New Method for Coupling Collagen on Biodegradable Polyurethane for Biomedical Application

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ABSTRACT: A two-step method was used in this article to synthesize a novel biodegradable polyurethane (PU) composed by L-lysine ethyl ester diisocyanate (LDI) hard segment, poly(ε-caprolactone) diols (PCL-diol) soft segment and 1,4:3,6-dianhydro-D-sorbitol (isosorbide) chain extender. FTIR, ^{1}H-NMR results revealed the products had typical PU structures. GPC result showed that the number average molecular weight of the PU was 36,800 with the heterodisperse index of 2.18. DSC result displayed the PU melting temperature was 42°C. Surface aminolyzing of the PU membrane was performed by reacting it with 1,3-propanediamine under alkaline condition. Then, type I collagen was grafted on the PU surface by EDC/NHS coupling method. Rhodamine B isothiocyanate (RBITC) fluorescence spectrum presented that both amino group (—NH_2) and type I collagen were successfully introduced on the PU surface. Furthermore, ninhydrin analysis showed that the maximum surface density of amino groups was 11.4×10^{-7} mol/cm^2 after aminolyzing in 12% 1,3-propanediamine aqueous solution for 1 h. Cell viability experiments indicated both PU-NH_2 and PU-Col had low cytotoxicity and supported the ATDC5 cell's proliferation on them. © 2012 Wiley Periodicals, Inc. J Appl Polym Sci 000: 000–000, 2012

Key words: biodegradable polyurethane; surface modification; aminolysis; type I collagen; cell viability

INTRODUCTION

Biodegradable polymers such as polylactides, polyglycolides, polyhydroxyacids, and their copolymers have been used as implant materials and tissue engineering scaffolds for a very long time. The degradable materials could avoid the implants surgical removing after patients recover and provide a free tissue ingrowth space after degrading. These merits make them popular in clinic and tissue engineering. However, the low elasticity and deformability properties prevent their further application in some soft tissue repairs such as tendon and cartilage.[1] In addition, the acidic degradable products causing aseptic inflammation after implanting was another big problem for clinic using.[2,3]

Segmented PU is a polymer with good tailorable mechanical properties which could be achieved by molecular design. When different hard, soft segment molecular and their ratio were designed, it could be fabricated into rigid or elastic materials with stable or degradable properties. PU has already been used as implant or scaffold for tissues such as cardiovascular implants, artificial skin, and nerve conduits.[4–8] However, the monomers used for traditional PU synthesis such as 4,4-diphenylmethane diisocyanate, 2,4-toluene diisocyanate have a considerable degree of cytotoxicity[9,10] and that kind of polymer has always been designed to be nondegradable for conventional clinic application.

The developing requirements of novel biodegradable polymers for tissue engineering scaffold arose since the drawbacks of those degradable polyesters were noticed. Degradable PU attracted lots of scientists' interest because its tailorable properties could be easily fulfilled by molecular design. There are increasingly efforts designing and synthesizing novel biodegradable PU. In those efforts, poly(ε-caprolactone) (PCL) diols which had degradable ester chemical structure and reaction hydroxyl end groups was always chosen as the soft segment. Low cytotoxicity aliphatic diisocyanates including 1,4-butane diisocyanate (BDI), hexamethylene diisocyanate (HDI),

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L-lysine ethyl (or methyl) ester diisocyanate (LDI), 1,4-trans-cyclohexane diisocyanate (CHDI), and isophorone diisocyanate (IPDI) were selected as hard segments. 1,4-butanediol and isosorbide were used as chain extender. The requirements for the balance between the degradation and mechanic properties could be harmonized by choosing a suitable ratio among the hard, soft segments, and chain extenter.[11–16] However, since the PU lacks the bioactive chemical structure, further modifications such as glow discharge or gas plasma treatment,[17,18] protein coating, gelatin impregnation, hyaluronic acid, and collagen immobilization were researched to improve its bioactivity.[19–21]

In this article, a new method of coupling type I collagen on the PU was researched. At first, PU with the number average molecular weight of 36,800 was synthesized. Then, the PU was aminolyzed to introduce the amino groups on it. Finally, type I collagen was coupled with the amino groups on the PU by EDC/NHS method. FTIR, H-NMR, XRD, GPC, and DSC were used for the PU structure characterization. Fluorescent and UV-vis spectrum were used for the qualitative and quantitative analyses of the amino groups and collagen presented on the PU surface. All results displayed that the amino groups and type I collagen were successfully grafted on the PU surface. The cell viability experiments results also revealed the modified PU could be used as substitute materials for the traditional degradable polyesters.

