Surface Modification of Polyurethanes with Covalent Immobilization of Heparin

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Summary: Thrombus formation and blood coagulation is a major problem associated with blood contacting products such as catheters, vascular grafts, arteries, artificial hearts and heart valves. An intense research is being conducted towards the synthesis of new hemocompatible materials and modifications of surfaces with biological molecules. In this study, polyurethane (PU) films were synthesized in medical purity from diisocyanate and polyol without using any other ingredients and their surfaces were modified by covalent immobilization of heparin. Two types of heparin, unfractionated (UFH) and low molecular weight heparin (LMWH), were immobilized to investigate their effect on cell adhesion. The surface properties of the modified PUs were examined with ESCA, ATR-FTIR and AFM. ESCA results demonstrated sulfur peaks indicating the presence of heparin and AFM results showed the alteration of surface structure after coating with heparin. Cell adhesion studies were conducted with heparinized whole human blood. The surfaces of the UFH immobilized films resulted in lesser red blood cell adhesion in comparison to LMWH demonstrating strong anti-thrombogenic activity of the latter.

Keywords: ESCA/XPS; hemocompatibility; heparin; polyurethane; surface modification

Introduction

Polyurethanes (PU) are highly preferred materials in the construction of biomedical devices due to their mechanical and physical properties, segmented polymeric structure, as well as very high blood and tissue compatibility. Their most common medical applications include vascular prostheses, blood filters, catheters, heart valves, cardiac assist devices, and artificial heart chambers.[1] In the recent years, PU-based wound dressing materials [2], porous scaffolds,[3] biodegradable[4] and bioinjectable[5] PU structures were produced for tissue engineering applications. There is also an intense research to improve the hemocompatibility of stents and catheters either by producing non-thrombogenic structures or by modifying their surfaces physically or chemically (eg. by linking bioactive molecules).[6–14]

Bio- and hemocompatibility of a material depends mostly on its surface properties; chemical structure, interfacial free energy, hydrophilic/hydrophobic balance, and surface topography. In some applications, even when the bulk may have the desired properties, the surface may need to be modified to improve its hemocompatibility. Various chemical methods such as oxidation, introduction of reactive groups through application of plasma glow discharge or by grafting of bioactive molecules, etc. can be used for this purpose.

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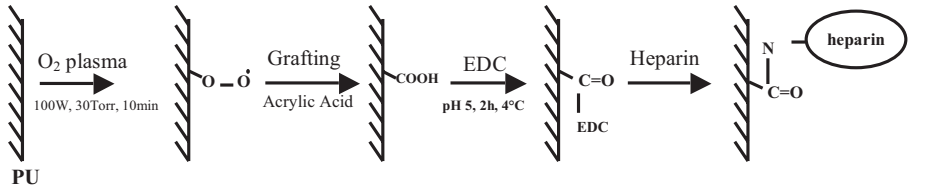

Figure 1.
Heparin immobilization scheme.

Some of these techniques have been designed to immobilize antithrombogenic biological molecules, such as heparin (inhibits the activation of blood clotting factors) or urokinase (initiates fibrinolysis or activate prostacyclin to suppress the aggregation of platelets).[15] Among these bioactive agents heparin is the most widely used. Heparin is a naturally occurring anticoagulant synthesized by the mast cells in the body and inhibits thrombin or some other coagulating proteases and prevents fibrin network formation. Unfractionated heparin (UFH) is a mixture of heparin chains with a molecular weight range of Mw ~ 17000–1900 Da. Low molecular weight heparins (LMWH) are produced by the chemical or enzymatic depolymerization of UFH and have the molecular weight about Mw ~ 3000 Da. In intravenous applications, at high doses, UFH lead to an unpredictable pharmacokinetic response; nonsaturable mechanism can lead to hemorraging. LMWHs are characterized by lower binding with plasma proteins, platelets, and the endothelium, longer half-life, and a more predictable anticoagulant response with lower rate of bleeding complications as compared with UFH.[16,17] In the literature heparin was found as a blend with PU in the form of a film,[18] bonded covalently to polyether urethaneurea containing quaternary ammonium groups in the side chain,[19] linked by other spacers such as diamine diisocyanate[20] and poly(aminoamine).[21] Grafting of functional groups by oxygen plasma followed by a reaction with acrylic acid,[22] use of acryloyl benzothiazole,[23] or polyethylene oxide[15] were other effective methods for immobilization of heparin. Heparinized polyurethanes demonstrated lower activation of platelets and plasma proteins, and thus, reduced thrombus formation as compared to unheparinized polyurethanes.[24] However, since no ultimate solution to the problem of hemocompatibility is known yet, the synthesis and modification of polyurethanes for improved biocompatibility is continuing.

