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Mechanisms of Platelet Adhesion on Elastic Polymer Surfaces: Protein Adsorption and Residence Effects

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Abstract : Platelet adhesion onto elastic polymeric biomaterials was tested *in vitro* by perfusing human whole blood at a shear rate of 100 sec^{-1} for possible verification of mechanisms of initial platelet adhesion. Perfusion of blood on the polymeric substrates was performed after treatments either with or without pre-adsorption of 1% blood plasma, and either with or without residence of the protein-preadsorbed substrate in phosphate buffered solution. The surfaces employed were elastic polymers such as poly(ether urethane urea), poly(ether urethane), silicone urethane copolymer, silicone rubber and poly(ether urethane) with the anti-calcifying agent hydroxyethane bisphosphate. Each polymer surface treated was exposed *in vitro* to the dynamic, heparinized whole blood perfused for upto 6 min and the surface area of platelets initially adhered was measured by employing *in situ* epifluorescence video microscopy. The blood perfusion was performed on the surfaces treated at the following three different conditions: directly on the bare surfaces, after protein pre-adsorption and after residence in buffer for 3 days of the surfaces protein pre-adsorbed for 2 h. The effects of blood plasma pre-adsorption on the initial platelet adhesion was surface-dependent. The amount of the adsorbed fibrinogen and the surface coverage area of the adhered platelets were dependent on the surface conditions whether substrates were bare surfaces or protein pre-adsorbed ones. To test an effect of possible morphological (re)orientations of the adsorbed proteins on the initial platelet adhesion, the polymeric substrate pre-adsorbed with 1% blood plasma was immersed in phosphate buffered solution for 3 days and then exposed to physiological blood perfusion. The surface area of the platelets adhered on these surfaces was significantly different from that of the surfaces treated with protein pre-adsorption only. These results indicated that platelet adhesion was dependent on the surface property itself and pre-treatment conditions such as blood perfusion without any pre-adsorption of proteins, and blood perfusion either after protein pre-adsorption or after subsequent substrate residence in buffer of the substrate pre-adsorbed with proteins. Understanding of these results may guide for better designs of blood-contacting materials based on protein behaviors.

Introduction

Understanding of biological interactions of blood cells such as platelets and polymorphonuclear cells with polymeric biomaterials has been drawing high interests in many blood-contacting biomedical areas including designs of vascular grafts,[1,2] prevention of blood vessel diseases,[3,4] and developments of biologically active biomaterials.[5] Implant of vascular grafts or other blood-contacting materials induces a series of biological events such as protein adsorption, platelet adhesion, inflammatory reactions and ultimately implant failures. Whereas adsorption of proteins on the implant surface occurs within seconds after implanted, cellular interaction such as platelet aggregation and inflammatory reactions are relatively long-term processes taking minutes to months. The behavior and compositions of the protein layers on the implant surface mediate subsequent blood cell interactions and may be dependent on surface chemistry and residence time.[6-8] Changes in the state of fibrinogen once adsorbed to Biomer, which has been well characterized and widely

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employed as biomaterials, have previously been detected.[9-16] In their studies, fibrinogen was adsorbed and then the fibrinogen-adsorbed surface was placed in buffer for a period of time ("residence time") prior to assay. The ability of the adsorbed fibrinogen to bind platelets or antibodies decreased with residence time,[9] indicating its postadsorptive behavior. Furthermore the elutability of the adsorbed fibrinogen by solution of sodium dodecyl sulfate and plasma also decreased with residence time.[11] Monoclonal antibodies had interacted with three different specific domains such as RGD and RGDS amino-acid sequences in the fibrinogen adsorbed[13] which are known to bind to platelets. From the observations of postadsorptive behavior and the existence of peptide domains specific to cell adhesion, those results suggested that the adsorbed proteins may undergo conformational changes such as spreading and unfolding on the surface, probably leading to conformational changes of platelet adhesion domains of the adsorbed proteins.

