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Synthesis and characterization of cycloaliphatic hydrophilic polyurethanes, modified with L-ascorbic acid, as materials for soft tissue regeneration

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a b s t r a c t

In this paper we described synthesis and characteristic of obtained hydrophilic polyurethanes (PURs) modified with ascorbic acid (commonly known as vitamin C). Such materials may find an application in the biomedical field, for example in the regenerative medicine of soft tissues, according to ascorbic acid wide influence on tissue regeneration Flora (2009), Szymańska-Pasternak et al. (2011), Taikarimi and Ibrahim (2011), Myrvik and Volk (1954), Li et al. (2001), Cursino et al. (2005) . Hydrophilic PURs were obtained with the use of amorphous α,ω-dihydroxy(ethylene-butylene adipate) (dHEBA) polyol, 1,4-butanediol (BDO) chain extender and aliphatic 4,4′-methylenebis(cyclohexyl isocyanate) (HMDI). HMDI was chosen as a nontoxic diisocyanate, suitable for bio-medical PUR synthesis. Modification with L-ascorbic acid (AA) was performed to improve obtained PUR materials biocompatibility. Chemical structure of obtained PURs was provided and confirmed by Fourier transform infrared spectroscopy (FTIR) and Proton nuclear magnetic resonance spectroscopy (^{1}HNMR). Differential scanning calo-rimetry (DSC) was used to indicate the influence of ascorbic acid modification on such parameters as glass tran-sition temperature, melting temperature and melting enthalpies of obtained materials. To determine how these materials may potentially behave, after implementation in tissue, degradation behavior of obtained PURs in var-ious chemical environments, which were represented by canola oil, saline solution, distilled water and phosphate buffered saline (PBS) was estimated. The influence of AA on hydrophilic-hydrophobic character of obtained PURs was established by contact angle study. This experiment revealed that ascorbic acid significantly improves hydro-philicity of obtained PUR materials and the same cause that they are more suitable candidates for biomedical ap-plications. Good hemocompatibility characteristic of studied PUR materials was confirmed by the hemocompatibility test with human blood. Microbiological tests were carried out to indicate the microbiological sensitivity of obtained PURs. Results of performed studies showed that obtained AA-modified PUR materials may find an application in soft tissue regeneration.

Keywords: Polyurethane, L-Ascorbic acid, Chemical structure, Physicochemical properties, Tissue regeneration

1. Introduction

PURs are one of the most extensively developed synthetic polymers for biomedical applications. The major advantage of PURs is the ease of their physicochemical and mechanical properties design, which may be performed by proper selection of raw materials used for their synthesis [7,8]. This is an important issue especially in the field of materials dedicated to regenerative medicine and tissue regeneration [9–11].

PURs consist of alternating soft (SS) and hard (HS) segments. Segmented structure of PURs led to the phase separation of these materials, which affect their physicochemical and thermomechanical properties. SS are formed by polyester, polyeter or polycarbonate polyol and HS are derived from diisocyanate and low molecular chain extender. SS provide elastomeric character of the PUR and HS provide good mechanical strength due to the hydrogen bonds formed between urethane linkages. Changing in chemical composition, the molecular weight and the ratio of HS and SS led to synthesize PURs of different physicochemical characteristic as well as biocompatibility and biodegradability [12–14].

Constantly growing interest of PURs application in biomedical field is related to their suitable biocompatibility, hemocompatibility and biodegradability [15–19]. The biodegradation rate is mainly dependent on the SS structure related directly to the type of used polyol [20]. Many different polyols are engaged in the synthesis of biomedical PURs. To the most commonly used polyols belong poly(caprolactone) (PCL), poly(propylene glycol) (PPG), poly(ethylene glycol) (PEG) and poly(glicolide) [21]. HS plays an important role in case of biocompatibility of PURs [22]. In the field of tissue engineering, where biodegradable

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scaffolds are produced, aliphatic diisocyanates are engaged to the synthesis of PURs. It is due to the reduced toxicity of PUR degradation products synthesized by using aliphatic diisocyanates (in comparison to aromatic diisocyanates) in in vivo conditions [23]. To the most commonly applied diisocyanates for biomedical PUR synthesis belong 1,4-butanediisocyanate (BDI), isophorone diisocyanate (IPDI), 1,6-hexamethylene diisocyanate (HDI) [7,22,24].

One of the most important parameters, which evaluate the applicability of synthetic polymers in biomedical applications, is their hydrophilic character. Hydrolytic degradation plays a key role in the development of materials dedicated to biomedical applications [25]. Degradation of poly(ester urethane)s was divided into three steps. First step is the incubation, where absorption of water occurs. In the second step the induction happens during which the polymer chains are broken via ester bonds. Last step is the erosion where water-soluble entities (such as PEG blocks and oligomers) are dissolved in the buffer solution, with corresponding polymer mass loss [26,27]. The hydrophilicity concept of synthetic polymers reports that surface characteristic of polymer should include the degree of water absorption to the polymer surface, what is associated with blood response. The suitable hydrophilic characteristic of polymeric surface prevents macrophages adhesion to their surface and the same prevents formation of blood clots, which presence could cause a serious system response [28,29]. An optimization of the hydrophobic-hydrophilic ratio of polymeric surface improves blood compatibility by reducing platelet adhesion [30]. Hydrophilic character of PURs is directly related to their chemical composition [28].

PURs may exhibit the hydrophobic surface properties, what is related to their composition, and due to this fact in such situation they need to be modified at variable levels to improve their hydrophilicity and the same the degree of their biocompatibility [31]. Literature data report many examples of PUR modifications performed directly to improve their antithrombotic character and biocompatibility [12]. For example, surface of PURs designed for tissue scaffolds may be functionalized to induce the cascade of biological processes, which lead to rebuilding and regeneration of destroyed tissue. Bioactive factors, which are used for this type of functionalization, may be introduced into the polymer matrix during the polymer synthesis [32], may also form a compatible protective covers (e.g. heparin, silicone) or bioactive factors may be physically, chemically or biologically functionalized onto the polymeric surface as for example phospholipids [33], biological anticoagulants (heparin) [34,35], anti-platelet factors such (glycoprotein inhibitor IIb/IIIa) [36] and also antiproliferative agents (rapamycin) or proliferation agents that improve cells growth [37,38].

To achieve the suitable hydrophilic characteristic and the degree of biocompatibility AA may also be used according to the fact that it improves tissue regeneration [7,39], AA is a strong antioxidant which prevents proteins oxidation [1] and improves function of ECM cells by stabilization of tetrahydrobiopterin, which is the main and necessary cofactor of nitric oxide synthase [2] Some references reported also that AA reveals the antibacterial activity against some microorganisms like *Streptococcus pneumonia*, *Escherichia coli* or *Pseudomonas aeruginosa* [3–6].