In this study, polyurethane was activated with oxygen plasma and then modified by covalent immobilization of heparin in the form of UFH and LMWH to investigate their effect on cell adhesion according to the scheme presented in Figure 1.

Surface modifications with plasma generally creates peroxide active groups which go further reactions with acrylic acid and then can linked to heparin with help of EDC. There are many clinical studies comparing the two heparin types in i.v. applications, however, this is the first study which compares the properties of UFH and LMWH immobilized on PU surfaces. The surface properties of the modified PUs were examined with goniometer, ESCA, ATR-FTIR and AFM, and blood cell adhesion was studied using heparinized, fresh, whole human blood.

Materials and Methods

Materials

Toluene diisocyanate (TDI, Dow Chemical Company Co. (USA), a mixture of 2,4- and 2,6-toluene diisocyanate in the ratio of 80:20) and polypropylene ethylene glycol (polyol, Dow Chemical Company Co. USA, $M_W \sim 3500$), were used in the synthesis of polyurethanes. UFH and LMWH were both from Sigma-Aldrich (H3393

Mw ~ 1900–17000 Da and H3400 Mw ~ 3000 Da, respectively). The acrylic acid and N′-3-(dimethylaminoproply)-N′-ethylcarbodiimidehydrochloride (EDC) were products of Sigma-Aldrich (USA). Human blood samples were obtained at METU Health Center from healthy volunteers.

Polyurethane Film Preparation

PU films were synthesized from toluene diisocynate and polypropylene ethylene glycol in medical purity without adding any other ingredients (solvent, catalyst or activator) in a closed vacuum system as briefly described in the earlier studies.[25–29] Briefly, in this process, polyol was placed in the reaction chamber, heated to about 80 °C and evacuated for 1 h in order to avoid volatile chemicals, especially water. A known quantity of TDI, which was kept under nitrogen atmosphere, was added dropwise and the total solution was stirred for 6 h at 90 °C under vacuum. The viscous prepolymer formed was poured into glass molds and placed into a vacuum oven where they were kept at 90 °C for complete curing. After solidification, polymer films about 1 mm thickness were separated from glass dishes by immersing into hot distilled water, rinsed few times with distilled water and used in the surface modification experiments.

Heparin Immobilization

PU membranes were cut into pieces (1×1 cm^2) and ultrasonically cleaned in isopropyl alcohol for 10 min. The cleaned and dried samples were treated with low temperature oxygen plasma (100 W, 10 min). After the plasma treatments, samples were immersed into acrylic acid (AA, Acros Organics, USA) and then dried.[30,31]. These AA-grafted PU films were immersed into citrate buffer (pH 5, 20 mL) containing 10 g/L of N-3-(dimethylaminoproply)-N-ethylcarbodiimide hydrochloride (EDC, Sigma, USA) and kept at 4 °C for 2 h. Afterwards, samples were immersed into either UFH or LMWH solution. In order to have a proper coating, various concentrations of heparin solutions and incubation times were applied and the surfaces were examined by ESCA. The best results were obtained when the samples were immersed into 1.25 g heparin/L in citrate buffer for 24 h at 4 °C. After the immobilization reaction of heparin, the samples were washed with distilled water and dried in a desiccator at room temperature.

