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The Synthesis and Evaluation of Pendant Oligosaccharide-Lipid Side Chain Copolymer

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Abstract: In this research, the *in vitro* anti-thrombogenecity of artificial materials was evaluated using hydrophilic/hydrophobic copolymers containing oligosaccharide as hydrophilic moiety and phospholipid as hydrophobic moiety respectively. *N*-(*p*-vinylbenzyl)-[*O*-α-*D*-glucopyranosyl-(1→4)]$_{n-1}$-D-glucoamide (VM7A) was adopted as hydrophilic oligosaccharide and 2-acryloxybutyl-2-(triethylammonium)ethyl phosphoric acid (HBA-choline) was adopted as hydrophobic phospholipid. Copolymers having various monomer feeding molar ratios were synthesized through radical polymerization. The synthesized copolymers were identified using FT-IR, ^{1}H-NMR, XPS, and DSC. The surface energy of the copolymers were evaluated by dynamic contact angle (DCA) method and checked different roles of VM7A as hydrophilic moiety and HBA-choline as hydrophobic moiety on surface. The surface morphological differences between hydrated and unhydrated surfaces of copolymers were observed and evaluated using AFM. The platelets were separated from canine whole blood by centrifugation and adopted to the anti-thromobogenecity test of the copolymers. From the results, we find out that as VM7A ratio increases, so did anti-thrombogenecity. Such results show the possibility of using these copolymers as blood compatible materials in living body.

Keywords: vinyl oligosaccharide, lipid containing monomer, anti-thrombogenecity, *in vitro* blood compatibility.

Introduction

When a foreign material is implanted into living body, the proteins adsorb to the surface of foreign material in the early stage, consequently causing platelets activation. This would cause the actual coagulation of the blood on the artificial materials surface.[1] Those phenomena occur critical problems when the polymer is applied as artificial blood vessel or organ substitutes. So there had been many attempts to overcome these undesired and unsatisfactory phenomena.[2-4]

Generally as hydrophilic characteristics of the material enhancing, the blood compatibility of that material turns to be better, in spite of leaching out of hydrophilic moieties from the materials when the material contact with body fluid in relatively long time. On the other hand hydrophobic materials would cause strong hydrophobic interaction with protein in body fluid, which eventually cause coagulation of the blood. Considering above characteristics depending materials surface, the amphiphilic materials were good candidates to solve such problems, but the physical properties (including solubility and mechanical properties) of the material still remained unsolved. Nakabayashi *et al.*,[5] reported that polymer containing lipid molecule in their molecular backbone would easily interact with phospholipid in our body fluid at first and could suppress the protein adsorption on lipid containing polymer surfaces. When vinyl lipid derivatives were copolymerized with hydrophobic polymer, physical properties and its prevention of protein adsorption were enhanced. And recently Ishihara[6] also reported the role of lipid moiety in synthetic copolymer and found out its preventing of platelet adhesion.

In our previous research,[7] to compensate high water-solubility of poly(vinyl oligosaccharide), we synthesized copolymer from vinyl oligosaccharide and hydrophobic monomer with different pendant groups. Other studies[8-10] indicate that the behavior of the polymer molecules on biomaterial surface, especially polymeric phospholipid molecules, reveals very important role in keeping biocompatibility during contact with body fluid (mainly water).

In this study, in contrast to Nakabayashi's work, we have synthesized novel vinyl phospholipid monomer (HBA-cho-

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line) which enhanced hydrophobicity by substituting methyl group in end point of lipid with ethyl group and copolymerized this monomer with hydrophilic monomer. And then we evaluated the prevention of platelet adhesion on these copolymer surfaces owing to lipid moieties and also confirmed their blood compatibility.

Experimental

Materials. *p*-Vinylbenzyl chloride, potassium phthalimide, acryloyl chloride, 2-chloro-1,3,2-dioxaphospholane-2-oxide (containing 10% of 2-chloro-1,3,2-dioxaphospholane, Aldrich, Milwaukee, USA), Ambelite IR-120 (Fluka, Buchs, Switzerland), diethyl ether, potassium hydroxide (Duksan Chemicals, Ahnsan, Korea) were purchased and used without purification. Triethylamine, acetonitrile, dimethyl sulfoxide (DMSO) (Duksan Chemical, Ahnsan, Korea), tetrahydrofuran (THF) (Daejung, Seoul, Korea) were dehydrated with molecular sieve (Aldrich, Milwaukee, USA) and purified by vacuum distillation before using.

