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Polyurethane modification with acrylic acid by Ce(IV)-initiated graft polymerization

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Abstract: This paper presents a method for polyurethane surface functionalization for tissue engineering applications. Functionalization has been carried out by grafting acrylic acid to the polyurethane surface with the use of radical polymerization with a Ce4+ initiator. Contrary to other papers suggesting that the presence of hydroxyl groups are essential for successful grafting via ceric ions, we propose a method with the omission of the surface hydroxylation step. The influence of reaction conditions: reaction time, reaction temperature and monomer concentration on carboxyl groups surface density has been analyzed and described. The quantity of carboxyl groups on the surface was determined with the use of the TBO method. Materials grafted with acrylic acid have been subjected to conjugation with a peptide using sulfoNHS/EDC chemistry. Successful incorporation of the peptide has been confirmed by an ELISA assay. Additionally, for better characterization, after each step of modification materials were subjected to SEM, FTIR-ATR, XPS and contact angle measurement analysis.

Keywords: polyurethane, surface modification, acrylic acid, peptide coating, cerium-induced grafting

1 Introduction

Polyurethanes (PUs) have been commonly used to fabricate blood-contacting implantable devices since the 1960s [1] because of good mechanical and physiochemical properties and acceptable hemocompatibility [2]. However, it has been known that any other artificial materials, a patient’s body rejects cardiovascular devices made of PUs. Small ions and protein adsorption takes place immediately after implantation, then blood cell adhesion causes chronic inflammation and fibrous tissue formation [3, 4]. Another issue is long-term thrombosis that is likely to occur as an effect of constant blood-biomaterial interaction [5]. Thus, there is a need for biomaterial modification to improve its features and enhance its interactions with tissues and blood.

One of the approaches assumes superficial modification of commercially available PUs. In this way, the modification process would not affect the bulk of the polymer and thus would not influence mechanical properties of the material. We have reported in previous works the increase of hydrophobicity of the surface by incorporation of hydrophobic agents [6] or hydrophilization of the surface by grafting poly(vinylpirrolidone) [7]. Nevertheless, these methods concern covering the surface of PU with a layer of another biocompatible polymer which leaves mechanical properties of a final product in doubt. Taking this into consideration, we have gained interest in grafting functionalized spacer molecules onto the PU surface. We focused on acrylic acid (AA) grafting since it is soluble in water, which enables water-based processes, free from toxic organic solvents. Carboxyl groups provided by AA could be subsequently utilized to bond biomolecules such as collagen [8] and adhesive peptides, e.g. RGD [9], that induce anchorage of endothelial or smooth muscle cells. In this way, it is expected that modified PU would act as a natural tissue.

In this paper, we present a method of acrylic acid (AA) grafting to a polyurethane (PU) surface via radical polymerization induced by ceric ion reduction. The method has been reported for chitosan [10], PET [11] or cellulose [12] surface modification with other compounds. There were also some trials with PU grafting triggered by Ce4+ reduction [14,15]. Significantly, many works claim that in order to successfully carry out this approach, superficial -OH groups are required [15,16] due to the proposed mechanism of preferable ceric ion reaction with primary -OH groups. The reaction is often described as -CH₂OH+ Ce⁴⁺ = -C*HOH +
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H^+ + Ce^{3+}, where the asterisk stands for free carbon radicals \[15\]. In contrast with this statement, we present a method of successful AA introduction onto the PU surface with the omission of the surface hydroxylation step. The AA-grafted PU can be subsequently conjugated with adhesive peptides, e.g. REDV, that induce anchorage of endothelial cells. It has to be mentioned that PAA is often used in biomaterials synthesis. Biocompatibility of AA is well-known \[16\]. A possibility of its polymerization in situ predestines AA to be used in obtaining polyelectrolytes for biomedical applications \[17\]. Furthermore, polymerized AA - poly(acrylic acid) (PAA) - not only provides COOH functional groups, but according to a recent study, also exhibits antimicrobial properties. It was demonstrated that PAA containing diblock copolymers prevents biofilm formation \[18\]. This is certainly a desired feature for a biomaterial.

