A novel urethane containing copolymer as a surface modification additive for blood contact materials

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Surface modification to develop a biomolecules-presenting surface is of interest, both from a scientific and an industrial point of view. In this research, a penta-block-coupling polymer of warfarin–PEO–MDI–PEO–warfarin was specially designed as the surface modifying additive (SMA). The warfarin-modified polyurethane surfaces were then prepared by dip-coating method. Attenuated total reflection fourier transform infra-red (ATR-FTIR) spectra revealed that the urethane segments in the SMA could penetrate into the hard block of segmented polyurethane (SPU) via intermolecular hydrogen bonds. The X-ray photoelectron spectroscopy (XPS) results indicated that the intermolecular hydrogen bonds were strong enough to form stable warfarin-PEO composite surfaces in an aqueous environment. Fibrinogen and albumin adsorption onto unmodified and SMA-modified SPU was investigated by the ^{125}I-labeled method. The surface for attracting and reversibly binding albumin, which was proved to suppress the platelet adhesion and prolong the clotting time, has been developed by the simple coating of the novel SMA in SPU.

1. Introduction

Segmented polyurethanes (SPU) are widely used as blood-contacting biomaterials because they exhibit excellent mechanical properties, resistance to biodegradation and favorable biocompatibility. Although their blood compatibility is acceptable for quite a number of situations, they are not adequate for more demanding applications. Besides thrombosis, the most severe complication that can arise from the implantation of SPU is the development of infection. One of the ways to develop anti-thrombogenic and anti-infective materials has been to focus on the design of surfaces to trigger specific adsorption of biological molecules from plasma [1]. According to the albumin passivation theory [2], a confluent layer of conformationally intact albumin will diminish the occurrence of thrombotic, inflammatory and infection. This presents two challenges to the investigator: firstly, albumin generally denatures on contact with foreign surfaces, and secondly, other proteins are also absorbed on the surfaces. To overcome these problems, it was decided to create a surface consisting of linear polyethylene oxide (PEO), which has low binding affinity for all proteins, and on the distal end attach a ligand that has a specific, non-denaturing binding site for albumin [3–8]

Since current biomedical devices generally have adequate mechanical properties and are relatively inexpensive, the inadequate surface properties were prompted by employing chemical, flame, photochemical, and plasma treatments [1, 9]. However, from a processing standpoint, surface modification through a simple coating approach may be more convenient. Typical SPU assumes a so-called ''micro-domain structure'', that is, hard segments interact with each other through hydrogen bonds and aggregate in the domains of the soft segments [10]. The hydrogen bonds between the urethanes in the hard segment domains are quite stable and may provide a bonding site for the urethane-containing materials [8].

In this study, warfarin, a well-known drug with high affinity to albumin [11], was used as a surface modification additive (SMA) by introducing urethane segments via PEO spacers. The urethane segments are expected to act as anchors to the hard segments of the SPU membrane and develop a stable warfarin–PEO composite layer [12, 13]. When the modified layer comes into contact with plasma, it is supposed that it binds the

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endogenous albumin selectively and reversibly. As a consequence, an improvement of the anti-thrombogenic and anti-infection properties is expected to occur.

2. Experiments

2.1. Synthesis and characterization of SMA

2.1.1. Synthesis of reactive tri-segmented block copolymer PEO-Urethane-PEO (EUE)

Tri-segmented block copolymers were prepared by a conventional two-step solution polymerization. Methyl isobutyl ketone (MIBK, Aldrich) was used as solvent and purified by distillation. The poly(ethylene oxide) (PEO, Mn = 1000, Aldrich) was dehydrated by azeotropic distillation with MIBK. 1,4-butanediol (BD) was used as a chain extender. It was refluxed with calcium hydride and distilled. The di-isocyanate employed was tolylene 2,4 di-isocyanate (TDI).

In the pre-polymer reaction, 0.01 mol of BD was made to react with 0.02 mol of TDI at 50 °C for 5 h in MIBK. The pre-polymer was then added to the 0.02 mol PEO solution in MIBK and reacted at 80 °C until the infrared spectra indicated that the di-isocyanate peak (2260 cm^{-1}) had disappeared. The entire synthesis was performed under a continuous purge of dried nitrogen. The tri-segmented block copolymer was precipitated by pouring the polymerization solution into heptanes and drying in a vacuum (lower than 1 bar) for 24 h at 80 °C.