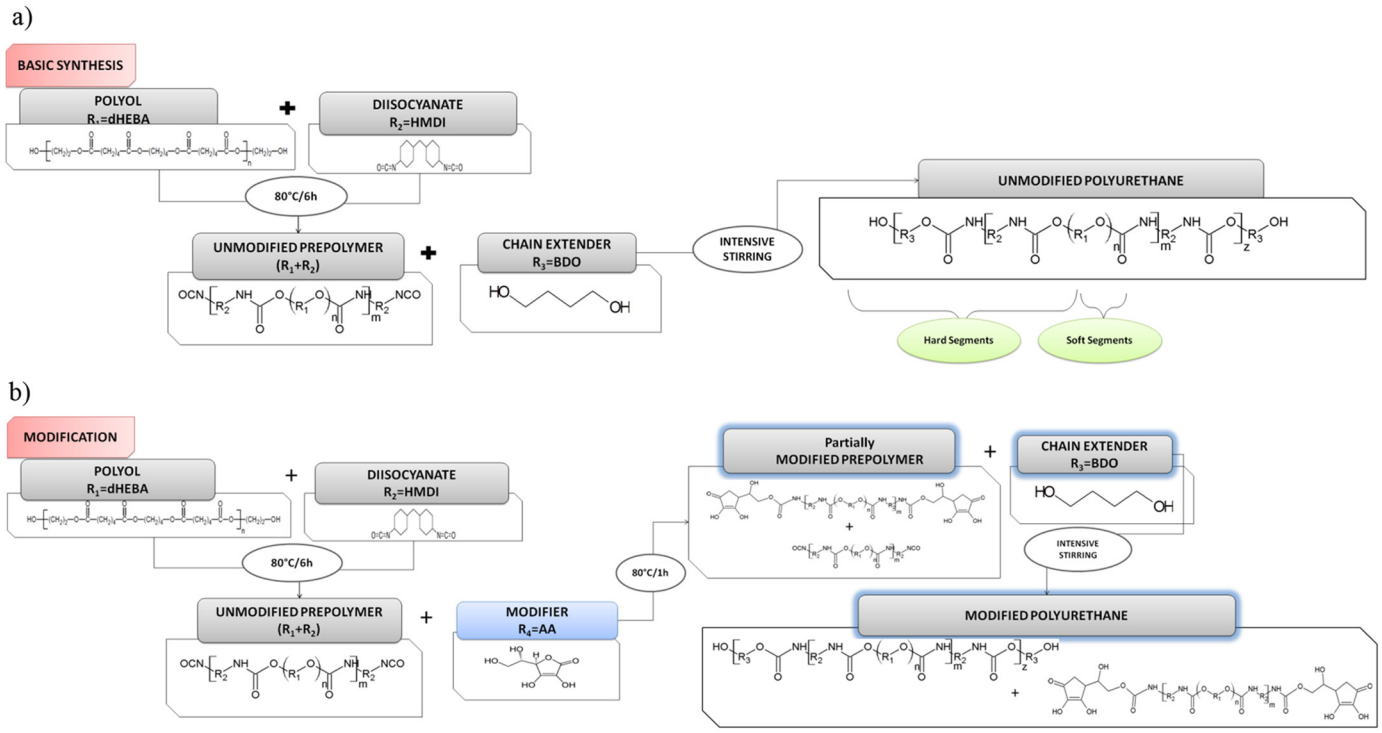
In this paper we described the synthesis and characteristic of hydrophilic PURs for biomedical applications such as regenerative medicine of soft tissues, what is the development of previously undertaken studies of our team [7]. To achieve proper hydrophilic characteristic PURs were obtained with the use of selected raw materials: amorphous polyester α,ω-dihydroxy(ethylene-butylene adipate) (dHEBA), difunctional chain extender 1,4-butanediol (BDO) and cycloaliphatic diisocyanate (4,4′-methylene bis(cyclohexyl isocyanate) (HMDI)). HMDI was chosen as a nontoxic diisocyanate widely reported in references as a suitable raw material for biomedical PUR synthesis. Moreover, the degradation products of aliphatic isocyanate-based PURs are considered as nontoxic for living organisms and so they are removed in natural life cycles [22]. To improve biomedical character of obtained PURs the modification with AA was performed, due to its wide influence on tissue regeneration [7,39]. Already described PUR-AA-based systems were designed for bones tissue regeneration and different raw materials were used in comparison to our study [40,41]. Due to our knowledge, there are no references yet describing AA influence on hydrophilic characteristic of PURs designed for biomedical applications. Chemical structure and composition was studied by FTIR and ^{1}HNMR spectroscopy. DSC analysis was performed to indicate the T_g, T_m and melting enthalpies of obtained PUR materials. To determine the degradation behavior of obtained PURs interactions with selected media (canola oil, saline solution, distilled water and phosphate buffered saline (PBS)) were studied. The characterization of materials' interactions with such media is commonly performed for medical-grade polymers [42]. Canola oil assay may be used to determine the in situ behavior of the biomaterials according to the lipids present in the living body [43]. The exposure of biomaterials to lipids may lead, in vivo conditions, to swelling and degradation in otherwise stable biomaterials [44]. Furthermore, drugs delivered to the body, encapsulated in a biodegradable polymer, are often introduced to the system as a lipid emulsion [45]. Contact angle was defined to estimate the degree of hydrophilic-hydrophobic characteristic of obtained PURs. Literature data require polymeric materials of contact angle in the range of 45–76°as most suitable for cells adhesion and proliferation [46]. Moreover, the ability of polymeric materials, of hydrophilic character, to form hydrogen bonds improves their biocompatibility by solvatation water molecules, which form at their surface water film that is biologically neutral [46]. To study microbiological sensitivity of obtained PURs the microbiological test was performed against selected microorganisms: *Staphylococcus aureus*, *P. aeruginosa* and *E. coli*. These microorganisms are dominant amongst the bacterium, which cause inflammatory complications [47] and examples of the common post-implantation infections [48].

2. Experimental

2.1. Polyurethane synthesis (PURs)

PURs were synthesized by standard two step polymerization procedure [49,50]. In the first step a prepolymer was obtained with 8% of free isocyanate groups. It was derived from oligomeric α,ω-dihydroxy(ethylene-butylene adipate) (dHEBA) polyester (trade name Polios 55/20; Purinova, Poland) (65 wt%) and aliphatic 4,4′-methylenebis(cyclohexyl isocyanate) (HMDI) (Sigma Aldrich, Poland) (35 wt%). The prepolymer reaction was carried out in the glass, 4-neck, reactor at 80 °C for 6 h. In the second step the chain extender - 1,4-butanediol (BDO) (POCH, Poland) - was added to obtain unmodified PURs with molar ratio of free isocyanate groups (NCO) (in the prepolymer) to hydroxyl groups (OH) of chain extender BDO NCO/OH = 0,9:1. Dibutyltin dilaurate (DBTDL), at the amount of 1 wt%, was used as a catalyst, commonly used for the synthesis of biomedical PURs [51,52]. The reaction mixture was subjected to intensive stirring and then transferred into a mold, set at 80 °C overnight. Then, the samples were left in a drier at 80 °C for 48 h to complete the reaction.

The synthesis of modified PUR was as follows: to the obtained prepolymer 2 wt% of solid AA (Sigma Aldrich), calculated per mass of the prepolymer (prepolymer: AA = 1:0,25), was added at room temperature. This amount of AA was established as most suitable in preliminary studies. After addition of the AA to the prepolymer, the temperature was raised to 80 °C and the reaction was continued for 1 h. Then the chain extender BDO was added to obtain AA-modified PURs. Reaction mixture, after vigorous stirring was transferred to a mold of 80 °C overnight. After this time, the AA-modified PUR was left in the drier for 48 h at 80 °C to complete the reaction. Thus samples of 2 mm thickness were obtained. Reaction of unmodified and AA-modified PURs was presented at Scheme 1. Table 1 presents symbols used to mark the obtained PURs and a brief explanation of them.



Scheme 1. Scheme of unmodified (a) and ascorbic acid modified (b) polyurethane synthesis path.

2.2. Characterization methods

2.2.1. Fourier transform infrared (FT-IR) spectroscopy

The FTIR of the solid PUR and AA-modified PUR was performed at FTIR Nicolet 8700 Spectrometer to characterize the chemical composition of obtained unmodified and modified with AA-modified PURs. The spectral range was from 4000 to 500 cm^{-1} averaging 254 scans per sample with a resolution of 4 cm^{-1}.

2.2.2. Nuclear magnetic resonance (^{1}HNMR) spectroscopy

^{1}HNMR spectra were obtained with the use of 500 MHz Varian Spectrometer Unity 500 Plus using deuterated dimethylsulfoxide (DMSO-d_6) as PUR solvent and tetramethylsilane (TMS) as the internal standard. The ^{1}HNMR was performed to determine the chemical structure of obtained PUR materials.

2.2.3. Differential scanning calorimetry (DSC)

DSC was performed on a NETZSCH DSC 204 F1 Phoenix apparatus using 10-mg samples at a temperature range of −30–250 °C and under N_2 atmosphere with a heating rate of the sample equal 5 °C/min. According to DSC curves was determined the melting point (Tm), enthalpy of melting (ΔHm) and the glass transition temperature (T_g) of obtained PUR materials.

