Blood compatibility of Poly(2-methoxyethyl acrylate)

—Design of a novel bio-interface—

Masaru Tanaka

Molecular Device Laboratory
Research Institute for Electronic Science
Hokkaido University

2003

CONTENTS

Chapter 1 Introduction

1-1 Research Background

Biocompatibility of biomaterials

Biomaterials are defined as materials which are used in contact with biological tissue : blood, cells, protein, and any another living substances (1). Since the early 1950s, polymers have been used in the medical field for a large number of important implants and devices (permanent, intermediate, or short term). They are used, for instance, as vascular, orthopedic and ophthalmologic implants, catheters, hemodialyzers, and blood bags (2-4). Multifaced aspects of research on biomedical polymers (1) are shown in **Table1-1**. End-use devices are manufactured starting from their original concept. Among many polymers having a potential for biomedical application, biocompatibility or blood compatibility (See **Table 1-1**), which is phenomenological concept, is the most important characteristic when a polymer is selected for practical use. On this account, it is necessary to find a suitable criteria for examining biocompatibility of targeted polymers. Indeed, many of the biomaterials are in contact with blood, and some undesirable events occur when blood proteins and cells interact with the polymers. Despite their extensive use, as well as numerous scientific and clinical investigations, the general problem of biocompatibility is not completely solved 50 years later (5-7).

When blood is exposed to artificial surfaces, several defense systems in organism, such as coagulation, complement, platelet, and immune systems, are activated. These systems are believed to be interrelated through common intermediates. To avoid the activation of the defense systems by the exposure of blood to foreign materials, many attempts to modify surfaces of them have been made (1,2). Many polymers have been developed in order to obtain blood compatible surfaces, and the mechanism of

Table 1. Multifaced aspects of research on biomedical polymers

Materials Design

Factors to be Controlled	**Biologically Derived Materials**	**Synthetic Materials**	**Biohybridized and Biomimicking Materials**
·hydrophilic ·hydrophobic ·microdomain ·cationic ·anionic	·celllosics ·chitin,chitosan ·dextran ·agarose ·colladgen (gelatin)	·polysiloxane ·polyester ·polyurethane · PEO (PEG) ·poly(methyl methacrylate) ·polyacrylamide ·PTFE ·polyolefin, polystyrene · <u>Newly designed materials</u>	·heparin conj. materials ·urokinase conj. materlas ·receptor mimicking materials ·biomimicking materials

Concept or Methodology

Implants	**Prosthesis**	**Blood Purification**	**Cell Separation**	**Biohybridization**	**Cell Cultivation**	**Drug Delivery**	**Bioanalysis Biosensing**	**Enzyme Immobilization**

Devices

·bone cements ·dental cement ·dressing ·contact lens ·ligament ·pacemaker	·vascular graft ·artificial heart ·stent	·artificial lung ·artificial kidney dialyzer filter ·heart valve ·LDL apheresis	·cell sorter	·artificial liver ·artificial skin ·artificial pancrea ·ligament ·pacemaker	→ Tissue Engineering		·bioreactor

Properties Demanded

Biocompatibility	**Biospecificity**	**Physical & Pysicochemical Capability**
·<u>blood compatibility</u> ·tissue compatibility ·bioinertness	·specific adsorption ·specific adhesion ·specific recognition ·cytokine production ·cell cycle determination	·mechanical strength ·selective permeation ·non-specific adsorption (adhesion) ·degradability

Fundamentals

Elucidation of structure-property relationship in interaction with biological elements

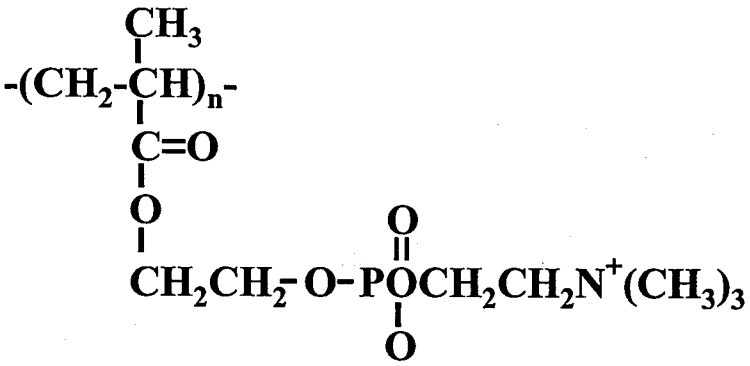
at molecular level
at molecular assembly level
at cellular level

expression of the compatibility responding to each the polymer has been proposed. These blood compatible surfaces can be classified into three main categories; hydrophilic surface (8-12), micro-phase-separated domain surface (13,14), and biomembrane like surface (15,16). (**Figure 1-1**).

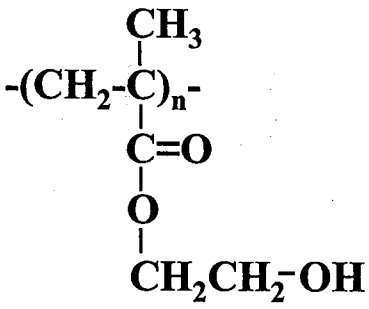
Since, the 1970s, a number of reports on biomaterials, providing us with evidence which shows the important role played by microdomain structures in realizing excellent biomedical properties. For example, segmented polyurethanes (17,18) and A-B-A type block copolymer (HEMA-St-HEMA) (13) (**Figure 1-1**) were shown to form microdomain structure and to exhibit excellent blood compatibility in both *in vitro* and *in vivo* examinations.

Nakabayashi and Ishihara et al. (15,16) have been studying blood compatible polymers with phospholipids polar groups (**Figure 1-1**). Their idea was to synthesize a polymer possessing a strong affinity for phospholipids from blood, which could be organized to form a biomembrane-like assemblage on the polymer surface. Phospholipid polymers were prepared by copolymerization of 2-methacryloyloxyethyl phosphorylcholine (MPC) with alkyl methacrylate.

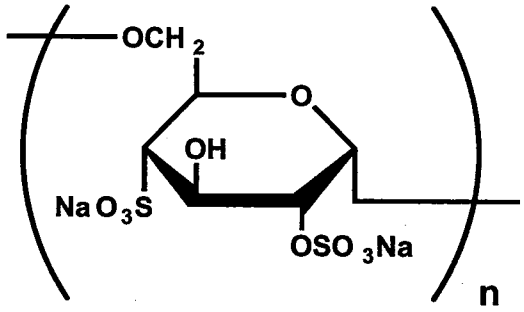
Hydrophilicity and hydrophobicity are the most fundamental properities to controlled for materials whatever they are utilized in biomedical devices. Poly(2-hydroxyethyl methacrylate) (PHEMA) (9) (**Figure 1-1**) is a widely used hydrophilic hydrogel in the medical device industry and is used in soft contact lenses and blood filters among others. Water soluble polymers were believed to be inert at the interface with any of the biological elements. A large number of researches have been carried out to obtain biocompatible hydrophilic surfaces by introducing water-soluble polymer grafts including poly(ethylene glycol) and polyacrylamide. However, it has occasionally been



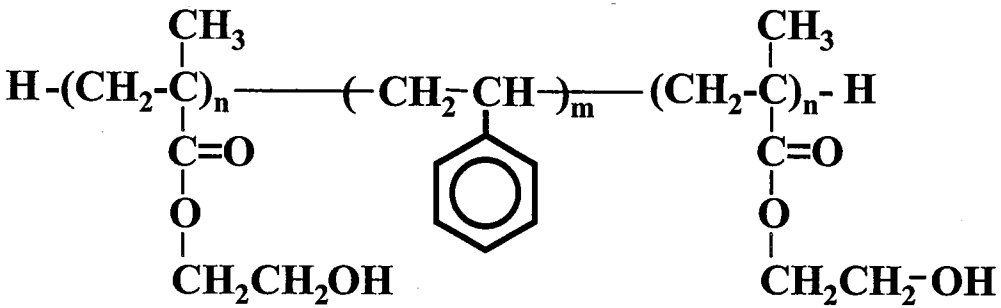
Poly(2-methacryloyloxyethyl phosphorylcholine)
(PPMC)
→ biomimicking surface



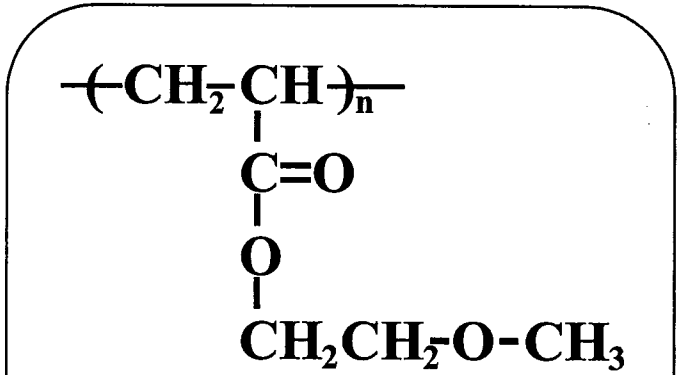
Poly(2-hydroxyethyl methacrylate)
(PHEMA)
→ hydrophilic surface



Polysaccharide
Biopolymer



HEMA-Styrene Triblock Copolymers
(HEMA-STY)
→ Microdomain surface



Poly (2-methoxyethyl acrylate)
PMEA
→ Newly designed surface

Figure 1-1. Chemical structures of blood compatible polymers.

observed that there is an optimum in the number of grafted chains for the surface to exhibit the best biocompatibility (9-11).

It is now well accepted that the initial rapid adsorption of proteins to polymeric surfaces affects the performance of these biomaterials. To minimize adverse reactions and to improve the biocompatibility of polymers, it is necessary to control the early stages of protein adsorption. Several surface modification techniques have thus been proposed to improve the biocompatibility of blood-contacting devices. These include the immobilization of biomolecules to prevent thrombus generation and platelet activation, the incorporation of hydrophilic grafts to reduce protein adsorption, the creation of microdomains to regulate cellular and protein adhesion, and chemical and physical surface modifications (19-26). The control of biocompatibility can also be achieved by the design of synthetic "biospecific" polymers, ie, polymers capable of biospecific molecular recognition (27).

It is now well established that protein adsorption is the first event following material-tissue contact, and the adsorption is generally nonspecific, with multiple binding sites on the proteins interacting with sites on the material surface (28,29). Nonspecific forces that must be considered are Van der Waal's forces, electrostatic double-layer forces, solvent-dependent interactions, hydrogen bonding, hydrophobic interactions, hydration forces, and steric forces (30). Moreover, depending on the chemical nature of the polymer, the interactions occur with various affinities, some being irreversible. Protein adsorption is the trigger for blood coagulation, which occurs when blood contacts polymeric biomaterials (31-33). Nonspecific protein adsorption on the surface occurs within minutes after the contact of the surface with blood (34). Blood is a complex mixture made up of water, salts, minerals, proteins, and cells. The latter

two components can interact with the foreign surface, be adsorbed, and be altered. Depending on the surface, protein adsorption varies in the thickness and the structure of the adsorbed layer. The more abundant proteins initially are adsorbed, but then are displaced by proteins that are less abundant but have a higher affinity for the surface. Protein adsorption to a biomaterial surface can be minimized by appropriate design of the interface, with modification of macroscopic surface properties such as the wettability. Such nonfouling surfaces can be obtained by coating with highly hydrated layers (35,36). Water-soluble polymers reduce thrombogenicity due to the unique hydrodynamic properties of the grafted macromolecules, and biomaterials grafted with polyethylene oxide or heparin are able to resist plasma protein adsorption and platelet adhesion, predominantly by a steric repulsion mechanism (37,38). Interestingly, Hubbell and colleagues also have reported the placement in situ of hydrogel materials by photochemical conversion of a liquid precursor to a solid hydrogel that can block cellular and biochemical interactions between the blood and the arterial intima (39,40). One of current ideas in the prevention of surface fouling by protein adsorption also involved tailoring the molecular interactions between the adsorbed proteins and the surface. The development of such bioactive biomaterials has been reviewed recently (26). For instance, biomaterials can be engineered by grafting peptides (41-43) or by using physical or chemical treatments (24,44,45) to create charges (eg, anionic groups, amino acids) on polymer surfaces, thereby mimicking sequences in natural molecules (27). Preparation of such biospecific polymers allows one to obtain new materials with specific biological requirements. The synthesis of biospecific random polymers has been applied successfully. The resulting biomaterials have been developed mainly to possess heparin-like anticoagulant properties or to modulate the immune response (46-49). For

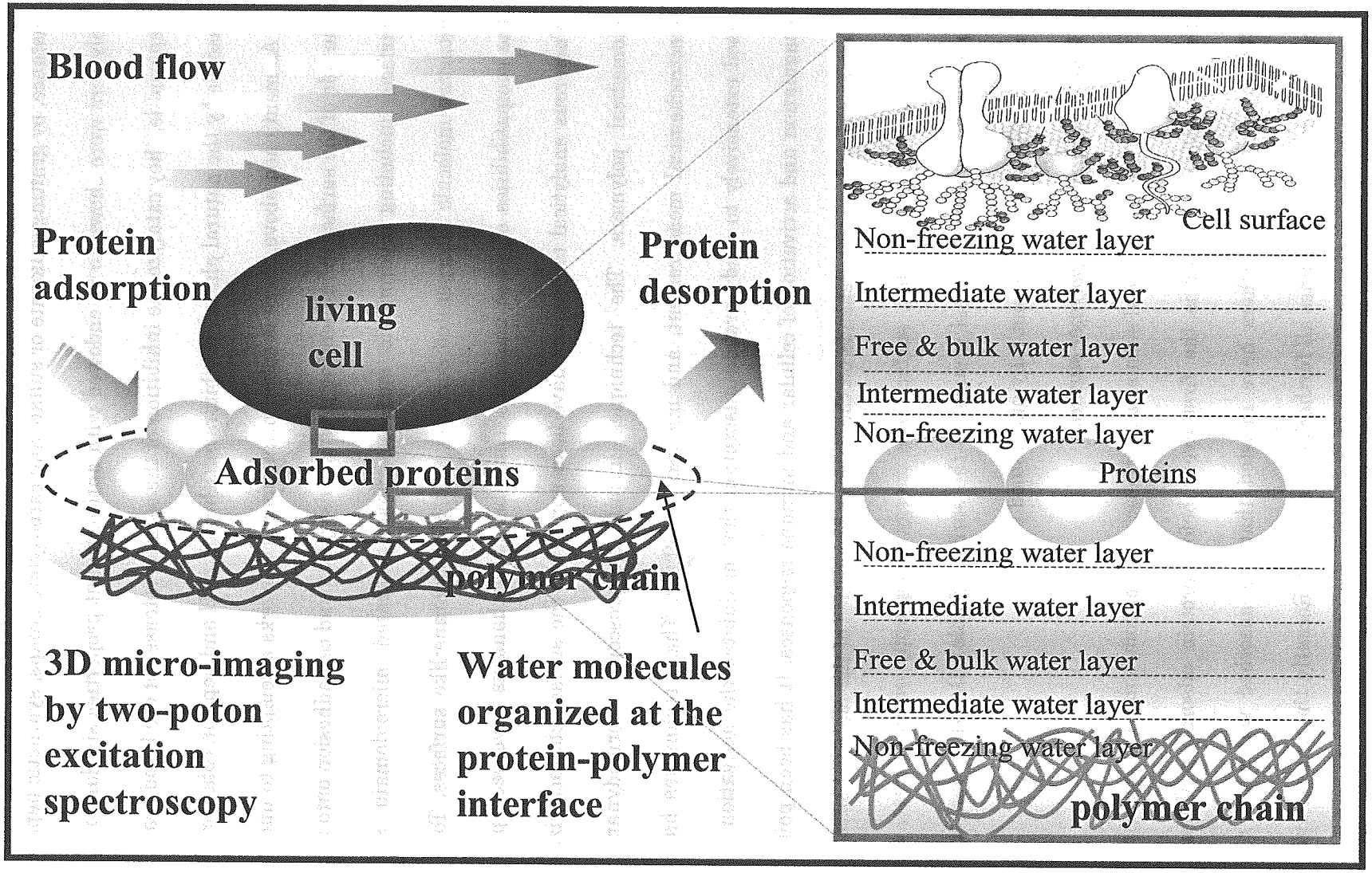


Figure 1-2. Schematic representation of the material and cell interface in blood.

instance, by grafting sulfonate or sulfate and carboxylate groups, synthetic heparin-like polymers were shown to be endowed with anticoagulant properties similar to those of heparin; ie, they catalyze the inhibition of the serine proteases of the blood coagulation cascade by the natural plasma inhibitors antithrombin III and heparin cofactor II.

As mentioned above, there is substantial amount of works attempting to understand the role of the surface in influencing protein adsorption and cell adhesion onto material surface, including hydrophilic surfaces, phase-separated micro-domain surfaces, bioactive molecule incorporated surfaces, and biomembrane-like surfaces. To explain the compatibilities of these materials a number of mechanisms have been proposed. Numerous analytical techniques have been applied to the surface characterization of biomedical polymers. The techniques include spectroscopy, thermodynamic and electrochemical measurement, and microscopy (50-53). These techniques have been used extensively to study protein adsorption that modulated the activation of blood coagulation and activation of cellular and immune responses. It has been pointed out that protein adsorption depends on the various properties of the material surface, e.g., surface charge, wettability, surface free energy, topography or roughness, and the presence of specific chemical groups on the surface (**Table1-1**). However, the blood compatibility of polymer is not fully understood (54).

Water structure on the polymer surface

When a foreign material such as a synthetic polymer comes into contact with blood, the adsorption of water is the first event before protein adsorption (**Figure 1-2**). Therefore, some researchers have insisted that the adsorbed water layer on the material surface determines all further events (55-60). Recently, the water structure on the polymer surface is one of the most important factors affecting the blood

compatibility (1,61-64). Most of the physical and the physiological properties of materials depend on the organization of water within and on the surface of the materials (65), and water molecule will take part in the reaction of cell such as adhesion, and morphological change. Many researchers have pointed out that the water structure on the material surface is an important factor for the material to express the good blood compatibility. Andrade et al. proposed that the important factor expressing the biocompatibility was not the amount of the adsorbed water on the surfaces but the structure of the adsorbed water (66). Tanzawa et al. suggested that the mobility of water correlates with biocompatibility (67-69). Recently, Israelachvili et al. (70) and Dequeiroz et al. (71) reported that the stability, density, and arrangement of the surface water molecules bring about the different capabilities of the polymer adhering to the proteins and blood cells. Grunze et al. suggested that the stability of the interfacial water layer could be one of the factors which resisted the protein adsorption (72-74). Kataoka et al. (75,76) and Ishihara et al. (77, 78) proposed that the total amount of the freezing bound water and free water content may be the key parameter for biocompatibility. On the other hand, it is well known that proteins and cells in blood have hydration shells composed of the non-freezing water, freezing bound water, and free water (**Figure 1-3**), and due to this hydration the components can keep their conformational structure stable (79,80). When the shell structure is changed or destroyed by contacting with a polymer material, they will be activated. What breaks the hydration structure of the shell? Park and Lu et al (81) indicated that when proteins adsorb to a polymer surface, hydrophobically-bound water molecules between the protein and the polymer surface need to be displaced (**Figure 1-4**). Thus, the major factor favoring resistance of protein adsorption will be the retention of the bound water.

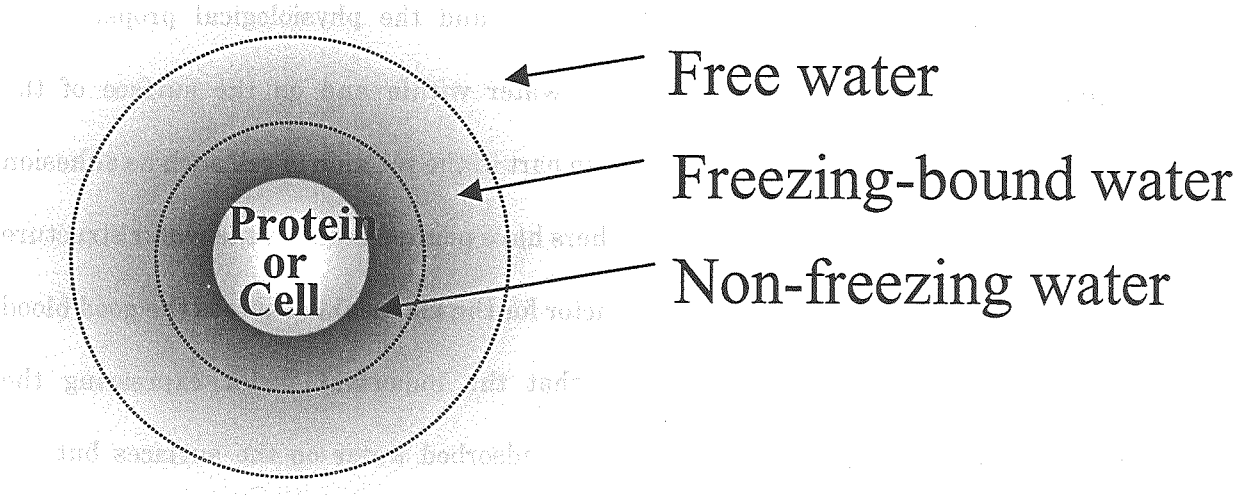


Figure 1-3. Three-state water structure model of hydrated materials.

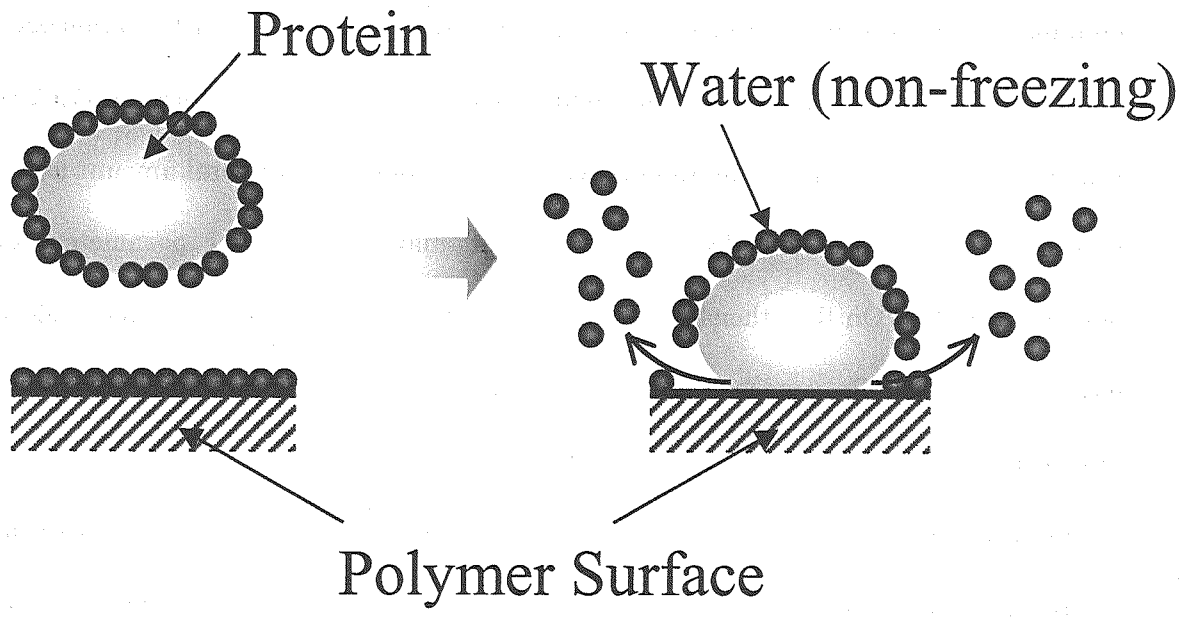


Figure 1-4. Schematic description of protein adsorption on the polymer surface.

In other words, the major factor favoring adsorption will be the release of the bound water, leading to a large entropy gain for the interface (82). The shorter the residence time of protein, the lower the chance for the protein adsorption and the conformational change. Thus the study on the nature of water on materials is very important to design more excellent biocompatible materials.

1-2 Purpose and Scope

Recently, we have developed a novel biocompatible surface prepared by poly(2-methoxyethylacrylate) (PMEA), and reported its blood compatibility. This polymer showed much-improved blood-compatibility with respect to such defense systems, and the absorbed proteins on PMEA surface scarcely denatured though those on other polymers denatured markedly. On the basis of these results, PMEA has been applied to an oxygenator (artificial lung) as a coating material, and the excellent blood compatibility as oxygenator was demonstrated in vitro evaluation test. Thus, the oxygenator coated with PMEA (X-coat®) recently commercialized in USA, Europe, and Japan. In order to clarify the reason why PMEA expresses the excellent blood compatibility, we have been characterizing PMEA from the viewpoint of the protein adsorption, the structure and the mobility of water in the polymer, and so on.

This doctoral thesis consists of 7 chapters. **Chapter 1** is "Introduction", in which the background of the research field is briefly summarized. In **Chapter 2**, we reported that PMEA showed excellent compatibility with platelets, white blood cells, coagulation system, and complement system. We investigated effects of PMEA-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass model. A PMEA-coated oxygenator was compared to a heparin-coated oxygenator and non-coated

oxygenator under conditions of in vivo circulation using human blood. **Chapter 3** is concerned with plasma protein adsorption onto polymer surfaces under a static condition. In addition, the conformational changes of the proteins on the polymer surfaces were investigated. The degree of the conformational changes of the proteins was discussed. The degree of conformational change of the protein adsorbed onto the surface was estimated by determining the α-helix content using CD. We report here the conformational changes of serum albumin and FNG adsorbed onto a PMEA surface and onto the various poly(meth)acrylates analogous polymer surfaces. We focused primarily upon the relationship between protein adsorption and platelet adhesion to the PMEA surface. **Chapter 4** deals with the adsorption behaviors of bovine serum albumin (BSA) and human fibrinogen onto the surfaces of PMEA were investigated by using a quartz crystal microbalance (QCM) under a dynamic condition. The QCM method could be applied to the analysis of the kinetics in the early stage of the adsorption reaction. We discuss the adsorption properties onto the PMEA and other polymers in terms of the maximum adsorption amounts as well as the apparent association constants, the adsorption and desorption rate constants of the proteins. In **Chapter 5**, we investigated the structural change of water in the PMEA using DSC in order to clarify the reason why PMEA exhibits low adsorption of proteins and low denaturation. Morerover, we characterized PMEA analogous poly(meth)acrylate, such as poly(2-hydroxyethyl methacrylate) and poly(2-methoxyethyl methacrylate) with respect to the chemical structure of the side chain, contact angle of water, water content, and water structure, and discussed the relationship between platelet compatibility and these properties. We also investigated the effect of the content of freezing bound water on the platelet compatibility by using poly(MEA-co-HEMA)s with various

copolymerization composition. We discuss the adhesion and spreading behaviors of platelet on the copolymer might be related to the amount of freezing bound water. **Chapter 6** describes the fabrication of highly regular porous-polymer films formed by simple casting technique and the applications for cell separators and cell-supported scaffolds. To simulate the leukocyte eliminating from human blood, the porous film which had been a novel biocompatible poly(2-methoxyethyl acrylate) coated was attached to a module. We discuss the biocompatible porous-polymer films for tissue engineering as novel biomedical interfaces. Concluding remarks are summarized in **Chapter 7.**

This thesis is the first report that the hypothesis for excellent blood compatibility of poly(2-methoxyethyl acrylate)(PMEA). The paper addresses an important issue related to the influence of the nature of bound/unbound water on blood compatibility. The most noticeable point is that the excellent blood compatibility is expressed by freezing bound water, which prevents the biocomponents from contacting the polymer surface or non-freezing water on the polymer surface.

1-3 References

(1) Tsuruta T. Contemporary topics in polymeric materials for biomedical applications. *Adv Polym Sci,* **126**:1 (1996)

(2) Horbett TA. Some background concepts. In: Biomaterials Science; An introduction to materials in medicine. Ratner BD, Schoen FJ., Lemons JE, ed. Academic Press., London. 1996;133-141,

(3) J.D. Andrade, in J.D. Andrade (Ed.), Surface and Interfacial Aspects of Biomedical Polymers, Plenum Publ., New York, 1985, p. 1.

(4) An YH, Friedman RI. Animal models of orthopedic implant infection. J Invest Surg 1998;11:139-146.

(5) Andrade JD. Needs, problems, and opportunities in biomaterials and biocompatibility. Clin Mater 1992;11:19-23.

(6) Courtney JM, Lamba NM, Sundaram S, Forbes CD. Biomaterials for blood-contacting applications. Biomaterials 1994;15:737-744.

(7) Ratner BD. Characterization of biomaterials surfaces. Cardiovasc Pathol 1993;2:87-100.

(8) Ratner, B. D.; Hoffman, A. S. *Hydrogels for Medical and Related Applications.* Andrade JD, Ed, ACS Symposium Series 31, American Chemical Society, Washington, D.C., 1976; pp1.

(9) Peppas, N. A. Ed. *Hydrogel in Medicine and Pharmacy, Vol.2*, CRC Press, Boca Raton, FL, 1987.

(10) Nagaoka, S.; Mori, Y.; Tanzawa, H.; Kikuchi, Y.; Inagaki, F.; Yokota, Y.; Noishiki, Y. *Trans. ASAIO.* **1987**, *10*, 76.

(11) Kulik, E.; Ikada, Y. *J. Biomed. Mater. Res.* **1996**, *30*, 295.

(12) Morra, M. J. *Biomater. Sci. Polymer Edn.* **2000**, *11*, 547.

(13) Okano, T.; Nishiyama, S.; Shinohara, I.;, Akaike, T.; Sakurai, Y.; Kataoka, K.; Tsuruta, T. *J. Biomed. Mater. Res.* **1981**, *15*, 393.

(14) Yui, N.; Sanui, K.; Ogata, N.; Kataoka, K.; Okano, T.; Sakurai, Y. *J. Biomed. Mater. Res.* **1986**, *20*, 929.

(15) Ueda, T.; Oshida, K.; Kurita, K.; Ishihara, K.; Nakabayashi, Y. *Polym. J.* **1992**, *24*, 1259.

(16) Iwasaki, Y.; Aiba, Y.; Morimoto, N.; Nakabayashi, Y.; Ishihara, K. *J. Biomed. Mater.*

Res. **2000**, *52*, 701.

(17) Flemming RG, Capelli CC, Cooper SL, Proctor RA Bacterial colonization of functionalized polyurethanes. Biomaterials 2000;21:273-281.

(18) John SF, Derrick MR, Jacob AE, Handley PS. The combined effects of plasma and hydrogel coating on adhesion of Staphylococcus epidermidis and Staphylococcus aureus to polyurethane catheters. FEMS Microbiol Lett 1996;144:241-247.

(19) Jacobs H, Grainger D, Okano T, Kim SW Surface modification for improved blood compatibility. Artif Organs 1988;12:506-507.

(20) Rubens FD, Weitz JI, Brash JL, Kinlough-Rathbone RL. The effect of antithrombin III-independent thrombin inhibitors and heparin on fibrin accretion onto fibrin-coated polyethylene. Thromb Haemost 1993;69:130-134.

(21) Hsu LC. Biocompatibility in heparin-coated extracorporeal circuits. Perfusion 1996;11:256-263.

(22) Okkema AZ, Yu XH, Cooper SL Physical and blood contacting characteristics of propyl sulphonate grafted Biomer. Biomaterials 1991;12:3-10.

(23) Skarja GA, Brash JL. Physicochemical properties and platelet interactions of segmented polyurethanes containing sulfonate groups in the hard segment. J Biomed Mater Res 1997;34:439-455.

(24) Ratner BD. Plasma deposition for biomedical applications: a brief review. J Biomater Sci Polym Edn 1992;4:3-11.

(25) Ito Y. Surface micropatterning to regulate cell functions. Biomaterials 1999;20:2333-2342.

(26) Hubbell JA. Bioactive biomaterials. Curr Opin Biotechnol 1999;10:123-129.

(27) Jozefowicz M, Jozefonvicz J. Randomness and biospecificity: random copolymers

are capable of biospecific molecular recognition in living systems. Biomaterials 1997;18:1633-1644.

(28) Brash JL, Ten Hove P Protein adsorption studies on 'standard' polymeric materials. J Biomater Sci Polym Edn 1993;4:591-599.

(29) Wojciechowski P, Brash JL. The Vroman effect in tube geometry: the influence of flow on protein adsorption measurements. J Biomater Sci Polym Edn 1991;2:203-216.

(30) Glantz PO, Arnebrant T, Nylander T, Baier RE. Bioadhesion-a phenomenon with multiple dimensions. Acta Odontol Stand 1999;57:238241.

(31) Courtney JM, Lamba NM, Sundaram S, Forbes CD. Biomaterials for blood-contacting applications. Biomaterials 1994;15:737-744.

(32) Brash JL, Scott CF, ten Hove P, Wojciechowski P, Colman RW Mechanism of transient adsorption of fibrinogen from plasma to solid surfaces: role of the contact and fibrinolytic systems. Blood 1988;71:932929.

(33) Yung LY, Colman RW, Cooper SL. The effect of high molecular weight kininogen on neutrophil adhesion to polymer surfaces. Immunopharmacology 1999;43:281-286.

(34) Cornelius RM, Brash JL. Adsorption from plasma and buffer of singleand two-chain high molecular weight kininogen to glass and sulfonated polyurethane surfaces. Biomaterials 1999;20:341-350.

(35) Hoffman AS. Non-fouling surface technologies. J Biomater Sci Polym Edn 1999;10:1011-1014.

(36) Peppas NA, Sahlin JJ. Hydrogels as mucoadhesive and bioadhesive materials: a review. Biomaterials 1996;17:1553-1561.

(37) Amiji M, Park K Surface modification of polymeric biomaterials with polyethylene

oxide), albumin, and heparin for reduced thrombogenicity. J Biomater Sci Polym Edn 1993;4:217-234.

(38) Leckband D, Sheth S, Halperin A. Grafted poly (ethylene oxide) brushes as nonfouling surface coatings. J Biomater Sci Polym Edn 1999;10:1125-1147.

(39) West JL, Hubbell JA. Separation of the arterial wall from blood contact using hydrogel barriers reduces intimal thickening after balloon injury in the rat: the roles of medial and luminal factors in arterial healing. Proc Natl Acad Sci (1 S A 1996;93:13188-13193.

(40) An Y, Hubbell JA Intraarterial protein delivery via intimally-adherent bilayer hydrogels. J Controlled Release 2000;64:205-215.

(41) Healy KE, Rezania A, Stile RA Designing biomaterials to direct biological responses. Ann N YAcad Sci 1999;875:24-35.

(42) Sakiyama SE, Schense JC, Hubbell JA. Incorporation of heparin-binding peptides into fibrin gels enhances neurite extension: an example of designer matrices in tissue engineering. FASEB J 1999;13:2214-2224.

(43) Sun X, Sheardown H, Tengvall P, Brash JL. Peptide modified gold-coated polyurethanes as thrombin scavenging surfaces. J Biomed Mater Res 2000;49:66-78.

(44) Stanislawski L, Serne H, Stanislawski M, Jozefowicz M. Conformational changes of fibronectin induced by polystyrene derivatives with a heparin-&ke function. J Biomed Mater Res 1993;27:619-626.

(45) McClung WG, Clapper DL, Hu SP, Brash JL. Adsorption of plasminogen from human plasma to lysine-containing surfaces. J Biomed Mater Res 2000;49:409-414.

(46) Migonney V, Fougnot C, Jozefowicz M. Heparin-like tubings. Biomaterials 1988;9:145-149.

(47) Migonney V, Souirti A, Jozefowicz M. Biospecific interactions of vitamin K-dependent factors with phospholipid-like polystyrene derivatives, part II: factor IX. Biomaterials 1997;18:1077-1084.

(48) Crepon B, Maillet F, Kazatchkine MD, Jozefonvicz J. Molecular weight dependency of the acquired anticomplementary and anticoagulant activities of specifically substituted dextrans. Biomaterials 1987;8:248-253.

(49) Thomas H, Maillet F, Letourneur D, Jozefonvicz J, Kazatchkine MD. Effect of substituted dextran derivative on complement activation in vivo. Biomaterials 1995;16:1163-1167.

(50) Werner C, Jacobasch Hj. Surface characterization of polymers for medical devices. Int J Artif Organs 1999;22:160-176.

(51) Siedlecki CA, Marchant RE. Atomic force microscopy for characterization of the biomaterial interface. Biomaterials 1998;19:441-454.

(52) Tengvall P, Lundstrom I, Liedberg B. Protein adsorption studies on model organic surfaces: an ellipsometric and infrared spectroscopic approach. Biomaterials 1998;19:407-422.

(53) Chittur HIC. FTIR/ATR for protein adsorption to biomaterial surfaces. Biomaterials 1998;19:357-369.

(54) Rathner, B.D. *J. Biomater. Sci. Polym. Edn.* **2000**, *11*, 1107.

(55) Rowland, S. P. Ed, *Water in polymer*, ACS Symposium Series 127, American Chemical Society, Washington, D.C., 1980.

(56) Ratner, B. D.; Horbett, T. A.; Hoffman, A. S.; Hauschka, S. D. *J. Biomed. Mater. Res.* **1975**, *9*, 407.

(57) Vogler, E. A. *J. Biomater. Sci. Polym. Ed.* **1999**, *10*, 1015.

(58) Vogler, E. A. *Adv. Colloid Interface Sci.* **1998**, *74*, 69.

(59) Sung YK, Gregonis DE, John MS, Andrade JD *J Appl Polym Sci* 26:3719 (1981)

(60) Lee HB, John MS, Andrade JD, *J Colloid Interface Sci*, 51: 225 (1975)

(61) *Water in Biomaterial Surface Science*; Morra, M., Ed.; JohnWiley & Sons, Ltd, Chichester 2001.

(62) Kitano, H.; Ichikawa, K.; Fukuda, M.; Mochizuki, A.; Tanaka, M. *J. Colloid Surface Sci.* **2001**, *242*, 133.

(63) Ichikawa, K.; Mori, T.; Kitano, H.; Fukuda, M.; Mochizuki, A.; Tanaka, M. *J. Polym. Sci., B Polym. Phys.* **2001**, *39*, 2175.

(64) Bajpai, A.K.; Shrivastava M. *J. Biomater. Sci. Polym. Ed.* **2002**, *13*, 237.

(65) Andrade, J. D.; Lee, H. B.; Jhon, M. S.; Kim, S. W.; Hibbs, J. B. *Trans. ASAIO*, **1973**, *19*, 1.

(66) Andrade, J. D.; Lee, H. B.; Jhon, M. S.; Kim, S. W.; Hibbs, J. B. *Trans. ASAIO*, **1973**, *19*, 1.

(67) Tanzawa, H. *Jpn. J. Artif. Organs* **1986**, *15*, 16.

(68) Yamada, N. A.; Ishikiriyama, K.; Todoki, M.; Tanzawa, H. *J. Appl. Polym. Sci.* **1990**, *39*, 2443.

(69) Yamada, N. A.; Tanzawa, H. *J. Appl. Polym. Sci.* **1991**, *43*, 1165.

(70) Israelachivili, J.; Wennerstrom, H. *Nature* **1996**, *379*, 219.

(71) Dequeiroz, A. A. A.; Barrak, E. R.; Decastro, S. C. *J. Mol. Struct. (THEOCHEM)* **1997**, *394*, 271.

(72) Wang, R. L. C.; Kreuzer, H. J.; Grunze, M.; *J. Phys. Chem.* B **1997**, *101*, 9767.

(73) Harder, P. Grunze. M.; Dahint, R.; Whitesides, G. M.; Laibins, P. E. *J. Phys. Chem.* B **1998**, *102*, 426.

(74) Feldam, K.; Hahner, G.; Spencer, N. D.; Harder, P.; Grunze, M. *J. Am. Chem. Soc.* **1999**, *121*, 10134.

(75) Kikuchi, A.; Karasawa. M.; Tsuruta, T.; Kataoka, K. *J. Colloid Interface Sci.* **1993**, *158*, 10.

(76) Kataoka, K.; Ito, H.; Amano, H.; Nagasaki, Y.; Kato, M.; Tsuruta, T.; Suzuki, K.; Okano, T.; Sakurai, Y. *J. Biomater. Sci. Polym. Ed.* **1998**, *9*, 111.

(77) Iwasaki, Y.; Fujiike, A.; Kurita, K.; Ishihara, K.; Nakabayashi, N. *J. Biomater. Sci. Polym. Ed.* **1996**, *8*, 91.

(78) Ishihara, K.; Nomura, H.; Mahara, T.; Kurita, K.; Iwasaki, Y.; Nakabayashi, N., *J. Biomed. Mater. Res.* **1998**, *39*, 323.

(79) Hazlewood, C.F.; Nicols. B. L.; Chamberlain, N. F. *Nature*, **1969**, *222*, 747.

(80) Uedaira, H. Water and metal cations in biological systems, Pullman, B.; Yagi. K. Eds, Japan Scientific Societies Press, Tokyo, 1980, pp47.

(81) Lu DR, Lee SJ, Park K, *J Biomater Sci Polymer Edn* 3:127-147,1991.

(82) Hoffman AS, *J Biomater Sci Polymer Edn* 10:1011-1014, 1999.

Chapter 2 Blood Compatibility of PMEA

2-1 Introduction

During the past decades synthetic polymers have been increasingly used in the fabrication of medical devices that make direct contact with human blood, for example, haemodialysis systems, extracorporeal circulation circuits, heart valves, blood by-pass tubes, prosthetic devices, catheters, etc. A major effort in this field of biomaterials technology has been directed towards developing biomaterials having improved blood ocompatibility. It is known that when blood contacts an artificial surface a complex series of interacting events occur: protein adsorption, cellular (mostly platelet) adhesion, activation and aggregation, activation of blood coagulation system, contact and complement activation and finally fibrin and thrombus formation. Many investigations have been carried out to prevent an artificial surface from provoking thrombus formation in order to form a blood compatible surface. Such investigations include the use of polymers with quite different properties such as for example hydrophilic, hydrophobic, zwitterionic and charged (anionic and cationic).

When blood contacting medical devices are used clinically, certain doses of an anticoagulant agent such as heparin, coumarine, etc. are administered in order to prevent thrombus formation and embolization. However, direct and systematic administration of these anticoagulants increases the risks of bleeding in the patient. This means that there is still an essential need to develop new compatible materials to be used in medical devices which will not need systemic anticoagulation as, for example, in the case of patients with acute renal failure having bleeding tendencies.

A significant part of current research for more blood compatible materials is focused on the surface modification of already existing materials with satisfying mechanical

properties rather than the development of new materials. Surface modification methods in order to improve the blood cmpatibility include chemical treatment (surface oxidation, fluorination, introduction of reactive groups, glow plasma discharge, surface grafting, etc.) (1-9)

More recently new surface modification techniques have been developed which include immobilization of specific biological molecules on the materials surface, mostly heparin and albumin (10-19). Heparin, an often-used anticoagulant is an anionic, highly sulfated mucopolysaccharide not having a uniform molecular structure. In an effort to counteract thrombogenicity while preventing bleeding caused by direct administration of heparin, many researchers tried to develop methods of attaching and binding heparin on biomaterials surface. Early work on antithrombogenic agents was done by Gott et al. (20). The method comprised treating a graphited surface first with Zephiran (benzalkonium chloride) and then with heparin providing a good non-thrombogenicity. Since then a lot of research has been done on this particular item so that a few years later review articles and books on this subject where published (21,22).

Generally, heparinized materials are of two major types:

Bulk heparinized materials, where heparin is included in or under a biostable or biodegradable polymeric material (depending on the application) throughout which it is progressively released at the blood–material interface. The efficacy of these materials depends on the duration of heparin release, which in vivo can range from weeks to months. And

Heparin coated materials having a surface layer of bound heparin which is thought to be capable of complexing in situ with Thrombin-antithrombin-III (AT III) and exerting

its anticoagulant effect.

Such heparin-coated materials can also be divided into three main categories:

(i) Materials formed simply by mixing heparin (normally in the form of heparin sodium) into a material. Such materials have the disadvantage that, while in contact with the blood, the heparin is washed out into the blood and the antithrombotic properties disappear in a short time.

(ii) Materials in which ionic bonding is effected between ionic residual groups in the heparin (COO^-, SO_4^{2-}, $NHSO_3^-$) and cationic residual groups in the material (fe. using ionic-bonded complexes of heparin and a polymerizable basic compound having a quaternary ammonium salt). Such a material has the disadvantage that the setting of conditions for polymerization of the ionic-bonded complex is difficult and discoloration of the material tends to occur, so obtaining an antithrombotic medical material of uniform product quality is difficult. Also attention must be paid that the ionic association of the cation and heparin anion must be sufficiently high to preclude blood from exchanging with the cation–heparin association and removing heparin from the surface of the polymer.

(iii) Materials in which covalent bonding is performed with the material, utilizing the hydroxyl, carboxyl or amino groups of the heparin. Usually, covalent immobilization of heparin to a biomaterial consists of activating the material in such a way that coupling between the biomaterial and functional groups on the heparin (–COOH, –OH, $–NH_2$) can be achieved.

However, the "perfect haemocompatible" material seems still an illusion in a field rather too complicated to provide simple and easy solutions.

Inflammatory response due to bioincompatibility of extracorporeal circuits is one of

the major clinical issues after a cardiopulmonary bypass (CPB). CPB has been linked to significant pathphysiological morbidity. Poor patient outcomes have been shown to be caused by perfusion and hemodynamic alterations (23-25), gas exchange. Many factors such as material-dependent bioincompatibility or material-independent ischemia-reperfusion or hypothermia, have been known to induce an inflammatory response during CPB : release of bradykinin, complement activation, leukocyte activation, increase in vascular permeability and subsequent leakage of endotoxin into the bloodstream (23,24). This inflammatory cascade may contribute to the development of postoperative complications, including respiratory failure, renal dysfunction, bleeding disorders and multiple organ failure. In particular, pulmonary edema associated lung injury is a problem of considerable clinical significance. During the last few decades, heparin-coated circuits for CPB have been studied, and their biocompatibility has been improved. Heparin-coated circuits are able to reduce complement activation and the subsequent release of cytokines which exert a well-known damaging effect on the lungs (24-38).

In this chapter, we reported that PMEA showed excellent compatibility with platelets, white blood cells, coagulation system, and complement system. We investigated effects of PMEA-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass model. A PMEA-coated oxygenator was compared to a heparin-coated oxygenator and non-coated oxygenator under conditions of in vivo circulation using human blood. We also investigated the platelet adhesion under a static condition.

2-2 Experimental

2-2-1 Materials

Various poly(meth)acrylates were prepared by radical polymerization using azobis-isobutyronitrile (AIBN) as the initiator. The monomers used were 2-methoxyethylacrylate (MEA), ethylacrylate (EA), 2-phenoxyethylacrylate (PEA), 2-hydroxyethylmethacrylate (HEMA), 2-hydroxyethylacrylate (HEA), and 2-ethylhexylacrylate (EHA). All monomers were obtained from Wako Pure Chemical Industries, Ltd., (Osaka, Japan). The molecular weights (Mw) of polymers were estimated by gel permeation chromatography (GPC), using polystyrene standards. Polypropylene (PP) and poly(ethyleneterephthalate) (PET) were used as polymer substrates. Methanol (MeOH) and tetrahydrofuran (THF) used as solvents were of analytical grade. Human plasma fibrinogen (FNG), was purchased from Sigma Co. Ltd., (St. Louis, MO), and used without further purification. Protein solutions were prepared in phosphate buffer (PBS, pH7.4). Deionized water from a Milli-Q (18MΩ-cm) system was used to prepare all aqueous solutions.

2-2-2 Preparation and characterization of the polymer surfaces

The samples of polymer surfaces for plasma protein adsorption and platelet adhesion were prepared as follows. The test polymers (PMEA, PHEMA, PHEA, PEA) were cast from a 0.1wt% MeOH solution, and PPEA and PEHA were cast from a 0.1wt% THF solution onto PP or PET plates, dried under air at room temperature, and then vacuum-dried for 24h. The wettability of polymer surfaces was characterized by contact angle measurement. PMEA and PHEMA were cast from a 0.1wt% MeOH solution, while PEHA was cast from a 0.1wt% THF solution. The polymers were then vacuum-dried for 24h. Prior to the experiment, the plate surfaces were washed three times with PBS.

2-2-3 Platelet adhesion test

Blood was drawn from healthy volunteers and mixed with a 1/9 volume of 3.8% sodium citrate. Platelet rich plasma (PRP) was obtained by centrifugation at 1200rpm for 5min, and platelet poor plasma (PPP) was obtained by centrifugation at 3000rpm for 10min (**Figure 2-1**). The blood was kept at 22°C before separation of the PRP. The platelet count in PRP was adjusted to 1×10^5 cells/μl by mixing PRP with PPP. The platelet concentration was determined by an automated hematology analyzer (Sysmex SE-9000, TOA MEDICAL ELECTRONICS, Tokyo, Japan). Then 200 μl (platelet number: 2×10^7) of the platelet suspension was placed on each polymer surface, and incubated for 30 min at 37°C. After washing the plates three times with PBS, the adhered platelets were fixed by immersing the paltes into 1% glutaraldehyde in PBS and allowing them to stand in the fixative for 60 min at 4°C. Samples were freeze-dried, and sputter-coated using a gold target (JUC-5000, JEOL Tokyo, Japan) prior to scanning electron microscopy (JSM-840, JEOL).