Surface Characterization

Chemical structures of the surfaces were examined with ATR-FTIR using a Perkin Elmer Spectrum BX-FTIR spectrometer equipped with a ZnSe crystal at 45 °. The samples were analyzed over the 500–4000 cm^{-1} range with the resolution of 4 cm^{-1}. All spectra were averaged over 32 scans. Surface atomic compositions of PU films were analyzed by using ESCA system equipped with MgKα at 1253 eV and 284 W power at the anode. The surface morphology of control, UFH and LMWH immobilized PUs were investigated by AFM (Quesant, USA) in non-contact mode, using standard non-contact cantilever with silicon tips.

Blood Cell Adhesion Studies

The films were placed into separate test tubes containing heparinized fresh human blood obtained from METU Health Center. The samples were kept at 37 °C for 15 min then rinsed with phosphate buffer and the cells attached to the surface were fixed with glutaraldehyde (4%, 30 min). Scanning Electron Microscopy (SEM) investigations were conducted with a JEOL JSM-6400 Electron Microscope.[32]

Results and Discussion

Polyurethanes are inherently hemocompatible materials and can be prepared in various structures ranging from very hard to soft, from sponge to films, from brittle to elastomers, and therefore, they are highly preferred for the design of many blood contacting devices. Upon modifications with oxygen plasma followed by heparin

coating, the properties (chemical, physical and biological) of the PU have changed and this change was dependent on the heparin type used.

Surface Characterization

The ATR-FTIR spectra of PU membranes after each surface modification step are shown in Figure 2. No significant difference is observed in the spectra of control polyurethane (PU) and oxygen plasma treated polyurethane (PU-plasma). This similarity in the spectra before and after plasma treatment may be the result of thickness of the surface analyzed being too large to reveal the changes at a depth of a few nanometers. After the acrylic acid binding the spectrum was still similar to that of PU; this may be due to the carbonyl adsorption of carboxylic acid groups of PU-AA overlapping with that of the urethane bond of PU. In the spectra of heparin immobilized polyurethanes the intensity of the C=O peak at 1735 cm^{-1} decreased and the small peak at 1690 cm^{-1} corresponding to the strong H-bonding disappeared. IR of both PU-LMWH and PU-UFH showed sharp shoulders around 1000–1100 cm^{-1}, compared with other samples, which were due to the symmetric stretching of S=O and C–O–C in the saccharide group of heparin. Also, the intensity of the CN stretching peak (1200 cm^{-1}) decreased and the absorbance at 1220 cm^{-1}, corresponding to sulfonic acid groups of heparin, significantly broadened. Due to the overlapping of the characteristic absorption peaks of SO_3^- and C–O–C in heparin with PU peaks at 1000–1220 cm^{-1} range, further surface characterizations of heparin immobilized PU surfaces, were carried out by ESCA.

In order to compare the pristine and heparinized PUs, the core level spectra of each atom of PU, PU-LMWH and PU-UFH were combined and the peaks corresponding to oxygen-1s core level (binding energy, 540–525 eV), nitrogen-1s core level (binding energy, 408–396 eV), carbon-1s core level (binding energy, 294–278 eV) and sulfur-2p core level (binding energy 174–162 eV) are presented in Figure 3. The oxygen-1s spectra (Figure 3-a) and nitrogen-1s spectra (Figure 3-b) of both PU-LMWH and PU-UFH are slightly broadened due to increase of oxygen and nitrogen content of heparinized surface. The carbon-1s core level spectra of all films shows a peak maximum at 285 eV resulting from hydrocarbon C–C backbone. After heparin

