Surface modification of polycaprolactone with poly(methacrylic acid) and gelatin covalent immobilization for promoting its cytocompatibility

Yabin Zhu, Changyou Gao*, Jiacong Shen
Department of Polymer Science and Engineering, Zhejiang University, Hangzhou 310027, China
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Abstract
Polycaprolactone (PCL) membrane was modified by grafting copolymerization of methacrylic acid (MAA) initiated under UV light. The covalent immobilization of gelatin on PCL-g-PMAA surface was consequently performed by using condensing agent, 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride. The occurrence of grafting copolymerization of PMAA and further immobilization of gelatin was confirmed by ATR-FTIR and X-ray photoelectron spectroscopy characterizations. The existence of carboxyl groups grafted on PCL surface was verified quantitatively by absorbance spectroscopy where rhodamine 6G was employed to react with carboxyl groups to generate an absorbance at 512 nm. The endothelial cell culture proved that the PCL membrane slightly modified with suitable amount of PMAA or gelatin had better cytocompatibility than control PCL or PCL membrane heavily modified with PMAA or gelatin.

Keywords: Polycaprolactone; Methacrylic acid; Grafting copolymerization; Cytocompatibility

1. Introduction
The biodegradable polymer scaffolds are paid more and more attentions in tissue engineering not only because they provide the necessary substance on which cells and tissues can adhere, but also because they can guide and regulate the proliferation and activities of the supported cells. However, the intrinsic hydrophobic property of these polymers restricts their applications as cell colonizing materials. For example, the surface of polycaprolactone (PCL) is hydrophobic and does not have any physiological activity, which makes it unfavorable for cell growth when it comes into contact with living body. Therefore, the cytocompatibility of these synthesized materials should be improved.

Various methods such as plasma treatment, ozone or photo-induced grafting and surface oxidation, etc., have been employed to introduce hydrophilic compounds onto polymeric scaffold surfaces [1–7]. Among which the photo-induced grafting has been frequently employed to produce hydrophilic layer onto biomedical polymers. Some hydrophilic macromolecules polymerized in situ from 2-dimethylaminoethyl methacrylate (DMA), 2-hydroxyl ethyl acrylate (HEA), acrylamide (AAm) and 2-hydroxylethyl methacrylate (HEMA) etc., have been grafted on poly(ester urethane) (PU) and poly(l-lactic acid) (PLLA) previously [8–11]. The introduction of the above-mentioned functional groups generates more favorable interaction with endothelial cells (ECs) and chondrocytes accompanying with the increase of surface hydrophilicity.

PCL, biodegradable aliphatic polyester [12,13], has been suggested for wide applications such as drug delivery system [14,15], tissue-engineered skin (plain film) and scaffolds for supporting fibroblasts and osteoblasts growth [16,17] etc. The present paper reports the grafting copolymerization of hydrophilic poly(methacrylic acid) (PMAA) onto PCL membrane surface under UV irradiation and the further immobilization of gelatin using a water soluble carbodiimide (WSC) as the condensing agent which can accelerate the reaction between \( \sim \text{COOH} \) from PMAA and \( \sim \text{NH}_2 \)....
from gelatin [18]. The variation of the hydrophilicity was determined by water absorbance and water contact angle (WCA) measurement. The culture of human umbilical ECs in vitro showed that the cytocompatibility of the PCL membrane grafted with PMAA or further immobilized with gelatin was improved obviously.

2. Materials and methods

2.1. Membrane preparation

PCL membrane was prepared by dissolving 5g PCL (Aldrich, Mn 80,000) in 40ml distilled 1,4-dioxane, and then spread onto a stainless plate. The solvent was evaporated at 30°C for 24h, and further dried under vacuum for another 24h at 30°C, yielding the translucent PCL membrane with a thickness of about 200μm. The membrane was cut into pieces of 2×2cm² for photo-oxidation and grafting copolymerization.

2.2. Photo-oxidation and grafting copolymerization

PCL membrane was immersed in alcohol/water (1/1 v/v) solution for 2–3h to clean oily dirt, then was washed with large amount of deionized water. The membrane was subsequently immersed in a quartz tube containing excessive hydrogen peroxide solution (30%). Under rotation by a motor, the tube was exposed to UV light generated from 250W high-pressure mercury lamp for a given time at 30°C. The photo-oxidized membrane was rinsed with deionized water thoroughly to eliminate the free hydrogen peroxide, and then placed in a copolymerization tube containing 4wt% methacrylic acid (MAA) aqueous solution. The oxygen in the tube was replaced by nitrogen. Grafting copolymerization was carried out under UV irradiation at a distance of 12.5cm from the mercury lamp for a given time at 30°C. The grafted membrane (PCL-g-PMAA) was rinsed with deionized water for at least 24h to remove the adsorbed homopolymer, and subsequently dried in vacuum at 30°C to constant weight.

