna Ca-P nie stwierdzono istotnych statystycznie różnic. Istotne statystycznie różnice ($p<0,05$) stwierdzono natomiast pomiędzy wartościami wytrzymałości na rozciąganie dla kompozytów 10m/90 i 20m/80 z udziałem 10% i 20% zmodyfikowanych włókien Ca-P oraz dla kompozytów 10m/90 i 30m/70 z udziałem 10% i 30% zmodyfikowanych włókien Ca-P.

Zaobserwowany wzrost wytrzymałości kompozytów z udziałem włókien modyfikowanych LA można przypisać poprawie połączenia międzyfazowego pomiędzy włóknami Ca-P i matrycą polimerową. Możliwe jest również, że utworzenie nierównej i chropowatej warstwy LA na powierzchni włókien spowodowało, iż potrzebne były większe siły do „wyciągania" zmodyfikowanych włókien z matrycy polimerowej w porównaniu do niezmodyfikowanych włókien o gładkiej powierzchni, w trakcie próby rozciągania.

Tensile strength tests of Ca-P whiskers/polylactide composites show that with the increase of whiskers amount, the tensile strength decreases. However, the composites based on modified whiskers show slightly higher tensile strength than the composites with unmodified whiskers (FIG. 3). No statistically significant differences were observed between the tensile strength values for composites containing unmodified Ca-P whiskers. However statistically significant differences ($p<0.05$) were observed between the tensile strength values for composites 10m/90 and 20m/80 containing 10% and 20% of modified Ca-P whiskers and for composites 10m/90 and 30m/70 containing 10% and 30% of modified Ca-P whiskers.

The observed increase in strength of composites with modified LA whiskers can be attributed to improvement of interfacial connection between Ca-P whiskers and polymer matrix. It is also possible that formation of an uneven and rough LA layer on the whiskers surface caused during the stretching test an increase in the pull forces of modified whiskers from the polymer matrix compared to whiskers with a smooth surface.



RYS. 4. Obrazy SEM widoku powierzchni próbek zerwanych podczas rozciągania kompozytów z 10%: a) niezmodyfikowanych włókien Ca-P, b) włókien Ca-P zmodyfikowanych kwasem laurylowym (strzałki wskazują na włókna wyciągnięte z matrycy polimerowej).
FIG. 4. SEM images of side view of tensile samples fractures of composites with 10% of: a) unmodified Ca-P whiskers, b) Ca-P whiskers modified with lauric acid (arrows indicate whiskers drawn out of the polymer matrix).

Obserwacje SEM (RYS. 4) pokazują, że podczas próby rozciągania, niezmodyfikowane włókna są wyciągane z matrycy polimerowej. Modyfikacja włókien kwasem laurylowym powoduje, że połączenie międzyfazowe jest silniejsze, a zatem warstwa międzyfazowa wytrzymuje większe naprężenia ścinające. Można zauważyć, że po modyfikacji, tylko niektóre z włókien są wyciągane z matrycy polimerowej, podczas gdy większość z nich pod obciążeniem jest zrywana. W ten sposób obciążenie jest częściowo przenoszone z matrycy do włóknistego wypełniacza i cały układ jest wzmocniony.

