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A new approach for the immobilization of poly(acrylic) acid as a chemically reactive cross-linker on the surface of poly(lactic) acid-based biomaterials

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ABSTRACT

A new approach for the immobilization of poly(acrylic) acid (PAA) as a chemically reactive cross-linker on the surface of poly(lactic) acid-based (PLA) biomaterials is described. The proposed technique includes non-covalent attachment of a PAA layer to the surface of PLA-based biomaterial via biomaterial surface treatment with solvent/non-solvent mixture followed by the entrapment of PAA from its solution. Surface morphology and wettability of the obtained PLA-PAA composite materials were investigated by AFM and the sitting drop method respectively. The amount of the carboxyl groups on the composites surface was determined by using the fluorescent compounds (2-(5-aminobenzo[*d*]oxazol-2-yl)phenol (ABO) and its acyl derivative N-(2-(2-hydroxyphenyl)benzo[*d*]oxazol-5-yl)acetamide (AcABO)). It was shown that it is possible to obtain PLA-PAA composites with various surface relief and tunable wettability (57°, 62° and 66°). The capacity of the created PAA layer could be varied from 1.5 nmol/cm^2 to 0.1 μmol/cm^2 depending on the modification conditions. Additionally, using bovine serum albumin (BSA) it was demonstrated that such composites could be modified with proteins with high binding density (around 0.18 nmol/cm^2). Obtained fluoro-labeled PLA-PAA materials, as well as PLA-PAA composites themselves, are valuable since they can be used for biodegradable polymer implants tracking in living systems and as drug delivery systems.

1. Introduction

Biomaterial surface properties are crucial for body–implant interaction [2]. Topography, surface chemical properties, ligand presentation, and also porosity are among the most important parameters that can influence cell response [3–6]. In order to control cell attachment, growth and differentiation, many biomaterial surface modification methods have been developed [2,7]. A majority of such strategies are related to biodegradable polymer materials. During the last decades these biomaterials were recognized as the best perspectives for the regenerative medicine and tissue engineering [8–10].

Poly(lactic acid) (PLA) is an ubiquitous biodegradable polymer used for scaffold, drug delivery system, implant, suture, and coating production owing to its biocompatibility and processibility [11]. However, PLA has several drawbacks such as hydrophobicity and a lack of good reactive groups on its surface [12].

Direct modification methods of the PLA-based biomaterial surface include both physical and chemical approaches. Coating [12–14], plasma treatment [15,16], chemical modification [17], including treatment with diazonium salts in the presence of reducing agents [18], grafting [19–21], and their combinations [22] are among the most common ones. However, PLA surface properties cannot be easily altered and modification often leads to surface chain degradation.

An alternative method that makes it possible to impart the desired physico-chemical and biological properties to PLA-based biomaterials is the attachment of biologically active compounds to their surface through the insertion of a cross-linker between PLA and the target modification agent. Cross-linker can bear any easily reactive group: carboxyl-, hydroxyl- or amino- groups, or a combination of them. For example, the bifunctional cross-linker - 4-aminobenzoic acid was used for covalent immobilization of chitosan on the PLA material surface via acylation of the chitosan amino-group by the linker, with subsequent formation of diazonium salt which then reacted with PLA under UV-light irradiation [23].

The ideal cross-linker should be non-toxic, inexpensive, show the equal distribution of active groups per square unit, and enhance PLA

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surface hydrophilicity. Poly(acrylic) acid (PAA) has one carboxylic group per monomer, is biocompatible and hydrophilic, thus it has good potential to be used as a cross-linker [24]. PAA is a polyanion and previously it was found that anionic surfaces demonstrate less cytotoxicity in comparison with cationic ones [4]. Mucoadhesive properties and complexation ability also make PAA a favorable material to be applied in wound healing and target biologically active compound delivery [25,26].

The aim of our work was to obtain a PLA-PAA composite biomaterial by non-covalent sustained attachment of PAA to the PLA-based biomaterial surface without any destructive treatment or the use of additional reagents. In such composite the attached PAA would act as a cross-linker between PLA and the desired biologically active compounds. Thus, PLA-PAA material could be applied as a well-performing substrate for the further modification by small organic molecules and proteins as well.

The proposed preparation method of the PLA-PAA composite is based on the idea of polymer treatment by a solvent/non-solvent mixture with subsequent entrapment of the desired compounds. During such treatment, the PLA-based biomaterial surface region reversibly swells, and while it is in its swollen state, it could absorb different compounds. When the surface dries these compounds remain entrapped on the biomaterial surface. This approach has been previously shown to be successful in entrapment of poly(ethylene glycol) [27–29], gelatine, alginate, chitosan [30], poly(aspartic acid) [31], and brilliant green dye [32] on the PLA-based biomaterial surface.

2. Materials and methods

2.1. Starting materials and instruments

PLA PURASORB® PL65 (M_w = 1,646,000 g/mol) was purchased from Purac (The Netherlands). PAA (M_v = 1,250,000 g/mol), ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were bought from Sigma-Aldrich (USA). Bovine serum albumin (BSA) (Albumin Fraction V, M_w = 68,000 g/mol) was purchased from PANREAC (Spain). Quick Start Bradford protein assay and Coomassie Brilliant Blue G-250 were bought from BIO-RAD (USA). 2-(5-aminobenzo[*d*]oxazol-2-yl)phenol (ABO) and its acyl derivative N-(2-(2-hydroxyphenyl)benzo[*d*]oxazol-5-yl)acetamide (AcABO) were synthesized according to methods previously described [1,33]. All solvents were ACS grade and used without further purification.