In this study, the effects of both protein adsorption and its residence time on platelet adhesion on elastic polymer surfaces were analyzed for their applications to the designs of better blood-contacting materials. Initial adhesion of platelets on the protein-adsorbed surfaces was investigated by perfusing whole blood in vitro over differently treated surfaces with *in situ* short term epifluorescence video-microscopy. The blood perfusion was performed at three different conditions: directly on bare polymer surfaces, after protein pre-adsorption and after residence in buffer of the surfaces protein pre-adsorbed. The results showed that the extent of the surface coverages of the platelets initially adhered was dependent on the treatment conditions, indicating that all the factors in this study such as polymeric surface properties, protein pre-adsorption and its residence seemed to have their own effects on adhesion of platelets.

Experimental

Materials. Silicon rubber (SR), silicon-urethane copolymer (SU), poly(ether urethane) (PEU), poly(ether-ether urethane) copolymer (PEEU) and poly(ether urethane) derivertized with the anti-calcifying agent hydroxyethane bisphosphate (PEU-HEDP) were employed as film surfaces coated on the glass coverslips. While a solution of PEEU (commercial brand: Biomer) was obtained from Ethicone Inc. (NJ, USA), the solutions of PEU, SR and SU were supplied by Carbomedics Inc. (TX, USA). All the polymers were supplied as solutions (2% w/v) and each polymer solution was cast on a glass coverslip (Fisher: 24×50 mm, thickness 0.17 mm) as described below.

Polymer Cast. Superficially clean coverslip of Fisher Scientific (PA; USA) was further cleaned by sonication in deionized water for 30 min, and then dried in vacuum. Each polymer solution was homogeneously cast using photoresist spinner of Headway Research Inc. (TX; USA) ranged 200 to 3,000 rpm dependent on the polymer solution properties. When a heterogeneous film was obtained, the surrounding temperature around the spin caster was controlled to get a homogeneous polymer phase by supplying heat during the casting process. The homogeneousity of the clear film was essential for an observation of blood cell behavior with an epifluorescence video-microscopy.

Sample Preparation. Adsorption of human blood plasma protein on the cast film surfaces was performed at the following three different conditions for the tests of initial platelet adhesion by epifluorescence video microscopy (EVM). The films were treated by immersing 1) in 50 mL phosphate buffered saline (PBS) at pH 7.4 at room temperature for 2 h, 2) in 1% (v/v) blood plasma in PBS for 2 h, 3) in PBS for 3 days which was in advance soaked in 1% blood plasma solution for 2 h. The PBS solution containing the samples was replaced with fresh one every day for 3 days.

Fibrinogen Adsorption on Elastic Polymer Substrates. In a separate experiment, an estimation of fibrinogen adsorption on the surfaces was performed by employing I^{125}-labeled fibrinogen as described in others.[9,10] Sufficient I^{125}-labeled fibrinogen was added to the plasma prior to dilution to give a specific activity of 2,000 cpm/μg and the labeled fibrinogen comprised 5% or less of the total fibrinogen pool. The amount of the fibrinogen adsorbed on the employed polymer surfaces was measured by counting I^{125}-labeled

fibrinogen after soaking the polymer substrate in the PBS solution containing 1% blood plasma serum for 2 h. The blood was obtained in one-tenth volume of 3.8% trisodium citrate. Plasma was prepared by centrifugation at 5,000 g and 4°C until cell-free, and stored frozen at -70°C. Fibrinogen was prepared from plasma using a technique developed by Horbett,[11] separating by poly(ethylene glycol) and β-alanine sedimentation. The fibrinogen obtained was pure, showing 97% clottability and no other proteins of blood plasma. Labelling of iodine isotope (I^{125}) was performed by sodium iodide technique.[17]

Epifluorescence Video Microscopy.