2.1.2. Immobilized warfarin onto EUE

0.005 mol EUE was dissolved in 1,4 dioxane (Aldrich) and dehydrated by azeotropic distillation. After approximately 10 ml had been distilled at the dry dioxane temperature 0.01 mol TDI was added into the solution and made to react at reflux temperature for 8 h. Then 0.308 g warfarin in 20 ml dry dioxane was added into the reaction system and made to react at reflux temperature until the infrared spectra indicated that the di-isocyanate peak (2260 cm^{-1}) had disappeared. The entire synthesis was performed under a continuous purge of dried nitrogen. The penta-segmented block copolymer, warfarin–PEO–Urethane–PEO–warfarin (WEUEW), was precipitated by pouring the polymerization solution into heptane and drying in a vacuum (lower than 1 bar) for 24 h at 80 °C. The structure of WEUEW was confirmed by ^{1}H-NMR (BRUKER 200M), FT-IR (Perkin Elmer system 2000 spectrometer) and UV spectrometry (Shimadzu UV 1201). ^{1}H–NMR ($CDCl_3$, chemical shifts, δ, in ppm): 3.40 ∼ 3.64 (–CH_2CH_2O–), 7.02–7.38 (H in aromatic ring); FT-IR wave numbers (cm^{-1}): 1100 (—C—O—C—), 1100–1260 (—COO—), 1571 (—NH—COO—), 1620 (aromatic ring), 1720(C═O), 2800–3000 (CH_2, CH_3), 3250–3350 and (—NHCOO—).

2.2. Film preparation and surface analysis

The polyurethane materials in our study originated from pellets of a commercially available biomedical grade poly(etherurethane). Pellethane 2363-80AE (PEL, DOW chemical). The material was prepared as a 10% (w/v) solution of PEL in tetrahydrofuran (THF). To the solutions of PEL were added 20% (w/w based on dry polymer) of SMA (PEO-1000, stearyl poly(ethylene oxide) (SPEO) 1000, and WEUEW). The films of the different polymers were prepared by solvent casting from the THF solutions onto clean Petri dishes. The films were cast in two layers with evaporation of the solvent between each casting (24 h at room temperature). The membranes were then immersed in distilled water for one day or seven days. Then the membranes were washed three times in ethanol and dried for 24 h in a vacuum (lower than 1 bar) at room temperature.

2.2.1. Attenuated total reflectance (ATR) infrared spectra

Attenuated total reflectance (ATR) infrared spectra were obtained with a Perkin Elmer system 2000 spectrometer using a germanium ATR element (3 × 10 × 50 mm, 45° aperture) supported on a variable ATR accessory (SPECAC P/N 11080). The sample was placed against the ATR element, and the spectra were collected in the range 1200–4000 cm^{-1} with a resolution of 1 cm^{-1}.

2.2.2. X-ray photoelectron spectroscopy

The chemical compositions of the surfaces were analyzed with an ESC Alab 200A spectrometer. The excitation X-ray source was Mg–K_α. High-resolution spectra of C_{1s}, N_{1s} and O_{1s} were run. The different band areas were normalized using Schofield section factors, $C_{1s} = 1.00$, $N_{1s} = 1.770$, and $O_{1s} = 2.85$. The C_{1s} peak was resolved into its constituent peaks by computer simulation.

2.3. Protein adsorption

Human proteins were obtained commercially. Human serum albumin was from Sigma and was 97% electrophoretically pure. Human fibrinogen was from Sigma and was 95% clottable.

Albumin and fibrinogen were labeled by iodine-125 using the iodine monochloride technique as described by Brash [14]. Experiments were conducted to verify that no dependence of the measured surface concentration on per cent labeled protein over a range of 1–20% existed. Amounts of ^{125}I-labeled protein and unlabeled protein were added to a tris-HCl (0.05 mol L, pH 7.4) buffer to give mixtures containing 5% labeled and 95% unlabeled protein.

Protein adsorption experiments utilized surfaces in the form of tubing segments of internal diameter in the range of 2–3 mm. The surfaces for protein adsorption were prepared by solution coating from a 5% w/v solution of the polymer (PEL or WEUEW/PEL = 1/5 in w/w) in THF on the inside of PVC medical grade tubes (diameter 3 mm, Pronefro Company, Porto). All surfaces were dried overnight in a vacuum (lower than 1 bar) at room temperature and were observed through an optical microscope to confirm that no flaw existed at the polymer membranes. All surfaces were equilibrated with a tris–HCl buffer for 24 h prior to measuring protein adsorption. The tubes were filled initially with a tris–HCl

buffer, which was subsequently displaced by a volume of protein solution that was 10 times the volume of tris–HCl, to guarantee that no air–solution interface made contact with the test surface. After the appropriate contact time, the solution was similarly displaced by a volume of buffer 30 times the original volume. All adsorptions were carried out under static conditions at 25 °C. Surface radioactivity was measured in a γ-counter and converted to mass of protein per cm^2 by comparison with an aliquot protein solution.

At least five experiments were conducted for each set of conditions. Standard deviations of measurements at steady state ranged from ± 10% of the average at low surface concentration, to ± 5% of the average at high surface concentration (indicated in more detail in Figs 7 and 8).