2.3. Immobilization of gelatin

The PCL-g-PMAA was further immersed in 10ml 1-ethyl-3-(3-dimethylamino propyl) carbodiimide hydrochloride (EDAC, Aldrich)/phosphate buffer solution (PBS, pH = 7.4) (10mg/ml) for 24h at 2-4°C, followed by rinsing with large amount of deionized water. The membrane was then incubated in gelatin/PBS solution (3mg/ml) for another 24h at room temperature. After rinsed with deionized water for at least 24h to remove free gelatin, the membrane was dried in vacuum at 30°C to constant weight. The advantage of this sequential procedure is that it may avoid the condensation intra- or inter-gelatin molecules to a great extent.

2.4. Determination of PMMA

Rhodamine 6G was dissolved in phosphate buffer solution (PBS, pH 11), and extracted with toluene [19]. PCL-g-PMAA was washed well with distilled water, dried, and dissolved in 1,4-dioxane. A known amount of the toluene solution of rhodamine 6G was added to the above 1,4-dioxane solution. After incubation in dark for 30min, the concentration of the carboxyl groups was determined by measuring the absorbance at 512nm. A calibration curve was obtained with 1,4-dioxane solution containing MAA of known concentration.

2.5. Human ECs culture

The ECs were isolated from the human umbilical cord veins of new born baby with 1.0 mg/ml collagenase (type I, Sigma)/phosphate buffer solution (PBS, pH = 7.4) for 20–25 min at room temperature [20,21]. The isolated ECs were routinely seeded in the beds that were pre-laid with control or modified PCL membranes as well as in the beds of tissue culture polystyrene (TCPS) (Nunc™, Denmark) as control. The ECs were incubated in a culture medium which consisted of 20% (v/v) little calf serum (Sijiqin Biotech. Co., China) and 80% RPM1640 (GibcoBrl Co.), supplemented with 100 unit/ml of penicillin and 100 μg/ml of streptomycin in a humidified air of 5% CO₂ at 37°C. After incubation for 24h, the culture medium was changed and then changed every 2d. The ECs were fixed with 2.5% glutaraldehyde for 30min for observation of cell morphology under scanning electric microscope (SEM) after cultured for 4d. The cell attachment and proliferation ratios were averaged from three parallel measurements at 12h and 4d, respectively, by trypsinization of the ECs and counting the cell number under a haemocytometer. The cell proliferation ratio was defined as (N₂−N₁)/N₁, where N₁ and N₂ represented the cell number per well at 12h and 4d, respectively.

2.6. Characterization

The attenuated total reflection (ATR)-FTIR spectra were obtained on a Nicolet Magnu-IR560 spectrometer. X-ray photoelectron spectroscopy (XPS) spectra were recorded on an ESCA LAB Mark II spectrometer employing Al Kα excitation radiation. The charging shift was referred to the C 1s line emitted from the saturated hydrocarbon. UV-Vis spectra were measured on UV-Visible Spectrophotometer (UV-1601, SHIMADZU). The wettability of the membrane was determined by using WCA and water absorbance methods. The water absorbance was defined as
(\(W_2 - W_1\))/\(W_1\), where \(W_1\) and \(W_2\) represented the weight of dried membrane and the membrane soaked in water for at least 24 h at 37°C, respectively. The WCA was measured at room temperature on a DSA10-MK2 Contact Angle Measuring System from Krüss, using the sessile drop method. The AFM image was obtained on Scanning Probe Microscopy (SPA400, Japan) with tapping mode. The cell morphology was observed under Scanning Electron Microscope (SEM, Stereoscan 260, Cambridge).

3. Results and discussion

Photo-oxidizing the PCL membrane in hydroperoxide solution will firstly generate macromolecular hydroperoxide groups (P–OOH, where P represents the PCL polymer chain) on its surface [22]. Under UV irradiation, the hydroperoxide groups will decompose into macromolecular oxygen radicals (P•O•) which have the ability to initiate the grafting copolymerization of methacrylic acid (MAA) on the substratum, and hydroxyl radicals (H•O•) which may initiate homopolymerization of MAA. A large amount of water was used to dissolve the homopolymer [9,10], yielding a PMAA grafted PCL (PCL-g-PMAA) as shown in Scheme 1. The PCL-g-PMAA membrane was further employed to covalently immobilize gelatin through the formation of amide bonds by the reaction of carboxyl groups from PMAA and amino groups from gelatin in the presence of condensation agent, EDAC, yielding PCL-g-PMAA-gelatin.

