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Effect of chain density and conformation on protein adsorption at PEG-grafted polyurethane surfaces

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Abstract

Polyurethanes were modified using monobenzyloxy polyethylene glycol (BPEG) which possesses a bulky hydrophobic benzyloxy group at one end and a hydroxyl group at the other end as a preconstructed BPEG layer, and poly(ethylene glycol) (PEG) and monomethoxyl poly(ethylene glycol) (MPEG) with various chain lengths as fillers. Our objective was to investigate the effect of PEG graft density and conformation on protein adsorption at PEGlated surface. The graft density was estimated by a chemical titration method. The combination of ATR-FTIR, AFM and titration results provide evidences that the graft density can be increased by backfilling PEG or MPEG to a BPEG layer. However, fibrinogen and albumin adsorption significantly increased on all surfaces after PEG or MPEG backfilling. We conclude that the conformation of hydrophobic benzyloxy end groups of the BPEG layer plays a key role. The benzyloxy end groups of preconstructed PEG chains stretch to the surface after PEG backfilling, which possibly accounts for the observed increase in protein adsorption. The BPEG conformation change after backfilling with PEG or MPEG was also suggested by contact angles. Additionally, protein adsorption was slightly influenced by the length of filler, suggesting a change in surface morphology.

Keywords: Graft density; PEG; Chain conformation; Protein adsorption; End groups

1. Introduction

Protein adsorption from a biological environment onto synthetic biomaterials may induce undesirable reactions in the body to the foreign materials, such as immune responses, blood coagulation, or bacterial adhesion [1,2]. To reduce foreign body responses, much attention has been paid on producing a nonspecific protein repelling surface by surface modification methods. A preferred strategy for blocking the adsorption of proteins is to immobilize hydrophilic polymers that shield the surface, thus introducing a high activation barrier to repel proteins [3]. One of the most effective approaches is to tether poly(ethylene glycol) to the surface [4,5].

Much theoretical work was generated to explain the early discovery that grafted PEG chains resist protein adsorption to a high degree. Excellent reviews and articles on this subject have appeared elsewhere [6–11]. However, each model is an incomplete description of a more complex reality since the adsorption mechanism is determined by many parameters, such as the size of the adsorbing protein, chain density, chain length, chain–protein interactions, protein–surface interactions and so on. It is believed that graft density is one of the most important parameters to protein repulsion [12,13]. SPR [14], XPS [6], and ellipsometry [13,15] were used to determine chain density on gold and silicon surfaces. Only a few studies on PEG chain density on a polymer surface have been reported in the literature mainly due to the difficulty of controlling and determining surface density of PEG on polymer surfaces.

Brash et al. grafted hydroxyl terminated-MPEG chains varying from 3 to 113 ethylene oxide units on the polyurethane surface to investigate the effect of chain length and surface density of PEG on protein adsorption. The graft density was assessed

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by XPS analysis [16]. Similarly, amine terminated-MPEG was used to modify polyurethane (PU) [17], and an increase in graft density on the resulting surface was shown by both XPS analysis and protein adsorption, compared to that modified by hydroxyl terminated MPEG. They suggested that increasing the density and chain length (up to mw 2000) of grafted PEG increased the protein repulsion properties of the PEGlated polyurethane surface. Since the above researches were conducted using PEGs without bulky hydrophobic end groups, the influence of chain conformation on protein adsorption was too small to observe.

In the work reported here, preconstructed benzyloxy terminated-PEGs layers were backfilled with various PEGs or MPEGs to create a mixed PEG layer with varying density and morphology on polyurethane for the purpose of studying protein resistance. The graft density of various surfaces was compared using a titration method, ATR-FTIR and AFM. Protein resistance by the modified surfaces was investigated by using radiolabelled fibrinogen and albumin as model proteins.