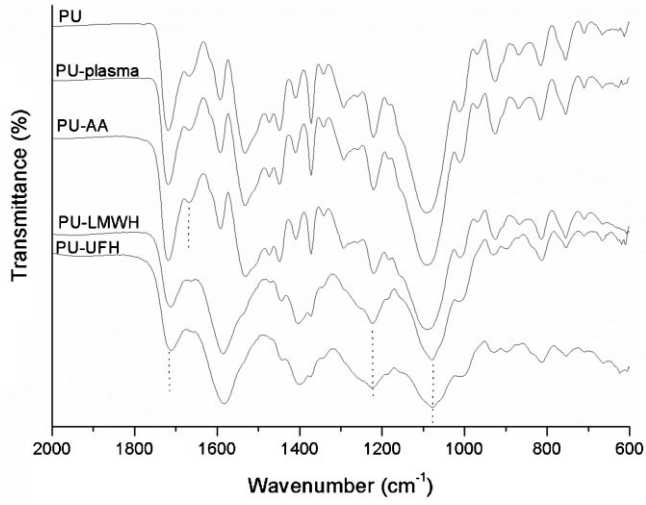

Figure 2.
ATR-FTIR spectra of control PU and surface modified PU samples.

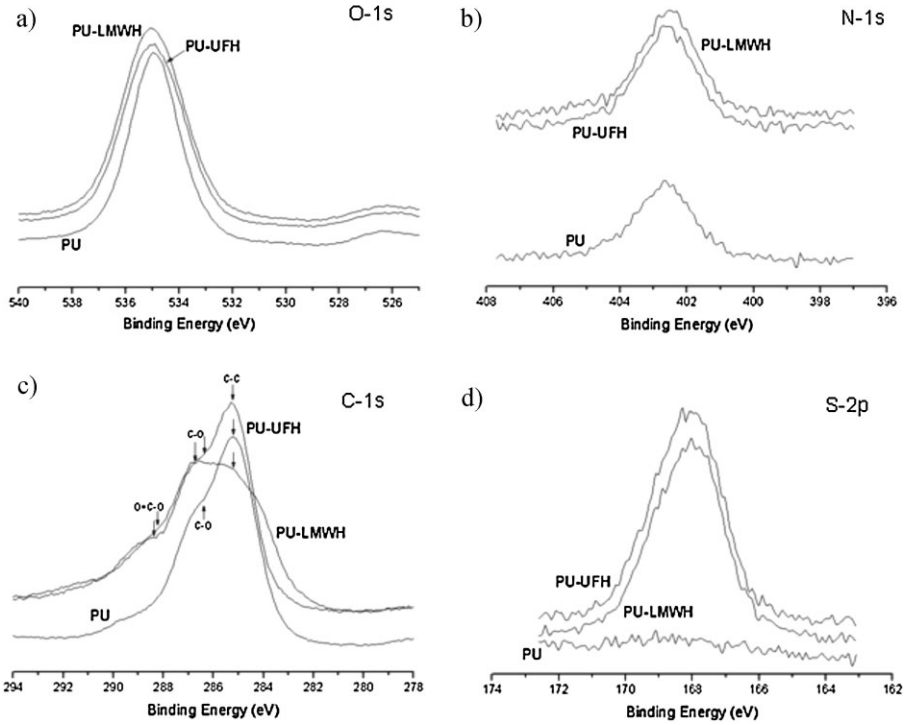

Figure 3.
ESCA spectra of the samples (a) O-1s, (b) N-1s, (c) C-1s, (d) S-2p core level.