2.4. *In vitro* hemo-compatibility test

2.4.1. Platelet adhesion experiment

The platelet reactivity of the materials was evaluated from the number of platelets and the total area of adherent platelets on the surface. Human platelet rich plasma (HPRP) was prepared from fresh human citrated blood. The PEL and SMA modified PEL films (15 × 15 mm) whose surfaces were cleaned and equilibrated with phosphate buffer solution (PBS, pH 7.2, I = 1.0, seven days) were incubated with HPRP for 1 h at 37 °C under static conditions. The surfaces of the samples were rinsed with PBS under gentle agitation to remove weakly adhered platelets. The platelets on the surface were fixed with 1.5 wt % glutaraldehyde solution and dehydrated by ethanol gradient dehydration (30%, 50%, 70%, 80%, 90%, and 100% ethanol in water). The morphology of the adherent platelets was observed using scanning electron microscopy (SEM) (JEOL, JSM-35C) following gold sputtering on the membrane surface. Each material was measured five times for reproducibility. The number and total area of adherent platelets was estimated by SEM images using the imaging analysis software developed by CEMUP (Centro Materials da University do Porto). Sixteen Different fields $(0.25 \times 0.16\,mm^2)$ were randomly counted for each sample.

2.4.2. Static clotting time

Clean glass test tubes (10 × 70 mm) were coated with 5% w/v solutions of PEL. SPEO/PEL = 1/5 (w/w) and WEUEW/PEL = 1/5 (w/w). The coatings were dried at atmospheric pressure for 2 h at room temperature and in a vacuum (lower than 1 bar) at 60 °C overnight. To each tube whose surface was equilibrated with PBS, 0.25 ml of plasma was added, followed by 0.04 ml of 0.1 M $CaCl_2$ solutions. The time for formation of a dense clot was measured. The tests were performed at 37 °C under agitation and a minimum of ten measurements was used to calculate the average clot time.

3. Results and discussion

3.1. Characterization of SMA

Tri-segmented block copolymers, PEO–Urethane–PEO (EUE), were prepared by a conventional two-step solution polymerization. The warfarin was attached to the end of the PEO chains by a TDI reaction (Fig. 1). The chemical structure of warfarin-coupled PEO–Urethane–

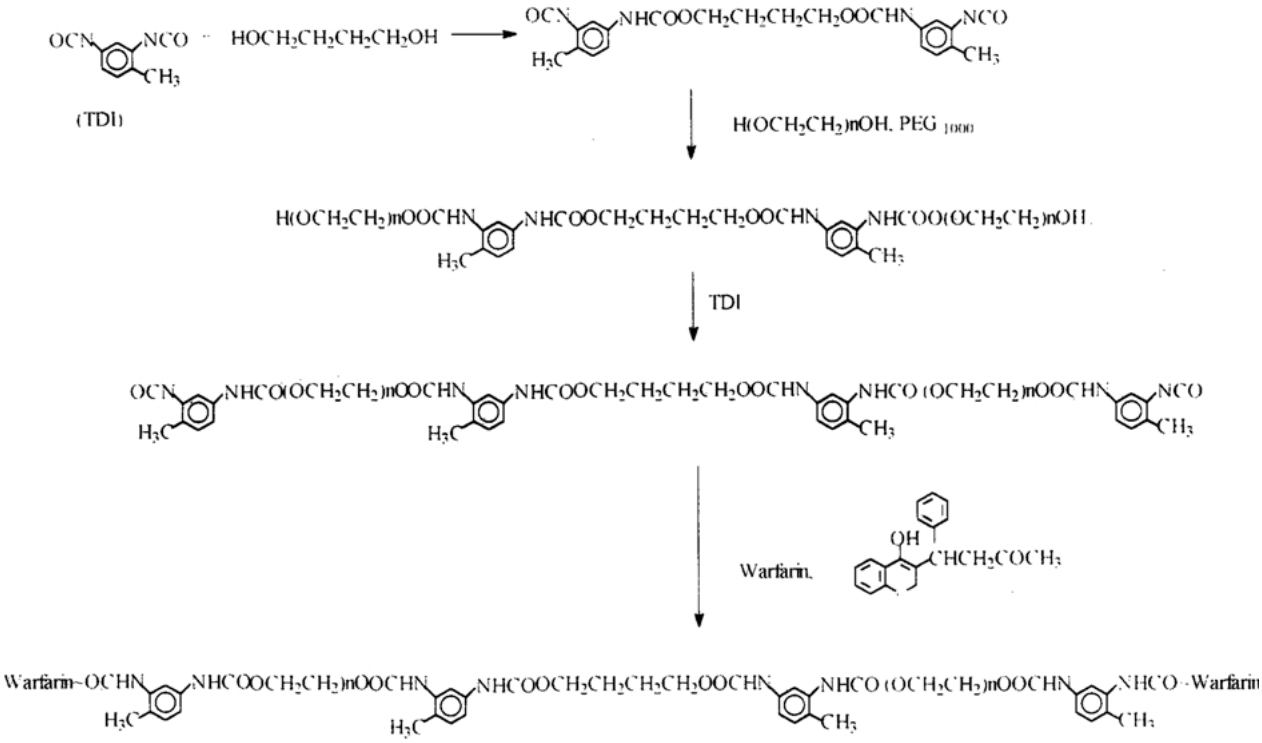

Figure 1 Synthetic route of warfarin–PEO–Urethane–PEO–warfarin.