Synthesis.

Modification of Oligosaccharide[7]: Maltodextrin (DP 4~7: Aldrich) was dissolved in water and ethanol was added to the solution. The solution was then stirred in the water bath for 2 hrs at 40 °C. Iodine (Duksan Chemicals, Ahnsan, Korea) was slowly added while stirring. Then potassium hydroxide (Daejung, Seoul, Korea) was added immediately until the color changed to white. The solution was recrystallized for overnight in refrigerator. The formed crystal was filtered and dissolved again in water and passed through the column filled with Amberlite IR-120 to neutralize the solution. This solution was then vacuum dried, and lactone type maltodextrin was obtained as final product.

Synthesis of *p*-Vinylbenzylamine[11]: *p*-Vinylbenzyl chloride was dissolved in *N,N*-dimethylforamide (Duksan Chemicals, Ahnsan, Korea), then potassium phthalimde was added to above solution and stirred for 4 hrs at 50 °C. DMF was evaporated and the residue was dispersed in chloroform afterward. The concentrated solution was rinsed with sodium hydroxide solution. Then methanol was added for solidification. The obtained crystal was filtered and dried under vacuum. The crystal was solved again in ethanol while stirring at 60 °C. The hydrazine hydrate ethanol solution was added to above solution and stirred additionally for 90 min. Then the precipitant was filtered and dried under vacuum for an overnight. The powdery precipitant was put into potassium hydroxide aqueous solution and extracted with diethyl ether. The mixture was rinsed with K_2CO_3 aqueous solution and solvent was evacuated under reduced pressure.

Synthesis of *N*-(*p*-vinylbenzyl)-[*O*-α-*D*-glucopyranosyl-(1→4)]$_{n-1}$-*D*-glucoamide (VM7A): Oligosaccharide aldonate was solved in ethylene glycol and *p*-vinylbenzylamine was added to the solution. The mixture was stirred for 13 hrs at 70 °C. Then the solution was precipitated in methanol and dried under vacuum after filtering.

Synthesis of Hydroxy Butyl Acrylate (HBA)[12]: Triethylamine (TEA) and 1,4-butandiol was mixed in tetrahydrofuran (THF) and stabilized with nitrogen gas. Then acryloyl chloride was added slowly for 5 hrs at 20 °C. The reactor was removed from the cooler and preceded the extra reaction for 4 hrs at room temperature. After reaction, the precipitant was removed by filtration and the residual solution was concentrated by evaporating and rinsed several times with *n*-hexane.

Synthesis of 2-HBA-chloro-1,3,2-dioxaphspholane-2-oxide: Synthesized HBA reagent was mixed with THF. Then the solution was stabilized with nitrogen gas and 2-chloro-1,3,2-dioxaphospholane-2-oxide was added for 4 hrs at 20 °C. The reactor was removed form the cooler and left at room temperature for 3 hrs. The precipitant filtered and the residue was recovered under reduced pressure.

Synthesis of HBA-choline and HBA Choline-*co*-VM7A: Acetonitrile and 2-HBA-chloro-1,3,2-dioxaphospholane-2-oxide was mixed and TEA was added to that solution. The mixture was stirred for 35 hrs at 65 °C. Then the mixture was kept in low temperature until white precipitates came out. The precipitant was separated form the solution and dried under vacuum (Figure 1).

The copolymerization of HBA-choline and VM7A was done by the molar feeding ratio of 9 : 1, 8 : 2, 7 : 3, 6 : 4 and 5 : 5. Each sample of various ratios of monomers was put into dimethylsulfoxide, degassed with nitrogen for 30 min and copolymerized using AIBN as initiator. The synthesized polymers were then precipitated in diethyl ether. For the case of 6 : 4 and 5 : 5 samples, the solution was first precipitated in methanol instead of diethylether and then rinsed with methanol (Figure 2).

Characterization.