2 Experimental procedure

2.1 Materials

For PU film preparation biodurable aromatic polycarbonate-based medical-grade polyurethane ChronoFlex C 75D (AdvanSource Biomaterial) was used. As a solvent for PU solubilization DMAC (N,N-dimethylacetamide, Merck) was applied. Reagents used for grafting were AA (acrylic acid anhydrous, 99%, Sigma Aldrich), HNO_3 (nitric acid 65%, Carlo Erba), (NH_4)_4Ce(SO_4)_2 \cdot 2 H_2O (ammonium cerium(IV) sulphate dehydrate, p.a. grade, Riedel-de Haen). Grafted PU films were rinsed with SDS (sodium dodecyl sulphate, 98%, Sigma Aldrich). For peptide molecule coupling to carboxylated PU films the following chemicals were utilized: peptide GSGREDVGSG that contains adhesive sequence REDV (99.52%, Novazym), sulfo-NHS (N-hydroxysulfosuccinimide, ≥98.0%, ThermoFisher), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, ≥98.0%, Sigma Aldrich), MES buffer (2-(N-morpholino)ethanesulfonic acid, ≥99.5%, Sigma Aldrich), PBS (phosphate-buffered saline (tablet, Sigma Aldrich)) and PBST (PBS containing TWEEN® 20, tablet, Sigma Aldrich) for rinsing. The surface density of COOH groups on PU were determined with the use of TBO (toluidine blue O, Sigma Aldrich). In order to confirm the presence of peptides on modified the PU surface an ELISA assay was applied: primary antibody - rabbit monoclonal anti-REDV antibodies (lyophilized, Novazym), secondary antibody - goat anti-rabbit IgG conjugated to peroxidase (lyophilized, Sigma Aldrich), substrate for the detection of peroxidase activity - SIGMAFAST™ OPD (tablet, Sigma Aldrich).

2.2 PU films preparation and grafting

PU pellets were purified in aqueous ethanol, dried at 37 °C and dissolved in DMAC (20% w/v). After complete dissolution of the granules the solution was poured onto glass surfaces and dried at 40 °C to completely evaporate the solvent. The sample discs were cut off from the film and modified with AA according to the following procedure: PU discs were immersed in a 0.4 M solution of HNO_3 in distilled water and heated to 25, 35 or 45 °C. After reaching this temperature (NH_4)_4Ce(SO_4)_2 \cdot 2 H_2O (0.1% w/v) and AA (1%, 3% or 5% v/v) were added to the solution. The mixture was left covered, stirring continuously for 0.5, 1, 1.5 or 2 h. The quantity of carboxyl groups was determined colorimetrically (see below). For further experiments, PU-COOH materials showing the largest amount of COOH groups were chosen. PU-COOH discs were washed with a 0.1% (w/v) SDS aqueous solution, rinsed 3 times with distilled water and placed in 0.05 M MES buffer (pH=6.0) for 1 h. Materials were transferred to a sulfo-NHS (5mM) and EDC (2 mM) solution in MES (pH=6.0) for 15 min, washed with MES buffer and placed in a GSGREDVGSG solution (0.1mM in PBS, pH=10.5) for 1 h, RT. Afterwards, discs were rinsed with PBST, followed by rinsing with PBS. As a control material samples physically coated with peptide (without activation with EDC and sulfo-NHS) were prepared.

2.3 Carboxyl groups determination

Effect of different process parameters (temperature, time, monomer concentration) on AA grafting yield was tested. Surface density of COOH groups on PU-COOH was measured using colorimetric reaction with toluidine blue (TBO) as described elsewhere \[19\]. The discs were put in TBO solution, pH=11, for 3 h, RT. Next, discs were dipped in distilled water and placed in 50% acetic acid solution, pH=1.5. Absorbance of the obtained solutions at 633 nm was measured using a plate reader. Each measurement was performed 10 times for each PU-COOH material. PU-COOH discs with the biggest amount of superficial COOH groups (PU-COOH_{max}) were submitted to REDV grafting.

2.4 Peptide presence confirmation

To confirm successful introduction of the peptide on PU-COOH_{max} an ELISA assay was performed. As a control material samples physically coated with peptide (without activation by EDC and sulfo-NHS) were used. The samples were placed in 24-well polystyrene plates, equilibrated...
with PBS and blocked with PBST (1 h, 37 °C). After blocking, samples were incubated with primary antibodies (1 h, 37 °C, rabbit monoclonal anti-REDV antibodies, dilution 1:1000) and secondary antibodies (1 hour, 37 °C, anti-rabbit IgG conjugated to peroxidase, dilution 1:20000) and transferred to fresh plates to eliminate the influence of the antibodies adsorbed to the well walls. After each assay step samples were rinsed with PBST (6 x 5 minutes on a shaker). In the last assay step samples were incubated with a peroxidase substrate solution – SigmaFast OPD (o-phenylenediamine dihydrochloride) in the dark at RT for 30 minutes. After the reaction, an aliquot of the solution (200 µl) from each well was transferred to a 96-well plate; the optical density of the solution was read at the 450 nm. Each measurement was performed 8 times for each type of material.