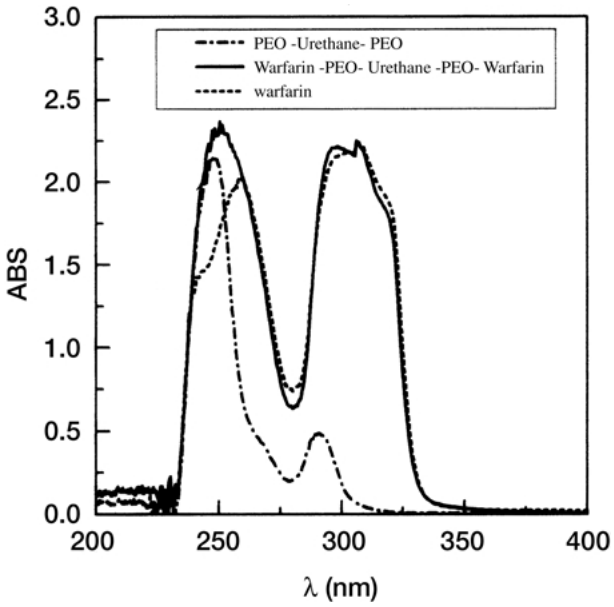

Figure 2 UV spectra of EUE, warfarin, and WEUEW.

PEO (WEUEW) was confirmed by FT-IR and $^{1}H-NMR$ (see details in the Experimental section).

UV spectrometry was utilized to determine the bulk composition of the copolymer. As shown in Fig. 2, the UV spectrum of EUE has no adsorption at 307 nm, whereas the peak of WEUEW at 307 nm corresponds well with that of pure warfarin. Two calibration curves relating absorbance at 307 nm to warfarin concentration were established using a set of warfarin solutions of varying concentration, with or without the presence of EUE.

The results (not presented here) show that the presence of EUE interferes with the quantitative measurement of warfarin content. In order to eliminate the unstructured background, the first derivative UV spectra [15] of warfarin, WEUEW, and of the blend of warfarin in EUE, were constructed. It was found that the undesirable interference mentioned above could be completely eliminated by this treatment and the warfarin concentration in WEUEW was determined as 18 wt %.

3.2. Surface analysis

As reported by Okkema *et al*. [16], the assignment of infrared H-bonding band in polyurethane is mainly performed in the —CO— stretching regions. The band of free carbonyl groups. —CO— (a), was approximately at 1730 cm^{-1} and the band of H-bonded (with —NH—) carbonyl groups, —CO— (b), was approximately at 1700 cm^{-1}.

As shown in Fig. 3, the WEUEW coating layer presents a single peak at 1732 cm^{-1}, indicating that most of the carbonyl groups of WEUEW were —CO— (a) and few H-bonds were formed in the pure SMA membrane. Both the original PEL membrane and the SMA-modified PEL membrane show two peaks at 1700 cm^{-1} and 1732 cm^{-1}.