Structural Analysis: The FT-IR (Mattson 5000, Wisconsin, USA), XPS (SSI 2803-S, VG Microtech, UK), and 500 MHz ^{1}H-NMR (Varian Unity Inova, Germany) were used to analyze polymer preparation. ^{1}H-NMR was used also to evaluate the monomer composition in synthesized copolymer. And XPS was used for elemental composition, such as

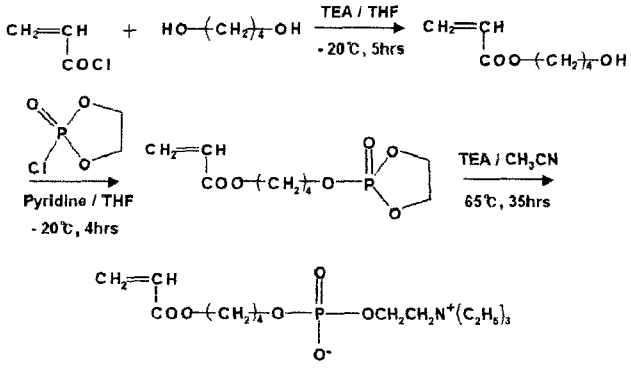

Figure 1. The synthetic scheme for HBA-choline.

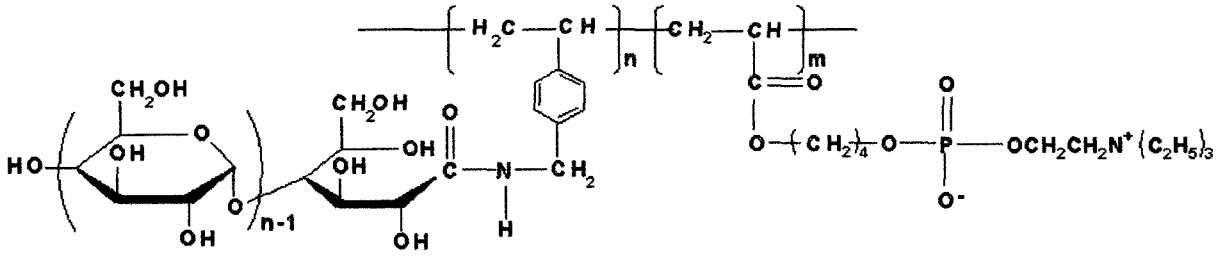

Figure 2. Chemical structure for poly(HBA-choline-*co*-VM7A).

nitrogen and phosphorus, which is originated from HBA-choline containing polymer.

Surface Characteristics Measurement: Dynamic contact angle (DCA) (DCA 322, Cahn Instrument, Germany) was measured to analyze the hydrophilicity of each samples. The synthesized copolymers were coated on glass plate and dried under vacuum. Then the samples were dipped into the water for 5 sec and pulled out using DCA, for recording advancing and receding contact angles. Thermal analysis was done using DSC (TA Instrument, Delaware, USA).

Surface Morphology Observation: The samples were coated on the glass plates using same method as mentioned above. The samples were divided into two groups, one was hydrated with buffer solution for 4 hrs and other was not. And the surface of the both samples were observed using AFM (Digital Instrument NanoScope III, Santa Barbara, USA).

Evaluation of *in vitro* Blood Compatibility and Platelet Adhesion Test: The platelet were separated from the canine whole blood and the total platelet number in PRP(platelet rich plasma) was controlled with PPP (platelet poor plasma) up to 1×10^5 cells/mL, which was counted deactivating platelet using hemacytometer (Marienfeld Co. Ltd., Germany). The glass beads were washed with chromatic acid, then with water, and finally with ethanol. The glasses were dried and coated with the samples that were solved in DMSO. Then the glasses were stabilized with nitrogen and dried in vacuum oven for an overnight.

PU (Pellethane 2363-80AE)-precoated glass beads were coated again with synthesized copolymers having various monomer composition, for the purpose of platelet adhesion test.