2. Materials and methods

2.1. Reagents and characterization methods

Poly(ethylene glycol)s (PEG, Mn = 400, 600, 2000) were obtained from Shanghai Chemical Reagent Co. Ltd. Monobenzyloxy poly(ethylene glycol) (BPEG, Mn = 700) was a gift from Nanjing Jutian Chemical Company. Monomethoxyl poly(ethylene glycol)s (MPEG, Mn = 350, 750, 2000) were purchased from Aldrich Chemical Co. All above reagents were dried before use. 4,4′-methylene-bis-(phenyl-isocyanate) purchased from Aldrich Chemical Co. and triethylamine from Shanghai Chemical Reagent Co. Ltd, were used without further purification. Pellets of polyurethane (PU) were obtained from Shanghai Pepson Polyurethane Co. Ltd. All solvents including toluene, isopropanol and dimethyl formamide (DMF), were purchased from Tianjin Bodi Chemical Co. Ltd., and thoroughly dried before use using standard methods. Fibrinogen was obtained as a solid lyophilized powder from Calbiochem (La Jolla, USA). Human serum albumin (HSA) was obtained from Sigma (St. Louis, USA).

2.1.1. Contact angle measurements

Advancing sessile drop water contact angles were measured on modified and unmodified surfaces using JJC-1 static contact angle equipment (Changchun No. 5 Optical Instrument Co. Ltd.). Milli-Q water was used with a drop volume of approximately 0.02 ml. Results are presented as an average of eight measurements on at least three different surfaces.

2.1.2. Attenuated total reflection-IR (ATR-IR)

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) measurements were carried out on a Nicolet Nexus FT-IR (Nicolet Instruments, USA) equipped with an ATR accessory MUP with GeS crystal. Sixty-four scans were collected for each sample.

2.1.3. AFM

A Digital Instruments Nanoscope IV (Veeco) atomic force microscope (AFM) was used to obtain images of the different surfaces in tapping mode.

2.2. Preparation of PEG-grafting polyurethane surfaces

2.2.1. Preparation of polyurethane films and surface functionalization

Commercially available polyurethanes were extracted by Soxhlet extraction for 24 h using toluene as the solvent to remove impurities. PU films were cast from a 5% (w/v) solution in DMF in glass dishes. Solvent was removed by drying at 65 °C for at least 48 h followed by 48 h drying under vacuum. The PU films were then punched into discs, which are approximately 5 mm in diameter and 0.5 mm thick.

To functionalize the PU surface, PU discs were immersed in a toluene solution containing 7.5% (w/v) MDI and 2.5% (w/v) triethylamine. After stirring at 50 °C for 100 min under N_2 protection, the PU surfaces were rinsed with toluene.

2.2.2. Grafting mixture of PEGs on the polyurethane surface

A two-step surface modification reaction, based on previously published protocols [17,18], was used to graft mixtures of PEG to the PU substrate. Briefly, the films were incubated in a toluene solution containing monobenzyloxy polyethylene glycol for 12 h. After rinsing with toluene, PEG or MPEG chains were added to the pre-grafted BPEG surface to increase the surface density of PEG. The BPEG-modified surface was then added to a stirred toluene solution containing PEG (Mn = 400, 600, 2000) or monomethoxyl-PEG (Mn = 350, 750, 2000) at 40 °C for another 12 h.

After the reaction, the films were washed thoroughly with toluene to remove unreacted PEG or MPEG. The films were characterized after drying under vacuum at 50 °C for 48 h. All reagents were dried extensively to avoid the reaction between MDI and traces of water.

2.2.3. The determination of NCO reduction on the surface

The polyurethane films modified by MDI, BPEG, BPEG/MPEG350, and BPEG/PEG400 were immersed in a toluene solution containing *N*-butyl-1-butanamine for 20 min respectivelly. Isopropanol was then added, and the resulting solution was titrated with hydrochloric acid using bromocresol green as the indicator. Each measurement was done in triplicate. The NCO content on the surfaces was calculated from the volume of hydrochloric acid used.