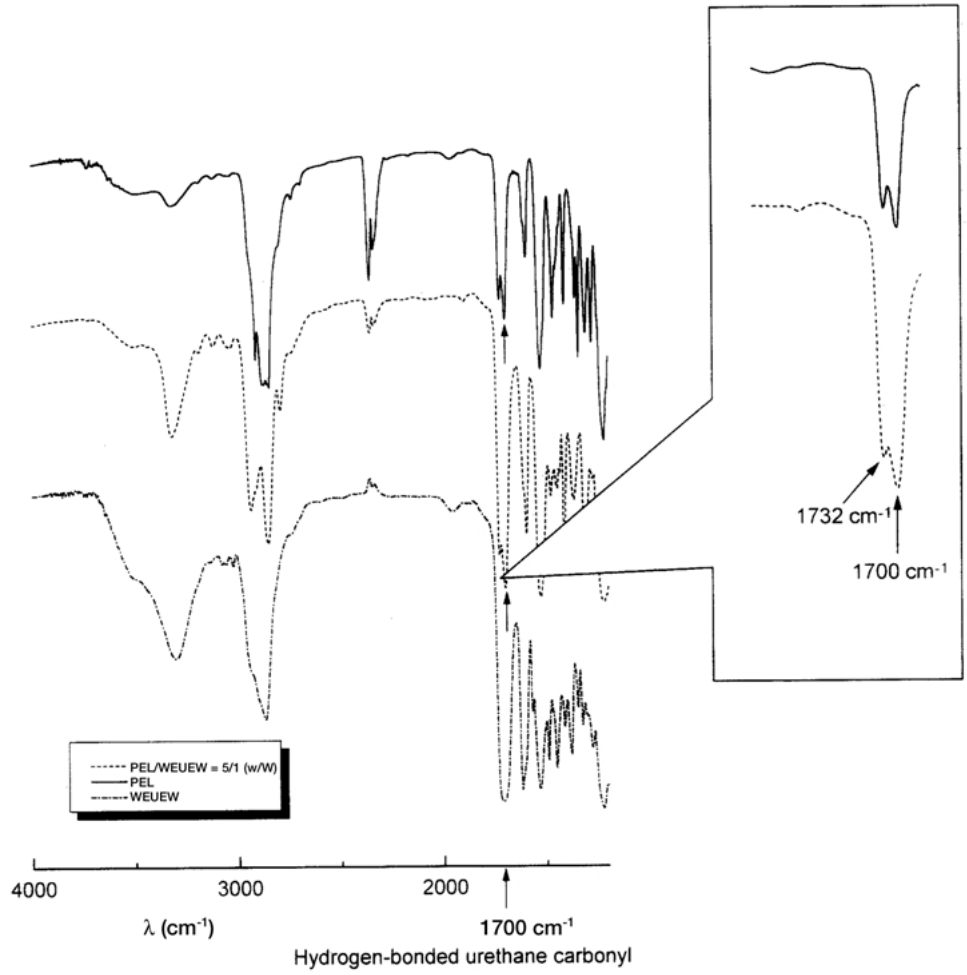

Figure 3 ATR-FTIR spectra of PEL, WEUEW, and WEUEW/PU = 1/5 in w/w.

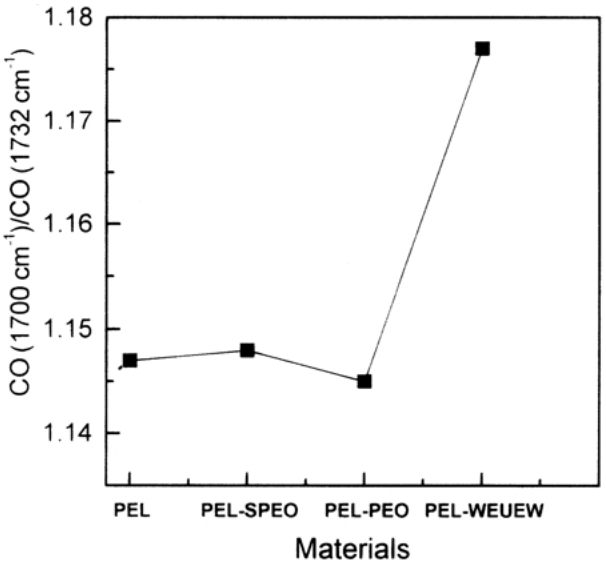

Figure 4 ATR-FTIR data ratios of the H-bonded carbonyl groups. —CO— (1700 cm^{-1}) and the free carbonyl groups —CO— (1732 cm^{-1}). The samples are PEL, PEO-1000/PEL = 1/5 and WEUEW/PEL = 1/5 in w/w.

As shown in Fig. 4, the ratio of —CO— (b)/—CO— (a) in PEO- and SPEO-modified PEL is almost the same as that in pure PEL. PEO does not contribute to the formation of hydrogen bonds. It is of interest to observe that the ratio —CO— (b)/—CO— (a) increases dramatically in the WEUEW-modified PEL membrane. Thus the conclusion can be deduced: in the surface layer of WEUEW-modified PEL membrane, the interaction between the SMA–WEUEW and the matrix-PEL was based on the H-bonds between the U-blocks of WEUEW and the hard blocks of PEU chains, which could be described in Fig. 5.

As the WEUEW-modified PEL membranes have been immersed in water for one week prior to being investigated by ATR-FTIR, one can expect that the stable warfarin/PEO composite surfaces have been obtained by penetrating the aromatic urethane segments of WEUEW into the hard segment of polyurethane.

The surface chemical composition of the membrane was determined by XPS. Representative XPS spectra of PEL and PEL modified by stearyl poly(ethyl oxide) (SPEO) and WEUEW are shown in Fig. 6. They were respectively C—C (aromatic) at 284.64 eV. C—C— (aliphatic) at 285.0 eV, C—N at 285.75 eV, C—O at 286.61 eV and C═O at 289.60 eV.

Since the hard segment ether carbon accounts for a very low percentage of the ether carbon in the polymer, the ratio of ether carbon to total carbon (C—O/C) provides a relative measurement of soft segment and PEO at the surface. As shown in Table I and Fig. 6, the C—O/C value increases significantly at the SPEO-modified surface. However, after immersion in water for one week, the C—O/C value decreases significantly and is almost the same as the PEL membrane. By contrast, the C—O/C value of the WEUEW-modified PEL membrane does not change significantly. A high value of C—O/C of 51.46% was obtained at WEUEU-modified PEL membrane.

It is well known that in order to obtain low interfacial energy, the hydrophilic component of a polymer will migrate to the polymer/water interface when the polymer is immersed in water. The results reported here show that the elution of SPEO occurred easily when the SPEO-modified membrane was immersed in water. However, the urethane segments of WEUEW are expected to act as anchors to the hard segments of PEL membrane. The intermolecular hydrogen bonds are strong enough to form a stable WEUEW-modified surface at the polymer/water interface.