0.2 g of copolymer-coated glass bead (PU coated bead for control) was inserted into syringe filled with 2 mL of PBS. After 1 hr hydration of coated glass beads, PBS was substituted with diluted platelet solution (0.5 mL) and incubated for predetermined time interval in shaking incubator (Daeil Engineering Co. Ltd., Korea) at 37 °C. Unreacted platelet was separated from above syringe and counted the numbers by hemacytometer with Nikon Inverted Microscope (DIPHOT-MD, Pola 4.0/0.55, ph3DL, Tokyo, Japan). This numbers of unreacted platelet was related to blood compatibility of coated polymeric materials, in other words biocompatibility.

Meanwhile, the PU coated glass plates immersed into the platelets solution which cured at 37 °C and stayed for 3 hrs. After incubation the coated glass plates were taken out and washed with buffer solution (pH = 7.38). Then the activated platelets were fixed with 2.5% glutaraldehyde solution. Then the coated glass plates were again washed with buffer solution and dehydrated with series of ethanol-water mixture. The platelet fixed glass plates were dried by critical point dryer(Hitachi HCP-2, Tokyo, Japan) and stayed for an overnight and evaluated platelet adhesion behavior with SEM (Hitachi S-2200, Tokyo, Japan) after platinum sputter-coating. The 64 and 55 samples were not evaluated because they showed too high hydrophilicity when they are exposed to water over 1 hr. PU coated glass bead was used as reference and this was also evaluated.

Results and Discussion

Chemical Structure Analysis. Figure 3 shows the result of XPS measurement. The calculated area of -COO- group which was originated from HBA-choline (showed at 289 eV) increased as the increment of HBA-choline moiety composition ratio in copolymers. This indicates that the monomeric composition in the synthesized copolymers was related with monomer feeding molar ratios in certain correctness under the consideration of their MW. This fact also supported by the results of Figure 4, which illustrated the XPS result of nitrogen and phosphorus atoms originated from HBA-choline moiety in synthesized copolymers, such that the calibrated area of the nitrogen and phosphorus increase with the increment of HBA-choline composition in copolymer respectively.

Surface Characteristics. Figure 5 showed the result of DCA measurement. As HBA-choline moiety decreases, the contact angle also decreases, corresponding to the increment of the hydrophilicity. The pendant ethyl groups in HBA-choline works strongly as hydrophobic characteristics of synthesized copolymer surfaces. By the previous report,[3] poly(HBA-choline) alone did not reveal as hydrophobic characteristics when contacted with water, and consequently showed good water solubility. But we found in this study that the hydrophobic behavior was only observed when HBA-choline was copolymerized with very hydrophilic

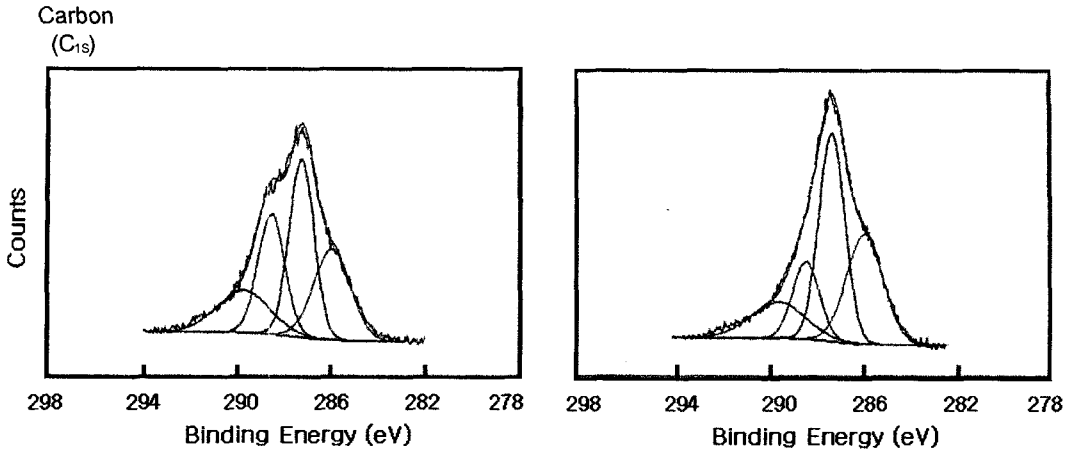

Figure 3. XPS results of the synthesized copolymers: (a) C_{1s} spectrum for copolymer 91 and (b) C_{1s} spectrum for copolymer 73.