immobilization, the C-1s spectra of PU-LMWH and PU-UFH became broadened as a result of overlapping of signals for many carbon sources (Figure 3-c). The broadening at 286.6 eV corresponds to C–O from both PU and grafted polymer, finally peak broadening at around 289 eV belongs to carboxyl groups of grafted acrylic acid.[11] Figure 3-d shows sulfur-2p core level spectra and the samples of PU-LMWH and PU-UFH exhibited a peak at 168 eV which is based on the sulfonate group ($-SO_3Na$) resulting from immobilization of heparin.[33] These results indicate that both types of heparin were covalently bonded onto the surfaces of the PU samples. The surface atomic compositions obtained from the ESCA survey scan spectra are given in Table 1. It can be seen that after the surface modification by acrylic acid grafting and heparin immobilization, the oxygen atomic content has slightly increased from 33% (PU) to 35.4% for PU-LMWH and 33.7% for PU-UFH. Also nitrogen content has increased from 2.5% (PU) to 3.1% and 2.8% for PU-LMWH and PU-UFH, respectively. Sulfur, which is an indicative atom for heparin immobilization, was found on both heparin modified surfaces to the level of ~1.7%.

Table 1.
The surface atomic compositions of heparinized PU samples calculated from ESCA survey scan spectra.

Sample	Atomic %			
	C 1s	O 1s	N 1s	S 2p
PU	64.5	33.0	2.5	–
PU-LMWH	59.8	35.4	3.1	1.7
PU-UFH	61.8	33.7	2.8	1.8

Heparin layer morphology, uniformity of coating, the part of the heparin molecule attached on the material surface and heparin molecule conformation play an important role in hemocompatibility of the material. The formation of a uniform heparin immobilization on the biomaterial

surface is very important for its performance, because the presence of incompletely heparinized areas or crevices permit platelet adherence and aggregation on the surface.[34] Surface topography was observed by AFM (Figure 4). The untreated PU surface demonstrated smoothness compared with heparin immobilized PUs. UFH