Video Hardware and Optics: Epifluorescence video microscopy equipment was in detail described elsewhere.[18,19] In brief, a Leitz Fluovert, inverted-stage microscope was employed with a 40x, oil immersion objective lens (Fluotar, Leitz; Germany). The illumination source was an 100 W mercury vapor lamp, and the light was filtered through an external neutral density filter (average transmittance 15%), an internal heat absorbing filter, and through an E3 epifluorescence filter cassette (cassette containing a band pass excitation filter at 436 nm with width at half-height of 7 nm; a reflection short pass filter dichroic mirror at 475 nm; and a long pass suppression filter at 490 nm). The illumination intensity under these conditions was measured through the objective at 325, 442, 514, and 633 nm with a power meter (Steintech model 45 pm; TX, USA), as was total illumination intensity. The size of the illumination beam through the objective was measure by projection on a piece of white paper in focus. A silicon-intensified target video camera (Dage-MTI SIT Model SIT-66X) was used to image the fluorescent objects. The microscope stage was steadily moved under the control of a DC motor attached to the stage gear system in custom fashion. The stage speed was controlled by varying the DC signal, which is provided by a DC power supply. The stage speed was controlled at a speed of 32 μm/s, which was in previous typically employed for observation of thrombosis on polymeric substrates without blood cell excitation.

Flow Chamber Preparation: A parallel-plate flow chamber was utilized as shown in Figure 1, similar to one previously described.[20-22] Briefly, a Lucite body with blood inlet and outlet contained flow distribution slots which directed the blood to one surface of the body, which surface formed

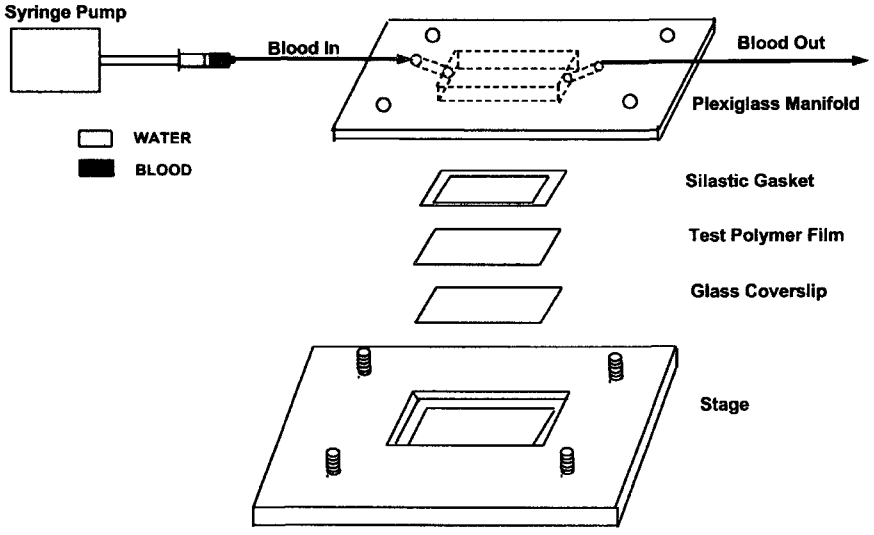

Figure 1. A view of the flow chamber employed in blood contact studies. All the stuffs of glass coverslip, test polymer film, silastic gasket and plexiglass manifold were assembled into the stage as shown and then the stage was tighten by screw and nut. The assembly on the stage was mounted directly on a microscopy for viewing with epifluorescence illumination and the images of platelets perfused by syringe pump were transferred into the image processer for an observation of their surface area.

one wall of the parallel plate chamber. A silastic gasket(24 mm × 50 mm, 0.25 mm thickness) was used to separate the body from a glass coverslip (24 mm × 50 mm) which formed the second wall. The test material was cast upon the coverslip as described above, the gasket provided mechanical support to keep the film from lifting off the coverslip. The thickness of the flow channel was determined by the depth of a milled-out deck within a metal base, which was bolted to the Lucite body to hold the coverslip in place. The depth of the deck was designed to be equal to the thickness of the coverslip (0.17 mm) plus the film thickness.

Blood Preparation. Human blood was withdrawn according to the previous protocol[20-22] from the antecubital vein of healthy nonsmoking adults, after informed consent, who had refrained from taking any drugs for 10 days. 20 mL blood was collected through a VenoJect butterfly set into a plastic syringe containing heparin (Sigma: MO, USA; final concentration in blood: 2 units/mL) and mepacrine (Sigma: final concentration in blood 2 M; absorbance maximum at 440 nm, emission maximum at 505 nm) in saline (<1% of blood volume). The anticoagulant and fluorophore were gently mixed by inversion of the syringe several times. The blood from donors had been previously demonstrated to not aggregate in response to heparin added. If aggregation did occur, it was obvious during perfusion, where the aggregates were readily observed attaching to the substrate.