EXPERIMENTS

Materials

The chemical reagents used were LDI (Adamas Reagent Co. Ltd), PCL diols with molecular weights of 2000 (PCL2000, ACROS ORGANICS), 1,4:3,6-dianhydro-D-sorbitol(isosorbide, TCI), dibutyltion dilaurate (DBDL, TCI). *N*,*N*-dimethylformamide (DMF, Guangzhou Chemical Reagents Co., Ltd), 1,3-propanediamine (Guangzhou Qiyun Reagent Co. Ltd). Cell Counting Kit-8(CCK-8) (Dojindo, Gaithersburg, MD) was used for the ATDC5 cell viability assessment.

Synthesis of the polyurethane

The polyurethane was bulk polymerized with the ratio of the diisocyanate, PCL diols and chain-extender 2 : 1 : 1. A two-step approach was used for the polymer synthesis.[22] First, the PCL diols was dried in a vacuum at 120°C for 2 h in a three-necked flask equipped with a stirrer. After the temperature dropped to 50°C, the given amount of HMDI and DBDL were added into the flask. Then the temperature increased to 70°C for the polymerization with an argon atmosphere protection. Fifty minutes later, the isosorbide was added into the reaction products and then the temperature increased to 110°C for another 5 more hours reaction. Finally, the polymers were dissolved in DMF and precipitated with methanol. The produced materials were dried at 70°C in a vacuum for 24 h.

PU membranes and scaffolds preparation

30 mL DMF dissolved with 3 g PU was casted into a teflon mold. The casted solution was evaporated at 70°C for 24 h and further dried in a vacuum for another 24 h at 35°C to remove residual solvent to get final membranes. Then, the membranes were cut into appropriate size and used for surface modification.

The PU scaffolds were produced via a salt-leaching–phase-inverse technique (salt leaching method combined with phase inverse approach).[12] Briefly, as the PU was totally dissolved in DMSO, the precipitated solvent (CH_3OH+H_2O) was added dropwise to the PU solution and then cooled down the solution temperature to form precipitation. Sodium chloride (NaCl) with a particle size range of 100–300 μm (sieved) was added and mixed with the deposition. After that, the produced polymer–NaCl paste was filtrated and transferred to a glass cylinder mould with both diameter and height of 1 cm and was solidified at the temperature of −20°C. The NaCl and DMSO were extracted from the cylinder PU scaffolds by dipping it in deionized water at room temperature. Finally, the scaffolds were dried in a vacuum at 40°C for 24 h.

Aminolysis of PU membranes (or scaffolds) and graft of type I collagen

The membranes (or scaffolds) were immersed in alcohol/water (1/1, v/v) solution for 2–3 h to remove oily contamination and then were washed with deionized water. Then, the membranes (or scaffolds) were immersed in the 1,3-propanediamine aqueous solution with different concentrations of 3, 6, 9, 12, and 15% for 1 h or with the same concentration of 9% for different reaction time (0.5, 1, 1.5, 2, and 2.5 h) at 30°C. After that, the membranes (or scaffolds) were taken out and rinsed with deionized water to remove free 1,3-propanediamine, and then dried in a vacuum at 35°C until a constant weight was obtained.[23]

For collagen grafting, the aminolyzed PU-NH_2 membranes (or scaffolds) were further rinsed by phosphate buffer solution (PBS, 50 m*M*, pH 7.0) after washing it with deionized water. After that, the membranes (or scaffolds) and type I collagen solution (1.0 mg/mL, in 0.1 *M* acetic acid) were added in a 20 mL EDC/NHS, 10 mg/mL in PBS (50 m*M*,

pH 7.0) solution for 30 min at 4°C, respectively. And then, the two solutions were mixed and kept for 24 h at 4°C. The collagen–covalent membranes (or scaffolds) (PU-Col) were taken out and washed with 0.1*M* acetic acid solution for three times and abundant deionized water to remove the adsorptive collagen. The modified membranes (or scaffolds) were dried in a vacuum at 30°C for 24 h.[24]

ATDC5 cell culture and cell proliferation assay

Small pieces of PU-NH_2, PU-Col scaffolds were placed in 96-well plates seeded with 5×10^3 ATDC5 cells every well and incubated in Dulbecco's modified Eagle's medium in a humidified atmosphere incubator of 5% CO_2 at 37°C. The tissue culture polystyrene (TCPS) seeded with same number of ATDC5 cells was used as control. After incubation for 24 h, the culture medium was changed, and then changed every 2 days. The cell proliferation was determined on 1 day, 3 days, 5 days, and 7 days, respectively, according to the manufacturer's instructions of CCK-8 colorimetric assay.

Characterization

Bulk property characterization

A Fourier transformation infrared spectrometer with the attenuated total reflection (FTIR-ATR, Vector 33, Bruker, Germany) was utilized to determinate the chemical structure of PU samples at 25°C. Wavenumber scan ranged between 400 and 4000 cm^{-1} with a step width of 4 cm^{-1}.