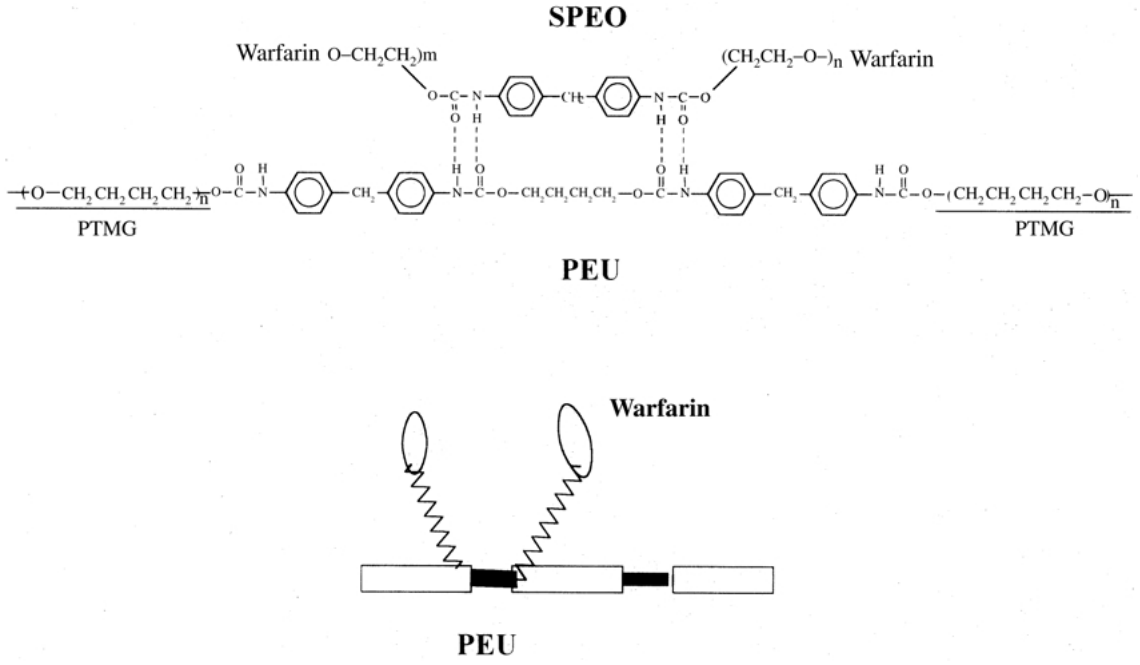

Figure 5 Incorporation of the U-block of WEUEW with hard block of PEU by the linkage of hydrogen binding.

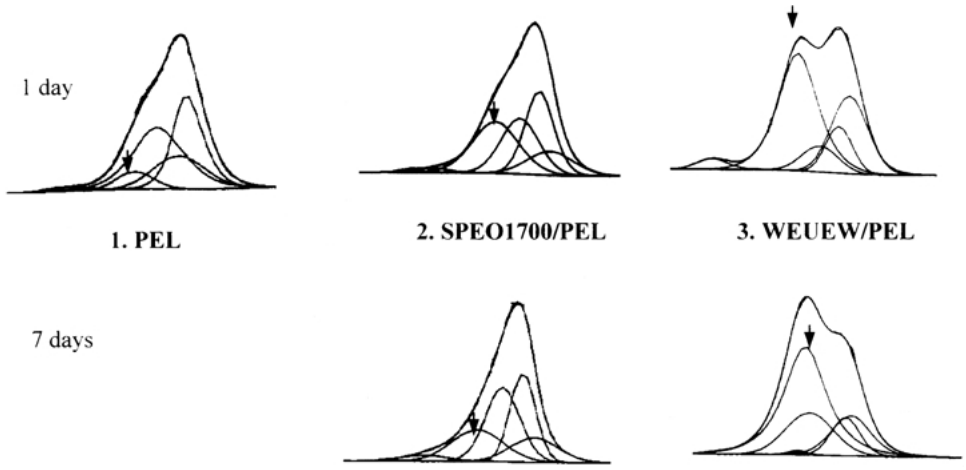

Figure 6 XPS spectrum of C_{1s} of (1) PEL (2) SPEO1700/PEL = 1/5 (w/w) 3) WEUEW/PEL = 1/5 (w/w) membranes after immersion in water for one day or one week.

TABLE I Surface chemical composition of PEL and SMA modified PEL membrane

	C–O/C($\times 10^{-2}$)		N/C($\times 10^{-2}$)[2]	C = O/C($\times 10^{-2}$)[2]
	One day[1]	Seven days[2]		
PEL	7.24	7.24	3.18	1.29
PEL–SPEO[3]	20.7	8.01	—	—
PEL–WEUEW[4]	48.27	51.46	4.92	3.17

[1]Data for the sample immersion in water for one day.
[2]Data for the sample immersion in water for seven days.
[3]The blend coating of PEL and SPEO (5/1(w/w)).
[4]The blend coating of PEL and wafarin-PEO–Urethane–PEO–warfarin (5/1(w/W)).