Atomic force microscopy (AFM) measurements were carried out on an AFM "Solver HV" (NT-MDT, Russia). Cantilevers of HA_HR from ETALON series (NT-MDT, Russia) were used as probe sensors. Surface wettability measurements were carried out on an EasyDrop installation (Krüss, Germany). Fluorescence spectra were recorded using the spectrofluorometer CM2203 (Solar, Belarus); all fluorescence intensity measurements were also performed by CM2203 (Solar, Belarus). The absorbance was measured using iMark Microplate Absorbance Reader (BIO-RAD, USA).

2.2. PLA films processing

PLA films were produced by solvent casting as previously described [32]. Briefly, an 18 ± 1 g of PLA solution in a mixture of dichloromethane and chloroform (40:60 v/v) with a concentration of 1.7% (mass.) was poured into a dry Petri dish, sealed and dried in solvent vapour over 72 h. The thickness of obtained films was 30 ± 2 μm.

2.3. Modification of the PLA films with PAA

PLA films with the size of 2 × 2 cm were dipped into the one of the following solvent/non-solvent miscible mixtures: toluene/ethanol (3:7 v/v) (MePh/EtOH), chloroform/ethylacetate (1/9 v/v) ($CHCl_3$/EtOAc) or dichloromethane/acetone (1:9 v/v) (CH_2Cl_2/acetone) for 12 min then rapidly transferred into a 0.05% (mass.) solution of PAA in water. Films were incubated in a PAA solution for 3 h. Thereafter, films were dried for 1 h, soaked in water for 24 h to remove extra PAA and then dried under a vacuum for 12 h.

2.4. Amine attachment to the PLA-PAA films

PLA films modified with PAA were dipped into thionyl chloride solution in hexane (1:10 v/v) for 1 h, then removed, extensively washed with hexane and dried under a vacuum for 6 h. After that, films were dipped in ABO solution in ethanol with a concentration of $1 \cdot 10^{-6}$ M, $1 \cdot 10^{-5}$ M, $1 \cdot 10^{-4}$ M or $1 \cdot 10^{-3}$ M for 5 h. The films were then removed, washed with ethanol and dried.

2.5. BSA attachment to the PLA-PAA films

BSA non-covalent attachment to the composites surface was performed as following. PLA films modified with PAA were equilibrated in 40 mM phosphate buffer (pH 7.2) for 3 h. Then the films were placed in BSA solution in 10 mM acetate buffer (pH 4.5) with a concentration of 0.1 mg/ml and incubated overnight. BSA solution was used as a control. BSA was solubilized in acetate buffer (pH 4.5) because at pH lower than its isoelectric point (4.7) BSA is charged positively. It might contribute to more efficient adsorption of BSA to the surface of PLA-PAA owing to electrostatic forces. The concentration of protein was measured in the BSA solution that remains after immobilization using Quick Start Bradford protein assay according to the manufacturer instructions (λ_{max} = 595 nm). The BSA amount attached to the PLA-PAA material surface was calculated as a difference between the initial protein concentration (0.1 mg/ml) in the solution and the protein concentration measured after the immobilization.

BSA covalent attachment to the composites surface was performed as following. PLA films modified with PAA were equilibrated in 40 mM phosphate buffer (pH 7.2) for 3 h. Then the films surface was subsequently activated using 0.4 M EDC and 0.1 M NHS solutions in water for 2 h at 4 °C. The activated PLA-PAA films were placed in BSA solution in 10 mM acetate buffer (pH 4.5) with a concentration of 0.1 mg/ml and incubated overnight. BSA solution was used as a control. The concentration of protein was measured in the BSA solution that remains after immobilization using Quick Start Bradford protein assay according to the manufacturer instructions (λ_{max} = 595 nm). The BSA amount attached to the PLA-PAA material surface was calculated as a difference between the initial protein concentration (0.1 mg/ml) in the solution and the protein concentration measured after the immobilization.

The experiment was repeated 3 times; the value is reported as mean ± SD in μg (or nmol) of BSA per cm^2 of the PLA-PAA film.

All the PLA-PAA films modified with BSA were stained with Coomassie Brilliant Blue G-250. The PLA-PAA and PLA films were used as a control ones.

2.6. Direct modification of PLA films with 2-(5-aminobenzo[d]oxazol-2-yl)phenol (ABO) and N-(2-(2-hydroxyphenyl)benzo[d]oxazol-5-yl)acetamide (AcBO)

PLA films with the size of 2 × 2 cm were dipped into the one of the following solvent/non-solvent miscible mixtures: toluene/ethanol (3:7 v/v), dichloromethane/acetone (1:9 v/v) or chloroform/ethylacetate (1:9 v/v) for 12 min then rapidly transferred into a 0.001 M solution of ABO or AcABO in ethanol. Films were incubated in the solution for 3 h. Thereafter, films were dried for 1 h, soaked in ethanol for 24 h to remove extra amine, and then dried under a vacuum for 12 h.

2.7. Atomic force microscopy (AFM)

Measurements of the surface's relief were performed in the semi-contact scanning mode with parallel investigation of phase contrast. The quantity of measurement points was 1024 × 1024 regardless of the width of the scanning area. All measurements were carried out in a high vacuum (10^{-7}–10^{-6} Torr).

2.8. Surface wettability measurements

Wettability of the sample surface was investigated using the "sitting" drop (volume of 20 μl) method. A drop of water was placed on the studied surface and after 1 min the contact angle was measured. Each experiment was repeated 3 times with 3 drops placed on different places on the material sample surface; the value is reported as mean ± SD ($n = 9$).