Table 1
Symbols given to the obtained polyurethanes with the brief explanation.

Symbol PUR	Explanation
PU-0,9/HMDI/AA0	PU-0,9 – polyurethane obtained at molar ratio of NCO:OH = 0.9:1 (at the stage of chain extending with BDO), HMDI – used diisocyanate, in this case 4,4′-methylene bis(cyclohexyl isocyanate), AA0 – polyurethane which was not modified with ascorbic acid
PU-0,9/HMDI/AA2	PU-0,9 – polyurethane obtained at molar ratio of NCO:OH = 0.9:1 (at the stage of chain extending with BDO), HMDI – used diisocyanate, in this case 4,4′-methylene bis(cyclohexyl isocyanate), AA2 – polyurethane modified with 2 wt% of ascorbic acid

2.2.4. Interactions with canola oil, saline, distilled water and phosphate buffered saline

PUR and AA-modified PUR materials were cut into 6 samples of a 1 cm^2 area and then dried and weighed in Thermobalance (RADWAG MAX50/SX) set at 60 °C. Prepared samples were placed in glass containers filled with canola oil, distilled water, saline or phosphate buffered saline (PBS) (each medium – volume of 3 mL). Samples were incubated in a specific media at room temperature. Changes of samples' weight were examined after 1 day of incubation for canola oil medium; after 1, 3, 7, 14 days and 1, 2, 3 months for distilled water, saline and PBS [42]. The measurement procedure was as follows: samples were taken from the container and placed between paper towels in order to reduce the excess of medium used in the test. The samples were then placed in a thermobalance (set at 60 °C) and weighed to the constant mass. The weight loss was calculated by the formula (1), where m_i - sample weight after 1, 3, 7, 14 days and 1, 2, 3 months of incubation (g), m_0 - sample weight before the test (g). The results are arithmetic average of four measurements. In case of PBS study pH solution was controlled every two weeks with the use of Metler Toledo pH-meter. The statistical analysis was performed with the use of the Origin Pro 8.5. To evaluate statistical differences the two-way ANOVA ($\alpha = 0{,}05$) and post-hoc Tukey test ($\alpha = 0{,}05$) were used.

$$S = \left(\frac{m_i - m_0}{m_0}\right) \cdot 100\% \quad (1)$$

2.2.5. Contact angle

Static contact angles of both basic PUR and AA-modified PUR were determined at room temperature with the use of Reichert Wien optical microscope (35× magnification). PURs were cut in 2 cm^2 samples, which surface was purified with n-hexane (POCH, Poland) before the measurement. To determine contact angle the Sessile Drop Method (SDM) was applied, using a 5 μL of distilled water droplet. For each

angle reported, at least five measurements on different surface locations were averaged. The width and the height of the Sessile Drop was indicated and the contact angle was determined according to the formulas (2)–(4). The statistical analysis was performed with the use of the Origin Pro 8.5. To evaluate statistical differences the two-way ANOVA ($\alpha = 0{,}05$) and post-hoc Tukey test ($\alpha = 0{,}05$) were used.

$$\mathrm{tg}\frac{\theta}{2} = \frac{2h}{d} \tag{2}$$

$$\mathrm{arctg}\frac{2h}{d} = \frac{\theta}{2} \tag{3}$$

$$\theta[^\circ] = \theta[\mathrm{rad}] \cdot \frac{180^\circ}{\pi} \tag{4}$$

2.2.6. Hemocompatibility

This test was performed to evaluate the biocompatibility of obtained hydrophilic PUR with the human blood (Gdansk University Clinical Center). The influence of PUR materials on blood parameters is measured with the use of blood clotting, hemolysis and platelet deposition tests [53,54]. Well designed PURs are known to be biocompatible with blood and prevent blood clotting [55]. Hemocompatibility was examined in Medical Laboratory with analyzer SYSMEX XS – 1000i. Sample of venous blood from two healthy women were used in this study. Biologic material, directly after being taken, was put into test-tube containing potassium acetate – agent which prevents blood clotting. Next step was obtaining reference parameters for blood morphology. After that to the test-tube were put samples with a size of 8 cm^2 of PUR or AA-modified PUR and 8 mL of blood was added. The samples before hemocompatibility test were sterilized with argon gas plasma generated over H_2O_2. The samples were incubated in blood for 15 min at room temperature. After this time they were removed and blood was hematologically analyzed. The statistical analysis was performed with the use of the Origin Pro 8.5. To evaluate statistical differences the two-way ANOVA ($\alpha = 0{,}05$) and post hock Tukey test ($\alpha = 0{,}05$) were used.

2.2.7. Microbiological studies

2.2.7.1. Microorganisms. Antibacterial activities of the prepared materials were tested by using *Escherichia coli* (Gram negative), *Staphylococcus aureus* (Gram positive) and Pseudomonas aeruginosa (Gram negative). All experiments were carried out with use of the same bacterial strains, obtained from collection of the Department of Molecular Biotechnology and Microbiology, Gdańsk University of Technology. Stock cultures were maintained by periodic subculture on nutrient agar slants which were stored at 4 °C. Before each experiment, strains were cultivated on fresh

LA plates and incubated for 24 h at 37 °C. For preparation of LA plates, LA medium containing, g/L: casein peptone 10.0; yeast extract 5.0; NaCl 10.0, agar 15.0 were dissolved in 1 L of deionized water and then autoclaved (121 °C, 1,5 atm., 20 min), cooled to 45 °C and poured into sterile Petri plates. Approximately 25 mL of sterile agar medium was poured into 90-mm disposable, sterile Petri dishes and allowed to solidify. All reagents were supplied by BTL Sp. z o.o., Lodz. Poland.

2.2.7.2. Bacterial tests. The startup of the experiments was obtained by inoculating 20 mL of sterile LB (Luria Broth) with microbial strains from LA plates. Cultivation was carried out in 200 mL Erlenmeyer flasks, on a rotary shaker at 170 rpm at 37 °C for 24 h. LB medium containing, g/L: casein peptone 10.0; yeast extract 5.0; NaCl 10.0, were dissolved in 1 L of deionized water and then

autoclaved (121 °C, 1,5 atm., 20 min) and cooled to room temperature. All reagents were supplied by BTL Sp. z o.o., Lodz. Poland.

After the incubation, 1 mL of each bacterial strain suspension was transferred into 100 mL sterile Erlenmeyer flasks containing 10 mL of the fresh LB medium. The cells were cultivated for 6–8 h at 37 °C on a rotary shaker at 170 rpm to get the log phase of bacteria.

For determination of potential antibacterial activities of selected PURs, 100 μL of each bacterial strain suspension were transferred and spread on LA plates with sterile glass rod. After of incubation of bacterial cultures on LA plates for 24 h at 37 °C, the sterile samples of examined PUR material were placed over the bacterial cultures on LA plates. For this purpose, samples surface (1 cm diameter) were sterilized by submerging in 70% ethanol and then rinsed thoroughly with sterile deionized water. Afterward, each side of PUR was exposed to UV radiation for 30 min. Sterile samples were dried out in Thermobalance (RADWAG MAX50/SX) set at 80 °C for 30 min and then placed with sterile tweezers on LA plates. Plates were incubated in 37 °C for 24 h. After the incubation, the presence or absence of zones of bacterial growth inhibition around samples of unmodified and modified PURs was checked.