3.1. The effect of preparation conditions on grafting degree

The grafting degree of PMAA on PCL membrane is influenced by the photo-oxidation time and temperature, the concentration of grafting solution, the grafting time and temperature etc. Considering the physical properties of PCL, the reaction temperature was always controlled at 30°C to avoid destroying the bulk properties (m.p. ~60°C). The concentration of MAA was chosen as 4wt% for low pH will accelerate the degradation of PCL.

Fig. 1 showed the influence of photo-oxidation time on the WCA and water absorbance of the final grafted PCL membrane. WCA decreased sharply from 81° to 48° at 2 h of photo-oxidation and then decreased slowly to a constant value, ~41°. The water absorbance increased correspondingly. This reduction of WCA is certainly caused by the introduction of PMAA. Therefore, it can reveal relatively the amount of PMAA on PCL surface.

At a given concentration of 4 wt% MAA solution, the influence of grafting copolymerization time on the WCA of grafted PCL membrane is shown in Fig. 2. When PCL membrane was grafted with PMAA for different time after photo-oxidized for 6 h, the WCA of PCL-g-PMAA membrane decreased sharply within 1 h to 45°.
and then decreased slowly to 38.8°. The WCA minimum value appeared at about 2 h and then slightly increased. This fluctuation is due to the partial degradation of PCL superficial layer at higher grafting degree. The concentration of carboxyl groups on the modified PCL membrane increased along with the grafting time, while the acid-induced degradation and dissolution of the superficial layer of PCL membrane were strengthened also because of the extended incubation in MAA solution. At a moment that the degraded superficial layer dissolved on somewhere of PCL, the substrate PCL exposed, leading to the increase of surface hydrophobicity.

The quantitative density of COOH groups grafted on PCL membrane was determined by the reaction with rhodamine 6G, generating the absorbance at 512 nm [19]. Fig. 2 showed that COOH groups increased rapidly within the first 1.5 h, which is coincidence with the WCA measurement. However, no corresponding decrease of COOH amount at longer grafting time was detected. The COOH density increased along with the grafting time steadily. This inconsistency between the WCA and the COOH density was attributed to the different aspects being revealed. The WCA reflects the superficial property of a material. Hence, the existence of hydrophilic–hydrophobic domains on a material surface will have great influence on its value. On the other hand, rhodamine 6G method reflects the total amount of COOH groups existed not only on the superficial layer, but also in the entire bulk materials. The steadily grafting copolymerization will generate a longer PMAA chain containing more carboxyl units. The hide units in the inner layer have no contribution to the WCA but have contribution to the total amount of COOH groups. Moreover, if the surface is not absolutely smooth or has pores, the grafting copolymerization can also occur in some depth in z-direction. AFM measurement (Fig. 3) showed that the control PCL membrane was rough and there existed many deep pores ranging from several tens to hundreds nanometers, which generated during the solvent evaporation process. After PMAA was grafted, the PCL membrane surface became rougher and the pore size decreased as well. In conclusion, after PMAA grafting, the surface property of PCL was greatly altered. The introduction of COOH groups provides also an opportunity to further modify the surface biocompatibility.

### 3.2. Confirmation of gelatin immobilization on PCL-g-PMAA

The change of chemical structure on the PCL membrane surface before and after PMAA grafting and gelatin immobilization was also investigated by ATR-FTIR spectroscopy (Fig. 4). Comparing with control PCL (a), the absorption at 2800 and 2900 cm⁻¹ was strengthened after PMAA grafting (b) because of the contribution of C–H stretching vibration of MAA. A new peak at 1600 cm⁻¹ that was attributed to the absorption of carboxylic groups appeared. After immobilization of gelatin, however, the absorption at 2900, 2800, 1600 cm⁻¹ on PCL-g-PMAA-gelatin(c) was obviously decreased, while a broad peak at 3300 cm⁻¹ and a weak absorption at 1540 cm⁻¹ designated to the amide groups (N–H vibration) in gelatin appeared. Semiquantitative analysis found that the area ratio $A(C=O)/A(C–H)$ of PCL-g-PMAA, 1.52 is bigger than that of PCL, 0.42, while the PCL-g-PMAA-gelatin’s is in between 1.24. This is well consistent with the above qualitative analysis.

The surface alteration after PMAA grafting and gelatin immobilization was further confirmed by XPS measurement. Fig. 5 showed that both control PCL (a) and PCL-g-PMAA (b) displayed the absorption of C₁s at 285.0 eV and O₁s at 531.0 eV, respectively. The peak area ratio of O₁s/C₁s is 0.35 for PCL (a) and 0.45 for PCL-g-PMAA (b), which is in good agreement with the theoretical values of [CH₂CH₂CH₂CH₂COO] in PCL (1/3) and [CH₂C(CH₃)COOH] in PMAA(1/2).