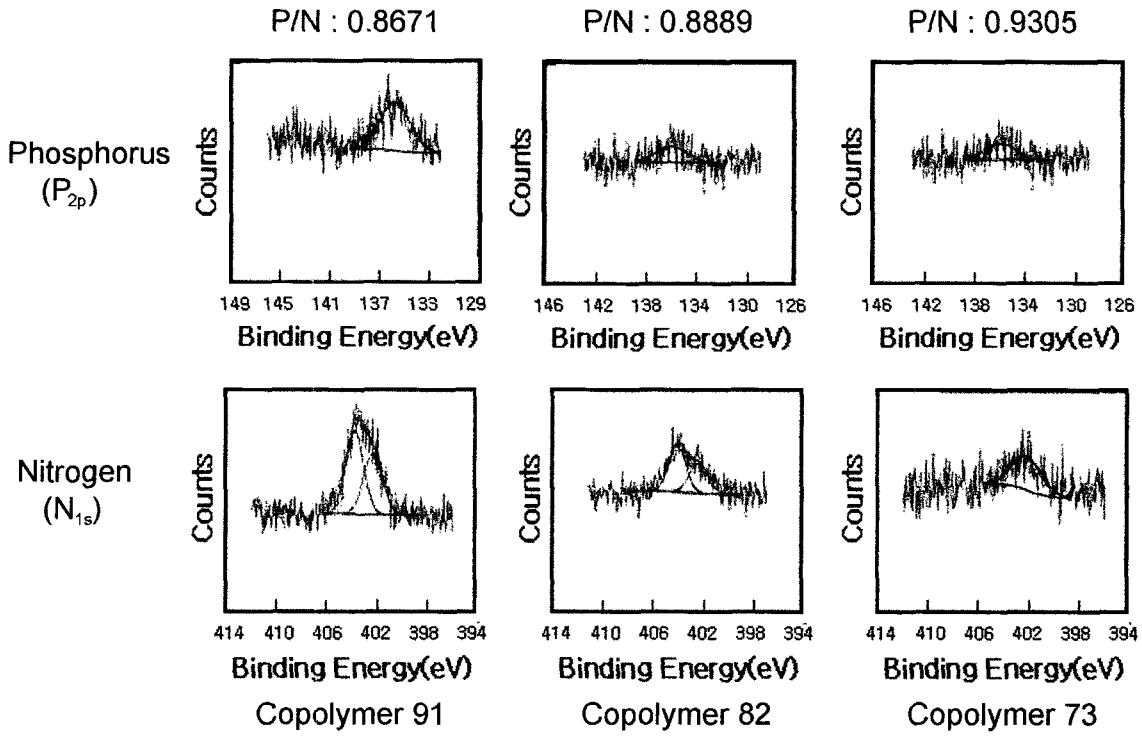

Figure 4. The XPS spectra for P_{2s} and N_{1s} of various copolymers.

monomer and its composition ratio was high enough than that of counter part in copolymer. Therefore in case of the 64 and 55 samples which had relatively low content of HBA-choline in copolymer, they showed water soluble behavior in water for their hydrophilic characteristics owing to high VM7A ratio, as expectedly. The contact angle of the 55 sample was the lowest, which can be interpreted as promising good blood compatibility, but that was limited only within an hour because of its good water solubility.

Thermal Behavior. DSC was used to characterize the thermal behavior of the copolymer. As shown in Figure 6, the increase of the hydrophilic characteristics equivalent to (i.e., VM7A content) copolymers (91 sample→73 sample) brought the increase of the endothermic energy, this tendency indicated that the obtained copolymer was turned to be random polymer and had partially crystalline region

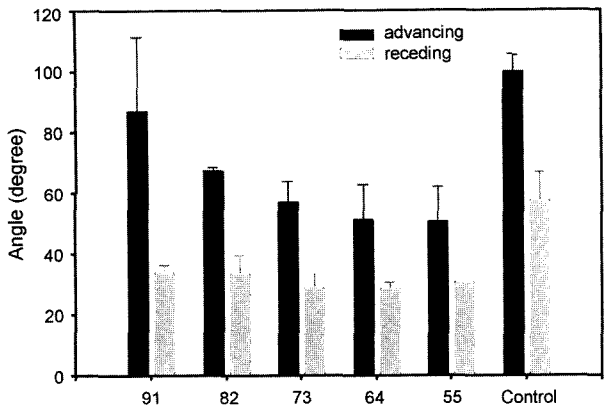

Figure 5. DCA measurement data for various copolymers and PU control.