2.9. Thermogravimetric analysis (TGA)

The investigation of the residual solvents content in the modified PLA films was performed by thermogravimetric analysis (TGA) according to the ISO 11358 using DSC/TG/DTA analyzer (SDT Q600, TA Instruments, USA). Samples were run from 25 to 500 °C at heating rate of 10 °C/min and 100 ml/min air flow in a dry air atmosphere. Each experiment was repeated 3 times; the value is reported as mean ± SD ($n = 3$).

2.10. Modified layer capacity determination

Modified layer capacity was determined by measuring the fluorescence intensity of ABO solutions before and after PLA-PAA films were immersed at an excitation wavelength of 340 nm. Quantification was performed using different calibration curves obtained for diluted (concentration range of $5 \cdot 10^{-7}$–$1 \cdot 10^{-4}$ M, registration wavelength of 500 nm) and concentrated (concentration range of $1 \cdot 10^{-4}$–$1 \cdot 10^{-3}$ M, registration wavelength of 550 nm) ethanol solutions of ABO. Each experiment was repeated 3 times with 3 repeats; the value is reported as mean ± SD ($n = 9$).

2.11. Statistics

To assess the statistical significance of the differences in data obtained for various material groups, the non-parametric Mann-Whitney test (U test) was used. Differences were considered significant at a significance level of $p < 0.05$.

3. Results and discussion

In order to obtain PLA-PAA composites, several approaches were applied before. The common technique includes dissolving PLA in acrylic acid with subsequent polymerization with or without polymerization initiators [34]. However, this method implies the insertion of PAA in a bulk of PLA that leads to undesirable changes in initial PLA properties.

Surface modification of PLA by PAA was performed by the polymerization of acrylic acid on the PLA surface resulting in a PAA layer - the so-called "grafting from" approach [23,35,36]. This technique has several advantages [35], however the use of UV-light, polymerization initiators and also complications with the resulting PAA molecular mass control make it less attractive.

High molecular PLA dissolves well in dichloromethane, chloroform and aromatic carbohydrates, while at the same time high molecular PAA is dissolved in water. Thus, it's impossible to prepare PLA/PAA solutions, as high molecular PLA and PAA are not compatible polymers. Simple casting of PAA solutions on the PLA-based materials is not effective as well, because the PAA layer cannot attach to the PLA surface as we have shown experimentally. Thereby, preliminary treatment of the PLA surface is a necessary step in composite production.

Pre-swelling of the PLA surface region is a simple approach [27–32], which allows for the formation of the active layer able to absorb PAA. In our work, three different solvent/non-solvent systems were chosen in order to obtain PLA-PAA materials: toluene/ethanol (3:7 v/v) (PLA-PAA MePh/EtOH), dichloromethane/acetone (1:9 v/v) (PLA-PAA CH_2Cl_2/acetone), and chloroform/ethylacetate (1:9 v/v) (PLA-PAA $CHCl_3$/EtOAc). Prior to PLA-PAA composites preparation the influence of solvent/non-solvent treatment on PLA was investigated. The images of the PLA surface after the treatment with solvent/non-solvent mixtures are shown on Fig.1.

The original PLA film has a rather smooth surface with dendritic-like structures (Fig. 1, a). The morphology of the PLA film treated with toluene/ethanol (3:7 v/v) (Fig. 1, a) is also represented by the dendritic-like structures with the slightly swollen branches. In addition, the hemispherical inclusions with different size could be found. On the surface of the PLA film treated with chloroform/ethylacetate (1:9 v/v) (Fig. 1, c) the dendritic-like structures with strongly swollen brunches are observed. The morphology of the PLA film treated with dichloromethane/acetone (1:9 v/v) (Fig. 1, d) differs from all the samples and characterized with hilly relief with a rather large (in comparison to other materials) difference in altitude. Apparently, the differences in surface morphology between the treated samples are caused by the differences in the vaporization enthalpy of the solvent/non-solvent systems. In particular, the use of the mixture with lower vaporization enthalpy leads to the formation of rougher surface.

Since the described modification technique requires the use of organic solvents, which are toxic for the human body, the residual solvent content in the PLA films treated with solvent/non-solvent mixtures was evaluated by TGA. All the used solvents - toluene (b.p. 110.6 °C), ethanol (b.p. 78.4 °C), dichloromethane (b.p. 39.6 °C), acetone (b.p. 56 °C), chloroform (b.p. 61.2 °C), and ethylacetate (b.p. 77.1 °C) have boiling points lower than the decomposition temperature of PLA film (330–340 °C). Thus, the sample weight loss in the temperature range of 30–150 °C observed in the TGA curve appears because of the evaporation of the solvents remaining in the biomaterial [37]. The quantity of the residual solvents (%) in the investigated materials is presented in Table 1.

The non-treated PLA film has a weight loss of (3.6 ± 0.8)% due to the evaporation of the dichloromethane and chloroform retained in the sample from the film preparation. The weight loss of the PLA materials treated with solvent/non-solvent mixtures does not vary significantly (Table 1) from the weight loss of the initial PLA film. Considering the fact that all the films were produced by solvent casting, it could be concluded that the preparation method mostly causes the residual solvents content. Thus, proposed surface modification method does not lead to an increase of the organic solvent amount in PLA-based biomaterial.

The images of the original PLA film surface and PLA film surface modified with PAA are shown in Fig. 2.

The PLA-PAA film obtained by using the toluene/ethanol (3:7 v/v) system has rather developed surface morphology with nano-scaled triangle-shaped convexities (Fig. 2, a). In comparison, the use of chloroform/ethylacetate (1:9 v/v) (Fig. 2, b) or dichloromethane/acetone (1:9 v/v) (Fig. 2, c) for pre-swelling, gives a material with wavy-shaped structures on the surface.