2.5 SEM imaging

Scanning electron microscope (SEM) imaging was performed for PU, PU-COOH_{max} and PU-REDV in order to visualize differences in surface topography after each modification step. SEM images were obtained with the use of a Zeiss Ultra Plus microscope equipped with a secondary electron detector SE2. Materials were fixed by dipping in a 2% (v/v) glutaraldehyde aqueous solution for 24 h rinsing with distilled water and dehydrating in ethanol solutions (ethanol concentrations form 50% to 100%). After fixation, materials were sputter-coated with carbon. The imaging was performed in at least 5 randomly selected spots on the analyzed material. Each material was prepared in triplicate. Representative images for each material were selected.

2.6 Wettability measurement

Surface wettability was measured for PU, PU-COOH_{max} and PU-REDV. Materials were glued to a glass slide and a drop of distilled water (5 µl) was placed on a clean and dry surface. The contact angle was measured automatically using Kruss DCA 100 software. The measurement was performed in at least 5 randomly selected spots on the analyzed material and each material was prepared in triplicate.

2.7 FTIR-ATR analysis

FTIR spectra were recorded with a Nicolet™ 6700 spectrometer (Thermo Scientific). All samples were detected in attenuated total reflection (ATR) mode. Spectra were analyzed with the use of OMNIC 8.3 software. Spectra were collected in at least 5 randomly selected spots and each material was prepared in triplicate. One representative spectrum for each material was selected.

2.8 XPS analysis

The changes of chemical composition during surface modification were determined by XPS on a VG Scientific ESCALAB photo-electron spectrometer. Spectra fitting and determination of atomic composition was performed with software provided by VG Scientific.

3 Results and discussion

3.1 Modification mechanism

The aim of this research was to develop a simple and effective method for the chemical attachment of AA to PU. The method may be utilized to improve the wetting properties of PU, which is essential as we discuss biomedical purposes. Also, AA provides carboxyl groups that can be used to couple biomolecules such as peptides. AA was chosen as a spacer molecule that provides connection and proper distance between the surface and bioactive molecule (peptide). PU belongs to a group of polymers with no surface functional groups that could be useful for chemical grafting. In order to attach biomolecules, the process of surface activation (which enriches surface in functional groups) must be carried out. It should be also kept in mind, that peptides or proteins immobilized on a polymer should posses appropriate conformation (orientation in space). This ensures that the biological activity and recognition by specific cell receptors of the peptide and/or protein is maintained. Therefore, some free space around them should be provided. In addition, when a peptide molecule and the surface of the polymer are too close to each other, unwanted electrostatic repulsion can occur. Consequently, biomolecules should be placed at the end of spacer molecules bonded to the surface of the polymer.

In many cases, photooxidation is a method of choice for PU surface activation [20–22]. The surface hydroxyl groups seemed to be essential especially for cerium-induced grafting [15]. However, our initial experiments demonstrated that the PU surface treated with UV was physically changed (in terms of topology and roughness)
which negatively affected surface adsorption properties. In order to minimize the invasiveness of the modification process, we have decided to abandon the oxidation step. The mechanism of grafting has been schematically shown in Figure 1, details have been described elsewhere [23]. Briefly, cerium initiator abstracts hydrogen from urethane groups and generates nitrogen radicals, which react with AA. In order to provide an acidic environment, which is necessary in this reaction, nitric acid has been added to the solution. As a polymerization initiator ammonium cerium(IV) sulphate has been used, which, apart from ammonium cerium nitrate, is one of the most common initiators utilized in cerium-induced grafting.

In the next modification step, AA-grafted materials with the largest amount of COOH (PU-COOH_max) were reacted with the peptide solution. After AA grafting, materials were subjected to reaction with sulfo-NHS/EDC (PU-NHS). First, activation of carboxyl groups with sulfo-NHS/EDC was conducted. Then, materials were immersed in the peptide solution, where activated carboxyl groups reacted with amine groups present in peptide chain (PU-NHS-REDV). In order to optimize the AA grafting yield the following parameters were tested: reaction temperature, reaction time and AA concentration. The materials preparation scheme is presented in Figure 2.