2.3. Protein adsorption

Fibrinogen and human serum albumin were labeled with ^{125}I (Isotope Company of China, Beijing, PR China) using the ICl method [19]. Labeled proteins were dialysed against Tris buffered saline TBS (pH 7.4) to remove any free iodide. For studies of protein adsorption from buffer, labeled proteins were mixed with unlabeled proteins at a 1:19 ratio (labeled:unlabeled

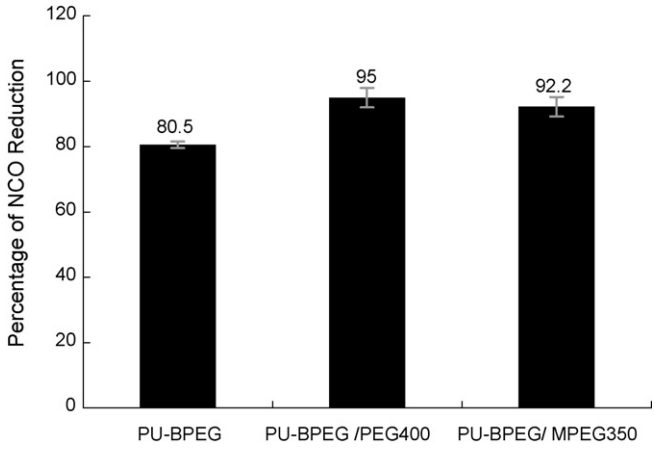


Fig. 1. The percentage reduction of NCO on the modified PU surface.

protein) to give a final concentration of 1 mg/ml. Serial dilutions were done in TBS (50 mM, pH 7.4) for other protein concentrations tested. Surfaces were then incubated in protein solutions for 2 h at room temperature, rinsed three times in TBS (10 min each), wicked onto filter paper, and then transferred to clean tubes for radioactivity measurement by gamma counting (Perkin-Elmer 1480, USA).

3. Results

3.1. Surface preparation and graft density determination

To form benzyloxy PEG-grafted polyurethane surfaces, the PU base was initially activated by diisocyanate, and then modified by the addition of monobenzyloxy PEG. The bulky benzyloxy end group on PEG allows us to control the graft density of PEG on the surface by steric interactions. A chemical titration method was employed here to compare PEG graft density on the modified PU surfaces. Briefly, quantitative imine was added to the system and consumed by NCO on the surface. The unreacted imine was determined by HCl titration, giving the amount of NCO bound to the surface. The PU–MDI surface was used as a standard to calculate the percentage reduction of NCO. The results are summarized in Fig. 1. The percentage reduction of NCO on the PU surface was 80% after grafting BPEG to the MDI-modified surface, and increased above 90% after further grafting with MPEG350 or PEG400.

3.2. Surface characterization

3.2.1. ATR-FTIR

ATR-FTIR spectra of PU–MDI, PU–BPEG, PU–BPEG/MPEG350 surfaces, as well as the unmodified surface are shown in Fig. 2. The most significant difference among these surfaces is the appearance of a relatively strong new band of –NCO stretching vibration at 2283 cm^{-1} on the PU–MDI surface, which becomes very weak on PU–BPEG surface and is not present on either the unmodified surface or PU–BPEG/MPEG350 surface. Following modification by PEG, there appears a broader CH_2 stretching vibration band around 2870 cm^{-1}, corresponding to the CH_2–O repeat unit in PU–BPEG and PU–BPEG/MPEG350 [20] though other bands of PEG are overlapped with features of the PU substrate. The above results suggest that isocyanate groups are formed on the polyurethane surface, which is subsequently modified by covalent grafting of monobenzyloxy PEG and then MPEG350. The weak band presented on the PU–BPEG surface almost disappeared completely after backfilling with MPEG350, a finding consistent with the titration results.

3.2.2. Water contact angle

Sessile water contact angles were measured on the control PU, BPEG-modified PU and various PEG combinations (Fig. 3). Unmodified PU showed characteristically high sessile water contact angles (78°), as expected, while PU–BPEG showed a lower water contact angle of 60°. It is interesting to note that the different PEG combinations of grafted surfaces had similarly high water contact angles compared to the unmodified PU sur-

