Research Article

Conjugated Linoleic Acid Grafting Improved Hemocompatibility of the Polycaprolactone Electrospun Membrane

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Polycaprolactone (PCL) is a versatile biomaterial with a wide range of medical applications, but its use in blood-contacting devices is hampered due to insufficient hemocompatibility. In this work, electrospun polycaprolactone (PCL) membranes were chemically grafted with conjugated linoleic acid (CLα) to prevent induced blood coagulation. The density of grafted CLα and its effects on the morphology and wettability of the membranes were examined. The study also investigated how the membrane interacted with human whole blood and platelets to determine its antithrombotic properties. As the results suggested, the grafting caused a negligible effect on the physical properties of the membrane but greatly improved its compatibility with blood, showing that the approach can be investigated further for blood-contacting applications.

1. Introduction

Polycaprolactone (PCL) is an FDA-approved resorbable and biocompatible polyester. Thus, this polymer is well used as a base material to produce a tissue-engineered scaffold [1]. Among the fabrication methods applied on PCL, electrospinning created nanofibers of PCL, which are highly controllable in terms of the morphological, mechanical, and biological properties [2–5]. These advantages also promote the application of these nanofibers in vascular tissue engineering and other blood-contacting applications [6–8]. However, the fibrous surface of PCL is prone to cause activation of platelets than flat sheets made from the same material [9]. Thus, an effective approach to improve the hemocompatibility of electrospun PCL without compromising other properties is required to expand its biomedical application.

To create hemocompatible PCL electrospun membranes, several studies had investigated the loading of antithrombotic agents and hemocompatible materials into the PCL fibers. Among them, heparinized materials attracted large research
interest. For instance, the incorporation of heparin-immobilized chitosan or coating of heparin-doped polypropylene fibers on the PCL fibers effectively improved its hemocompatibility [10, 11]. Dou et al. proposed the co-electrospinning of sulfonated keratin with PCL to create nanofibrous mats for vascular tissue engineering [12]. Other studies probing the effect of nanomaterials embedded in PCL, such as multi-walled carbon nanotubes [13] or europium hydroxide nanorods [14], have also yielded promising results.

Herein, this study endeavors to employ a similar approach without the use of other polymers and nanomaterials. Thus, we aim to investigate the application of conjugated linoleic acid (CLA)—a known antithrombotic fatty acid that interferes with platelet activation [15]. CLA has been incorporated to improve the hemocompatibility of a given surface for different biomedical applications [16, 17]. CLA can be incorporated into electrospun materials either by direct loading into the solution before the electrospinning process or by chemical grafting on the surface after the spinning. As shown by our previous studies [18, 19], the grafting of CLA outperformed the loading approach. Grafted CLA mostly resided on the surface of the fibers, so their bioactivity was preserved, helping to reduce the required amount of CLA, and did not alter other properties of the membrane. In contrast, direct-loaded CLA had lowered activity while weakened the structural integrity of the membrane as it was contained inside the fibers.

Thus, this study is aimed at improving the hemocompatibility of PCL membranes by chemically grafting CLA on its surface using a carbodiimide crosslinker. The plain and treated PCL membranes were analyzed on their physicochemical properties and interaction with blood to examine the effect of the process.

2. Materials and Methods

2.1. Fabrication of the CLA-Grafted PCL Membrane

2.1.1. Materials. Acetone (99.5%) was purchased from Xilong Chemical Co., Ltd. (China). Poly (e-caprolactone) (PCL, Mn 80,000), conjugated linoleic acid (CLA), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), and glutaraldehyde were obtained from Sigma-Aldrich Co. (USA). Absolute ethanol and acetone were bought from Xilong, China. All other chemicals are of analytical grade.

2.1.2. Electrospinning of the PCL Membrane. The PCL membrane was prepared according to a previous study [20]. Briefly, PCL pellets were dissolved in pure AC to create a 15% w/v PCL solution. The solution was horizontally electrospun at 15 kV voltage with a flow rate of 1 mL/h using a peristatic syringe pump (55–2226, Harvard Apparatus 22, USA) onto a collector 10 cm away.

2.1.3. Grafting of CLA on the PCL Membrane. The electrospun PCL membrane was plasma-treated using the Plasma Cleaner (PDC-32G-2, Harrick Plasma, USA). The membranes were treated for 60 seconds under a high RF setting (18 W) in low pressure. Then, a 1 x 1 cm membrane was immersed in 500 μL solution containing 0.4 mM CLA and 8 mM EDC for 24 h at 4°C. Finally, the sample was rinsed thrice in ethanol and twice in distilled water before being dried. The experiment was conducted in a dark room. The plain PCL membrane, the PCL membrane treated with plasma, and the PCL membrane immersed in CLA without a crosslinker (C2-E0) were also investigated for comparison purposes.