3. Results

3.1. Fourier transform infrared (FT-IR) spectroscopy

The FT-IR spectra of solid samples were presented in Fig. 1. In the interpretation of the particular bands (Table 2) helped us Panicker et al. [55] scientific paper and the books of Silverstein et al. [56] and Socrates, G [57]. The spectra of PUR and AA-modified PUR (Fig. 1) are similar to some extent. The weak absorption peaks, observed at 3319 cm^{-1}, were assigned to stretching vibrations of N—H groups present in both types of PUR spectra. The wide base of the indentified peak of NH stretching may be related with presence of hydrogen bonds in the PUR and AA-modified PUR structure. The asymmetric and symmetric stretching vibrations of CH_2 groups were noted between 2930 and 2854 cm^{-1} in both cases of studied materials (Table 2). The very strong carbonyl stretching, in case of obtained PURs and AA-modified PURs, appeared at 1726 cm^{-1} and it is directly related with presence of substantial amount of non-hydrogen bonded or poorly organized hydrogen bonded carbonyl urethane groups; and at 1655 cm^{-1}, which concerns well ordered and strongly hydrogen bonded urethane groups in PURs

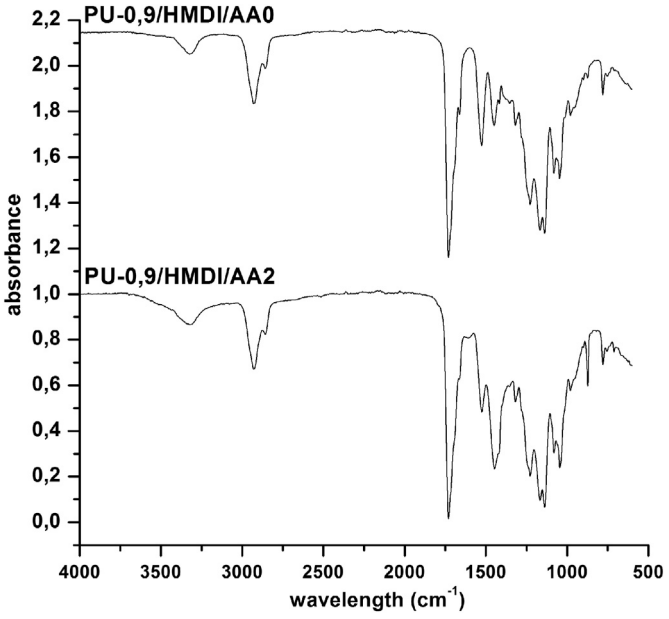


Fig. 1. FTIR spectra of obtained unmodified and ascorbic acid modified poly(ester urethane)s.

Table 2
Spectral data[a] and band assignments of FTIR analysis presented in Fig. 1.

PU-0,9/HMDI/AA0		PU-0,9/HMDI/AA2	
Wavelength (cm^{-1})	Assignments	Wavelength (cm^{-1})	Assignments
3319w	N—H stretching, (urethane bonding)	3319w	N—H stretching, (urethane bonding)
2930w, 2859w	CH_3; CH_2 (aliphatic) asymmetric and symmetric stretching bonding	2930w, 2854w	CH_3; CH_2 (aliphatic) asymmetric and symmetric stretching bonding
1726vs, 1655w	C═O stretching of non-hydrogen bonded urethane groups and well ordered and strongly hydrogen bonded urethane groups respectively	1735vs, 1659w	C═O stretching of non-hydrogen bonded urethane groups and well ordered and strongly hydrogen bonded urethane groups respectively
1527m	Deformation bond N—H	1522m	Deformation bond N—H
1451w, 1420vw, 1354vw, 1314s	Planar bonds of symmetric and asymmetric CH_2	1446s, 1323w	Planary bonds of symmetric and asymmetric CH_2
1221s	N—C stretching (urethane bonding)	1217s	N—C stretching (urethane bonding)
1168s, 1138s	CO—O stretching of ester	1164s, 1129s	CO—O stretching of ester
1080m, 1036m	C—O stretching of urethane groups	1076m, 1036m	C—O stretching of urethane
969vw, 943vw, 871vw, 779w	Out of plane bondings of C—H (bending), CH2 scissoring; CH2 wagging; NH and OH scissoring and wagging	998vw, 863w, 779vw, 717vw	Out of plane bondings of C—H (bending), CH2 scissoring; CH2 wagging; NH and OH scissoring and wagging

[a] w - weak, vw – very weak, m - medium, s - strong, vs - very strong.

and AA-modified PURs [58]. Peak present at 1522–1527 cm^{-1} is related with NH groups deformation vibrations. Signals, indicated in the range of 1451–1314 cm^{-1} concerns planar vibrations of symmetric and asymmetric CH_2 groups. Peaks observed at 1221–1217 cm^{-1} are related with N—C stretching (urethane bonding). Peaks at 1168 cm^{-1}, 1138 cm^{-1} and 1164 cm^{-1}, 1129 cm^{-1}, respectively for PURs and AA-modified PURs, corresponds to the CO-O stretching of ester groups of dHEBA, and peaks in the range 969–779 cm^{-1} are related with out of plane bondings vibrations of C—H bending, CH_2 scissoring; CH_2 wagging; NH and OH scissoring and wagging. The FTIR analysis confirmed that the performed synthesis lead to obtain PURs.

3.2. Nuclear magnetic resonance (^{1}HNMR)

The chemical structure of solid unmodified (Fig. 2) and AA-modified PURs (Fig. 3) was determined by ^{1}HNMR spectra. In making assignments of spectra helped us Silverstein et al. [56] and Socrates G [57]. The ^{1}HNMR spectra of PUR (Fig. 2) and AA-modified PUR (Fig. 3) are similar to some extent. The presence of proton signals of soft and hard segments and formed urethane bonding were confirmed and presented in Figs. 2 and 3. The spectra interpretation was performed and presented in Table 3. The HNMR spectra confirmed the formation of PUR and presence of AA in PURs chains, which was partially incorporated into

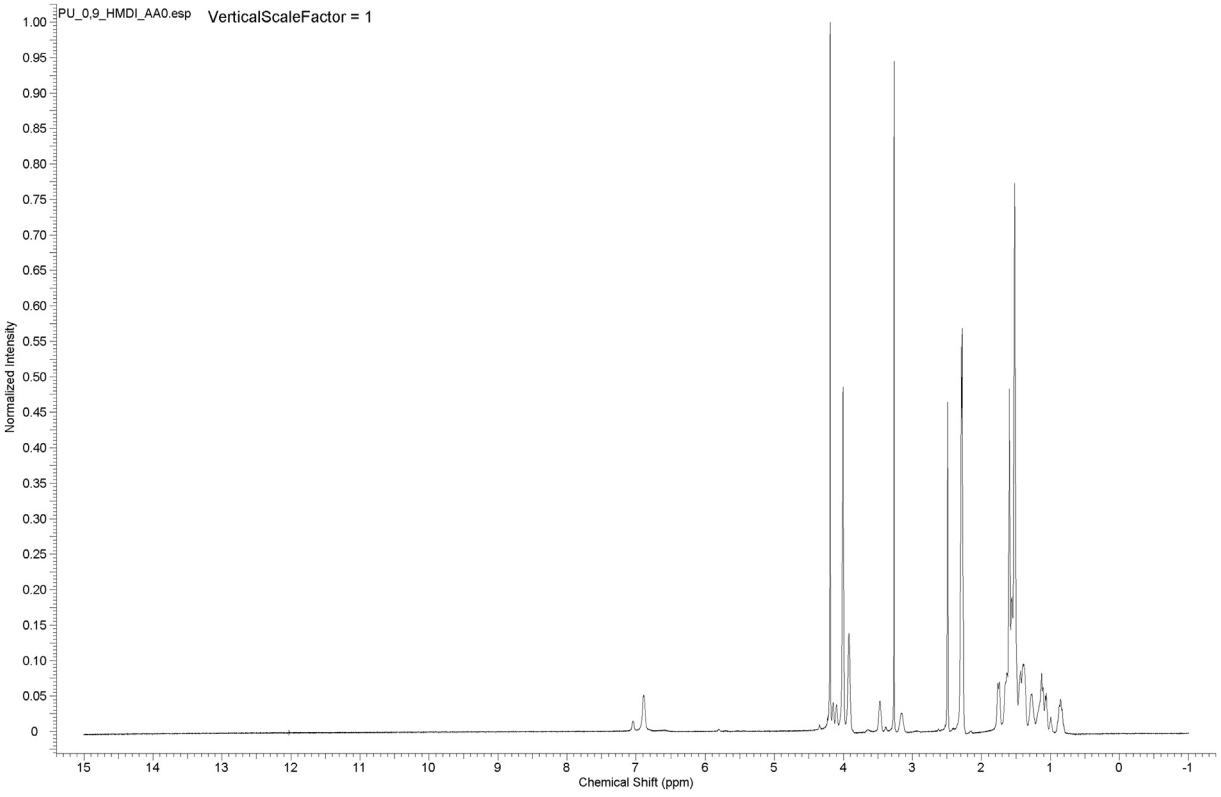


Fig. 2. The HNMR spectra of unmodified PURs.