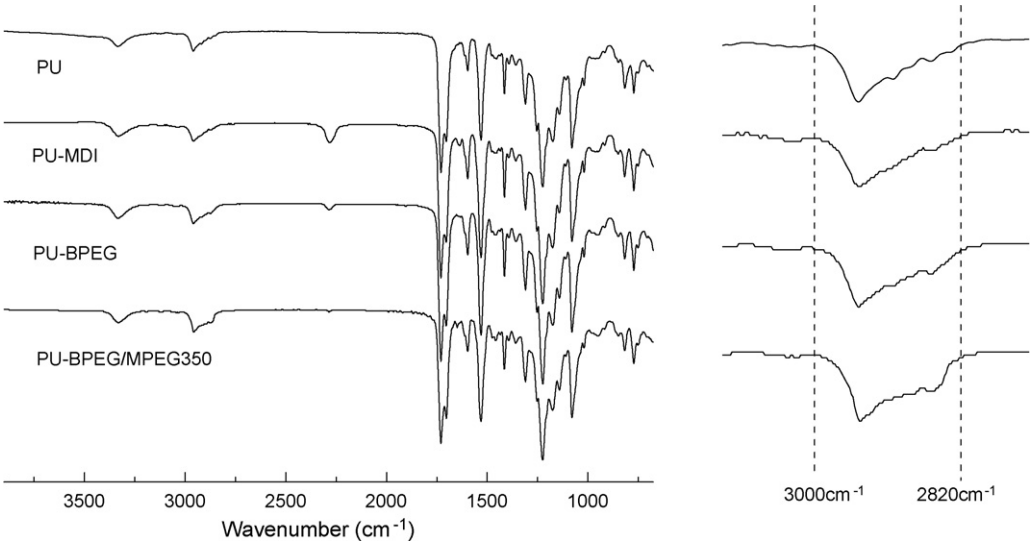


Fig. 2. ATR-FTIR spectra for the control PU, MDI-, BPEG-, and BPEG/MPEO-modified surfaces.

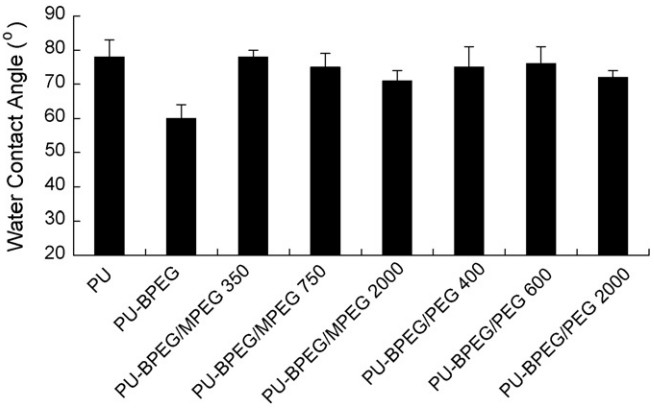


Fig. 3. Data of Water Contact Angle Measurements.

face. This suggests that the various backfilled PEG-modified PU surfaces were more hydrophobic than the sole BPEG-modified PU surface. This result is contrary to previous studies in which the hydrophilicity of PEG-modified surfaces increased with increasing graft density.

3.2.3. Atomic force microscopy (AFM)

The surface topographies of the control, BPEG- and BPEG/PEG400-grafted surfaces were examined by AFM. As shown in Fig. 4A, the control surface was relatively smooth, with a mean roughness of 3.3 nm. After modified by MDI, the roughness was slightly increased to 10.4 nm as shown in Fig. 4B. The BPEG-grafted surface (Fig. 4C) had a significantly increased roughness value of 34.7 nm, accompanied by the formation of domains, probably due to the aggregation of BPEG on the surface. However, the BPEG/PEG400-grafted surface (Fig. 4D) showed a decrease in roughness to 22.4 nm, most likely the result of backfilling the BPEG layer with PEG400.