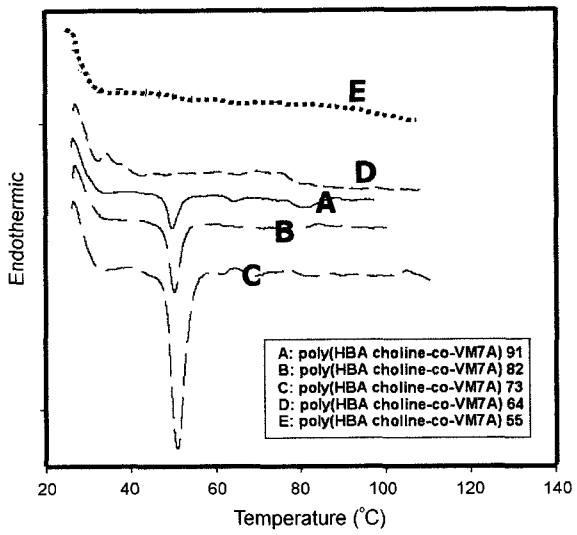

Figure 6. DSC measurement data for various copolymers.

owing to interactions between side chains in copolymers.

Measured molecular weight by GPC of 64 and 55 samples were revealed to 40,000-50,000 and this value was much higher than that of 91, 82 and 73 sample (6,000-7,000). Due to its low MW in 91, 82 and 73 samples, molecular structure of copolymer was easily turn to be crystallized by molecular chain interaction (including side chain interaction). This can be explained as follows; there were strong repulsive interaction between the different two monomeric unit in above 91, 82 and 73 copolymer molecule and this force also found in intermolecular interactions. Therefore large endothermic peak were observed in those three copolymers shown in Figure 6, often observed in the DSC curves of polymers containing bulky side chains. On the contrary, 64 and 55 copolymers did not show such endothermic peak in Figure 6, those copolymers did not have crystalline structure in its molecular structure owing to lack of bulky oligosaccharide side chain. This result is related with good solubility in water because the lack of strong repulsive interaction in 64 and 55 copolymers.

Surface Morphology Characterization. The surfaces of the hydrated and unhydrated copolymers were shown in Figure 7. There were noticeable differences between hydrated and unhydrated copolymer surfaces. First of all, the surfaces of the hydrated ((d), (e), (f) in Figure 7) were smoother than dried samples surfaces ((a), (b), (c) in Figure 7) and the peaks on the surface were clearly distinguishable. This difference is very clearly for 91 copolymer. Small peaks that were shown around the large mountain for unhydrated sample (Figure 7(a)), but in Figure 7(b) these small peaks disappeared when they were hydrated and the big mountain was divided into small plateau, indicating that there were some changes within it. It was probably due to the water that had been trapped in hydrophilic moieties of copolymer surface while hydrating with water, and the change of surface environment and final rearrangement of the molecular chains in copolymer surface. This rearrangement also are expectable other copolymer samples. During hydration, the mountains were stuck up as if they have been aggregated each other. If so, this means that there were non-crystalline oligosaccharide groups existing at the surface after hydration and they can easily aggregated at the moment of water contacting. The surfaces of the samples are very important that they are the exact part which will eventually in contact with body fluid.

Therefore the phospholipid (HBA-choline) group located on the surface enhances the water-durability and preventing protein adsorption, VM7A group on the surface contribute

Table I. Chemical Composition for Synthesized a Poly(HBA choline-*co*-VM7A) and Their M_w

Samples	Monomer Molar Composition in Feed (HBA-choline : VM7A)	Monomer Molar Composition in Copolymer (HBA-choline : VM7A)	Molecular Weight (by GPC)
poly(HBA choline-*co*-VM7A) 91	9 : 1	11.4 : 1	6,400
poly(HBA choline-*co*-VM7A) 82	8 : 2 (4 : 1)	4.9 : 1	7,000
poly(HBA choline-*co*-VM7A) 73	7 : 3 (2.3 : 1)	3.1 : 1	8,500
poly(HBA choline-*co*-VM7A) 64	6 : 4 (1.5 : 1)	2.6 : 1	40,000
poly(HBA choline-*co*-VM7A) 55	5 : 5 (1 : 1)	0.7 : 1	53,000