2-2-4 Washed platelets adhesion test

To prepare washed platelets, the PRP obtained as mentioned above under the section *2-3*, was diluted using on RCD solution (pH 6.5, 36mM citric acid, glucose 5mM, $MgCl_2$ 1mM, KCl 5mM, NaCl 100mM) containing 40ng/ml prostaglandin E_1(PGE_1) to prevent platelet aggregation (7). The diluted PRP was then centrifugated at 300rpm for 10 min. This procedure was repeated once more. After washing, the platelets were pooled and resuspended at a cell density of 1×10^5cells/μl in 50mM Tris/HCl buffer (pH 7.4, Tris/HCl 50mM, NaCl 140mM, glucose 5mM, $MgCl_2$ 2mM, PGE_1 40ng/ml). Then 200 μl (platelet number:2×10^7) of this platelet suspension was placed onto each polymer

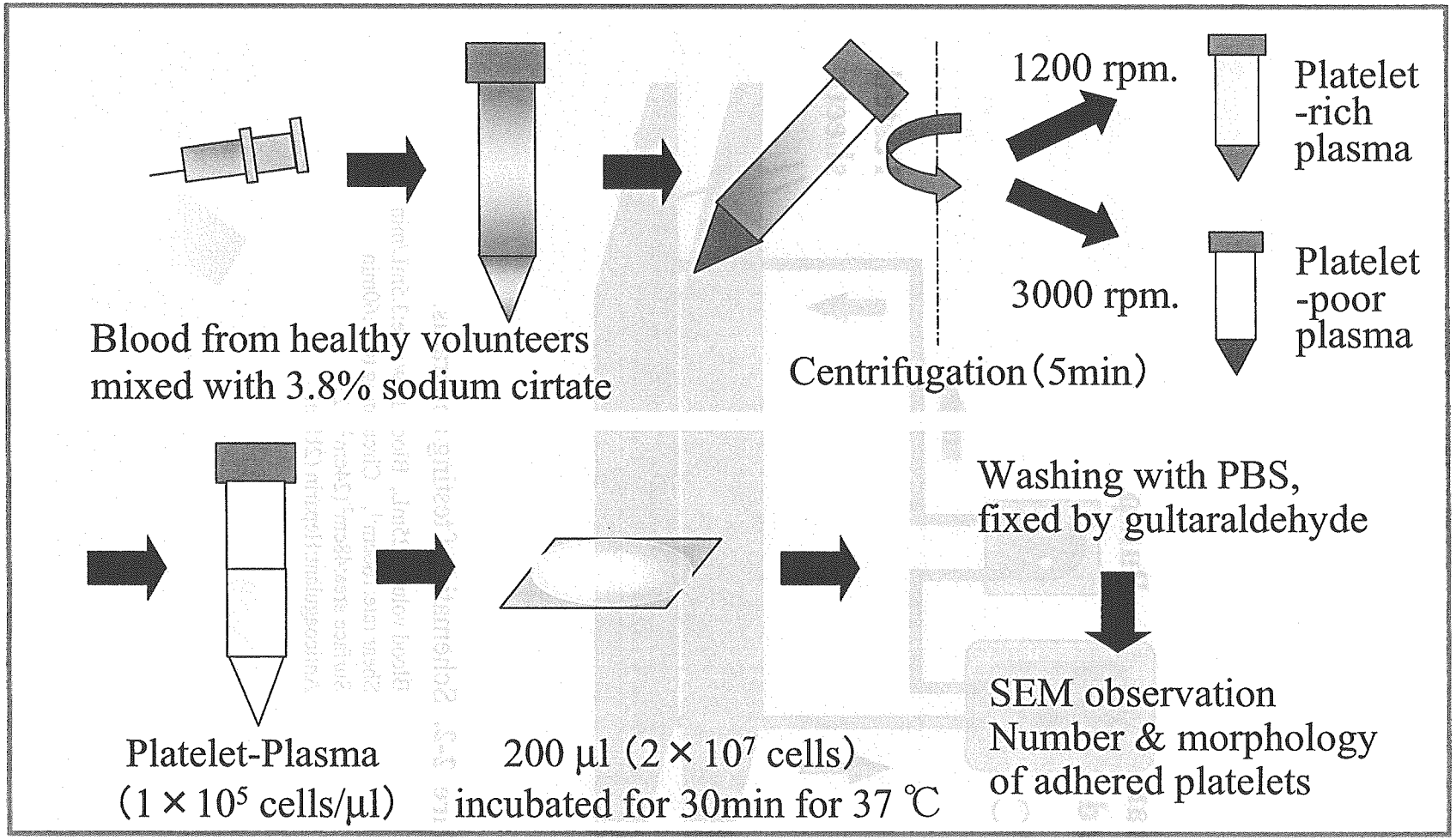
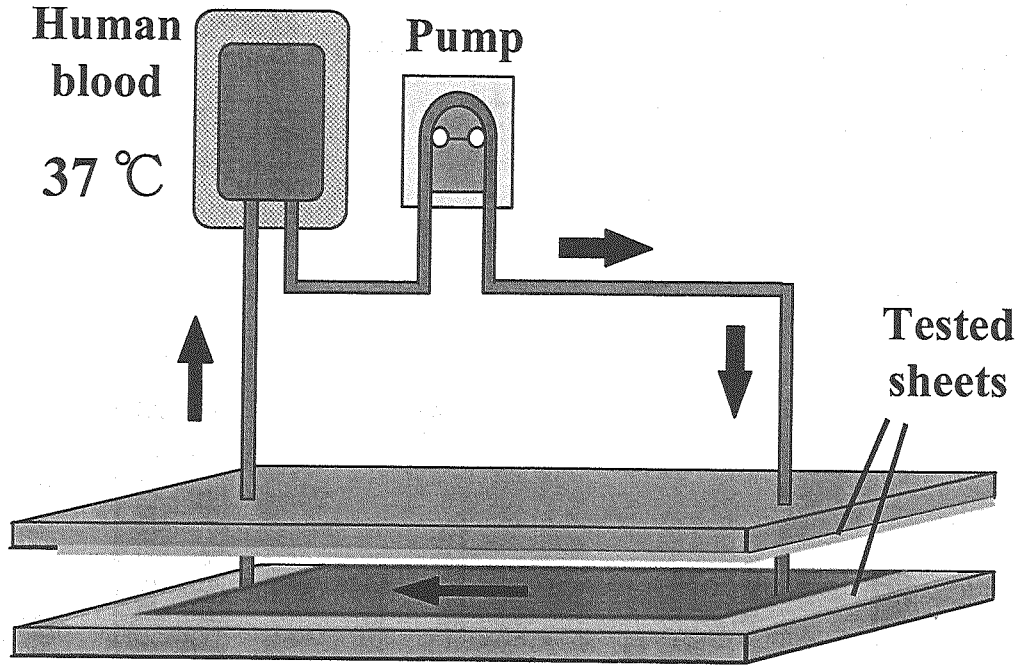


Figure 2-1. Human platelet adhesion test.



Fiure 2-2. Schematic of testing apparatus.

Blood volume:25mL, Blood flow rate:3.5mL/min
Shear rate:160cm^{-1}, Circulating time:60min
Surface area:48cm^2 (24cm^2 × 2)
Anticoagulant:Heparin (2U/mL)

surface precoated with FNG or noncoated, and incubated for 30 min at 37°C. The plate was coated by immersing it into FNG solution (0.3mg/dl). After washing the plate three times with PBS, the number of platelets adhered to the surface adhesion and the morphology of the adhered platelets were also evaluated by SEM.

2-2-5 Blood compatibility on circuits

First, we investigated blood compatibility of PMEA surface compared to various poly(meth)acrylate surface during human blood flow model with blood bag (**Figure 2-2**). In the present study we modified an in vitro closed-loop model with fresh human whole blood for the preclinical evaluation of the blood compatibility of PMEA. Each single loop consists of two polycarbonate connectors joined together by two pieces of silicone tubes. After circulation of 60 min, release of beta-thromboglobulin (β-TG) and lacoferrin, and production of thrombin-antithrombin-III complex (TAT) and terminal complement complex (TCC), as a indicator of platelet activation, leukocyte activation, coagulation and complement activation were measured.

Secondly, we investigated effects of PMEA-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass model (**Figure 2-3**). A PMEA-coated oxygenator (**Figure 2-4**) was compared to a heparin-coated oxygenator and non-coated oxygenator under conditions of in vivo circulation using human blood. The circuit consisted of polyvinyl chloride tubing connected to a soft-shell venous reservoir (Terumo, Tokyo, Japan). The entire blood-contacting surface was not coated. Blood flow rate was maintained at 1-2 L/min.

The uncoated group: The circuit consisted of polyvinyl chloride tubing connected to the soft-shell venous reservoir and a hollow-fiber membrane oxygenator CAPIOX® SX18

(Terumo). The entire blood-contacting surface was not coated.

The PMEA-coated group: The circuit consisted of polyvinyl chloride tubing connected to the soft-shell venous reservoir and the oxygenator CAPIOX® SX18. The entire blood-contacting surface was coated with PMEA.

Blood samples were taken after anesthetization but before injection of heparin, and at 120 minutes after starting the blood circulation. After 0-6 h recirculation, platelet and leukocyte loss, release of β-TG and granulocyte elastase and production of TAT were measured. Samples of plasma bradykinin were drawn in ice-cooled tubes containing the inhibitors: aprotinin, soybean trypsin inhibitor, protamine sulfate and EDTA, and were centrifuged at 1,000 × g at 4 °C. After centrifugation, the supernatant was separated and stored at –80 °C. Plasma bradykinin levels were determined by radioimmunoassay. Samples for plasma endotoxin were drawn in heparinized endotoxin-free tubes, and plasma endotoxin levels were determined by the *Limulus* amoebate lysate assay. We also measured adhered platelets after the blood circulation

Data were processed with Sigma STAT® for Windows (SPSS Inc., Chicago, IL, USA), and the results were expressed as mean ± standard error. One-way analysis of variance (ANOVA) followed by the Tukey test was used for comparing variables among experimental groups. Statistical significance was assumed for values of $p < 0.05$.

2-3 Results and Discussion

Platelet adhesion

Figure 2-5 shows the chemical structures of the poly(meth)acrylates used in this work and **Table 2-1** shows their molecular weights (Mw) and contact angles. The number of platelets adhered to the surface of adhesion on poly(meth)acrylates is shown in **Figure**

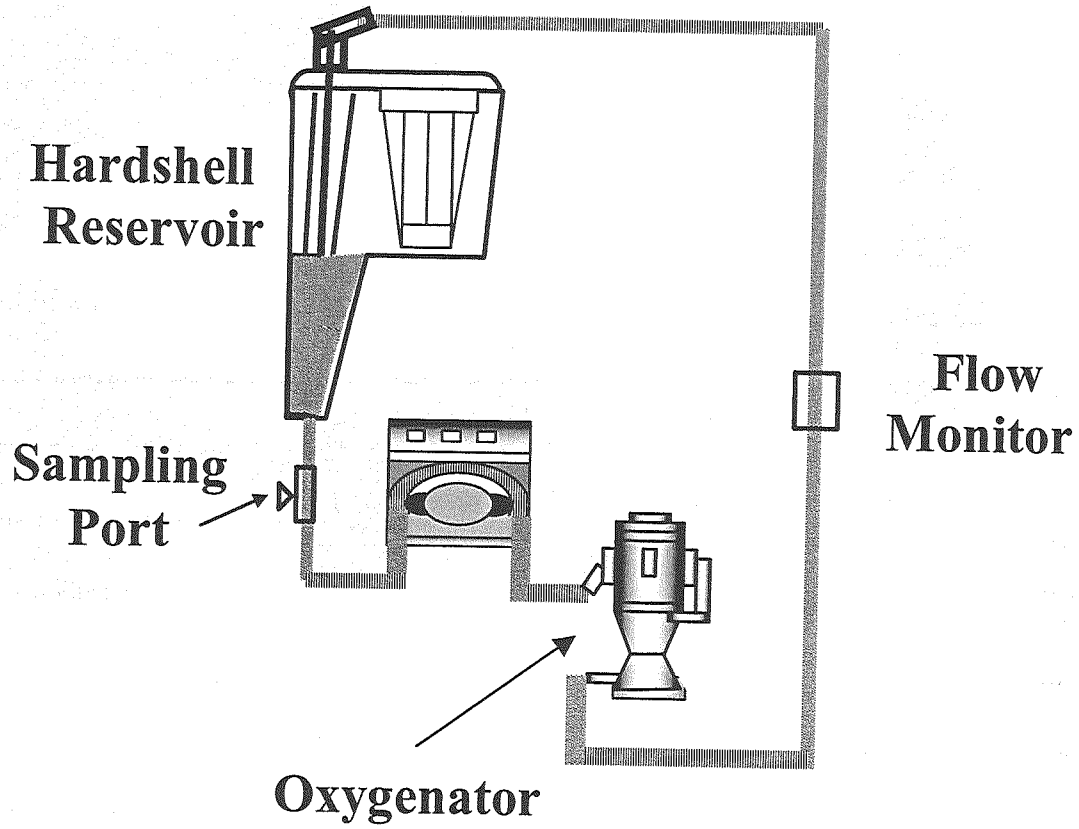


Figure 2-3. *In vitro* circulation using human blood.

Blood volume : 4 L, Blood flow rate : 2.0 L/min
Circulating time : 0-360 min.
Anticoagulant:Heparin (3.8 U/mL)
Blood tempreture : 37 ℃

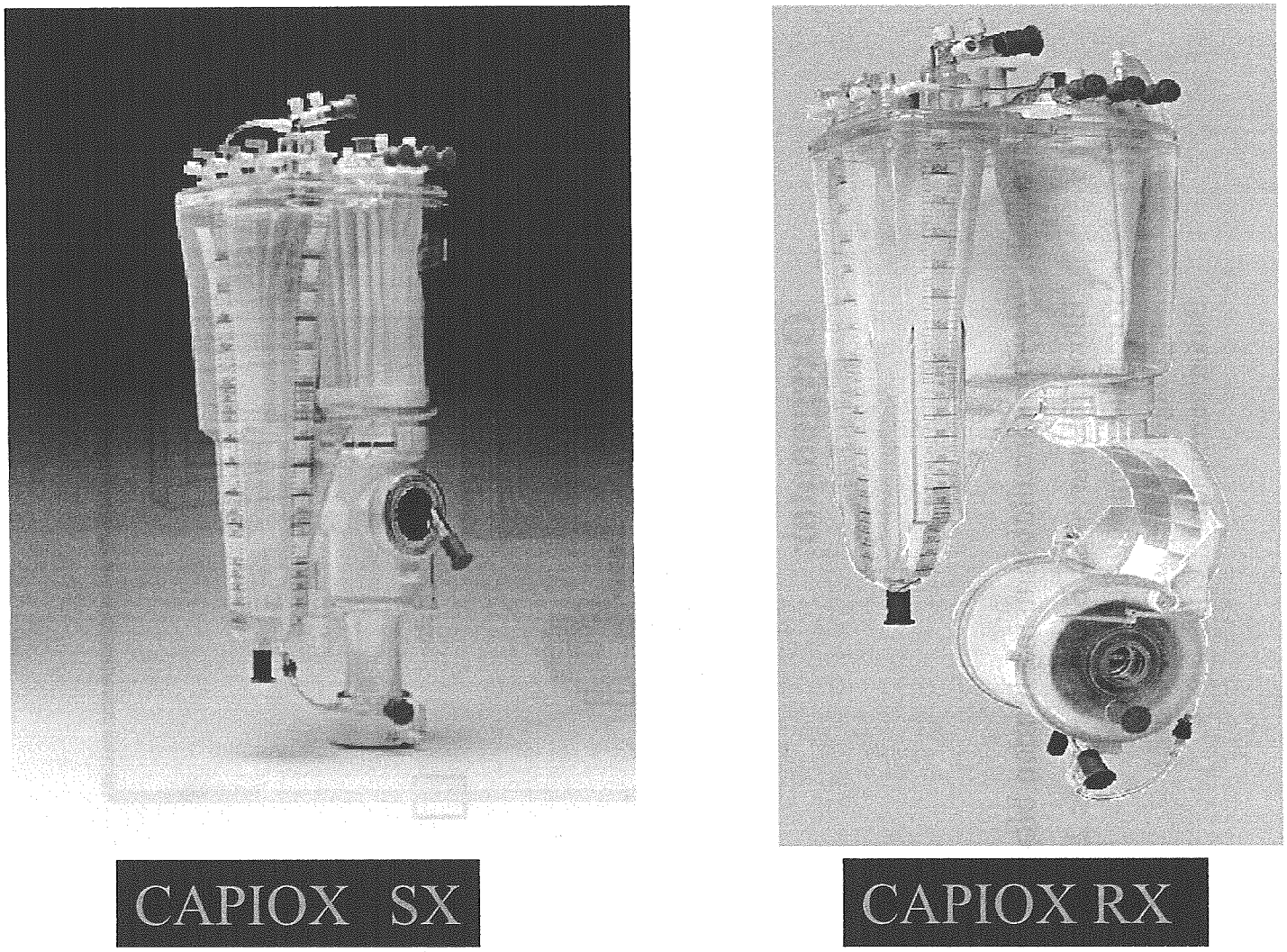
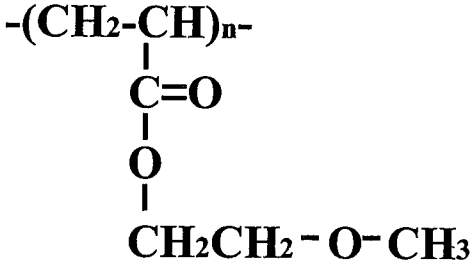


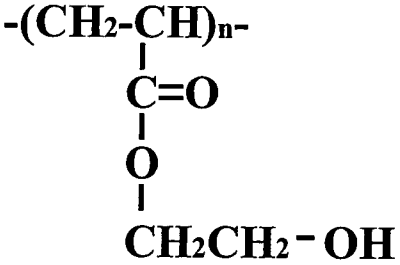
Figure 2-4. Blood compatibility of PMEA Coating (Capiox SX and RX).

Table 2-1. Molecular weights (Mw) and contact angle of poly(meth)acrylates (Mean±standard deviation, n=6) .

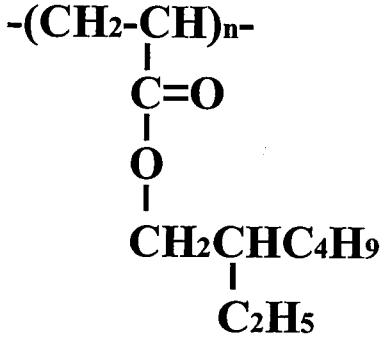
Sample	Mw	* Contact Angle (degree)
PMEA	85,000	49.8 (±4.3)
PHEA	109,800	17.3 (±4.7)
PEA	85,000	79.0 (±2.9)
PEHA	110,000	82.1 (±1.2)
PPEA	50,200	86.7 (±1.8)
PHEMA	111,000	30.6 (±3.5)



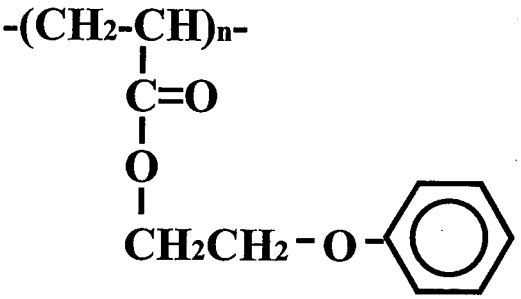
Poly(2-methoxyethyl acrylate)
PMEA



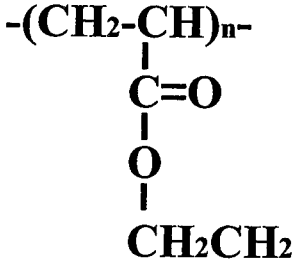
Poly(2-hydroxyethyl acrylate)
PHEA



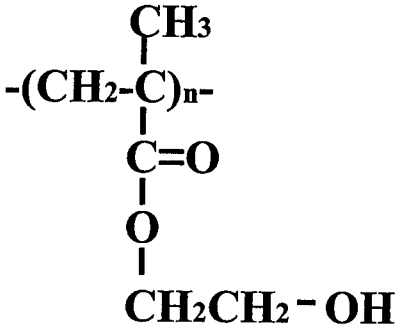
Poly(2-ethylhexyl acrylate)
PEHA



Poly(2-phenoxyethyl acrylate)
PPEA



Poly(ethyl acrylate)
PEA



Poly(2-hydroxyethyl methacrylate)
PHEMA

Figure 2-5. Chemical structures of poly(meth)acrylates.

2-6. The number of platelets adhered to the PMEA surface was the least. The composition in the copolymer affects significantly the platelet adhesion. The number of platelets adhering on PMEA surface, 2.5 cells/10^4 μm^2, is markedly small when compared with those of other polymers, over 14 cells/10^4 μm^2. These results suggest that the PMEA analogous polymers investigated activate platelet more than PMEA.

As the morphology of the platelet is one of the indexes expressing the degree of the platelet activation, the morphology was observed under SEM. The platelet adhered on the surfaces of PMEA keep its original spherical shape. This result means that the surfaces do not activate the platelet. In the case of HEMA, slightly small change in the morphology can be found. For the other polymers, it is clearly observed that the platelet has pseudopod and/or spread, which indicates the activation of the platelet. When both the results, the number and the shape of the adhered platelet, are combined, it is concluded that the platelet compatibility of the polymer changes drastically.

Washed platelets adhesion

Next, the adhesion of washed platelets onto PMEA and PHEMA is discussed. It is well known that platelet adhesion takes place when the material surface is coated with FNG. In order to clarify the relationship between adsorbed FNG and platelet adhesion, in this experiment, platelet adhesion to surfaces noncoated and precoated with FNG was investigated using washed human platelet. Here, PMEA and PHEMA were examined because they showed almost the same amount of adsorbed plasma protein and the amounts were lower than those adsorbed by other polyacrylates (Chapter 3).

Figure 2-7 shows the number of washed platelets adhered to the surface of polymers noncoated and precoated with FNG. In the absence of FNG, the adhesion of platelets

to the surface of PMEA and PHEMA was completely inhibited. On the other hand, in case of surfaces precoated with FNG, much platelet adhesion and spreading were observed on PHEMA, but little platelet adhesion was recognized on PMEA. The platelets adhered to PMEA maintained their original round shape compared with those adhered to PHEMA (**Figure 2-8**).

Next, the morphology of the adhered platelet is described. The SEM images of the platelet morphology on PMEA, PHEMA and PMEMA are shown in **Figure 2-8** as representatives. The platelet adhering on PHEMA and PMEMA spread markedly, while in the case of PMEA the platelet kept its original shape. For other poly(meth)acrylates the similar drastic morphological change of platelet was also observed. From these results on the platelet number and the morphology, it is concluded that PMEA analogous polymers activate platelet markedly and PMEA is very inert for the activation of platelet.

When PMEA and PHEMA surfaces were not coated with FNG, platelet adhesion was inhibited. On the other hand, when the surfaces were precoated with FNG, the numbers of adhered platelets considerably differed (**Figure 2-7**). The number of platelets adhered to PMEA was considerably smaller than that of platelets adhered to PHEMA. The platelets adhered to PMEA but not those adhered to PHEMA, maintained their original round shape (**Fig. 2-8**). The platelets adhered onto PHEMA appeared spread. These findings indicated that activation of platelets was significantly inhibited on PMEA but accelerated on PHEMA and also indicated that the conformation of adsorbed FNG was closely related to platelet adhesion.

Blood compatibility of PMEA on circuits

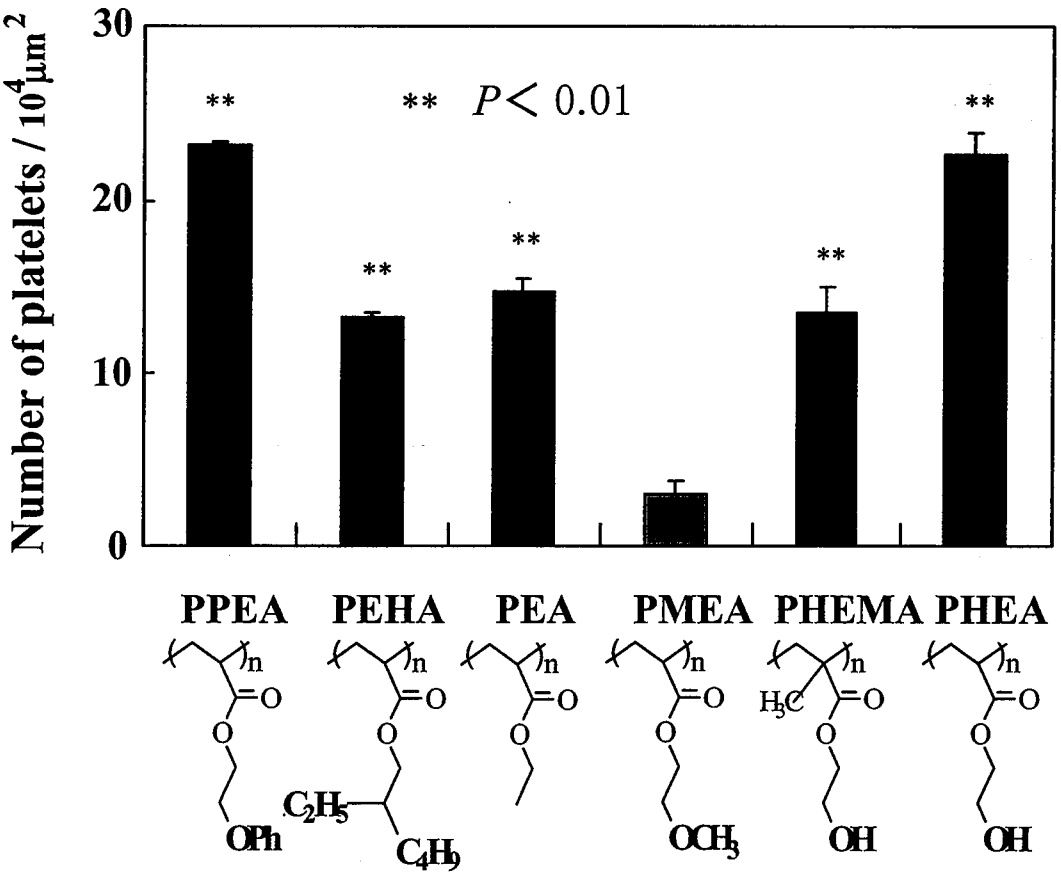


Figure 2-6. Number of platelets adhered to the surface of poly(meth)acrylates. (**P<0.01 vs PMEA, Mean±standard deviation, n=5)

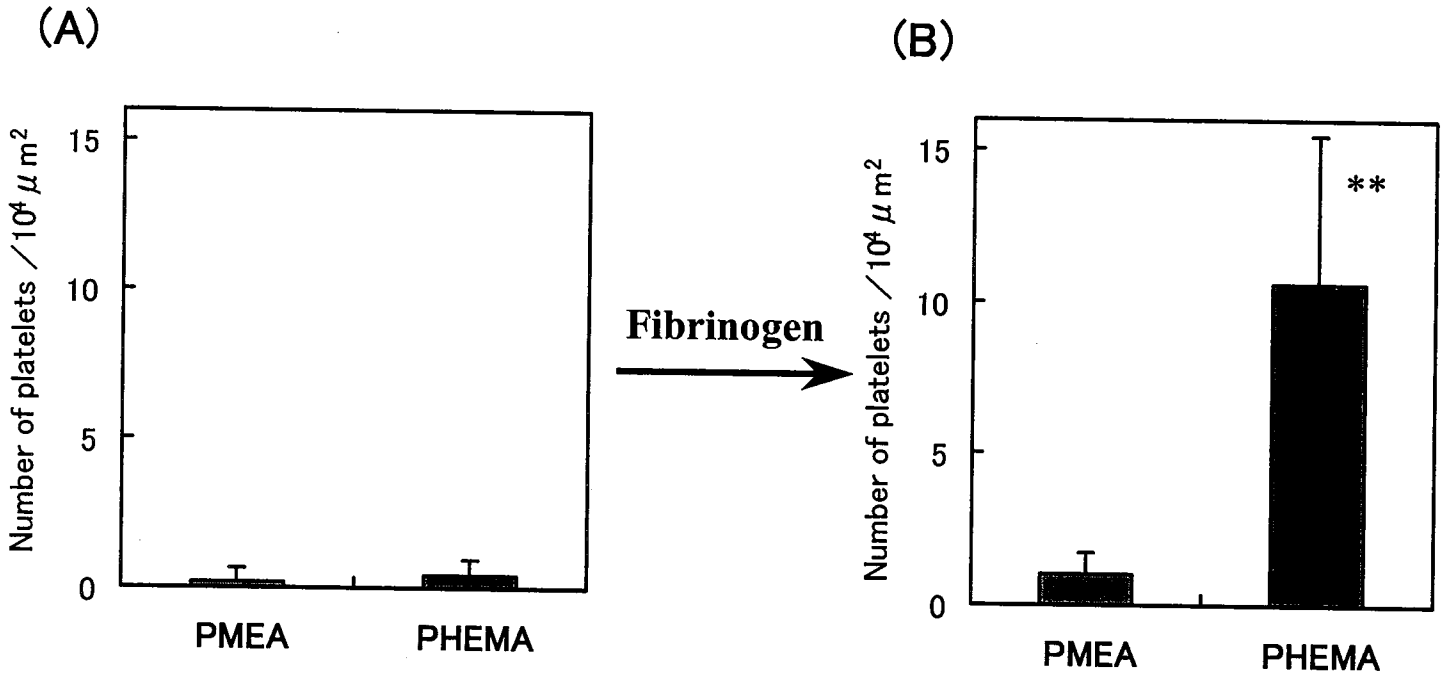


Figure 2-7. Adhesion of washed platelets to the surface of polymers noncoated (A) and precoated (B) with FNG (**P＜0.01 vs PMEA, Mean±standard deviation, n=5)

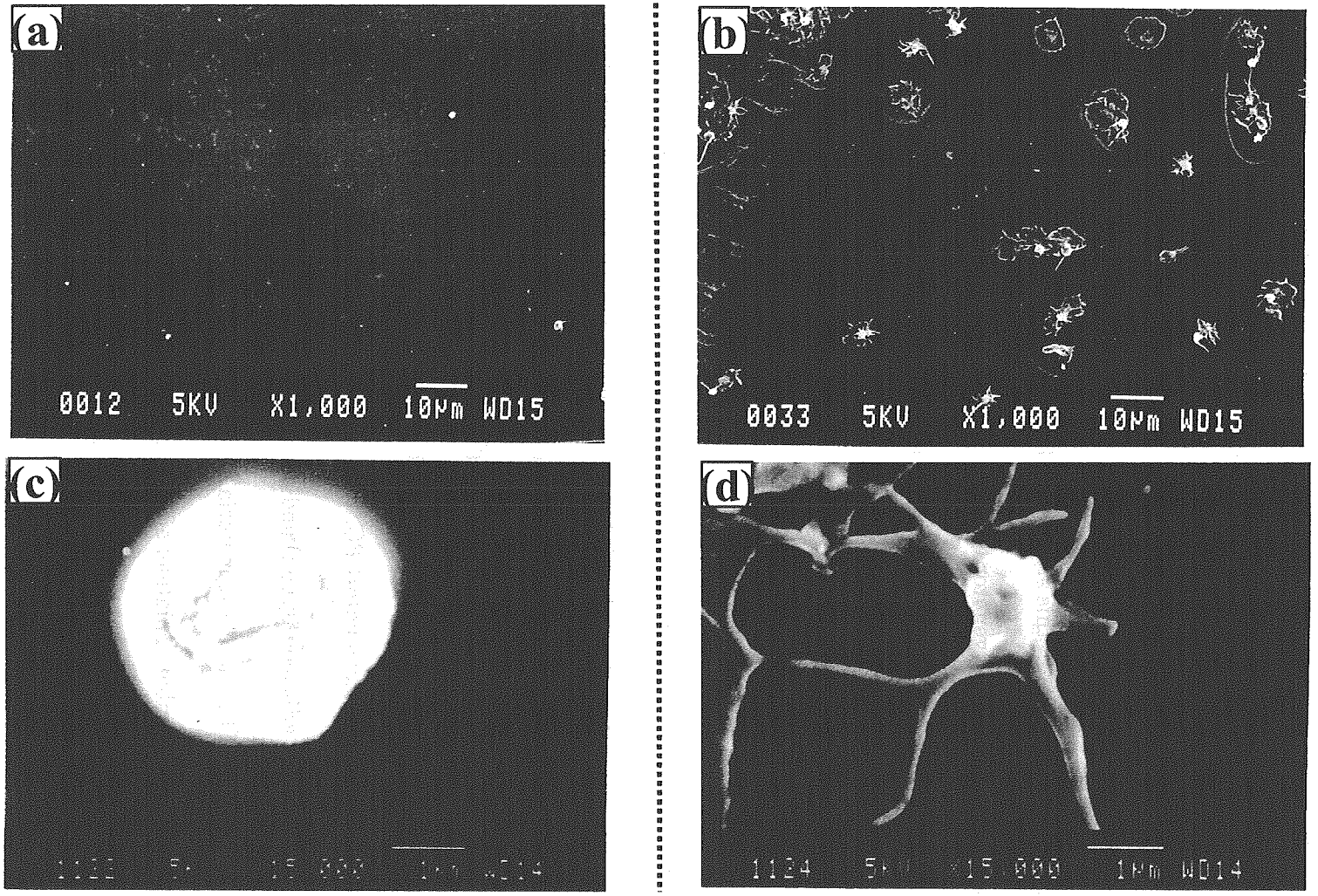


Figure 2-8. Scanning electron micrographs of washed platelets adhered to polymer precoated with FNG or noncoated. (a)PMEA (b)PHEMA (magnification × 1,000) (c)PMEA (d)PHEMA (magnification × 15,000)

Figure 2-9 show release of beta-thromboglobulin (β-TG) and lacoferrin, and production of Thrombin-antithrombin-III complex (TAT) and terminal complement complex (TCC). PMEA shows excellent blood compatibility with respect to platelet, leukocyte, coagulation, and complement systems when compared with other polymers. **Figure 2-10** show the effects of PMEA-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass model. In this study, a PMEA-coated oxygenator (Group P) was compared to a heparin-coated oxygenator (Group H) and non-coated oxygenator (Group N) under conditions of in vivo circulation using human blood. The results of this study indicated that in comparison to Group N, Group P allowed retention of a significantly smaller amount of platelet and leukocyte loss, release of β-TG and granulocyte elastase, production of TAT and bradikinin (**Figure 2-11**). There were no significant differences between Group P and Group H. After 6h, circulation adsorbed protein on the oxygenator was extracted and analyzed by immunoblotting. The amount of the adsorbed fibrinogen on the Group P membranes was smaller than that of Group N. This indicate that biocompatibility of the Group P is equal to that of Group H. **Figure 2-12** show the results of platelet adhesion after the blood circulation. Different materials which are component of the oxygenator; PC, PVC, PP, PU, PET and SUS, generate a varying degree of platelet adhesion (type I) and activation (type II). Polycarbonate (PC) can be classified as one of the harshest materials in relations to platelet adhesion and activation. However, the number of platelet adhesion was reduced when PMEA is coated the surface of materials. The platelet adhered on the surfaces coated with PMEA keep its original spherical shape (**Figure 2-13**). These findings indicated that activation of platelets was significantly inhibited on PMEA after the blood circulation.

Based on the above view points, PMEA-coated circuits could be regarded as

biocompatible circuits distinguished by their low platelet, leukocyte and complement activation ability and the low bradykinin level, similar to heparin-coated circuits. This leads us to the question of how PMEA-coated circuits show such biocompatibility, particularly in terms of reducing complement activation, as heparin-coated circuits show. The mechanism by which surface-coated heparin reduces complement activation and leukocyte activation is, however, not well known. A more feasible explanation for the reduction of complement activation and the subsequent reduction of cellular activation by coated heparin is the direct effect of the heparin molecule, particularly its inhibition of the alternative pathway of complement activation (36,38,39). On the other hand, coating of PMEA on a biocompatible surface suppresses the adhesion or adsorption of biocomponents, irrespective of the humoral (e.g., adhesive protein, coagulation factors and complement factors) or cellular components (e.g., leukocytes and platelets). The biocompatibility of a surface is generally considered to be related to the protein adsorption process that involves FXII, FXI, high-molecular-weight kininogen (HMWK), complement C3, platelets, and others. The approach of PMEA coating is based on the hypothesis that a surface whose interaction with biocomponents is minimized, namely less protein adsorption onto the surface, might be biocompatible. In fact, Saito et al investigated that the amount of protein adsorbed on PMEA-coated circuits was significantly lower than that on uncoated circuits: 3.42 $\mu g/cm^2$ for uncoated circuits and 0.30 $\mu g/cm^2$ for PMEA-coated circuits (40). Niimi et al. reported the amount of protein adsorbed on some extracorporeal circuits *in vitro*, as follows: 2.32 $\mu g/cm^2$ for an uncoated polypropylene surface, 1.40 $\mu g/cm^2$ for a silicone-coated surface, and 1.84 $\mu g/cm^2$ for polyolefin-coated surface (41). These figures prove clearly that the PMEA coating has an ability to suppress protein adsorption onto the surface compared with

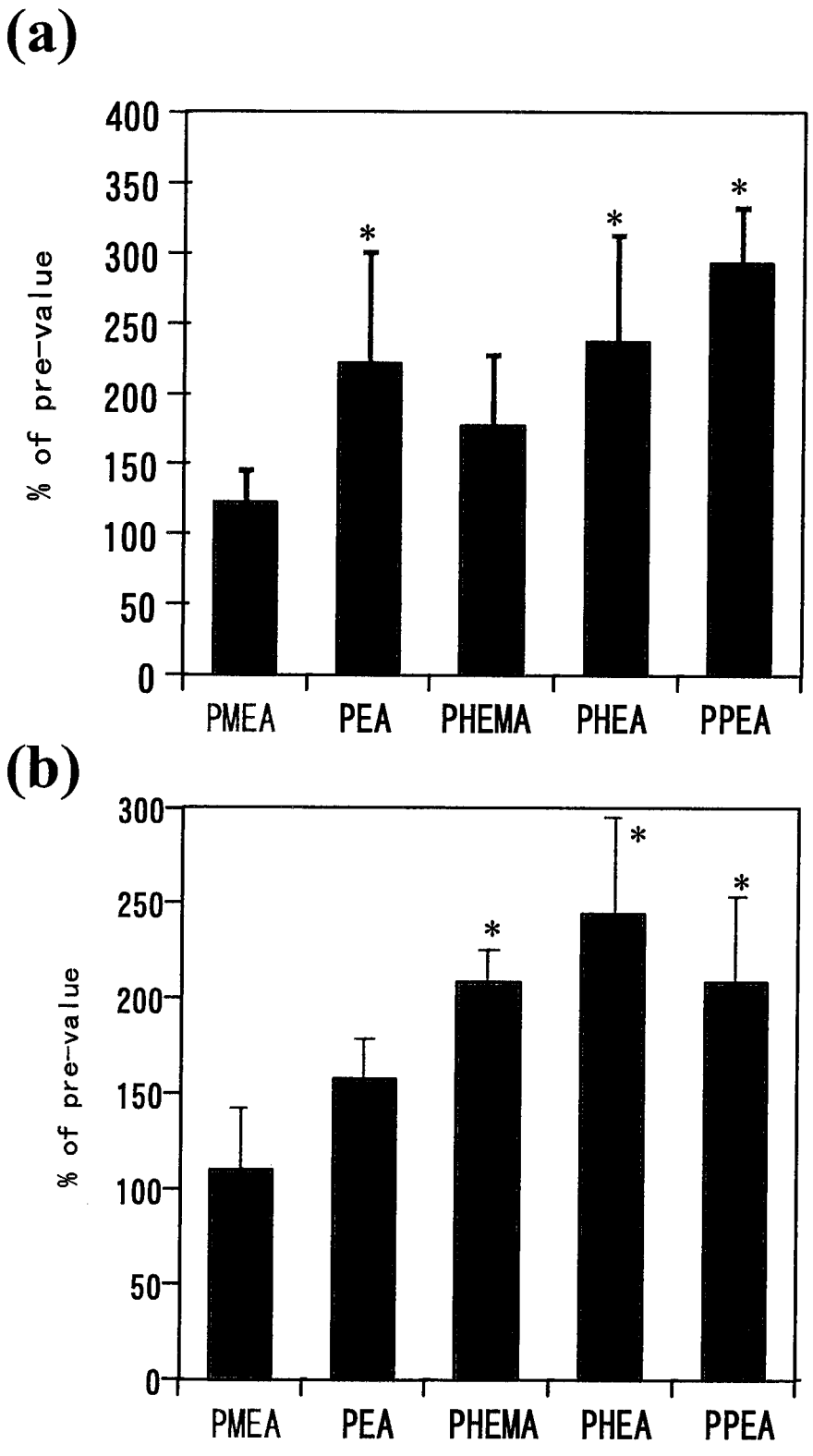


Figure 2-9. Release of β -TG (a) and lactoferrin (b). (*P＜0.05 vs PMEA, Mean±standard deviation, n=6)

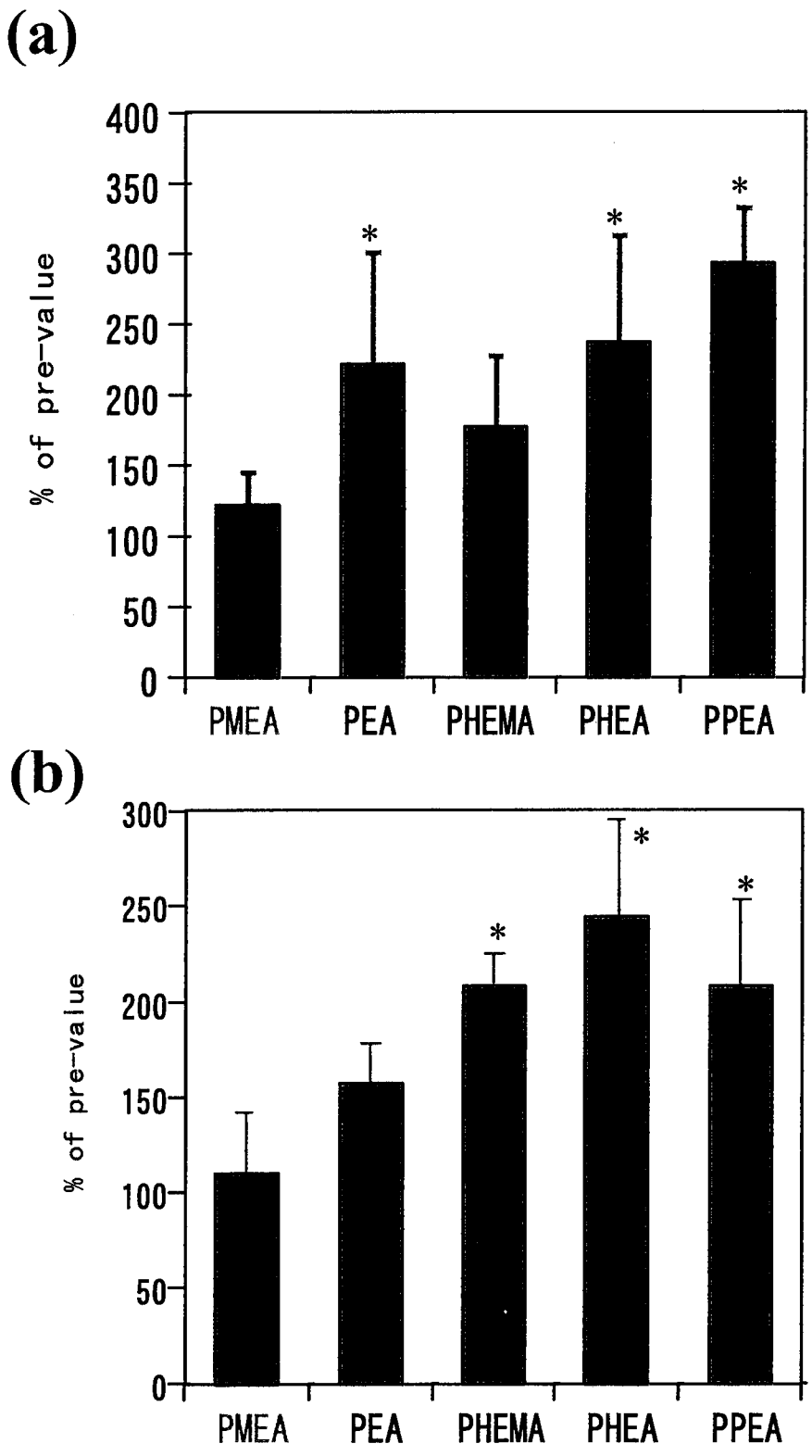
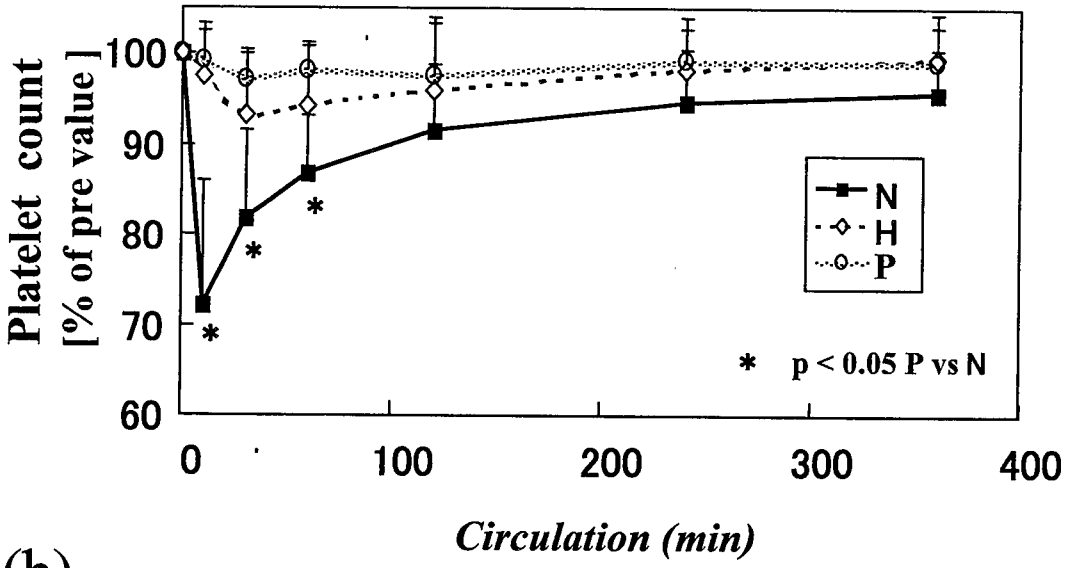


Figure 2-9. Release of β -TG (a) and lactoferrin (b).
(*P＜0.05 vs PMEA, Mean±standard deviation, n=6)

(a)



(b)

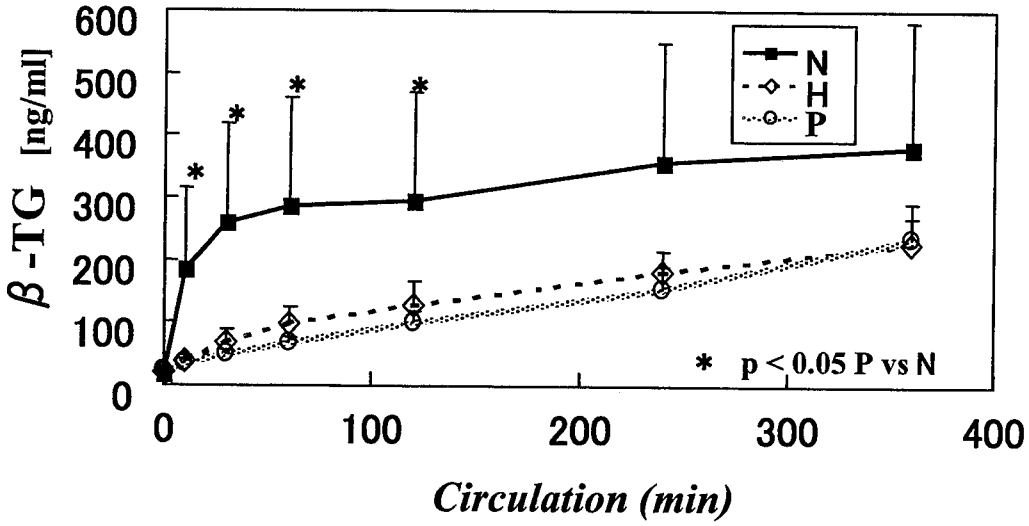
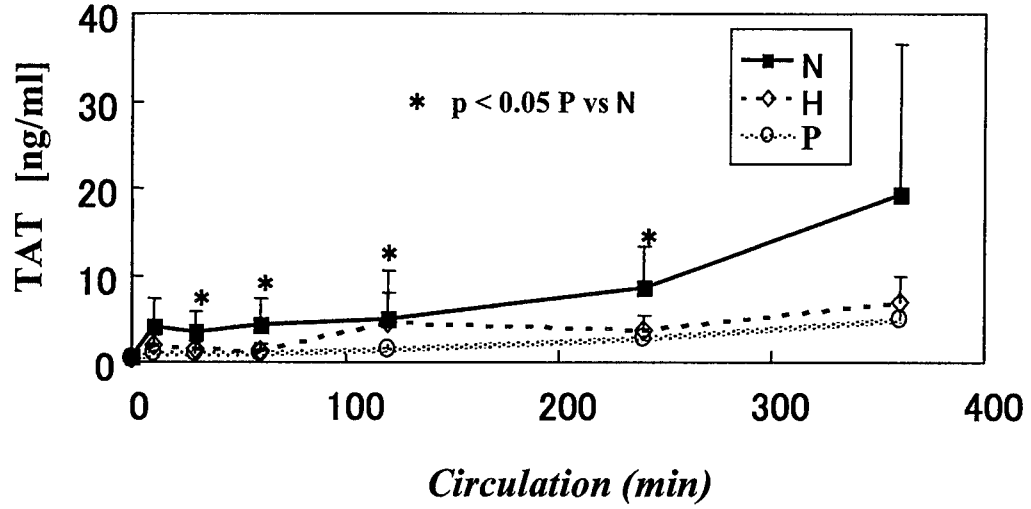


Figure 2-10. *In vitro* circulation using human blood.
(a) Platelet count (b) β -TG (c) TAT (d) Leukocyte count
(e) Granulocyte elastase

(c)



(d)

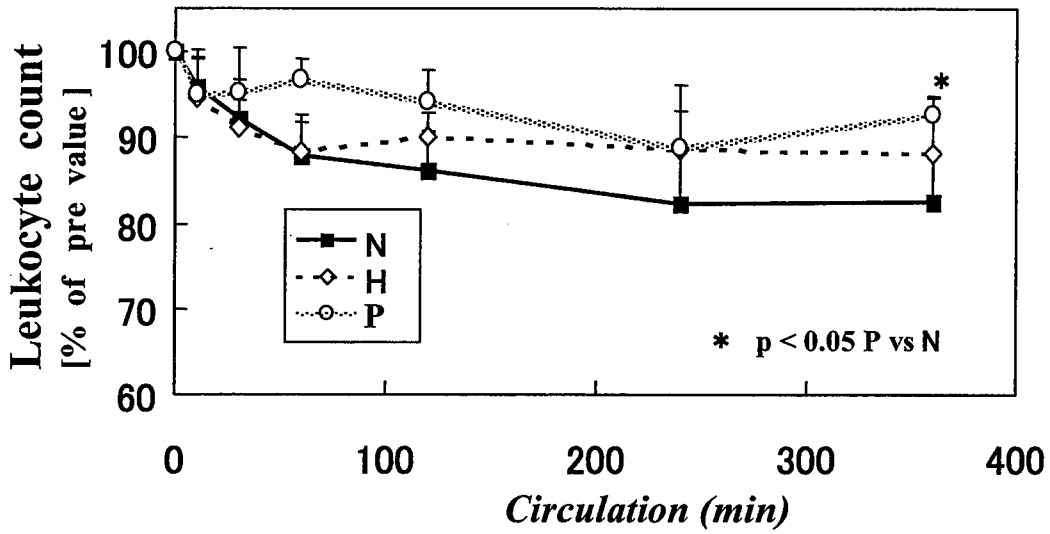


Figure 2-10. Continued

(e)