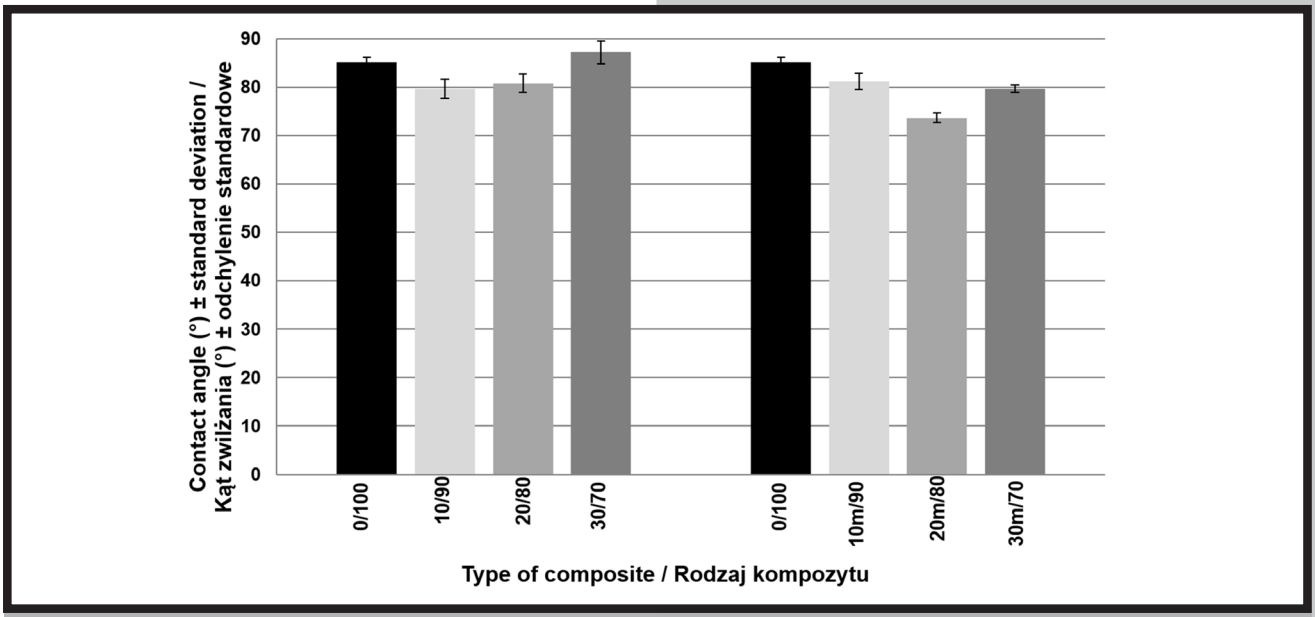
Poza wytrzymałością mechaniczną materiałów do niektórych zastosowań implantacyjnych, niezwykle istotna jest również zwilżalność powierzchni implantów, która wpływa na oddziaływanie komórka-implant. Wcześniejsze badania wykazały, że sekwencja procesów adhezji komórkowej, takich jak: kontakt, przyłączanie, rozprzestrzenianie i proliferacja, jest znacznie opóźniona i osłabiona na powierzchniach hydrofobowych (słabo zwilżalnych w wodzie) [29]. Powierzchnie hydrofilowe wykazują lepszą skuteczność przyłączania i krótsze okresy indukcji fazy wzrostu komórek osteoblastycznych w porównaniu z powierzchniami hydrofobowymi [29].

Zmiana hydrofilowości powierzchni kompozytów polimerowych pod wpływem dodatku napełniacza była już opisywana wielokrotnie wcześniej przez innych autorów [30-33]. Wiadomo, że stosowane dodatki, a zwłaszcza nanocząstki mogą modyfikować zwilżalność powierzchni polimeru za pomocą dwóch mechanizmów: (a) mogą modyfikować skład chemiczny powierzchni, wpływając w ten sposób bezpośrednio na oddziaływania międzycząsteczkowe pomiędzy ciałem stałym i wodą, (b) mogą modyfikować morfologię powierzchni.

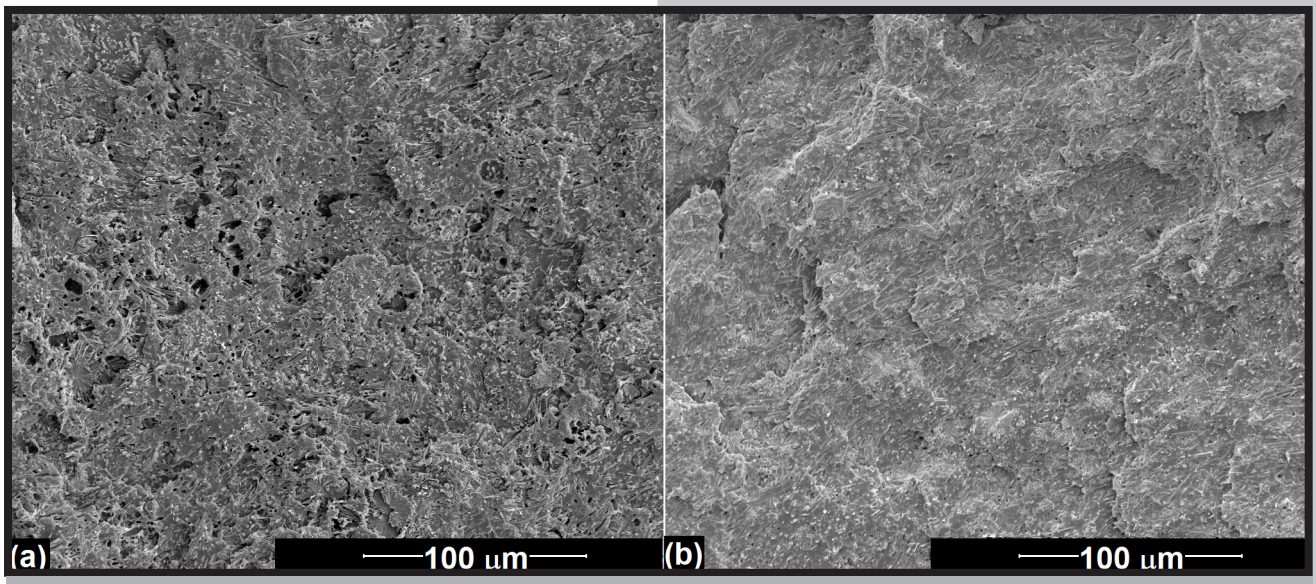
SEM observations (FIG. 4) show that during the tensile test, unmodified whiskers are drawn out of the polymer matrix. Modification of the whiskers with lauric acid makes the interfacial bonding stronger and therefore interface withstands higher shear stresses. It can be seen that after modification only some of the whiskers are drawn out of the polymer matrix, while most of them under the load are broken. In this way, the load is partially transferred from the matrix to the whisker filler and the system is reinforced.