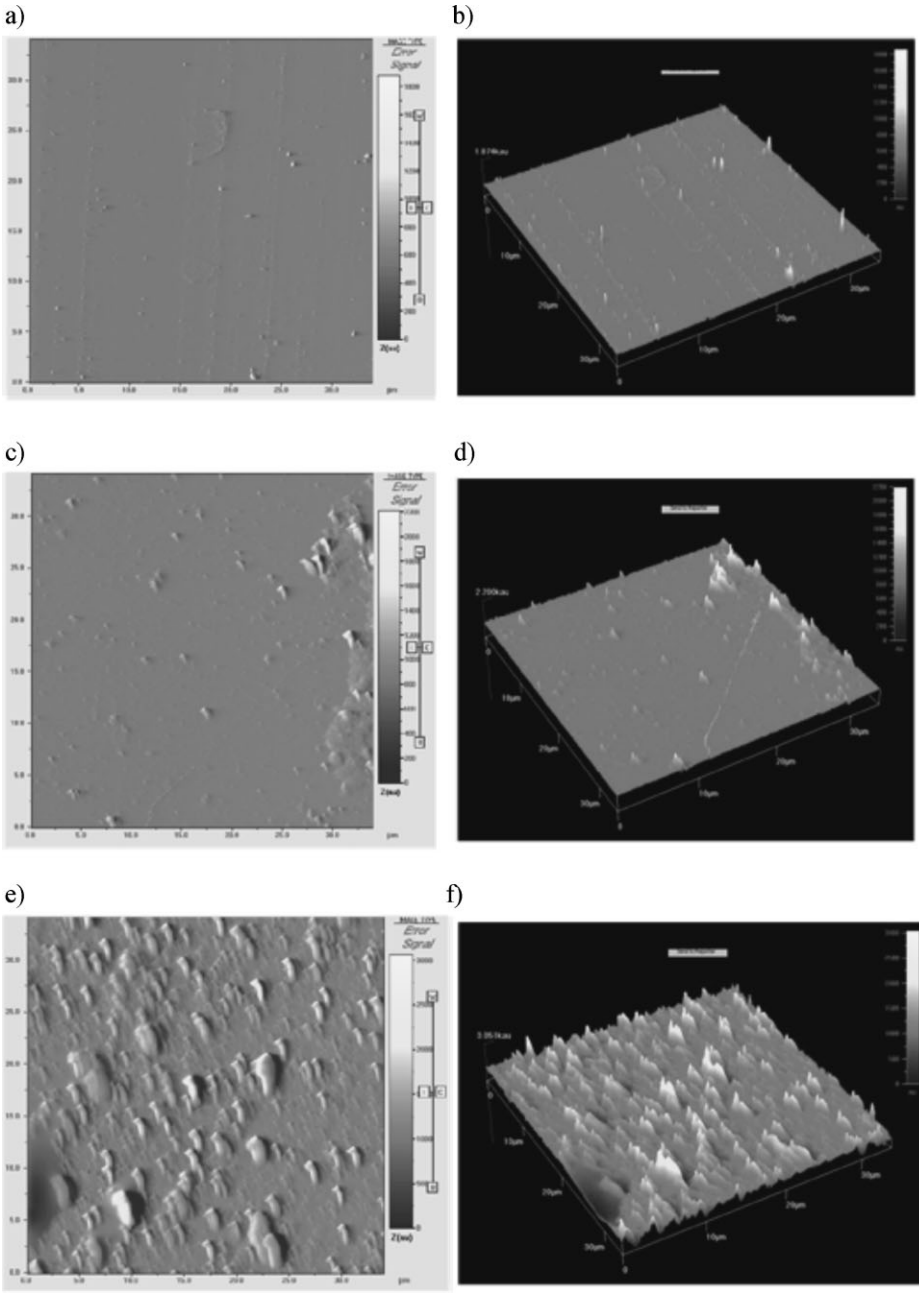

Figure 4.
AFM images in non contact mode for surfaces of (a-b) PU, (c-d) PU-LMWH, (e-f) PU-UFH. The scan size is 30 μm × 30 μm.

immobilized PU surface shows a dendrite-like morphology of high molecular weight heparin chains with uniform distribution of heparin layer. Similar surface topographies were reported for heparin.[35] LMWH immobilized PU showed less uniform heparin coating compared with PU-UFH and this may be an important factor affecting the blood cell adhesion evaluations.

Blood Cell Adhesion Studies with Heparinized Polyurethane Membranes

It is known that heparin prevents cell aggregation and platelet adherence. Blood cell adhesion studies of polyurethane