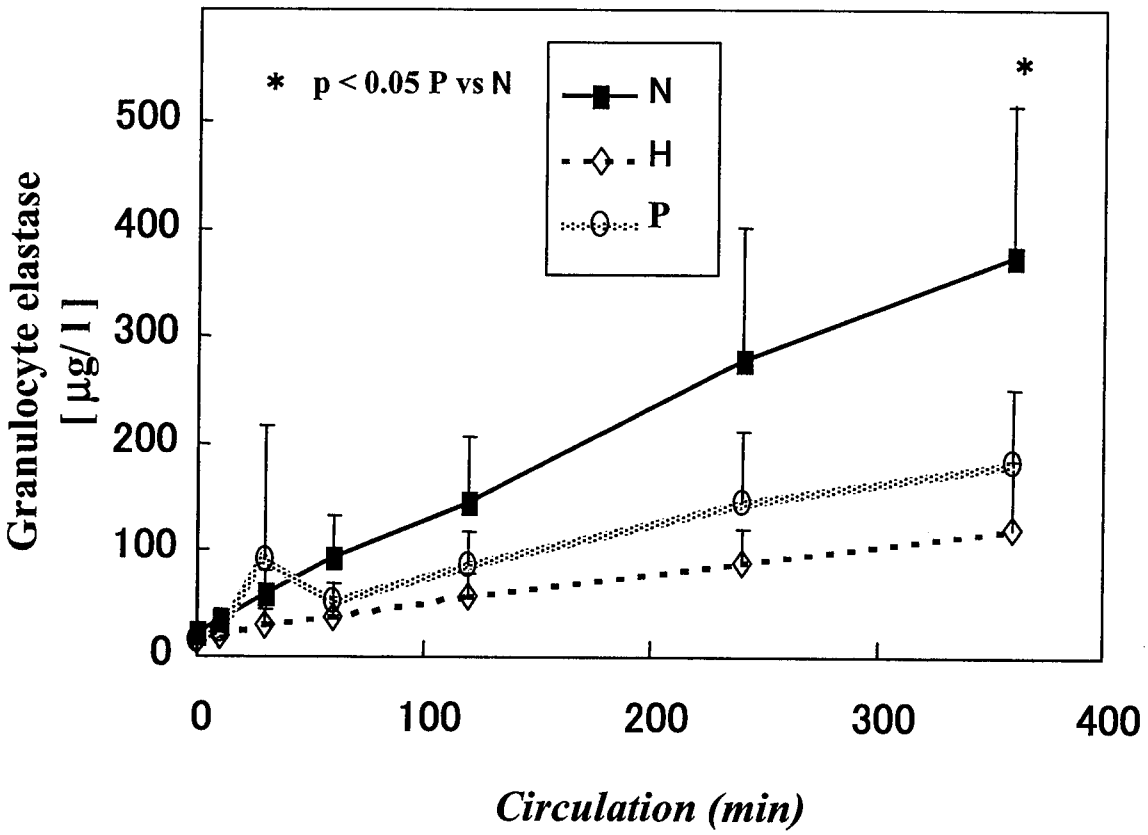


Figure 2-10. Continued

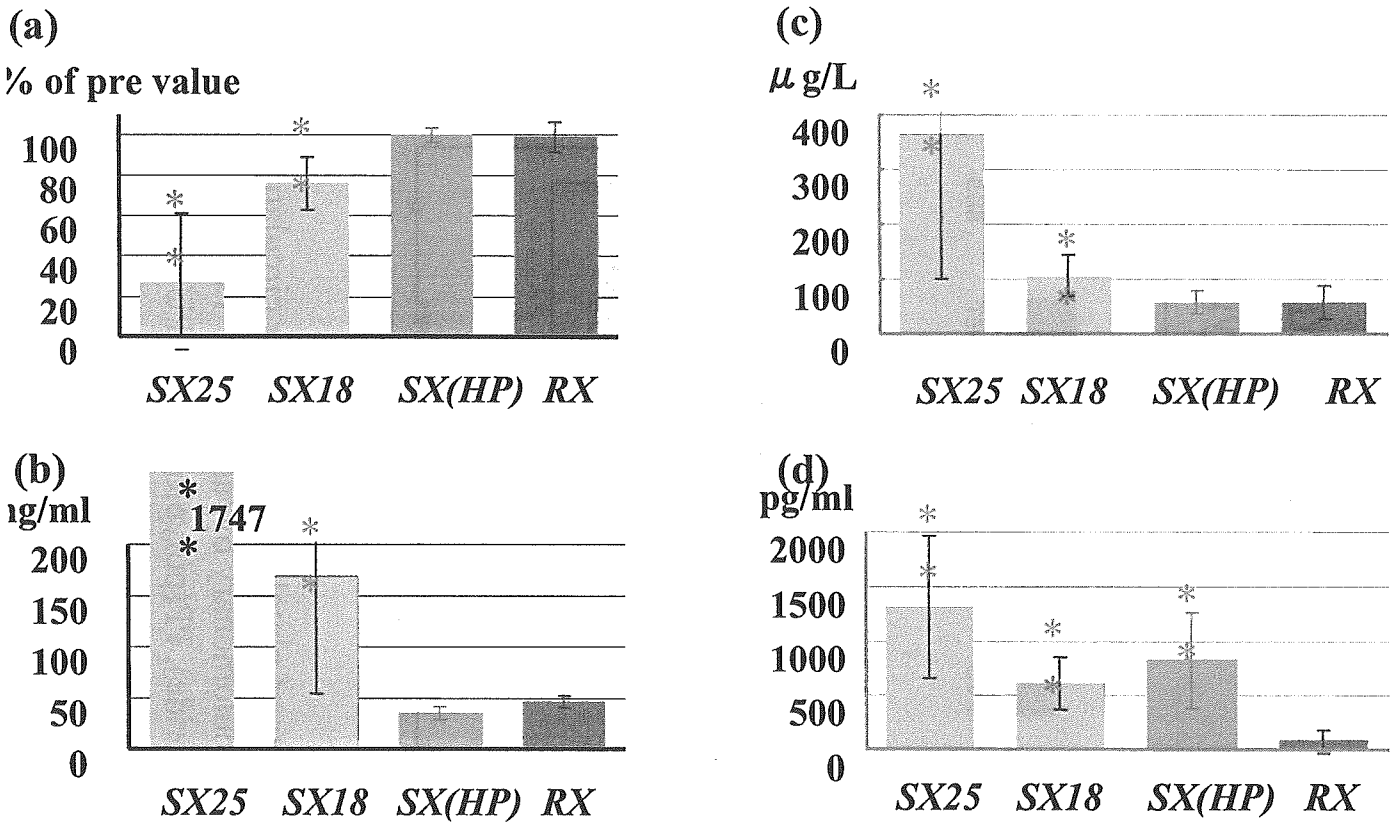


Figure 2-11. Effect of *in vitro* circuits on blood comaptibility during cardiopulomonary bypass model (after 2hours of circulation).
(a)Platelet count (b) β -TG (c) Granulocyte elastase (d)Bradikinin ** $P<0.01$

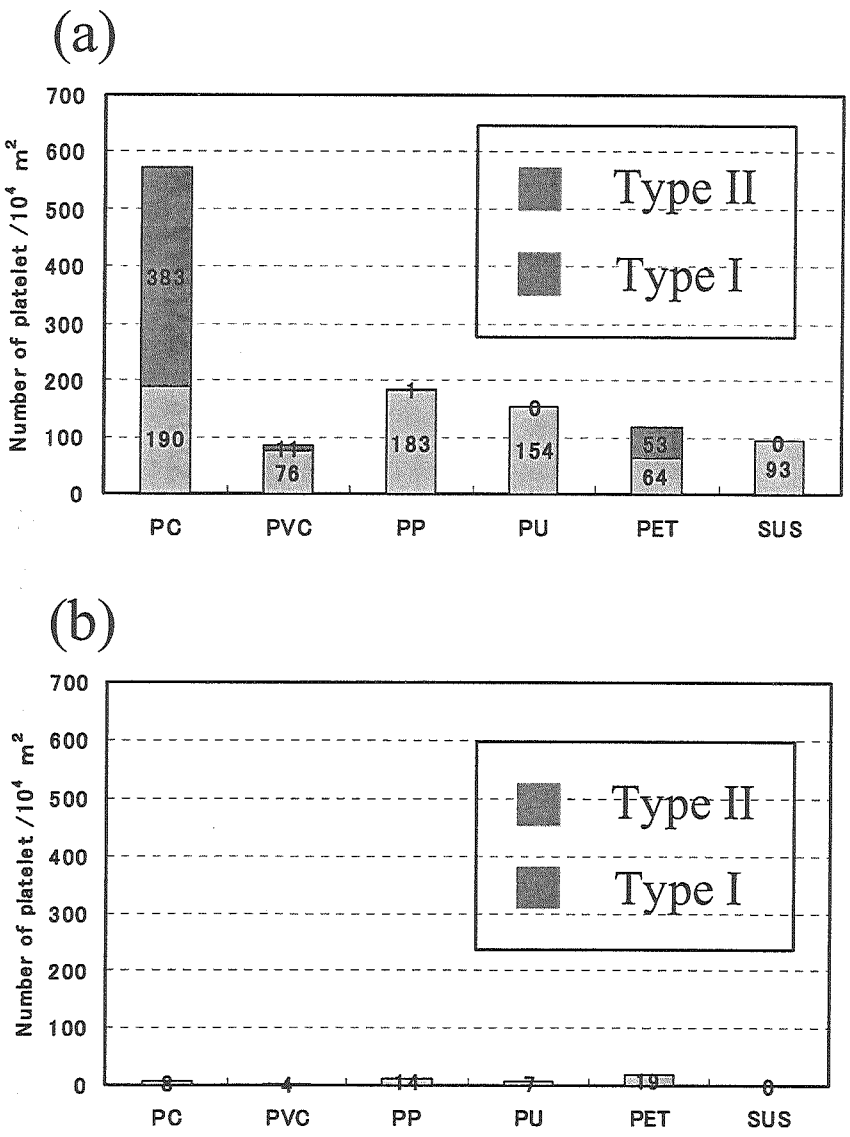


Figure 2-12. Results of platelet adhesion after circulation of blood by the cardiopulomonary bypass model. PC(polycarbonate), PVC(polyvinyl chloride), PP(polyplopylene), PU(polyurethae), PET(polyethyleneterephtalate), SUS(stainless steel)
(a) with PMEAcoating (b) without PMEA coating.
Type I : round shape, Type II : spreading

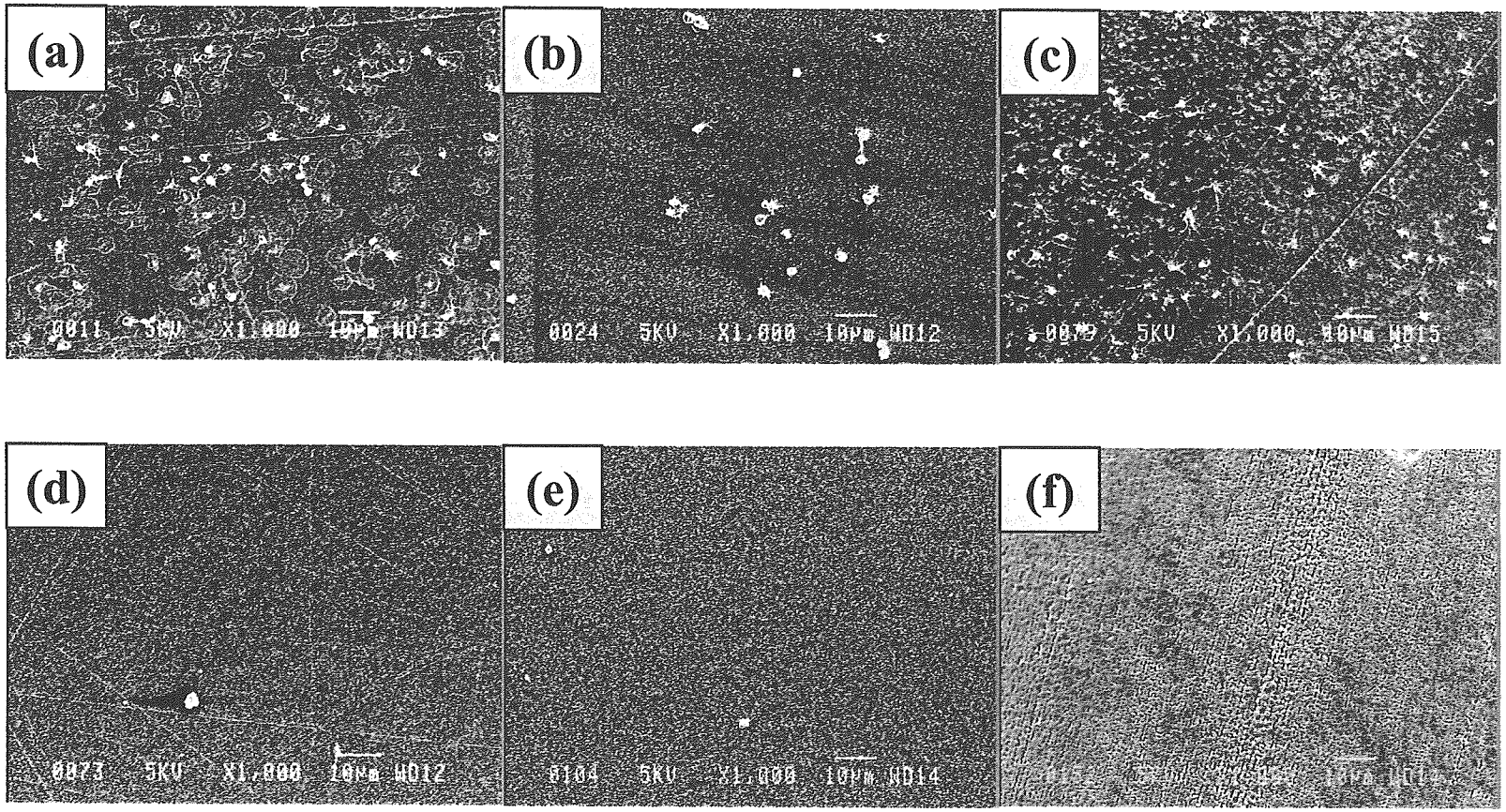


Figure 2-13. SEM of platelet adhered to various materials after circulation of blood by the cardiopulomonary bypass model. (a, d) PC(polycarbonate), (b,e) PVC(polyvinyl chloride), (c, e) PP(polyplopylene)
(a,b,c) without PMEA coating, (d,e,f) with PMEA coating

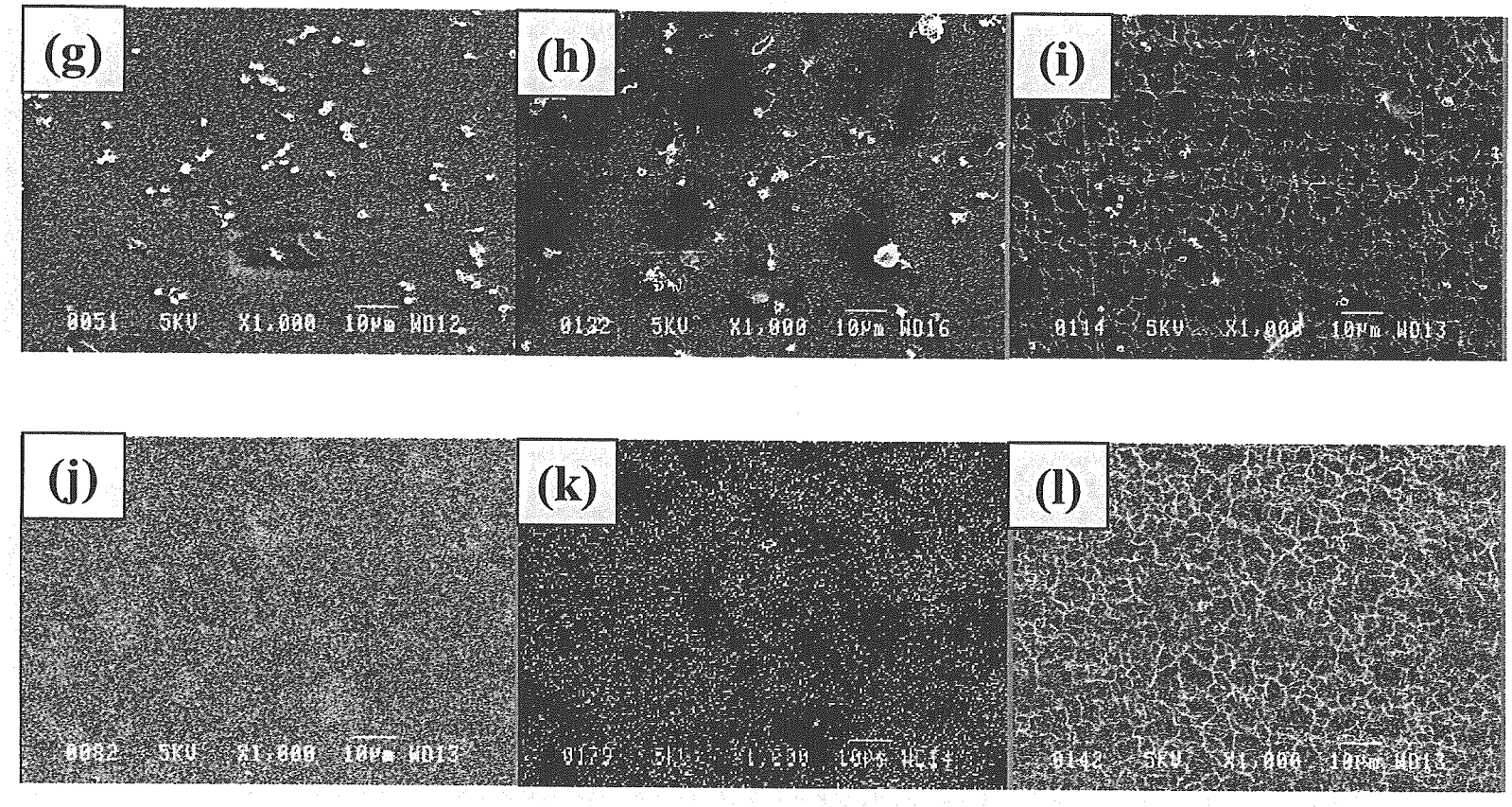


Figure 2-13. (Continued) (g, j) PU(polyurethae), (h, k) PET(polyethyleneterephtalate), (i, l) SUS(stainless steel) (g,h,i) without PMEA coating, (j,k,l) with PMEA coating

other circuits surfaces.

Bradykinin is released from HMWK to form complexes with FXII and FXI on the surface, particularly on negatively charged surfaces; this is the so-called "contact blood activation". Therefore, it could be said that the low bradykinin level of PMEA-coated circuits results from the minimization of the interaction of the PMEA-coated surface with FXII, FXI and HMWK. Based on a similar hypothesis, many basic studies on biocompatible surface designs have been done (42). This "PMEA coating" may, however, be the first coating material that is applied to commercialized cardiovascular devices.

Moreover, complement-associated protein, also known as apo-J or clusterin or SP-40,40 (43), was detected as the major protein adsorbed on PMEA-coated circuits, and may be worth mentioning. Like vitronectin, this complement-associated protein inhibits C5b-6 initiated complement hemolysis, and both proteins combine with nascent C5b-7, preventing membrane insertion and cell lysis. Moen et al. showed that the plasma concentrations of both proteins significantly decreased during CPB, and speculated that the decrease might due to the adsorption on extracorporeal circuits (44). In this study, we showed that complement-associated protein adsorbed on PMEA-coated circuits during the bypass. This protein adsorbed on PMEA coating might be associated with the reduction of complement activation.

In addition Suhara et al. proved the excellent blood compatibility of PMEA in the extracorporeal circulation of the PMEA-coated oxygenator using a pig in terms of TAT, bradykinin, ATIII, and the retention of platelet number (45). On the basis of these results, we concluded that the platelet adhesion test has a sufficient validity to estimate the blood compatibility. However, we have only limited information on its function in the complement system. Further studies to determine what the adsorption of

complement-associated protein on PMEA-coated surface means are required.

When an artificial surface comes into contact with blood, plasma proteins adsorb in seconds to the surface, followed by platelet thrombus formation. At the molecular level, the surface structure has an effect on the adsorption of proteins, and these adsorbed proteins play a major biological role in platelet adhesion. It is now well established that protein adsorption is the first event following material-tissue contact, and the adsorption is generally nonspecific, with multiple binding sites on the proteins interacting with sites on the material surface (46,47).Nonspecific forces that must be considered are Van der Waal's forces, electrostatic double-layer forces, solvent-dependent interactions, hydrogen bonding, hydrophobic interactions, hydration forces, and steric forces (48). Moreover, depending on the chemical nature of the polymer, the interactions occur with various affinities, some being irreversible. Protein adsorption is the trigger for blood coagulation, which occurs when blood contacts polymeric biomaterials (49-51). Nonspecific protein adsorption on the surface occurs within minutes after the contact of the surface with blood (52). Blood is a complex mixture made up of water, salts, minerals, proteins, and cells. The latter two components can interact with the foreign surface, be adsorbed, and be altered. Depending on the surface, protein adsorption varies in the thickness and the structure of the adsorbed layer. The more abundant proteins initially are adsorbed, but then are displaced by proteins that are less abundant but have a higher affinity for the surface. As we will see chapter 3, protein adsorption is also the first step leading to bacterial adhesion on biomaterial surfaces. The relationship between blood compatibility and protein adsorption will discuss in next chapter.

2-4 Conclusion

PMEA showed excellent blood compatibility in terms of platelet and leukocyte adhesion/activation (release of β-TG, and granulocyte elastase), complement activation (production of TCC) and coagulation (production of TAT). We showed that the plasma bradykinin level in the PMEA-coated group were significantly lower than those in the uncoated group. PMEA-coated circuits, distinguished by the low complement activation and the low bradykinin level, would reduce inflammatory responses including pulmonary edema after CPB compared with uncoated circuits, similar to heparin coated circuits.

2-5 References

(1) C. Pusineri and J.P. Cazenave , Techniques for modifying artificial surfaces. In: J.P. Cazenave, J. Davies, M.D. Kazatchkine and W.G. van Aken, Editors, *Blood–surface interactions: biological principles underlying haemocompatibility with artificial materials*, Elsevier Science Publishers, Amsterdam (1986), pp. 209–235.

(2) A.S. Hoffman , Modification of material surfaces to affect how they interact with blood. *Ann NY Acad Sci* 516 (1987), pp. 96–101.

(3) S.W. Kim and S. Feijen , Surface modification of polymers for improved blood compatibility. In: D.F. Williams, Editor, *CRC critical reviews in biocompatibility VI I3*, CRC Press, Boca Raton, FL (1985), pp. 229–260.

(4) C.J. Van Delden, J.P. Lens, R.P.H. Kooyman, G.H.M. Engbers and J. Feijen , Heparinization of gas–plasma modified polystyrene surfaces and the interaction of these surfaces with proteins studied with surface plasmon resonance. *Biomaterials* **12** (1997), pp. 845–852.

(5) B.D. Ratner , Surface modification of polymeric biomedical applications: chemical, biological, and surface analytical challenges. In: B.D. Ratner and D.G. Castner, Editors, *Surface modification of*

(6) M.S. Sheu, A.S. Hoffman, B.D. Ratner, J. Feijen and J.M. Harris , Immobilization of polyethylene oxide surfactants for non-fouling biomaterial surfaces using an argon glow discharge treatment. *J Adhes Sci Technol* 7 10 (1993), pp. 1065–1076.

(7) B.D. Ratner , Biomaterials science: overview and opportunities with special reference to organic and polymeric glow discharge plasma treatments. In: R. D'Agostino, Editor, *Plasma treatments and depositions of polymers*, Kluwer Academic Publishers, Dordrecht (1997), pp. 453–464.

(8) B.D. Ratner , Surface modification of polymers: chemical, biological and surface analytical challenges. *Biosensors Bioelectron* **10** (1995), pp. 797–804.

(9) Ratner BD, Castner DG, editors. Surface modification of polymeric biomaterials. New York, NY: Plenum Press, 1997. p. 206.

(10) J. Hoffman, O. Larm and E. Scholander , A new method for covalent coupling of heparin and other glycosaminoglycans to substances containing primary amino groups. *Carbohydr Res* **117** (1983), pp. 328–331.

(11) E. Brynda, M. Houska, M. Jirouskova and J.E. Dyr , Albumin and heparin multilayer coatings for blood-contacting material devices. *J Biomed Mater Res* **2** (

(12) C.J. Van Delden, G.H.M. Engbers and J. Feijen , Interaction of ATIII with surface immobilized albumin heparin conjugates. *J Biomed Mater Res* **29** (1995), pp. 1317–1329.

(13) H. Jacobs, D. Grainger, T. Okano and S.W. Kim , Surface modification for improved blood compatibility. *Artif Organs* **6** (1988), pp. 506–507.

(14) H.F.M. Cremers, G. Kwon, Y.H. Bae, S.W. Kim, R. Verrijh, H.P.J.M. Noteborn

(15) W.E. Hennink, S.W. Kim and J. Feijen , Inhibition of surface induced coagulation by preadsorption of albumin–heparin conjugates. *J Biomed Mater Res* 18 (1984), pp. 911–926.

(16) C.S. Brazel and N.A. Peppas , Pulsatile local delivery of thrombolytic and antithrombotic agents using poly(*N*-isopropylacrylamide-co-methacrylic acid)

(17) W.L.J. Hinrichs, H.W.M. ten Hoopen, M.J.B. Wissink, G.H.M. Engbers and J. Feijen , Desighn of a new type of coating

(18) M.R. Kreitz, J.A. Domm and E. Mathiowitz , Controlled delivery of therapeutics from microporous membranes. II. In vitro defradation and release of heparin-loaded poly(D , L -lactide-co-glycolactide). *Biomaterials* 24 (1997), pp. 1645-1655.

(19) Y.J. Kim, I-.K. Kang, M.W. Huh and S-.C. Yoon , Surface characterization and in vitro blood compatibility of poly(ethylene teraphthalate) immobilized with insulin and/or heparin using plasma glow discharge. *Biomaterials* 2 (2000), pp. 121–130.

(20) V.L. Gott, J.D. Whiffen and R.C. Dutton , Heparin bonding on colloidal graphite surfaces. *Science* 142 (1963), pp. 1297–1298.

(21) J.E. Wilson , Heparinised polymers as thromboresistant biomaterials. *Polym Plast Technol Eng* 16 2 (1981), pp. 119–208.

(22) Lane DA, Undahl U, editors. Heparin. Boca Raton, FL: CRC Press, 1989.

(23) Wan S, LeClerc JL, Vincent JL. Inflammatory response to cardiopulmonary bypass. Chest 1997;112:676-92.

(24) Butler J, Rocker GM, Westaby S. Inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 1993;55:552-9.

(25) Moen O, Høgåsen K, Fosse E, Dregelid E, Brockmeier V, Venge P, Harboe M, Mollnes TE. Attenuation of changes in leukocyte surface markers and complement activation with heparin-coated cardiopulmonary bypass. Ann Thorac Surg 1997;63:105-11.

(26) Gu YJ, von Oeveren W, Akkerman C, Boonstra PW, Huyzen RJ, Wildevuur CRH. Heparin-coated circuits reduce the inflammatory response to cardiopulmonary bypass. Ann Thorac Surg 1993;55:917-22.

(27) Te Velthuis H, Jansen PG, Hack CE, Eijsman L, Wildevuur RH. Specific complement inhibition with heparin-coated extracorporeal circuits. Ann Thorac Surg 1996;61:1153-7.

(28) Lazar HL, Zhang X, Hamasaki T, Memmelo CA, Treanor P, Rivers S, Aldea GS, Bernard SA, Shemin RJ. Heparin-bonded circuits decrease myocardial ischemic damage: an experimental study. Ann Thorac Surg 1997;63:1701-5.

(29) Fearon DT. Identification of the membrane glycoprotein that is the C3b receptor of the human erythrocyte, polymorphonuclear leukocyte, B lymphocyte, and monocyte. J Exp Med 1980;152:20-30.

(30) Dentener MA, Bazil V, von Asmuth EJU, Ceska M, Buurman WA. Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-a, IL-6 and IL-8 release by human monocytes and alveolar mocrophages. J Immunol 1993;150:2885-91.

(31) Jean-Charles S, Ron DA, Oliver G, Christian P, Florence R, Amos B, Denis FH. Inside SWISS-2DPAGE database Electrophoresis 1995;16:1131-1151.

(32) Karl-Erik S, Gösta A, Ulf RN. Purification and characterization of porcine C3. Studies of the biologically active protein and its split products. Vet Immunol Immunopathol 1992;34:47-61.

(33) Track BF, Janatova J, Thomas ML, Harrison RA, Hammer CH, The third, fourth and fifth components of human complement: isolation and biochemical properties. Lorand L eds. Proteolytic Enzymes Part C. New York: Academic Press, 1981:64-101. (Colowick SP, Kaplan NO ed. Methods in Enzymology volume 80).

(34) Pang LM, Stalcup SA, Lipset JS, Hayes CH, Bowman OF, Millins RB. Increased circulating bradykinin during hypothermia and cardiopulmonary bypass in children. Circulation 1979;60:1503-7

(35) Lazar HL, Hamasaki T, Bao Y, Rivers S, Bernard S, Shemin RJ. Soluble complement receptor type I limits damage during revascularization of ischemic myocardium. Ann Thorac Surg 1998;65:973-7.

(36) Garred P, Mollnes TE. Immobilized heparin inhibits the increase in leukocyte surface expression of adhesion molecules. Artif Organs 1997;21:293-99.

(37) Fingerle-Rowson G, Auers J, Kreuzer E, Labeta M, Schmidt B, Samtleben W, Ziegler-Heitbrock HW, Blumenstein M. Down-regulation of surface monocyte lipopolysaccharide-receptor CD14 in patients on cardiopulmonary bypass undergoing aorta-coronary bypass operation. J Thorac Cardiovasc Surg 1998;115:1172-8.

(38) Kazatchkine MD, Fearon DT, Metcalfe DD, Rosenberg RD, Austen KF. Structural determinants of the capacity of heparin to inhibit the formation of the human amplification C3 convertase. J Clin Invest 1981;223-8.

(39) Maillet F, Kazatchkine MD, Glotz D, Fischer E, Rowe M. Heparin prevents formation of the human C3 amplification convertase by inhibiting the binding site for B on C3b. Mol Immunol 1983;20:1401-4.

(40) Saito, N.; Motoyama, S.; Sawamoto, J. Effects of new polymer-coated

extracorporeal circuits on biocompatibility during cardiopulmonary bypass *Artif. Organs* 2000, *24*, 547.

(41) Niimi Y, Yamane S, Yamaji K, Tayama E, Sueoka A, Nosé Y. Protein adsorption and platelet adhesion on the surface of an oxygenator membrane. ASAIO Journal 1997;43:M706-710.

(42) Ratner BD. Correlations of material surface properties with biological responses. In: Ratner BD, Hoffman AS, Schoen FJ, Lemons JE eds. Biomaterials Science. San Diego: Academic Press, 1996:445-51.

(43) Murphy BF, Saunders JR, O'Bryan MK, Kirszbaum L, Walker ID, d'Apice AJF. SP-40,40 is an inhibitor of C5b-6-initiated hemolysis. Int Immunol 1989;1:551-554.

(44) Moen O, Fosse E, Brockmeier V, Andersson C, Mollnes TE, Høgåsen K, Venge P. Disparity in blood activation by two different heparin-coated cardiopulmonary bypass systems. Ann Thorac Surg 1995;60:1317-23.

(45) Suhara, H.; Sawa, Y.; Nishimura, M.; Oshiyama, H.; Yokoyama, K.; Saito, N.; Matsuda, H *Ann. Thoracic. Surg.* 2001, *71*, 1603.

(46) Brash JL, Ten Hove P Protein adsorption studies on 'standard' polymeric materials. J Biomater Sci Polym Edn 1993;4:591-599.

(47) Wojciechowski P, Brash JL. The Vroman effect in tube geometry: the influence of flow on protein adsorption measurements. J Biomater Sci Polym Edn 1991;2:203-216.

(48) Glantz PO, Arnebrant T, Nylander T, Baier RE. Bioadhesion-a phenomenon with multiple dimensions. Acta Odontol Stand 1999;57:238241.

(49) Courtney JM, Lamba NM, Sundaram S, Forbes CD. Biomaterials for blood-contacting applications. Biomaterials 1994;15:737-744.

(50) Brash JL, Scott CF, ten Hove P, Wojciechowski P, Colman RW Mechanism of transient adsorption of fibrinogen from plasma to solid surfaces: role of the contact and fibrinolytic systems. Blood 1988;71:932929.

(51) Yung LY, Colman RW, Cooper SL. The effect of high molecular weight kininogen on neutrophil adhesion to polymer surfaces. Immunopharmacology 1999;43:281-286.

(52) Cornelius RM, Brash JL. Adsorption from plasma and buffer of singleand two-chain high molecular weight kininogen to glass and sulfonated polyurethane surfaces. Biomaterials 1999;20:341-350.

Chapter 3 Protein Adsorption onto a PMEA Surface

3-1 Introduction

When a foreign material comes into contact with blood it will rapidly adsorb proteins onto its surface, and the adsorbed protein layer will determine all further events, namely, platelet adhesion, aggregation and coagulation (1-3). Therefore, understanding the mechanism of protein adsorption is very important for surface design of not necessary biomedical materials. Protein adsorption onto various kinds of polymer surfaces has been extensively investigated, and many studies have focused on the development of synthetic materials for blood compatible devices (4). Several studies have suggested that both the amount of adsorbed protein and conformational change play an important role in platelet adhesion. In particular, conformational change influences the functions of proteins themselves. Thus, determining the degree of conformational change in adsorbed protein is considered to be one of the important aspects affecting blood compatibility (5-7).

Previously, the conformational change of adsorbed protein on a polymer surface was analyzed using the ATR-FT-IR flow cell method (8-10), CD (11-13), and calorimetric analysis (14). Recently, Ishihara et al. evaluated conformational changes of proteins adsorbed onto the surface of a phospholipid polymer using CD in terms of changes of α-helix content of the protein (15). Their experiments were carried out using serum albumin and fibrinogen (FNG) since they are the major plasma proteins. The former is one of the most important transport proteins. The latter is an adhesive protein, the same as fibronectin, von Willebrand factor, and thrombospondin, which regulate or modulate the adhesive response of platelets. FNG has been well investigated from the viewpoint of a mediator of platelet adhesion via direct interactions with platelet receptors such as glycoprotein GPIIb/IIIa. Several groups have reported a monoclonal antibody that binds to the functional region of the platelet receptor (e.g. RGDS or

C-terminal dodecapeptide of the γ chain) and reacts with FNG adsorbed onto a surface, but not with FNG in solution (16,17). They concluded that the conformation of FNG adsorbed onto the surface is changed. Moreover, other studies have suggested that not only the total amount of adsorbed FNG but also the conformation or orientation of adsorbed FNG play an important role in determining platelet adhesion to biomaterials (18,19).

In order to produce with artificial organs a novel surface, such as an artificial lung, we have developed a poly(2-methoxyethylacrylate) (PMEA) surface. In our preliminary evaluation, PMEA showed excellent blood compatibility in terms of platelet and leukocyte adhesion/activation (release of β-TG, PMN elastase, and lactoferrin), complement activation (production of C3a and TCC) and coagulation (production of TAT) (20).

The advantageous properties of PMEA for biomedical applications are as follows: blood compatibility, low toxicity, adhesive property, economical production by radical polymerization on large scale, easiness to copolymerize, and easiness to control quality. However, we have not found the reason for the blood compatibility exhibited by PMEA as yet.

In this chapter, to understand the reason why PMEA exhibits blood compatibility, we investigated the amount of plasma protein adsorbed on PMEA. We focused primarily upon the relationship between protein adsorption and platelet adhesion to the PMEA surface. The degree of conformational change of the protein adsorbed onto the surface was estimated by determining the α-helix content using CD. We report here the conformational changes of serum albumin and FNG adsorbed onto a PMEA surface and onto the various poly(meth)acrylates analogous polymer surfaces.

3-2 Experimental

3-2-1 Analysis of protein adsorbed onto PMEA

Whole blood was separated into plasma and cells by centrifugation at 3000 rpm for 10min. A PET or PP plate coated with the poly(meth)acrylate polymer was immersed in the plasma and placed at 37°C for 60min without moving it, then it was rinsed five times with PBS for 30 seconds taking care of the air-water interface (21,22). The protein adsorbed onto the plate is desorbed by exposing the plate to an SDS solution (0.125M Tris/HCl, pH6.8 with 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue) for 15min at 100°C. The total amount of adsorbed protein was measured by the gold stain dot-blotting method (23). In a microsyringe 1 μl of sample was taken up and applied to nitrocellulose paper (ADVANTEC MFS, INC.) . After the sample had dried, the paper was washed for 20 min at room temperature in Tris buffer (20mM Tris, 500mM NaCl, 0.3%Tween-20, pH7.5), rinsed in water, and stained colloidal gold stain reagent (BIO-RAD Laboratories. Inc.) for 60min at room temperature. After a brief wash in water, the stained nitrocellulose paper was scanned on a laser densitometer (Image Master DTS, Pharmacia LKB).

3-2-2 Conformational changes of adsorbed proteins

The conformation of the protein adsorbed onto the polymer surface was determined by the circular dichroism (CD) method. The CD spectra was recorded from 195 to 250nm on a CD spectrophotometer (J-720WI, JASCO Co., Ltd., Tokyo, Japan), using a 1cm quartz cuvette (**Figure 3-1**). The sample temperature was kept constant at 37℃ by a cell holder incorporating a Peltier device (PTC-343, JASCO Co., Ltd.), and the sample compartment was continuously flushed with N_2. The spectrum was the average of 10 scans, using a bandwidth of 1mm, and a stepwidth of 1 mm. The concentrations of proteins were 4.0g/dL for BSA, and 0.3g/dL for FNG which were nearly equal to the physiological concentrations. The polymer-coated quartz plate was immersed into the protein solution and incubated for 60min

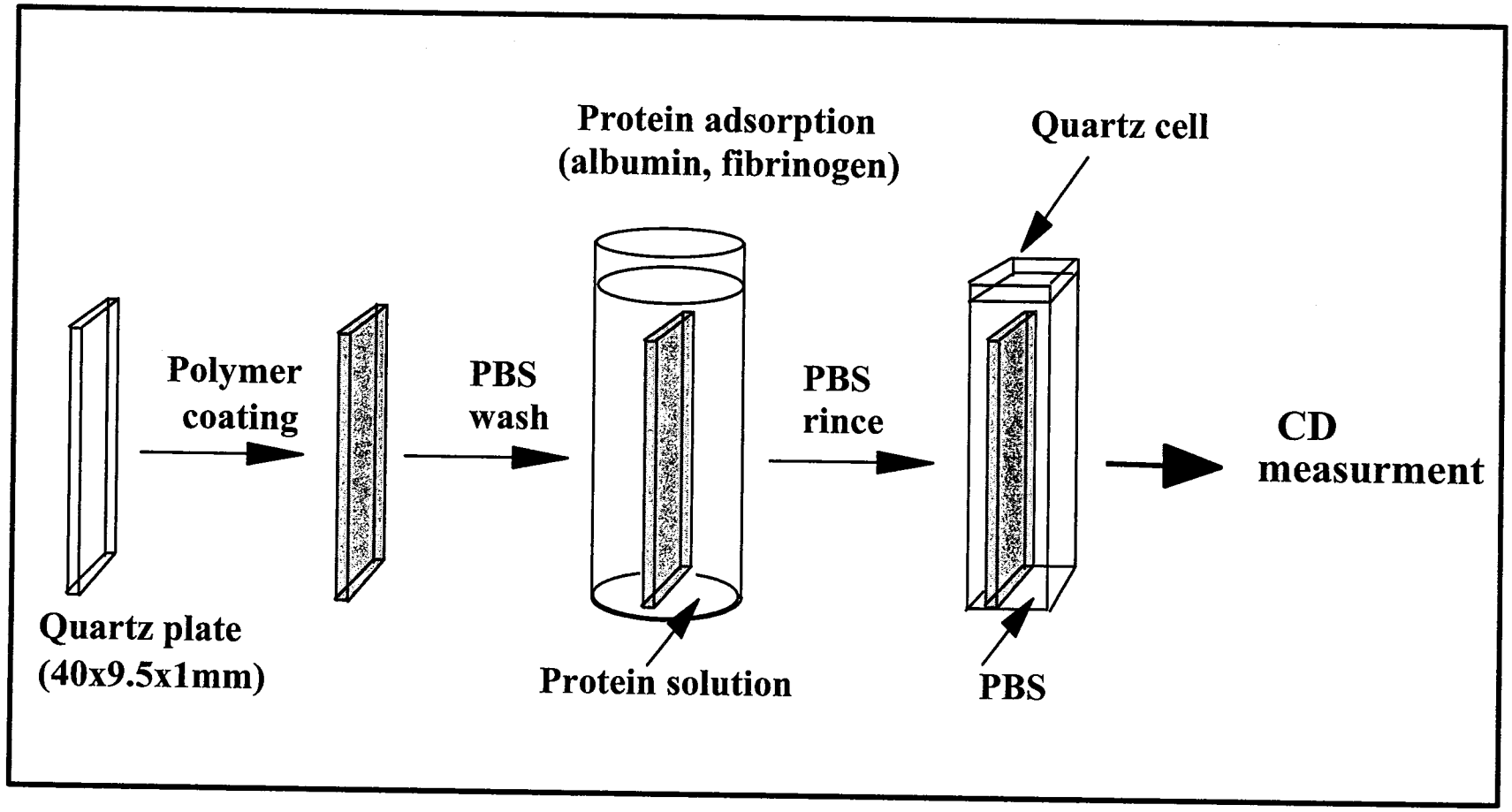


Figure 3-1. CD measurement of protein adsorbed onto the surface of polymers.

at 37°C. Then, the plate was rinsed with 20ml of PBS (pH 7.4) for 10 seconds to displace the bulk protein solution and to remove loosely bound protein from the plate (8). Then, the plate was gently rinsed with PBS for 2 seconds and was used for CD spectroscopic measurements. The α-helix content of the native and adsorbed proteins was determined from the ellipticity at 222nm. The ellipticity of poly-L-lysine, which has a 100% α-helical structure, was approximately -40,000 (deg cm^2 $dmol^{-1}$), and was used as the standard (24). The α-helix content of the adsorbed protein was calculated using the following equation according to Akaike et al. (25).

$$[\theta] = \theta/(10 \times C \times L) \text{(degree cm}^2 \text{ dmol}^{-1}\text{)}$$

$$C = (C_0/M_0) \times 1000$$

$$\alpha\text{-helix content (\%)} = [\theta]_{222}/(-40000)$$

where θ is the ellipticity in degree cm^2 $dmol^{-1}$, L is the optical pathlength in cm, C is the molar concentration in M, C_0 is the surface concentration (μg/ml), and M_0 is the mean residue weight (=118). The total amount of the protein adsorbed onto the polymer surface was measured by the gold stain method (23) as mentioned above. The stability of base line of CD depended on the polymer species. PMEA hardly affects the base line, and we chose the polymers that affected hardly the base line of the CD as the reference samples.

The results were statistically analyzed using analysis of variance (ANOVA) and Student's *t* test. The level of significance was set at $P<0.05$.

3-4 Results and Discussion

Protein adsorption

Figure 3-2 shows the adsorption of human plasma proteins onto six meth(acrylate)polymers. This figure clearly shows that the adsorption profiles differed from one another, being influenced by the different nature of the polymers surface. The total amount of plasma proteins adsorbed onto PMEA was 0.26 $\mu g/cm^2$, which was lower than that adsorbed onto PEA, PEHA, PPEA or PHEA. The amount of protein adsorbed onto PHEMA was almost the same as that adsorbed onto PMEA.

For the CD measurements, the amounts of adsorbed BSA and FNG from their mono-component solutions onto the surface of polymers were investigated (**Figure 3-3**). The amounts of BSA and FNG adsorbed onto PMEA were lower than those adsorbed onto PEHA, but they were very similar to those adsorbed onto PHEMA. This pattern was similar to that observed for protein adsorption from human plasma, but the amounts of proteins adsorbed differed (**Figure 3-3**). One possible reason for the difference is the effects of rinsing. In the case of CD measurement, we adopted a 10-second gentle rinsing procedure because this was enough to eliminate the dipping effect (8). On the other hand, we rinsed the plates five times for 30 seconds to measure the amount of strongly adsorbed plasma protein.

Figure 3-4 shows the CD spectra of BSA and FNG in PBS, which are assumed to have a native secondary structure. The CD curves showed a minimal value at 208nm and a shoulder at around 222nm which corresponded to an α-helical structure. The α-helical contents of native BSA and FNG in PBS were calculated to be 51% and 27%, respectively. The α-helix content of BSA was about twice that of FNG. These results agreed well with those reported in the literature (26). **Figures 3-5** and **3-6** show the CD spectra of BSA and FNG adsorbed onto the surface of polymers. The degree of conformational change of the adsorbed protein

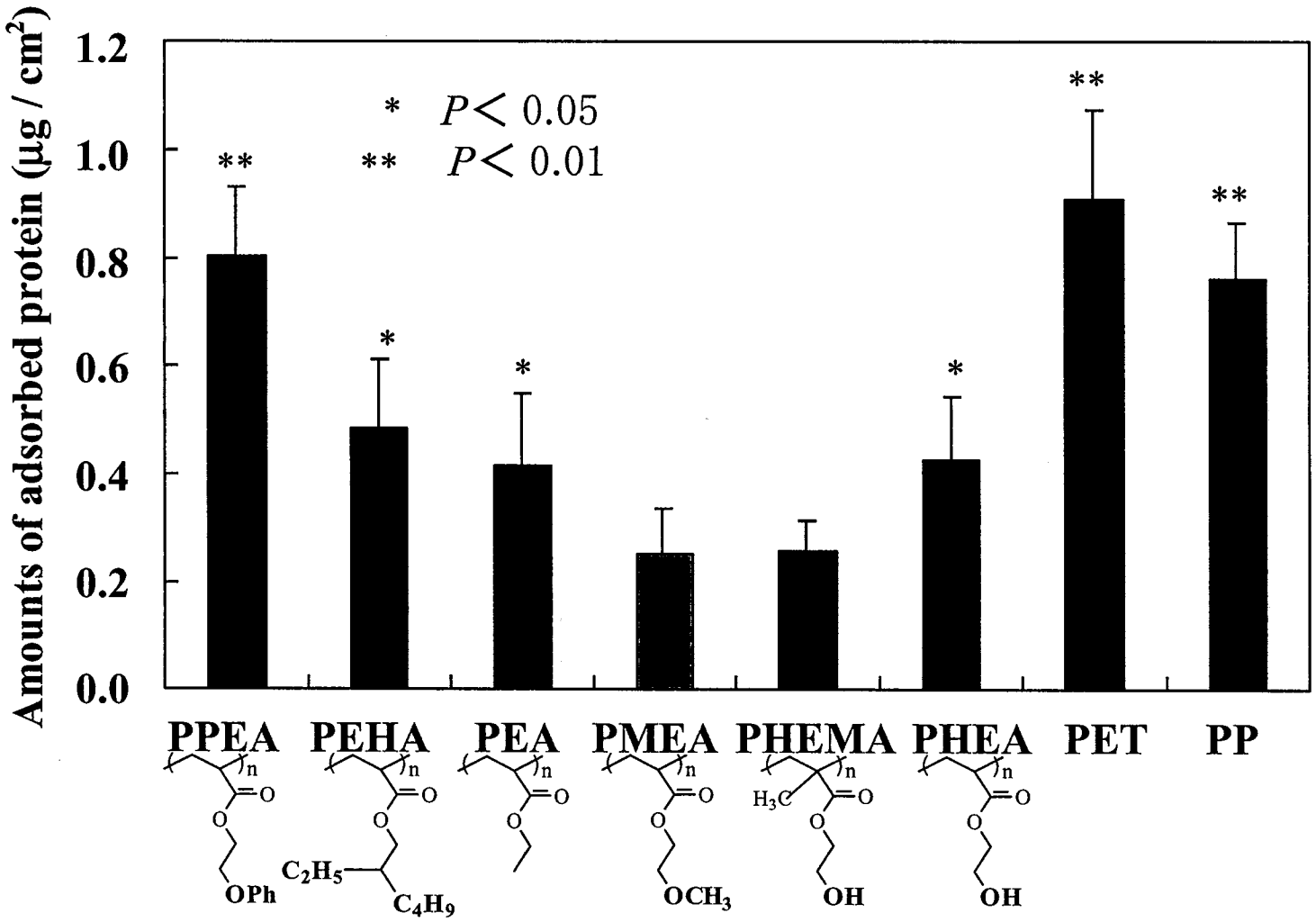


Figure 3-2. Total amount of proteins from human plasma adsorbed onto the surface of polymers. (*P<0.05 vs PMEA; **P<0.01 vs PMEA, Mean±standard deviation, n=5)

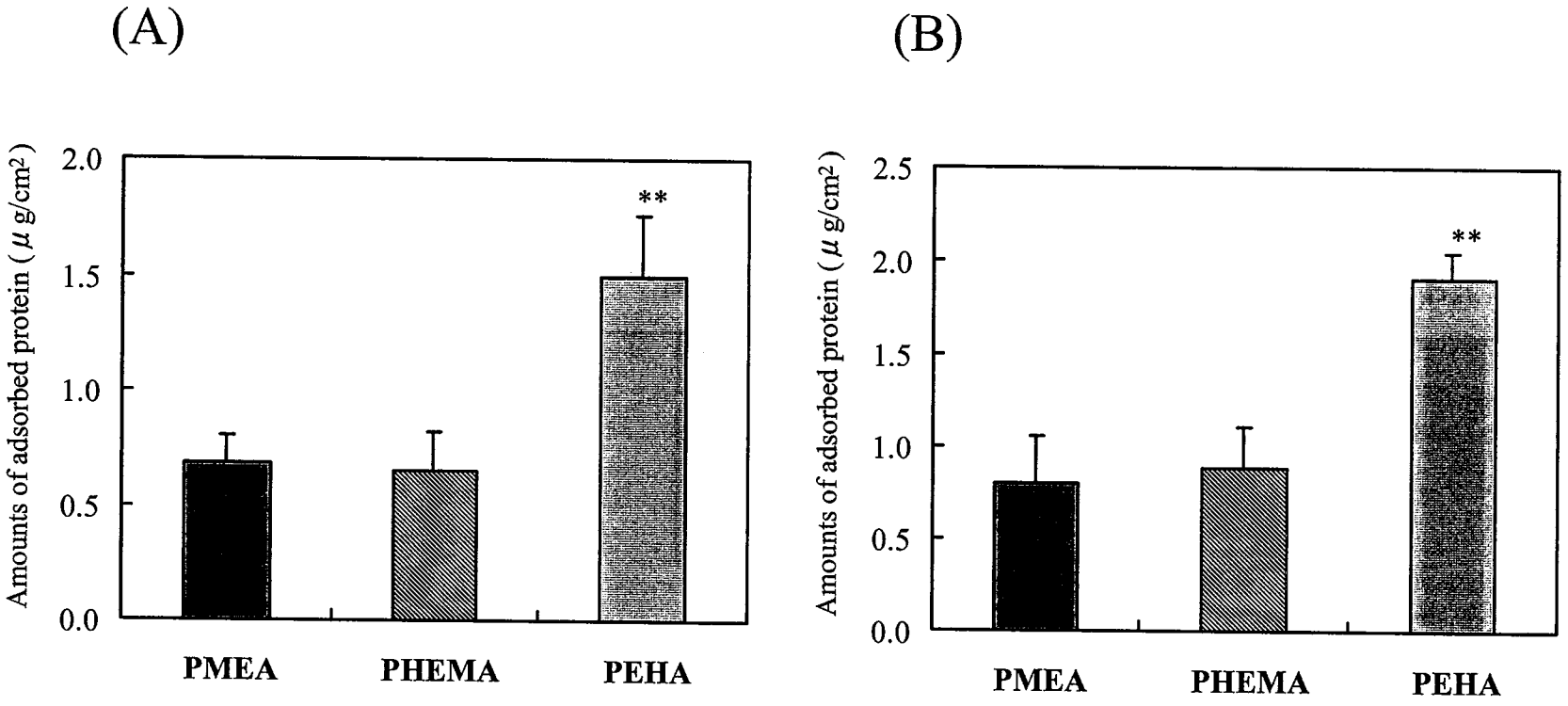


Figure 3-3. Total amount of protein adsorbed onto the surface of polymers. (A)BSA (B)FNG (**P＜0.01 vs PMEA, Mean±standard deviation, n=5)

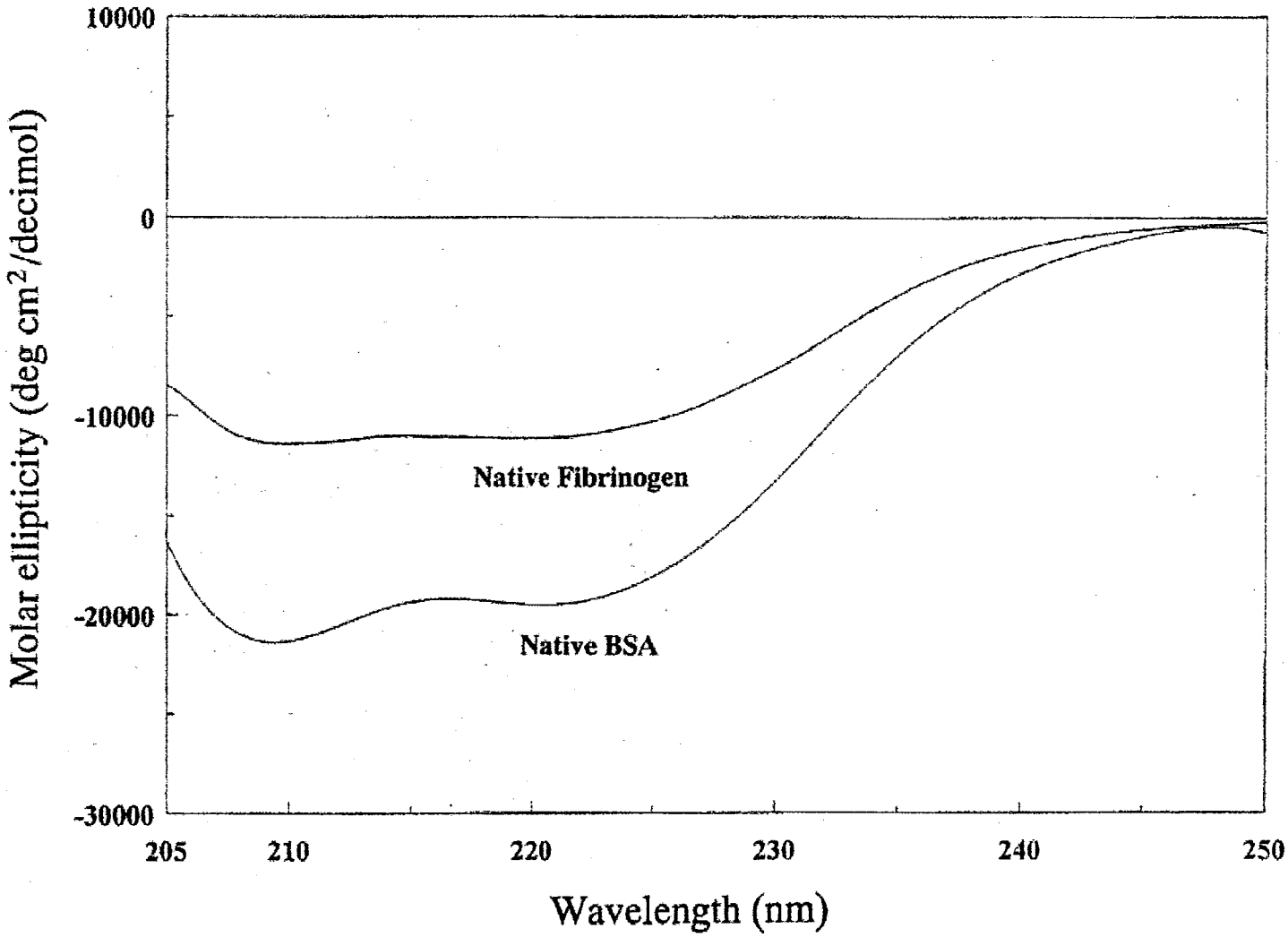


Figure 3-4. CD spectra of native BSA and FNG in PBS (pH 7.4, 37°C).