The surface morphology of the PLA-PAA materials obtained using different solvent/non-solvent systems differs from that of PLA films treated with the corresponding mixtures and of the original PLA. This indicates that the observed surface structural features of PLA-PAA materials are caused by PAA entrapment.

Thus, pre-swelling of PLA material in solvent/non-solvent mixtures with different composition provides an ability to design PLA-PAA composites with diverse surface relief.

Surface wettability measurements of PLA-PAA samples are summarized in Table 2. PLA-PAA materials have improved hydrophilicity that differs among composites obtained from various solvent/non-solvent

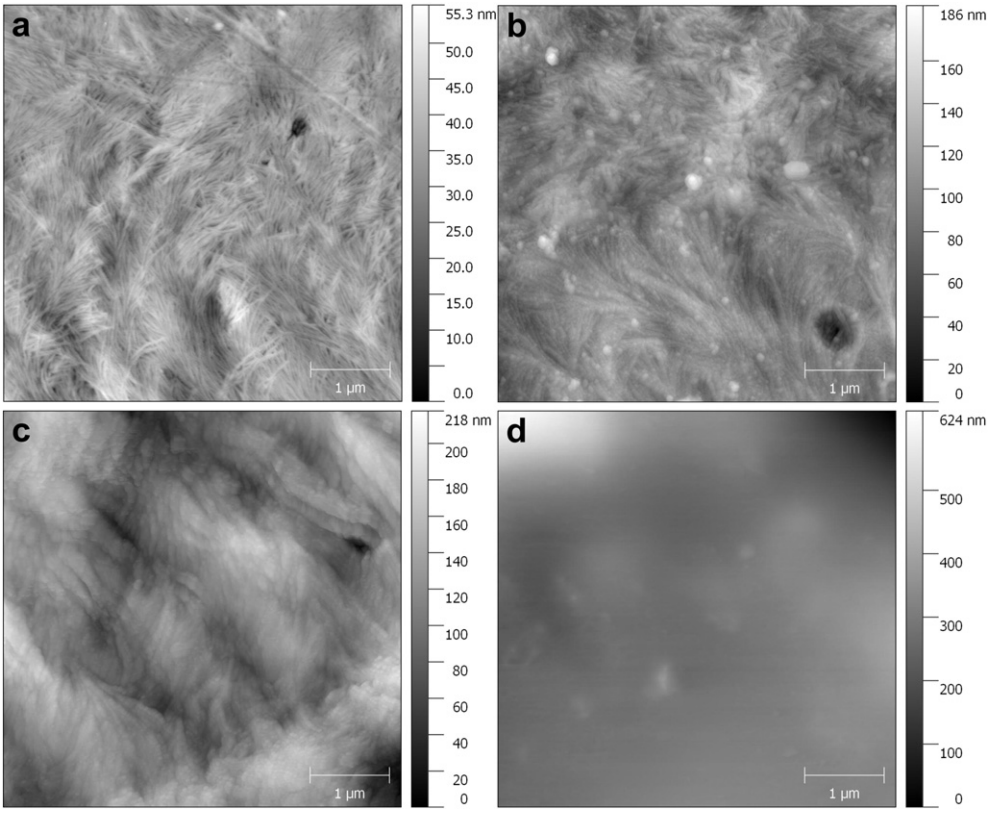

Fig. 1. Images of PLA material surface after the treatment with different solvent/non-solvent systems obtained by AFM (width of the scanning area is 5 μm): a) non-treated PLA; b) PLA treated with MePh/EtOH (3:7 v/v); c) PLA treated with $CHCl_3$/EtOAc (1:9 v/v); d) PLA treated with CH_2Cl_2/acetone (1:9 v/v).

systems. The most hydrophilic ones are PLA-PAA obtained after the PLA treatment with chloroform/ethylacetate (1:9 v/v). Thus, composites with tunable wettability can be produced.

Assessment of PAA quantity/concentration on the PLA film surface is a quite difficult task because of the little amount of PAA entrapped. However, the quantity of carboxylic groups represented by the cross-linker on the PLA-PAA composite surface can be measured through acylation reactions with the amine group containing substances. In order to evaluate the amount of carboxylic groups and, simultaneously, the amount of molecules which can be covalently linked to the PLA-PAA materials surface, the fluorescent amine ABO and its acyl derivative - AcABO - were used. These two substances were chosen because of the significant blue shift of the fluorescence band maximum that appears after ABO acylation (fluorescence band maximum for AcABO is 476 nm and for ABO is 503 nm) (Fig. 3). Also, one can notice that the AcABO ethanol solution fluorescence spectrum has a short-wavelength "shoulder" near 430 nm, which is absent in the ABO spectrum. Thus, we can easily detect which kind of bond has been formed between the PLA-PAA surface and the amine by means of fluorescence spectra. Moreover, this method of high sensitivity allows for the measurement of small differences in concentration of the ABO solution before and after PLA-PAA film immersion and, hence, anchored amine concentration on the PLA-PAA material surface. Additionally, fluoro-labeled PLA-PAA materials have practical value since they can be used for non-invasive implant fluorescent imaging. The ABO was also selected owing to the fact that 2-arylbenzoxazoles have biological activity [1]. This can be regarded as an example of biologically active amine immobilized on the PLA surface using PAA as a chemically reactive cross-linker.

Table 1
Estimated residual solvents content in the PLA films treated with different solvent/non-solvent mixtures (TGA, temperature range of 30–150 °C).