3.3. Selectivity and reversibility of albumin binding

The protein adsorption onto unmodified and SMA-modified SPU was determined by using mixtures of ^{125}I-labeled albumin/fibrinogen (or ^{125}I-labeled fibrinogen/albumin). Fibrinogen was used as a competitive adsorption protein because it is known to be readily adsorbed onto implant surfaces and because surfaces that avidly adsorb fibrinogen may be highly thrombogenic.

As shown in Fig. 7(a), WEUEW imparts significant resistance to both albumin and fibrinogen adsorption. It is of interest that the modified surfaces resist fibrinogen adsorption much more effectively than albumin adsorption. The ratio of adsorbed albumin to fibrinogen Fig. 7(b) can be used to indicate the preference for protein adsorption. The WEUEW-modified PEL was found to improve the albumin selectivity significantly.

Reversibility of bound albumin was estimated by

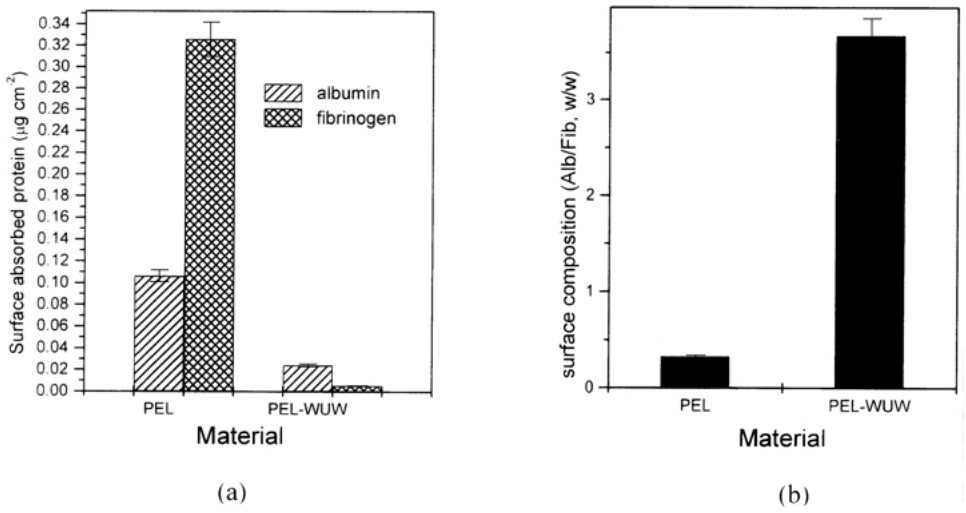

Figure 7 Protein competitive adsorption from a multi-component buffer. Samples were exposed to an ^{125}I-labeled albumin/fibrinogen or ^{125}I-labeled fibrinogen/albumin mixture (fibrinogen 0.03 mg ml^{-1}, albumin 0.4 mg ml^{-1}, 1% concentration of fibrinogen and albumin in plasma) for 2 h at 25 °C. The samples were then washed by a 30 times volume buffer and counted in a γ-counter. Each point represents the average of five experiments with standard deviation from 5.0% to 7.0% within each set.

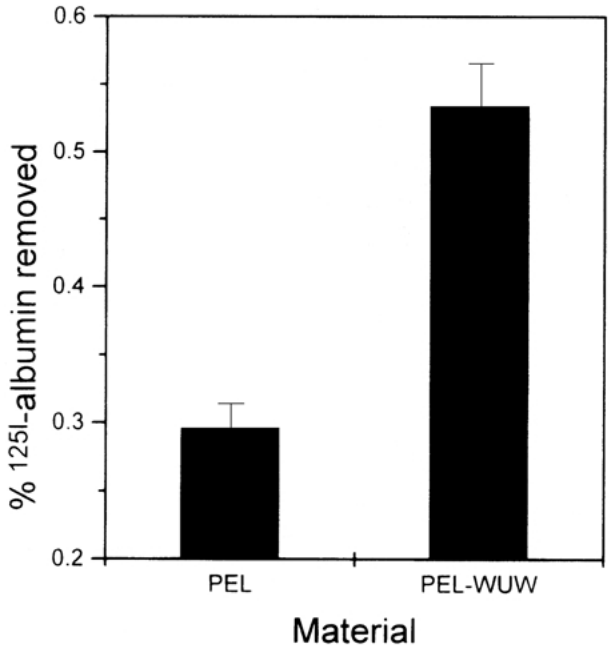

Figure 8 Competitive displacement of pre-adsorbed ^{125}I-albumin by unlabeled albumin. Samples were exposed to ^{125}I-labeled albumin (0.4 mg ml^{-1}, 4 h at 25 °C) and then displaced by a 30 times volume buffer. Next, the unlabeled albumin solution (0.4 mg ml^{-1}) was injected into the tube bearing pre-adsorbed ^{125}I-labeled albumin for 4 h at 25 °C and then washed by 30 times volume buffer. Each point represents the average of five experiments with standard deviation from 5.0% to 7.0% within each set.