3.3. Protein adsorption

Figs. 5 and 6 show isotherms for the adsorption of fibrinogen and albumin to the control PU, BPEG-grafted and other surfaces backfilled with PEG or MPEG of varying chain length. It is clear that specific surface differences strongly influence the adsorption of both proteins studied. All PEG-modified surfaces showed some decrease in fibrinogen and albumin adsorption compared to the control surface, and the sole BPEG-modified surface had the lowest adsorption value. As shown in Fig. 5A, at a fibrinogen concentration of 0.01 mg/ml, fibrinogen adsorption decreased from 370 ng/cm^2 on the control PU surface to 78 ng/cm^2 on the BPEG-modified surface, a reduction of approximately 80%. Other modified surfaces showed smaller reductions. Increased protein adsorption was observed on all the modified surfaces backfilled with PEGs of various chain lengths when compared to the BPEG-modified surface. The fibrinogen adsorption levels on the BPEG/PEG400 and BPEG/PEG600 surfaces were virtually identical and higher than that on BPEG/PEG2000 surface along the entire course for the isotherms. Although BPEG/PEG2000 possessed the lowest fibrinogen adsorption values among these three surfaces, significantly more protein adsorption was found on the BPEG/PEG2000 surface when compared to the sole BPEG-modified surface (0.532–0.398 μg/cm^2, respectively at 1 mg/ml). The plateaus are at levels of about 1.092 μg/cm^2 for the control surface, 0.398 μg/cm^2 for the BPEG-modified surface and 0.532 μg/cm^2 for the BPEG/PEG2000 surface. Similar

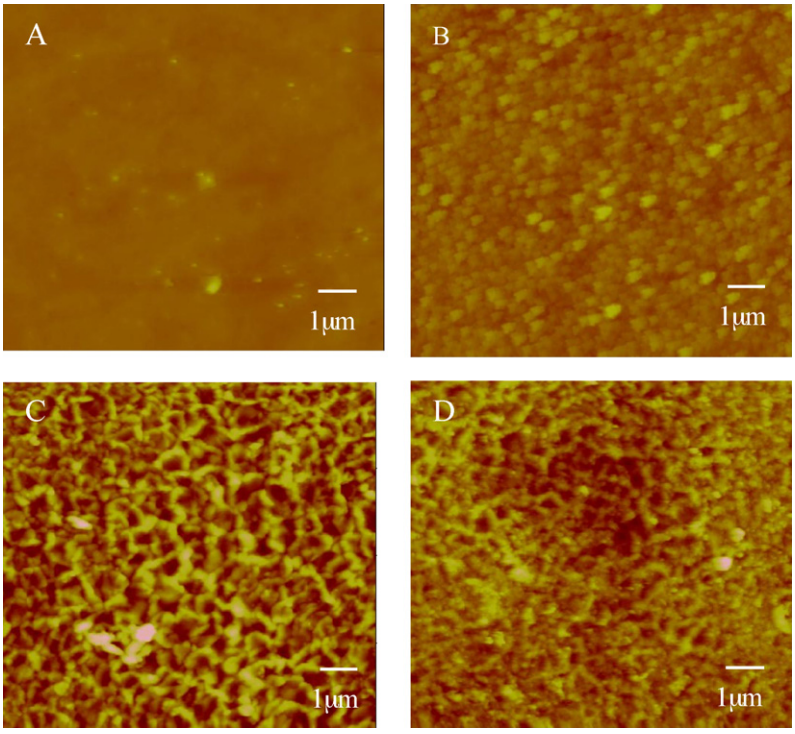


Fig. 4. AFM images of control PU (A), PU–MDI (B), PU–BPEG (C) and PU–BPEG/PEG400 (D) surfaces (scan size = 10 μm × 10 μm, data scale = 300 nm).