^{1}H-NMR spectra of the polymer in deuterated dimethyl sulfoxide (DMSO) was performed at 25°C with a AVANCE Digital 400-MHz NMR spectrometer (Bruker, Germany). Tetramethylsilane was used as a reference. The delay time was 2 s. The scan number was 16 times.

The molecular weight and molecular weight distribution (polydispersity index) of the polymer were determined by size exclusion chromatography (SEC) analyses carried out on a Waters modular GPC system (Waters, USA) consisting of a 515 pump, 717 injector, and the 2414 refractive index detector. Calibration was carried out with polystyrene molecular weight standards. Approximately 120 mg of polymer in 10 mL fluent was used to ensure good representation of the polymer. A flow rate of 1.0 mL/min was applied.

X-ray diffraction (XRD) was used to observe the crystallinity of PU film. The sample was analyzed by X-ray scanning with X-ray diffractometer (X'pert, Netherlands).The X-ray source was Cu/Kα radiation. Two theta (θ, θ being the angle of diffraction) ranged from 10° to 50° with a step width of 0.02° and a scanning rate of 30°/min.

Differential scanning calorimetry (DSC) was conducted by using a TA Instruments DSC Q20 thermal analyzer (Waters, USA) with N_2 purging (20 mL/min) at a rate of 10°C/min. The sample weight was about 10 mg. The samples were scanned two times. (A first ramp from 25 to 200, followed by a ramp down to −60°C; the second ramp from −60°C to 200°C).

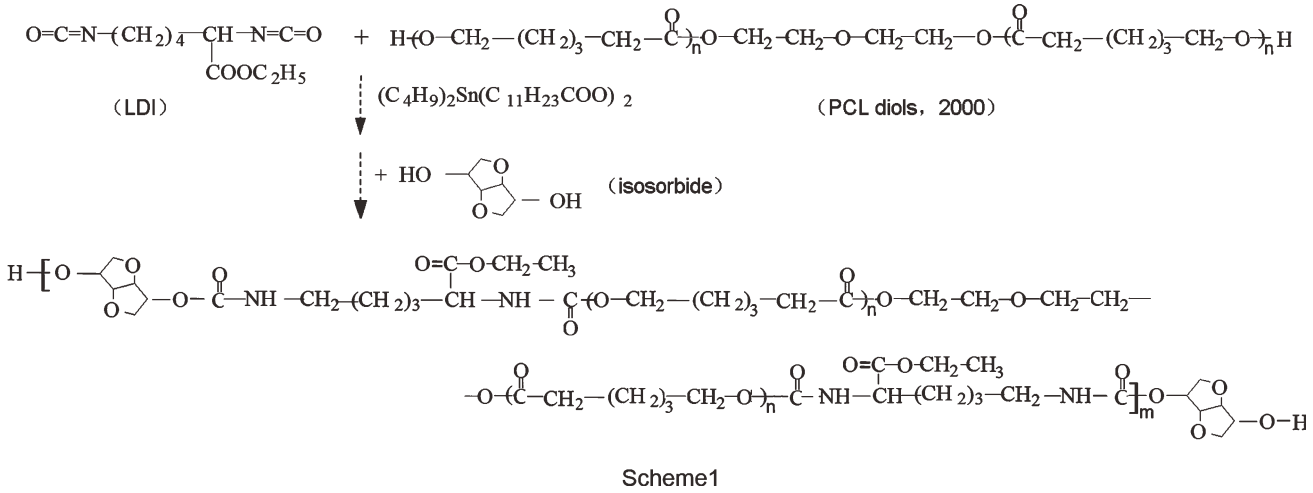
Qualitative and quantitative analysis of the amino groups on the PU-NH_2 membranes

Rhodamine B isothiocyanate (RBITC) was used to label the amino groups on the PU membranes for qualitative analysis by a fluorescence spectrophotometer (F-7000 Spectrophotometer, Hitach, Japan). First, the PU-NH_2 membranes were immersed in 0.1 mg/mL RBITC solution for 24 h at 4°C. Then the RBITC-labeled PU membranes were rinsed with deionized water for 24 h at 4°C to remove free RBITC and finally dried under vacuum for another 24 h at 30°C. To prevent fluorescent from quenching, the entire procedure was processed in a dark environment.[25] The membranes had a maximum wavelength at $\sim$ 580 nm arising from the RBITC group (excitation wavelength was 543 nm).