2.2. Surface Characterization

2.2.1. Detection and Quantification of Grafted CLA. The infrared spectra of the membranes were captured to detect the presence of CLA by Fourier transform infrared spectroscopy (Spectrum GX, PerkinElmer, USA).

For quantification, CLA grafted on the surface was removed by deesterification and then measured using UV-Vis spectroscopy. Briefly, a 0.5 M solution of HCl was prepared in ethanol-water (9:1 v/v). Then, the CLA-grafted membranes were immersed in this solution and incubated at 37°C to deesterify CLA from the surface. After 3 hours, the solution was collected, and its UV-Vis absorbance was measured at 233 nm wavelength. The concentration of CLA was determined based on a previously built calibration curve.

2.2.2. Surface Morphology of PCL Membranes. Dried membranes (1 x 1 cm) were sputter-coated with gold. Then, the membranes were captured using a scanning electron microscope (SEM, JSM-IT100, JEOL, Japan) at the accelerating voltage of 10 kV. The diameters of the fibers were measured using ImageJ software (National Institutes of Health, USA).

2.2.3. Surface Wettability of PCL Membranes. The water contact angle was measured at room temperature to determine the wettability of the PCL surfaces. Water droplets were dispensed from a 25G needle onto the membranes, and their shape was recorded using a DSLR camera (Canon) and analyzed with ImageJ software (National Institutes of Health, USA).

2.2.4. Mechanical Characterization. The tensile strength of the membranes was evaluated using the Texture Analyser (TA.XTPlus, Stable Micro Systems, USA). Specimens were clamped at both ends so that the stress area had dimensions of 10 x 30 mm and were prepared for each measurement. The measurements were triplicated, and representative stress-strain curves of each sample were illustrated.

2.3. Blood-Material Interaction Assays. The study complied with the ethical regulation of the Institutional Review Board. Fresh human whole blood—donated by volunteers with informed consent—was immediately drawn into tubes of 3.2% v/v sodium citrate solution (so that the blood:sodium citrate v/v ratio is 9:1) to prevent blood clotting. The blood was used for at most 2 hours after collection.

2.3.1. Blood Clotting Assay. Citrated human whole blood was recalculated to contain 0.04 M calcium cation by adding 4.4 mg of CaCl2 per mL blood. Then, 20 μL of the blood was dropped on the surface of each 1 x 1 cm sample and incubated at 37°C for varied durations. A 1 x 1 cm glass surface was used as the negative control. After the incubation periods, the specimens...
were immersed for 10 minutes in 5 mL of distilled water to induce the eruption of uncoagulated red blood cells. The absorbance of the supernatant at 540 nm was then measured using a microplate reader (Varioskan™, Thermo Scientific, USA) to determine the concentration of free hemoglobin. The 100% reference value was determined as the absorbance of 20 μL citrated whole blood lysed in 5 mL distilled water. The anticlotting index (ACI) of the samples was calculated as follows:

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ACI = \frac{\text{Absorbance of the supernatant of blood contacted with sample}}{\text{Absorbance of citrated whole blood lysed in distilled water}} \times 100.
\]

2.3.2. Platelet Adhesion Assay. Fresh citrated human whole blood was centrifuged at 1200 rpm for 5 minutes to obtain platelet-rich plasma (PRP) and then centrifuged at 3500 rpm for 10 minutes to obtain platelet-poor plasma (PPP). PRP and PPP were mixed according to their platelet density to adjust the final density to 10^6 cells/μL. 200 μL of this platelet suspension was dropped on the surface of each sample, which was then incubated statically at 37°C. After 2 hours, the membranes were rinsed with phosphate-buffered saline (PBS) (1X, pH 7.4) to remove thoroughly plasma and the weakly adhered platelets. Then, the samples were immersed in 2.5 wt% glutaraldehyde for 2 hours for fixation and then washed consecutively with PBS, 50% PBS, and distilled water. Finally, the samples were dried in a safety cabinet and observed by SEM.

2.4. In Vitro Cytotoxicity Assay. Murine fibroblast L929 cells were cultured in DMEM (10% fetal bovine serum and 1% penicillin/streptomycin). The culture condition was set at 37°C with a 5% CO₂ atmosphere; the subculture was performed when the cell confluence reached 85-90%.

The resazurin assay was performed based on ISO 10993 to evaluate the cytotoxicity of the membranes’ extract on L929 cells. For each sample, 6 cm² of the membrane was immersed in 1 mL of media and incubated for 24 h at 37°C to prepare the extract solution. The cells were seeded at a density of 1 × 10⁵ cells/mL and cultured for 24 h. Then, the extract solution was removed and diluted into different concentrations (50%, 25%, and 12.5%) with media before being added into the cell-seeded wells and cultured for another 24 h. After that, the extracts were removed, and 1 mL of culture medium with resazurin (0.02 mg/mL) was added to each well. The cell culture was incubated for another 4 h before fluorescence measurement. The fluorescence of the solutions was excited at 530 nm, and the emitted signal was measured at 590 nm using a microplate reader (Varioskan™, Thermo Scientific, USA). The signal of cells cultured in normal media was considered to indicate 100% viability. Each sample was replicated 4 times.