Blood Perfusion. Perfusion of the chamber followed previous protocol[20-22] and was accomplished by employing an infusion mode syringe pump connected to the flow chamber shown in Figure 1. The wall shear rate was controlled at 100 sec^{-1} with a DC motor, a shear rate characteristic of the venous system. The sample stage was moved forth and back several fields of view for upto 6 min, and additionally video was recorded. This permitted acquisition of images of nearby fields after extended illumination and without prior illumination. On each of the substrates at the exposure times utilized, the surface area of platelets adhered within fields of view could be quantified by digitally image processing measurement. The ratio of these values on each field was computed by dividing the surface area of the adhered platelets at the end of the extended exposure period by the whole surface of each field. For minimization of experimental error both the beginning and ending portions of the polymeric substrate were neglected, thus obtaining as experimental results the middle portions only of the sample surfaces.

Experimental Results

Measurement of short term fibrinogen adsorption on the elastic polymer surfaces was performed to understand adsorption behavior of blood plasma protein and its mediating effects on initial platelet adhesion. Since fibrinogen is the major protein among numerous plasma proteins in blood to induce platelet adhesion, it was chosen as a model protein in plasma proteins. The adsorption of fibrinogen was clearly observed by measuring the I^{125}-labeled fibrinogen adsorbed on the surface which was immersed in PBS containing 1% plasma for 2 h. This result showed that the amount of fibrinogen adsorbed was dependent on the chemical compositions of the substrates employed (Figure 2). While both silicone-rubber (SR) and silicone-urethane (SU) substrates induced least amount of fibrinogen adsorption, poly(ether urethane) (PEU) and poly(ether urethane) with the anti-calcifying agent hydroxyethane bisphosphate

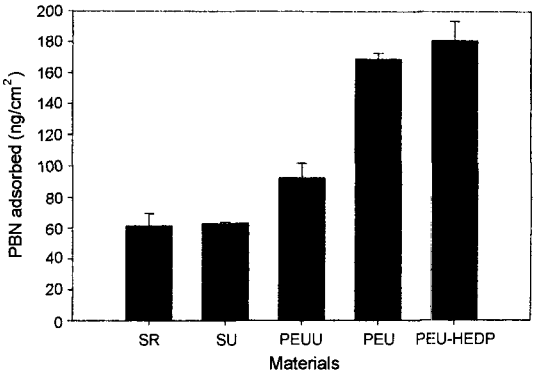

Figure 2. Amount of I^{125} labelled fibrinogen (FBN) adsorbed for 2 h on the elastic polymeric surfaces. The materials were the polymeric substrates coated on the glass coverslip, where SR is silicon rubber, SU silicon-urethane copolymer, PEU poly(ether urethane), PEEU poly(ether-ether urethane) copolymer and PEU-HEDP poly(ether urethane) derivertized with the anti-calcifying agent hydroxyethane bisphosphate (PEU-HEDP).

(PEU-HEDP) induced largest amount of fibrinogen adsorption. Poly(ether-ether urethane) copolymer (PEEU) showed medium amount of fibrinogen adsorption among the surfaces employed.

The combined effects on platelet adhesion of both polymer's bare surface property itself and instantaneous protein adsorption on its bare surface during blood perfusion were tested. The surfaces without previous protein adsorption, i. e. no previous protein adsorption and no residence in PBS, were vacuum-dried to remove solvent in the substrates and then *in vitro* tested after soaking in PBS for by blood perfusion at 100 sec^{-1} venous flow rate for 6 min. Figure 3 shows the results of the surface area of initial platelet adhesion observed by EVM on the five different fields of elastic polymer surfaces without previous adsorption of human plasma proteins. Their platelet adhesion results showed dependence of surface properties, leading to differences in the surface area of initial platelet adhesion. The SU surface induced the largest surface area of platelet adhesion compared to the surface area induced by others (7 times higher than the others). The surfaces of SR, PEUU, PEU and PEU-HEDP substrates induced relatively smaller surface areas of initial platelet adhesion and there were no big difference among them. Adhesion of platelets was mediated by the effects of both surface property and proteins instantaneously adsorbed from the perfusing blood.