The ninhydrin analysis method was used to quantitatively determine the amount of free amino groups on the PU-NH_2 membranes. The membranes were immersed in 1.0*M* ninhydrin/ethanol solution for 1 min and then they were extracted and placed into a cuvette to heat at 80°C for 15 min to accelerate the reaction between ninhydrin and amino groups on PU-NH_2 membranes. Then, 5 mL of 1,4-dioxane was added into the tube to dissolve the membranes and the solution presented Ruhemann's purple color. Another 5 mL of isopropane was added into the solution to stabilize the purple compound. The absorbance at 570 nm of this mixture was measured by a Varioskan Flash (Thermo Scientific, America). A calibration curve was firstly obtained by a series of standard 1,3-propanediamine in 1,4-dioxane/isopropane (1 : 1, v : v) solutionvent.[23–26]

Qualitative analysis of the collagen immobilization on PU-NH_2 membranes

Rhodamine-labeled collagen (Rd-Col) was prepared by mixing 0.2 mg/mL rhodamine B isothiocyanate into 3 mg/mL collagen solutions at 4°C for 48 h. The free dyes were dialyzed off in 0.5 m acetic acid for 2 weeks.[25] Rd-Col was grafted on the surface of the PU-NH_2 membranes with EDC/NHS as a cross-linking agent by fluorescence test (without EDC/NHS as control). Then the PU-NH_2 membranes coupled with rhodamine-labeled collagen (Rd-Col) were qualitatively analyzed by a fluorescence



Scheme 1 The schematic representation of synthesis of polyurethane.

spectrophotometer (F-7000 Spectrophotometer, Hitach, Japan).

Contact-angle measurements

The water contact angle was tested with a sessile drop at room temperature on the PU membranes or the modified PU membranes by a water contact angle measurer (OCA15, DATAPHYSICS, Germany). Five measurements were carried out for each sample. The data resulted were the mean of five measurements.

RESULTS AND DISCUSSION

Bulk characterization

The polyurethane was synthesized by PCL diols (Mn = 2000), LDI, and isosorbide as shown in Scheme 1.

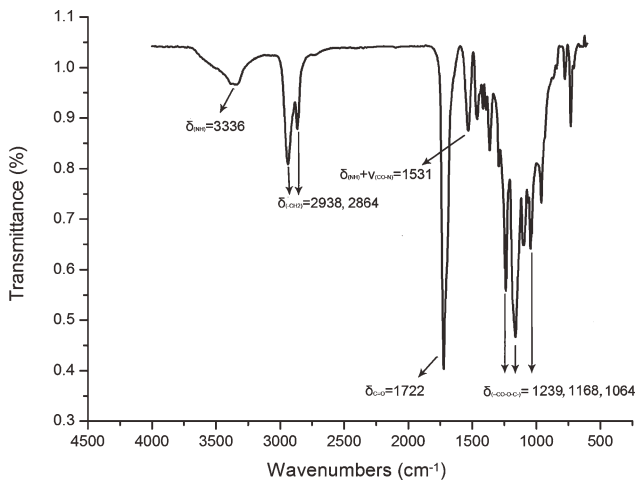


Figure 1 FTIR graph of polyurethane.

The chemical structure of the polymer was confirmed by FTIR and ^{1}H-NMR spectroscopy as Figures 1 and 2 shown. The absorption band at 1722 cm^{-1} was assigned to C=O stretching (urethane). The absorption band at 1531 cm^{-1} corresponded to $\delta_{(NH)} + \nu_{(CO\text{-}N)}$, which was the characteristic absorption peak of amide II for poly(ester urethane).[15] The absorption band at 1262 cm^{-1} corresponded to $\nu_{(C\text{-}N)}$ with $\delta_{(NH)}$ of aliphatic —R—NH—COO— groups. The absorption bands at 1239, 1168, and 1064 cm^{-1} were assigned to the stretching vibration of the ester group —CO—O—C—. The absorption band at 775 cm^{-1} was assigned to the out-of-plane bending of the ester group. The absorption band at 3336 cm^{-1} was due to N—H stretching (urethane). The absorption bands at 2938 and 2864 cm^{-1} were associated with asymmetric and symmetric —CH_2 groups.[27]

Figure 2 was the ^{1}H-NMR spectra of polyurethane. The peaks of "b,c,f" were strong which corresponded to proton peaks of the PCL. The peak of "a" (at 1.2 ppm) corresponded to proton peak of the —CH_3 on LDI chain.

Figure 3 was the GPC spectrum of the synthesized alternative block copolymers. It displayed a narrow, unimodal molecular weight distribution of the PU. The number average molecular weight and heterodisperse index calculated according to the figure were 36,800 and 2.18, respectively.

The FTIR, ^{1}H-NMR, and GPC characterizations of synthesized polymer showed that polyurethane was synthesized.

Figure 4 was the XRD diffraction patterns of PCL and PU. The PU produced similar diffraction patterns as PCL, but getting quite weak intensity compared to PCL, as that diffraction peak at 21.8° shown in Figure 4. This result indicated that the crystal structure in PU was mainly attributed to the ordered structure of soft chain segment of PCL.