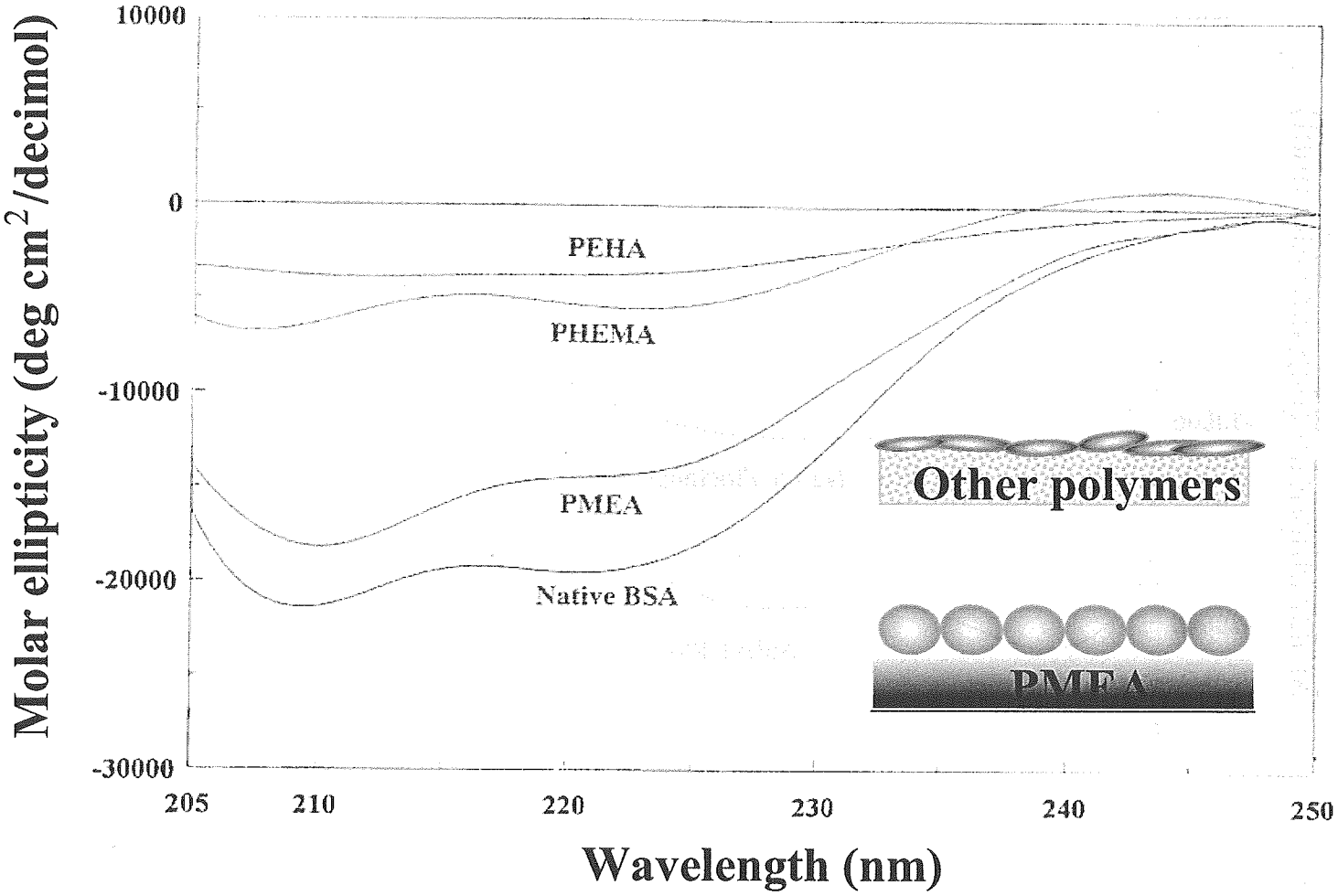


Figure 3-5. CD spectra of BSA adsorbed onto the surface of polymers (pH 7.4, 37 °C)

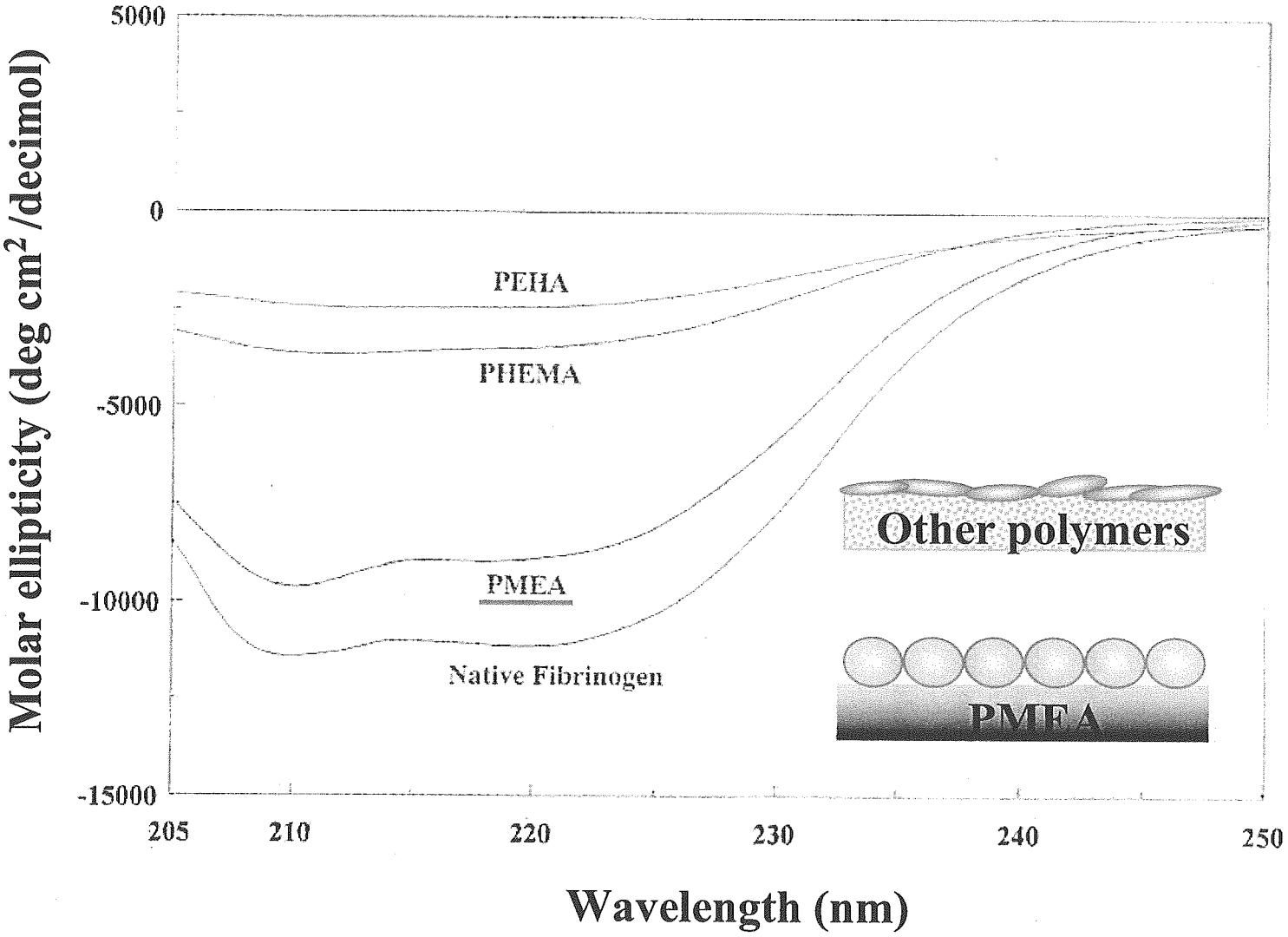


Figure 3-6. CD spectra of FNG adsorbed onto the surface of polymers (pH 7.4, 37 °C).

depended on the property of the polymer surface. The percentages of α-helix content in BSA adsorbed onto the surface of polymers and in PBS are summarized in Fig. 10, and those of α-helix content of BSA adsorbed onto the surface of PMEA, PHEMA and PEHA were 37, 15 and 14%, respectively.

Similar results were observed for FNG (**Figure 3-6**). The α-helix contents decrease from 27 to 22, 8 and 6% for PMEA, PHEMA and PEHA, respectively (**Figure 3-7**). The α-helix content of FNG adsorbed onto PHEMA and PEHA was strongly reduced. On the other hand, the α-helix content of FNG adsorbed onto PMEA was nearly equal to that of native FNG.

Platelet adhesion

When an artificial surface comes into contact with blood, plasma proteins adsorb in seconds to the surface, followed by platelet thrombus formation. At the molecular level, the surface structure has an effect on the adsorption of proteins, and these adsorbed proteins play a major biological role in platelet adhesion. Thus, platelet adhesion was investigated in terms of the amount and conformation of the proteins adsorbed onto the surface of PMEA and analogous polymers.

The number of adhered platelets onto the surface of PMEA was the least among all polymers examined (See Chapter 2 Figure 2-5). To clarify the reason, first, the adsorption of proteins is discussed. As shown in **Figure 3-2** the total amounts of proteins from human plasma adsorbed onto PMEA and PHEMA were lower than those adsorbed onto PPEA, PEHA, PEA and hydrophilic PHEA. These high adsorption of protein onto PPEA, PEHA and PEA was probably due to the hydrophobic property of their surface as demonstrated by their contact angles (See **Table 2-1**). The reason why PHEA adsorb a lot of protein is not clear, but Holly et al. also reported that the amount of protein adsorbed onto PHEA was higher than that adsorbed onto PHEMA (27, 28). These results indicated that PMEA and PHEMA surfaces did not

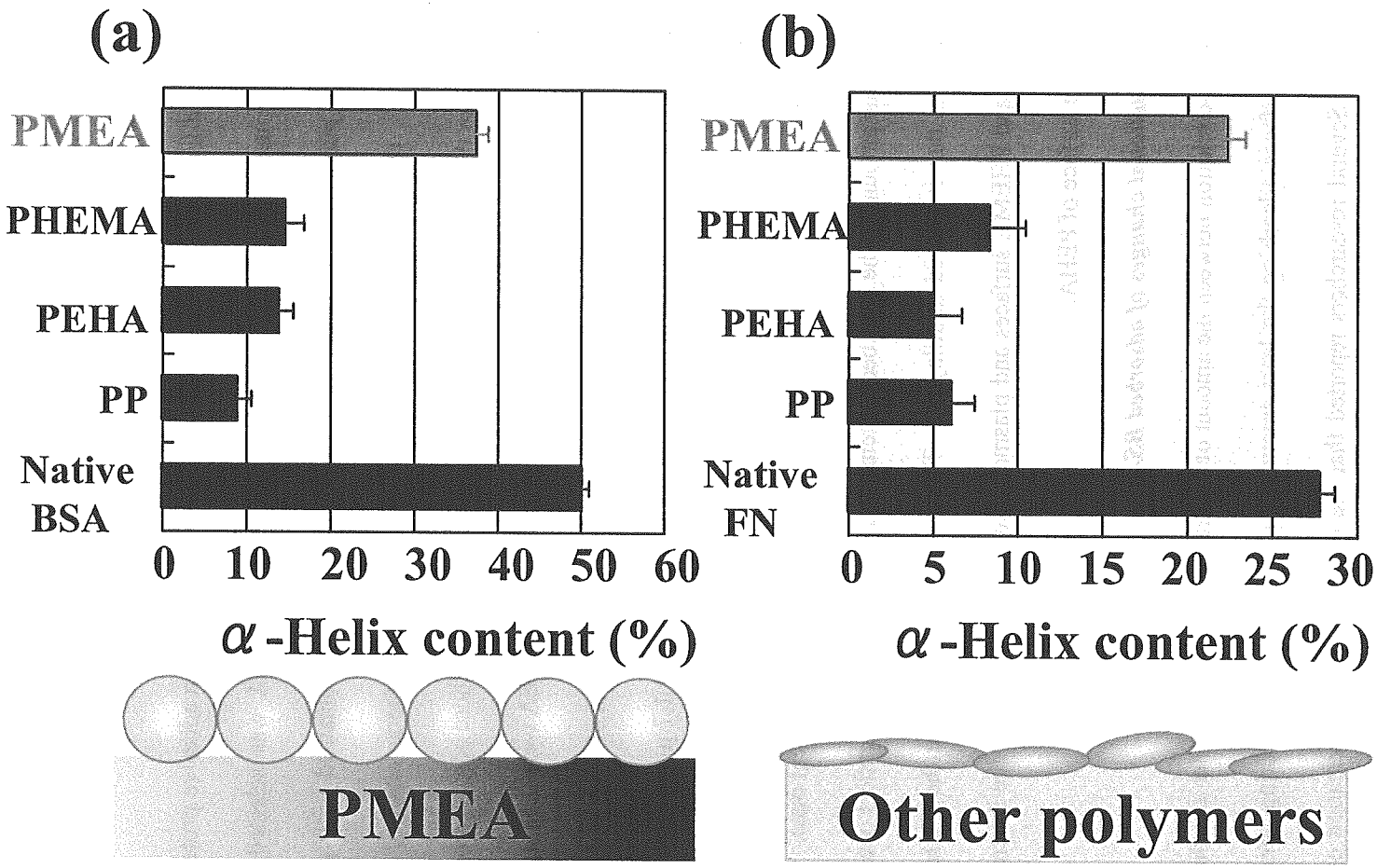


Figure 3-7. Percentage of α-helix content in BSA (A) and FNG (B) adsorbed onto the surface of polymers and in PBS. (**P＜0.01 vs PMEA, Mean±standard deviation, n=5)

induce plasma protein adsorption, compared with other polymer surfaces.

Conformational changes of adsorbed protein

We described the adsorption of proteins from a mono-component (BSA or FNG) protein solution instead of plasma protein for the CD measurements.

BSA is one of the most important transport proteins. FNG is an adhesive protein. Here, the polyacrylates used were PMEA, PHEMA and PEHA. PEHA was selected as a type of hydrophobic polyacrylate. The results were similar to those obtained with plasma protein (**Figure 3-2**). On the PMEA surface, as shown in **Figure 3-3**, the amounts of adsorbed BSA and FNG from their mono-component solutions were similar to those adsorbed onto the surface of PHEMA. The amounts of BSA and FNG adsorbed onto PMEA were 0.68 $\mu g/cm^2$ and 0.80 $\mu g/cm^2$, respectively. It was reported that the theoretical amounts of BSA and FNG adsorbed on a surface in the end-on monolayered state are 0.9 $\mu g/cm^2$ and 1.7 $\mu g/cm^2$, respectively (29). Our results imply that BSA and FNG adsorbed onto PMEA and PHEMA did not form multilayers because the amounts adsorbed were similar to the theoretical ones. In the case of PEHA, as the amount of the adsorbed proteins was larger than the theoretical ones (**Figure 3-3**), it is assumed that it formed multilayers. These results indicated that the interaction between the PMEA and PHEMA surfaces and plasma protein was weaker than the interaction that took place on the surface of PEHA.

1) Conformational changes of adsorbed BSA

Is there no relation between the amount of adsorbed protein and platelet adhesion? In general, polymer surfaces adsorbing the least amount of plasma protein will bring about less platelet adhesion. Several researchers reported that the conformation or orientation of the adsorbed protein rather than the total amount of the adsorbed protein played an important role in determining platelet adhesion onto biomaterials (30-32). The conformation of the protein

adsorbed onto the polyacrylates surface was investigated by CD spectroscopy with attention to the α-helix content. When a protein absorbes to the surface of a polymer, its secondary structure changs. A decrease of the α-helix content and an increase of the random fraction and/or β-region occur. Our data proved that different polymers induced different degrees of conformational change of the adsorbed protein. In comparison with the native protein, it was apparent that the conformational changes of BSA adsorbed onto the surface of PHEMA and PEHA were remarkable. In the case of PEHA, the amount of adsorbed protein and the conformational changes of BSA were larger than those observed in case of PHEMA and PEHA. Whereas, in the case of PMEA, little change was observed (**Figure 3-5**). These results indicated that the conformation of BSA adsorbed onto the surface of PMEA was closer to that of native BSA compared with BSA adsorbed onto the surface of PHEMA and PEHA. It is well known that platelet adhesion and spreading do not occur on an albumin-coated surface, and that with increasing degree of albumin denaturation, platelet adhesion and activation are enhanced (33). Thus, one of the reasons for the low platelet adhesion and spreading observed on PMEA may be the low degree of the conformational change of the adsorbed BSA.

2) Conformational changes of adsorbed FNG

FNG is known as a blood-clotting protein and as a universal cofactor for platelet aggregation and adhesion. The dissolved, native FNG does not bind to the adhesion receptors of platelets unless platelets are appropriately stimulated. Whereas unstimulated platelets can adhere to FNG adsorbed onto a polymer surface, it appears that adsorption of FNG to the surface accentuates and modulates the adhesion receptor and FNG interaction (2). Moreover, platelet adhesion and activation will strongly depend not only on the presence of adsorbed FNG, but also on the conformational change of the adsorbed FNG (34).

In order to provide the further supporting evidence for the hypothesis that FNG adsorbed onto

PMEA is a key player in determining platelet adhesion, the state of FNG adsorbed on PMEA was also examined by measuring the amount of adsorbed fibrinogen and its conformation. Although PMEA and PHEMA exhibited almost the same amount of adsorbed FNG, the α-helix content of FNG adsorbed onto PMEA was higher than that of FNG adsorbed onto PHEMA or PEHA (**Figure 3-7**). In the case of PEHA, the amount of adsorbed protein and the conformational change of FNG were larger than those observed in case of PHEMA and PEHA. The FNG adsorbed onto PMEA was similar to native FNG which will not be recognized by platelets. Results from these observations supported the hypothesis that conformation of the adsorbed FNG is an important factor for the adhesion of platelets onto polymer surfaces. Taken together, these results also support the concept that platelet adhesion is dominated by both the conformational change and the amount of the adsorbed BSA and FNG.

Figure 3-8 shows a schematic representation of the relationship between the state of the adsorbed protein and platelet adhesion. The PMEA surface suppresses platelet adhesion, because exposure of the binding sites of platelets is induced by denaturation of the adsorbed protein (35). When the adsorbed protein in close to the native protein, this does not take part in platelet adhesion and aggregation. Thus, the PMEA surface showed excellent blood compatibility. As for the PHEMA surface, the amount of adsorbed proteins was almost equal to that absorbed by PMEA. The conformation of the adsorbed protein was considerably altered and platelet adhesion and spreading occurred. These findings would imply that the conformational change of the protein adsorbed onto PHEMA induced the exposure of binding sites for platelets. The denaturation of the adsorbed protein could lead to platelet activation and subsequent thrombus formation. In other words, when the protein molecule which adsorbs onto a polymer surface retains its native conformation, platelets cannot adhere to the surface. If we were able to develop a surface that does not denature proteins, that surface would be blood

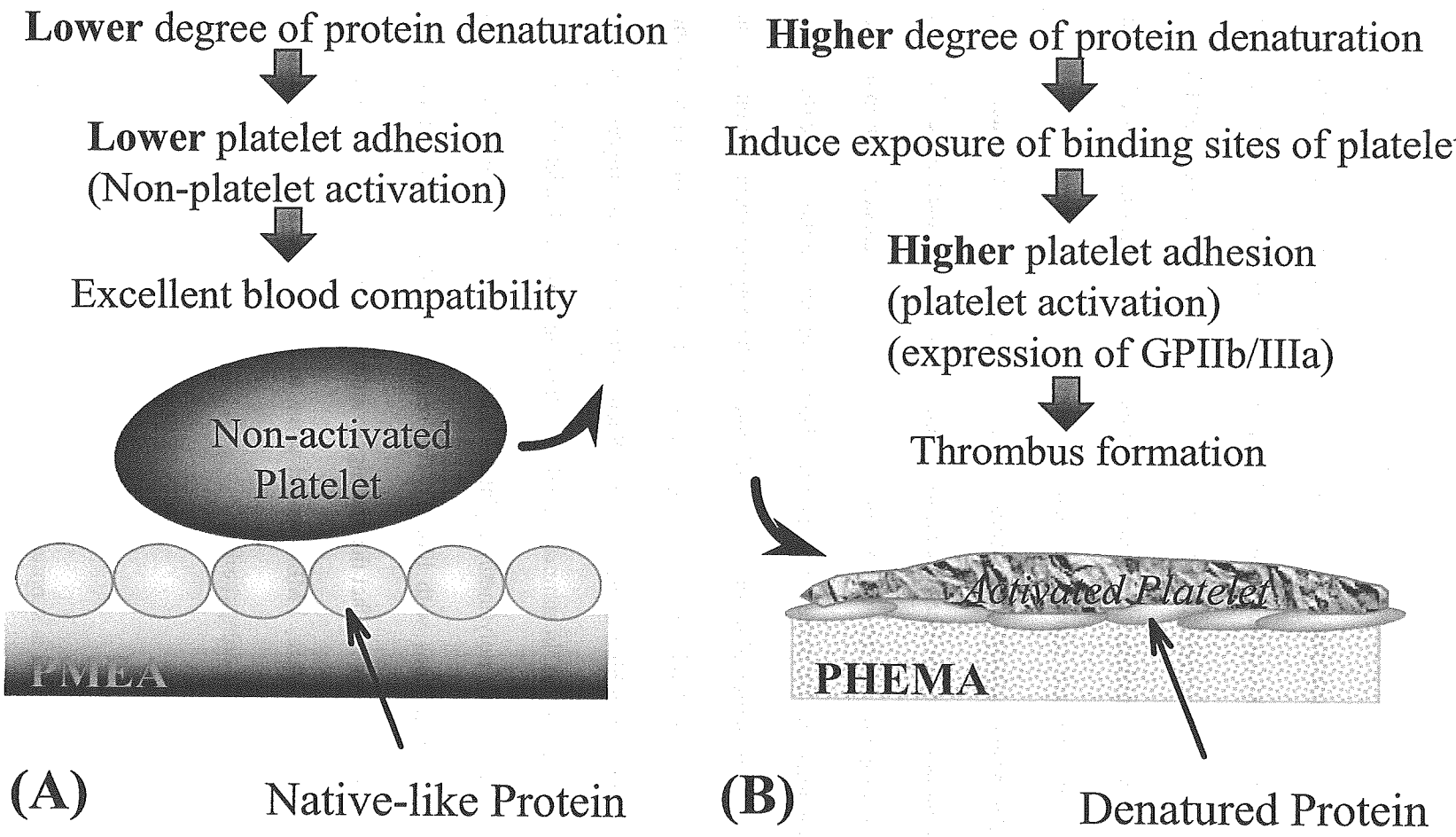


Figure 3-8. Schematic representation of the assumed adsorption state of FNG and platelet adhesion to and spreading behavior. PMEA (A) and PHEMA (B)

compatible.

Some hypotheses have been proposed to design blood compatible surfaces, which included hydrophilic surfaces, phase-separated microdomain surfaces, bioactive molecule incorporated surfaces, and biomembrane-like surfaces (36). Though the PMEA surface does not belong to these categories, the surface suppresses the denaturation of the adsorbed proteins and shows excellent compatibility for platelets. Why are the conformational changes proteins adsorbed on PMEA suppressed? Protein adsorption includes many complicated phenomena. It has been pointed out that protein adsorption depends on the various properties of the material, e.g., surface charge, wettability, surface free energy, topography or roughness, the balance between hydrophobic and hydrophilic groups, chain mobility, crystallinity, the capacity for ionic and hydrophobic interactions, and the presence of specific chemical groups on the surface (6,37,38). Recently, the environment surrounding the water molecule on the polymer surface is one of the great aspects to understand blood-contacting properties (4). How is water structured on the PMEA surface and how does it bind to the surface? Such studies are now in progress and will be reported in subsequent articles. In summary, PMEA surface suppressed platelet adhesion and spreading as compared to other poly(meth)acrylates. These results indicated that the PMEA surface minimized its interactions with blood. The reason why PMEA showed excellent compatibility with platelet lies on the fact that the higher-order structure of the adsorbed plasma proteins is maintained. Therefore, PMEA would serve to modify the surface of artificial materials to attain a higher blood compatibility.

3-5 Conclusion

Platelet adhesion and spreading is suppressed when a Poly(2-methoxyethylacrylate) (PMEA) surface is used, compared with other polymer surfaces. To clarify the reason for this

suppression, the relationship among the amount of the plasma protein adsorbed onto PMEA, its secondary structure and platelet adhesion was investigated. Poly(2-hydroxyethylmethacrylate) (PHEMA) and polyacrylate analogous were used as references. The amount of protein adsorbed onto PMEA was very low and similar to that absorbed onto PHEMA. Circular dichroism spectroscopy was applied to examine changes in the secondary structure of the proteins after adsorption onto the polymer surface. The conformation of the proteins adsorbed onto PHEMA changed considerably, but that of proteins adsorbed onto PMEA differed only a little from the native one. These results suggest that low platelet adhesion and spreading are closely related to the low degree of the denaturation of the protein adsorbed onto PMEA. PMEA could be developed as a promising material to produce a useful blood contacting surface for medical devices.

3-6 References

(1) Brash JL, Horbett TA. In: Proteins at interfaces: Physicochemical and Biochemical Studies, ACS Symposium Series 343, Washington, DC, 1987;1-33

(2) Andrade JD. Principles of protein adsorption. in surface and interfacial aspects of biomedical polymers, Andrade JD, ed. Plenum Publ., New York 1985 1-80

(3) Vroman L, Adams AL. Identification of rapid changes at plasma-solid interfaces. J Biomed Mater Res 1969; 3:43-67

(4) Tsuruta T. Contemporary topics in polymeric materials for biomedical applications. Adv Polym Sci, 1996;126:1-51

(5) Horbett TA. Some background concepts. In: Biomaterials Science; An introduction to materials in medicine. Ratner BD, Schoen FJ., Lemons JE, ed. Academic Press., London. 1996;133-141,

(6) Horbett TA, Brash JL, ed. Proteins at interfaces II. Fundamental and applications. ACS Symposium Series 602, Washington, DC, 1995

(7) Nagai H, Handa M, Kawai Y, Watanabe K, Ikeda Y. Evidence that plasma fibrinogen and platelet membrane GPIIb-IIIa are involved in the adhesion of platelets to an artificial surface exposed to plasma. Thromb Res 1993;71:467-477

(8) Ito Y, Shishido M, Imanishi Y. Adsorption of plasma proteins to the derivatives of polyetherurethaneurea carrying tertiary amino groups in the side chains. J Biomed Mater Res 1986;20:1139-1155

(9) Sanada T, Ito Y, Shishido M, Imanishi Y. Adsorption of plasma proteins to the derivatives of polyaminoetherurethaneurea. The effect of hydrogen-bonding property of the material surface. J Biomed Mater Res 1986;20:1179-1195

(10) Lenk TJ, Ratner BD, Gendreau RM, Chittur KK. IR spectral changes of bovine serum albumin upon surface adsorption. J Biomed Mater Res 1989;23:549-569

(11) McMillin CR, Walton AG. A circular dicroism technique for the study of adsorbed protein structure. J Colloid Interface Sci 1974;48:345-349

(12) Norde W, Favier JP. Structure of adsorbed and desorbed proteins. Colloids and Surfaces 1992;64:87-93

(13) Castillo EJ, Koenig JL, Anderson JM, Lo J. Characterization of protein adsorption on soft contact lenses. I. Conformational changes of adsorbed human serum albumin. Biomaterials 1984;5:319-325

(14) Nyilas E, Chiu TH, Herzlinger GA. Thermodynamics of native protein/fibrinogen surface interactions I. Calorimetry of the human γ-globulin/glass system. Trans ASAIO 1974;20:480-490

(15) Ishihara K, Nomura H, Mihara T, Kurita K, Iwasaki Y, Nakabayashi N. Why do

phospholipid polymers reduce protein adsorption?. J Biomed Mater Res 1998;39:323-330

(16) Shiba E, Lindon JN, Kushner L, Kloczewiak M, Hawiger J, Matsueda G, Kudryk B, Salzman EW. Conformational changes in fibrinogen adsorbed on polymer surfaces detected by polyclonal and monoclonal antibodies. in Fibrinogen 3, Moseson MW, Ambani DL, Siebenlist, DiOrio JR,. ed, Elsevier Science, New York, 1988;239-244

(17) Zamarron C, Ginsberg MH, Plow EF. Monoclonal antibodies specific for a conformationally altered state of fibrinogen. Thromb. Haemostas 1990;64;41-46

(18) Lindon JN, McManama G, Kushner L, Merrill EW, Salzman EW. Does the conformation of adsorbed fibrinogen dictate platelet interactions with artificial surfaces?. Blood 1986; 68: 355-62

(19) Kiaei D, Hoffman, AS, Horbett TA, Lew KR. Platelet and monoclonal antibody binding to fibrinogen adsorbed on glow-discharge-deposited polymers. J Biomed Mater Res 1995; 29: 729-39

(20) Onishi M, Tnaka M, Kawada M, Anzai T, Kasori Y, Shimura. J Biomater Sci Polym Edn, submitted for publication.

(21) Dong DE, Andrade JD, Coleman DL. Adsorption of low density lipoproteins onto selected biomedical polymers. J Biomed Mater Res 1987; 21: 683-700

(22) Chandy T, Sharma CP. Effects of lipoproteins on protein/platelet interaction on polymers. J Biomed Mater Res 1991; 25: 1085-1094

(23) Rohringer R, Holden DW. Quantification of proteins in the low nanogram range by staining with the colloidal gold stain aurodye. Anal Biochem 1985;144:118-127

(24) Greenfield N, Fasman G.D. Computed circular dichroism spectra for the evaluation of protein conformation. Biochemistry 1969;8:4108

(25) Akaike T, Sakurai Y, Kosuge K, Senba Y, Kuwana K, Miyata S, Kataoka K, Tsuruta T.

Study on the interaction between plasma proteins and polyion complex by circular dicroism and ultraviolet spectroscopy. Koubunshi Ronbunshu 1979;36:217-222

(26) Soderquist ME, Walton AG. Structural change in protein adsorbed on polymer surfaces. J Colloid Interface Sci 1980;75:386-397

(27) Holly FJ, Refojo MF. In: Hydrogels for Medical and Related Applications. Andrade JD, ed, ACS Symposium Series 31, Washington, D.C.,1976, p252

(28) Holly FJ. Protein and lipid adsorption by acrylic hydrogels and their relation to water wettability. J Polym Sci Polym Symp 1979;66:409-417

(29) Baszkin A, Lyman DJ. The interaction of plasma proteins with polymers. I. Relationship between polymer surface energy and protein adsorption/desorption. J. Biomed Mater Res 1980;14:393-398

(30) Chinn JA, Horbett TA, Ratner BD. Baboon fibrinogen adsorption and platelet adhesion to polymeric materials. Thromb Haemostas 1991;65: 608-617

(31) Tsai WB, Grunkemeier JM, Horbett TA. Human plasma fibrinogen adsorption and platelet adhesion to polystyrene. J Biomed Mater Res 1999; 44: 130-139

(32) Balasubramanian V, Grusin NK, Bucher RW, Turitto VT, Slack SM. Residence-time dependent changes in fibrinogen adsorbed to polymeric biomaterials. J Biomed Mater Res 1999; 44: 253-260

(33) Ito Y, Shishido M, Imanishi Y. Adsorption of plasma proteins onto novel polyetherurethaneureas-relationship between denaturation of adsorbed proteins and platelet adhesion. J Biomed Mater Res 1990;24:227-242

(34) Horbett TA. Principles underlying the role of adsorbed plasma proteins in blood interactions with foreign materials. Cardiovascular Pathology 1993;2:137S-148S

(35) Tomioka M, Iwamoto M, Olsson P, Soderman S, Blomback B. On the platelet fibrinogen

interaction. Thromb Res.1980;19: 869-876

(36) Akaike T, Okano T, Akashi M, Terano M, Yui N, ed. Advances in Polymeric Biomaterials Science, CMC Co., LTD, Tokyo, 1997

(37) Brier-Russell D, Salzman EW, Lindon J, Merrill EW, Dincer AK, Wu JS. In vitro assessment of blood with model surfaces: acrylates and methacrylates. J Colloid Interface Sci 1981;81:311-318

(38) Haynes CA, Norde W. Globular proteins at solid/liquid interfaces. Colloids Surfaces B. Biointerfaces 1994; 2: 517-566

Chapter 4 Kinetics of Protein Adsorption and Desorption onto a PMEA Surface by a *In Situ* Quartz Crystal Microbalance

4-1 Introduction

In Chapter 2 and 3, we showed that PMEA had the excellent compatibility with platelet, white blood cell, coagulation and complement systems, and that the protein adsorption and protein denaturation were in the lowest level when compared with other polymers (1- 7).

It is pointed out by many researchers that adsorption properties of plasma proteins onto the polymer surfaces affect the blood compatibility of polymers (8-10). One of the important properties is the adsorption amount of the proteins on the material surface. The well-known methods to determine the adsorption amount were ultraviolet spectroscopy (UV), infrared spectroscopy (IR), X-ray photoelectron spectroscopy (XPS) and radioisotope-labeled immunoassay (RI) (10-12). However, these methods cannot provide information about the real time adsorption behavior of the proteins. In other words, these methods cannot observe the adsorbing process and thus cannot evaluate directly the polymer-protein interactions. As *in situ* studies on the adsorption properties, radioisotope-labeling and fluorescent-labeling techniques have been reported (11, 12). One of the methods which has been successfully applied to get information on an absorbed proteins is total internal reflection fluorescence spectroscopy (TIRF) (13, 14). The high sensitivity of fluorescence spectroscopy enables the quantification of small amounts of the adsorbed proteins, including the competitive adsorption, interfacial conformation changes, and the surface mobility of the adsorbed proteins. These methods, however, have some problems. One is the complicated procedure to introduce the radioisotope- or fluorescent-labels to the proteins as pre- or post- treatment, and moreover this procedure has a possibility of denaturing the protein. Another problem is that it takes relatively long time to obtain the results. Recently, a new methodology, surface plasmon resonance, has been applied to

in situ detection of the adsorbed protein. However, it requires large and expensive equipment and has some difficulties to obtain quantitative results. As an effective and easy method to analyze the *in situ* biomolecular interaction, quartz crystal microbalance (QCM) has been recommended (15-19). QCM is known to be very sensitive mass-measuring device in the air (20, 21) and in the aqueous solution (22-25). The resonance frequency of QCM electrode decreases linearly with the increase in the mass of the electrode due to the adsorption of some compound, and the sensitivity is in nanogram level. Several researchers have reported the interaction between polymeric biomaterial and protein by using this method, but the investigations have been limited to the qualitative ones (26). We have adopted this method to perform the quantitative analysis of adsorption behavior of protein onto the PMEA surface.

When an artificial material comes into contact with blood it will rapidly adsorb proteins onto its surface, and the adsorbed protein layer will determine all further events, namely, platelet adhesion, aggregation and coagulation, as mentioned above. Protein adsorption behaviors on various kinds of polymer surfaces have been extensively investigated. It is reported that the important factor expressing the biocompatibility is not the amount of adsorbed proteins on the surfaces but the structure or orientation of the adsorbed proteins (27-34). One of the attractive objects is whether the protein adsorption is reversible or not, and many researches on it have been carried out. There are many reports that insisted on the irreversible adsorption of proteins on the polymer surface (35-37), whereas some researchers reported the reversible adsorption (38). Thus, it is important to analyze the kinetics during adsorption of protein on polymer surface in addition to the adsorption amount of the protein when the blood compatibility of polymers are discussed (chapter 2 and 3).

In this chapter, we report the comparison of the adsorption properties of proteins to the polymer surfaces using the saturation method of QCM. That is, we discuss the adsorption

properties onto the PMEA, poly(2-hydroxyethylmethacrylate) (PHEMA), and polypropylene (PP) in terms of the maximum adsorption amounts and the apparent association constants of the proteins (39). PHEMA and PP were selected as the representatives of the hydrophilic and hydrophobic polymers, respectively because the former is known as one of the most popular biomedical polymer and the latter is used in many medical devices. We also report the investigation of early stage of the protein adsorption behaviors on PMEA and other common polymer surfaces by the curve fitting method of QCM (40).

4-2 Experimental

4-2-1 Coating of a QCM electrode with polymer and characterization of it

Materials

PMEA and PHEMA were prepared by radical polymerization and their weight average molecular weights were 85,000 and 110,000, respectively (by GPC with polystyrene standard). Polypropylene (PP) was purchased from Aldrich Chemical, Ltd. (Milwaukee, WI). Methanol and xylene used as solvents were of analytical grade.

Proteins, bovine serum albumin (BSA) and human plasma fibrinogen, were purchased from Sigma Co. Ltd. (St. Louis, MO), and used without further purification. Protein solutions with the concentration mentioned below were prepared using a phosphate-buffered saline (PBS, pH7.4). The concentrations of BSA were varied in the range of 0 to 27×10^{-6} M, and those of fibrinogen were from 0 to 32×10^{-8} M, , respectively.

4-2-2 Measurement of QCM

QCM-apparatus

Figure 4-1 shows a schematic illustration of a QCM equipment. The 9 MHz QCM employed

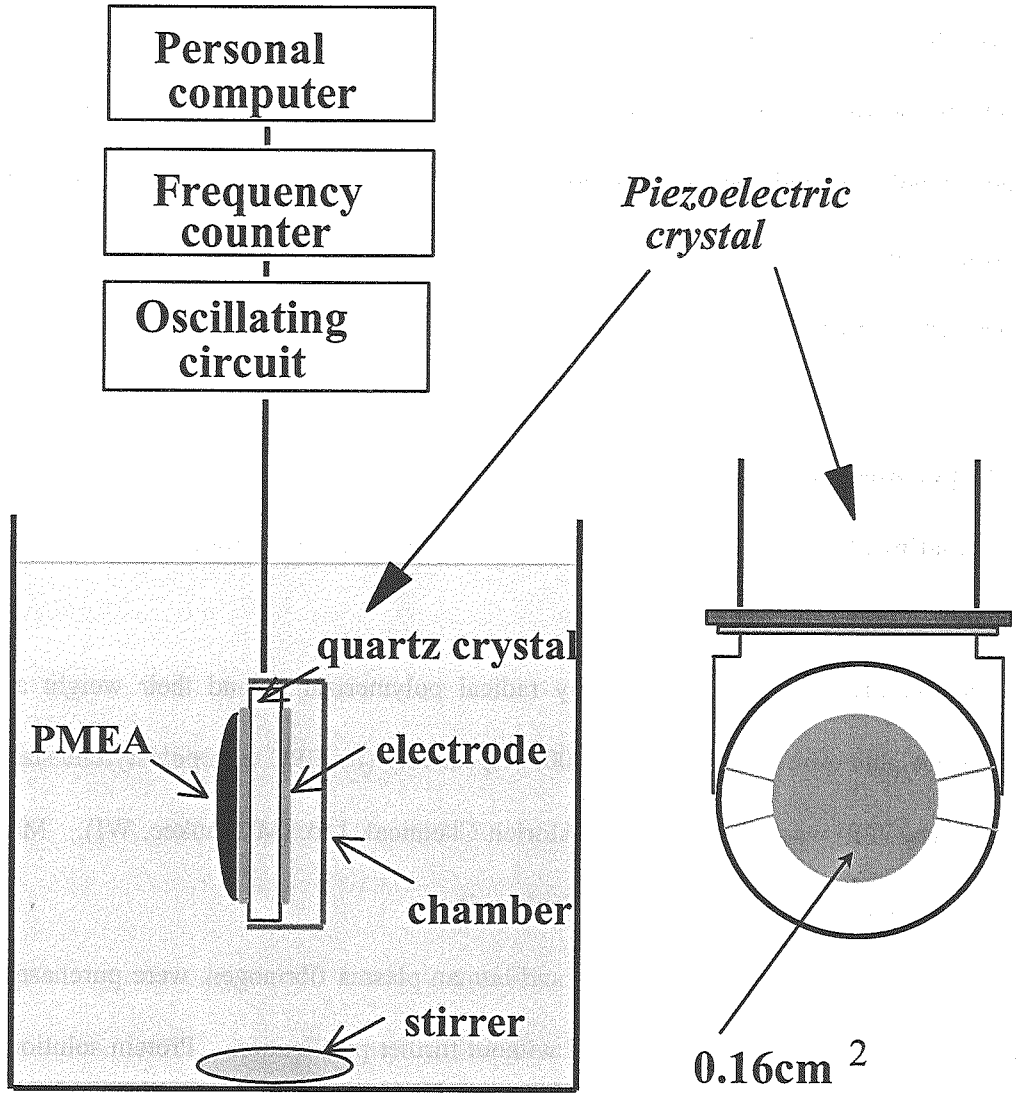


Figure 4-1. Schematic Diagram of QCM System.

was a commercially available from Ukou Electronics Co. Ltd., (Saitama, Japan). Au electrode (area 0.16 cm^2) was deposited on both sides of the AT-cut quartz (18 mm diameter). The one side of the QCM electrode was covered with a rubber sealing in order to insulate from the ionic aqueous protein solution, while the other was exposed to the aqueous solution. The electrode was connected to an oscillation circuit designed to drive it. The frequency changes were followed by a universal frequency counter (Iwatsu Co., model SC-7201, Tokyo, Japan) attached to a personal computer system (NEC Co., Model PC 9801, Tokyo, Japan). The data of the frequency change during the experiment were put into the personal computer in every second, and treated. The stability or the drift of the 9 MHz QCM frequency in the aqueous solution and in the air was in the range of ±1 Hz for 3 h at 37 ℃.

The relationship between the frequency change and the change of the quartz electrode mass in the air is presented by Sauerbrey equation **[1]** (15)

$$\Delta F = \frac{-2F_0^2}{A\sqrt{\rho d}}\Delta m \qquad [1]$$

where ΔF = the measured frequency change (Hz), F_0 = the parent frequency of QCM (9×10^6 Hz), Δm = the adsorption amount (ng), ρ = the density of quartz (2.65 g/cm^3), **d** = the shear modulus of quartz (2.95×10^{11} dyn/cm^2), and **A** = the effective area of the electrode (0.16 cm^2). Substituting these values of F_0, ρ, d, and A for eq [1] yields eq [2].

$$-\Delta F = (2.1\pm0.1)\Delta m \qquad [2]$$

When QCM is employed in an aqueous solution, eq [1] cannot be simply applied due to the

effects of interfacial liquid properties such as viscosity, density and conductivity (41-43). It is, however, known that eq [1] can be used for the solution system existing under specific condition where the influence of the visco-elasticity of the polymer is negligible (23-25). In our experiments, a linear relationship was observed between the change of mass and frequency change of the QCM both in the air and in the aqueous solution. This means that the Sauerbrey equation can be applied in our aqueous solution system, and the frequency change of 1 Hz corresponds to the mass increase of 0.5 ng on the QCM electrode (23-25).

Coating of a QCM electrode with polymer and characterization of it

The bare Au plate side of the QCM was cleaned with a dilute sulfuric acid (H_2SO_4/H_2O_2=3/1 (v/v)) (piranha solution) and was rinsed with ultra-pure water and $CHCl_3$/MeOH=2/1 (v/v). PMEA or PHEMA was simply coated on the cleaned Au surface by casting the MeOH solution of 0.1wt% polymer, while polypropylene (PP) was cast from the xylene solution of 0.1wt% polymer. The coated QCM electrode was dried under air at room temperature, and dried in vacuum oven for 24h to remove the residual solvent.

The surfaces of these coated electrodes were analyzed by X-ray photoelectron spectroscopy (XPS) (Jeol, JPS-90SX, Tokyo, Japan) to confirm the state of the coated polymer. MgKα X-ray source (1254 eV) was used for this measurement, and the take-off angle was 90°.

Contact angle was measured by the captive-bubble technique (44). The height (h) and width (d) of the air bubble in contact with the polymer surface were observed by a microscope. Contact angle (θ) was calculated according to the following equation;

$$\theta = \arccos(2h/d - 1)$$

Measurement of QCM

At first, the stability of the frequency of the QCM electrode in the PBS buffer at 37 °C was confirmed and then the adsorption experiment was started by the addition of an adequate protein solution into the PBS buffer. The solution was stirred at 1000 rpm. It was confirmed that the stirring did not disturb the stability of QCM. Monitoring the frequency change was continued for 3000 sec. During the experiments, the PBS buffer was stirred gently in order to avoid the effect of the diffusion process of the protein, and the stirring was confirmed not to affect the stability of QCM.

4-2-3 Analysis of QCM Data (curve fitting method)

The protein adsorption on polymer surface is described by the following Eq 1.

$$[\text{Polymer}] + [\text{Protein}] \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} [\text{Polymer–Protein}] * \underset{k_{-2}}{\overset{k_2}{\rightleftarrows}} [\text{Polymer–Protein}] \qquad 1$$

In the equation, [Polymer-Protein]* means the meta-stable complex composed of the polymer surface and the protein, and is formed by just the collision of the protein with the surface at the rate, k_1. In the meta-stabel complex the protein on the polymer surface can be detached at k-1 until the reorientation of the protein, which minimize the interaction energy between the protein and the polymer surface, is occurred (i.e.: This reaction is reversible), or changes to the final product, adsorbed protein, at k_2 (mainly irreversible).

In the early stage of the reaction, it is assumed that the production of meta-stable complex was predominant and the final product is negligible. Thus, Eq 1 could be approximated by Eq 1'.

$$\frac{d[\text{Polymer–Protein}]}{dt} = k_1[\text{Polymer}][\text{Protein}] - k_{-1}[\text{Polymer–Protein}]* \qquad 1'$$

where the concentration of polymer, [Polymer], means the number of the absorption site on the polymer surface.