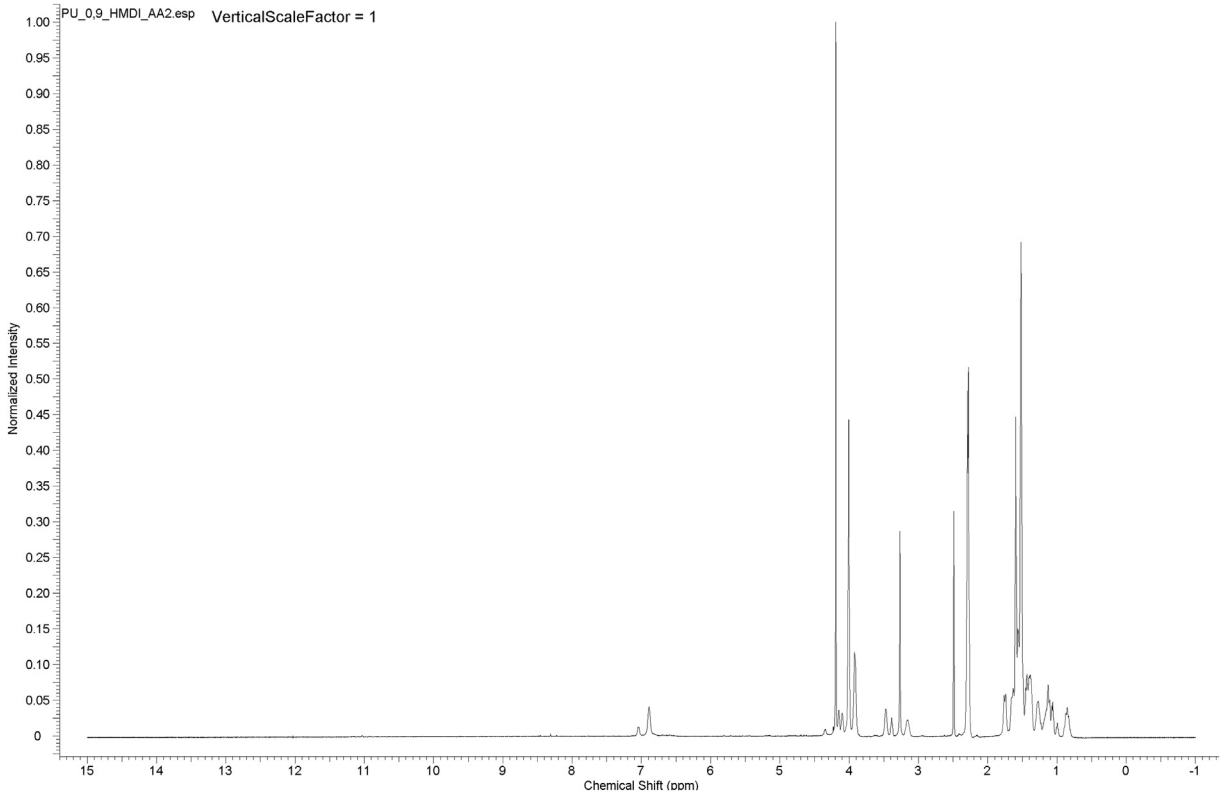


Fig. 3. The H1NMR spectra of modified with ascorbic acid AA-PURs

PURs chemical structure. The AA excess was enclosed in PUR matrix and might be useful tool after implantation of the PUR scaffold according to its significant influence on tissue regeneration [59–62].

3.3. Differential scanning calorimetry (DSC)

Fig. 4 presents DSC curves of PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2 obtained at the first heating and in the Table 4 were gathered the most important data taken of this experiment.

DSC study was carried out to characterize changes in the melting point (T_m) and glass transition temperature (T_g) caused by the effect of AA addition into the PUR systems (Table 4). DSC curves, for both PUR unmodified and modified samples, exhibited similar behavior with the temperature increase. Melting point temperatures were noted for unmodified (T_{m1} = 90.3 °C) and modified with AA-modified PURs (T_{m1} = 95.8 °C) (Fig. 4, Table 4). Also for the prepared samples enthalpies of melting were analyzed, which were comparable in both cases. As shown in Fig. 4 for PU-0.9/HMDI/AA2, the temperature at about 191.4 °C was related to the peak maximum of AA melting temperature (T_m). The glass transition temperature, detected for PU-0.9/HMDI/AA0 and PU-0.9/HMDI/AA2 samples, showed very similar values: T_g = −45 °C and was assigned to PUR soft segments (SS). Thus, the interactions between PUR soft segments and AA modifier were weak. Glass transition temperature (T_g) of BDO-HMDI PUR hard segments was detected at 70 °C (Fig. 4). Results of DSC analysis of obtained PUR and AA-modified PUR samples were similar to the literature data [63, 64]. AA did not influence temperature and enthalpy of melting. Application of amorphous polyol and HMDI provide stable chemical structure of obtained PURs of predictable and repetitive manufacturing procedure what is a desired feature.

Table 3
Spectral data* and band assignments of FTIR analysis presented in Figs. 2 and 3.

Chemical shift (ppm)	Assignments
0,5–1	Protons of OH groups present in PUR structure and protons of aliphatic CH_2 groups located in the middle of the PUR chain in direct neighborhood of other protons of aliphatic origin (CH_2 groups).
1–1,5	Protons of aliphatic CH_2 groups present in soft and hard segments of PURs structure closer to electron withdrawing groups (EWG) like –CO—O– and –O—
1,5–2,2	Protons of aliphatic CH_2 groups present in polyester dHEBA in closest neighborhood of C═O (β position)
2,5	DMSOd6 solvent
3–3,5	Protons of CH_2 groups in closest neighborhood with –NH—CO—O– urethane bonds at the NH site and protons which are present in ascorbic acid
4,0	Protons of aliphatic CH_2 groups present in polyester –CO—O– groups in closer neighborhood of –O—
6,5–7,5	Protons of NH groups, which form intermolecular hydrogen bonding with PUR chain and with ascorbic acid

3.4. Interactions with canola oil, saline, distilled water and phosphate buffered saline

The observed interactions of PURs and AA-modified PURs with canola oil, water, saline and phosphate buffered saline were presented at Fig. 5. In case of canola oil (Fig. 5) observed sorption values were comparable for both types of obtained PURs (PU-0,9/HMDI/AA0 = 1.75 ± 0,1%; PU-0,9/HMDI/AA2 = 2.00 ± 0,1%).