Sample	Weight loss, %
PLA	4.6 ± 0.8
PLA treated with MePh/EtOH (3:7 v/v)	4.7 ± 1.3
PLA treated with $CHCl_3$/EtOAc (1:9 v/v)	4.6 ± 0.8
PLA treated with CH_2Cl_2/acetone (1:9 v/v)	4.5 ± 1.0

Firstly, original PLA films were directly non-covalently modified with ABO and its acyl derivative AcBO using the same solvent/non-solvent mixtures (Fig. 4, a) in order to obtain fluorescence spectra of the solid fluoro-labeled PLA materials (Fig. 5). Alternatively, PLA-PAA material surface were activated by $SOCl_2$ and reacted with ABO. Fluorescence spectra from the materials (Fig. 5) were recorded and compared with directly modified ones. The general scheme of PLA surface treatment, modification with PAA, and further labelling with fluorescent compound is shown in Fig. 4, b.

Obtained spectra correspond to acyl amine derivative modified PLA materials, thus an acylation reaction took place. The spectrum of PLA-PAA-ABO also has a short-wavelength "shoulder" near 430 nm similar to the one observed in the spectrum of the AcABO ethanol solution.

Results of the created PAA surface layer capacity determination are shown in Fig. 6. The use of the ABO solution with a higher concentration leads to the creation of materials with higher fluorescent amine quantity (Fig. 6). From the ABO solutions with a concentration of $1 \cdot 10^{-6}$ M, materials containing from 1.5 to 4.3 nmol/cm^2 ABO on the surface could be obtained. The use of ABO solutions with a concentration of $1 \cdot 10^{-5}$ M leads to the slight increase in ABO concentration on PLA-based film surface up to 4.6–7.6 nmol/cm^2. Higher concentrations of ABO ($1 \cdot 10^{-4}$ M) allows for the preparation of materials containing from 9.2 to 21 nmol/cm^2. In addition, when ABO solutions with a concentration of $1 \cdot 10^{-3}$ M are used, the amount of ABO on PLA-based material surface is significantly increased up to 0.1 μmol/cm^2. Apparently, it is caused by the fact that at the concentration of $1 \cdot 10^{-3}$ M ABO is attached to all the available reaction sites on the PLA-PAA surface.

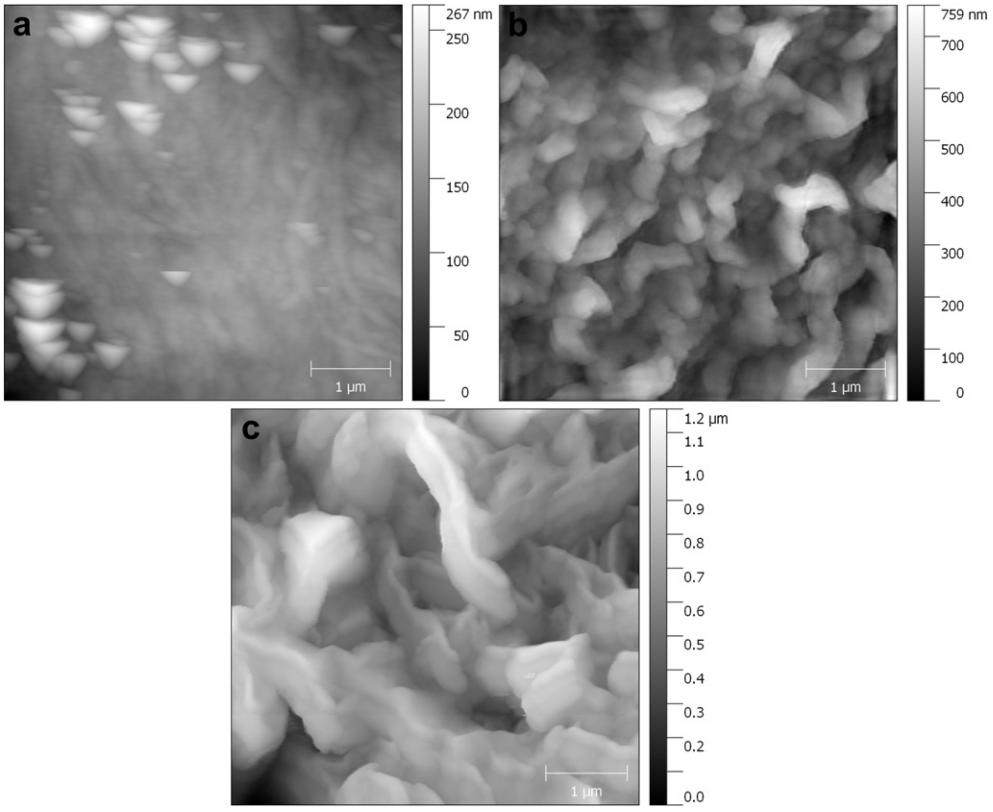

Fig. 2. Images of PLA-PAA material surface obtained by AFM (width of the scanning area is 5 μm): a) PLA-PAA MePh/EtOH (the estimated amount of PAA is 9.2 ± 1.7 μg/cm^2), b) PLA-PAA $CHCl_3$/EtOAc (the estimated amount of PAA is 9.9 ± 1.2 μg/cm^2), and c) PLA-PAA CH_2Cl_2/acetone (the estimated amount of PAA is 8.6 ± 0.3 μg/cm^2).