Besides of mechanical strength of materials for some implant applications, the wettability of the implant surface is also very important property, which affects the cell-implant interaction. Previous studies have shown that the sequence of cell adhesion events such as contact, attachment, spreading and proliferation is significantly delayed and impaired on hydrophobic surfaces (poorly water-wettable) [29]. Hydrophilic surfaces show better attachment efficiency and shorter induction periods of osteoblastic cell growth phase compared to hydrophobic surfaces [29].

The change of the hydrophilicity of the polymer composites surface with the filler addition has already been described many times before by other authors [30-33]. It is known that the additives used, especially nanoparticles, can modify the wettability of the polymer surface by means of two mechanisms: (a) by modifying the chemical composition of the surface, thus directly affecting intermolecular interactions between solid and water, (b) by modifying the surface morphology.



RYS. 5. Kąt zwilżania w zależności od rodzaju kompozytu.
FIG. 5. Water contact angle depending on the type of composite.



RYS. 6. Obrazy SEM powierzchni kompozytów z 30% wag.: (a) niezmodyfikowanych włókien Ca-P, (b) włókien Ca-P zmodyfikowanych kwasem laurylowym.
FIG. 6. SEM images of composites surface with 30% of: a) unmodified Ca-P whiskers, b) Ca-P whiskers modified with lauric acid.

W celu określenia zwilżalności powierzchni kompozytów otrzymanych w niniejszej pracy, wyznaczono ich kąty zwilżania wodą. Wartości tych kątów przedstawiono na RYS. 5. Jak można zauważyć w porównaniu z czystą próbką polilaktydu, hydrofilowość kompozytów początkowo rosła po dodaniu napełniacza, po czym malała w różnym tempie w zależności od tego czy włókna Ca-P były zmodyfikowane czy nie. Kompozyty zawierające niezmodyfikowane włókna Ca-P wykazały największą hydrofilowość przy zawartości włókien równej 10% wag., a kompozyty zawierające zmodyfikowane włókna Ca-P przy zawartości włókien równej 20% wag. Istotnych statystycznie różnic pomiędzy wartościami kątów zwilżania nie stwierdzono jedynie dla kompozytów 10/90 i 20/80 z udziałem 10% i 20% niezmodyfikowanych włókien Ca-P oraz dla kompozytów 10m/90 i 30m/70 z udziałem 10% i 30% zmodyfikowanych włókien Ca-P.

In order to determine the surface wettability of the obtained composites, their water contact angles were evaluated. Values of the water contact angles are shown in FIG. 5. As can be seen in comparison with pure PLLA sample, hydrophilicity of the composites increased initially after addition of the filler, and then decreased at different rates depending on whether the whiskers were modified or not. Composites containing unmodified Ca-P whiskers showed the highest hydrophilicity at the whiskers content of 10 wt% and composites containing modified Ca-P whiskers at the whiskers content of 20 wt%. Statistically significant differences between the contact angle values were not observed only for composites 10/90 and 20/80 containing 10% and 20% of unmodified Ca-P whiskers and for composites 10m/90 and 30m/70 containing 10% and 30% of modified Ca-P whiskers.