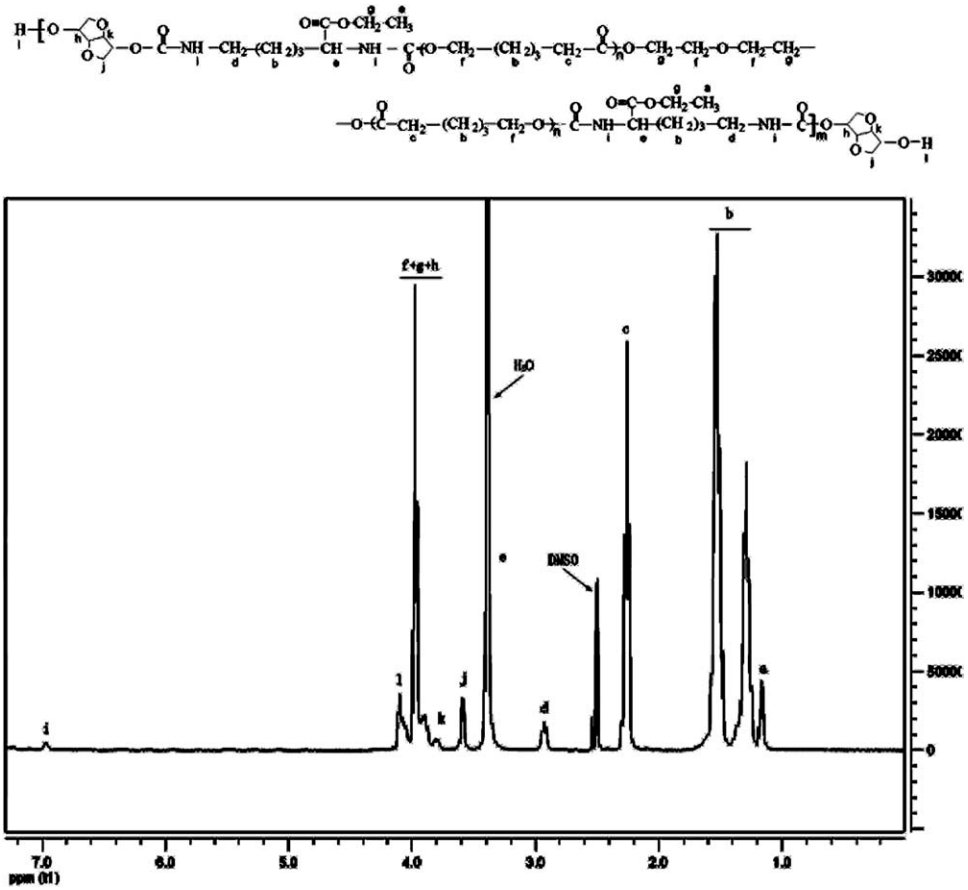


Figure 2 ^{1}H-NMR spectrum of polyurethane.

Figure 5 was the DSC thermograms of PU. From the graphs, below −50°C should have a transition which was attributed to the glass transition. The transition from −10°C to 4°C was attributed to the crystallization exothermic peaks of soft segments of PCL, which corresponded to the result of XRD. The thermal transition from 25 to 55°C was assigned to the melting of the soft and hard segments.

Surface modification of PU by aminolysis

The schematic of polyurethane membranes surface aminolysis was showed in Scheme 2.

Figures 6 and 7 displayed the fluorescence spectrum of RBITC-immobilized PU-NH_2 membranes. RBITC emitted a 580 nm wavelength light when excited by the 543 nm incident light. Figure 6 displayed the relative fluorescence intensity of RBITC-

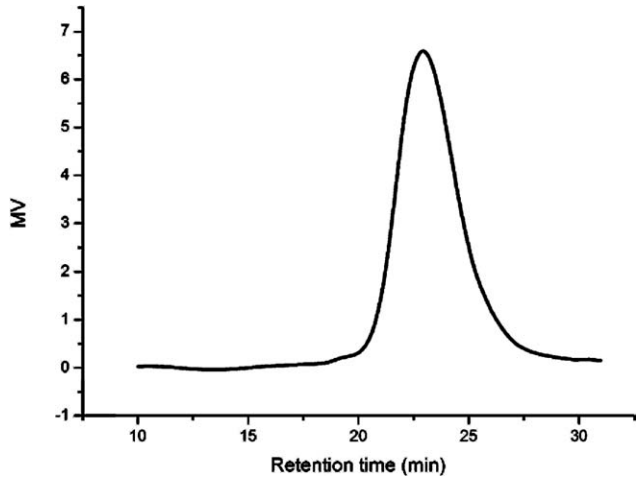


Figure 3 GPC graph of polyurethane.