Amine concentrations of more than $1 \cdot 10^{-3}$ M were not checked because of the limited solubility of ABO in ethanol. Thus, the capacity of the created PAA layer could be varied from 1.5 nmol/cm^2 to almost 0.1 μmol/cm^2, which means that around 10^{15} carboxylic groups/cm^2 are able to react with amines. Surface morphology does not greatly affect the capacity of the PAA layer as there is no significant difference among materials belonging to the same group (except ones labeled using $1 \cdot 10^{-4}$ M amine solution).

The surface morphology investigation of fluoro-labeled PLA-PAA materials is shown in Fig. 7. Images demonstrate that surface relief changes after the labelling process.

PLA-PAA $CHCl_3$/EtOAc materials have a hemispherical densely packed structures on the surface (Fig. 7, c, d). The surface of the PLA-PAA CH_2Cl_2/acetone composites are represented by "stalagmite-like" structures mixed with spherical agglomerated particles (Fig. 7, e, f). PLA-PAA MePh/EtOH materials have blurred curled patterns on the surface (Fig. 7, a, b).

Thus, we have shown that the obtained composite materials surface can be successfully modified with the small amine-bearing group biologically active compounds. However, there are many examples when the presence of biomolecules of protein nature on the material surface is more favorable [2,7]. In order to demonstrate that the PAA can serve as a chemically reactive cross-linker between the PLA and proteins as well BSA was covalently and non-covalently attached to PLA-PAA materials surface. The covalent modification procedure was similar to one shown in Fig. 4, b, but instead of $SOCl_2$ the EDC/NHS were used for the surface activation. For the non-covalent BSA attachment PLA-PAA without any treatment were incubated in the protein solution. The presence of BSA on the surface of the composite materials was qualitatively

Table 2
Surface wettability of produced PLA-PAA materials (water).

Sample	Contact angle (water)
PLA	74.9 ± 0.6
PLA-PAA MePh/EtOH	62.2 ± 2.2[a,b]
PLA-PAA $CHCl_3$/EtOAc	57.7 ± 1.3[a,b]
PLA-PAA CH_2Cl_2/acetone	66.4 ± 1.7[a,b]

[a] $p < 0.05$, PLA in comparison with PLA-PAA.
[b] $p < 0.05$, among PLA-PAA groups.

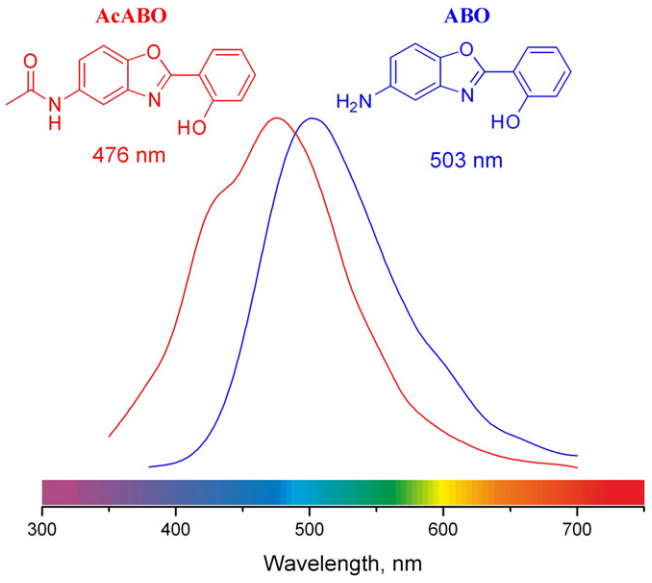

Fig. 3. Fluorescence spectra of ABO (visualized in blue) and AcABO (visualized in red) in ethanol.