3.2 Effect of temperature on AA grafting yield

As presented in Figure 3a) the concentration of surface carboxyl groups increases with the increase of temperature. That trend can be observed for lower concentrations monomer (1% and 3%). The increase in grafting efficiency may be due to: (i) increased decomposition of Ce⁴⁺-PU complex and (ii) increased diffusion of monomer to the active sites. For the highest AA concentration analyzed (5%) the density of COOH increases with temperature and reaches a maximum at 35 °C. Further increase in the temperature resulted in a rapid decrease in grafting efficiency. This decrease may be due to: (i) increased monomer hydrolysis, (ii) acceleration of chain termination processes and (iii) acceleration of chain transfer processes leading to increased homopolymerization [24,25].

3.3 Effect of time on AA grafting yield

AA grafting yield strongly depended on the reaction time (Figure 3b). It has been observed that with an increase in reaction time from 30 to 60 minutes the graft yield increases. The further course of the curve was different depending on monomer concentration. For small AA concentration (1%) the increase does not significantly influence the grafting density. In the case of 3% AA, grafting yield increases with increasing time, reaching a maximum grafting yield after 90 minutes. A further increase in time did not alter the grafting density. The course of the curve was different in the case of the higher monomer concentration analyzed (5%). Density of surface carboxyl groups reached a maximum in 60 minutes. A further increase in time leads to a decrease in the grafting yield. This trend has been observed in similar studies [13,25,26]. As other authors suggest, this effect can be due to termination or destruction of growing chains by active radicals, which decreases grafting yield [24,27].
3.4 Effect of the monomer concentration on AA grafting yield

The effect of monomer concentration on grafting density is presented in Figure 3c). The grafting yield increases together with the increasing AA concentration from 0.14 M to 0.70 M. This trend can be simply explained: the more AA molecules in solution, the bigger chance for growing polyacrylic chains to encounter more monomer units.

3.5 Selection of PU-COOH material for further experiments

For REDV coupling and characterization PU-COOH with the largest volume of COOH groups (PU-COOH\textsubscript{max}) was selected. PU-COOH\textsubscript{max} was obtained for the following parameters of AA grafting: 35 °C, 1.5 h and 5% (v/v) AA. For this set of parameters, the procedure seemed to be the most efficient and repeatable.

3.6 Peptide presence confirmation

The aim of the presented surface modifications was the introduction of peptide sequences onto the PU surface. For peptide coupling, PU with the maximum volume of COOH groups (PU-COOH\textsubscript{max}) was used. In order to confirm peptide presence, an ELISA assay was carried out. The results are shown in Figure 3d). As expected, materials after AA grafting (PU-NHS) gave negligible responses. Similar results were observed for materials grafted with AA and immersed in the peptide solution without previous activation of carboxyl groups (PU-REDV). This result proves that creating a stable peptide coating requires a chemical reaction between the amine and carboxyl groups. Peptides that were physically adsorbed on the surface, without a chemical bond, were washed away during washing step. On the other hand, materials marked as “PU-NHS-REDV” gave a higher response. In this case, carboxyl groups present on the materials surface were activated with sulfoNHS. This step allows the chemical reaction between COOH and NH\textsubscript{2} groups to occur. The positive result of the ELISA assay confirmed that the proposed surface modification method can be applied to chemically conjugate peptide molecules to spacer-grafted PU. What is more, the presence of surface hydroxyl groups is not essential for the reaction.
3.7 SEM imaging

Scanning electron microscope (SEM) imaging was performed for PU, PU-COOH_\text{max}, and PU-REDV in order to visualize changes in topography after each modification step. Figure 4a-c shows SEM images for these surfaces. It can be noticed that the PU-COOH_\text{max} surface presented more irregularities than the PU surface. There were some particles on the PU-COOH_\text{max} surface (Fig. 4b) seen as sharp and bright points which were caused by monomer (AA) polymerization [14]. Figure 4c presents PU-REDV surfaces on which peptide aggregates are clearly seen. There are similar SEM images of PU grafted with peptides in other works [29], which suggests that such clusters might represent peptide molecules. We assume that peptide molecules bonded to PU in the course of chemical grafting are not seen on the SEM images. Those visible aggregates might be formed as a result of crosslinking between additional peptides adhered to the PU surface and glutaraldehyde used for materials fixation. This is probably the result of insufficient rinsing of materials after the peptide-coupling step.