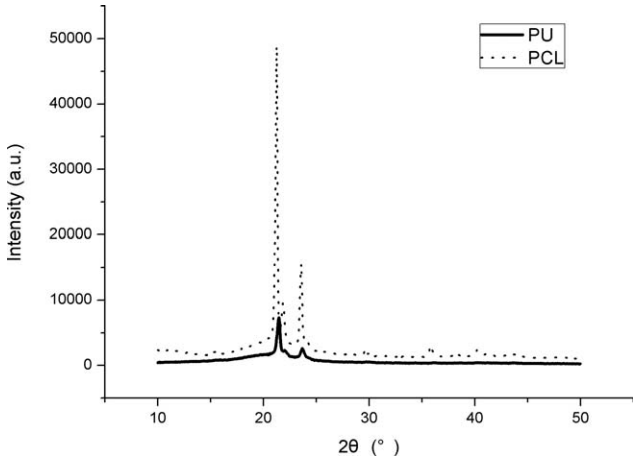


Figure 4 XRD graph of polyurethane & PCL2000.

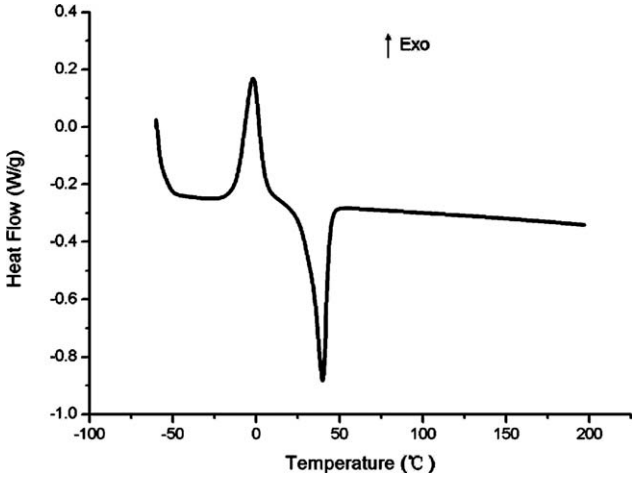
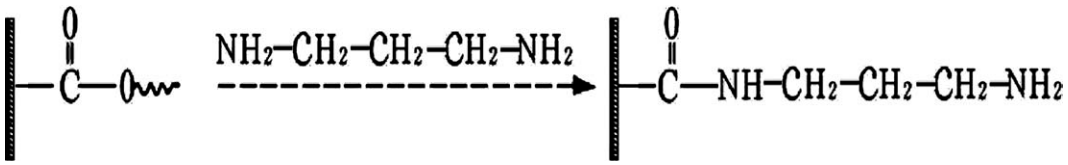


Figure 5 DSC thermograms of polyurethane.

immobilized PU-NH_2 membranes aminolyzed by the 9% 1,3-propanediamine aqueous solution at different time. The intensity increased with aminolyzing time but decreased 2 h later. This would be caused by too long aminolyzing time that led to the degradation of the superficial layer. This resulted in the free surface amino groups concentration decreasing and the fluorescence intensity decreasing either. Similar regulation could also be observed when PU membranes were aminolyzed for 1 h by different higher concentration 1,3-propanediamine aqueous solution. It was noticed in Figure 7, the fluorescence intensity decreased when the solution concentration was higher than 12%. That could be the same reason caused by strong alkalescence solution treatment that resulted in the surface degradation.

Figures 8 and 9 were the curves of the quantitative analysis of the amino groups on the PU-NH_2 membranes aminolyzed by 1,3-propanediamine aqueous solution with different concentration and time. As shown in Figure 8, the highest surface NH_2 density (7×10^{-7} mol/cm^2) was presented at aminolyzing time of 2 h. That was similar to the qualitative analysis results displayed in Figure 6. The maximum of NH_2 density was 11.4×10^{-7}mol/cm^2 presented at 12% solution concentration aminolyzing for 1 h.

We presumed that the membrane surface was absolutely smooth and the amino groups immobilized on the membrane as a single layer. In this way, the maximum density of amino groups should be $\sim 1.0 \times 10^{-8}$mol/cm^2, which is much smaller than 11.4×10^{-7}mol/cm^2 we got when the film was aminolyzed in the 12% 1,3-propanediamine solution for 1 h. It was obviously unreasonable and improper and hence the hypothesis was incorrect. This was because the aminolysis occurred not only on the rough surface layer but also in the deep interface layer since the solution could penetrate into the membrane surface as similarly as the results of the literature.[23]



Scheme 2 The schematic representation of aminolysis on polyurethane membrane.

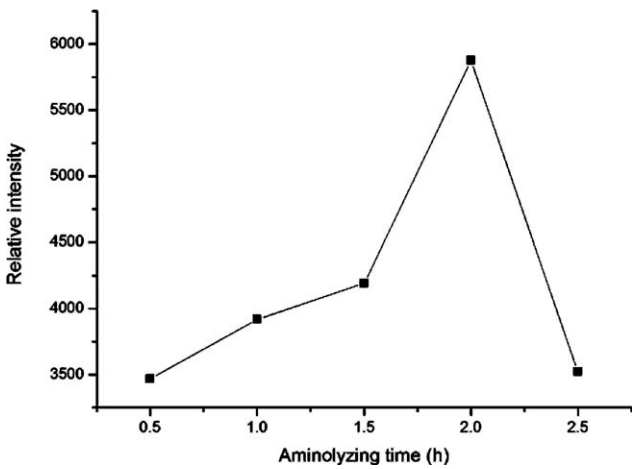


Figure 6 The fluorescence relative intensity of the RBITC-immobilized PU membranes as a function of aminolyzing time at a concentration of 9% 1,3-propanediamine solution.