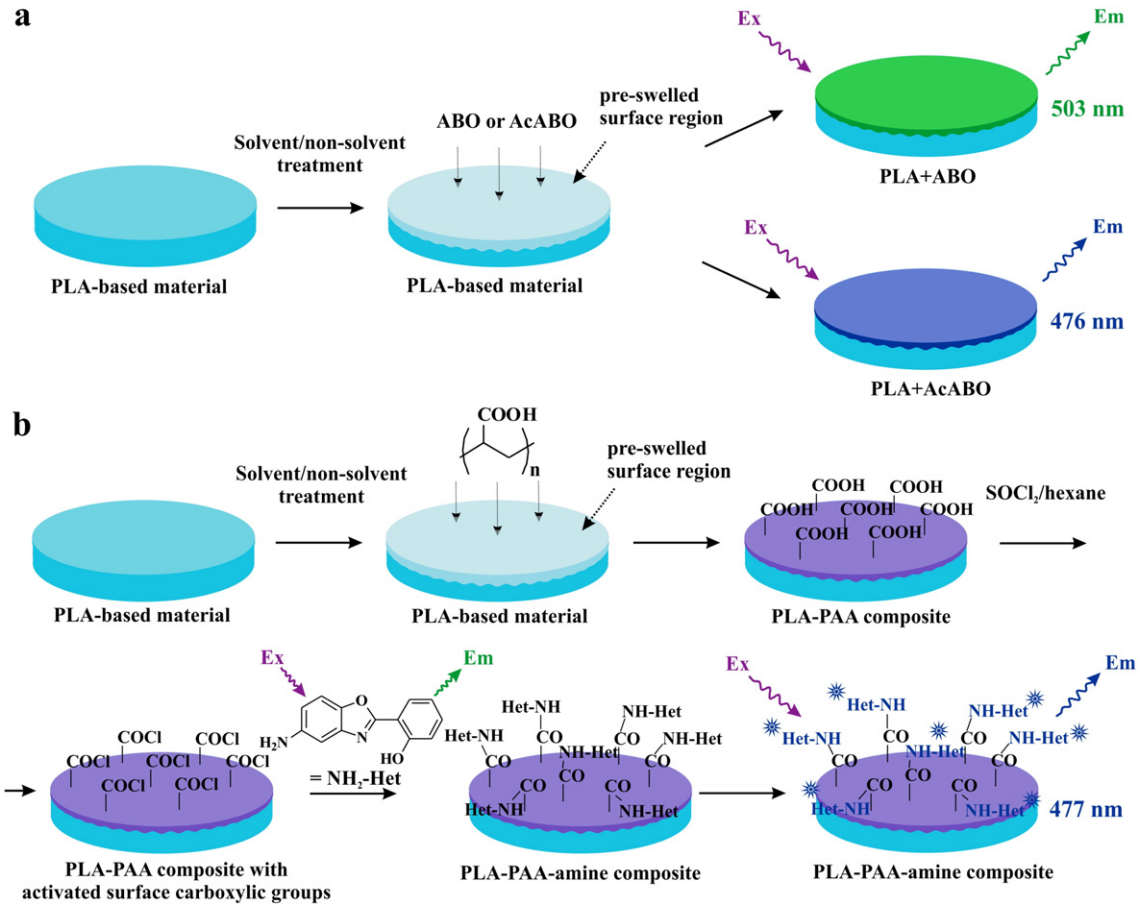

Fig. 4. a) Direct modification of PLA-based biomaterials with ABO and AcABO; b) Modification of PLA-based biomaterials with fluorescent ABO compound using PAA as a cross-linker.

confirmed by staining with Coomassie Brilliant Blue G-250. The results are presented in Table 3. During the BSA covalent attachment the protein adsorption to the material surface also occurs. Taking into account the fact that the amount of BSA on the PLA-PAA surface was calculated as a difference in BSA concentration in its solution before and after the material immersion, it includes both covalently and non-covalently attached protein and in Table 3 is assigned as BSA total. In order to assess the amount of covalently bound BSA the average concentration of the BSA adsorbed was subtracted from the BSA total and assigned as BSA covalent.

According to the data presented, PLA materials modified with PAA are able to adsorb BSA from the solution without any additional treatment owing to the presence of the large number of the carboxylic groups on their surface. It is observed that the highest amount of the BSA was adsorbed to PLA-PAA MePh/EtOH material with the least rough surface (Table 3). It might be explained by the fact that multilayer adsorption of BSA occurs more easily on the smooth surface while on the rough surfaces the adsorbate-adsorbate interactions could be hindered due to the steric reasons.

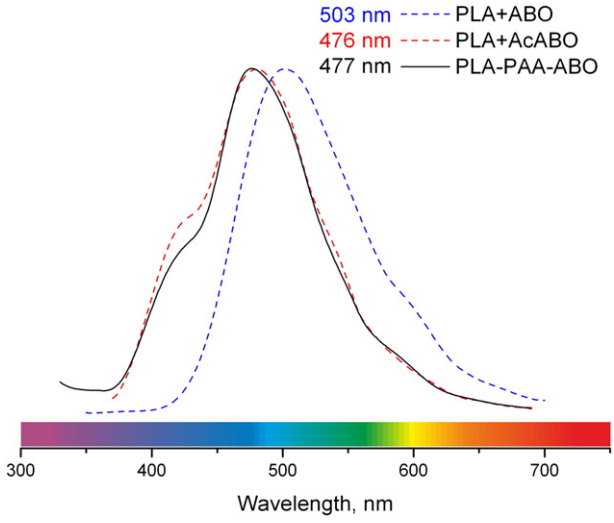

Fig. 5. Fluorescence spectra of PLA films directly modified with amines ABO (PLA + ABO) and AcABO (PLA + AcABO), and PLA-PAA films reacted with ABO (PLA-PAA-ABO).

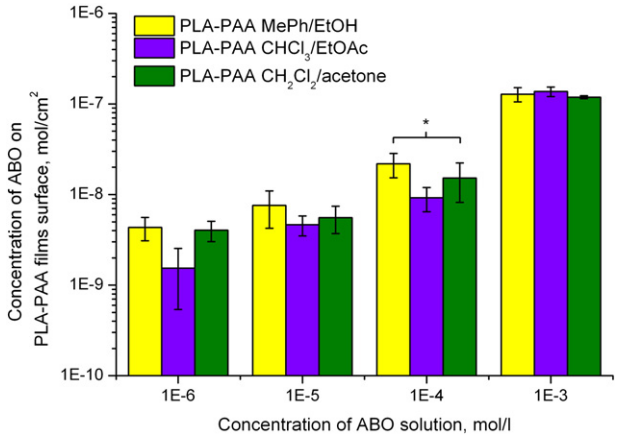

Fig. 6. Results of PLA-PAA surface layer capacity determination in double log reciprocal (* - $p < 0.05$, Mann-Whitney test).