On the basis of Eq 1', the production rate of meta-stable complex can be expressed by the following Eq.

$$d[\text{Polymer-Protein}]/dt = k_1[\text{Polymer}][\text{Protein}] - k_{-1}[\text{Polymer-Protein}]* \qquad 2$$

The concentration of polymer is expressed as follows.

$$[\text{Polymer}] = [\text{Polymer}]_0 - [\text{Polymer–Protein}]* \qquad 3$$

where $[\text{Polymer}]_0$ is the initial concentration of polymer. From Eqs 2 and 3, following equation 4 is given.

$$\frac{d[\text{Polymer–Protein}]*}{dt} = -(k_1[\text{Protein}] + k_{-1})\left\{\frac{[\text{Polymer–Protein}]* - k_1[\text{Polymer}]_0[\text{Protein}]}{(k_1[\text{Protein}] + k_{-1})}\right\} \qquad 4$$

When the equation in the braces of the right side in the Eq 4 is abbreviated as [Polymer-Protein]*' (Eq 5), Eq 4 can be changed to new equation (Eq 6)

$$[\text{Polymer–Protein}]*' = \frac{[\text{Polymer–Protein}] - k_1[\text{Polymer}]_0[\text{Protein}]}{k_1[\text{Protein}] + k_{-1}} \qquad 5$$

$$\frac{d[\text{Polymer–Protein}]*}{dt} = -(k_1[\text{Protein}] + k_{-1})[\text{Polymer–Protein}]*' \quad 6$$

In this experiment, we supposed that [Protein] approximated to $[\text{Protein}]_0$,

$$[\text{Protein}] = [\text{Protein}]_0 \quad 7$$

because the amount of the adsorbed protein was significantly small compared with the initial amount of the protein in the solution. Thus, Eq 5 can be converted to the following equation.

$$[\text{Polymer–Protein}]*' = \frac{[\text{Polymer–Protein}]* - k_1[\text{Polymer}]_0}{k_1[\text{Protein}]_0 + k_{-1}} \quad 8$$

Both sides of Eq 8 are differentiated and Eq 9 is given.

$$\frac{d[\text{Polymer–Protein}]*'}{dt} = \frac{d[\text{Polymer–Protein}]*}{dt} \quad 9$$

From Eqs 6,7 and 9, Eq 10 is given

$$\frac{d[\text{Polymer–Protein}]*'}{dt} = -(k_1[\text{Protein}]_0 + k_{-1})[\text{Polymer–Protein}]*' \quad 10$$

Integrating Eq 10 by time t gives Eq 11

$$[\text{Polymer–Protein}]*' = Ae^{(-1/\tau)t} \quad 11$$

Where τ is the relaxation time of the adsorption, and the reciprocal of τ is defined by Eq 12.

$$\tau^{-1} = k_1\,[\text{Protein}]_0 + k_{-1} \qquad \textbf{12}$$

From Eqs 5 and 11,

$$[\text{Polymer–Protein}]* = \frac{k_1[\text{Polymer}]_0[\text{Protein}]}{k_1[\text{Protein}] + k_{-1}} + Ae^{(-1/\tau)t} \qquad \textbf{13}$$

When the time t is zero, [Polymer-Protein] is also zero. Consequently, from the Eq 13, following equation is obtained.

$$A = -\frac{k_1[\text{Polymer}]_0[\text{Protein}]}{k_1[\text{Protein}] + k_{-1}} \qquad \textbf{14}$$

By combing Eqs 13 and 14, the amount of polymer-protein complex formed at time t is given by Eq 15 (19-22).

$$[\text{Polymer–Protein}]* = \left\{\frac{k_1[\text{Polymer}]_0[\text{Protein}]}{(k_1[\text{Protein}] + k_{-1})}\right\}(1 - e^{(-1/\tau)t}) = [\text{Polymer–Protein}]_{t\to\infty}(1 - e^{(-1/\tau)t}) \qquad \textbf{15}$$

where $[\text{Polymer-Protein}]^*_{t\to\infty}$ is the concentration of meta-stable polymer-protein complex at theoretical time ∞.

The absorbed mass of the protein on QCM electrode can be substituted for the concentration of the complex in Eq 15, and thus Eq 15 is expressed as

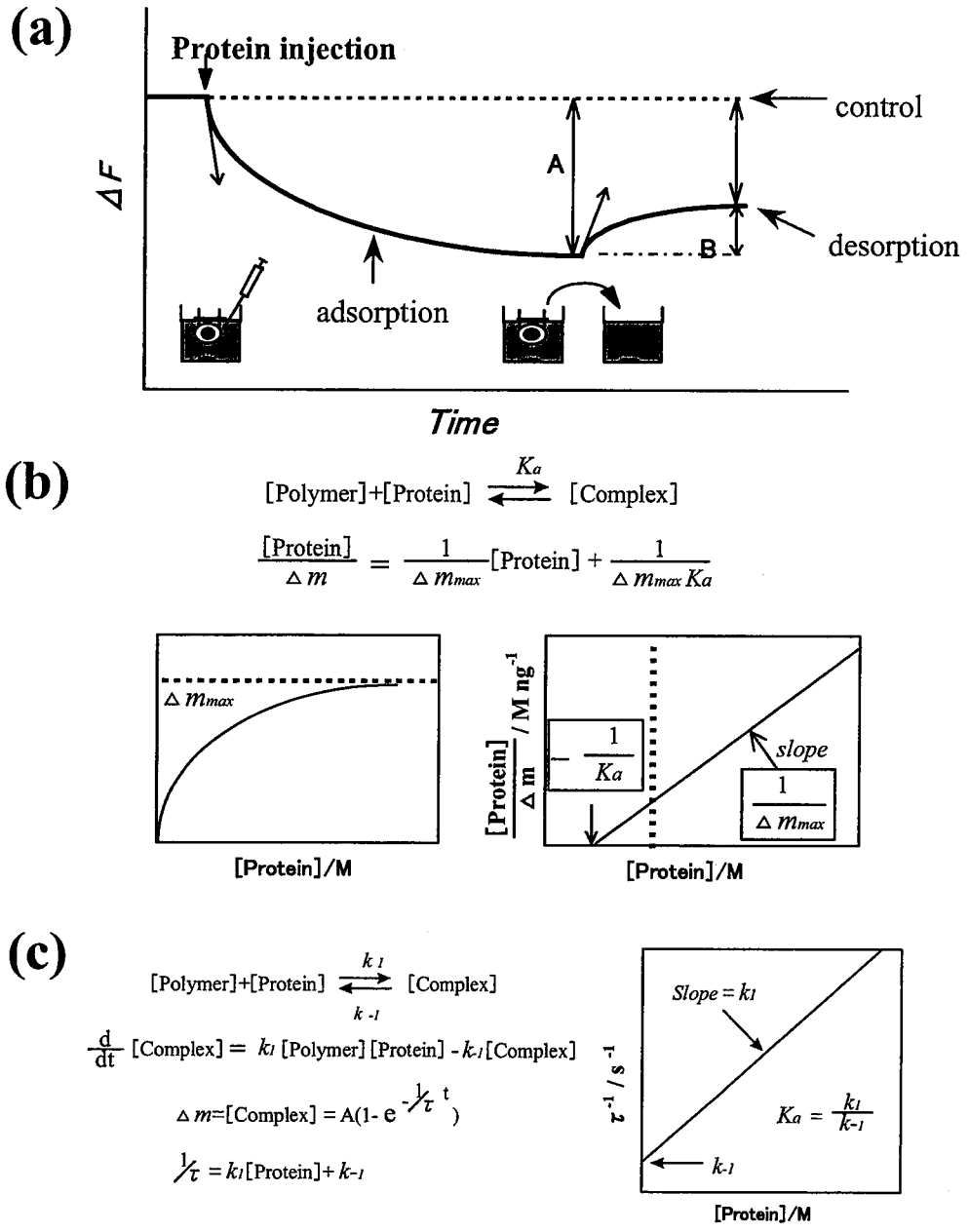


Figure 4-2 (a) Real-time monitoring of protein adsorption to polymer surfaces (37 ℃ , PBS) A: Amount of adsorbed protein B: Amount of desorbed protein. Kinetic measurements by using a QCM method. Saturation method (b) and Curve fitting method (c)

$$\Delta m_t = \Delta m_{met}(1-e^{(-1/T)t}) \qquad 16$$

where Δm_t is the mass increase of the QCM electrode at time *t, and* Δm_{met} is the mass increase at 1300 sec.

Figure 4-2 shows schematic representation of the frequency change curve of the adsorption and desorption process in the time course and the kinetic measurements by QCM method.

4-3 Results and Discussion

Protein adsorption on polymer surface (saturation method)

Figure 4-3 shows the XPS spectrum of surface of the QCM electrode coated with PMEA. C1s and O1s peaks based on the polymer were observed, and Au peak could not be observed. The XPS spectra of other electrodes coated with PHEMA and PP also showed no Au peak. This result indicates that the electrode surface is completely covered with the polymer.

The contact angles of the air bubble in contact with the polymer surface are presented in **Table 4-1**. These data indicate that the hydrophilicity of PMEA is between PP and PHEMA. **Figure 4-4** shows a typical time-course of frequency changes (ΔF) caused by the adsorptions of BSA onto the PMEA-coated QCM. Almost the same time-course curves were obtained for the adsorption of fibrinogen except the degree of the frequency change. On the basis of the frequency changes in the equilibrium state of the adsorption, Δm, the amount of each protein adsorbed onto the electrode could be calculated. The results are shown in **Figure 4-5**, where the adsorption amounts are plotted against the protein concentration in the PBS buffer. In both the protein systems, Δm increased sharply on injecting the protein solution to the PBS buffer, and reached the plateau (**Figure 4-4**). It is known that the adsorption onto the polymer surface is

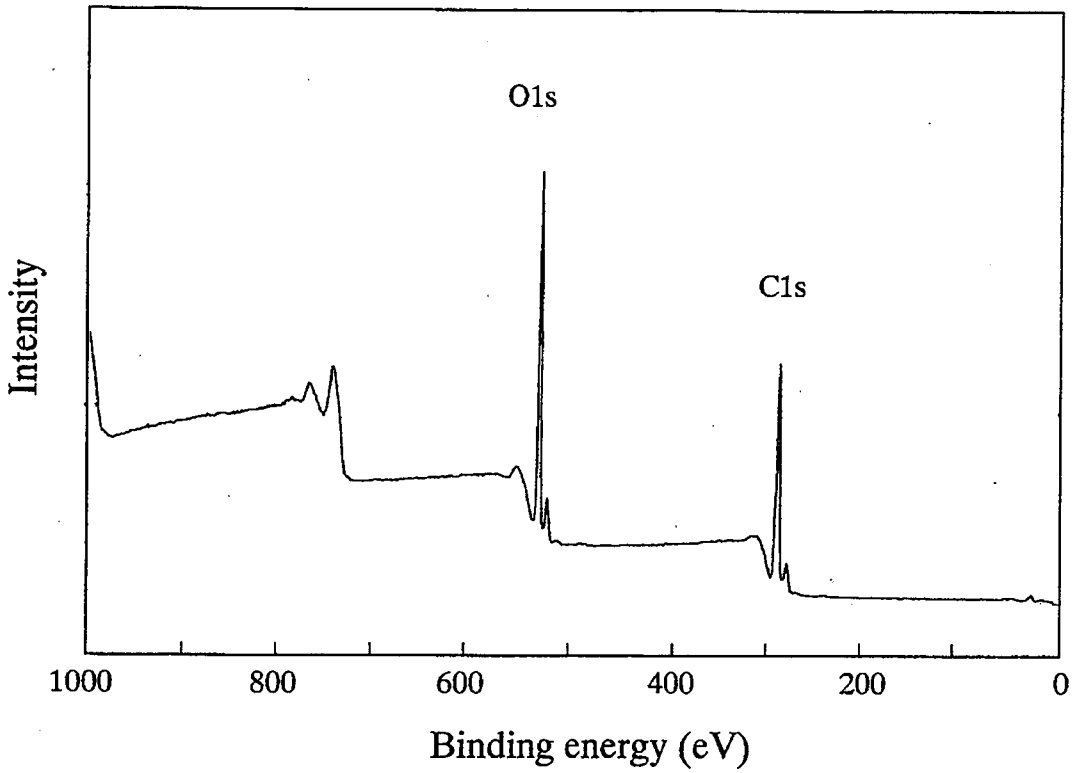


Figure 4-3. XPS spectra of PMEA coated on Au surface.

Table 4-1 Contact angle of air bubble on polymer surface

Surface	θ c	(degree)
PMEA	**50.1**	**(±3.2)**
PHEMA	**29.7**	**(±3.9)**
PP	**92.3**	**(±2.2)**

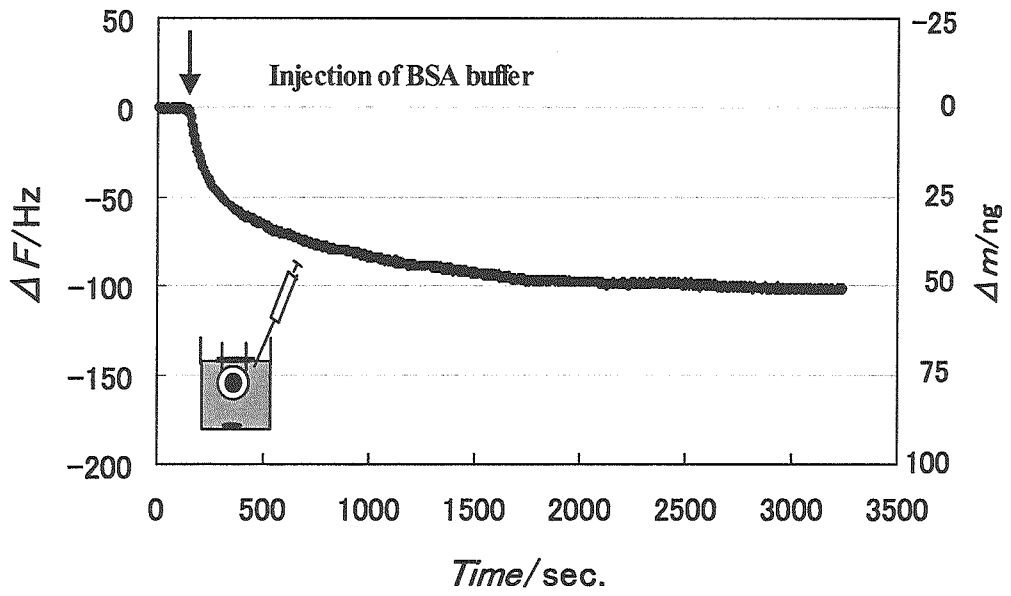


Figure 4-4. Typical time courses of frequency decrease (mass increase) of QCM due to BSA Adsorption. The concentration of BSA is 1×10^{-5} M.

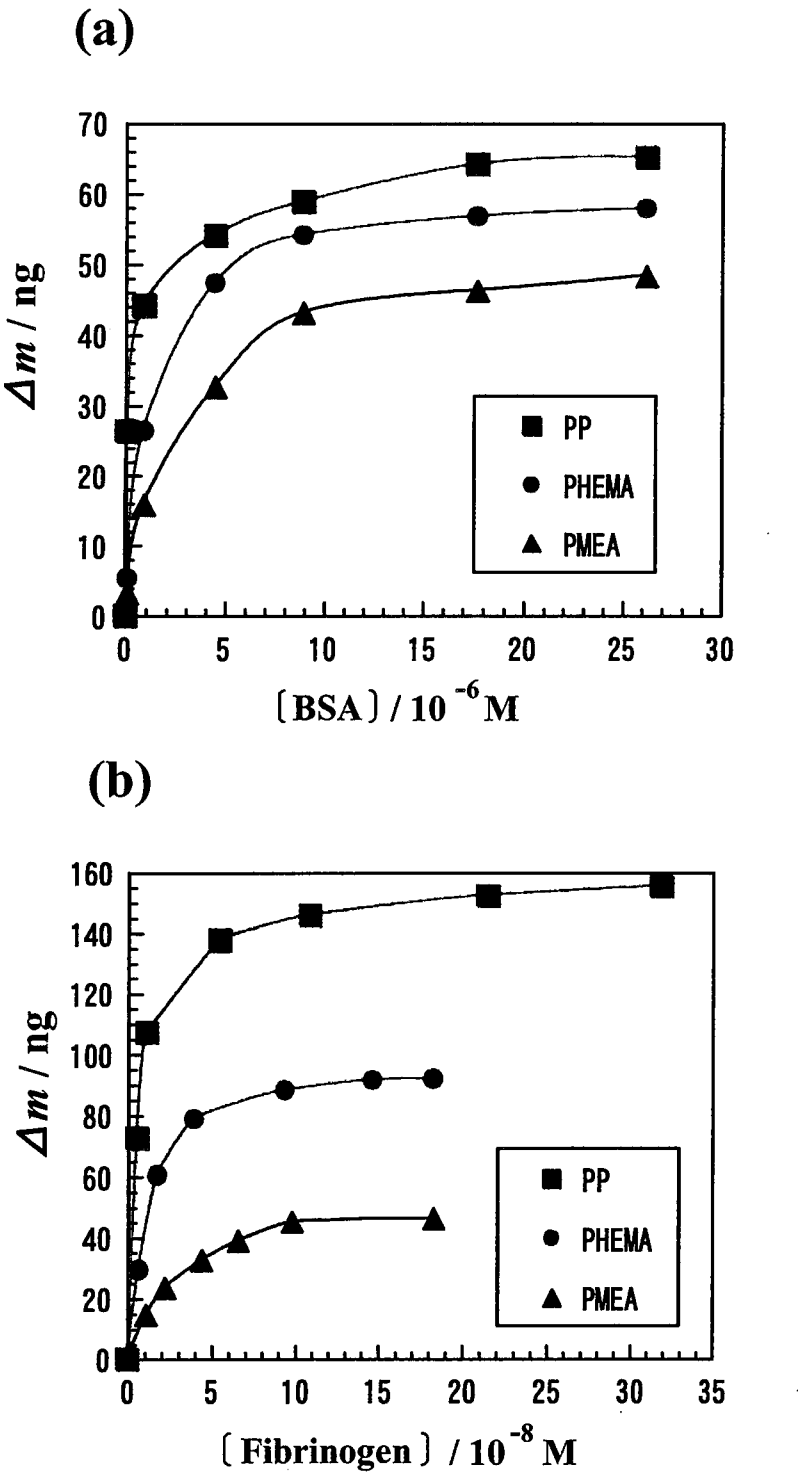


Figure 4-5. Saturation adsorption behaviors of protein on polymer surfaces. (a) BSA and (b)Fibrinogen

the monomolecular adsorption when the Langmuir type adsorption is observed (9, 10) and that proteins adsorb onto a polymer surface with a monomolecular layer structure (45). Therefore, Δm and the concentration of the protein were plotted according to Langmuir's adsorption isotherm eq [3].

$$C / \Delta m = C / \Delta m_{max} + 1 / (\Delta m_{max} K_a) \qquad [3]$$

where C = the concentration of the protein, Δm_{max} = the maximum adsorption amount, and K_a = apparent association constant of protein

The results are shown in **Figure 4-6**. In both cases of BSA and fibrinogen, one can notice the perfect linear relationship between the $C/\Delta m$ and C. These results indicate that the adsorption of the proteins occurs in the mode of Langmuir type adsorption, and therefore, it is concluded that the monolayer adsorption of the protein proceeds in this experiment. The Δm_{max} and K_a were calculated from the slope and intercept of each linear line in **Figure 4-6**, respectively, and the results are summarized in **Table 4-2**. The Δm_{max} values of BSA for PMEA, PHEMA, and PP were 0.33, 0.38, and 0.41 μg/cm^2, respectively, and the Δm_{max} values of fibrinogen for them were 0.34, 0.62 and 0.99 μg/cm^2, respectively (**Table 4-2**). In the case of the hydrophilic protein, BSA, PMEA shows indeed the lowest adsorption, but the Δm_{max} values for three surfaces are in the same level. It is implied that the adsorption of BSA onto the polymer is hardly influenced by the hydrophobicity / hydrophilicity of them under this experimental condition. On the other hand, the hydrophobic protein, fibrinogen, shows marked different adsorption behavior toward the polymer surfaces. The large value for PP and rather small value for PHEMA will be attributed to the hydrophobic/hydrophilic interaction between the polymers and fibrinogen. The Δm_{max} values for PMEA is half and one-third as large as those

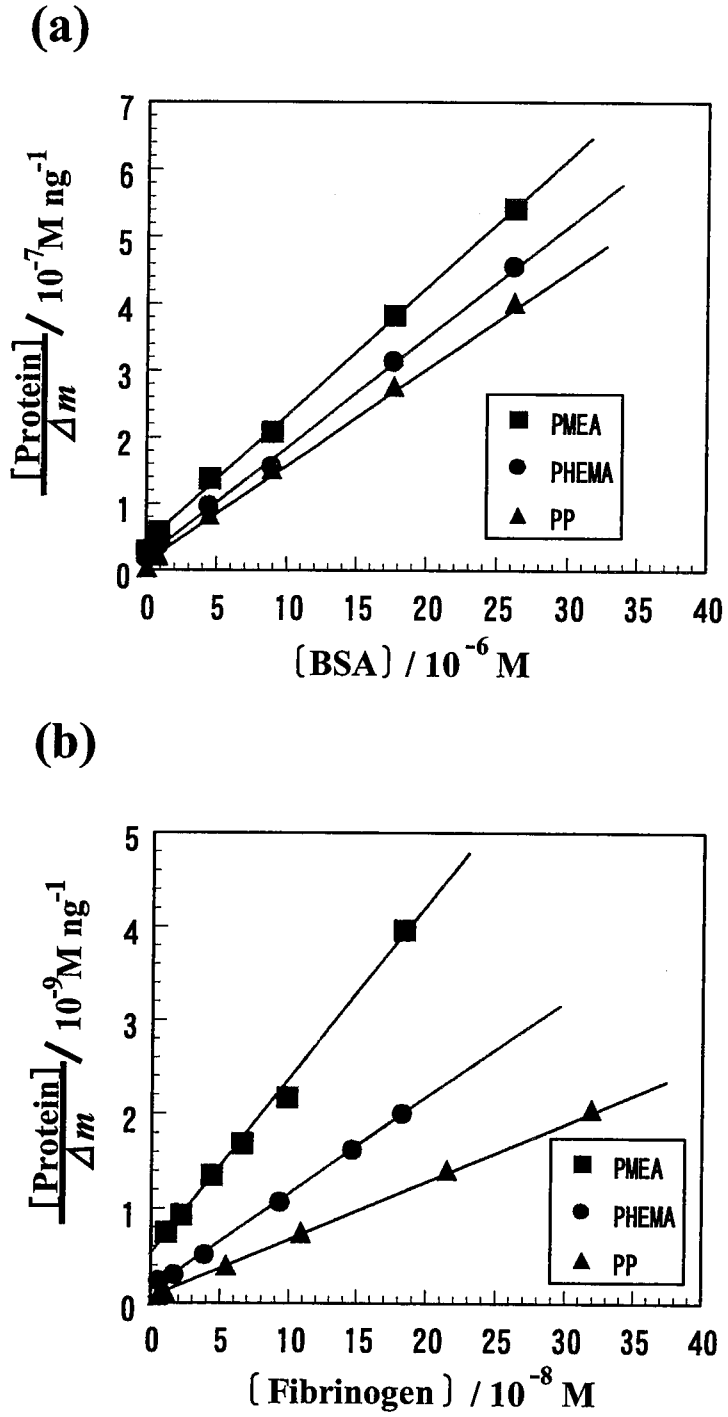


Figure 4-6. Langmuir adsorption isotherm (eq. 3) for (a) BSA and (b)Fibrinogen

Table 4-2 Maximum adsorption amounts (Δm_{max}) and Association aonstants (K_a)

	K_a ($10^5 M^{-1}$)	Δm_{max} (ng/QCM)	Δm_{max} ($\mu g/cm^2$)
PMEA	**5.1**	**52**	**0.33**
PHEMA	**9.2**	**60**	**0.38**
PP	**16.0**	**66**	**0.41**

	K_a ($10^7 M^{-1}$)	Δm_{max} (ng/QCM)	Δm_{max} ($\mu g/cm^2$)
PMEA	**3.7**	**54**	**0.34**
PHEMA	**8.1**	**99**	**0.62**
PP	**14.0**	**159**	**0.99**

on PHEMA and PP, respectively.

It is well established that protein adsorption on hydrophilic surface is relatively small, whereas the adsorption of proteins onto hydrophobic surfaces is very strong and often partially irreversible (8-10, 46). Moreover, it is known that the intermediate hydrophilic surface where the water contact angle is in the range of 60-80° is most favorable for cell adhesion (47, 48). It is surprising fact that the amount of the fibrinogen absorbed on PMEA is smaller than that on PHEMA though PHEMA is significantly more hydrophilic than PMEA as mentioned above.

On the basis of the Δm_{max} the structure of the adsorbed protein layer will be discussed below. From the Δm_{max} and the occupation area of the protein molecule, it can be predicted which type of the adsorption occurs, in the end-on type or the side-on type adsorption. The theoretical amounts of the monomolecular BSA adsorption are ca. 0.25 μ g/cm^2 for the side-on type adsorption and ca. 0.9 μ g/cm^2 for the end-on type (**Table 4-3**)(45). The theoretical one of fibrinogen for end-on type adsorption is 1.7 μ g/cm^2, and that for side-on type is 0.18 μg/cm^2 (**Table 4-3**)(45). As mentioned above, the Δm_{max} values of BSA are in the range of 0.33 to 0.41 μg/cm^2 and the Δm_{max} values of fibrinogen are in the range of 0.34 to 0.99 μg/cm^2 for these three polymers. From the comparison of these values with the theoretical adsorption amounts of BSA and fibrinogen it will be concluded that the end-on type adsorption occurs, resulting in the mono-layered structure in our experiment.

The tendencies of K_a values observed for both the proteins are similar to those of Δm_{max} values. Thus, the K_a values of both the proteins for the polymers increase in the following order, PMEA<PHEMA<PP. K_a values for PHEMA are about twice and those for PP are about three times as large as those for PMEA, as shown in Table 2. These results indicate that PMEA surface has a weaker interaction with the proteins than HEMA and PP surfaces in spite of the middle hydrophobicity. In addition, the table shows that K_a of fibrinogen for each polymer is

Table 4-3 Dimensional data for plasma proteins

Protein	Molecular Weight Diam/Length(Å)	Projected Area Ocupied (Å2)		Projected Conc. (μg/cm2)		Plasma Conc. (g/dl)	Function
		End-on	Side-on	**End-on**	Side-on		
Albumin	67,500 40×115	1,256	4,600	**0.9**	0.25	4.5	Carrier
Fibrinogen	340,000 65×475	3,317	30,875	**1.7**	0.18	0.3	Clotting

markedly large, about 100 times higher than that of BSA for the corresponding polymer though Δm_{max} values of BSA and fibrinogen are in the same order. This implies that the interaction between fibrinogen and the polymer surface is much stronger than that between the surface and BSA. These results will relate the properties of the protein such as hydrophobicity, electrostatic potentials, molecular size, protein structure and structural stability as well as the nature of the surface. Considering that fibrinogen plays an important role in the coagulation system, the fact that the interaction of fibrinogen with the PMEA surface is small may be one of the main factors to express the excellent blood compatibility.

Kinetics in the early stage of the protein adsorption on polymer surface (curve-fitting method)

We have already confirmed the quantification of the adsorbed protein on PMEA in terms of the apparent association constant (K_a) and adsorption amount by the saturation method of quartz crystal microbalance (QCM) (22). Next, we report the investigation of early stage of the protein adsorption behaviors on PMEA and other common polymer surfaces by the curve fitting method of QCM.

Protein adsorption on polymer surface is complex phenomenon and involves many dynamic steps as mentioned above. Considering these facts, we assumed the adsorption model in the analysis of kinetics of protein adsorption as follows. First, the protein adsorption proceeds according to equation 1. In the process, we defined the meta-stable complex, [Polymer-Protein]*, which is the intermediate formed by just the collision of the protein with the polymer surface at the rate, k_1. In the meta-stabel complex the protein on the surface can be detached at k_{-1} until the reorientation of the protein, which minimize the interaction energy between the protein and the surface, is occurred (i.e.: This reaction is reversible). By the reorientation or the conformational change of the protein, the intermediate changes to the final product at k_2. The extent of stability of the final product will determine whether the adsorption

is reversible or irreversible, and it will depends on the properties of the surface structure (i.e. electric charge, hydrophobicity, hydrogen-bond and so on). We conceived that the meta-stable complex would affect the adsorption behaviors and the amount of the final product (e.g: adsorbed protein), which involved the blood compatibility of the materials. Therefore, we focused our attention on the kinetics of production of the meta-stable complex. In the early stage of the adsorption reaction, we assumed that the production of meta-stable complex was predominant, and that the equation 1 could be approximated by the equation 1'. That is, in the early stage of the reaction, the observed mass increase of the QCM cell is due to the meta-stable complex, and the amount of the final product could be negligible.

$$\Delta m \propto [\text{Polymer-Protein}]^* \qquad \textbf{(1')}$$

The time presenting the early stage of the reaction was defined to be within 1300 sec, because within this time the data points obtained by QCM could be curve-fitted well by the equation 16. When the measuring time was over 1300 sec, the data points could not curve-fitted. These results will strongly support that the assumption that in early stage of the adsorption reaction the reversible formation of the meta-satable complex is predominant is right.

It is known commonly that in the analysis of the adsorption kinetics of the protein on solid surface the diffusive effect sometimes affect the apparent result. However, the diffusion effect was shown to be negligible if well-stirred system was adopted (49-51). In the QCM measurement we confirmed that stirring at under 500 rpm did not give a reproducible results, but over 500 rpm the values observed were stable and were the same regardless of the stirring rate. Therefore, we stirred the solution at 1000 rpm in this study. We thought this condition gave a sufficient stirring efficiency and the diffusion effect could be negligible.

The attachment and detachment constant rates (k_1 and k_{-1}, respectively) of the protein on polymer surfaces could be calculated as follows. At first, from curve-fitting the time courses of mass

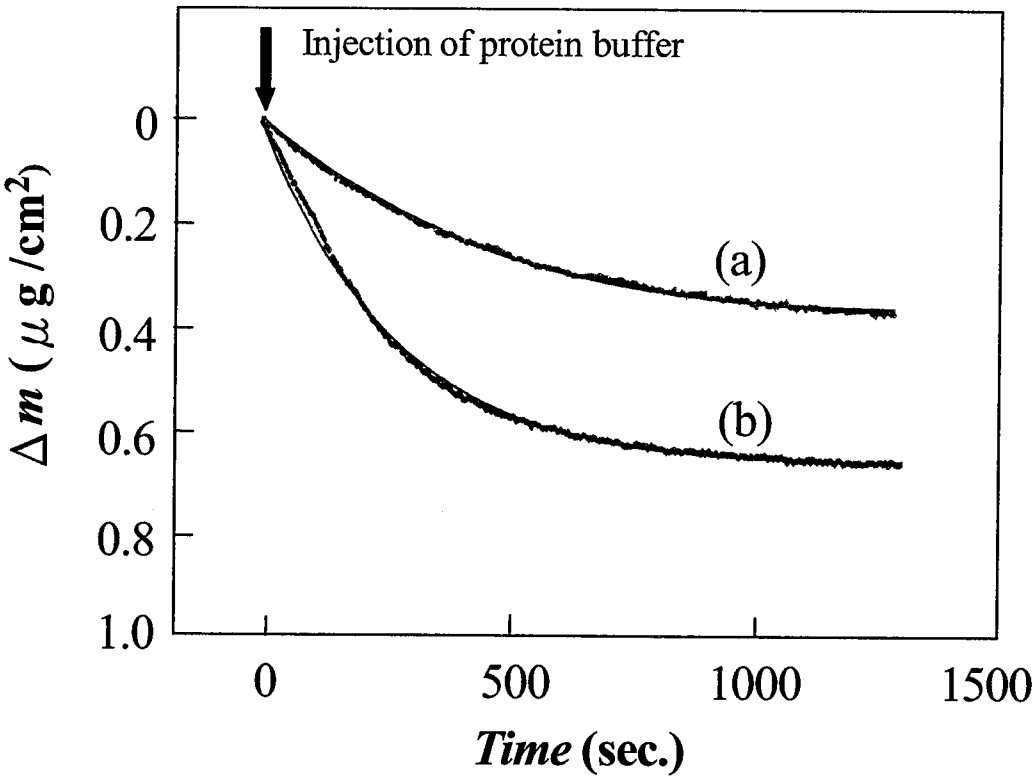


Figure 4-7. Typical time dependence of mass changes responding to adsorption of fibrinogen onto polymer surfaces. (a) PMEA (b) PHEMA The conentration of fibrinogen was 1.1×10^{-7} M (37℃, phosphate buffer, pH 7.4).

increases (frequency decreases) in **Figure 4-7** by Eq 5, the reciprocal of the relaxation time of the adsorption $1/\tau$ was obtained for each concentration of the protein. Next, plotting the $1/\tau$ against the protein concentration (eq 4) gave a linear line (**Figure 4-8**), and thus k_1 and k_{-1} were obtained from the slope and the intercept of the **Figure 4-8**, respectively. Association constant, K_a, was presented by the ratio of k_1/k_{-1}.

The parameters of the kinetics (k_1 and k_{-1}) and the association constant, K_a, are summarized in **Table 4-4**. In this work, the QCM measurement for each sample was carried out 4-6 times, and they were almost the same time courses of frequency changes (see the error bar in the **Figure 4-8**). The plot of the relaxation time τ obtained by curve-fitting the time course curve to equation 16 against the protein concentration in the solution gave a linear line with good coefficient of correlation, 0.99 (**Figure 4-8**). Therefore, it is concluded from these results that the k_1 and k_{-1} were well reproducible.

In the case of BSA which is the major component of plasma proteins, the rate of the attachment, k_1, for PMEA is almost equal to that for PHEMA and they are close to the half of that for PP. However, the desorption rate constant, k_{-1}, for the PMEA was three times larger than those for PHEMA and PP. In the case of fibrinogen which plays an important role in coagulation system, k_1 for PMEA is indeed smaller than two third times of those for PHEMA and PP. However, it will be concluded that k_1's for the three polymers are in the same level. On the contrary, PMEA showed the highest k_{-1} value among the three polymers. The k_{-1} for PMEA was three and six times larger than those for PHEMA and PP, respectively.

We have already reported the investigation of amounts of the absorbed proteins on the polymer surfaces by using QCM (39). In the article, we showed that the maximum adsorption amounts (Δm_{max}) of BSA on both PMEA and PHEMA surfaces were the same level and that the Δm_{max} of fibrinogen on PMEA surface was significantly smaller than those on PHEMA and PP surfaces

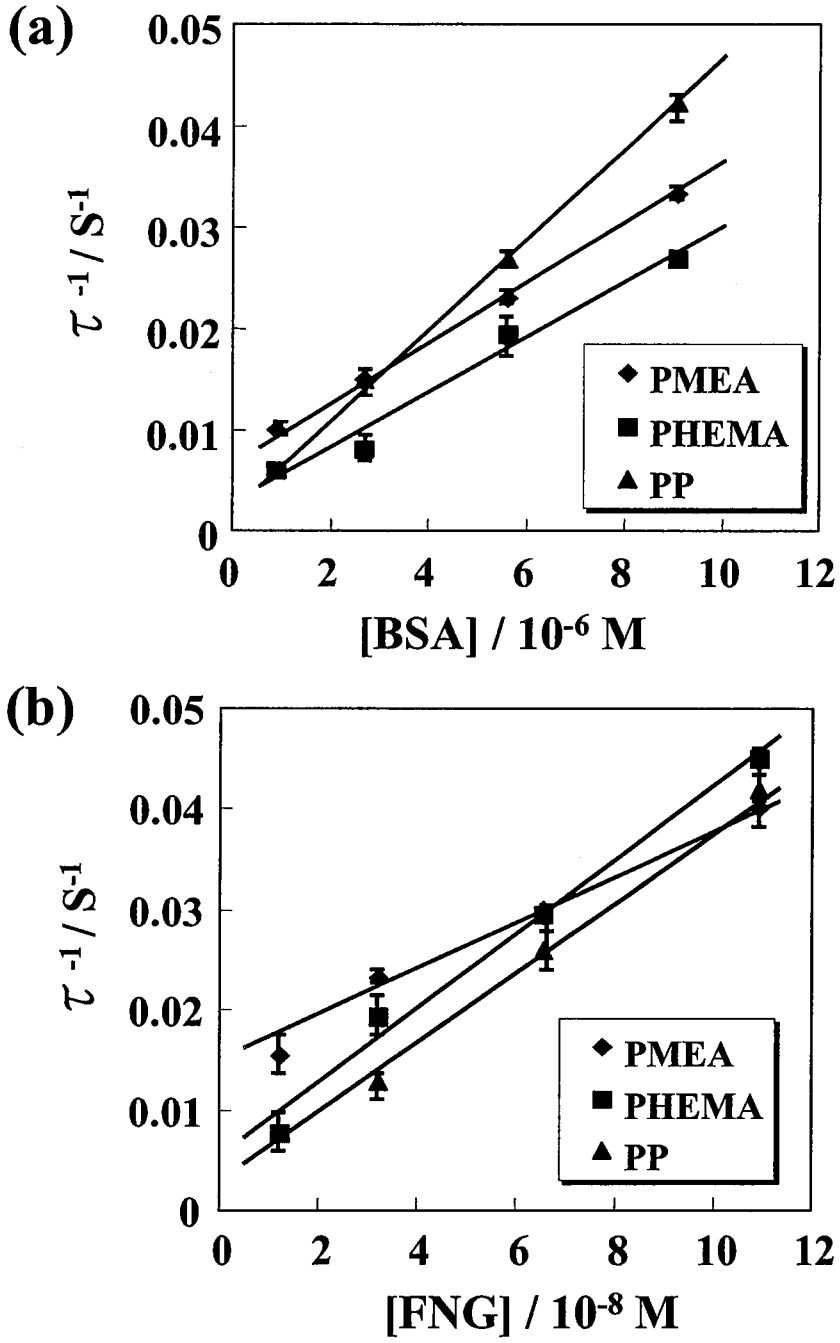


Figure 4-8. Reciprocal plots of relaxation time (τ) against concentrations of protein (n=4-6, mean $\pm$ SD). (a) BSA (b)Fibrinogen

Table 4-4 Adsorption behaviors of BSA and fibrinogen on polymer surfaces.

Proteins	Polymers	Δm_{max} ($\mu g/cm^2$)	k_1 ($10^3 M^{-1}s^{-1}$)	k_{-1} ($10^{-3} s^{-1}$)	$K_a = k_1/k_{-1}$ ($10^6 M^{-1}$)
BSA	PMEA	0.33	2.9	7.3	0.40
	PHEMA	0.38	2.6	2.8	0.93
	PP	0.41	4.4	2.4	1.8
Fibrinogen	PMEA	0.34	240	14	17
	PHEMA	0.62	370	5.0	74
	PP	0.99	360	2.3	160

Δm_{max} : maximum adsorption amount
k_1 : adsorption rate constant
k_{-1} : desorption rate constant
K_a : association constant

as shown in **Table 4-4**. When the phenomenon is considered from the viewpoint of the kinetic parameter in the formation of the meta-stable complex, the marked low protein adsorption on PMEA surface will be explained by the difference of the detachment rate (k_{-1}) of the proteins observed for each polymer surface.

The K_a^*'s defined as the ratio of the attachment and detachment rate constants are summarized in **Table 4-4.** K_a^* for PMEA is the smallest among the investigated polymers. This is due to the large desorption rate constant. Therefore, it will be clear that the feature of the PMEA surface is related to the large desorption rate constant of protein compared with those for other polymers. In the results, one should notice that differences of the hydrophobicity in proteins and polymers have little or no influence on the adsorption behavior (39). That is, the hydrophobicity of the polymers increased in the following order; PHEMA<PMEA<<PP, and this trend does not agree with the trend of the kinetics observed in the polymers.

In fact, as indicated **Figure 4-9**, the adsorbed BSA and fibrinogen on PMEA were slower than those of PHEMA and PP. While, when the protein injected buffer solution was changed to a new buffer solution at the arrow in the figure, the frequency was increased (mass decreased) due to desorption of the adsorbed protein. The desorption of the adsorbed protein onto PMEA was faster than those of PHEMA and PP. This means that while rinsing, the protein adsorbed on PMEA desorbed more easily than those of PHEMA and PP. By investigating the behavior, protein adsorbed on PMEA gave higher degree of reversibility than those of PHEMA and PP. If the protein molecule which adsorbs onto a polymer surface retains its native conformation, adsorbed protein could remove from the surface easily.

Next, the conformation of BSA and fibrinogen adsorbed on the polymer surfaces are discussed on the basis of the CD results (as mentioned chapter 3). In this study the polymer surface was contacted with the protein solution for 60 min and thus the results would not the one for the

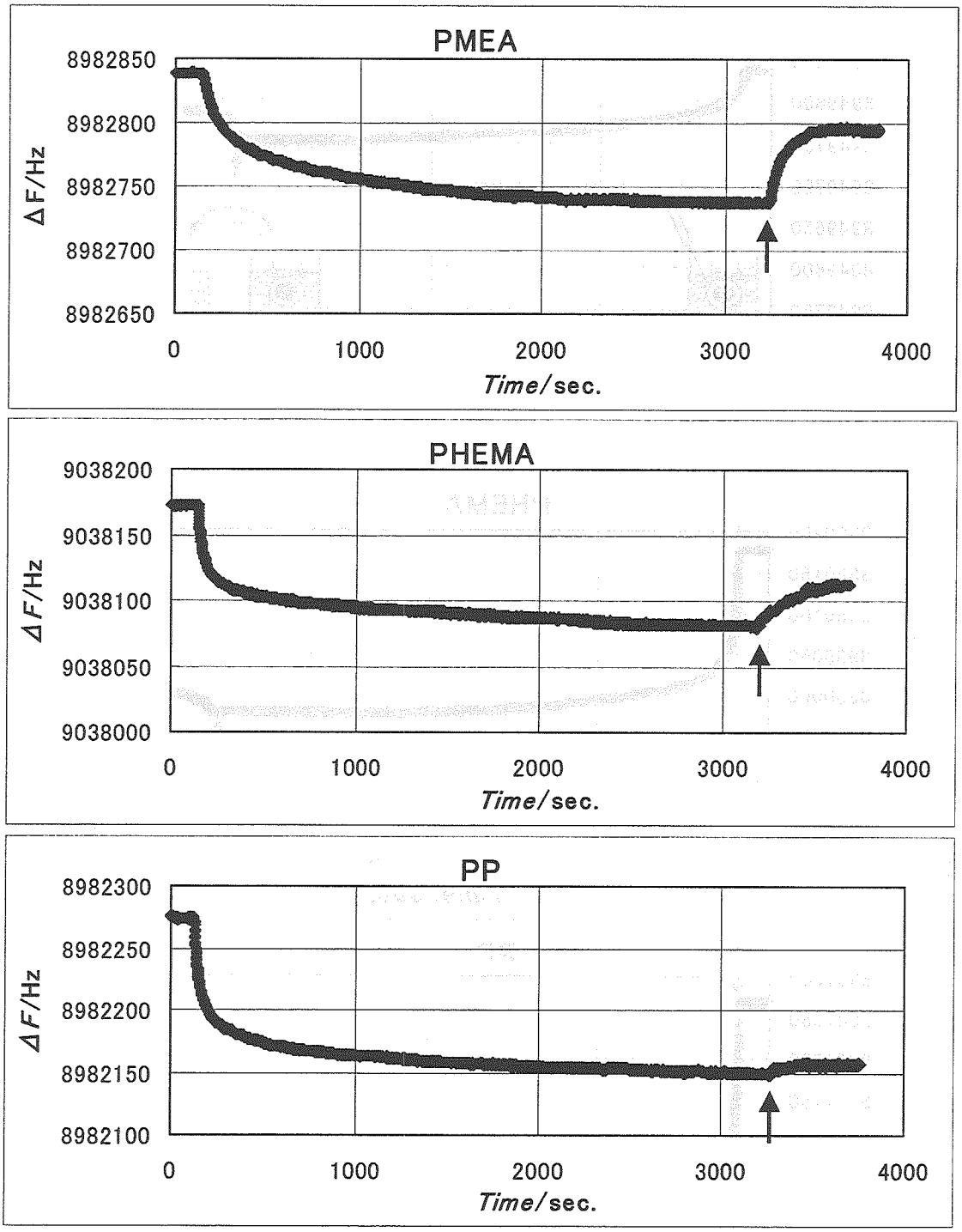


Figure 4-9(a). Typical time courses of frequency decrease (mass increase) of BSA adsption on polymer-coated QCM.

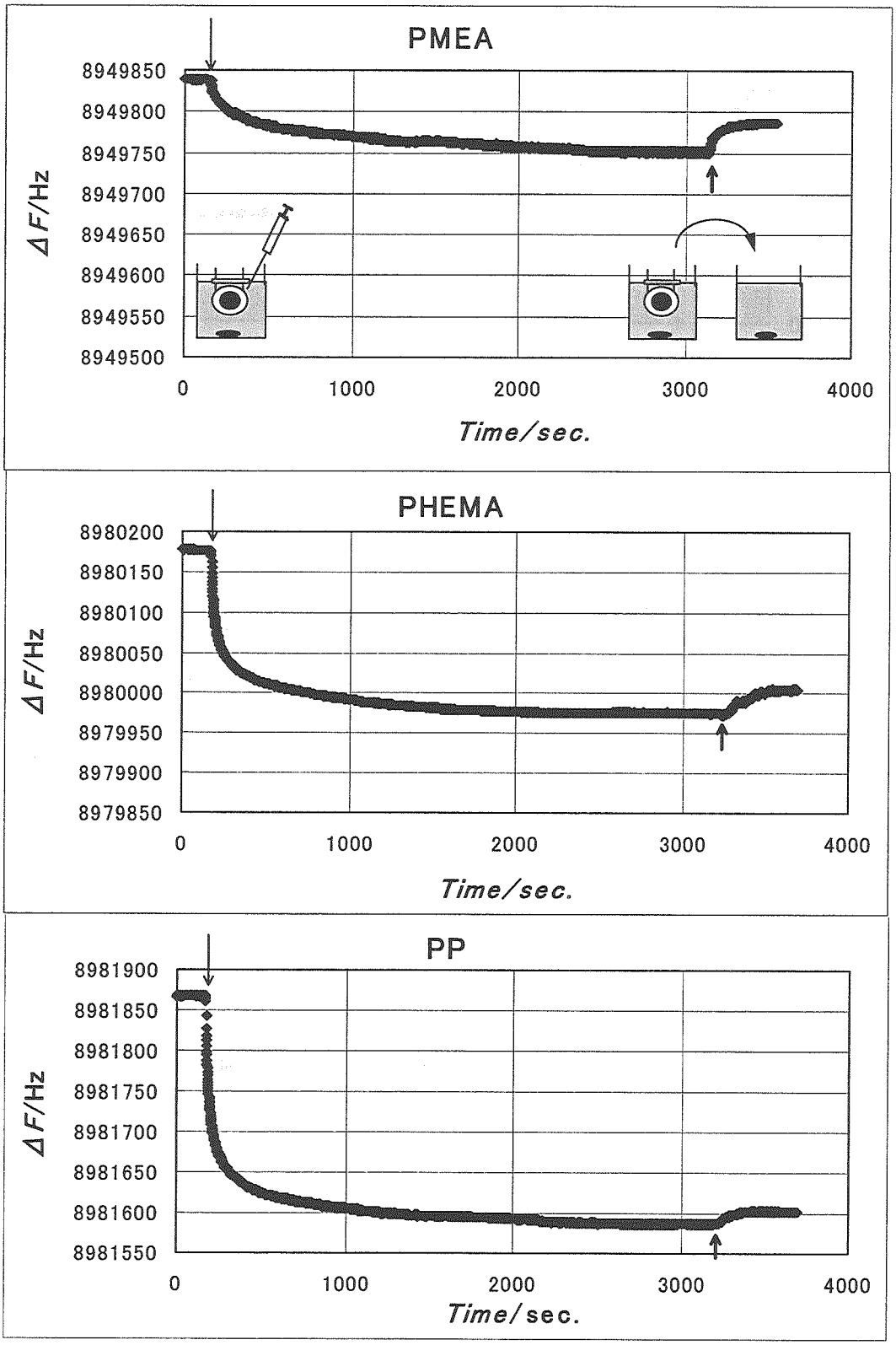


Figure 4-9(b). Typical frequency decrease (mass increase) of fibrinogen adsorption on polymer-coated QCM.

the early stage of the adsorption reaction. In the sample for the early stage reaction, the amount of the protein on the polymer surface was little, so that we could not observed CD spectra. The CD spectra of the proteins are shown in **Figure 4-10**. It is found that PP and PHEMA surfaces strongly denature the conformations of BSA and fibrinogen and that the conformations of both the proteins on PMEA surface are close to the native ones, where the native conformation means the one observed for the protein in the phosphate buffer. The contents of α-helix in the proteins adsorbed on the polymer surfaces, which will present the degree of the denaturation quantitatively and are calculated on the basis of chapter 3, are shown in **Figure 4-11**. The figure indicates that the degrees of denaturation of both the proteins increase in the following order; PMEA<<HEMA<PP. From these results of the kinetics and conformational changes, it is concluded that the interaction between PMEA and the proteins is weaker than those between the other polymers and proteins, and thus the denaturation is small. These properties will bring about the excellent blood compatibility of PMEA. On the basis of the results described above, the schematic representation of adsorption-desorption behavior of albumin and fibrinogen onto polymer surfaces is illustrated in **Figure 4-12**. Here, the thicker arrows mean the larger adsorption and desorption rate constants. The study on the long-term protein adsorption and desorption onto PMEA surface is now in progress and will be published elsewhere. The reason for the marked low adsorption of protein observed on PMEA surface has not been well understood. We believe that the water structure observed as cold crystallization in DSC measurement as mentioned next chapter is an important factor for blood compatibility (chapter 5). To prove the hypothesis, more detailed researches described below are now in progress. The mobilities of water molecule in PMEA and other polymers and their polymer chains are investigated by means of NMR. The thermal behaviors and the hydrogen bonding states of water in PMEA and the some other polymers are analyzed by DSC and FT-IR

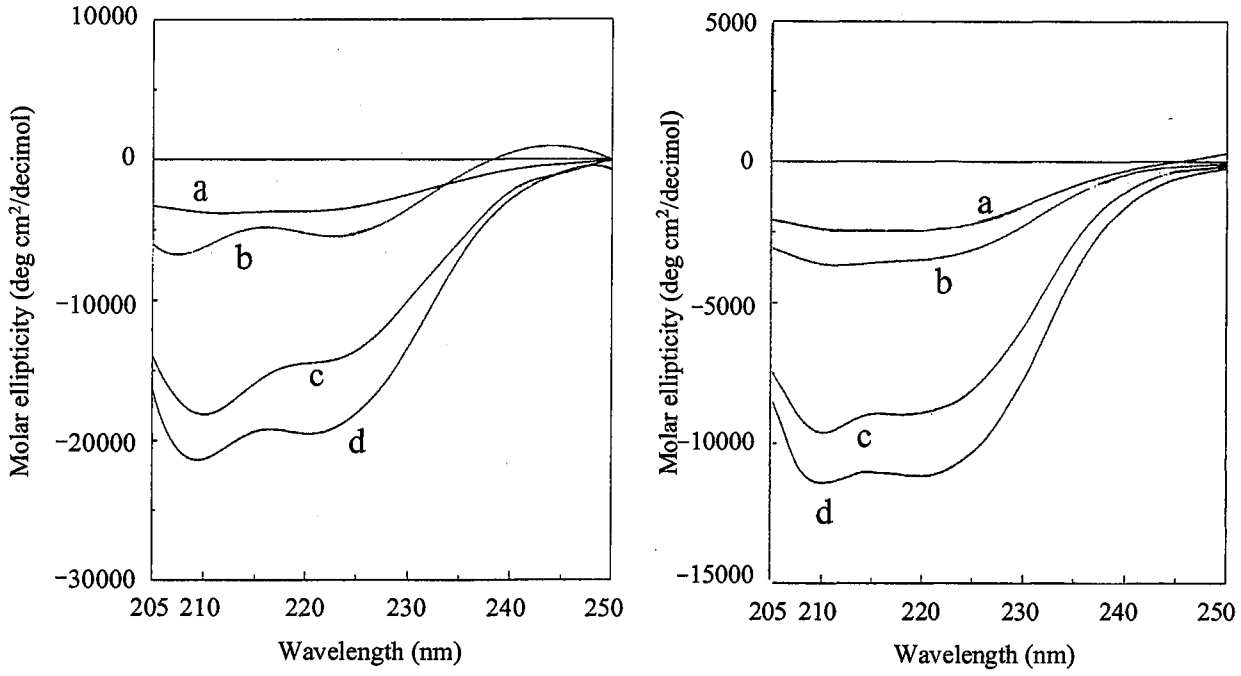


Figure 4-10. CD spectra of proteins adsorbed on polymer surfaces. (a) BSA (b) Fibrinogen, Native protein dissolved in phosphate buffer solution (37℃, pH 7.4). a:PP b:PHEMA c:PMEA d: native protein.