Studied interactions of obtained PURs and AA-modified PURs with water-based media: distilled water, saline, phosphate buffered saline showed that after 3 months of incubation slightly higher values of media sorption were observed in case of AA-modified PURs in comparison to unmodified PURs. In case of distilled water the sorption values noted, after 3 months of incubation, were 2,1 ± 0,2% and 2,65 ± 0,2% respectively for PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2. Noted sorption values for saline sorption (after 3 months of incubation) were 2,0 ± 0,2% for unmodified PURs (PU-0,9/HMDI/AA0) and 2,79 ± 0,2% for AA-modified PURs (PU-0,9/HMDI/AA2). In case of PBS interactions determined sorption values, after 3 months of incubation, were 2,00 ±

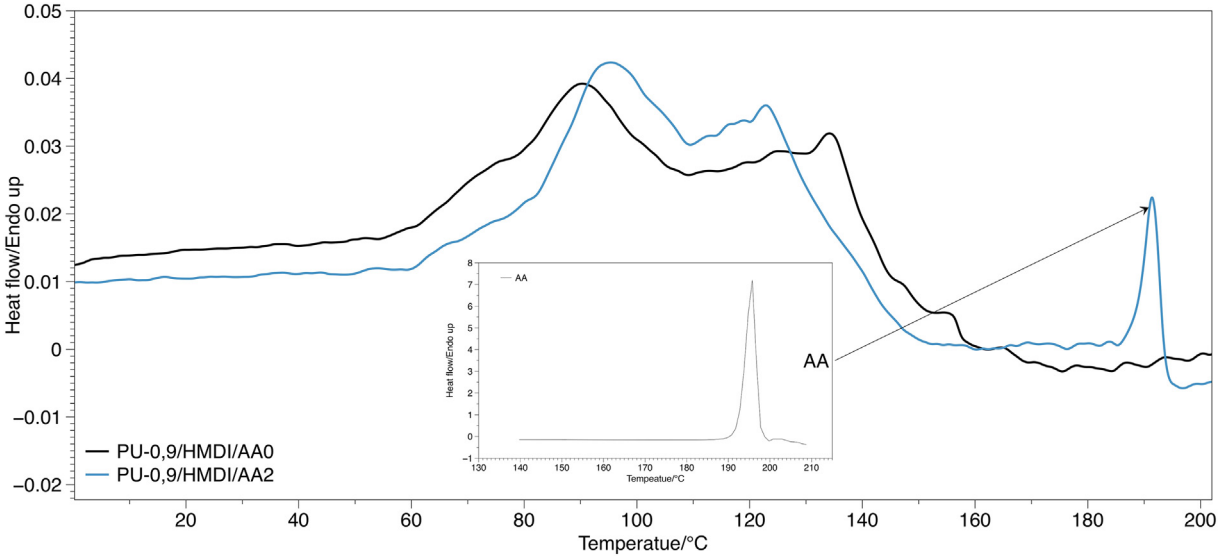


Fig. 4. DSC curves of PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2 (first heating).

0,2% and 2,50 ± 0,2% respectively for PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2. Obtained PUR and AA-modified PUR are stable up to 3 months, what is suitable for tissue regeneration, according to the fact that biological processes requires that the degree of degradation have to be controlled in such a way that the scaffold retains its physico-chemical and mechanical properties for at least 3–6 months. During 1–-3 months cells are constantly proliferating and between 3 and 6 months regeneration occurs in situ. Henceforth, the scaffold matrix may start losing its mechanical properties and should be metabolized by the body without foreign body re action after 12–18 months [64–66]. The statistical analysis showed that obtained results for different materials: PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2 are significantly different. Thus, it can be concluded that AA has a significant influence on PUR materials degradation.

3.5. Contact angle

Contact angle of unmodified and AA modified PURs was presented in the Fig. 6. Contact angle (CA) study, of unmodified PURs, revealed that basic PUR material had hydrophobic characteristic (74 ± 12°) (Fig. 6). It is on the threshold of desirable value. The contact angle of polymeric surface in the range of 45–76° is the most suitable for mammalian cells adhesion and proliferation [46] Established contact angle of AA modified PURs was decreased in comparison to unmodified PUR samples (58 ± 3°). The difference of contact angle values obtained for both materials, PU-0,9/HMDI/AA0 and PU-0,9/HMDI/AA2, were statistically significant, what means that AA modification improves values of CA towards biomedical applications of PURs. Thus, AA incorporation into the PUR system decreased the contact angle of 16° what was suitable for polymeric materials for biomedical applications.

Moreover, the ability of polymeric materials, of hydrophilic character, to form hydrogen bonds improves their biocompatibility by solvatation water molecules, which form at their surface water film that is biologically neutral [46].

3.6. Hemocompatibility

Table 5 presents the reference values of blood parameters and their values for blood taken for hemocompatibility test and Fig. 7 presents the results of hemocompatibility test performed for PUR and AA-modified PUR. It can be noted that both types of obtained PURs are biocompatible with blood and do not significantly influence their values which after the study stayed in the reference range (Fig. 7). It has to be pointed out that obtained results showed that blood parameter PCT was slightly increased, but still was in the reference range. PCT is released to the system when inflammatory response occurs, but it takes place each time after biomaterial implantation. It is worth mentioning that the inflammatory response may occur but it cannot be prolonged in time, because in other way it may cause serious system inflammation and implant rejection [28], parameters of MCHC, PLT, RDW-CV, MPV and PDW were slightly decreased, but were also still in reference range. Such parameters as WBC, RBC, Hgh/Hb, HCT, MCV, RDW-SD, PLCR stayed unchanged. The hemocompatibility of obtained unmodified and AA-modified PURs was suitable for biomedical applications. Moreover, obtained PUR materials did not significantly affect blood parameters. Obtained results were not statistically different, what means that both materials, do not have significant impact on blood parameters.

3.7. Microbiology studies

The interactions of obtained PUR and AA-modified PUR with selected microorganisms was showed at Fig. 8. Results of culturing of *Staphylococcus aureus*, *Escherichia coli* and Pseudomonas aeruginosa in the presence of modified (PU-0,9/HMDI/AA2) and unmodified PURs (PU-0,9/HMDI/AA0) revealed the lack of the antimicrobial effect of AA on the analyzed bacterial species (Fig. 8). Interestingly, after one week storage of analyzed LA plates in a refrigerator at 8 °C we found the presence of *S. aureus*, *P. aeruginosa* and E.coli colonies on PUR surface of PU-0,9/

Table 4
DSC data of PU-0,9/HMDI/AA0, PU-0,9/HMDI/AA2, solid AA and BDO-HMDI hard segments (PUR HS) of obtained PURs.

Sample	T_{m1}/°C	ΔH_{m1}/J·g^{-1}	T_{m2}/°C	ΔH_{m1}/J·g^{-1}	T_{m3}/°C	ΔH_{m3}/J·g^{-1}	T_g/°C
PU-0.9/HMDI/AA0	90.3	2.9	134.1	2.9	–		−45.4
PU-0.9/HMDI/AA2	95.8	2.5	123.2	1.2	191.4	1.1	−45.1
AA	195.7	271	–				–
BDO-HMDI (PUR HS)	–	–	–				70.0