The effect of an in advance protein adsorption on initial platelet adhesion was clearly observed by comparing the differences in initial surface area of platelet adhesion on the substrates soaked in 1% blood plasma for 2 h but treated no further (Figure 4). All the substrates except the SU surface with protein pre-adsorbed, i. e. 1% blood plasma pre-adsorption for 2 h with no subsequent residence in PBS, induced more platelet adhesion compared to the results of the blood perfusion of the corresponding bare surfaces (Figure 4 vs Figure 3). Interestingly, the SU surface with protein pre-adsorbed showed less surface area of platelet adhesion than that without protein pre-adsorbed, indicating that the bare SU surface was more *thrombogenic*. On the other hand the PEU-HEDP surface showed similar amount of platelet surface coverage compared to that without protein pre-

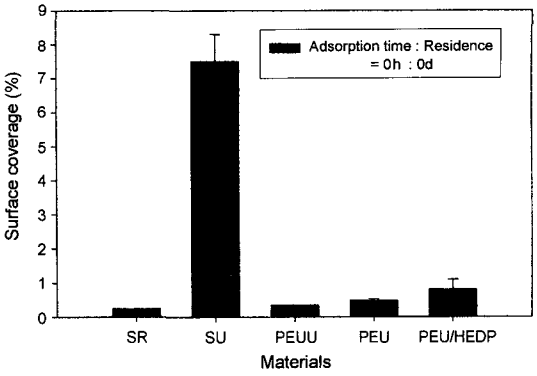

Figure 3. Initial surface area of the platelets adhered on the elastic polymer surfaces. Each surface without any protein pre-adsorbed was exposed to the dynamic blood, thus inducing instantaneously both adsorption of plasma proteins and adhesion of platelets. The surface areas of platelets adhered was fractioned over each field observed by epifluorescence video microscopy. The indications of the samples are the same as in Figure 2.

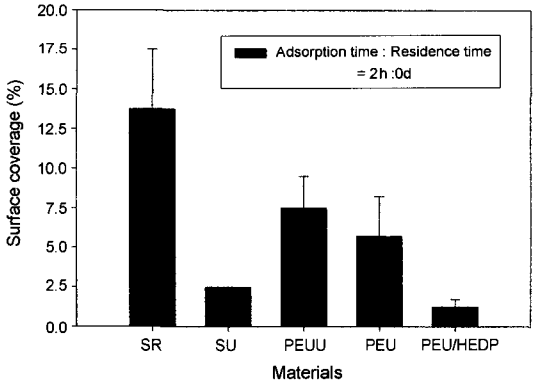

Figure 4. Initial surface area of the platelets adhered on the elastic polymer surfaces after its immersion in 1% blood plasma protein for 2 h. Each sample was exposed to the dynamic blood and the surface areas with platelets adhered was fractionated over each field observed by epifluorescence video microscopy. The indications of the samples are the same as in Figure 2.

adsorbed, showing no significant effect of protein adsorption on initial adhesion of platelets.

The effect on the initial platelet adhesion of morphological reorientation or/and possible desorption of the proteins adsorbed over residence in buffer solution was clearly observed by perfusing blood on the substrate after residing the protein-adsorbed substrate in PBS for 3 days (Figure

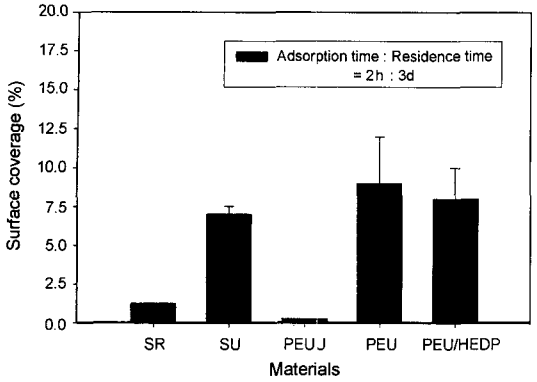

Figure 5. Initial surface area of the platelets adhered on the elastic polymer surfaces after sample's immersion in 1% blood plasma for 2 h and its subsequent residence in PBS for 3 days. Each sample was exposed to the dynamic blood flow and the surface areas with platelets adhered was fractioned over each field observed by epifluorescence video microscopy. The indications of the samples are the same as in Figure 2.