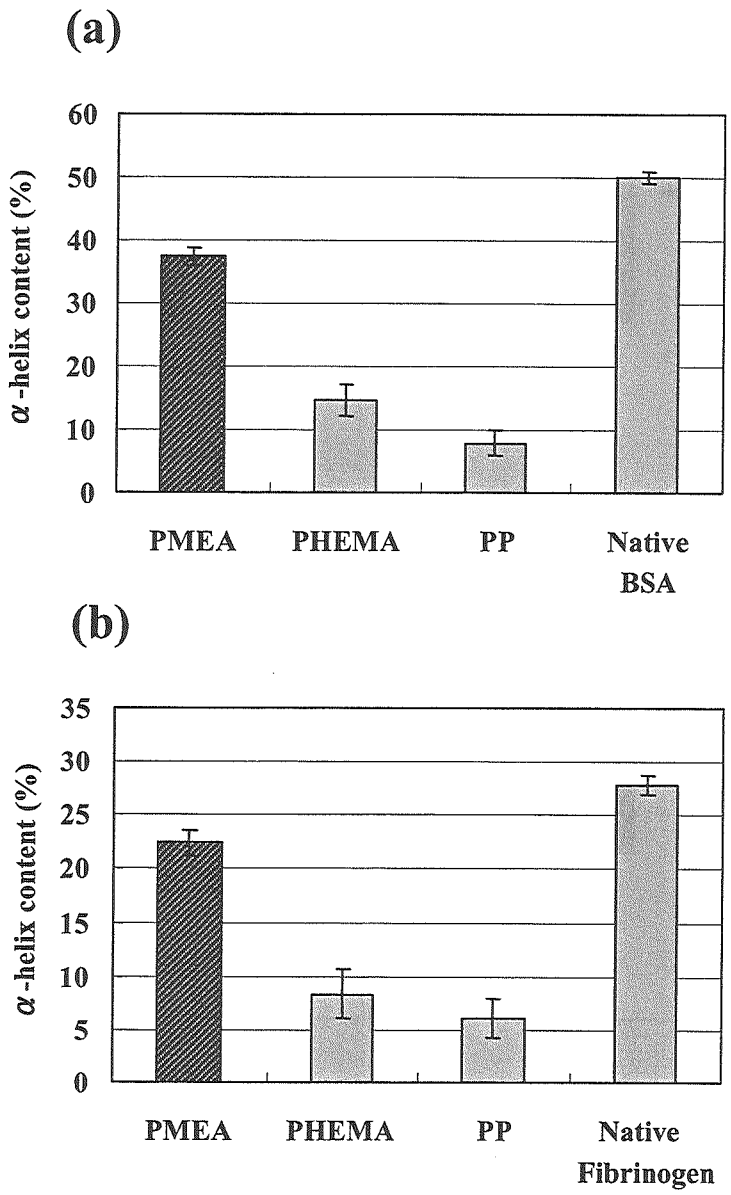


Figure 4-11. Percentage of α-helix content in proteins adsorbed onto polymer surfaces (n=5, mean ± SD). (a) BSA (b) Fibrinogen, Native protein dissolved in phosphate buffer solution (37℃, pH 7.4). The concentrations of proteins were 4.0g/dL for BSA, and 0.3g/dL for FNG. The contact time is 60 min.

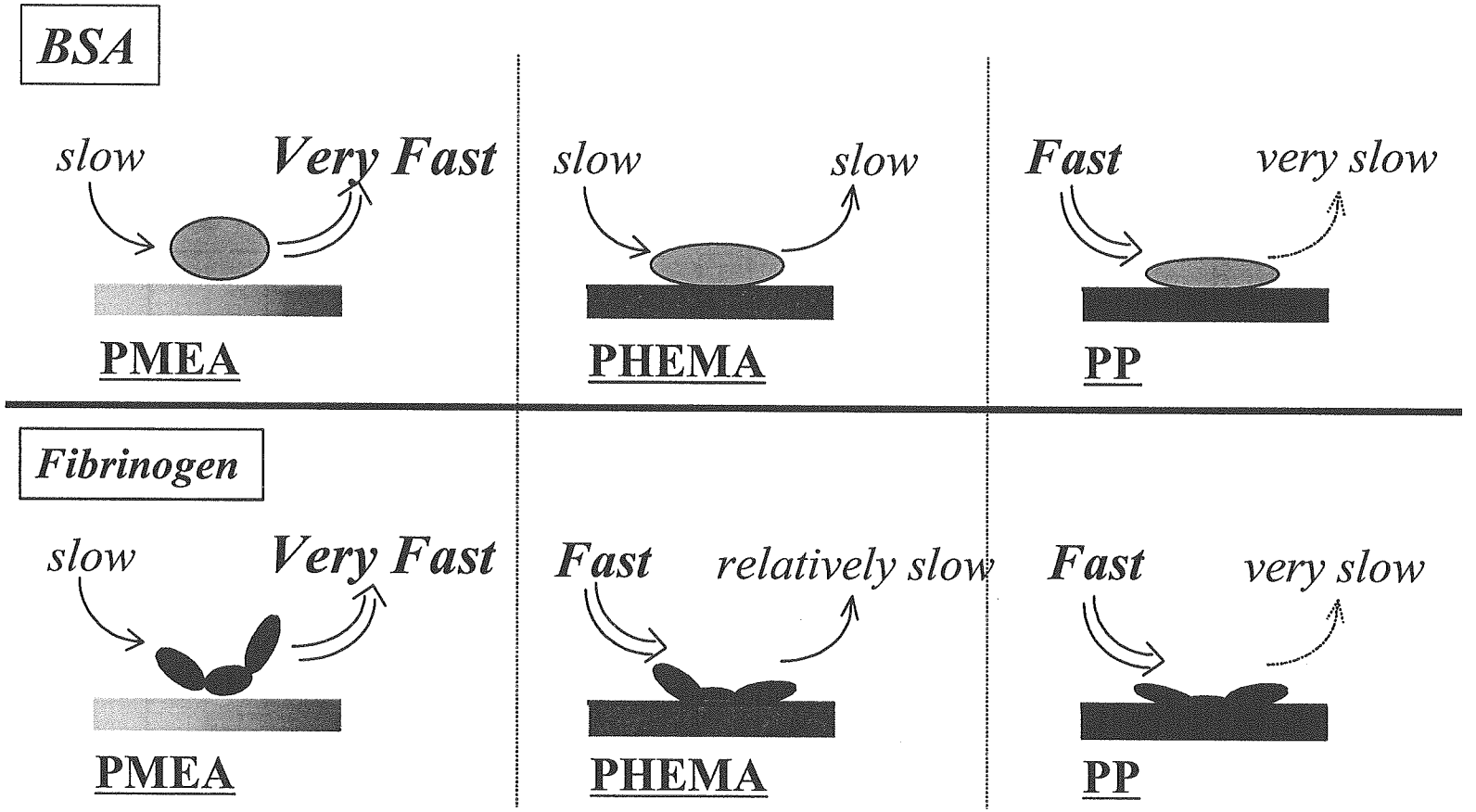


Figure 4-12. Schematic representation of binding behavior structure and surface adsorption of albumin and fibrinogen.

spectroscopy, respectively. These results will be reported elsewhere.

4-4 Conclusion

We adopted a QCM technique to quantify the protein adsorption on PMEA surface. The amounts of the absorbed proteins on PMEA were markedly smaller than those on PHEMA and PP. The adsorption behaviors of the proteins obey the Langmuir's adsorption isotherm and this fact indicates the mono-layered adsorption occurs. On the basis of the adsorption isotherm, the apparent association constants (K_a) and maximum adsorption amounts (Δm_{max}) of BSA and fibrinogen for PMEA, PHEMA, and PP were obtained. Δm_{max} and K_a values of both proteins increase in the following order, PMEA<PHEMA<PP. These results indicate that the PMEA surface is less active than PHEMA and PP for both the hydrophobic and hydrophilic proteins, though its hydophobicity is between PP and PHEMA. This article is the first report that QCM system could be applied to investigating kinetics in the early stage of the protein adsorption on polymer surface, where the property of PMEA was compared with those of PHEMA and PP using BSA and fibrinogen. Our experiment revealed that the detachment rates of protein from PMEA surface (k_{-1}'s) were much higher than those from the other polymer surfaces though the difference of k_1's for the three polymers were not so large. Therefore the association constants, K_a's (the ratios of k_1 / k_{-1}), for PMEA were lower than those for PHEMA and PP (schematically showed **Figure 4-10)**. Moreover, the investigation of conformation changes of the adsorbed proteins on the polymer surfaces showed that the degree of protein denaturation decreased in the following order: PP>PHEMA>>PMEA. These results suggest that the interaction between PMEA and the proteins is weaker than those observed in the other polymers. This weak interaction on PMEA surface will be the cause of its excellent blood compatibility.

4-5 References

1. M. Tanaka, T. Motomura, M. Kawada, T.Anzai, Y. Kasori, T. Shiroya, K. Shimura, M Onishi and A. Mochizuki, *Biomaterials*, 21 (2000) 1471.
2. M. Tanaka, T. Motomura, M. Kawada, T.Anzai, Y. Kasori, T. Shiroya, K. Shimura, M Onishi, A. Mochizuki and Y. Okahata, *Jpn. J. Artif. Organs*, 9 (2000) 209.
3. T. Anzai, A. Okumura, M. Kawaura, K. Yokoyma, H. Oshiyama, T. Kido and C. Nojiri, *Jpn. J. Artif. Organs*, 9 (2000) 73.
4. M. Tanaka, T. Motomura, N. Ishii, K. Shimura, M Onishi, A. Mochizuki and T. Hatakeyama, *Polym. Int*, 49 (2000) 1709.
5. M. Tanaka, T. Motomura, M. Kawada, T.Anzai, Y. Kasori, T. Shiroya, K. Shimura, M Onishi, A. Mochizuki and Y. Okahata, in C Jameson (Ed.), *Recent Advances in Enviromentally Compatible Polymer,* 193 (2001) 547.
6. N. Saito, S. Motoyama and J. Sawamoto, *Artif. Organs*, 24 (2000) 547.
7. M. Tanaka, A. Mochizuki, N. Ishii, T. Motomura and T. Hatakeyama, *Biomacromolecules*, 3 (2002)36.
8 J.D. Andrade, in J.D. Andrade (Ed.), *Surface and Interfacial Aspects of Biomedical Polymers*, Plenum Publ., New York, 1985, p. 1.
9 J.L. Brash and T.A. Horbett, in J.L. Brash and T.A. Horbett (Eds.), *Proteins at Interfaces: Physicochemical and Biochemical Studies*, ACS Symposium Series 343, American Chemical Society, Washington, DC, 1987, p. 1.
10 T.A. Horbett and J.L. Brash (Eds.), *Proteins at Interfaces II. Fundamental and Applications.* ACS Symposium Series 602, American Chemical Society, Washington, DC, 1995.
11 P. Huetz, P. Schaaf, J.C. Voegel, E. K. Mann, B. Miras, V. Ball, M. Freund and J. P.

Cazenave, in T.A. Horbett and J.L. Brash (Eds.), *Proteins at Interfaces* II; ACS Symposium Series 602, American Chemical Society, Washington, DC, 1995, p. 334.

12 C. H. Ho and V. Hlady, in T.A. Horbett and J.L. Brash (Eds.), *Proteins at Interfaces II*; ACS Symposium Series 602, American Chemical Society, Washington, DC, 1995, p. 371.

13 B. Lassen and M. Malmsten, *J. Colloid Interface Sci.* 179 (1996) 470.

14 J. Buijs, D.W. Britt and V. Hlady, *Langmuir*, 14, (1998) 335.

15 G. Sauerbrey, *Z. Phys*, 155 (1959) 206.

16 G.G.Guilbault, *Anal. Chem*, 55 (1983) 1682.

17 Y. Okahata, H. Ebato and K. Taguchi, *J. Chem. Soc. Chem. Commun,* (1987) 1363.

18 Y. Okahata, K. Kimura and K. Ariga, *J. Am. Chem. Soc*, 111 (1989) 9190.

19 S.A. Darst, M. Ahlers, P.H. Meller, E.W. Kubalek, R. Blankenburg, H.O. Ribi, H. Ringsdorf and R.D. Kornberg, *Biophys. J,* 59 (1991) 387.

20 Y. Okahata, K. Matsuura and Y. Ebara, *Supramol. Sci*, 3 (1996) 165.

21 K. Matsuura, Y. Ebara and Y. Okahata, *Langmuir*, 13 (1997) 814.

22 Y. Okahata, K. Ijiro and Y. Matsuzaki, *Langmuir*, 9 (1993) 19.

23 Y. Ebara and Y. Okahata, *J. Am. Chem. Soc*, 116 (1994) 11209.

24 T. Sato, T. Serizawa and Y. Okahata, *Biochim. Biophys. Acta*, 1285 (1996) 14.

25 Y. Okahata, M. Kawase, K. Niikura, F. Ohtake, H. Furusawa and Y. Ebara, *Anal. Chem*, 70 (1998) 1288.

26 N. Yui, Y. Suzuki, H. Mori and M. Terano, *Polym. J*, 27 (1995) 614.

27 B.D. Ratner, in B.D. Ratner, A.S. Hoffman, F.J. Schoen and J.E. Lemons (Eds.), *Biomaterials Science*, Academic Press, San Diego, 1996, p. 445.

28 T. Tsuruta, *Adv. Polym. Sci*, 126 (1996) 1.

29 T. Akaike, T. Okano, M. Akashi, M. Terano and N. Yui (Eds.), *Advances in Polymeric*

Biomaterials Science, CMC Co., LTD, Tokyo, 1997.

30 J.N. Lindon, G. McManama, L. Kushner, E.W. Merrill and E.W. Salzman, *Blood*, 68 (1986) 355.

31 J.A. Chinn, T.A. Horbett and B.D. Ratner, *Thromb. Haemostas*, 65 (1991) 608.

32 D. Kiaei, A.S. Hoffman, T.A. Horbett and K.R. Lew, *J. Biomed. Mater. Res*, 29 (1995) 729.

33 W.B. Tsai, J. M. Grunkemeier and T.A. Horbett, *J. Biomed. Mater. Res*, 44 (1999) 130.

34 V. Balasubramanian, N.K. Grusin, R.W. Bucher, V.T. Turitto and S.M. Slack, *J. Biomed. Mater. Res*, 44 (1999) 253.

35 F. Fang and I Szleifer, *Biophys. J*, 80 (2001) 2568.

36 J. Talbot, G. Tarjus, P.R. Van Tassel, P. Viot, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 165 (2000) 287.

37 J. J. Ramsden, *Chem. Soc. Rev*, (1995) 73.

38 A. P. Minton, *Biophys. J*, 80 (2001) 1641.

39 M. Tanaka, A. Mochizuki A, T. Motomura, K. Shimura, M. Onishi and Y. Okahata, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 193 (2001) 145.

40 M. Tanaka, A. Mochizuki A, T. Shiroya, T. Motomura, K. Shimura, M. Onishi and Y. Okahata, *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 203,(2002) 195.

41 T. Nomura and M. Iijima, *Anal. Chim. Acta*, 131 (1981) 97.

42 K.K. Kanazawa and J.G. Gordon, *Anal. Chim*, 57 (1985) 1770.

43 S. Bruckenstein and M. Shay, *Electrochim. Acta*, 30 (1985) 1295.

44 J.D. Andrade, L.M. Smith and D.E. Gregonis, in J.D. Andrade (Ed.), *Surface and Interfacial Aspects of Biomedical Polymers. Vol.1, Surface Chemistry and Physics*, Plenum Publ., New York, 1985, Chapter 7.

45 A. Baszkin and D.J. Lyman, *J. Biomed. Mater. Res*, 14 (1980) 393.

46 C.M. Cunannan, N.M. Tarbaux and P.M. Knight, J. Cataract. *Refract. Surg*, 17 (1991) 767.

47 Y. Tamada and Y. Ikada, in E. Cjiellini, P. Giusti, C. Migliaresi and L. Nicolais (Eds), *Polymers in Medicine II*, Plenum Pres, New York, 1986, p.101.

48 Y. Tamada and Y. Ikada, *J. Biomed. Mater. Res*, 28 (1994) 783.

49 E. Mandrusov, J. D. Yang, N. Pfeiffer, L .Vroman, E. Puszkin and F. Lnard, *AICh J*, 44 (1998) 233.

50 R. M. Corenellius, P. Wojciechowswki and J. L. Brash, *J Colloid Interf. Sci.*, 150 (1992) 121.

51 F. W. Christian and Maria M. S, *Langmuir*, 15 (1999) 8884.

Chapter 5 Water Structure of PMEA -The Role of Water in the Mechanism of Biocompatibility-

5-1 Introduction

Among many polymers having a potential for biomedical application [1-10], biocompatibility is the most important characteristic when a polymer is selected for practical use. On this account, it is necessary to find a suitable criteria for examining biocompatibility of targeted polymers. Biological activity always occurs in aqueous environments. When a polymer comes into contact with blood, the polymer rapidly adsorbs plasma proteins onto its surface, following the adsorption of water molecules [11]. The above situation suggests that the water-polymer interaction plays a crucial role in biological processes. It is thought that structural change of water on the polymer interface is one of the important aspects affecting blood compatibility [12-16].

From the viewpoint of water structures in polymers, a large number of studies have been performed using various experimental methods, such as nuclear magnetic resonance spectroscopy (NMR), dilatometry, differential scanning calorimetry (DSC), Raman spectroscopy and infrared spectroscopy [17-20]. DSC is one of the most promising methods, since it is accurate and the measurement is relatively easy. DSC has enabled us to analyze quantitatively the structures of water in the polymers from their enthalpies of melting and crystallization.

We have already reported that poly(2-methoxyethylacrylate) (PMEA) shows excellent blood compatibility (chapter 2). It is found that induced denaturation of the adsorbed proteins on the PMEA surface is less than that of other polymers (chapter 3) [21-25] although the reason for this is not clear.

In this chapter, we investigated the structural change of water in the PMEA using DSC in

order to clarify the reason why PMEA exhibits low adsorption of proteins and low denaturation. Morerover, we characterized PMEA analogous poly(meth)acrylate, such as poly(2-hydroxyethyl methacrylate), poly(2-methoxyethyl methacrylate), and copolymers (poly(MEA-co-HEMA)) with respect to the chemical structure of the side chain, contact angle of water, water content, and water structure, and discussed the relationship between platelet compatibility and these properties.

5-2 Experimental

5-2-1 Materials

The initiator, 2,2'-azobis-isobutyronitrile (AIBN) purchased from Wako Pure Chemical Industries (Osaka, Japan), was recrystallized from methanol. The monomers used were 2-methoxyethyl acrylate (MEA), 2-methoxymethyl metacrylate (MEMA), ethyl acrylate (EA), 2-phenoxyethyl acrylate (PEA), 2-ethylhexyl acrylate (EHA), n-butyl acrylate (BA), 2-hydroxyethyl methacrylate (HEMA) and 2-hydroxyethyl acrylate (HEA). MEA, EA, PEA, EHA and BA were obtained from Osaka Organic Chemical Industry (Osaka, Japan). HEMA and HEA were obtained from Tokyo Chemical Industry (Tokyo, Japan). MEMA was obtained from Aldrich (Milwaukee, USA). Methanol (MeOH), 1,4-dioxane, N,N-dimethylformamide (DMF), tetrahydrofuran (THF), and diethylether used as solvents were of analytical grade (Kanto Chemicals, (Tokyo, Japan)). Poly(ethyleneterephthalate) (PET) film (Futamura kagaku (Osaka, Japan)) was used as a substrate for coating the polymers. Deionized water from a Milli-Q (18MΩ-cm) system was used to prepare all aqueous solutions.

Co-polymerization of poly(MEA-co-HEMA)

Poly(2-methoxyethylacrylate)(PMEA), poly(2-hydroxyethylmethacrylate)(PHEMA)

and methoxyethylacrylate-hydroxyethylmethacrylate copolymers (poly(MEA-co-HEMA)) with five different compositions were prepared by free-radical polymerization initiated by AIBN. The polymerizations of MEA and the copolymerization of MEA with HEMA were carried out in 1,4-dioxane at 75 °C for 8 h. After the reaction, the reaction solution was poured into an excess of hexane, and then the polymer was obtained as precipitate. The polymerization of HEMA was performed in DMF at 70 °C for 8 h, and subsequent precipitation was carried out in diethyl ether. The polymers were purified by three-time precipitation of THF solution for PMEA and the copolymers, and of MeOH solution for PHEMA in hexane and diethylether, respectively. The feed compositions of the monomers were MEA:HEMA=100:0, 90:10, 80:20, 70:30, 60:40, 50:50, and 0:100 (mol/mol), and the polymer yields were 61, 68, 73, 60, 72, 78, and 62 %, respectively.

Polymerization of PMEA analogous poly(meth)acrylate

The radical polymerizations of MEA, MEMA, EA, PEA, BA and EHA were carried out in 1,4-dioxane at 75 °C for 8 h using AIBN as initiator under nitrogen. After the reaction, the reaction solution was poured into an excess of hexane, and then the polymer was obtained as precipitate. The polymerization of HEMA and HEA were performed in DMF at 70 °C for 8 h, and the subsequent precipitation was carried out in diethyl ether. The polymers were purified by three-time precipitation from THF solution for PMEA, PMEMA, PEA, PPEA, PBA and PEHA, and from MeOH solution for PHEMA and PHEA.

Characterization

The average molecular weights of the polymers were measured by using gel permeation chromatography (GPC) with polystyrene (Aldrich, Milwaukee, USA) as a standard. GPC measurement was carried out using a Tosoh SC-8020 equipped with refractive index detector (RI-8020) (Tosoh, Tokyo, Japan), and the column used was a Waters Styragel

HR4E (Waters, Milford, USA). THF was used as mobile phase at a flow rate of 1.0 ml/min.

The composition of the copolymer was determined by 400 MHz proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy using a Varian Unity Plus 400 spectrometer. The NMR solvents were $CDCl_3$ for PMEA and the copolymers containing up to 40 mol% of HEMA, and DMSO-d_6 for PHEMA and the copolymer with 50 mol% of HEMA. The reference used was tetramethylsilane (TMS).

Equilibrium water content

PMEA is insoluble in water. The water content (Wc) and The equilibrium water content (EWC) of the polymer were defined as defined by the following equation (26, 27).

$$W\text{c (wt \%)} = \{ (W_1 - W_0) / W_1 \} \text{X } 100 \text{ (\%)} \qquad \text{eq.(1)}$$

where W_0 and W_1 are the weights of the dry sample and the hydrated sample, respectively.

The EWC was obtained by the method shown below. The polymer was soaked in distilled water for 7 days at room temperature, taken out of water, and wiped with filter paper to remove excess water on the surface. The fully hydrated polymer was weighed quickly (W_1), and then the polymer was dried at 120 °C *in vacuo* until the weight (W_0) became constant.

5-2-2 Measurement of DSC

The different water contents were obtained by evaporation method [18, 27]. After the hydrated sample was weighed in a DSC aluminum sample pan, the water of hydrated sample was slowly evaporated till the weight of the sample become to be close to the

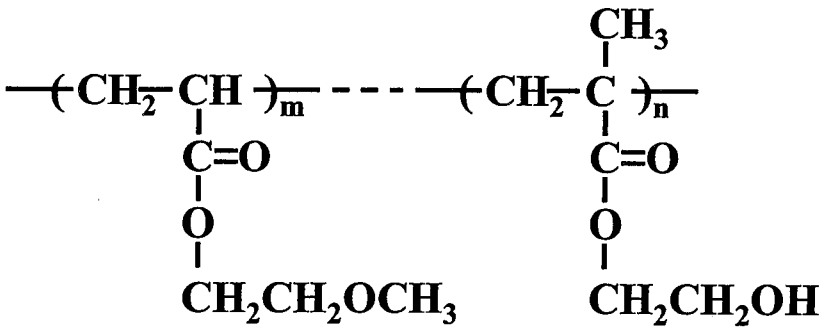
expectant value. After sealing the DSC sample pan, the sample was kept at room temperature for a few days to stabilize the sample. The dry sample weight was obtained as follows: After puncturing the sealed sample pan, the sample was dried at 100 °C *in vacuo* and then was weighed. A sample with *W*c=36 wt % was prepared without removing the excess water on the surface.

The phase transition of water in PMEA, PMEA analogous poly(meth)acrylate and p(MEA-co-HEMA) were measured under nitrogen atmosphere with DSC (DSC-50, Shimadzu Co., Kyoto, Japan) equipped with a low temperature cooling apparatus (TAC-50, Shimadzu Co., Kyoto, Japan). The hydrated sample was placed in an aluminum pan and hermetically sealed. The weight of the sample used was 5-6 mg, and no weight loss was observed during the measurement. The hydrated sample was first cooled to -100 °C at the rate of 2.5 °C /min, held at -100 °C for 10 min and then heated to 50 °C at the same rate under nitrogen atmosphere. The heating process was monitored. It was confirmed that there was no weight loss during the measurement.

5-2-3 Platelet adhesion test of MEA-HEMA copolymers and PMEA analogous poly(meth)acrylate

PET sheet (7 mm×7 mm) was cleaned thoroughly using THF and then dipped into MeOH solution of the polymer, whose concentration was 10 mg/ml. The PET sheet coated with the polymer was dried overnight at room temperature. The sample sheet was washed three times with phosphate-buffered saline (PBS) before the platelet adhesion test.

Human blood was drawn from healthy volunteers and mixed with a 1/9 volume of acid citrate dextrose (ACD). Platelet rich plasma (PRP) and platelet poor plasma (PPP) were obtained by centrifugation of the blood at 1200 rpm for 5min and at 3000 rpm for 10min,



MEA-HEMA copolymer

Figure 5-1. Chemical structure of poly(MEA-co-HEMA). m=1-0.5, and 0, n=0-0.5, and 1.

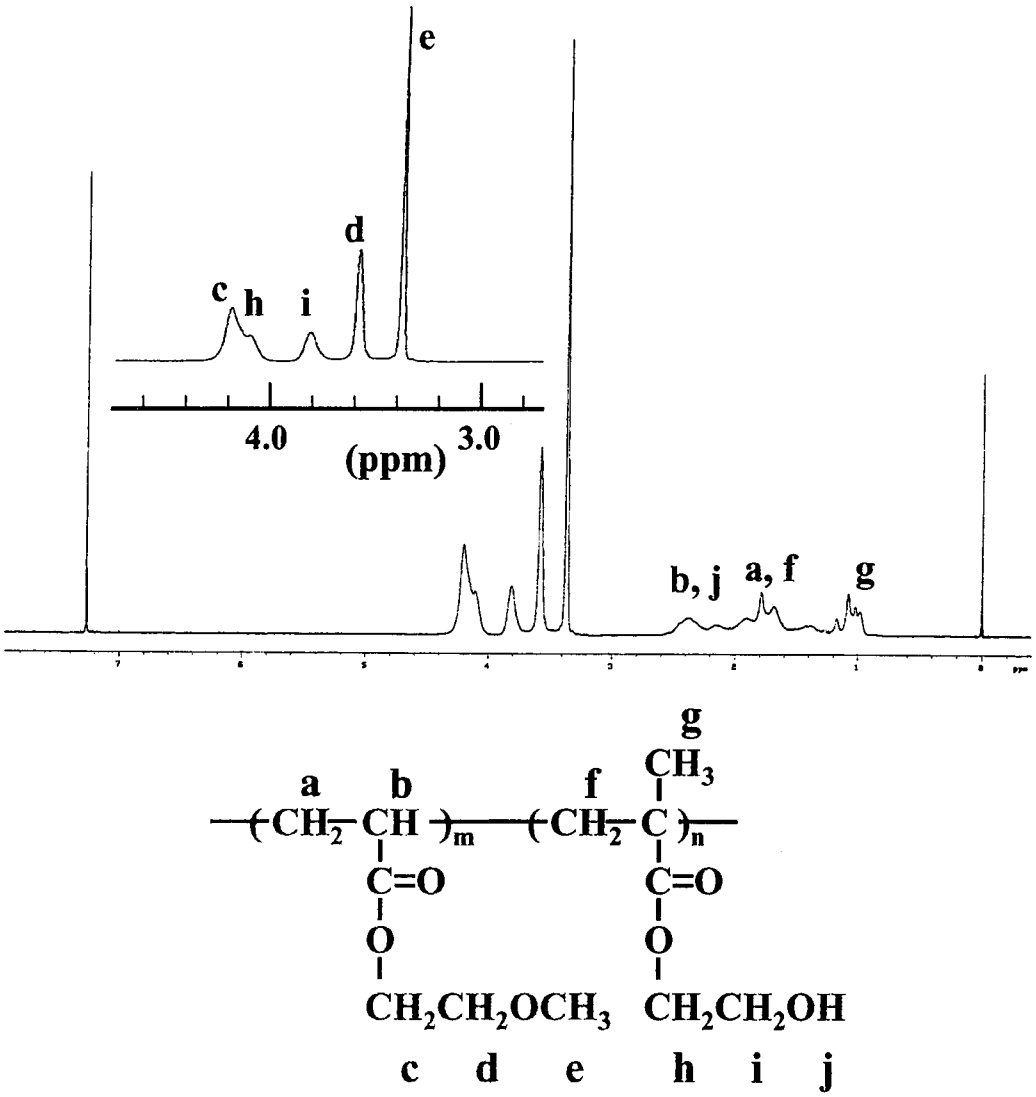


Figure 5-2. 1H-NMR spectrum of poly(MEA-co-HEMA) containing 30 mol% of HEMA.

respectively. The platelet suspension plasma containing 1×10^5 cells/μl of platelet was prepared by mixing PRP with PPP. The platelet concentration was determined by an automated hematology analyzer (Sysmex SE-9000, TOA MEDICAL ELECTRONICS, Tokyo, Japan). Then, 200 μl (platelet number : 2×10^7) of the platelet suspension plasma was placed on the polymer surface of the sheet, and incubated for 60 min at 37 °C. After the sheet was washed three times with PBS, it was immersed into 1 % glutaraldehyde of PBS for 60 min at 4 °C to fix the adhered platelet. The sample was freeze-dried, and sputter-coated using gold (JUC-5000, JEOL Tokyo, Japan) prior to observation under scanning electron microscopy (JSM-840, JEOL, Tokyo, Japan).

5-3 Results and Discussion

Characterization of poly(MEA-co-HEMA)

The polymers used in this study were PMEA, PHEMA, and poly(MEA-co-HEMA), and the chemical formula of poly(MEA-co-HEMA) is shown in **Figure 5-1**. The NMR spectrum of the copolymer with 30 mol% of HEMA is shown in **Figure 5-2** as a representative of the copolymers. The assignment of each proton peak is also shown in the Figure. The monomer composition of the copolymer was calculated from the equations 2 and 3.

2X + 2Y = Peak intensity based on *c* and *h* protons in the Figure **eq.(2)**

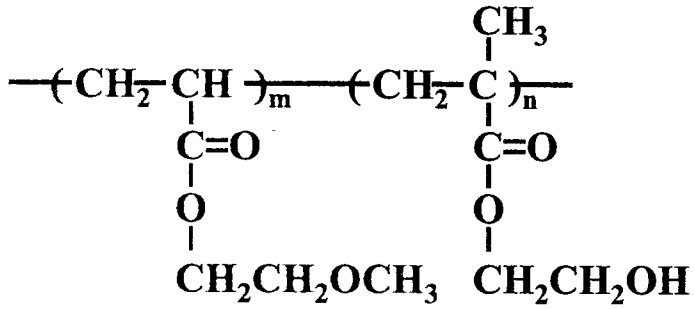
5X + 2Y = Peak intensity based on *d, e* and *i* protons in the Figure **eq.(3)**

where X and Y are the MEA and the HEMA contents in the copolymer, respectively. The

Table 5-1. Result of Copolymerization of MEA with HEMA

Sample	Feed composition		Yield	Copolymer composition [a]		Mw [b]	Mw/Mn [b]
	MEA mol%	HEMA mol%	wt%	Content of MEA mol%	Content of HEMA mol%		
PMEA	100	0	61.1	100	0	78,000	2.2
PMEA90	90	10	68.3	90.9	9.1	68,000	1.8
PMEA80	80	20	73.2	80.1	19.9	65,000	2.1
PMEA70	70	30	60.5	68.2	31.8	80,000	1.6
PMEA60	60	40	72.3	60.8	39.2	78,000	2.3
PMEA50	50	50	77.6	48.5	51.5	66,000	2.4
PHEMA	0	100	62.0	0	100	75,000	1.6

a Determined by ^{1}H-NMR
b Determined by GPC



poly(MEA-co-HEMA)

results are shown in **Table 5-1** together with the results of GPC analysis. The Table shows that the monomer composition in the copolymer agrees well with the feed composition regardless of the polymer yield, and that the molecular weights of these polymers are in the same level (Mw=65,000-80,000).

PMEA analogous poly(meth)acrylate Properties related to Water

Figure 5-3 shows the chemical structures of the poly(meth)acrylates used in this work and their abbreviations. Contact angle of water droplet on the polymer surface is listed in **Table 5-2**. The contact angle reflects the surface property of the polymer: hydrophobic or hydrophilic. On the basis of the result of the contact angle one can classify these polymers into three categories. The one is the hydrophobic polymer with more than 70° of contact angle and it has alkyl, methoxyethyl, or phenoxyethyl group as a ester side chain of the polymer. The second is the hydrophilic polymer with less than ca. 30° of contact angle and PHEMA and PHEA with hydroxy group are the representatives. The last is the intermediate hydrophilic polymer with ca. 50° of contact angle and is represented by PMEA that has methoxyethyl group. The difference in the contact angle will be caused mainly by the chemical structures of the side chains (the ester side chain and the methyl group in α-position in acrylate monomer unit).

In **Table 5-2** the total equilibrium water contents (EWC) of the polymers are also listed. From the result of EWC the polymers can be categorized into three groups again like the case of the contact angle; the one has below 5 % of EWC (hydrophobic polymer), the second has high EWC, over 30% (hydrophilic polymer), and the third has the intermediate EWC, ca. 10 % (intermediate hydrophilic polymer). This classification is in good agreement with the one by the contact angle. That is, the polymer species belonging to

Table 5-2 Properties of poly(meth)acrylates

	Mw[1)]	Contact Angle[2)]	EWC (wt%)
PMEA	85,000	48.0° (±4.0)	9.0
PMEMA	108,500	70.1° (±2.3)	4.2
PHEMA	75,000	30.2° (±3.6)	39
PHEA	109,800	17.3° (±4.7)	60
PEA	87,000	79.0° (±2.9)	4.8
PPEA	80,000	86.8° (±2.3)	3.3
PBA	105,000	81.1° (±3.3)	1.9
PEHA	110,000	82.1° (±1.2)	2.1

[1)]: GPC [2)]:means±SD, n=6

each group classified by EWC or by the contact angle are the same. However, these results imply that EWC does not relate strictly to the contact angle. The increasing order of hydrophilicity or hydrophobicity is different between in the polymer group classified by contact angle and in the one by EWC. For example, the hydrophobicity determined by contact angle increases in the following order; PEA<PBA<PEHA<PPEA, while that by EWC is ; PEA<PPEA<PEHA<PBA. In addition, it is found that methyl group in α-position of the monomer unit strongly decreases EWC. When EWCs of PMEA and PHEA are compared with PMEMA and PHEMA, respectively, the marked decrease of EWC in the polymethacrylates is observed.

Water structure in PMEA

Figure 5-4 shows DSC heating curves for PMEA-water systems, whose W_c was varied from 0.2 to 36 wt %. In the samples with W_c of 0.2 and 1.8 wt %, baseline deviation due to glass transition was observed. A large and distinct exothermic peak was observed for the samples with W_c 3.0 to 36 wt %. For the samples with W_c 3.0 and 5.8 wt %, an endothermic peak at –4 ~ –14 °C was also observed. . In the case of the samples with

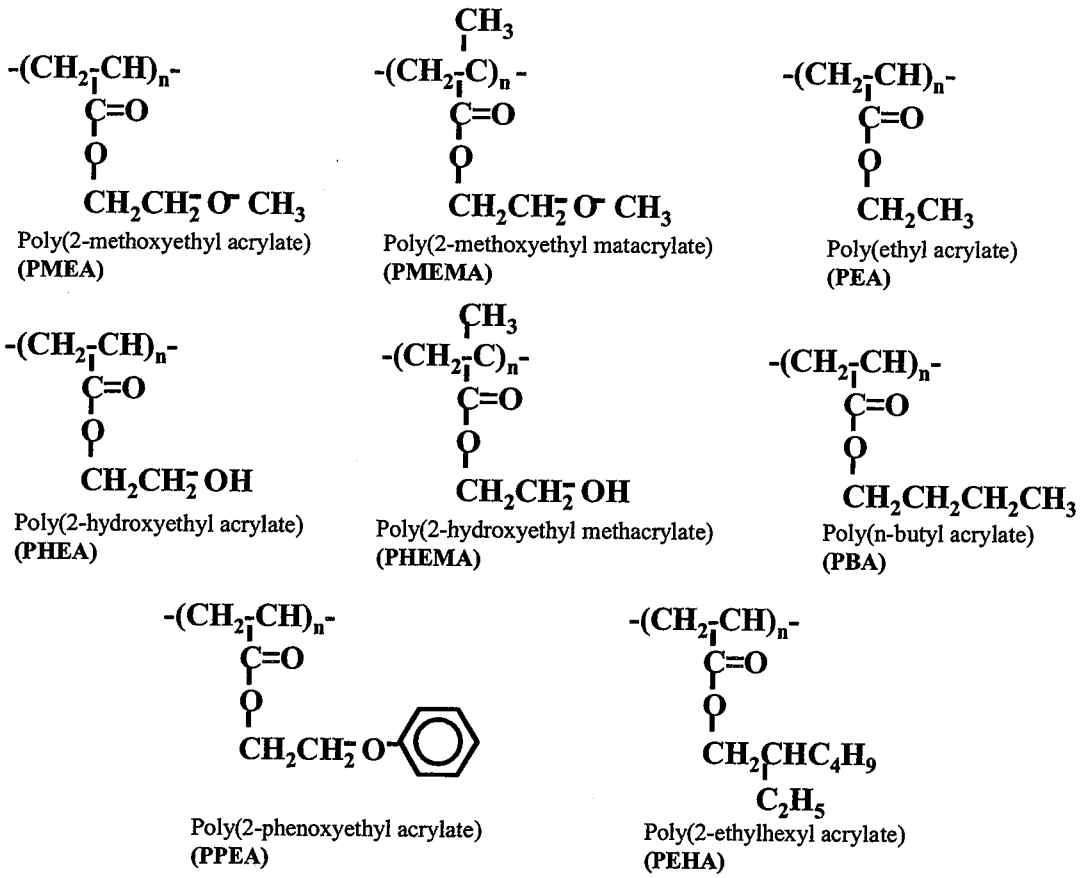


Figure 5-3. Chemical structures of poly(meth)acrylates.

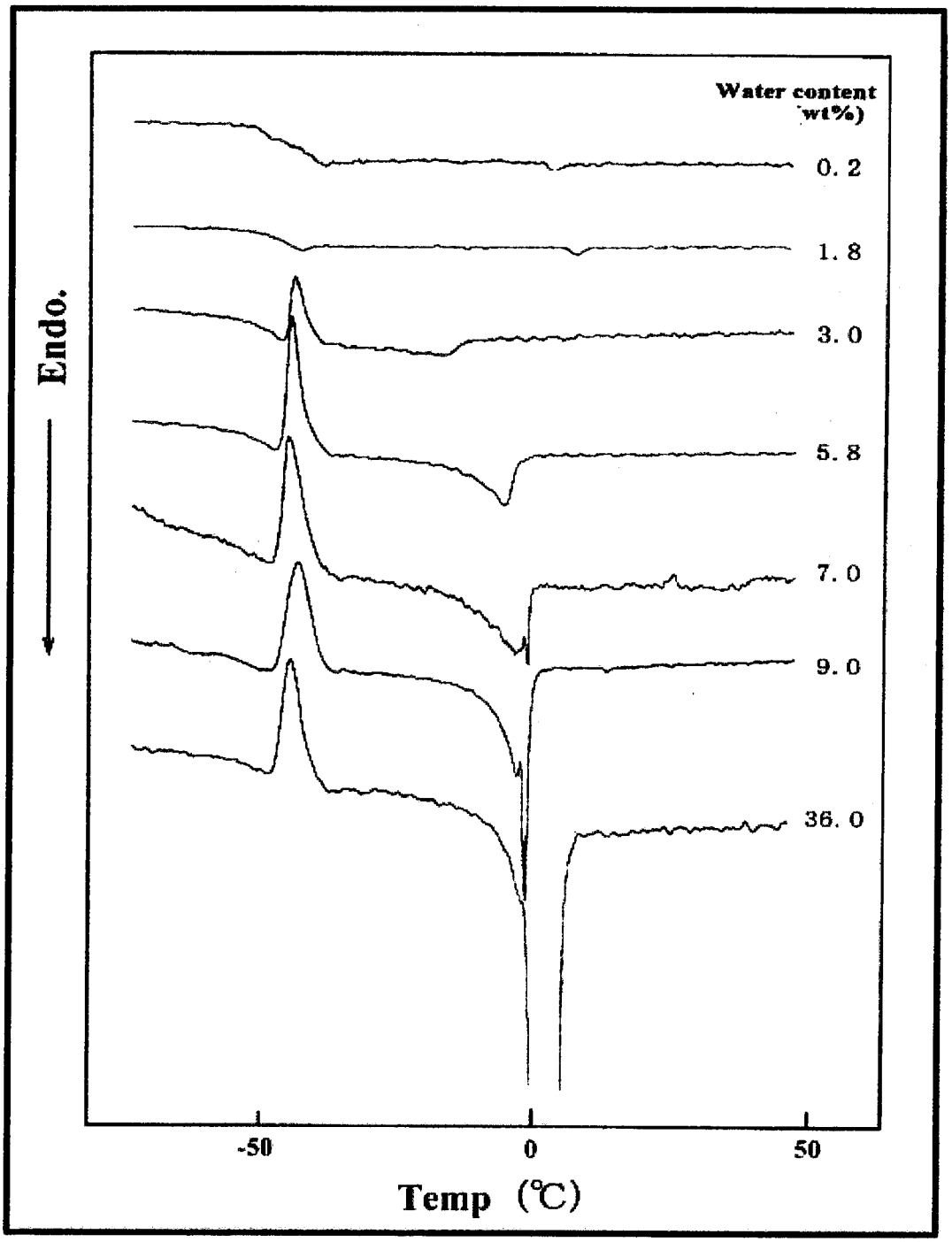


Figure 5-4. DSC heating curves of PMEA-water system : heating rate=2.5 ℃ / min.

over 7.0 wt % of W_c, the exothermic peaks at –42 °C and two endothermic peaks at –3 °C and –1 °C were detected. As a reference, **Figure 5-5** shows DSC heating curves for PHEMA-water systems, whose W_c was varied from 4.9 to 42.8 wt %. The exothermic peak was not observed. This means the water structure of hydrated PHEMA and that of PMEA is different.

It is well known that there are two kinds of water in a hydrated polymer. The water which shows first-order phase transition such as crystallization and melting, is called freezing water, and the water which never crystallizes even at –100 °C is called "***Non-freezing water***". "***Non-freezing water***" is strongly bound to polymers. Freezing water is classified into two types of water. One is "***Free water***" crystallizing at ca. 0 °C. The other is "***Freezing bound water***"crystallizing below 0 °C which interacts with the polymer weakly. **Figure 5-6** shows the classification of water structure in a hydrated polymer (28-30). On the basis of the classification (28-33), the freezing water in PMEA can be divided into two groups. One is the "***Freezing bound water***" showing the melting point at –4 °C ~ –14 °C (T_{m1}). The other is the free water melting at –1 °C (T_{m2}).

Low temperature melting peak (T_{m1}) rises with an increase in W_c in the range of 3.0~9.0 wt %. The rise of Tm1 is explained as follows. The interaction of water molecules with the polymer molecules will be weakened by the increase of W_c because the number of water molecules per PMEA repeating unit increases.

The exothermic peak clearly observed in the samples with more than 3.0 wt % of W_c is attributed to the cold crystallization of water (**Fig 5-4**). It is reported that cold crystallization of water is the transition of amorphous ice to crystalline ice [18, 27]. On cooling, the water vitrifies without crystallization. Therefore, the exothermic peek corresponding to the cold crystallization is never observed at –100 °C ~ 0 °C. On heating, the water molecule

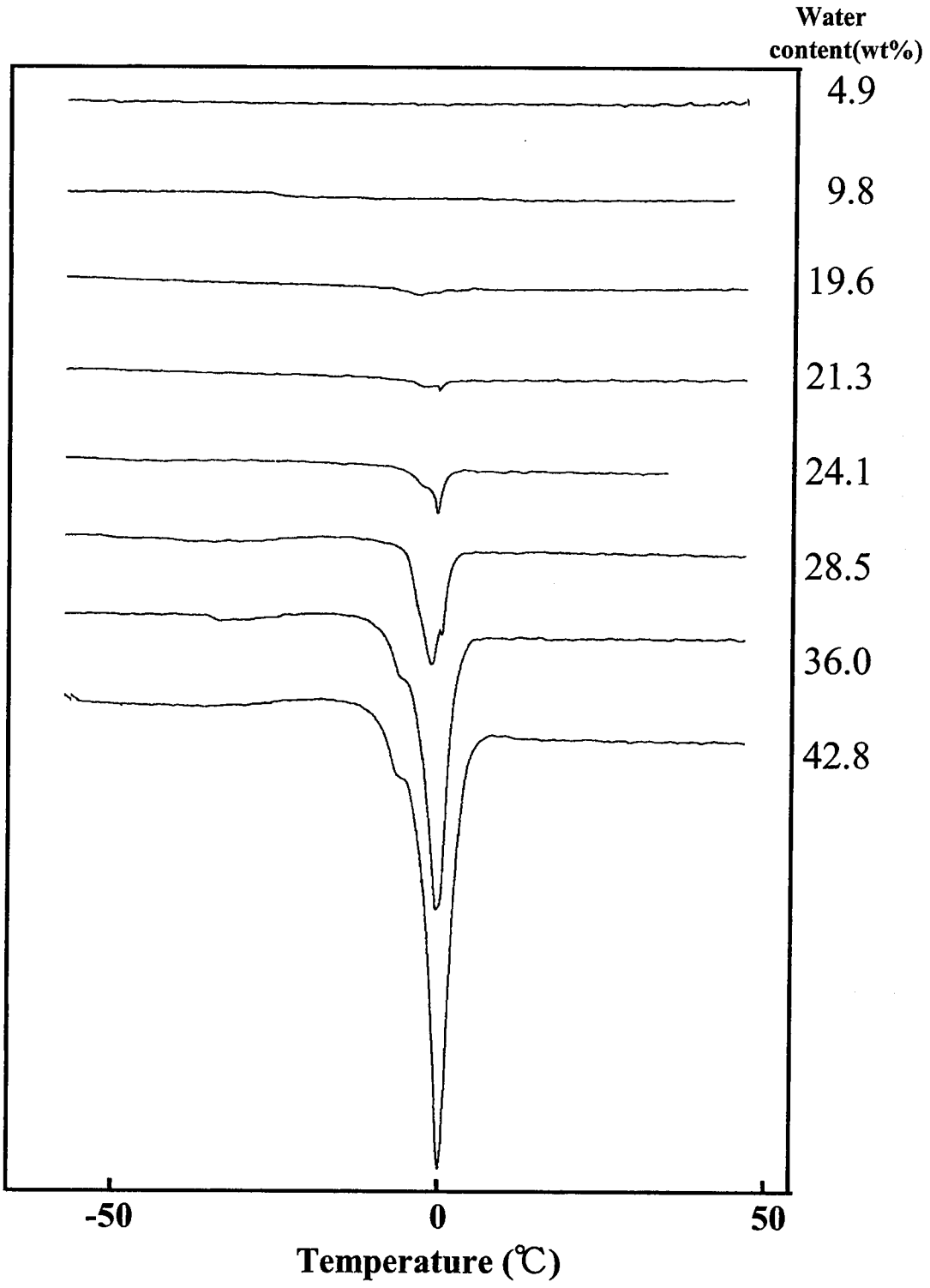


Figure 5-5. DSC curves of the PHEMA-water systems with various water contents; heating rate=2.5 ℃ / min.

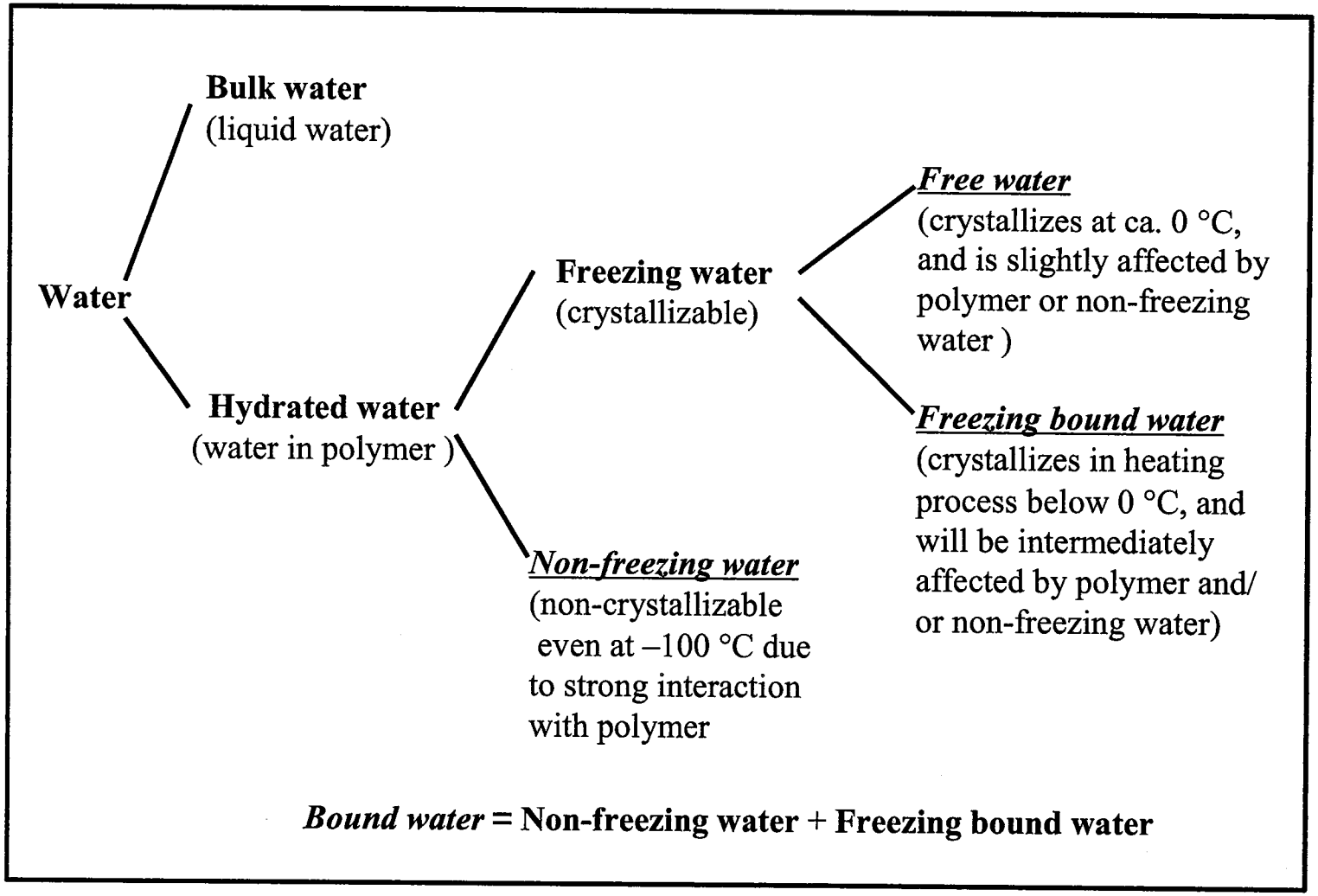


Figure 5-6. Classification of water in hydrated polymer based on DSC analysis.

solidified as an amorphous ice can be mobilized when the temperature rises above the glass transition (Tg), and successive heating enhances the molecular motion. The ice starts to crystallize at ca. –50 °C. In the case of PMEA, enthalpy of the cold crystallization (ΔH_{cc}) increases with increase in W_c from 3.0 to 7.0 wt % and reaches a plateau at ca. 14 J/g (**Table 5-3**). For the samples with 3.0 and 5.8 wt % of W_c, the ΔH_{cc} is almost equal to the enthalpy of melting (ΔH_m). This indicates that the cold crystallizable water is assigned to freezing bound water.