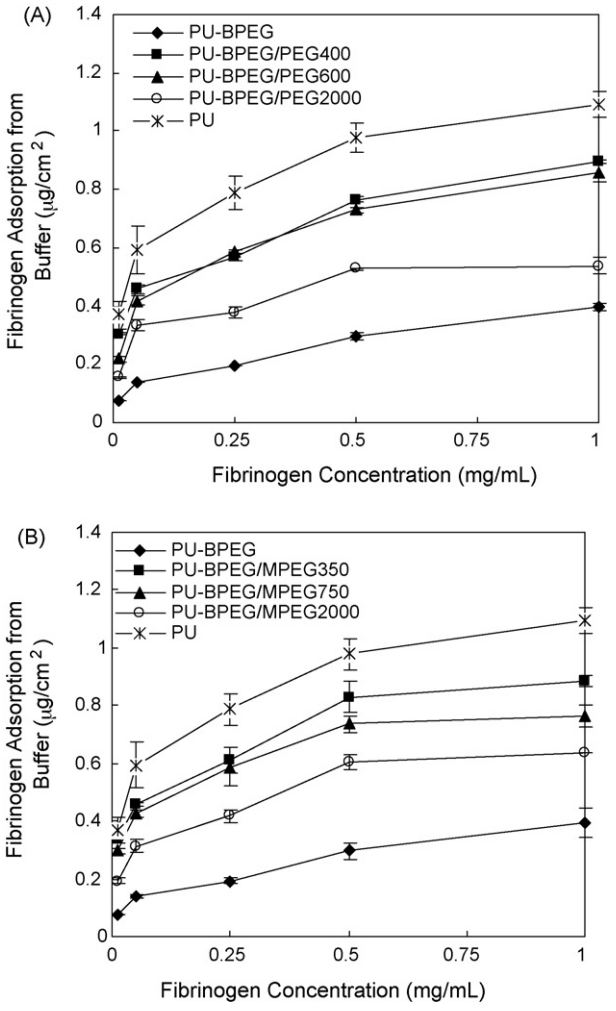


Fig. 5. Fibrinogen adsorption on the control PU and PEG-modified surfaces from TBS buffer. (A) Modified by BPEG and backfilled by different mw PEGs. (B) Modified by BPEG and backfilled by different mw MPEGs.