After gelatin immobilization there appeared a peak at 399.8 eV, which is attributed to the nitrogen element (N₁s) in gelatin. The area ratio of O₁s/C₁s in PCL-g-PMAA-gelatin, 0.37 is quite close to the mean value of gelatin molecule, 0.4 [23]. From these results, one can
conclude the occurrence of PMAA grafting and gelatin immobilization. It is worth to note that the immobilized gelatin amount is related to the amount of COOH groups existed on PCL surface. For example, the WCA of PCL-g-PMAA changed from 69.8°, 53.2° and 30.8° to 62.6°, 50.6° and 32.2° after gelatin immobilization, respectively.

3.3. The cell compatibility of modified PCL membranes

The introduction of the hydrophilic groups onto PCL membrane surfaces after PMAA grafting or further gelatin immobilization provides the possibility of cytocompatibility improvement. The endothelial cell culture results showed that the cytocompatibility of the modified PCL membrane was related to the degree of PMAA grafting and gelatin immobilization (Fig. 6).

The PCL-g-PMAA membranes with moderate grafting degree that correspond to WCA of 69.9° and 53.2° (Fig. 6c and d) have shown an improved cell attachment and proliferation ability than the control. With still higher grafting degree (WCA 32.2°), however, both the cell attachment and proliferation properties became worse. Similar cell morphology on PCL-g-PMAA with WCA of 69.9° to that on TCPS was also observed as shown in Fig. 7, where the ECs presented a flat and spreading shape to form a confluent cell layer. On the other hand, there were few cells observed under SEM when ECs have been cultured for 4d on both PCL-g-PMAA with WCA 53.2° and 30.8°. After gelatin covalent immobilization, all the cell attachment and proliferation rates of the PCL membranes were apparently improved (Fig. 6f–h). Whilst the PCL-g-PMAA-gelatin membrane with WCA of 62.6° which corresponds to WCA of 69.9° for the precursor PCL-g-PMAA membrane had the highest attachment

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**Fig. 4.** ATR-FTIR spectra of control PCL (a), PCL grafted with PMAA at a MAA concentration of 4 wt% for 1 h after it was photo-oxidized for 6 h at 30°C (b), and PCL further immobilized with gelatin (c).

**Fig. 5.** Survey XPS spectra of control PCL (a), PCL grafted with PMAA at a MAA concentration of 4 wt% for 1 h after it was photo-oxidized for 6 h at 30°C (b), and PCL further immobilized with gelatin (c).

**Fig. 6.** The ECs attachment (■) and proliferation (□) ratio (%) after cultured for 12 h and 4 d on TCPS (a), control PCL (b, WCA 81.0°), PCL-g-PMAA with WCA 69.9° (c), 53.2° (d) and 30.8° (e), PCL-g-PMAA-gelatin with WCA 62.6° (f), 50.6° (g) and 32.2° (h), respectively. Cell seeding density 15 × 10⁴ cm⁻².
and proliferation ratios, which were quite close to those of TCPS. SEM observation demonstrated that the ECs also presented a better cell morphology (Fig. 7c). Considering the comprehensive results of cell attachment, proliferation and morphology, the PCL-g-PMAA with WCA of 69.9° and PCL-g-PMAA-gelatin with WCA of 62.6° possessed the best cytocompatibility.

Hence, it can be concluded that PCL membrane modified slightly with PMAA or gelatin is quite effective to improve the PCL cytocompatibility to human ECs. The reason why the cytocompatibility became worse at a higher grafting degree is not very clear. The reasonable explanation could be that the higher mobility of the hydrophilic molecular chains would retard the cell attachment, thus not favorable for cell anchor as on a surface with proper density of hydrophilic groups. It is worth noting that a suitable balance of hydrophilicity/hydrophobicity is required for many cell categories to sustain their better activity in vitro [24]. The surface microstructure alteration may also have an influence on cell growth. It was observed in our previous experiments that the ECs showed a better cytocompatibility on polyurethane surface with a nano-scale roughness than on plane or micro-scale pores [25]. However, due to the small difference of the surface morphology among the three PMAA grafted PCL membranes, this is not the main reason for the poor cytocompatibility of the PCL-g-PMAA with higher grafting degree.

4. Conclusions

PMAA was successfully grafted onto PCL surface by combining the application of photo-oxidation and grafting copolymerization under UV irradiation. Biomacromolecule such as gelatin was further immobilized on the grafted PCL surface through condensing agent, EDAC. The measurement of WCA demonstrated the improvement of hydrophilicity of modified PCL membranes. The degree of grafting copolymerization could be regulated by the control of the preparation condition, e.g. grafting time. The introduction of hydrophilic PMAA and biocompatible gelatin had a positive effect on modifying the ECs cytocompatibility of PCL membrane when a suitable degree of PMAA grafting and gelatin immobilization was achieved.

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References