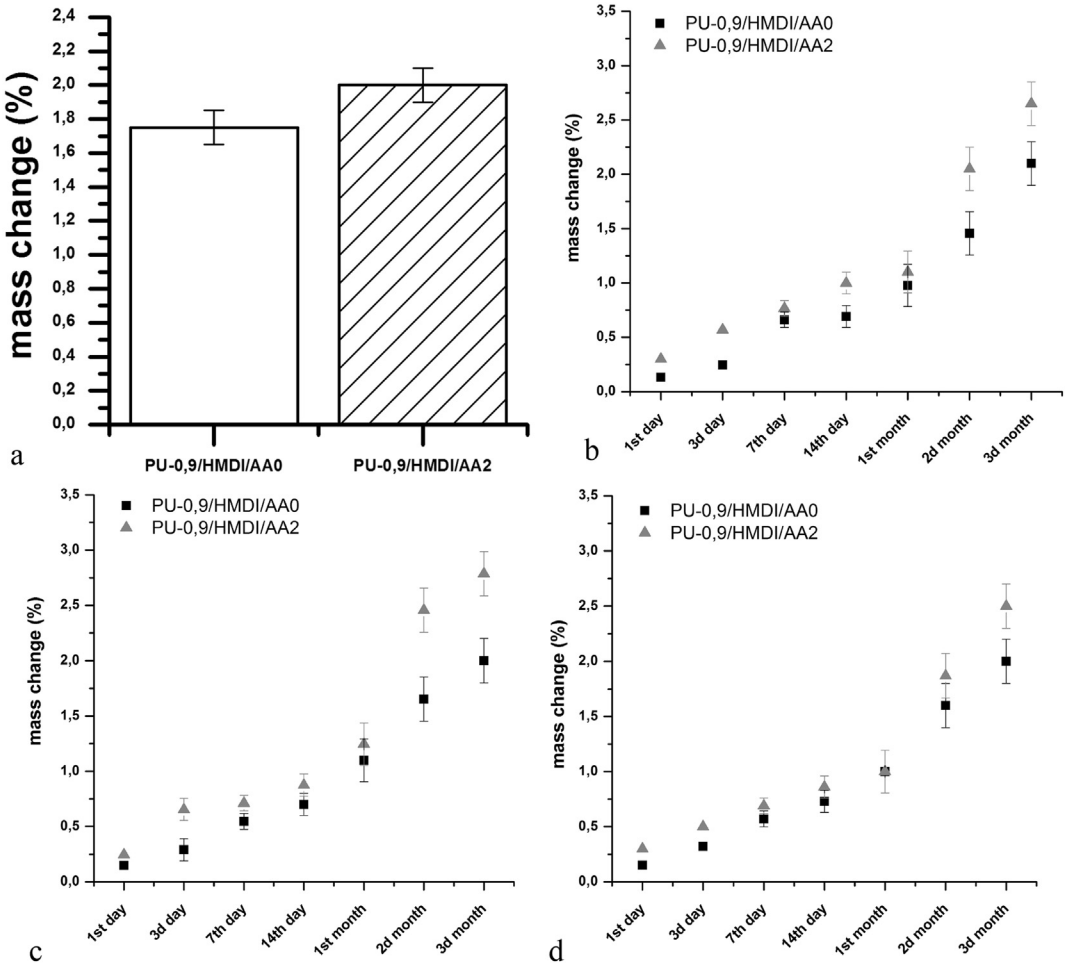


Fig. 5. Interactions of unmodified and modified with 2 wt% of ascorbic acid polyurethanes with (a) canola oil, (b) distilled water, (c) saline, (d) PBS after 3 months of incubation.

HMDI/AA2. In this case the presence of vitamin C in analyzed samples seems to promote the growth of bacterial cells on its surface (Fig. 8) On the other hand, absence of inhibition zones around the control samples indicates that unmodified PURs seem to be not toxic to the cells of studied bacterial cultures.

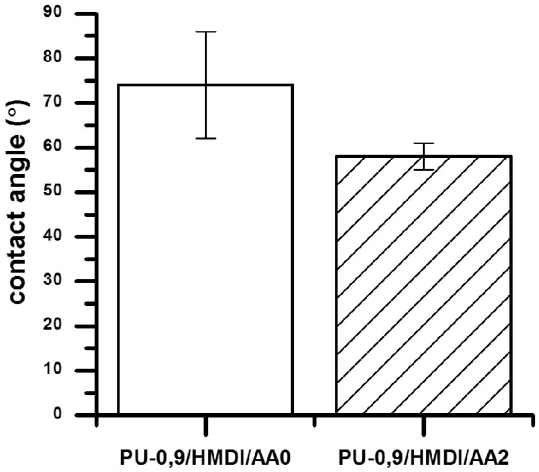


Fig. 6. Contact angle of unmodified and AA-modified PURs.

4. Discussion

In this paper we report the synthesis and characteristic of novel hydrophilic PURs for biomedical applications like regenerative medicine of soft tissues. PURs were obtained with the use of cycloaliphatic HMDI

Table 5
Reference values of blood parameters and their value for blood taken to the studies of unmodified and modified with 2 wt% of ascorbic acid polyurethanes hemocompatibility.

Parameters	Unit	Reference range	Initial blood parameter value
WBC	$10^3/\mu L$	3.00–14.00	8,11
RBC	$10^6/\mu L$	4.00–5.90	4,83
PCT	mg/dL	0.17–0.35	0,27
Hgh/Hb	g/dL	12.00–17.00	13,2
Hct	%	36.00–50.00	39,0
MCV	%	83.00–103.00	90,55
MCHC	g/dL	32.00–36.00	33,8
PLT	$10^3/\mu L$	140–440	243
RDW-CV	%	11.50–14.50	13,6
RDW-SD	fL	37.00–54.00	39,4
MPV	fL	9.00–13.00	11,1
PDW	fL	9.00–17.00	13,6
P-LCR	%	13.00–43.00	34,5

WBC - white blood cells (leucocytes); RBC - red blood cells (erythrocytes); PCT - percentage of platelets in whole blood volume; Hgh/Hb - hemoglobin; Hct – hematocrit; MCV mean corpuscular volume; MCHC - mean concentration of hemoglobin in blood cells; PLT - platelet amount (thrombocytes); RDW-CV/RDW-SD - distribution volume of red blood cells; MPV - mean platelet volume; PDW - indicator of platelet volume distribution; P-LCR - platelet larger cell ratio.

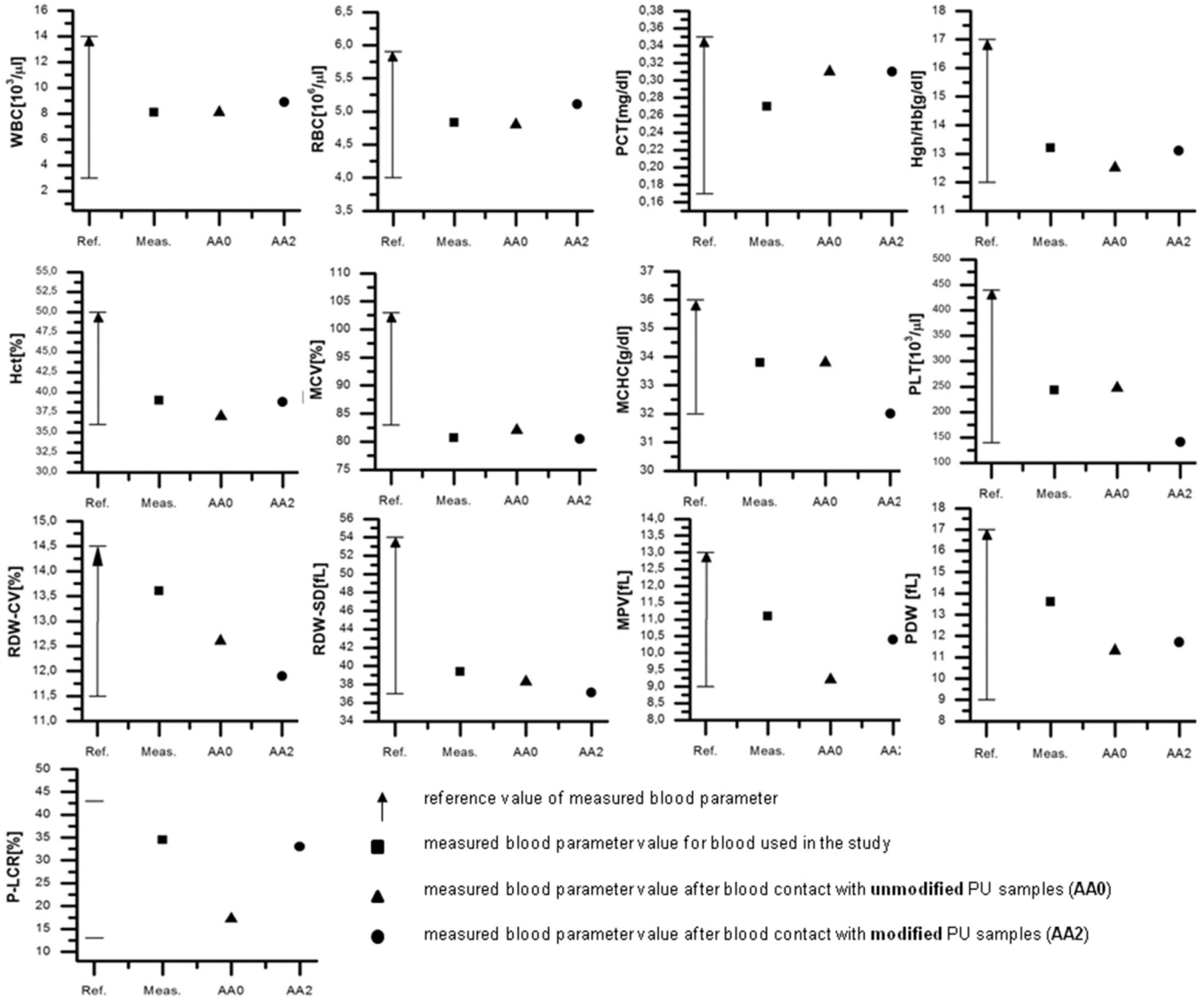


Fig. 7. Blood parameters values: reference values (Ref), values of measured blood parameter of taken blood for further hemocompatibility study (Meas.), and blood parameter values measured after contact with unmodified (AA0) and modified with 2 wt% ascorbic acid (AA2) poly(ester urethane)s.