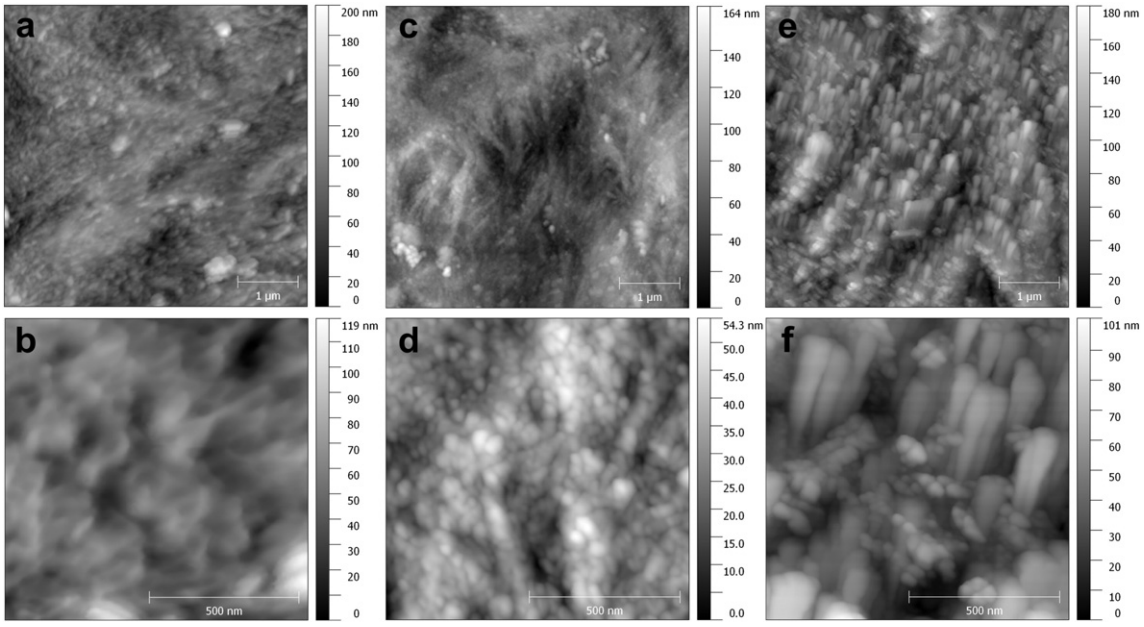

Fig. 7. Images of PLA-PAA-ABO material surface obtained by AFM: a)-b) PLA-PAA-ABO MePh/EtOH (the estimated amount of ABO is 128.2 ± 2.3 nmol/cm^2), c)-d) PLA-PAA-ABO $CHCl_3$/EtOAc (the estimated amount of ABO is 137.3 ± 1.6 nmol/cm^2), and e)-f) PLA-PAA-ABO CH_2Cl_2/acetone (the estimated amount of ABO is 118.7 ± 0.4 nmol/cm^2). For a), c), and e) the width of the scanning area is 5 μm, while for b), d), and f) the width of the scanning area is 1 μm. The concentration of ABO used to modify PLA-PAA surfaces was 10^{-3} M.

The amount of BSA covalently linked to the PLA-PAA materials surface does not vary significantly among the materials types. BSA could be attached to all the PLA-PAA composites with the average binding density of 0.18 nmol/cm^2 (Table 3). It should be noted that the amount of the protein attached to the developed PLA-PAA composites is two times higher than the amount of BSA linked to the PLA-PAA materials produced by the polymerization of acrylic acid on the PLA surface [24]. However, such a concentration is still much less that the concentration of the ABO covalently linked to the corresponding material surface. It may be caused by fact that single BSA molecule bears a number of amino-groups and could react with the several carboxyl groups on the PLA-PAA surface at once. Nevertheless, taking into account the previous studies [24] we can conclude that the surface of the produced PLA-PAA composites could be modified with proteins (BSA) with high binding density.

In order to investigate how firmly the PAA layer is attached to the PLA surface, PLA-ABO-PAA materials were obtained. Briefly, PLA-based films were modified step-wise with ABO first and then PAA using the modification method described above. These materials were continuously immersed in water solutions with different pH (1.0, 7.0 and 10.0) for 1 week in each and the fluorescence of the solutions was measured. If the PAA layer remains on the PLA surface during the experiment it should prevent the output of the ABO to solution. After 3 weeks, no fluorescence was detected, which means that the PAA layer firmly connected to the PLA surface.

4. Conclusions

PLA-PAA composite biomaterials were obtained via non-covalent attachment of the PAA to PLA surface without any destructive treatment or the use of additional reagents. The described technique allows for the preparation of the materials with various surface morphology and improved tunable wettability. The use of the organic solvents for the PLA surface modification does not lead to an increase of the residual solvent amount in biomaterial. Obtained PLA-PAA biomaterials can be further modified with amine group containing compounds, which was shown by fluorescent labelling. The capacity of the created PAA layer could be varied from 1.5 nmol/cm^2 to almost 0.1 μmol/cm^2. Moreover, produced composites could be modified with proteins (as it was shown on BSA example) with high binding density (around 0.18 nmol/cm^2). Obtained fluoro-labeled PLA-PAA materials, as well as PLA-PAA composites themselves, are valuable since they can be used for biodegradable polymer implants tracking in living systems and as drug delivery systems. The use of fluorescent amines for the quantification of active functional groups on the surface of the composite materials is a more affordable alternative method to XPS.

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Table 3
The amount of the covalently linked and adsorbed BSA on the PLA-PAA composites surface.

Material type	Qualitative test	BSA adsorbed, μg/cm^2	BSA total, μg/cm^2	BSA covalent (total -adsorbed), μg/cm^2	BSA covalent (total -adsorbed), nmol/cm^2
PLA-PAA MePh/EtOH	+	0.49 ± 0.09 [a]	13.19 ± 2.28	12.70 ± 2.37	0.19 ± 0.04
PLA-PAA $CHCl_3$/EtOAc	+	0.12 ± 0.05	11.95 ± 1.34	11.83 ± 1.39	0.17 ± 0.02
PLA-PAA CH_2Cl_2/acetone	+	0.15 ± 0.06	13.22 ± 1.78	13.07 ± 1.84	0.19 ± 0.03

[a] - $p < 0.05$ in comparison with other material types, Mann-Whitney test.

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