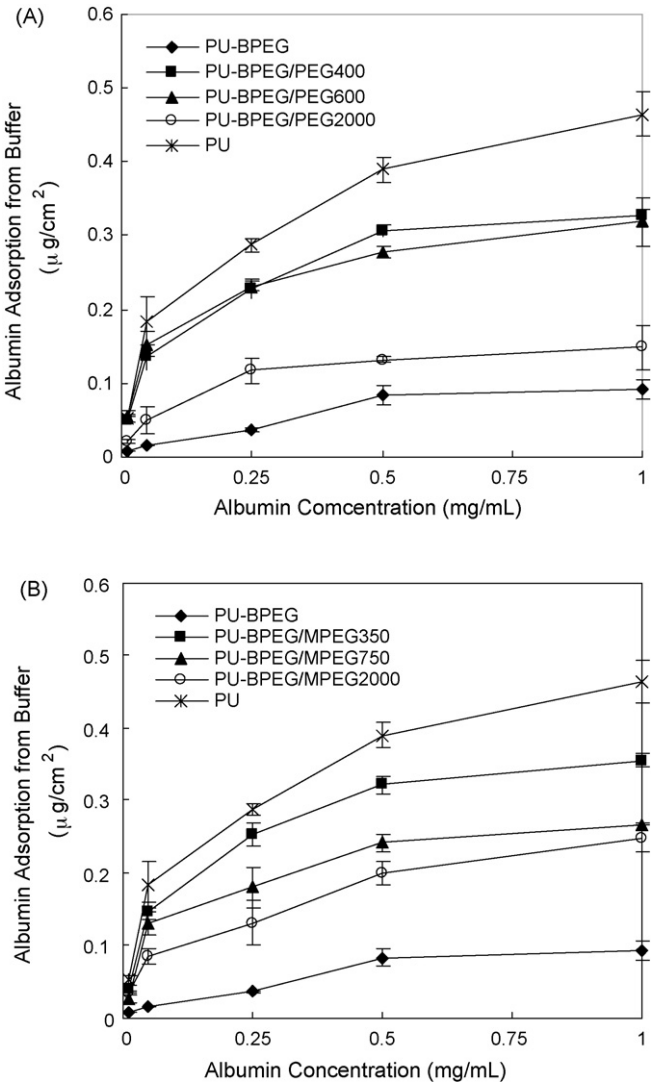


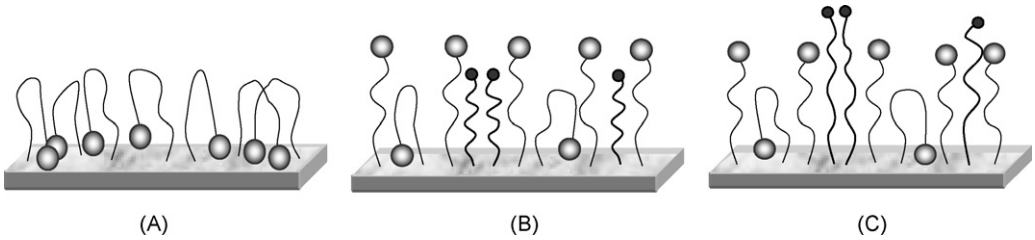
Fig. 6. Human serum albumin adsorption on control and PEGs-modified surfaces from TBS (pH 7.4) buffer. (A) Modified by BPEG and backfilled by different mw PEGs. (B) Modified by BPEG and backfilled by different mw MPEGs.

trends in the adsorption isotherms and in the effects of filler's chain length were found in Fig. 5B, in which the BPEG-modified surface was backfilled with MPEG of various chain lengths. It was observed that fibrinogen adsorption is slightly higher on BPEG/MPEG2000 surface than on BPEG/PEG2000 surface, probably due to the fact that the methoxyl end groups of MPEG2000 are hydrophobic, while the hydroxyl end groups of PEG2000 are hydrophilic. The results reveal that fibrinogen adsorption was slightly influenced by the type and the chain length of the filler.

The amounts of adsorbed HSA were significantly lower on all the surfaces compared to fibrinogen. As shown in Fig. 6A, albumin adsorption on the control surface was approximately 464 ng/cm^2 at an albumin concentration of 1 mg/ml, with a decrease of 80% (93 ng/cm^2) for the BPEG surfaces and 68% (148 ng/cm^2) for the BPEG/PEG2000 surfaces. When the filler was changed from PEG2000 to MPEG2000, albumin adsorption increased from 148 ng/cm^2 to 249 ng/cm^2. These trends are consistent with those for fibrinogen, suggesting that the protein adsorption resistance effects of PEG are not dependent on the molecular weight of the protein.

4. Discussion

PU was activated by reaction with diisocyanate to form an isocyanate surface as reported [17,18]. Based on this method, we grafted different combinations of mixed PEGs to the polyurethane surface by multiple steps. In order to control the graft density, a monobenzyloxy PEG (BPEG) with a bulky group was used to form preconstructed PEG layers on the surface. Each BPEG chain possesses two different end groups, the hydroxyl group at one end can react with the NCO group of the pre-functionalized polyurethane surface, while the benzyloxy group at the other end is a bulky and very hydrophobic group. It was expected that the graft density of BPEG-modified surfaces would not be very high due to the steric effect of the bulky BPEG end group and that there would be some unreacted NCO groups remaining on the surface. It is naturally reasonable to speculate that by backfilling with another PEG with small end groups, a pre-grafted BPEG layer with increased surface graft density



Scheme 1. Possible morphology of PEG derivative modified surfaces. (A) BPEG-grafted surface. (B) Shorter PEG or MPEG as fillers in BPEG layer. (C) Longer PEG or MPEG as fillers in BPEG layer.

could be produced. For this purpose, PEG or methoxyl-PEG with various molecular weights were used as fillers to increase PEG surface density. Although the chain density of PEGs on various surfaces can be determined by SPR, XPS, ellipsometry [6,13–15], it is difficult to characterize PEG graft density on a polymer substrate. A chemical titration method was employed here to compare the graft density after each step. Using this method, we could calculate the amount of NCO on the surface, thereby estimating the PEGs graft density by the reduction of unreacted NCO. It should be noted that minute traces of water and other impurities in the titration system could easily consume unreacted NCO on the polymer surface, resulting in higher estimated PEG graft densities. This implies that the graft density are over estimated by using the reduction results of NCO measurements presented in Fig. 1, but the relative values are still quite helpful in comparing the PEG graft density. The percentage reduction of NCO from 80% for PU–BPEG to over 90% for BPEG/400PEG and BPEG/MPEG350 is consistent with the ATR-FTIR results, where a strong band of –NCO stretching vibration at 2283 cm^{-1} was present on the PU–MDI surface and became weaker on the PU–BPEG surface, then almost disappeared completely after backfilling with MPEG350. Besides, AFM results show that the roughness of the control surface slightly increased to 10.4 nm after MDI modification and then increased to 34.7 nm after BPEG grafting, but the roughness of the PU/BPEG surface slightly decreased after backfilling with PEG400. From the above results, it is concluded that mixed PEG-modified surfaces possess higher graft densities than surfaces modified solely with BPEG.