3.8 Wettability measurement

The contact angle (CA) values of the surface of various types of materials are presented in Figure 5. AA grafting resulted in a decrease in CA value: from average value CA = 101.4±2.8° (PU) to CA = 66.4±5.0° (PU-COOH_\text{max}). This result was expected since polymerized AA (PAA) grafted to the PU surface is completely dissociated at neutral pH. After peptide coupling (PU-REDV) the hydrophilicity of the surface was further increased (CA = 52.6 ±2.6°). The increase in hydrophilicity is due to the coupling of the peptide chain, which consists of highly hydrophilic amino acids: arginine, aspartic acid and glutamic acid. All three of them have polar side chains that are dissociated at neutral pH. Such an increase in hydrophilicity after REDV incorporation on the modified PU surface was reported in our previous work [28].

3.9 FTIR-ATR analysis

Figure 6 shows the comparison of FTIR-ATR spectra obtained for PU, PU-COOH_\text{max}, and PU-REDV surfaces. A characteristic stretching vibration band at 3700-3400 cm\(^{-1}\) that relates to NH_\text{r}, -OH and -C=O groups is shown [18]. The signal was more intense for PU-REDV than for other materials, which indicates the presence of peptide containing amine groups. On the other hand, there was barely any difference in this range for PU and PU-COOH_\text{max}. Nevertheless, it should be noted that according to the manufacturer of the Nicolet 6700 spectrometer, the depth of laser beam penetration for ATR mode is approx. 2 µm. This means that the band at 3700-3400 cm\(^{-1}\) came from the PU surface more than from grafted AA. This is why bands assigned to C=O and -OH groups present in the PU hid bands assigned to C=O and -OH in COOH groups from AA. Nevertheless, the presence of COOH on the PU-COOH_\text{max} surface was confirmed by other methods used in this work.

3.10 XPS analysis

The atomic composition of PU, PU-COOH_\text{max}, and PU-REDV was determined with the use of XPS (Table 1). As expected,
after AA grafting the percentage of oxygen atoms increased from 23.7% in PU to 30.2% in PU-COOH\textsubscript{max} due to the introduction of COOH groups on the surface. It proves the assumed course of the AA grafting reaction. The decrease of nitrogen content on PU-COOH\textsubscript{max} is a result of AA presence on the surface. Since AA does not contain N atoms, nitrogen percentage dropped from 4.3% in PU to 0.8% in PU-COOH\textsubscript{max}. However, nitrogen could still be detected in PU-COOH\textsubscript{max}, thus the PU surface was not completely covered with PAA. In PU-REDV nitrogen percentage rose to 3.5%. This confirms that peptide molecules of relatively high nitrogen content (amine groups and guanidine group in arginine) were successfully coupled.

4 Conclusion

In this work PU films were modified with peptides in order to prepare specific scaffolds for tissue engineering applications. AA has been grafted to PU using a cerium
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initiator. On the contrary to other papers suggesting that the presence of hydroxyl groups is essential for successful grafting with ceric ions, we propose a method with the omission of the PU hydroxylation step. The process of AA grafting has been analyzed in terms of reaction time, temperature and AA concentration. Successful peptide conjugation has been confirmed with ELISA assays. PU, PU-COOH_{max} and PU-REDV materials were additionally characterized with SEM, XPS, FTIR-ATR and contact angle measurements. All these methods indicated that PU was efficiently grafted with AA and that it was possible to attach peptides to the PU-COOH surface. In continuation of this work, prepared materials will be contacted with human endothelial cells in order to assess their potential as scaffolds for in situ endothelialization.

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Abbreviations

AA: acrylic acid;
COOH: carboxyl group;
DMAC: N,N-dimethylacetamide;
EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide;
ELISA: Enzyme-Linked Immunosorbent Assay;
FTIR-ATR: Fourier- transform infrared spectroscopy attenuated total reflectance;
GSGREDVGSG: peptide sequence: glycine-serine-glycine-glutamate-aspartate-valine-glycine-serine-glycine;
MES: buffering agent, 2-(N-morpholino)ethanesulfonic acid;
NH_{2}: amine group;
NHS: N-hydroxysuccinimide;
OPD: o-phenylenediamine dihydrochloride;
PAA: polyacrylic acid;
PBS: phosphate-buffered saline;
PBST: phosphate-buffered saline with TWEEN® 20 (detergent);
PET: polyethylene terephthalate;
PU: polyurethane;
REDV: peptide sequence: arginine-glutamate-aspartate-valine;
RT: room temperature;
SEM: scanning electron microscope;
sulfo- NHS: N-hydroxysulfosuccinimide;
SDS: sodium dodecyl sulphate;
TBO: toluidine blue O;
XPS: X-ray photoelectron spectroscopy.

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