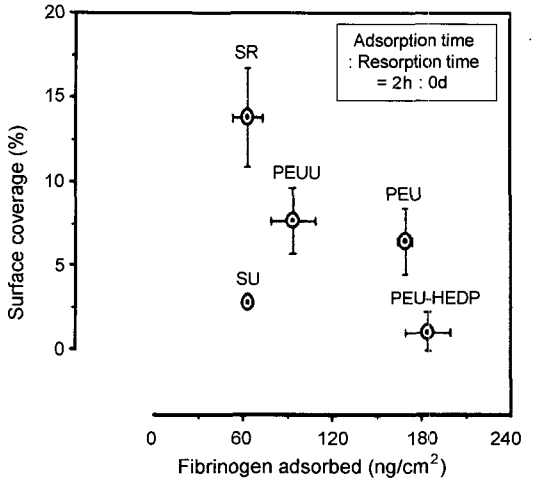

Figure 6. Relationship between the amount of fibrinogen adsorbed on the elastic polymer surfaces for 2 h and their initial surface coverages of platelets observed by an epifluorescence video microscopy. Each sample was exposed to the dynamic blood and the surface areas with platelets adhered was fractioned over each field observed by epifluorescence video microscopy. The indications of the samples are the same as in Figure 2.

5: 2 h blood plasma pre-adsorption and then its residence in PBS for 3 days). Residence in buffer made changes in platelet surface coverages. The SR and PEEU substrates showed decreases of platelet surface coverages from 14% and 7.5% when soaked in the 1% plasma solution (Figure 4) to 1% and 0.3%, respectively, when further resided in PBS for 3 days (Figure 5). On the contrary, while the SU and PEU-HEDP substrates showed approximately 2.5% and 1.3% surface coverage when soaked in the 1% plasma solution, those increased to 7.0% and 8.0%, respectively, when the protein-preadsorbed substrates further resided in PBS for 3 days. Their results were significantly different in statistics, verified with ANOVA comparison at $\alpha = 0.05$. Changes in protein morphology on PEUs were previously inferred from decreased platelet adhesion[9] as well as decreased fibrinogen elutability and anti-fibrinogen binding.[12] The 7.0% platelet surface coverage of the SU substrate after its residence in PBS solution was similar to that of its bare surface. On the other hand, there was nearly no difference in platelet-adhered surface coverages of the protein-preadsorbed PEU substrate whether it resided in PBS or not. These results suggested that initial platelet adhesion was dependent on each conditions such as substrate properties, protein pre-adsorption, subsequent residence as observed in Figure 3, 4 and 5.

An overall relationship between the amount of fibrinogen pre-adsorbed for 2 h and the initial EVM surface coverage of platelets adhered on all their substrates was comprehensively plotted on Figure 6. Striking results were shown on the substrates of the PEU-HEDP and SR substrates in terms of platelet-inducing fibrinogen adsorption and initial platelet adhesion. The PEU-HEDP substrate attracted the most amount of fibrinogen from the 1% plasma solution but its induction of initial platelet adhesion was minimal among the substrates tested. Reversely the SR substrate induced minimal amount of fibrinogen but it showed maximal amount surface coverage of platelets adhered, probably indicating importance of orientations of protein domains inducing platelet adhesion.