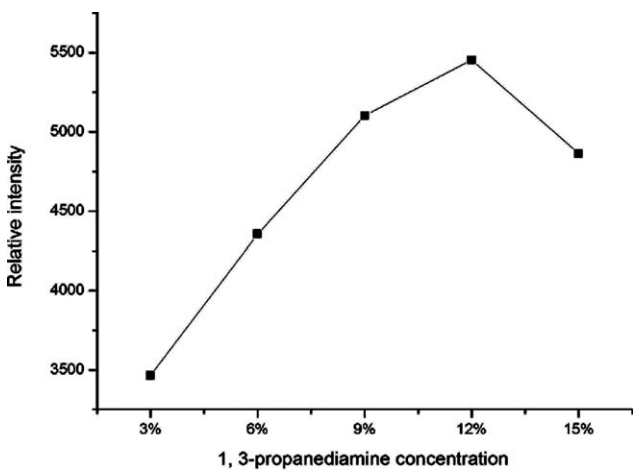


Figure 7 The fluorescence relative intensity of the RBITC-immobilized PU membranes aminolyzed for 1 h as a function of 1,3-propanediamine concentration.

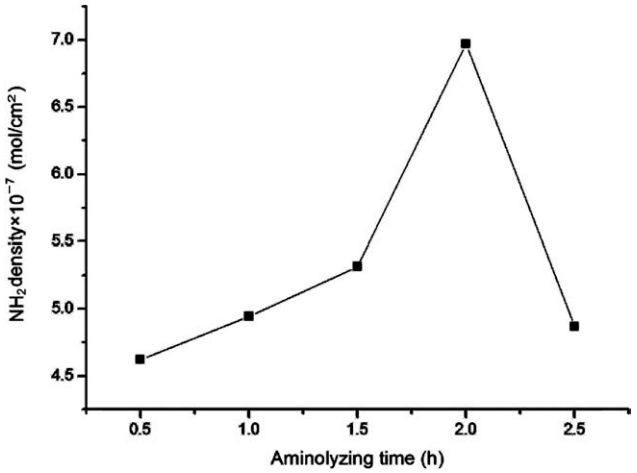
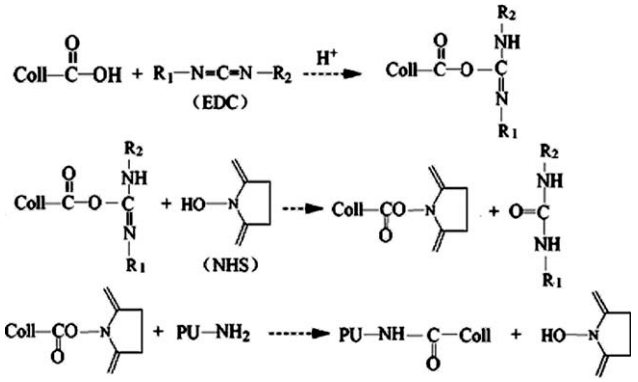


Figure 8 The absorbance at 570 nm of the PU membranes aminolyzed treated with ninhydrin as a function of aminolyzing time at a concentration of 10% 1,3-propanediamine solution.



Scheme 3 The schematic representation of collagen immobilization on PU membrane with EDC/NHS.

Type I collagen immobilization on the PU-NH_2 membranes

Type I collagen was introduced on the PU-NH_2 membranes as described in Scheme 3. Figure 10 displayed that after the rhodamine-labeled collagen being grafted on the PU-NH_2 membranes surface by EDC/NHS coupling method, the relative intensity got a high value compared to the membranes without EDC/NHS treatment.

Water contact-angle

Results presented in Table I were the mean of five measurements. Hydrophilicity of the PU and modified PU membranes was evaluated by water contact angle measurement. The contact angle of the modified PU membranes after aminolysis and subsequently immobilized by collagen was smaller than that of the PU membranes, which meant that the hydrophilicity of the modified PU membranes increased by aminolysis or collagen grafting.

The ATDC5 cell proliferation on PU-NH_2 and PU-Col

Figure 11 showed the ATDC5 cell proliferation behavior on PU-NH_2 and PU-Col scaffolds and TCPS. The cells seeded on the scaffolds showed a higher viability compared to the control. However, the viability of ATDC5 on the PU-Col scaffolds was not significantly different from that on the PU-NH_2 scaffolds. The results demonstrated that the PU-NH_2 and PU-Col could support the ATDC5 cells to live and proliferate.