Table 5-3 Enthalpy changes of melting (ΔH_m) and enthalpy changes of crystallization (ΔH_{cc}) of PMEA-water system.

W_c wt%	ΔH_{cc} J/g	ΔH_m J/g
0.2	-*	-
1.8	-	-
3.0	4.6	4.6
5.8	11.8	11.8
7.0	14.1	14.4
9.0	14.0	20.1
36.0	13.6	108

* not observed

At the same time, as shown in **Figures 5-4 and 5-7**, the cold crystallization temperature (T_{cc}) maintains a constant temperature regardless of the W_c. The above facts can be attributed to the correlation between Tg and W_c. As shown in **Figure 5-4**, Tg was clearly observed at ca. -50 °C for all the samples. For the samples with less than 1.8 wt % of W_c, the transition was clearly observed. In the case of the samples with over 3.0 wt % of W_c, Tg

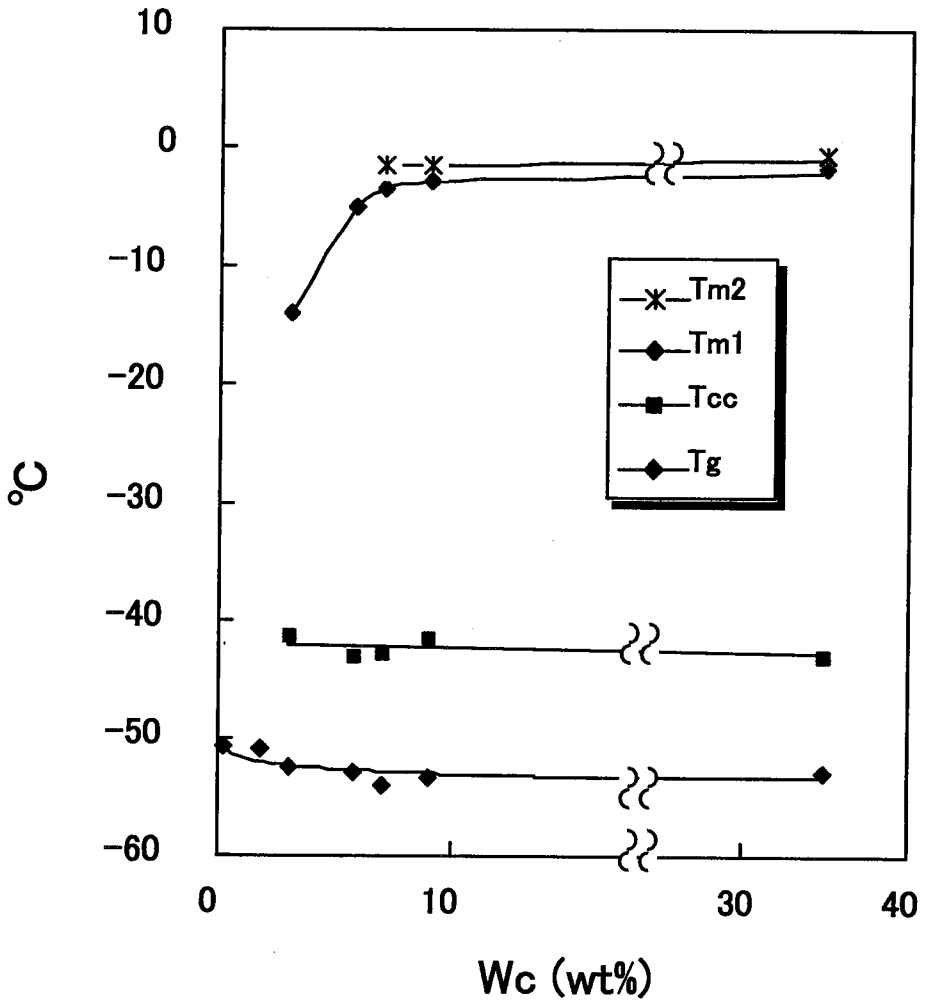


Figure 5-7. Phase diagram of PMEA-water system.
Tm1 : lower melting temperature,
Tm2 : higher melting temperature,
Tcc : cold crystallization temperature,
Tg : glass transition temperature.

is also at around ca. -50 °C observed, although the endothermic deviation partially overlaps with the cold crystallization peak. This fact indicates that W_c has little or no effect on the *Tg*. On this account, it is thought that the molecular motion of PMEA is not affected by the presence of water. Therefore, constant *Tg* values regardless of W_c suggests that the cold crystallization is induced by the molecular motion of PMEA.

Next, the state of the water in PMEA is discussed below in detail in terms of the ratio of the free water (W_f), freezing bond water (W_{fb}) and non-freezing water (W_{nf}). W_{fb} and W_f correspond to the water which crystallizes near -50 °C in the heating process and the water that freezes near -0 °C, respectively. W_{nf} is the water that shows no transition over the range from -100 to 0°C. The relation of these parameters is represented by the following equation;

$$W_c\,(\mathrm{wt\%}) = W_{nf}\,(\mathrm{wt\%}) + W_{fb}\,(\mathrm{wt\%}) + W_f\,(\mathrm{wt\%}) \qquad \text{eq.(4)}$$

The amount of the water corresponding to Wfb can be obtained by the entharpy of the cold crystallization (ΔHcc) and the heat of fusion of the water, where it is assumed to be equal to the heat of fusion for pure water, 334 J/g (18). As the fusion entharpy observed near 0 °C is the summation of the waters corresponding to the Wfb and Wf, the amount of freezing water was calculated by subtracting ΔHcc from the entharpy of melting (ΔHm). Wnf was obtained by subtracting Wfb and Wf from Wc.

The results are shown in **Figure 5-8**. *W*nf and *W*fb reach plateau values, 3.0 wt% and 4.4 wt% at ca. 3.0 and 7.0 wt % of *W*c, respectively, while *W*f firstly appeared at ca. 7.0 wt % of *W*c, and increases with increasing *W*c. When the *W*c is below 3.0 wt %, all of the water exists as the *W*nf. The EWC includes32 % *W*nf, 48 % *W*fb and 20 % *W*f, respectively.

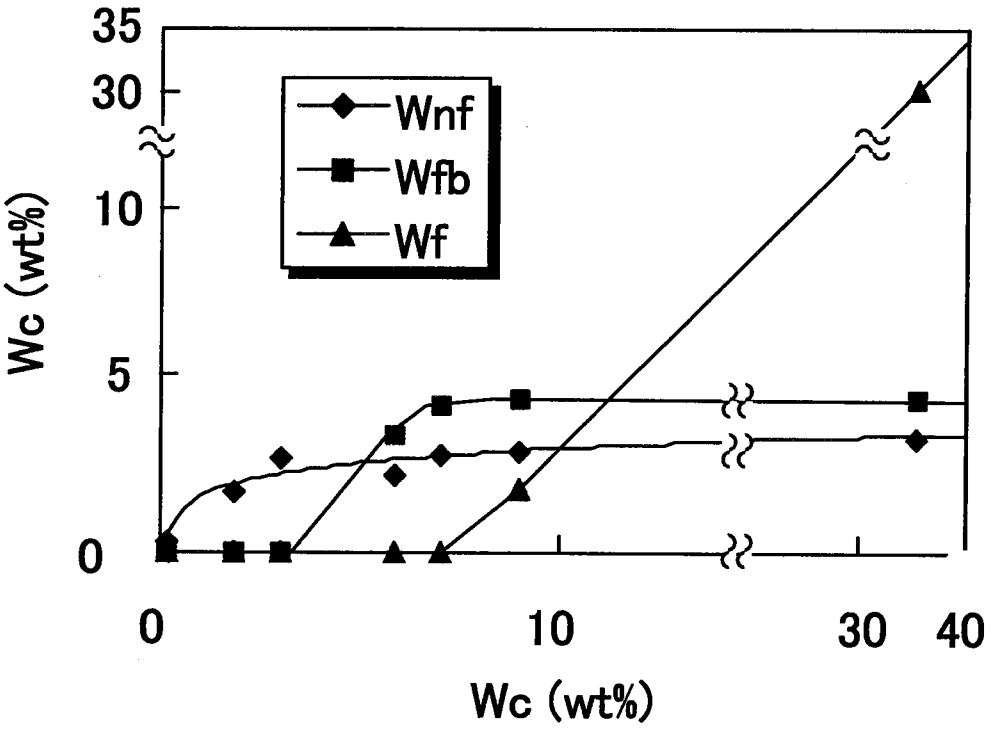


Figure 5-8. Relationship between amounts of free water (Wf), freezing bound water (Wfb), non-freezing water (Wnf) and Wc of PMEA-water systems.

This means that the major part of the hydrated water in the equilibrium state is *W*fb.

There are a few reports on cold crystallization of water in the hydrated polymers, such as polysaccharide-water, polyethylene oxide (PEO)-water and gelatin-water systems [27, 34-38]. PMEA is similar to PEO, polysaccharide and gelatin in that the hydrated polymers show the phenomenon of the cold crystallization. PEO is known as one of the most biocompatible synthetic polymers at the present. Polysaccharides are components of extra cellular matrix. The polysaccharide moieties in the outer regions of the cell surface play a fundamental role in cell interfacial phenomena. Gelatin is one of the most important biocompatible biopolymers. The water content in PMEA is smaller than in these biomedical hydrogels [39]. However, blood compatibility of PMEA is successfully confirmed in our previous paper [21-25]. In general, cold crystallization is observed in a very narrow *W*c range and in some cases lacks the thermal reversibility. As for the PMEA, cold crystallization is observed over a wide *W*c range and is well reproducible. This indicates that amorphous ice in PMEA is stable, although the reason for the stability of the amorphous ice in the PMEA is not clear yet. It is reported that the important factor expressing the biocompatibility is not the amount of water bound in the systems but the structure or orientation of the water in the polymer [20]. Considering these facts, the water structure appeared as cold crystallization may play an important role in the expression of the blood compatibility. The final goal of this research is to establish the relationship between water structure and blood compatibility, and to design biomedical materials. We have not enough information to prove the hypothesis that water observed cold crystallization is an important factor for blood compatibility. More detailed research is now in progress.

Water structure in p(MEA-co-HEMA)

One of the important characteristics of the polymers in this study is the equilibrium water content (EWC), and the results are shown in **Figure 5-9**. The EWC of PMEA was 9 wt%. The EWC of the copolymer increases linearly with an increase of the HEMA content up to 50 mol%, and levels off. The EWC of the copolymer with 50 mol% of HEMA is almost equal to the EWC of PHEMA, 40 wt%.

It is well known that water in a polymer can be classified into three types of water : non-freezing water, freezing bound water, and free water (28-33). The relationship between EWC and the three types of water can be expressed by equation 4. (EWC (wt%) = *W*nf (wt%) + *W*fb (wt%) + *W*f (wt%)) where *W*nf, *W*fb, and *W*f are the contents of non-freezing, freezing bound, and free waters, respectively. These three kinds of water will be described latter.

The thermal behavior observed by DSC for the hydrated polymers with the EWC is discussed in the following. The thermograms in the heating process are shown in **Figure 5-10**. A characteristic exothermic peak was found for the copolymers with 0-30 mol% of HEMA at around -50 °C, and this peak is due to the cold crystallization of water in the hydrated polymer. The large endothermic peak with a shoulder appearing in the range of -15 to 0 °C is based on the melt of the ices, where the ices are derived from free water and freezing bound water. The cold crystallization of water mentioned above is interpreted as the phase transition from the amorphous ice to crystalline ice, the former of which will be originated from the freezing bound water in the polymer (18, 26, 27). Generally, the crystallization occurs in the cooling process from liquid to solid. On the other hand, the cold crystallization is defined as the crystallization occurring in heating process in the solid state. Cold crystallization is commonly observed in the solid (amorphous) state substance

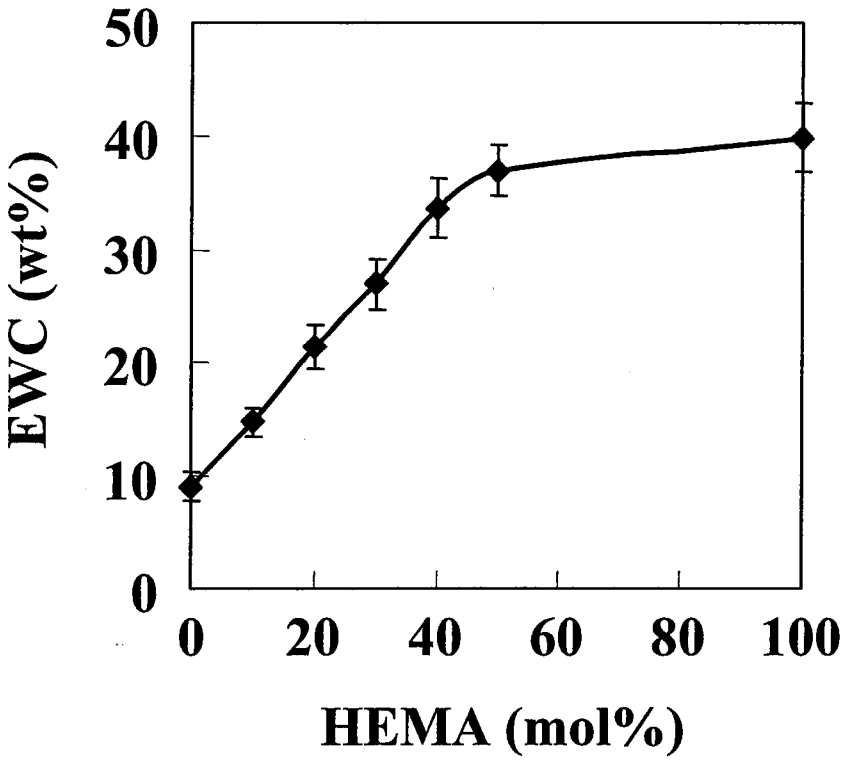


Figure 5-9. EWC of poly(MEA-co-HEMA). (mean ± SD, n=4)

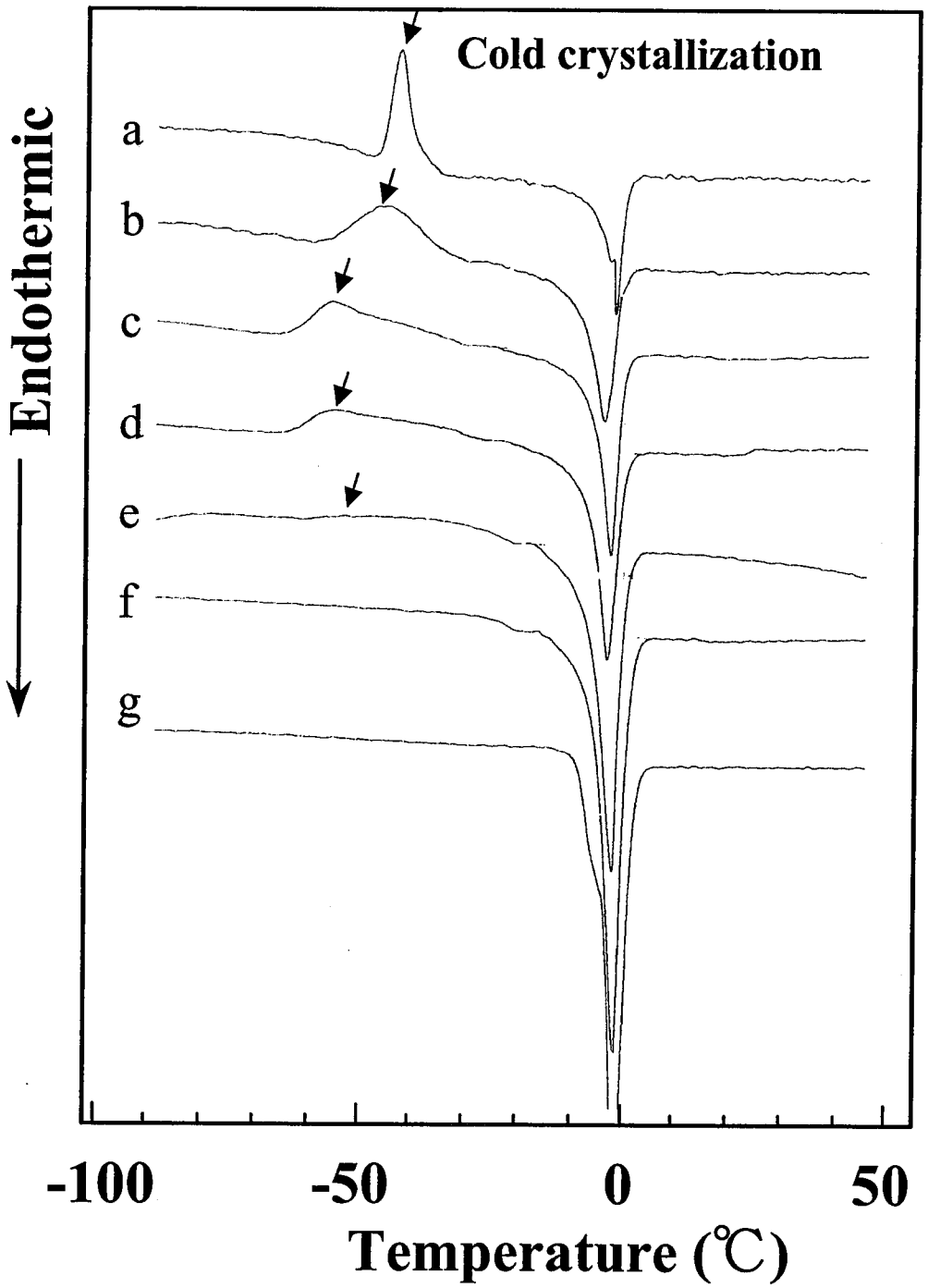


Figure 5-10. DSC heating curves of poly(MEA-co-HEMA) with EWC. heating rate=2.5 ℃/min. HEMA content in copolymer (mol%) : (a) 0 (PMEA), (b)10, (c)20, (d)30, (e)40, (f)50, (g)100 (PHEMA).

formed by very rapid cooling of the liquid state. In our experiment, as the rapid cooling was never carried out, the reason for the cold crystallization of water will be due to the specific interaction with PMEA molecule. The cold crystallization peak was observed clearly till the HEMA content increases to 30 mol%, but the peak became broader with an increase of the HEMA content from 0 to 30 mol%. In the case of the copolymer with 40 mol% of HEMA, the peak could barely be observed, and in 50 mol% of HEMA the peak disappears thoroughly. The broadening of the cold crystallization peak with an increase of the HEMA content will be attributed to the change of the water environment : the mobility of the polymer chain and the hydrogen bonding by the hydroxyl group of HEMA.

On the basis of EWC, ΔH_{cc}, ΔH_m, and equation 4, we can quantify the amount of the three kinds of water : non-freezing, freezing-bound, and free water, where ΔH_{cc} is the enthalpy of the cold crystallization and ΔH_m is the fusion enthalpy of ice observed by DSC. To determine the content of each water we assume the followings : 1) The cold crystallizable water is composed of the freezing bound water (Wfb$\propto\Delta H_{cc}$), and ΔH_m is the summation of the fusion enthalpies of the ices, which derive from the free water and the freezing bound water in the polymer ((Wfb + Wf)$\propto\Delta H_m$). 2) The fusion enthalpies of the ices are equal to the fusion enthalpy of ice derived from bulk pure water, 334J/g (18). The enthalpy of the cold crystallization is also 334 J/g. The results are shown in **Figures 5-11 and 5-12** where the Wnf, Wfb, and Wf are plotted against the HEMA content in the polymers. *W*nf and Wf increase steadily over the range of HEMA content, 0-50 mol%, and the Wnf and Wf at 50 mol% of HEMA are close to those of PHEMA. The polymers with 30-100 mol% of HEMA content have non-freezing water as major water. On the other hand, Wfb reaches a maximum at 30 mol% of HEMA, decreases harshly with increasing HEMA content from 30 mol%, and disappears at 50 mol%. It should be noticed that

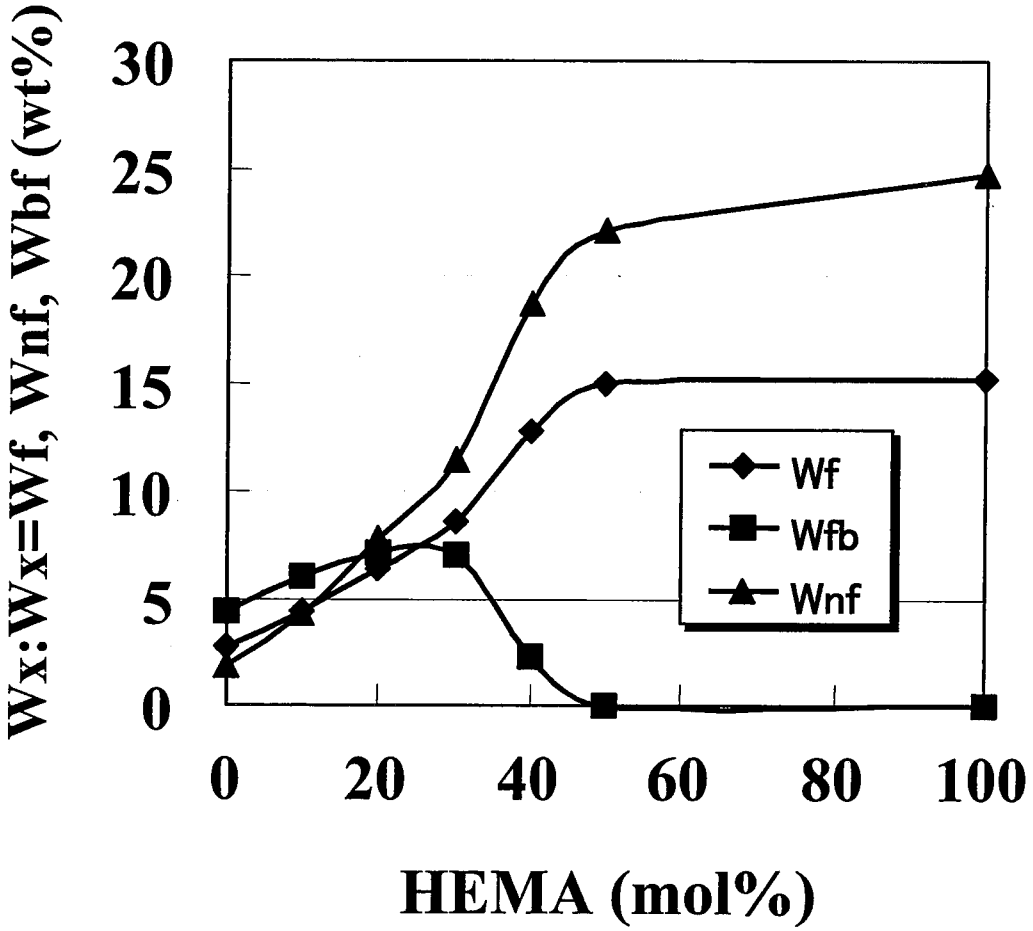


Figure 5-11. Relationship between the amounts of three types of water and HEMA content in poly(MEA-co-HEMA). Wf : free water, Wfb : freezing bound water, Wnf : non-freezing water.

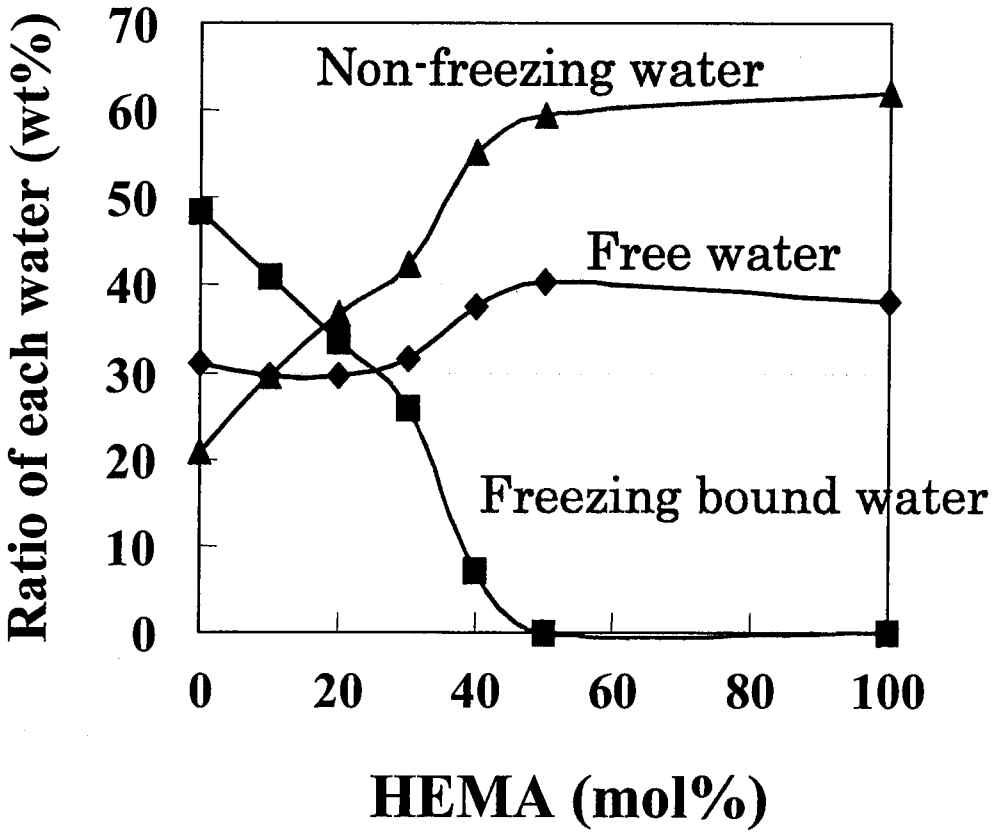


Figure 5-12. Relationship between the ratios of three waters and HEMA content in poly(MEA-co-HEMA). (Wfb/EWC)×100, nf : non-freezing water (Wnf/EWC)×100.

freezing water is major water in the polymers with 0-20 mol% of HEMA content.

Water structure in PMEA analogous poly(meth)acrylate

The structure of water in the polymer with EWC was investigated by DSC. The thermograms in heating process are shown in **Figures 5-13**. It can be recognized that the significant difference between the thermogram of PMEA and other polymer's ones. That is, PMEA shows clear exothermic peak at -42 ℃, and the other polymers never show such exothermic peak. We have attributed this peak to cold-crystallization of water (defined as freezing bound water), and defined it as freezing bound water (35). In addition, it is found that there is a small difference in the shape of the endothermic peak at the range of -15 to 0 ℃ due to the melt of ice, when all of the thermograms are compared. Except PMEA and PHEMA, the peak is single and symmetrical. PMEA has two endothermic peaks and PHEMA has a peak with shoulder. In the case of PMEA, the endothermic peak at lower temperature will be due to the melt of the cold-crystallized water. The shoulder observed in PHEMA peak will be caused by the water interacting weakly with the polymer molecule. The peak appering at higher temperature in both the cases is due to the melt of ice based on free water. As non-freezing water is defined as the water which never crystallizes even at -150 °C, it does not appear in the DSC curve.

As mentioned above, the relationships between EWC and the three waters (non-freezing water, freezing bound water, and free water) and between the enthalpy change and the content of the water can be described as equations 4-6.

$$\text{EWC (wt\%)} = W\text{nf (wt\%)} + W\text{fb (wt\%)} + W\text{f (wt\%)} \qquad \textbf{eq.(4)}$$

$$\Delta H_{cc} \propto W\text{fb} \qquad \textbf{eq.(5)}$$

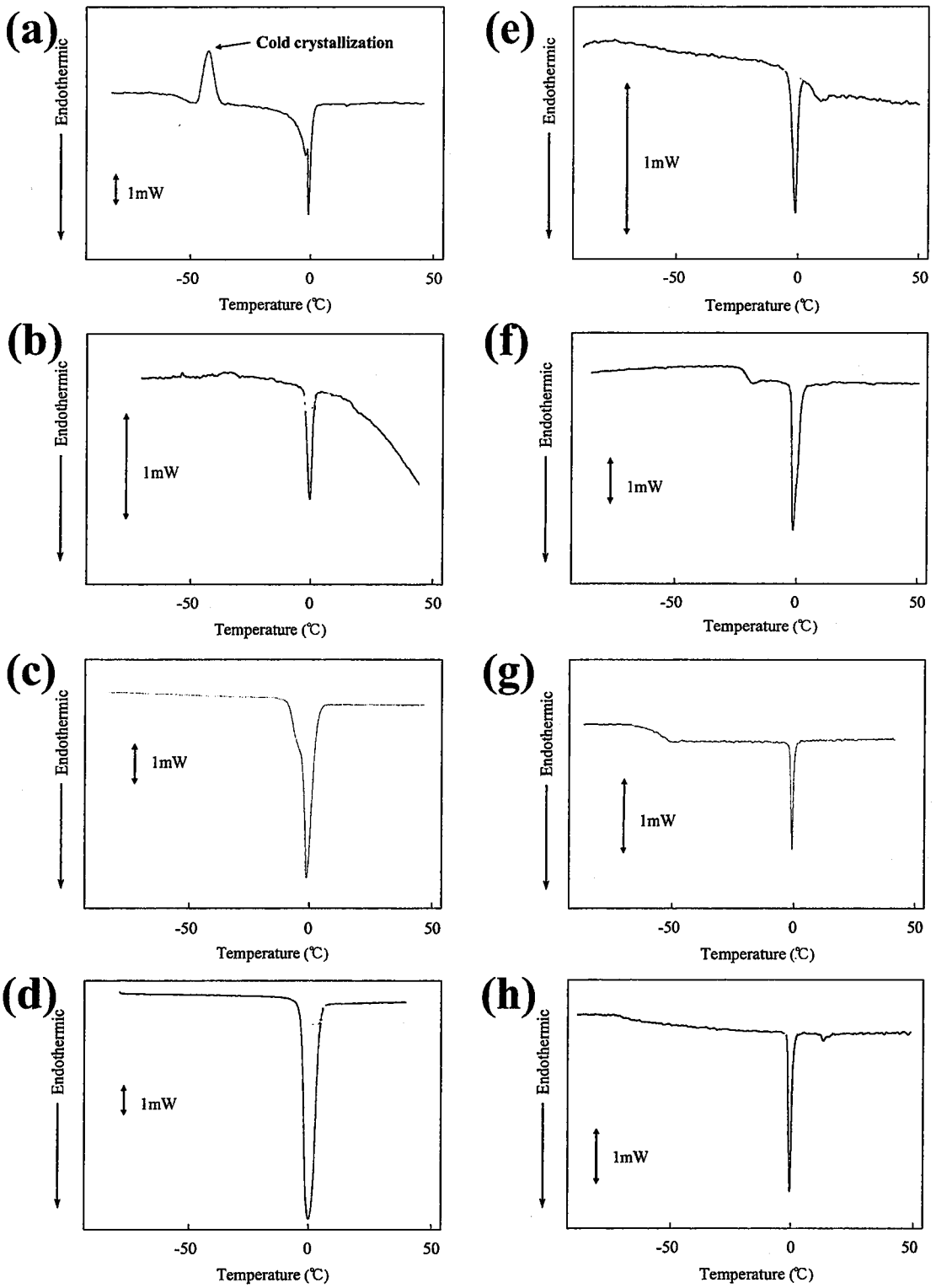


Figure 5-13. DSC thermogram of poly(meth)acrylate with EWC. a)PMEA, b)PMEMA, c)PHEMA, d)PHEA, e)PPEA, f)PEA, g)PBA, h)PEHA heating rate:2.5℃/min.

$$\Delta H_m \propto (Wf + Wfb) \qquad \text{eq.(6)}$$

where Wnf, Wfb, and Wf are the contents of the non-freezing, freezing bound, and free waters, respectively, and ΔH_{cc} and ΔH_m are enthalpy changes in the cold-crystallization and the melt of ice at ca. –15 to 0 °C, respectively.

When the water contents were calculated from the equations, it was assumed that the heat capacities in ΔH_{cc} and ΔH_m are 334J/g [36]. EWC and the content of each water in the polymers, Wnf, Wfb, and Wf, are listed in **Table 5-4**.

Table 5-4 Contents of three waters in poly(meth)acrylates.

	EWC (wt%)	free water:Wf (wt%)	freezing bound water:Wfb (wt%)	non-freezing water :Wnf (wt%)
PMEA	9.0	2.7	4.5	1.8
PMEMA	4.2	0.4	0	3.8
PHEMA	39	24	0	15
PHEA	60	29	0	31
PEA	4.8	3.4	0	1.4
PPEA	3.3	2.2	0	1.1
PBA	1.9	0.4	0	1.5
PEHA	2.1	0.6	0	1.5

EWC = Wf + Wfb + Wnf

From these results it is found that only PMEA contains a large amount of freezing bound water, and that water in other poly(meth)acrylates is composed of two waters: non-freezing water and free water. Moreover, the table indicates that the polymers except PMEA are classified into three groups, the polymer containing non-freezing water as main water (PMEMA, PBA and PEHA), the polymer having free water as main water (PHEMA,

PEA and PPEA) and the polymer having both the waters equally (PHEA). The content of the water will be described below more quantitatively. Non-freezing water can be defined as the water bound to specific groups of the polymer directly and strongly, and thus its content is small, 0.8-3.7 wt% except PHEMA and PHEA. This phenomenon will be explained from the viewpoint that water molecule composing non-freezing water mainly interacts with the ester group or the methoxy group of the side chain. On the other hand, the content of non-freezing water in PHEMA and PHEA is large, in the range of 15-31 wt%, which depends on the formation of the strong hydrogen-bonding between water molecule and the hydroxy group in the polymer. The water having little interaction with these functional groups in the polymer exists as free water, and its content varies from 0.42 (PBA) to 28.8 wt%(PHEMA).

When the reason for the excellent blood compatibility of PMEA is clarified, it is important to reveal the water structure on the polymer surface. It should be noticed that the information obtained from DSC regards the structure of water in the polymer, and does not exhibit the structure of the water on the polymer surface, while the contact angle reflects the surface structure of the polymer. The above-described fact that the classification of the polymers by EWC agrees approximately with the one by the contact angle suggests a possibility that the water structure on the polymer surface relates to the one in the polymer. Therefore, the relationships between the results of the contact angle and the water in the polymer were investigated in detail. The results of plotting the content of the waters determined by DSC against the contact angle are shown in **Figure 5-14**. This figure indicates strongly that the content of the bound water corresponds to the contact angle, where the word, "bound water" includes both freezing bound water and non-freezing water. If the contact angle is plotted against only the content of non-freezing bound water, it is

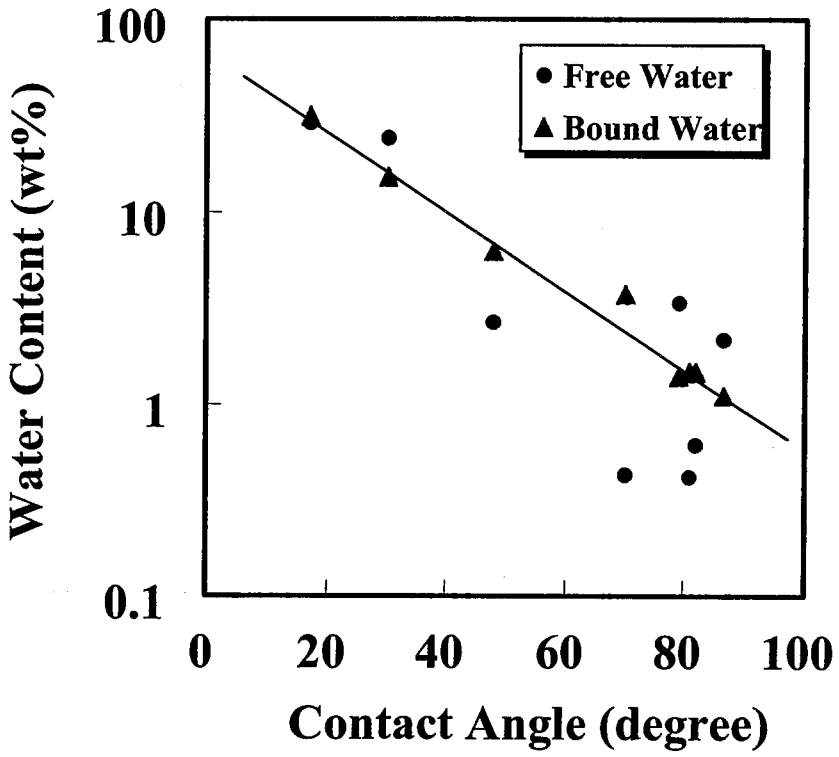


Figure 5-14. Relationship between contact angle and water in polymer. a)plotted against free water b)plotted against bound water (non-freezing water +freezing bound water).

obvious that the point of PMEA is not on the linear line drawn as the function of the content of the bound water. Moreover, this figure shows that the relation of free water in the polymer to the contact wangle is poor when compared with the bound water. Therefore, these results indicate that the amount of the bound water existing on the polymer surface determines the contact angle, or the surface property (hydrophobicity/hydrophilicity), and thus it will be concluded that the bound water exists on the surface stably.

Platelet adhesion and spreading of polymers

(a) p(MEA-co-HEMA)

We have already reported the excellent blood compatibility of PMEA by estimating the early stage reactions of platelet (platelet adhesion test and β-TG), leukocyte, complement system, and coagulation system which were induced by the contact of PMEA surface with human blood for 30-60 min in vitro (21-25). Anzai and Saito *et al.* showed that PMEA had a possibility of applying PMEA to clinical usage (23). That is, they coated an oxygenator with PMEA and evaluated the blood compatibility under the circulation of human blood for 6 h in terms of the activation of platelet (retention of the number and β-TG), coagulation system (bradykinin and TAT), and leukocyte (retention of the number, and PMN-elastase) (23). After the circulation, they analyzed the adsorbed proteins on the oxygenator and concluded that the surface of the oxygenator had significantly small amount of proteins compared with the non-coated one (23, 25). In addition Suhara *et al.* proved the excellent blood compatibility of PMEA in the extracorporeal circulation of the PMEA-coated oxygenator using a pig in terms of TAT, bradykinin, ATIII, and the retention of platelet number (40). On the basis of these results, we concluded that the platelet adhesion test has a sufficient validity to estimate the blood compatibility, and thus, we

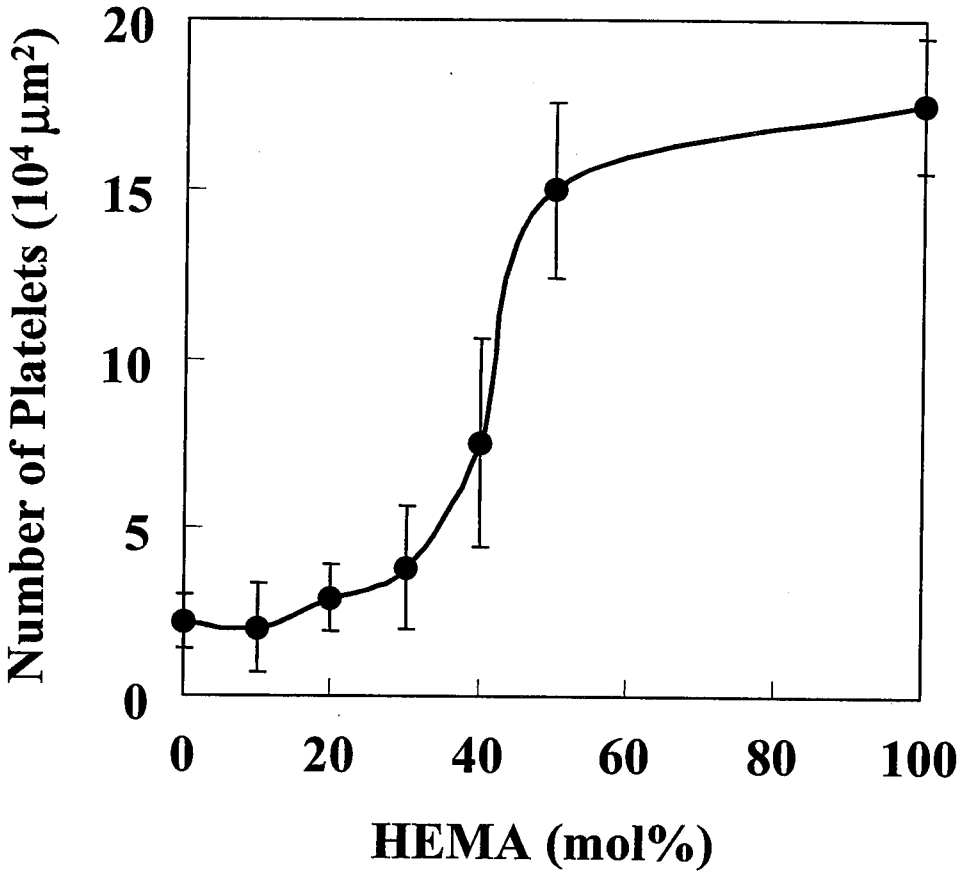


Figure 5-15. Number of adhered platelet onto the surface of poly(MEA-co-HEMA). (**P<0.01 vs PMEA, Mean±standard deviation, n=5)

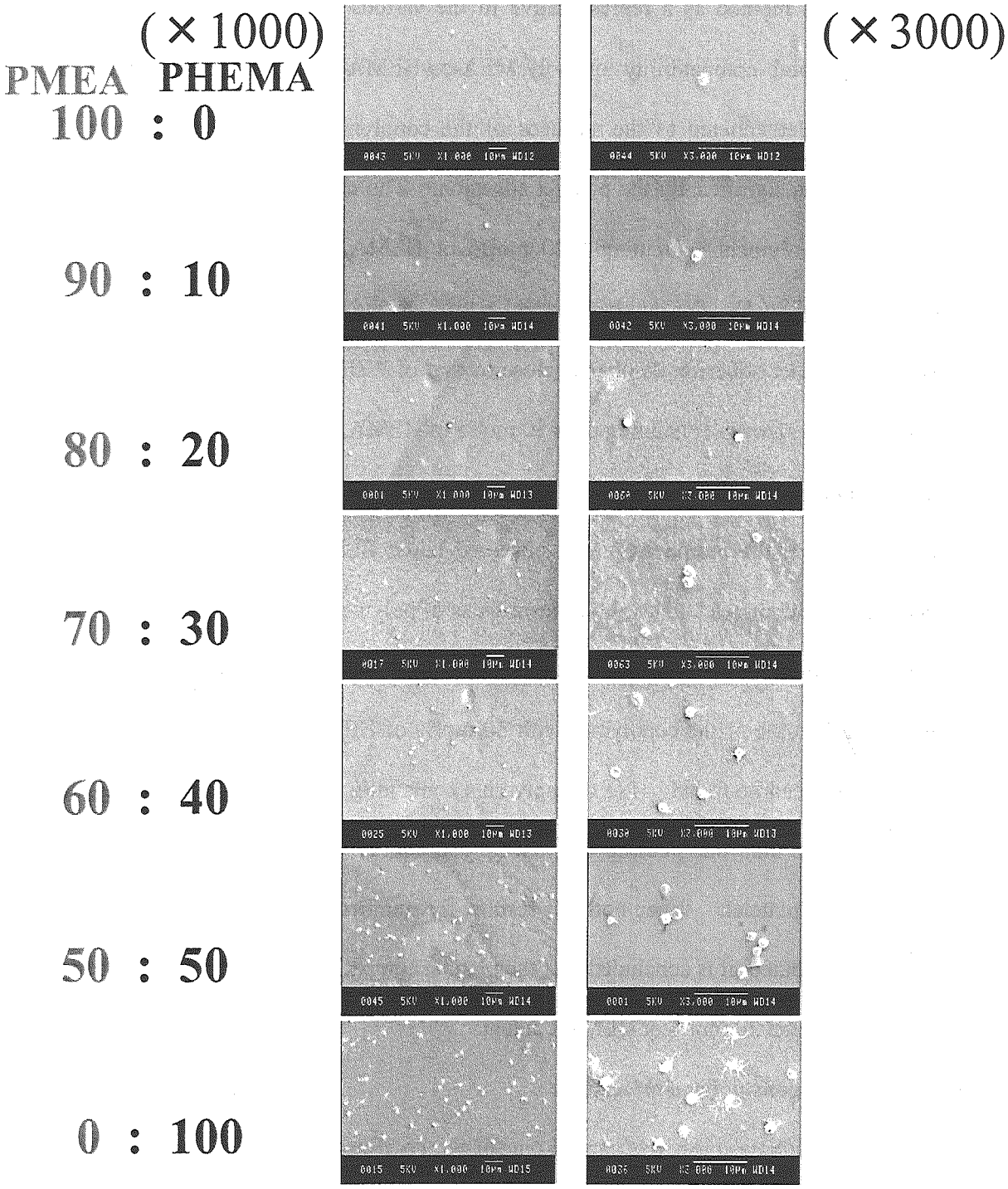


Figure 5-16. Scanning electron micrographs of platelet adhered onto the surface of poly(MEA-co-HEMA). magnification left side image: × 1,000 right side image : × 3,000 HEMA content in copolymer (mol%) (A,H):0(PMEA), (B,I):10, (C,J):20, (D,K):30, (E,L):40, (F,M):50, (G,N):100 (PHEMA).

adopted this test method as a representative in the various blood-compatible indexes to evaluate the blood compatibility of poly(MEA-co-HEMA). **Figure 5-15** shows the number of platelet adhered to the surfaces of the copolymers. The composition in the copolymer affects significantly the platelet adhesion. The number of the adhered platelet is least in the polymers containing 0-20 mol% of HEMA. When the HEMA content becomes over 30 mol%, the number sharply rises. In the copolymer with 50 mol% of HEMA, the platelet adhesion number is close to that of PHEMA. These results strongly suggest that the polymers containing over 30 mol% of HEMA activate the platelet.

As the morphology of the platelet is one of the indexes expressing the degree of the platelet activation, the morphology was observed under SEM. The results are shown in **Figure 5-16**. The platelet adhered on the surfaces of polymers with 0-20 mol% of HEMA keep its original spherical shape. This result means that the surfaces do not activate the platelet. In the case of the copolymer with 30 mol% of HEMA, slightly small change in the morphology can be found. For the polymers containing 40-100 mol% of HEMA, it is clearly observed that the platelet has pseudopod and/or spread, which indicates the activation of the platelet. When both the results, the number and the shape of the adhered platelet, are combined, it is concluded that the platelet compatibility of the polymer changes drastically at around 30 mol% of HEMA content.

(b) PMEA analogous poly(meth)acrylate

In vitro platelet adhesion test was carried out for the poly(meth)acrylate polymers from the viewpoint of the adhesion number and the morphology, and the results are shown in **Figures 5-17 and 5-18**. The number of platelets adhering on PMEA surface, 2.5 cells/10^4 μm^2, is markedly small when compared with those of other polymers, over 14 cells/10^4 μm^2. These results suggest that the PMEA analogous polymers investigated activate platelet more

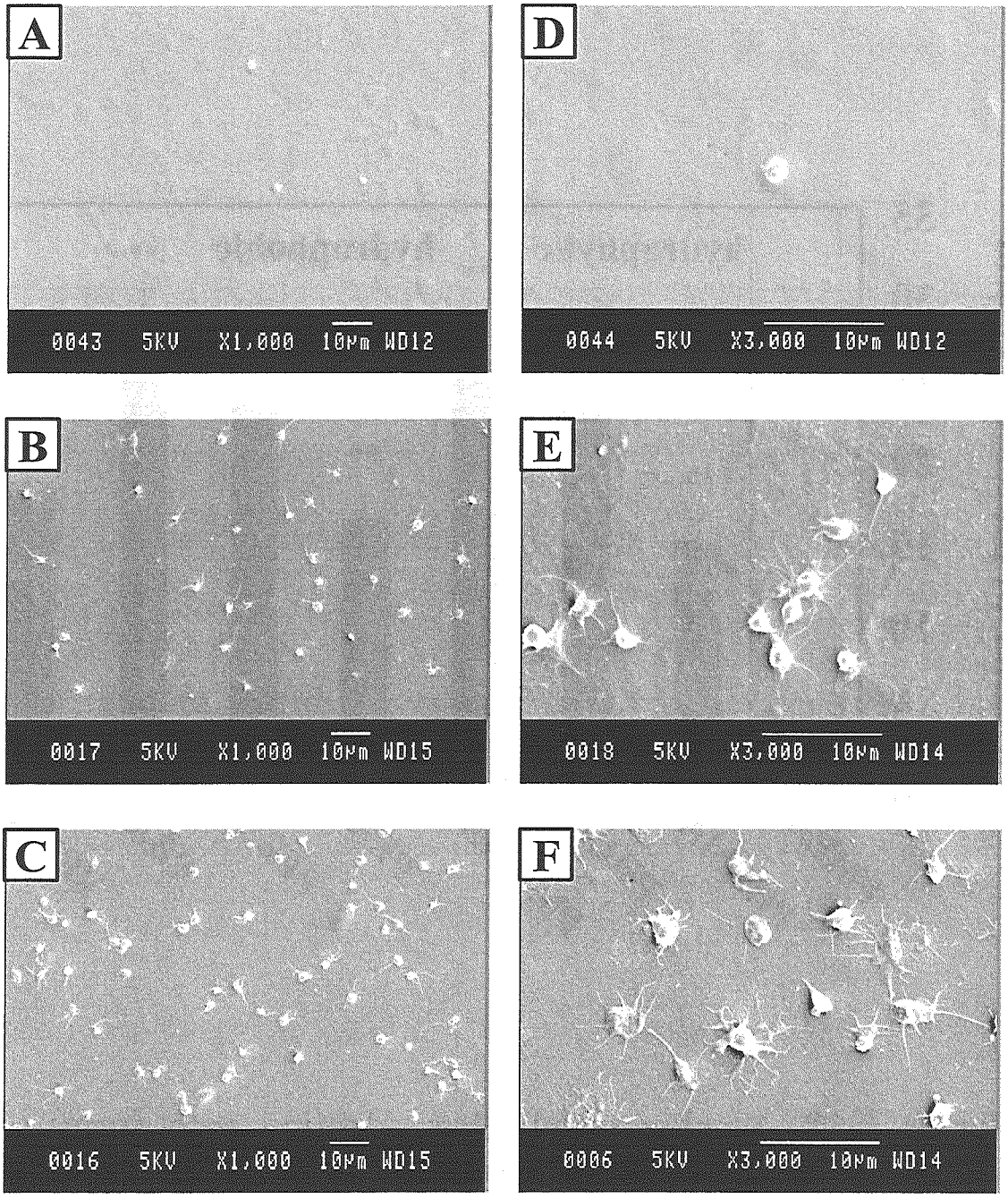


Figure 5-17. SEM images of adhered platelets to polymer surface. left side image magnification 1000×, right side image magnification 3000×, A,D)PMEA, B,E)PHEMA, C,F)PMEMA.