Discussion

Understanding of protein adsorption and its subsequent morphological (re)orientation on the implant is important since the layers and cell

adhesion domains of the adsorbed proteins mediate cellular interactions with implants. Interaction and subsequent morphological organization of the adsorbed proteins are dependent on the chemical composition of implant surfaces, and surface receptors of each cell including platelets differently responded to their ligand protein domains. While fibrinogen in blood plasma is a well-known protein that enhances adhesion and aggregation of platelets when adsorbed, but not when fluidized. Fibrinogen was in this study employed as a model protein for an observation of adsorption of platelet adhesion-medicating proteins in numerous proteins in blood, and protein pre-adsorption and substrate residence in buffer were employed as factors influencing its morphological changes of the proteins on the initial platelet adhesion of medically important some elastic polymeric surfaces such as urethane and silicone derivatives. Understanding of the relationships of adsorption of plasma proteins and adhesion of platelets may guide for the development of better blood-compatible implants.

Adsorption of plasma proteins was clearly surface chemistry-dependent from the measurement of I^{-125} labelled fibrinogen adsorbed (Figure 2). The amount of fibrinogen adsorbed on the corresponding surface was strikingly different from the surface coverage of platelet adhered (Figure 3 and 4), when whole blood was perfused on each surface treated either with or without pre-plasma. The SR surface which showed least amount of fibrinogen adsorption induced largest surface area of platelets when its bare surface, i.e. with no pretreatment with plasma, was exposed to the perfusing whole blood (Figure 6). On the other hand the SU surface was quite the reverse. While the SU surface showed least amount of fibrinogen adsorption and platelet adhesion when its bare surface was exposed directly on the perfusing blood, it induced largest surface area of platelet adhesion when its surface was pre-adsorbed with dilute plasma for 2 h. These contrast results indicated that induction of platelet adhesion was dependent on the treatment conditions and fibrinogen itself may be not the only factor to induce adhesion of platelets. If the layer of fibrinogen adsorbed is the only factor to control platelet adhesion, the surface area of the platelets initially adhered would follow the order of the amount of the adsorbed fibrinogen. However, from the above three observations of the surface area of initial platelet adhesion and the amount of the absorbed fibrinogen, it is concluded that some factors other than just fibrinogen adsorption may interfere with platelet-surface interaction. Since normally numerous proteins exist in the blood plasma and there are unique cell adhesion domains in these proteins, e. g. two arginine-glycine-aspartic acid sequences in both ends of the fibrinogen's long chain, the initial orientation of the adsorbed proteins inducing platelet adhesion may be important as postulated in Figure 7. Even though the adsorbed fibrinogen was initially large in quantity (Figure 7A), if the orientation of its cell adhesion domains was not adequate to induce adhesion of perfusing platelets (Figure 7B), the possibility of its induction of initial platelet adhesion would be less than that of properly oriented fibrinogen could (Figure 7C). Reversely even though cell adhesion proteins were not large in amount, if their cell adhesion domains were properly exposed to the perfusing platelets the surfaces might induce adhesion of more platelets. This may be one of the reasons why the amount of fibrinogen adsorption and the surface coverage areas of platelet adhesion on the corresponding surfaces were not correlated well as shown in Figure 2, 3, 4 and 5.

The results of platelet adhesion on the surfaces pre-adsorbed with dilute plasma for 2 h was very different from those of the corresponding surfaces by further residence in buffer for 3 days, Figure 4 vs. Figure 5; i. e. 3 days substrate residence in PBS vs. no residence). The amount of platelet adhesion on both the SR and PEUU substrates decreased significantly after their residence in PBS. On the other hand while the PEU surface was nearly the same extent of the surface area of the initial platelets adhered even after its residence in PBS, the SU and PEU-HEDP surfaces showed even in-creases in the surface area of the initial platelet adhesion after their residence in PBS. These contrary results of initial platelet adhesion after their residence in buffer may be from morphological reorientation of platelet adhesion domains of the proteins, i.e. covering