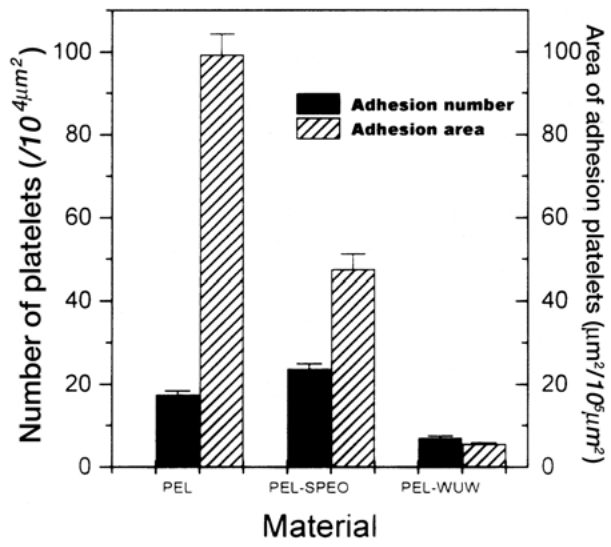

Figure 9 The adhesion number and total area of adherent platelet on PEL. SPEO1700/PEL = 1/5 (in w/w) and WEUEW/PEL = 1/5 (in w/w).

measuring the extent of competitive elution of the pre-bound ^{125}I-labeled albumin by the unlabeled albumin. As shown in Fig. 8 more than 50% of albumin adsorbed on the WEUEW-modified PEL was displaceable by unlabeled albumin, whereas less than 30% of albumin bound to PEL could be removed.

3.4. *In vitro* hemo-compatibility

The platelet reactivity of unmodified and SMA-modified PEL was evaluated here on the basis of the number and

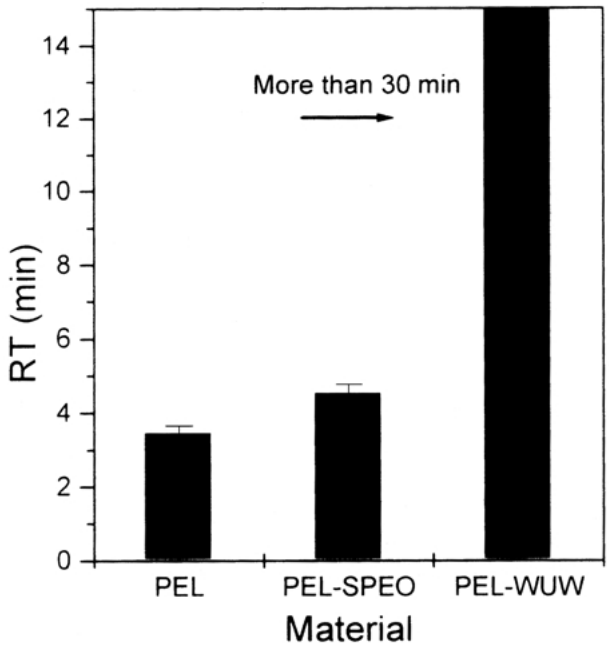

Figure 10 The clotting time of PEL and SPEO1700/PEL = 1/5 (in w/w) and WEUEW/PEL = 1/5 (in w/w).

total area of adherent platelets. As reported in previous studies [17], the deformation of adherent platelets proceeds in the order of (I) attachment of cells at a point of contact with the substratum; (II) centrifugal growth of filopodia; and (III) cytoplasmic webbing and flattening of the central mass, so that ultimately thrombi are formed. If the interaction between blood platelets and the substratum is very weak, the surface presents low adhesion numbers and the adherent platelets maintain their discoidal shape with low spreading area.

Both PEL- and SPEO-modified PEL membranes (Fig. 9) adsorb a high number of platelets. The number of platelets adhering to SPEO-modified PEL is even higher than that to PEL. This is not surprising if we examine the present data with respect to the stability of SPEO-modified PEL. As shown by XPS (Table I), the SPEO easily eluted from the surface when the SPEO-modified PEL was immersed in water. The surface composition of SPEO-modified PEL is almost the same as PEL after seven days in contact with water. Furthermore, the elution of SPEO from the membrane was found to increase the surface roughness, which will induce platelet adhesion.

WEUEW-modified PEL membranes are found to suppress the platelet adhesion (Fig. 9). In addition, the total area of binding platelets is quite low, which indicates that the shape change in adherent platelets was also suppressed. The WEUEW-modified surfaces represent low platelet activity. Furthermore, the stable coating prolongs the clotting times dramatically. It is an exciting observation that no clots form at the WEUEW-modified PEL in 30 min, whereas the clotting time of the original PEL membrane is only 3.5 min (Fig. 10).

As was described above, a novel surface modification additive, warfarin-coupled PEO–Urethane–PEO, was specifically designed to obtain a surface capable of triggering albumin adsorption. Through a simple coating process, a stable composite layer of PEO and warfarin

was obtained due to the hydrogen bond between the urethane segments in the additive and the hard block of PEL. The composite coating showed enough stability in aqueous medium and attracted a layer of renewable albumin. As a result, it inhibited platelet adhesion and prolonged the clotting time. The simple coating approach may be of great interest, especially from a technological point of view, for producing complex three-dimensional medical devices.

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