2.5. Statistical Analysis. All experiments were replicated three times unless stated otherwise. Data were presented as average ± standard deviation and analyzed using SPSS Statistics software (IBM). One-way ANOVA followed by the Tukey-Kramer post hoc test was used to compare three or more groups. A p value < 0.05 was considered significant.

3. Results and Discussion

3.1. Detection and Quantification of Grafted CLA. Regarding the grafting of CLA, we hypothesized that plasma treatment can introduce hydroxyl groups onto the surface of the PCL membrane. Then, CLA can form a complex with the carbodiimide crosslinker EDC and bind to this -OH group via esterification [19]. Firstly, FT-IR spectroscopy was conducted to verify the presence of CLA. The FT-IR spectra of 4 samples (Figure 1) were similar except for a peak at 1602 cm⁻¹, which was only detected in C2-E0 and C2-E8. As the IR spectra of plain PCL did not contain this peak [21], it could be associated with the alkene C=C bond of CLA, confirming its presence on the surface of the membrane. In the study of CLA-grafted acrylonitrile, Kung and Yang also detected and attributed a similar IR peak to this double bond [16]. However, FT-IR spectra cannot confirm the formation of an ester bond as its signal could be overlapped by the C=O bond in the structure of PCL.

However, the quantification results suggested effective grafting. As shown in Table 1, C2-E0 and C2-E8 differed in quantified CLA density. Using the deesterification of the CLA from the surface, we observed a CLA density of 18.96 ± 1.52 nmol/cm² on C2-E8 while C2-E0 only exhibited a density of 5.78 ± 0.92 nmol/cm². The small amount of CLA on C2-E0 inferred that there was CLA already physically adhered to the surface after the wash, and the chemical crosslinker helped immobilize more of the fatty acid (Table 1). The CLA density of C2-E8 was approximately 9.5% of the hypothetical maximum, illustrating constraints in reaction efficiency. This density was also in accordance with other studies that employed different quantification methods [16, 22]. As an antithrombotic agent, the density of CLA would play a critical role in the hemocompatibility of the treated membranes.

3.2. Morphology and Surface Properties. Morphology and surface properties are crucial factors influencing the blood-material interaction. Thus, the intervention to enhance antithrombotic activity should not negatively affect these characteristics of the initial materials. The samples after each step of treatment were observed using SEM to detect any undesired alteration. SEM images of the three treated samples exhibited morphological features similar to that of the plain PCL membrane: nonwoven matrix of microfibers and no beads (Figure 2(a)). There was also no significant difference in fiber diameters. Thus, the plasma treatment and chemical graft of CLA and EDC caused a negligible effect on the morphology of the membrane and individual fibers.

As shown in Figure 2(b), the plain PCL membrane was moderately hydrophobic. After the introduction of -OH groups on its surface by plasma treatment, the membranes exhibited high wettability (water contact angle approximated to 40°). However, after being coated (C2-E0) or grafted (C2-E8) with CLA, the hydrophobicity of the membranes increased significantly. We attributed this decrease to the
presence of CLA and the loss of plasma effect. CLA is an unsaturated fatty acid with a hydrophobic tail. As its carboxyl group formed ester bonds with the surface, the hydrophobic tails of CLA would contribute to the decrease in the membrane’s wettability. Moreover, the effect of plasma would be diminished after the membranes were constantly treated and washed. As hydrophobicity significantly impacts the blood-material and cell-material interaction, especially on the adhesion of protein, platelets, and cells [23, 24], this change could also contribute to the antithrombotic effect of CLA on the membrane surface. For application in vascular tissue engineering, hydrophobicity that favors endothelialization while preventing platelet adhesion would be ideal. Thus, further investigation on the relation between CLA density and the surface wettability would be beneficial.

3.3. Mechanical Properties. Table 2 presents the mechanical properties of the membranes, and their stress-strain behaviors are illustrated in Figure 2(c). Generally, the membranes exhibited similar behavior when stressed, indicating no significant alteration in mechanical properties after the treatment ($p > 0.05$). The membranes can withstand the stress of 11 MPa and can be deformed up to approximately 158% of their original lengths before rupture. Regarding their elasticity, the membranes all had yield stress around 6 MPa, the limit where they started to deform irreversibly.

These parameters were in accordance with our previous reported PCL electrospun membrane using acetone as the solvent [20], indicating insignificant effects of the CLA grafting. As plasma treatment introduced functional groups on the surface of the PCL fibers, CLA was expected to bind exclusively to the surface with no interference to the structural integrity of the membrane. Moreover, the EDC crosslinker and used solvent are also undamaging to the polymeric materials.