Zaobserwowana różnica pomiędzy składem kompozytów z udziałem włókien niezmodyfikowanych i zmodyfikowanych, dla których zarejestrowano najmniejsze kąty zwilżania wydaje się być ciekawa, biorąc pod uwagę hydrofobowy charakter kwasu laurylowego. Wytłumaczeniem obserwowanego zjawiska może być różnica w jakości powierzchni uzyskanych kompozytów. Z literatury wiadomo, że na zwilżalność powierzchni wpływa jej chropowatość [34,35]. Zakładając, że woda nie zwilża głębokich zagłębień chropowatej powierzchni ponieważ pewna ilość otaczającego gazu jest uwięziona w tych zagłębieniach, uzyskuje się w rezultacie wzrost wartości kąta zwilżania. RYS. 6 przedstawia powierzchnię kompozytów utworzonych z udziałem (a) włókien niezmodyfikowanych i (b) zmodyfikowanych. Zauważyć można, że niezmodyfikowane włókna Ca-P mają tendencję do nierównomiernego rozmieszczenia w matrycy polimerowej, co powoduje powstawanie ich skupisk i wielu porów, podczas gdy zmodyfikowane włókna rozkładają się bardziej jednorodnie, a powierzchnia kompozytu jest mniej chropowata. Stąd prawdopodobnie wynika również większy spadek kąta zwilżania dla kompozytów z udziałem włókien zmodyfikowanych aniżeli dla kompozytów z udziałem włókien niezmodyfikowanych.

Biorąc pod uwagę otrzymane wyniki oraz wspomniane powyżej dane literaturowe [29], można przypuszczać, że adhezja i wzrost komórek osteoblastycznych w ewentualnych zastosowaniach implantacyjnych dla niektórych z kompozytów uzyskanych przy udziale włókien zmodyfikowanych kwasem laurylowym, mogą być zwiększone.

Wnioski

Modyfikacja powierzchni włókien fosforanowo-wapniowych kwasem laurylowym pozwala na uzyskanie lepszego połączenia międzyfazowego pomiędzy tymi włóknami a matrycą z polilaktydu. W rezultacie, otrzymane kompozyty charakteryzują się lepszą wytrzymałością na rozciąganie. Zastosowanie kwasu laurylowego jako modyfikatora powierzchni włókien pozwala również uzyskać kompozyty o zwiększonej hydrofilowości, co ma znaczenie dla przyłączania i wzrostu komórek. Dodatkowo, biorąc pod uwagę właściwości przeciwbakteryjne kwasu laurylowego, wydaje się on obiecującym środkiem modyfikującym do zastosowań w medycynie.

Podziękowania

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The observed difference between the composition of composites with unmodified and modified whiskers, for which the lowest contact angles were recorded, seems to be interesting, taking into account the hydrophobic nature of lauric acid. The difference in surface quality of the obtained composites may be the explanation for the observed phenomenon. It is known from the literature that the wettability of the surface is affected by its roughness [34,35]. Assuming that the water does not wet the deep hollows of the rough surface because a certain amount of the surrounding gas is trapped in these hollows, the result is an increase in the value of the contact angle. FIG. 6 shows the surface of composites formed with unmodified (a) and modified (b) whiskers. It can be noticed that the unmodified whiskers tend to have uneven distribution in the polymer matrix which results in the formation of clusters and many pores, while the modified whiskers distribute more homogenously and the composite surface is less rough. It is probably also the reason of greater decrease of the contact angle for composites with modified whiskers than for composites with unmodified whiskers.

Considering the results obtained and the abovementioned literature data [29], it can be assumed that the adhesion and growth of osteoblastic cells in possible implantation applications for some of the composites obtained with the participation of whiskers modified with lauric acid may be increased.

Conclusions

The modification of calcium-phosphate whiskers surface with lauric acid allows for a better interfacial connection between these whiskers and the polylactide matrix. As a result, the obtained composites are characterized by better tensile strength. The use of lauric acid as a surface modifier of the whiskers also allows to obtain composites with increased hydrophilicity, which is important for the attachment and growth of cells. In addition, lauric acid appears to be a promising modifier for applications in medicine considering its antibacterial properties.

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