diisocyanate as a nontoxic diisocyanate, reported in references as a suitable raw material for biomedical PUR synthesis [22,45]. Due to our knowledge, biomedical PUR systems composed of amorphous polyol, HMDI diisocyanate and modified with AA were not described yet for soft tissue regeneration. PURs were modified with AA to improve biomedical characteristic of obtained materials. AA significant influence on tissue regeneration was described in the literature data [1,2,7]. Performed FTIR and ^{1}HNMR studies confirmed that obtained materials had chemical structure and composition of PURs [7,39]. The FTIR and ^{1}HNMR spectra analysis revealed also, according to the chemical shifts of detected signals, that AA was partially introduced into the chemical structure of obtained PURs. This data are consistent with literature data [7,39]. Excess of AA, enclosed in the PUR matrix, might be released to the environment surrounding the implant and perform protective function due to its antioxidative properties. Moreover, AA may improve tissue regeneration by forcing cells to produce more nitric oxide, which is necessary to collagen fibers formation [60–62]. DSC curves, for both unmodified PURs and AA-modified PUR samples represented similar behavior with the temperature increase. Melting point temperatures and enthalpy of melting were comparable for unmodified PURs and AA-modified PURs. AA did not influence studied temperatures and enthalpies of melting. Glass transition of soft segments occurred at −45 °C and for hard segments at 70 °C. These data were similar with those reported at references [63,67]. Application of amorphous polyol and HMDI provides stable chemical structure of obtained PURs of predictable and repetitive manufacturing procedure what is a desired feature. To determine how these materials may potentially behave after implantation into living organisms, we estimated the degradation behavior of obtained unmodified PURs and AA-modified PURs in various chemical environments, which were represented by canola oil, saline solution, distilled water and PBS. Obtained materials occurred to be stable up to 3 months, what is favorable for materials dedicated for tissue engineering applications. This was recognized as suitable behavior of the implant according to the literature data [64–66]. It was established that obtained AA-modified PURs caused significant decrease of contact angle from 74 ± 12° to the 58 ± 3°. Thus, according to the references [46], the AA-modified PURs reached the optimal hydrophilicity, suitable for polymers dedicated to the biomedical applications (45–76°). Such contact angle enables proper surface adhesion of mammalian cells and further their growth and proliferation [46]. Moreover, highly hydrophilic polymers enable formation of hydrogen bonds, which improves their biocompatibility by solvatation of water molecules, which form on their

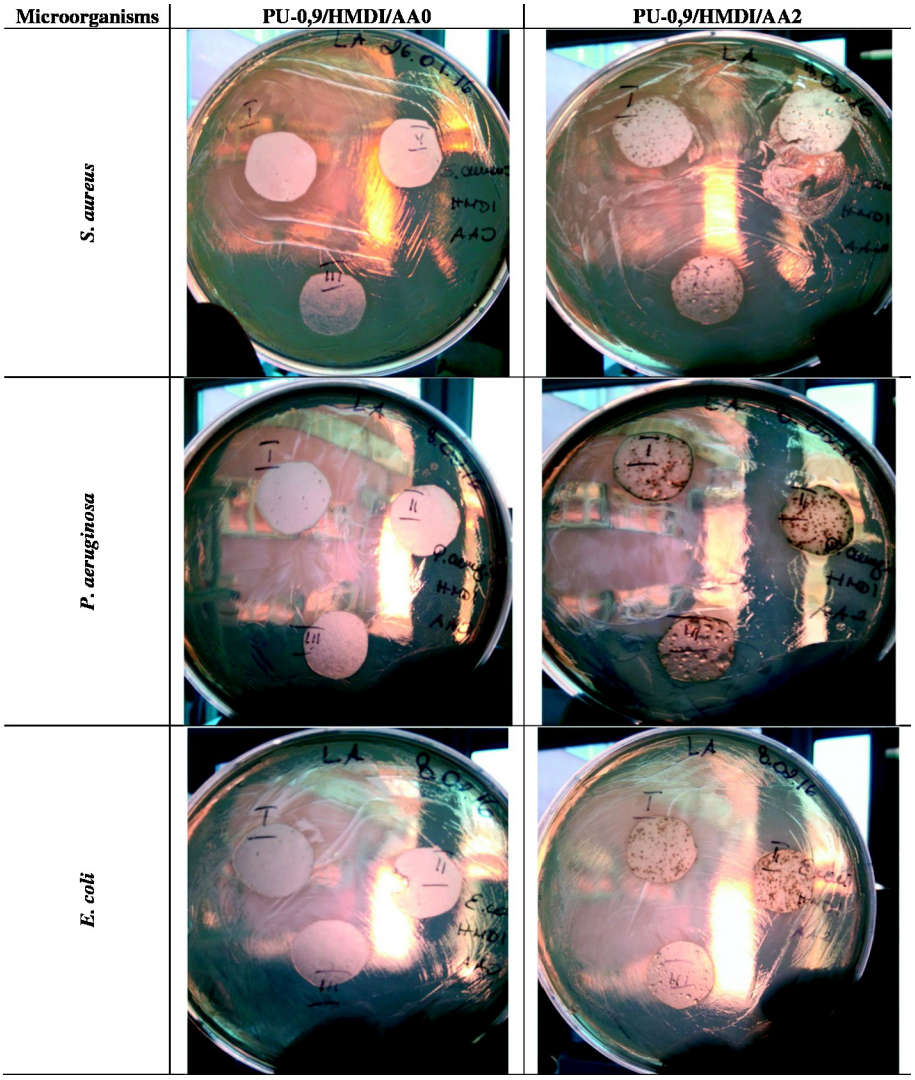


Fig. 8. Sensitivity of obtained PUR and AA-PUR on selected microorganisms a) *S. aureus*, b) *P. aeruginosa*, c) *E. coli*.

surface water film and cause their biological neutrality [46]. Obtained unmodified PURs and AA-modified PURs possessed good hemocompatibility with human blood. Studied PURs did not significantly affect blood parameters. Performed microbiological test revealed that obtained PURs were sensitive to the bacteria such as *S. aureus*, *P. aeruginosa* and *E. coli*. Moreover, AA modification seems to promote the growth of bacterial cells and their colonization on the material. On the other hand, absence of inhibition zones, around the control samples (PU-0,9/HMDI/AA0), indicates that PURs seem to be not toxic to the cells of studied bacterial cultures. PURs obtained with the use of cycloaliphatic diisocyanate HMDI possessed comparable properties to the PUR systems obtained with the use of HDI diisocyanate reported in our previous work [7,39]. Obtained materials might be developed as suitable candidates for biomedical applications such as regenerative medicine of soft tissues.

5. Conclusions

In this paper we reported the synthesis and characteristic of PURs, which were synthesized with the use of cycloaliphatic diisocyanate, HMDI, and modified with AA to improve PURs biocompatibility. Results of performed studies showed that AA significantly improved hydrophilic characteristic of AA-modified PURs what was suitable for regenerative medicine applications. Moreover, AA modification of PUR didn't cause significant changes in performed hemocompatibility test. Thus obtained AA-modified PURs were compatible with human blood. The microbiological studies confirmed that AA-modified PUR were not toxic to the cells of studied bacterial cultures. In this case, obtained AA-modified PURs might be further developed as material of potential use in regenerative medicine.

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