It is interesting to notice that protein adsorption increased with increasing graft density in our case. This is contrary to the result in literature. Nagasaki reported that formation of a short, filler layer of PEG (2 kDa) in a preconstructed longer PEG brushed layer (5 kDa) on the surface of a plasmon resonance (SPR) sensor chip almost completely prevented nonspecific protein adsorption [14]. Their results suggest that protein repulsion was due to increasing PEG graft density. We believe that the hydrophobic end groups of the monobenzyloxy PEG used here in a pre-grafted PEG layer, where chain conformation was changed by fillers, led to an increase in protein adsorption despite of an increase in PEG graft density. The orientation of hydrophobic end groups will largely influence the protein adsorption. Based on the above observations, we have proposed the following model to explain our protein adsorption results.

The end groups of grafted BPEG are assumed to orient toward the PU substrate surface and away from the aqueous protein solution. This assumption was supported by the result of Somorjai et al., who found that hydrophobic end groups tend to migrate to the polymer surface in air while the hydrophilic end groups remain in the bulk by using the surface sensitive SFG method [21]. In contrast (as shown in Scheme 1A), in an aqueous environment, hydrophilic groups extend out to the aqueous surface while hydrophobic end groups move away from the aqueous layer.

As shown in Scheme 1A, when the PU surface is grafted with only monobenzyloxy PEG at a relatively low density, there will be enough room for the PEG chain to adopt its preferred conformation—the hydrophobic end groups migrate towards the PU surface. The morphology of the modified surface is similar to that of PEG chains tethered in a loop conformation. It was suggested that PEG chains tethered in a loop conformation behave similarly to PEG chains tethered at one end [22]. So the sole monobenzyloxy PEG-grafted surface reduces protein adsorption from 1.092 $\mu g/cm^2$ to 0.398 $\mu g/cm^2$ for fibrinogen and from 0.464 $\mu g/cm^2$ to 0.093 $\mu g/cm^2$ for HSA at 1 mg/ml concentration, even though it contains a very hydrophobic end group. After backfilling with PEGs of various chain length, the benzyloxy PEG chain is forced to take an extended conformation with the hydrophobic end group exposed to the outer layer as shown in Scheme 1B and C. In Scheme 1B, the filler chain is shorter than the preconstructed BPEG chain, implying that the surface is most likely to consist of hydrophobic end groups, with an increased protein adsorption as expected. If the filler has longer chains, as shown in Scheme 1C, some hydrophobic end groups of BPEG may be sheltered by the longer filler chains, leading to a lower protein adsorption. This conclusion has been verified by our experimental results that fibrinogen and albumin adsorption was greater on the conformation described in Scheme 1B than on Scheme 1C. All the surfaces modified with mixed PEGs possessed higher amount of protein adsorption than the surface grafted with sole monobenzyloxy PEG. Additionally, it was found that BPEG-modified surface exhibited the lowest water contact angle (60°) in all modified surface. This result supported our proposed model as well.

5. Conclusion

An increase in PEG graft density on a preconstructed BPEG layer when backfilled with various PEGs was demonstrated by ATR-FTIR, AFM and a chemical titration method. All the surfaces modified with various combinations of PEGs were more hydrophobic and had higher protein adsorption levels than the

sole BPEG-modified surface. This result suggests that the conformation of monobenzyloxy PEG was changed by the fillers, and the extremely hydrophobic end group of BPEG played a key role. Also, both chain length and type of filler influenced the amount of adsorbed protein and a similar trend was found for both fibrinogen and albumin adsorption on all the modified surfaces.

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