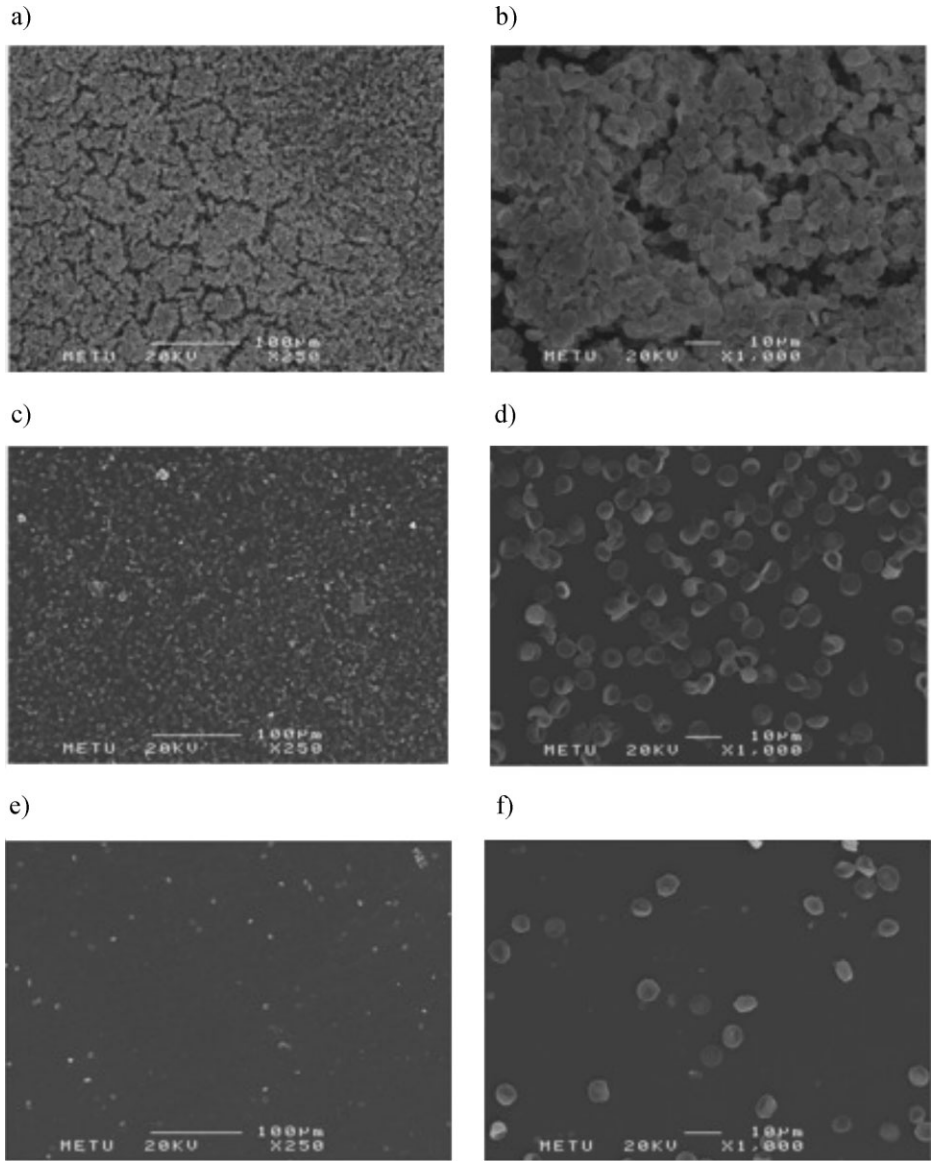

Figure 5.
SEM images after blood contact (a) PU (×100), (b) PU (×1000), (c) PU-LMWH (×100), (d) PU-LMWH (×1000) (e) PU-UFH (×100), (f) PU-UFH (×1000).

surfaces covalently coated with heparin demonstrated that cell adhesion was reduced and blood compatibility of the material was improved (Figure 5).

Pristine samples demonstrated very high adherence of red blood cells (Figure 5-a and b) while the cell adherence decreased for PU-LMWH (Figure 5-c and d), and only a little adhesion has occurred for PU-UFH (Figure 5-e and f). From these results, it may be concluded that UFH has a more significant anti-adhesion effect on the surface than LMWH. This may be due to the more uniform heparin layer of the PU-UFH surface or a result of the different biological activities of LMWH and UFH. Like UFH, LMWHs produce their major anticoagulant effect by activating antithrombin. Their interaction with antithrombin is mediated by a unique pentasaccharide sequence found on less than one third of LMWH molecules. A minimum chain length of 18 saccharides is required for ternary complex formation of heparin, antithrombin, and thrombin. Therefore, only the 25% to 50% of LMWH species that are above this critical chain length, are able to inactivate thrombin. In contrast to LMWH, all UFH molecules contain at least 18 saccharide units. The longer chain, unfractionated heparin UFH is also able to inactivate factor IIa through formation of a tertiary complex, unlike LMWH.[36] The difference in the cell adhesion onto the surface may be due to the biological activity difference between the heparins.

Conclusion

Polyurethanes are the most widely used polymeric materials in the design and production of blood contacting devices because of their inherent non-thrombogenic properties. Surface is very important because it is the part of an implant or device that triggers cell adhesion or blood coagulation, and therefore, surface modifications are needed to improve these properties. Therefore, an intense research is conducted to modify the surfaces to improve blood compatibilities of polyurethanes. With the modification the surface chemistry of PU cell adhesion levels changed. The surface of UFH immobilized films showed lower red blood cell adhesion compared with LMWH because of the more extensive coverage of the surface and higher anti-thrombogenic activity.

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