CONCLUSIONS

A novel biodegradable polyurethane was obtained from PCL diols, L-lysine ethyl ester diisocyanate,

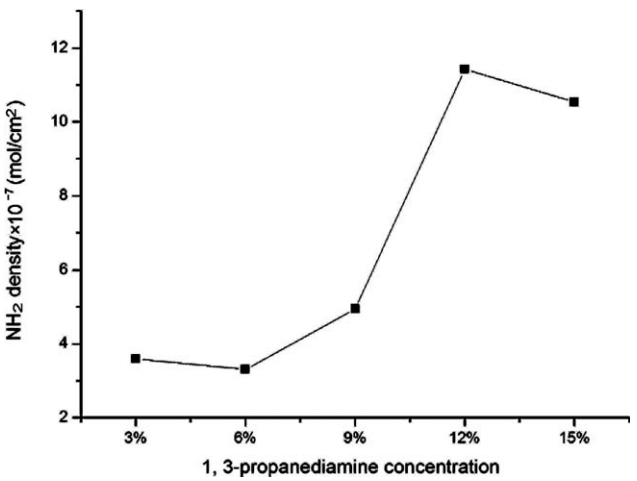


Figure 9 The absorbance at 570 nm of the PU membranes aminolyzed for 1 h treated with ninhydrin as a function of 1,3-propanediamine concentration.

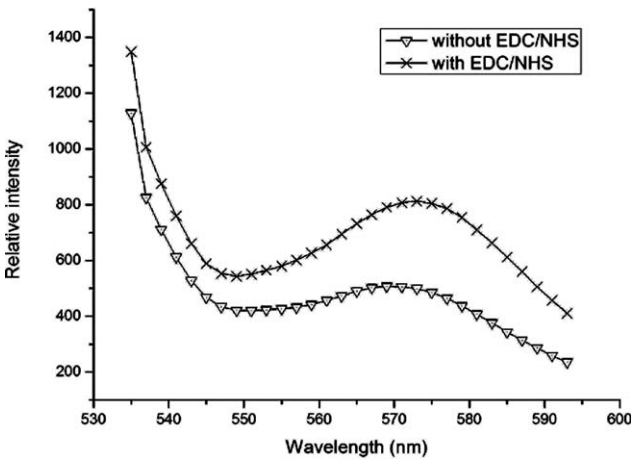


Figure 10 The absorbance at 570 nm of the PU-NH_2 membranes immobilized by Rd-Col with EDC/NHS or without EDC/NHS.

TABLE I
The Water Contact Angle of the Membranes

Samples	Contact angle (°)
PU membranes	85.5
PU-NH_2 membranes	78.8
PU-Col membranes	71.1

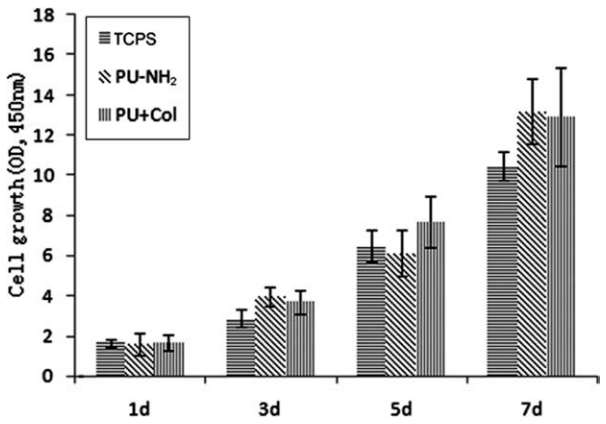


Figure 11 The cell proliferation on PU-NH_2, PU-Col scaffolds as well as on TCPS as control at 1, 3, 5, and 7 days after cell seeding.

and isosorbide in this article. The FTIR and ^{1}H-NMR determination showed that the polymer had the typical PU structures. The GPC result showed the synthesized polyurethane had narrow, unimodal molecular weight distribution with the number average molecular weight 36,800 and a polydispersity of 2.18. The Rhodamine B isothiocyanate (RBITC) fluorescence spectrum showed that both amino groups and type I collagen were successfully introduced on the PU surface. The ninhydrin analysis result displayed that the aminolysis function acted not only on the surface but also in some depth of PU. The surface modification could improve the hydrophilicity of the PU surface. And the cell viability experiment results showed that both the PU-NH_2 and PU-Col had low cytotoxicity and could support the ATDC5 cells to live and proliferate on them. This new coupling method can also be applied to graft polysaccharide such as glucosamine, hyaluronic acid, and chondroitin sulfate to PU surface and enhance polymeric materials biological activity. In addition, the cationic polyurethane with the pendent amino group had potential bio-feature and positive charge which can be developed for nonviral vectors.[28]

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