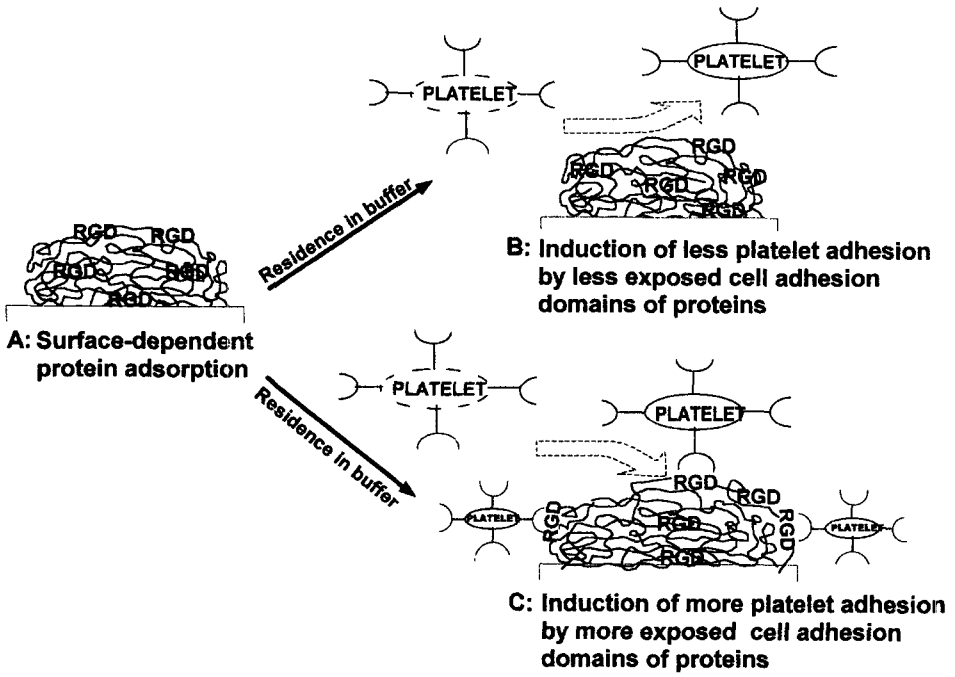

Figure 7. A putative mechanism of platelet adhesion on the elastic polymer surfaces. Proteins may adsorb on the surfaces dependent on their surface properties (A). The amount, layers and morphological orientations of platelet adhesion domains of the adsorbed proteins may affect on the surface coverage of platelets adhered. The adsorbed proteins may expose or cover the cell adhesion domains to the perfusing platelets dependent on the surface composition or residence in physiological environment. The surfaces containing more exposed cell adhesion domains may induce more platelet adhesion (B) but those containing less exposed ones do less platelet adhesion (C) independent of the adsorbed cell adhesion proteins such as fibrinogen.

and exposing effects of cell adhesion domains of the proteins over their residences in buffer (Figure 7B and C). The SR and PEUU surfaces may let the cell adhesion domains such as arginic acid-glycine-aspartic acid amino acid sequences and other platelet adhesion domains of the adsorbed proteins expose outside over their residence, thus inducing adhesion of more platelets after residence than those without residence. According to this hypothesis, while the previously exposed cell adhesion domains of the SU and PEU-HEDP surfaces may be covered with non-platelet activating or platelet-passivating domains of proteins over their residence, those of the SU and PEU/HEDP surfaces did the reverse. These morphological changes of cell adhesion domains may come from the mobilities of the adsorbed, platelet-activating proteins and the adsorbed other proteins, such as platelet-inert albumin, in plasma as well. These proteins may cover or expose already adsorbed platelet adhesion-related proteins over residence time, making the surfaces less or thrombogenic dependent on their environmental conditions such as surface property and adsorbed protein behavior.

Conclusion

We tested by EVM initial adhesion of platelets on the elastic biomedical polymeric surfaces treated at different blood perfusion conditions such as bare surface property, protein pre-adsorption and further residence in buffer. Their results showed that both protein adsorption and initial platelet adhesion were dependent on the substrate surface properties and possibly subsequent morphological orientation of the pre-adsorbed proteins. From these results we postulated possible mecha-

nisms of platelet adhesion, that is initial platelet adhesion was controlled by the initial layers of platelet adhesion proteins, the initial orientation of platelet-activating domains and subsequent reorientation of the adsorbed proteins over their residence in buffer. This observation of initial platelet adhesion on the polymeric surfaces is expected to help understand mechanisms of biological interactions of implanted medical devices and may lead to better designs of biomaterials contacting with circulating bloods such as vascular grafts.

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