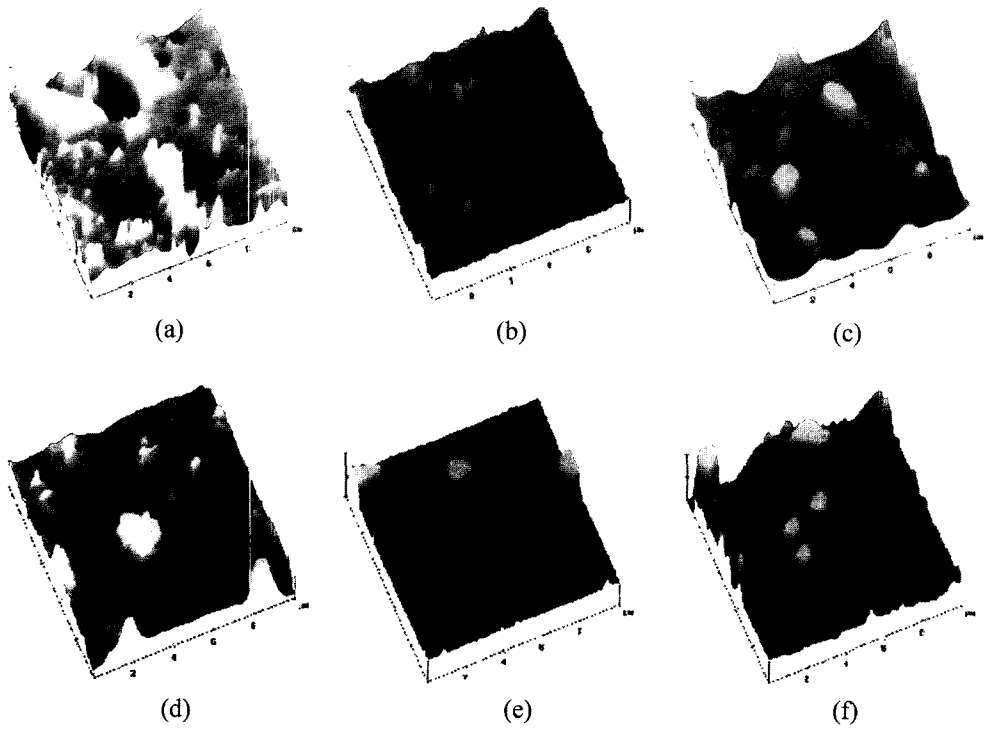

Figure 7. The AFM images of various copolymers: (a), (b) and (c) images for unhydrated 91, 82 and 73 copolymer surfaces, (d), (e) and (f) images for hydrated 91, 82 and 73 copolymer surfaces.

to suppress the platelet activation.

Platelet Adhesion Behavior and Blood Compatibility. In Figure 8, the amount of adhered platelets on copolymer coated glass beads was decreased with increment of HBA-choline composition in synthesized copolymers. Such results were greatly related to the decrease of contacting angles in 91, 82 and 73 copolymers according to the increment of HBA-choline composition. The coagulation of the platelets was suppressed as the VM7A moiety went up. This result clearly shows that phospholipid functioned as hydrophobic polymer, and the phospholipid molecules possess good blood compatibility, the synergetic effect of HBA-choline and VM7A contribute to divulge good biocompatibility of synthesized copolymer. In this study, we considered that it would be possible to synthesize balanced amphiphilic polymer materials through copolymerization of HBA-choline and VM7A under control of monomer feeding ratios. And we also expected that HBA-choline moiety was working as hydrophobic group in poly(HBA-choline-*co*-VM7A) and would show good protein repellency. On the basis of our results, when HBA-choline content in copolymer was over 1/3, water solubility of copolymer was extremely increased and could not investigate platelet adhesion behavior in film status. This was also related to thermal characteristics in Figure 6(64 and 55 copolymers did not show T_c peak in DSC curves), those amorphous structure and good water solubility.