Although mechanical behaviors do not significantly influence the interaction of materials with blood, they are prerequisites in many blood-contacting applications, especially of a vascular graft and medical catheter. Thus, this approach would be promising to improve the hemocompatibility of materials as its effects on their mechanical properties were negligible.

3.4. Blood-Material Interaction. To test the effect of CLA on the hemocompatibility of the PCL membrane, the blood clotting assay and platelet adhesion test were conducted under static condition. In the blood clotting assay (Figure 3(a)), the antithrombotic activity was represented by the ACI value, where a diminishing value indicated that blood coagulation occurred. The ACI value of 100, which corresponded to the absorbance of fresh whole blood in distilled water, is considered to indicate no clotting.
As illustrated in Figure 3(a), ACI of the glass surface and PCL-plasma reduced rapidly due to their coagulation-induced nature and high hydrophilicity. After 50 minutes of exposure, there were only 13.5% and 28.9% of uncoagulated blood on the glass and PCL-plasma surface, respectively. In contrast, CLA-containing samples exhibited slower blood clotting. C2-E0 initially exhibited ACI values similar to those of C2-E8 but gradually converged to the ACI of PCL. 84.5% of blood exposed to the C2-E8 surface remained uncoagulated after 50 minutes of exposure. In comparison, ACI of PCL dropped to 59.5 after 50 minutes.

**Table 2: Mechanical properties of the membranes.**

| Sample    | Peak stress (MPa) | Strain at break (%) | Yield stress (MPa) |
|-----------|-------------------|---------------------|-------------------|
| PCL       | 11.3 ± 0.4        | 154 ± 8             | 6.9 ± 0.7         |
| PCL-plasma| 11.1 ± 0.8        | 159 ± 4             | 6.2 ± 0.8         |
| C2-E0     | 11.0 ± 0.7        | 155 ± 3             | 7.0 ± 0.6         |
| C2-E8     | 11.4 ± 1.1        | 157 ± 7             | 6.3 ± 1.0         |

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while ACI of C2-E0 was recorded at 63.6. Thus, it can be deduced that CLA grafting is a promising approach to lower the blood clotting rate.

Figure 3(b) shows the SEM images of the membranes after being exposed to platelets. The PCL and PCL-plasma membranes were thrombogenic: the platelets were activated and adhered strongly to the surface. Large aggregations of platelets were observed on the surface of PCL-plasma, indicating the effect of its high hydrophilicity. This result was following the clotting assay, where PCL-plasma strongly induced blood coagulation compared to other membrane samples. However, the membranes that contained CLA effectively reduced platelet activation and adhesion. On the surface of C2-E0, platelets remained in their inactivated spherical shape whereas virtually no platelet was found on the surface of C2-E8. Thus, CLA did prevent blood clotting on the surface of the PCL membrane by mitigating platelet activation. Taking into account the quantified CLA density, this effect also corresponds to the CLA amount of the samples. The number of adhered platelets on C2-E8 was also comparable or lower regarding other studies on hemocompatibility of fibrous PCL structures [10, 12]. Therefore,
further investigation on variation and optimization of CLA density would be necessary to realize its potential application.

3.5. In Vitro Biocompatibility of the Membranes. Since PCL and CLA are highly biocompatible, while byproducts of the crosslinking procedure can be removed effectively by washing, the membranes are expected to exhibit no toxicity. In vitro biocompatibility of the membranes was examined based on the guidelines of ISO 10993-5 and is illustrated in Figure 4. The viability of cells cultured in diluted extracts mostly exceeded 100%; but for cells in the initial extract, this figure of PCL-plasma, C2-E0, and C2-E8 samples dropped to approximately 95%. Nevertheless, according to ISO, cell viability higher than 70% in the 100% extract indicated sufficient biocompatibility for further in vivo investigations. Thus, all samples including the grafted membranes (C2-E8) showed no sign of cytotoxicity, showing the potential of this grafting approach for materials with blood-contacting application.

4. Conclusion
The CLA-grafted membrane was fabricated successfully using plasma treatment and a carbodiimide crosslinker. The grafting of CLA did not alter the morphology and the mechanical properties of the PCL membrane, but it caused a significant impact on the hydrophobicity of the samples. Regarding hemocompatibility, the presence of CLA effectively prevented the adhesion and activation of platelets on the membranes. The grafted membrane also mitigated blood coagulation and exhibited high biocompatibility via the in vitro assay. Thus, the membrane has potential for further investigations as an antithrombotic material for blood-contacting applications.

Data Availability
The data used to support the findings of this study are included in the article.

Conflicts of Interest
The authors declare that there is no conflict of interest regarding the publication of this paper.

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