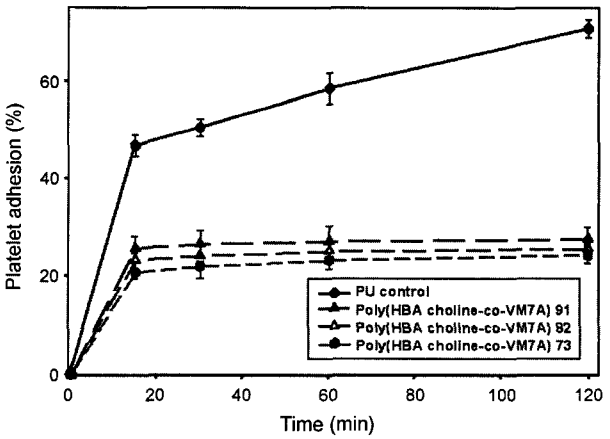

Figure 8. Platelet adhesion behaviors on PU and copolymers coated glass beads.

The coagulated platelets on copolymer coated glass beads were observed by SEM. As shown in Figure 9, the adhered platelets on the copolymer were diminished than that of control PU-coated glass beads. And the platelet activation was also suppressed in 73, 82 and 91 copolymer compared to PU control. The pseudo-pods were not developed in all copolymers and this indicates that there were not strong hydrophobic interactions between the surface of copolymers and the platelets during contacting for incubation time.

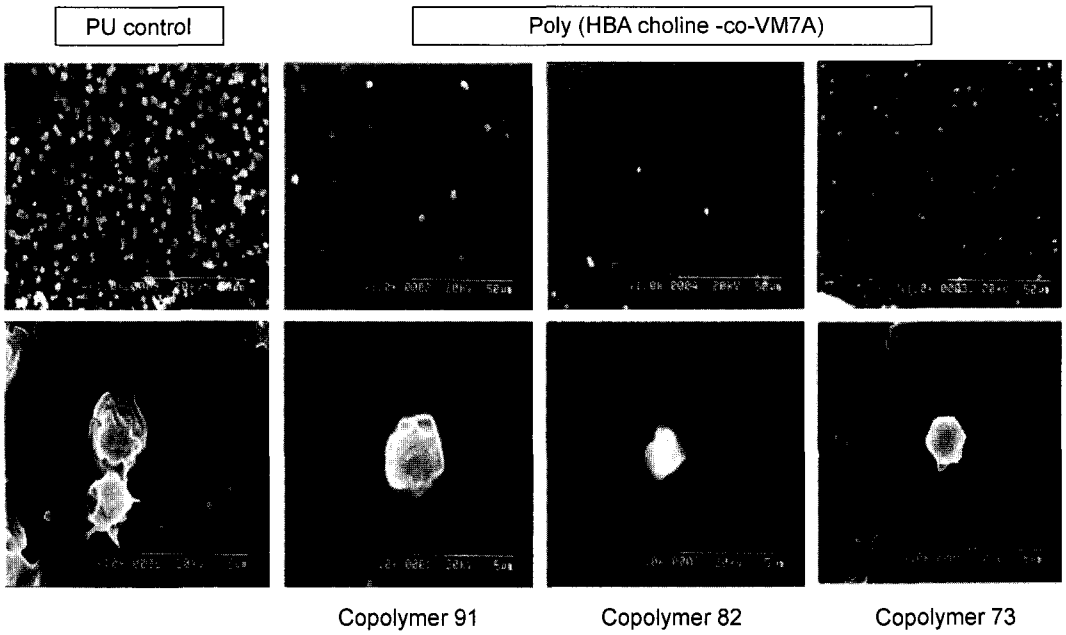

Figure 9. SEM images for PU and copolymers coated glass plate after platelet adhesion test.

Conclusions

The results showed that the synthesized materials should be applicable good coating material for improving blood compatibility. From *in vitro* test results, 55 sample would show the best blood compatibility but it showed unsatisfied water solubility when immersed in water over an hour. The materials themselves excluding 55 sample are very suitable for biomaterial and can also be applied as artificial organs.

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