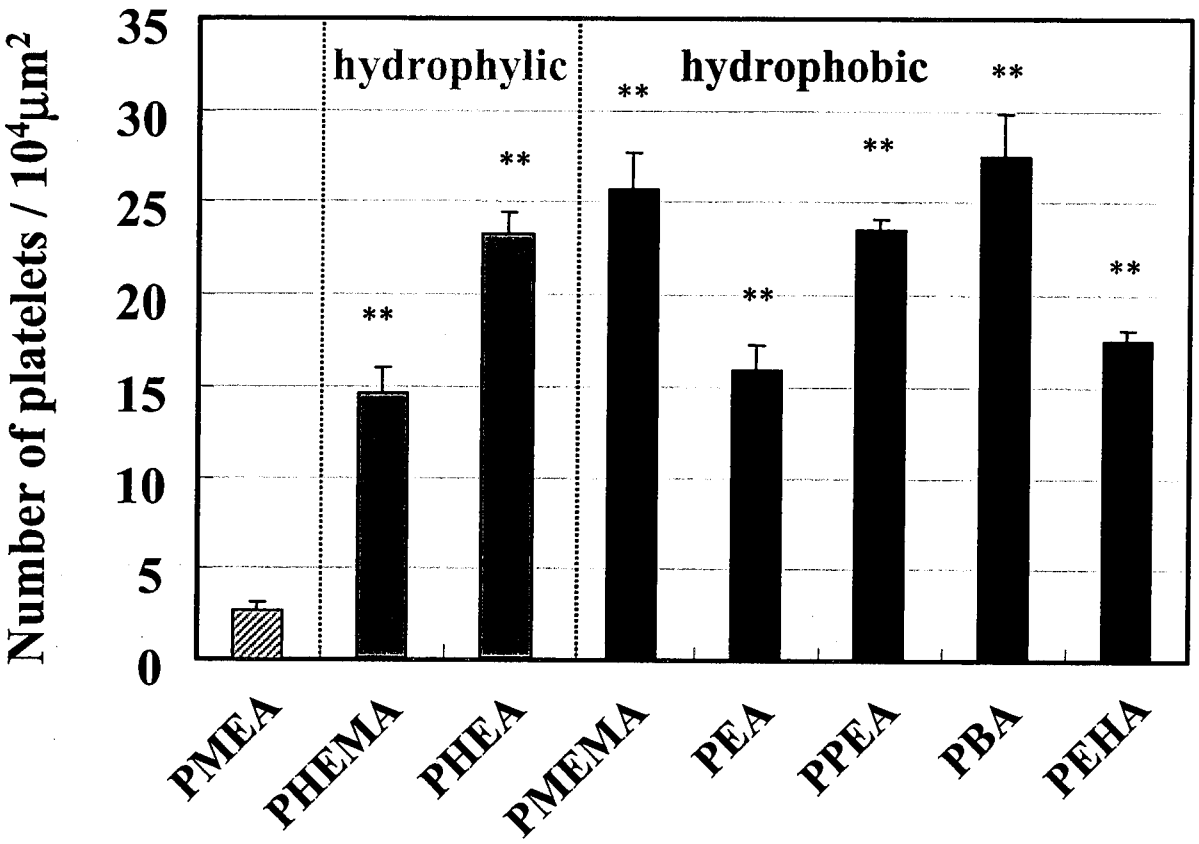


Figure 5-18. Number of platelets adhering onto surface of poly(meth)acrylates. (**P＜0.01vsPMEA, Mean±standard deviation, n=5)

than PMEA. Next, the morphology of the adhered platelet is described. The SEM images of the platelet morphology on PMEA, PHEMA and PMEMA are shown in **Figure 5-17** as representatives. The platelet adhering on PHEMA and PMEMA spread markedly, while in the case of PMEA the platelet kept its original shape. For other poly(meth)acrylates the similar drastic morphological change of platelet was also observed. From these results on the platelet number and the morphology, it is concluded that PMEA analogous polymers activate platelet markedly and PMEA is very inert for the activation of platelet.

Relationship between water structure and platelet adhesion

(a) p(MEA-co-HEMA)

To clarify the reason for the drastic change of the compatibility of poly(MEA-co-HEMA) at around 30 mol% of HEMA content, the relationship between the amount and state of water in the polymer and the platelet adhesion behavior is discussed on the basis of results shown in **Figures 5-11** and **5-12**. From both the figures it is found that when HEMA content exceeds 30 mol% the amount of the freezing bound water decreases markedly and the platelet was activated, which indicates that the amount of the freezing bound water will play an important role in determining the platelet compatibility.

The water structure described above is essentially limited to the water in the polymer, because the water structure was analyzed by DSC. However, one can anticipate easily that the water adsorbed on the polymer surface will have the same water structure in the bulk polymer. That is, the polymer surface will be covered with the three kinds of water layers. The bottom layer contacting with the polymer surface, outer layer, and outermost layer are composed of non-freezing water, the freezing bound water, and the free water,

respectively.

Many researchers have pointed out that the water structure on the material surface is an important factor for the material to express the good blood compatibility. Bruck indicated that there exists a critical level of water in PHEMA and poly(acrylamide) hydorogels to achieve blood compatibility (41). Andrade *et al.* proposed that the nature of the PHEMA hydrogel interface affects blood compatibility (42). Ratner and Hoffman have found that the important factor expressing the blood compatibility of grafted PHEMA and poly(acrylamide) hydorogels were not the amount of the adsorbed water on the surfaces but the structure of the water (43). Tanzawa *et al.* suggested that the mobility of polymer chains and water in poly(ethyleneglycol methacrylate) hydrogel correlated with blood compatibility (44-46). The importance of surface bound water on protein adsorption was indicated by Lu *et al.* (47) with respect of hydration interaction energies of polymer surfaces and proteins. When proteins adsorb onto polymer surfaces water molecules on their surfaces need to be replaced. Water molecules on polymer surfaces are attracted by hydrophilic amino residue on the protein and subsequently proteins adsorb onto the surfaces (see **Figure 1-4**). Israelachvili *et al.* (48) and Dequeiroz et al. (49) reported that the stability, density, and arrangement of the surface water molecules bring about the different capabilities of the lipid bilayer and poly(N,N-dimethylacrylamide) to adsorb the proteins and blood cells. Yui *et al.* (50) confirmed that polypropylene surfaces with a particular crystalline (amorphous microstructure) reduce protein adsorption and platelet adhesion. Although the wettability such as dynamic contact angle and surface free energy components were almost constant, the structure of water sorbed into the polymer was changed. They suggests that the water structure is important on the blood-contacting properties of the polymer surface. Recently, Grunze et al. studied the protein adsorption

properties of the oligo(ethylenglycol)-terminated self-assembled membrane experimentally and computationally, resulting in that the protein resistance was caused by the formation of structured or tightly bound layer water at the interface, which prevented direct contact between the proteins and the surface.(51-55) Kataoka et al. (56,57) found the excellent platelet compatibility in poly(HEMA-block-4-bis(trimethylsilil)methylstyrene) and concluded that this property was attributed to the high free water content and the increased mobility of PHEMA segments. Ishihara et al. (58-60) also proposed that with respect to the expression of the excellent blood compatibility of poly(2-methacryloylethyl phosporylcholine) the amount of the free water would be the key parameter. In addition, it is well known that proteins and cells in blood have hydration shells composed of non-freezing water, intermediate water, and free water (61-66), and due to this hydration structure the components can keep themselves stable (see **Figure 1-3**). On the basis of these facts, we considered that the trigger of activation of the biological component is the change or the destruction of the hydration structure by contacting directly the non-freezing water on the materials.

The above discussions implies that the activation of the biological components can be avoided if water layer exists between the biological components and polymer surface, and prevents the hydration shell of the component from contacting directly with the non-freezing water layer of the polymer material. Can free water act as the barrier layer? As the free water moves almost freely, it cannot act as this layer. We speculate that the freezing bound water which is present as the cold crystallizable water can play a role in shielding the non-freezing water layer of the polymer surface from the hydration shell of the biological components. Since the motion of the freezing bound water is only weakly restrained, this water layer can exist easily between the hydration shell and the non-freezing

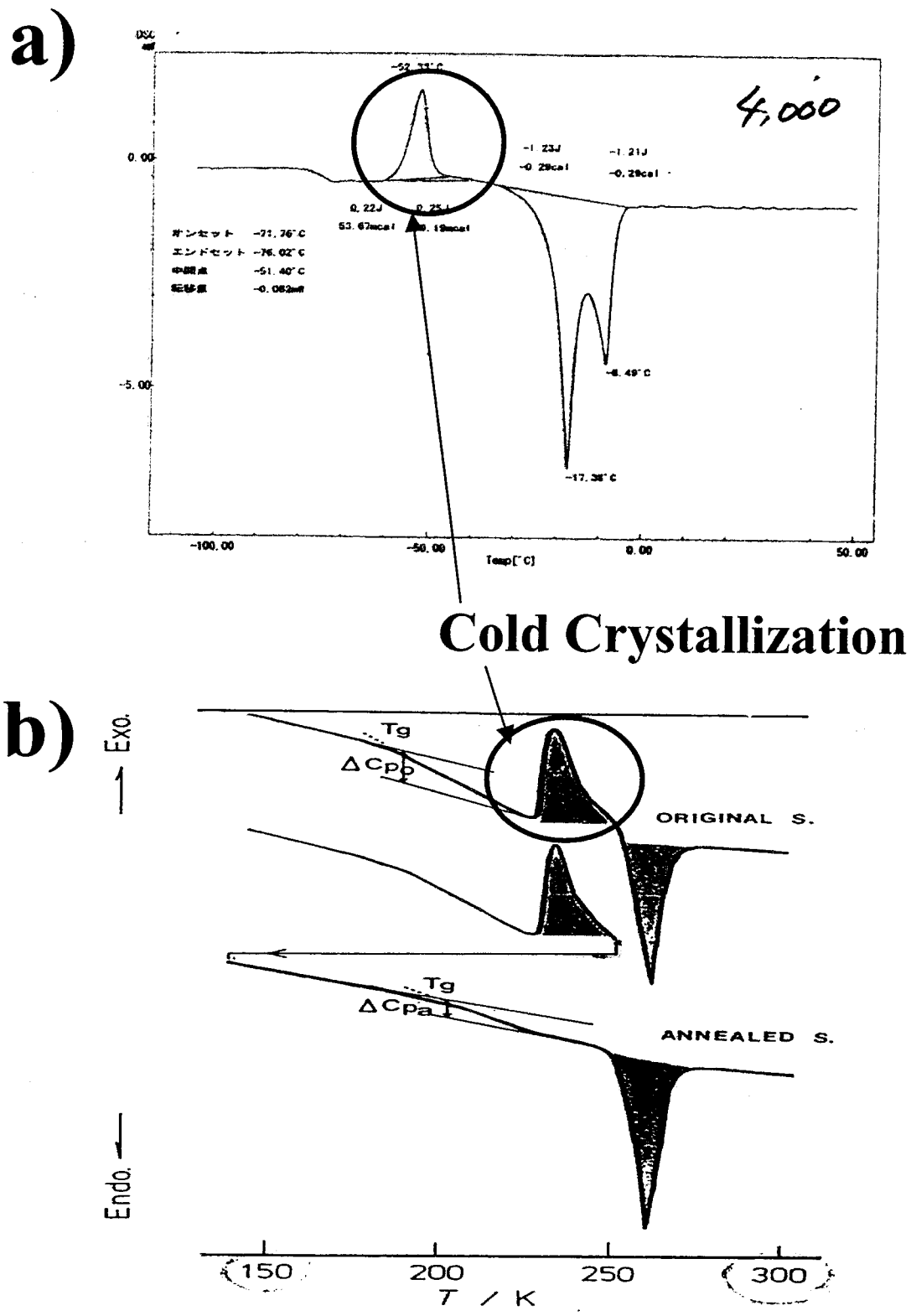


Figure 5-19. DSC heating curves for (a) PEG- and (b) polysaccharide-water systems.

water layer on the polymer surface. This speculation will be supported by the fact that the plasma proteins adsorbed onto the PMEA surface have the large adsorption/desorption rates and the small denaturation degree compared with those onto the surfaces of PHEMA and other acrylate polymers (21). It is important that the polymer surface has a sufficient amount of freezing bound water compared with the amount of the non-freezing water in order to prevent protein and cell from their recognizing the polymer surface or the non-freezing water layer as foreign substances. Thus, as PMEA and the copolymer with 0-20 mol% of HEMA have the freezing bound water as a major structured water excellent blood compatibility is expressed.

Our hypothesis, described above, may be supported by other reports that poly(ethylene glycol), gelatin and some polysaccharides which are well-known as good biomaterials have cold crystallizable water (27, 34-38). Figure **5-19** shows DSC heating curves for PEG- and polysaccharide-water systems. A large and distinct exothermic peak was observed for the samples.

(b) PMEA analogous poly(meth)acrylate

The effects of the physico-chemical properties of the polymers on the platelet compatibility are discussed. **Figure 5-18** indicates that the basic property of the polymer, hydrophilicity/hydrophobicity, has little effect on the compatibility. Moreover these results do not indicate that the chemical structure of PMEA is effective for the compatibility because both PMEA and PMEMA show the quite different compatibility though they have the same methoxyethyl group in the ester side chain of the monomer unit.

Next, from the viewpoint of quantitative properties related to water, (EWC, the types of water and their content in the polymer) the platelet compatibility is described. As described earlier, we revealed that water structure on the polymer surface is related to the

amount of the bound water in the polymer determined by DSC. Focusing our attention on the bound water, we will discuss the platelet compatibility of the polymer. Before this main subject, the relationship of free water with the compatibility is mentioned briefly. As free water in the polymer does not have good correlation with the contact angle (surface structure), one cannot predict that it is valuable to link them to the platelet compatibility. Surely the results shown in **Figure 20-a** indicate that there is no relationship between free water and platelet activation. Next, the main issue is described. The number of the adhered platelet on the polymer was plotted against the content of the bound water, which is shown in **Figure 20-b.** This result indicates that the platelet compatibility is not interdependent on the amount of the bound water. Considering that the bound water is non-freezing water in the polymers except PMEA, one can conclude that both non-freezing water and the polymer surface are involved with the activation of platelet. The activation by non-freezing water will be proved by the following. In the cases of PHEMA and PHEA the number of water molecule bounded to the monomer unit of the polymer is estimated to be in the range of 2-3 from Wnf and molecular weight of the monomer unit, and thus the surfaces of the polymers is expected to be almost covered with non-freezing water. Moreover, it is found that there is no relationship between the platelet compatibility of the polymer and which water is predominant in the polymer. As mention earlier, the polymers except PMEA are classified into two type by the main water. When PMEMA is compared with PEA, their platelet compatibilities are in the same level though the types of the main water in the polymers are different. Therefore, it is important to pay attention to the difference of the water structure on the polymer surface between PMEA and other polymers. The difference is that the main bound water in PMEA is freezing bound water while the others have only non-freezing water.

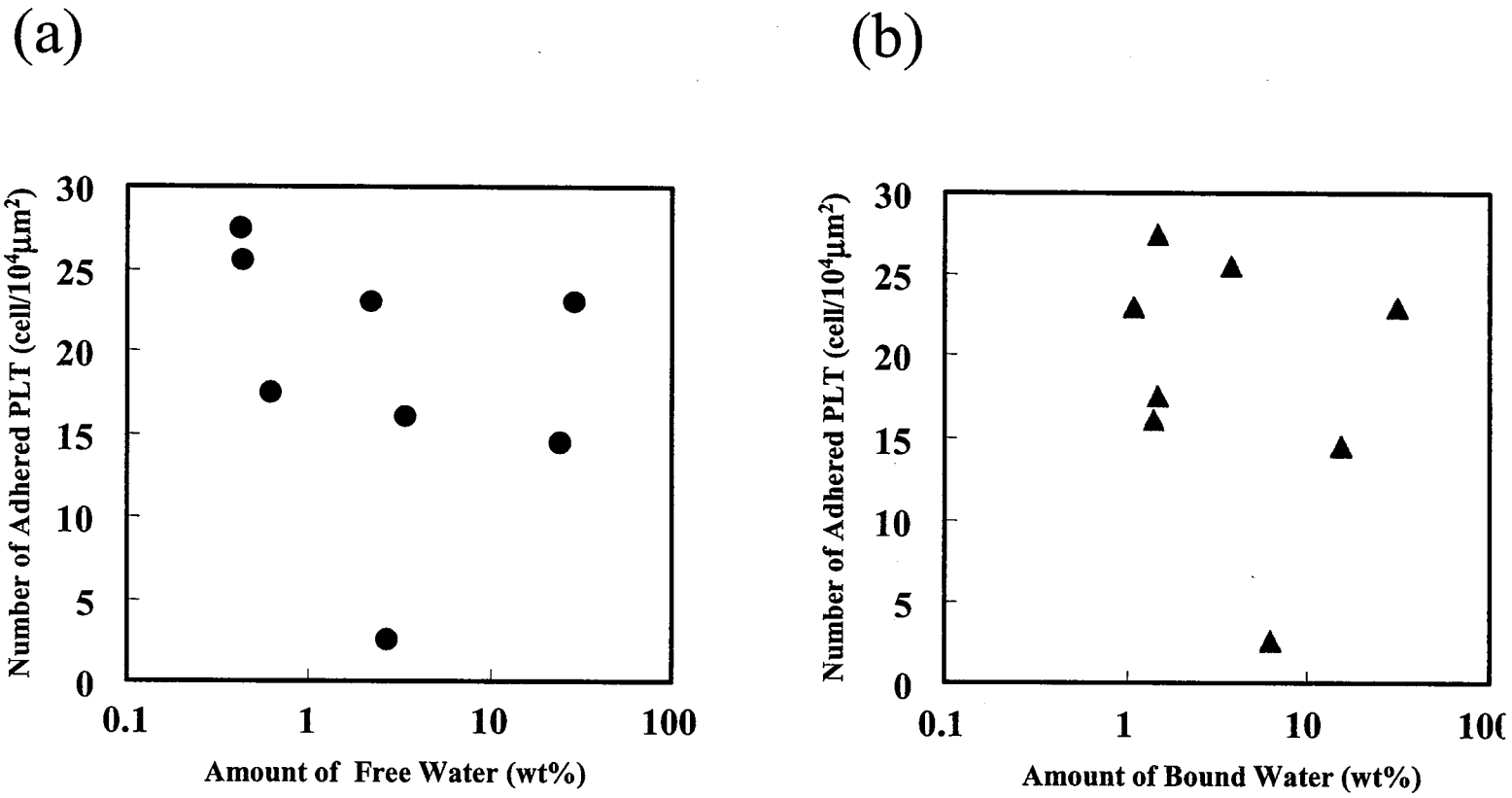


Figure 5-20. Effect of water type on number of adhered platelet.
a) free water, b) bound water (non-freezing water + freezing bound water)

In previous work we investigated the platelet compatibility of poly(MEA-co-HEMA) and the water structure by using DSC, and found that the compatibility worsend with a decrease in the content of freezing bound water or with an increase in the content of non-freezing water in the copolymer (67). In the work, however, we could not clarify which water affected the platelet compatibility.

By combining the results obtained from the study of the copolymer and the results from the present work, we can conclude that freezing bound water affects the blood compatibility. Namely, the worsening of platelet compatibility of poly(MEA-co-HEMA) with an increase of HEMA content is not attributed to the increase of non-freezing water but attributed to the decrease of the freezing bound water. On the basis of these discussion we propose the mechanism for the expression of excellent blood compatibility of PMEA below. The interactions of the proteins or the membrane of the cells relating to defensive response of the living system with non-freezing water on the polymer surface and/or with the polymer surface affect strongly the structure of the protein and the membrane, resulting in activating the bio-components. Therefore, the important factor to express the excellent blood compatibility is how to prevent the bio-components from contacting them directly. When the polymer surface contacts with blood, first of all the polymer surface will absorb water, and a specific structure of water will be formed on the surface. Supposed that the structure formed is layer one, the layers will be in the following order; polymer surface → non-freezing water → (freezing bound water) → free water → bulk water. When freezing bound water layer has enough thickness, it prevents the cell or the protein from contacting directly the polymer surface or non-freezing water. Freezing bound water is weakly bounded to the polymer molecule or non-freezing water, so that this layer will make a rather stable structure compared with that of free water (**Figure 5-21**). We speculated

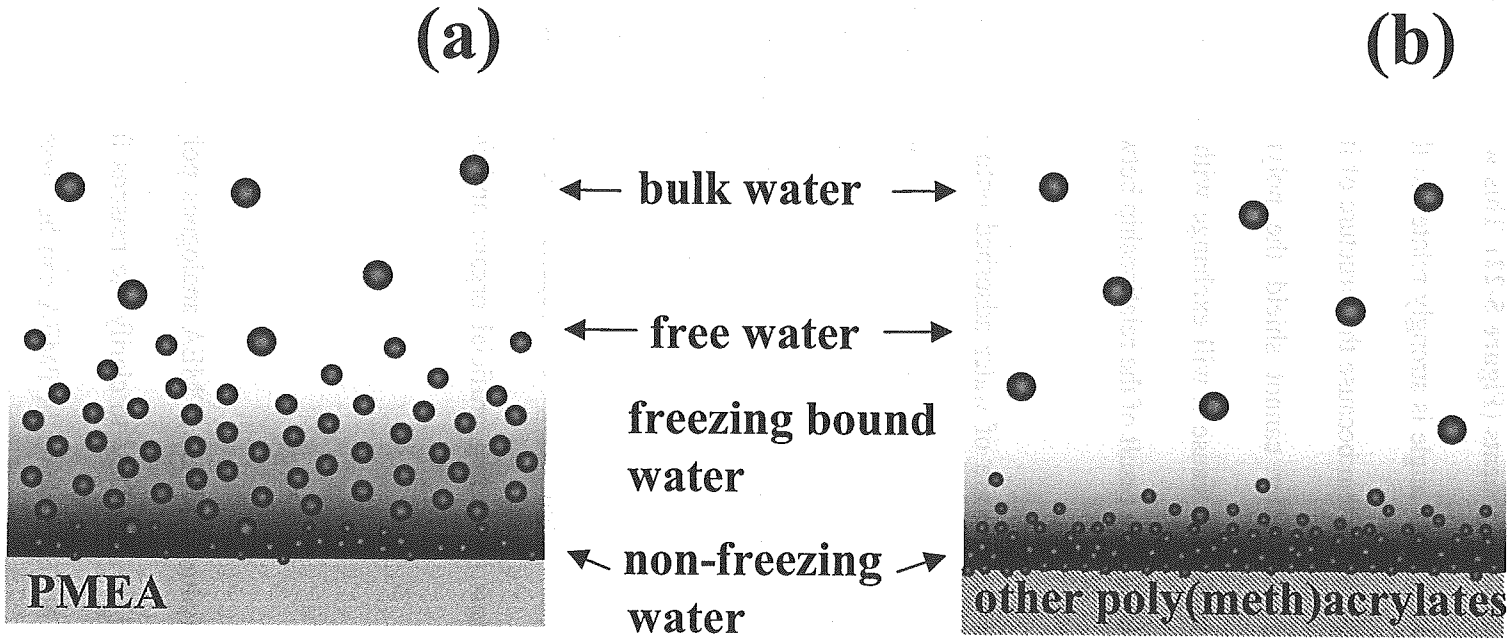


Figure 5-21. Imaginative water state on polymer.
(a) PMEA, (b) other poly(meth)acrylates.

that the changes in the structure or the mobility in freezing bound water is small and does not bring about the activation of the bio-components (**Figure 5-22**). This will be supported by the above-mentioned result that the contact angle is strongly related to the bound water. As for free water it will never activate the system because the structure of it will be similar to that of bulk water. However, free water cannot shield the polymer surface or non-freezing water on the polymer surface, because it will exchange with the bulk water very freely. This will be supported also by the result of the relationship between the contact angle and free water.

More detailed investigations such as the state of water adsorbed onto PMEA surface, mobilities of PMEA molecule and water in PMEA by NMR are now in progress, and I will investigate the multi-layer structure of biomolecules organaized at the living cell surface by a two-photon laser-scanning microscope combined with infrared-femtosecond laser (**Figure 5-23**). The final goal of this research is to establish the key role in blood compatibility of PMEA and molecular design of novel biomaterials. I also plan to design of novel biocompatible polymers for tissue engineering in regenerative medicine and minimam-invasive medical instruments (including artificial organs and implant devices) (see next chapter).

5-4 Conclusion

The structure of water in the hydrated PMEA, PMEA analogous poly(meth)acrylate and p(MEA-co-HEMA) were investigated by DSC to clarify the reasons for the excellent blood compatibility of PMEA. The hydrated water in PMEA can be classified into three types, non-freezing water, freezing bound water, and free water on the basis of the equilibrium water content (EWC) and the enthalpy changes due to the phase transition

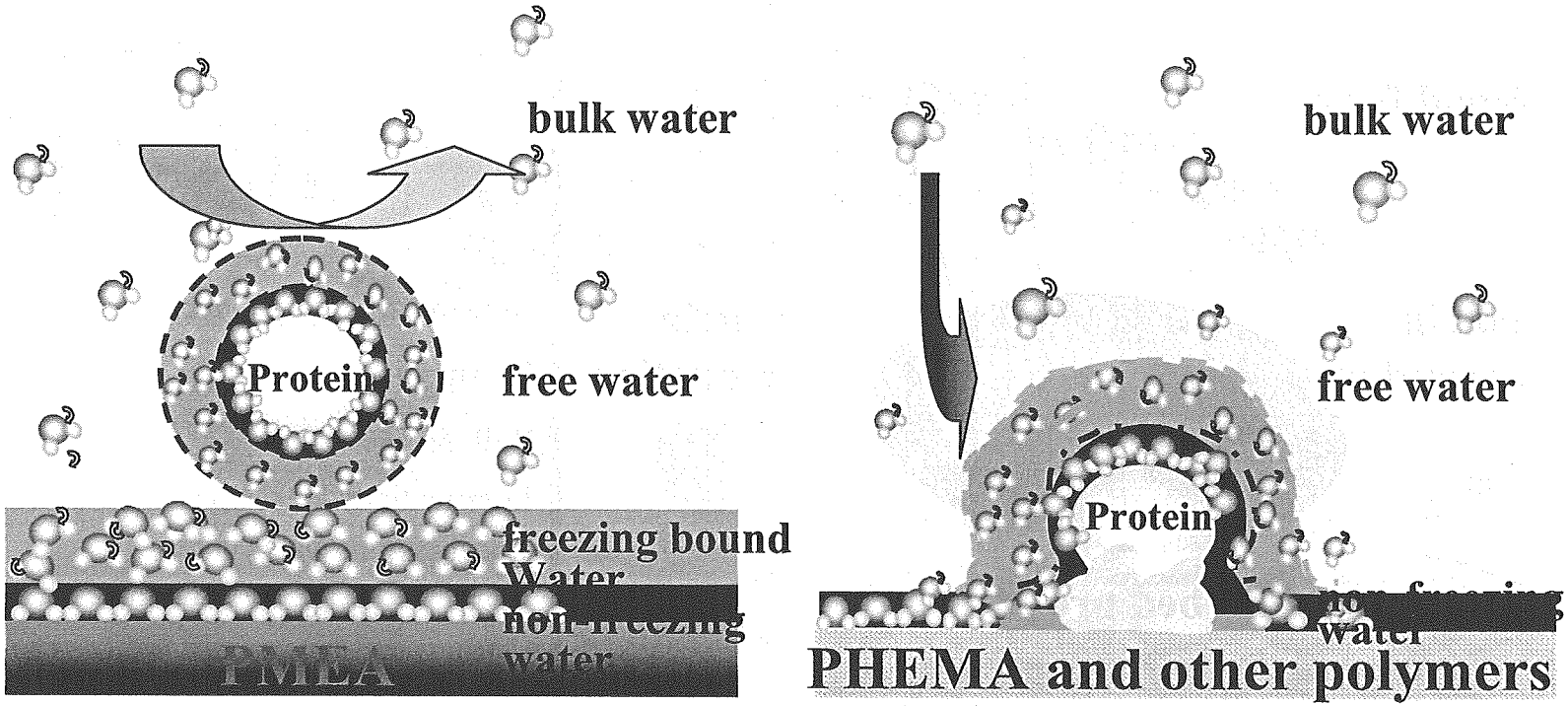


Figure 5-22. Schematic representation of the assumed bio-interface. The factor preventing the blood-components from contacting non-freezing water directly is one of the important procedure for the polymer to express the excellent blood compatibility is how. If freezing bound water layer exists with enough thickness, the contact can be avoided. Freezing bound water weakly interacts with non-freezing water, so that this layer can exist more stable than free water layer. Moreover, as the net-work structure or the mobility of freezing bound water is in between that of non-freezing water and that of free water, it will not bring about the activation of the bio-components. As for free water it will never activate the system because the structure of it will be similar to that of bulk water. However, free water cannot shield the non-freezing water on the polymer surface, because it will exchange with bulk water very freely.

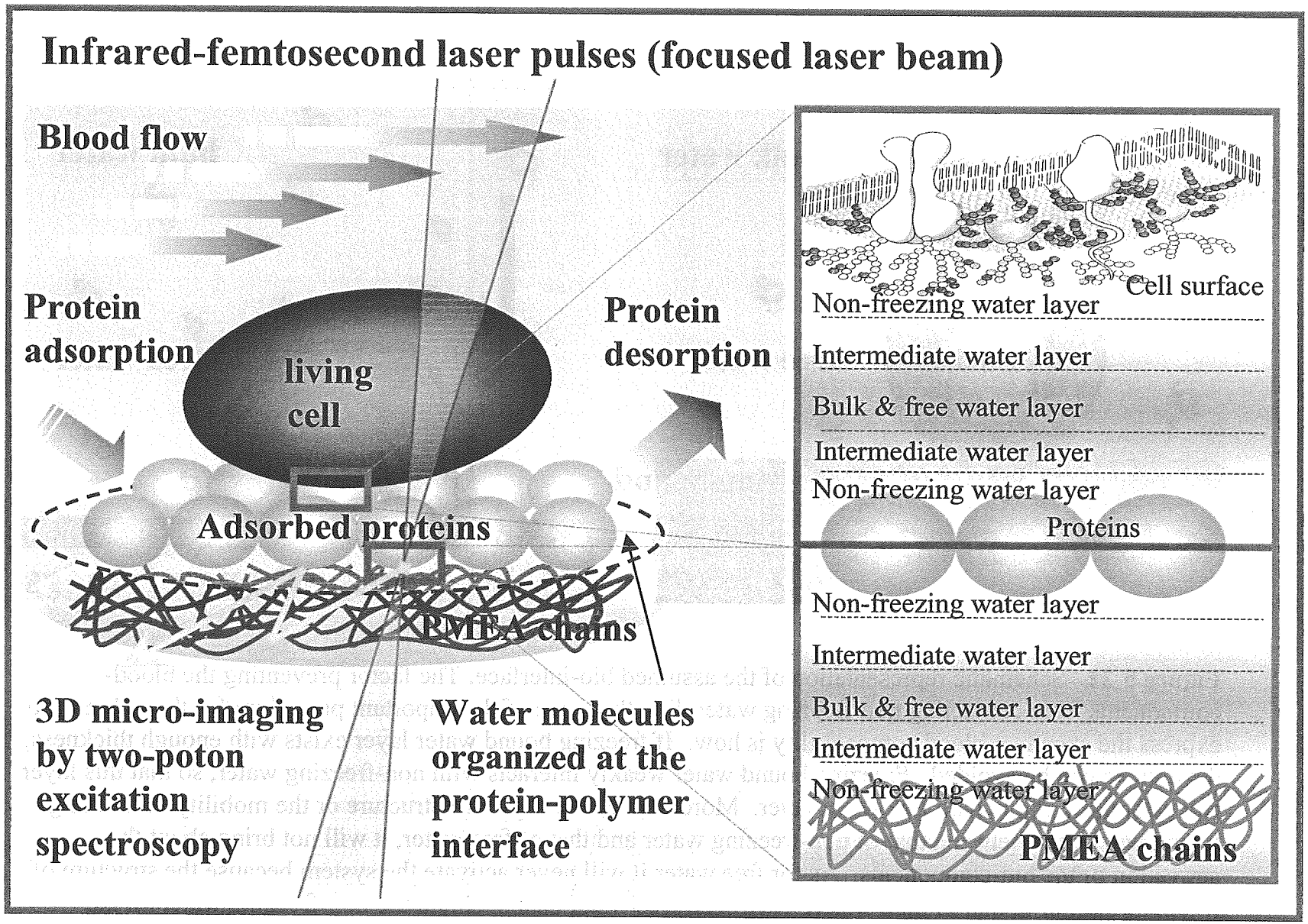


Figure 5-23. Schematic representation of the assumed bio-interface.

observed by DSC.

Cold crystallization of water in heating process was clearly observed at ca. -42°C when the water content is more than 3.0 wt %. The cold crystallizable water will be assigned to the *W*fb. At the EWC, the relative proportions of *W*fb is 48% of all the water in hydrated PMEA. In this chapter, we focused our investigation on the relationship between the structure of water in the hydrated polymer and the adhesion of platelet onto the polymer surfaces, where the polymers used were PMEA, poly(MEA-co-HEMA), and PHEMA. EWC of the polymers increased with an increase in the HEMA content in the polymer from 9 (for PMEA) to 40 wt% (for PHEMA). The typical feature of the hydrated polymers is that the cold crystallization peak appeared clearly in the DSC curve for polymers with 0-30mol % of HEMA. This cold crystallizable water was assigned to the freezing bound water, and was major water in the polymers with 0-20 mol% of HEMA. On the other hand, the polymers with 30-100 mol% of HEMA had non-freezing water as major water. In evaluation of the platelet compatibility, the polymers with 0-20 mol% of HEMA showed good performance in terms of the adhesion number and its morphology, while the polymers with 40-100 mol% of HEMA showed poor compatibility. In the polymer with 30 mol% of HEMA, the slight activation of platelet was observed. In addition, The PMEA analogous polymers investigated have typical functional group as ester side chain such as methoxyethyl, hydroxyethyl, phenoxyethyl, and alkyl groups. The polymers could be classified by EWC or the contact angle of water into three types; hydrophobic, hydrophilic, and intermediate hydrrophilic polymers. This property was governed by the functional group. The water structure in PMEA defined by DSC was quite different from those of other polymers. PMEA had three types of water: free water, freezing bound water, and non-freezing water while the analogous polymers had just two types: free and non-freezing

waters regardless of the chemical structure of the side chain. The amount ratios of these waters in the polymers did not depend on only the chemical structure of the side ester chain. It was found that the contact angle related to the content of the bound water (non-freezing water + freezing bound water), which indicated that the surface property was strongly affected by the amount of the bound water. The platelet compatibility of the polymer in *in vitro* test did not relate to free water or the bound water. We conclude that the amount of the freezing bound water relates to the platelet compatibility of the polymer and the excellent compatibility is expressed by freezing bound water, which prevents the biocomponents from contacting the polymer surface or non-freezing water on the polymer surface.

5-5 References

(1) Horbett, T. A. *Biomaterials Science; An introduction to materials in medicine*. Ratner, B. D, Hoffman A. S.; Schoen, F. J.; Lemons, J. E., Eds. Academic Press: London, 1996; pp133.

(2) Ratner, B. D.; Hoffman, A. S. *Hydrogels for Medical and Related Applications*. Andrade JD, Ed, ACS Symposium Series 31, American Chemical Society, Washington, D.C., 1976; pp1.

(3) Peppas, N. A. Ed. *Hydrogel in Medicine and Pharmacy, Vol.2*, CRC Press, Boca Raton, FL, 1987.

(4) Mori, Y.; Nagaoka, S.; Takiguchi T, Kikuchi, T, Noguchi N, Tanzawa, H.; Noishiki, Y. A new antithrombogenic material with long polyethylene oxide chains, *Trans. ASAIO*. **1982**, *28,* 459-463.

(5) Kulik, E.; Ikada, Y. *In vitro* platelet adhesion to nonionic and ionic hydrogels with different water contents., *J. Biomed. Mater. Res*. **1996**, *30*, 295-304.

(6) Morra, M. On the molecular basis of fouling resistance, *J. Biomater. Sci. Polymer Edn.* **2000**, *11*, 547-569.

(7) Ishihara, K , Ueda, T.; Nakabayashi, Y. Preparation of phospholipids polymers and their properities as hydrogel membrane. *Polym. J.* **1990**, *22*, 355-360.

(8) Iwasaki, Y.; Aiba, Y.; Morimoto, N.; Nakabayashi, Y.; Ishihara, K. Semi-interpenetrating polymer networks composed of biocompatible phospholipids polymer and segmented polyurethane. *J. Biomed. Mater. Res.* **2000**, *52*, 701-708.

(9) Okano, T.; Nishiyama, S.; Shinohara, I.;, Akaike, T.; Sakurai, Y.; Kataoka, K.; Tsuruta, T. Effect of hydrophilic and hydrophobic microdomains on mode of interaction between block copolymer and blob platelets, *J. Biomed. Mater. Res.* **1981**, *15*, 393-402.

(10) Yui, N.; Sanui, K.; Ogata, N.; Kataoka, K.; Okano, T.; Sakurai, Y. Effect of microstructure of poly(propylene-oxide)-segmented polyamides on platelet adhesion, *J. Biomed. Mater. Res.* **1986**, *20*, 929 -943.

(11) Andrade JD, ed, *Principles of protein adsorption. in surface and interfacial aspects of biomedical polymers,* Plenum Publ., New York , 1 (1985)

(12) Rowland SP, ed, *Water in polymer*, ACS Symposium Series 127, American Chemical Society, Washington, DC (1980)

(13) Ratner BD, Horbett TA, Hoffman AS, Hauschka SD, Cell adhesion to polymeric materials: implications with respect to biocompatibility. *J Biomed Mater Res* **9**:407-422 (1975)

(14)Tsuruta T. Contemporary topics in polymeric materials for biomedical applications. *Adv Polym Sci,* **126**:1-51 (1996)

(15) Vogler, E. A., Water and acute biological response to surface, *J. Biomater. Sci. Polym. Ed.* **1999**, *10*, 1015-.1045.

(16) Vogler, E. A. Structure and reactivity of water at biomaterial surface. *Adv. Colloid Interface Sci.* **1998**, *74*, 69-117.

(17) Sung YK, Gregonis DE, John MS, Andrade JD, Thermal and pulse NMR analysis of water in poly(2-hydroxyethyl methacrylate). *J Appl Polym Sci* **26**:3719-3728 (1981)

(18) Wolfgang GG, Hatakeyama H, ed, *Viscoelasticity of Biomaterials*, ACS Symposium Series **489**, American Chemical Society, Washington, DC (1992)

(19) Lee HB, John MS, Andrade JD, Nature of water in synthetic hydrogels, *J Colloid Interface Sci*, **51**: 225-231 (1975)

(20) Andrade JD, Lee HB, Jhon MS, Kim SW, Hibbs JB, Water as a biomaterial, *Trans ASAIO*, **19**:1-7 (1973)

(21)Tanaka M, Motomura T, Kawada M, Anzai T, Kasori Y, Onishi M, Shiroya T, Shimura K, Mochizuki A, Blood compatible aspects of Poly(2-methoxyethylacrylate) (PMEA) -Relationship between protein adsorption and platelet adhesion on PMEA surface-, *Biomaterials*, **21**:1471 (2000)

(22)Tanaka M, Motomura T, Kawada M, Anzai T, Kasori Y, Shimura K, Onishi M, Mochizuki A, Okahata Y, A new blood-compatible surface prepared by Poly(2-methoxyethylacrylate) (PMEA)coating -Protein adsorption on PMEA surface-, *Jpn J Artif Organs*, **9**:209 (2000)

(23)Anzai T, Okumura A, Kawaura M, Yokoyma K, Oshiyama H, Kido T, Nojiri C, Evaluation of the biocompatibility of an in vitro test using a poly(2-methoxyathyacrylate) coated oxygenators. *Jpn J Artif Organs*, **9**:73 (2000)

(24)Tanaka M, Motomura T, Kawada M, Anzai T, Kasori Y, Shimura K, Onishi M, Mochizuki A, Okahata Y, Biocompatible aspects of Poly(2-methoxyethylacrylate) (PMEA) -The relationship among conformational change of adsorbed protein and platelet

adhesion on PMEA-, *Recent Advances in Enviromentally Compatible Polymer*, J.F.Kennedy, G.O.Phillips, P.A.Williams and H. Hatakeyma eds., Woodhead Publishing Ltd., Cambridge, U.K., 405-410, 2001.

(25)Saito N, Motoyama S, Sawamoto J, Effects of new polymer-coated extracorporeal circuits on biocompatibility during cardiopulmonary bypass. Artif Organs 2000 Jul;24(7):547-54

(26)Ling G.N. Zhang Z.L *Physiol Chem Phys*, Studies on the physical state of water in living cells and model systems. V., **15**: 407-415 (1983)

(27)Hatakeyama H, Hatakeyama T, Interaction between water and hydrophilic polymers., *Thermochim. Acta*, **308**:3 -22(1995)

(28) Aizawa, M.; Suzuki, S. Properties of water in macromolecular gels. III. Dilatometric studies of the properties of water in macromolecular gels, *Bull. Chem. Soc. Jpn.* **1971**, *44*, 2967-2971.

(29)Jhon, M. S.; Andrade, J. D. Water and hydrogels. *J. Biomed. Mater. Res* . **1973**, *7*, 509-522.

(30)Y.K. Sung, D.G.Gregonis, M.S.John, Andrade, J. D., Thermal and puls NMR analysis of water in poly(2-hydroxyethyl methacrylate), *J. Appl. Polym. Sci.*, 26,3719-3728,1981.

(31)Philip HC, Alan MJ, Chiong ON, Brian JT, Synthetic hydrogels : 1. Hydroxyalkul acrylate and mrthacrylate copolymers-water binding studies, *Polymer*, **28**, 1758-1766, 1987.

(32)A. Higuchi, T. Iijima, DSC investigation of states of water in poly(vinyl alchol) membranes, *Polymer*, 26, 1207-1211, 1985.

(33)P.E.M. Allen, D.J.Bennett, D.R.G.Williams, Water in methacrylates-IV. Structures and organization in poly(2-hydroxyethyl methacrylate-co-grycol dimethacrylate) networks, *Eur. Polym. J.*, 29, 231-236,1993.

(34)R. M. Hodge, Graham H Edward, G. P. Simon, Water adsorption and states of water in semicrystalline poly(vinyl alchohol) films, *Polymer*, 37,1371-1376, 1996.

(35)Harris JM, ed, *Poly(ethylene glycol) chemistry, Biotechnical and Biomedical Applications* Plenum Press, New York (1992).

(36)L. Hang, K. Nishinari, Interaction between poly(ethylene glycol) and water as studied by differential scanning calorimetry, *J. Polym. Sci. B : Polym Phys*, 39, 496-506, 2001.

(37)Nishinari K, Watase M, Hatakeyama T, Effects of polyols and sugars on the structure of water in concentrated gelatin gels as studied by low temperature differential scanning calorimetry, *Colloid Polym Sci* **275**:1078-1082 (1997)

(38)J. Ratto, T. Hatakeyama, R.B. Blumstein, Differential scanning calorimetry investigation of phase transition in water / chitosan systems, *Polymer*, 36, 2915-2919, 1995.

(39)A.A. Apostolov, S. Fakirov, E. Vassileva, R.D. Patil, J.E. Mark, DSC and TGA studies of behavior of water in native and crosslinked gelatin, *J. Appl. Polym. Sci.*, 71, 465-470,1999.

(40) Andrade JD, ed, *Hydrogels for Medical and Related Applications*. ACS Symposium Series **31**, American Chemical Society, Washington, DC (1976).

(41) Suhara, H.; Sawa, Y.; Nishimura, M.; Oshiyama, H.; Yokoyama, K.; Saito, N.; Matsuda, H Efficacy of a new coating material, PMEA, for cardiopulmonary bypass circuits in a porcine model. *Annals Thoracic Surgery* 2001, *71*, 19703

(42) S.D. Bruck, Aspect of three types of hydrogels for biomedical applications, *J. Biomed. Mater. Res.* **1973**, *7*,387-404.

(43)Andrade JD, Lee HB, Jhon MS, Kim SW, Hibbs JB, Water as a biomaterial, *Trans ASAIO*, **19**:1 (1973)

(44)B.D.Ratner, A.S Hoffman, S.R.Hanson, L.A.Harker, J.D.Whiffen, Blood

compatibility-water content relationships for radiation-grafted hydrogels, *J. Polym. Sci.* 66, 363-375, 1979.

(45)Tanzawa, H. Blood compatibility of synthetic hydrogels, *Jpn. J. Artif. Organs* **1986**, *15*, 16-18.

(46)Yamada, N. A.; Ishikiriyama, K.; Todoki, M.; Tanzawa, H. ^{1}H-NMR studies on water in methacrylate hydrogels. I, *J. Appl. Polym. Sci.* **1990**, *39*, 2443-2452.

(47)Yamada, N. A.; Tanzawa, H. ^{1}H-NMR studies on water in methacrylate hydrogels. II, *J. Appl. Polym. Sci.* **1991**, *43*, 1165-1170.

(48)D.R.Lu, S.J.Lee, K, Park, Calculation of salvation interaction energies for protein adsorption on polymer surfaces, *J. Biomater. Sci. Polym. Ed.* **1991**, 2, 127-147.

(49)Israelachivili, J.; Wennerstrom, H. Role of hydration and water structure in biological and colloidal interactions, *Nature,* **1996**, *379*, 219-225.

(50)Dequeiroz, A. A. A.; Barrak, E. R.; Decastro, S. C. Thermodynamics analisis of the surface of biomaterials. *J. Mol. Struct. (THEOCHEM)* **1997**, *394*, 271.

(51)N. Kawamoto, H. Mori, N, Yui, M. Terano, Mechanistic aspects of blood –contacting properties of polyplropylene surfaces-from the viewpoint of macromolecular entanglement and hydrophobic interaction via water molecules, *J. Biomater. Sci. Polym. Ed.* **1998**,9, 543-559.

(52)Wang, R. L. C.; Kreuzer, H. J.; Grunze, M.; Molecular conformation and salvation of oligo(ethylene glycole)-terminated self-assembled monolayers and their resistance to protein adsorption. *J. Phys. Chem.* B **1997**, *101*, 9767-9773.

(53)Harder, P. Grunze. M.; Dahint, R.; Whitesides, G. M.; Laibins, P. E. Molecular conformation in oligo(ethylene glycole)-terminated self-assembled monolayers on gold and silver surfaces determines their ability to resist protein adsorption. *J. Phys. Chem.* B **1998**,

102, 426-436.

(54)Feldman, K.; Hahner, G.; Spencer, N. D.; Harder, P.; Grunze, M. Probing resistance to protein adsorption on oligo(ethylene glycole)-terminated self-assembled monolayers by scanning force microscopy. *J. Am. Chem. Soc.* **1999**, *121*, 10134.

(55)D. Schwendel, R. Dahint, S. Herrwerth, M. Schloerholz, W. Eck, M. Grunze, Temperature dependence of the protein resistance of poly- and oligo(ethylene glycole)-terminated self-assembled monolayers., *Langmuir*, 17, 5717-5720, 2001.

(56)R.G. Chapman, E. Ostumi, S. Takayama, R.E. Holmlin, L. Yan, G.M. Whitesides, Surveying for surfaces that resist the adsorption of proteins, *J. Am. Chem. Soc.* **2000**, *122*, 8303-8304.

(57)Kataoka, K.; Ito, H.; Amano, H.; Nagasaki, Y.; Kato, M.; Tsuruta, T.; Suzuki, K.; Okano, T.; Sakurai, Y. Minimized platelet interaction with poly(2-hydroxyethyl mathacrylate-block 4-bis(trimethylsililyl)methylstyrene) hydrogel showing anomalously high free water content. *Biomater. Sci. Polym. Ed.* **1998**, *9*, 111-129.

(58)Kikuchi, A.; Karasawa. M.; Tsuruta, T.; Kataoka, K. Differential affinity of lymphocyte subpopulations toward PHEMA surface derivatized with a small amont of amino groups-evaluation under the regulated shear stress, *J. Colloid Interface Sci.* **1993**, *158*, 10-18.

(59)Iwasaki, Y.; Fujiike, A.; Kurita, K.; Ishihara, K.; Nakabayashi, N. Protein adsorption and platelet adhesion on polymer surfaces having phospholipid polar group connected with oxyethylene chain. *J. Biomater. Sci. Polym. Ed.* **1996**, *8*, 91-102.

(60)Ishihara K, Nomura H, Mihara T, Kurita K, Iwasaki Y, Nakabayashi N. Why do phospholipid polymers reduce protein adsorption?. *J Biomed Mater Res* 1998;39:323-330

(61) Ishihara K, Ishikawa E, Iwasaki Y, Nakabayashi N. Inhibition of fibroblast cell

adhesion on substrate by coating with 2-methacryloyloxyethyl phosphorylcholine polymers. *J.Biomater.Sci.,Polymer Edn*, 10(10)1047-1061 (1999)

(62)Hazlewood, C.F.; Nichols. B. L.; Chamberlain, N. F. Evidence for the existence of a minimum of two phases of ordered water in skeletal muscle. *Nature*, **1969**, *222*, 747-750.

(63) Kuntz ID Jr, Brassfield TS, Law GD, Purcell GV, Hydration of macromolecules., *Science* 1969, 21;163(873):1329-31

(64) Kuntz ID Jr, Kauzmann W., Hydration of proteins and polypeptides., *Adv Protein Chem* 1974;28:239-345.

(65) Cooke R, Kuntz ID. The properties of water in biological systems., *Annu Rev Biophys Bioeng* 1974;3(0):95-126

(66) Uedaira, H. Water in biological systems, in Water and metal cations in biological systems, Pullman, B.; Yagi. K. Eds, Japan Scientific Societies Press, Tokyo, 1980, pp47-56.

(67) Pal SK, Peon J, Zewail AH., Biological water at the protein surface: dynamical solvation probed directly with femtosecond resolution. Pal SK, Peon J, Zewail AH. Proc Natl Acad Sci U S A 2002 Feb 19;99(4):1763-8

(68) Tanaka M, Mochizuki A, Ishii N, Motomura T, Hatakeyama T : Study on Blood Compatibility of Poly(2-Methoxyethylacrylate) －Relationship between Water Structure andPlatelet compatibility in Poly(2-Methoxyethylacrylate-co-2-Hydroxyethyle methacrylate). *Biomacromolecules* 2002, 3 : 36-41.

Chapter 7 Conclusion

Polymers are widely used for a large range of medical devices used as biomaterials on a temporary, intermittent, and long-term basis. Blood compatibility of materials is an important factor in the development of medical devices. We have reported that poly(2-methoxyethyl acrylate) (PMEA) shows excellent blood compatibility with respect to coagulation, complement, and platelet systems when compared with other polymers. In addition, we have revealed that the amount of protein adsorbed onto its surface is very small, and that the denaturation of adsorbed proteins is much lower than those on the surfaces of other conventional polymer surfaces. To search the mechanism of the excellent blood compatibility, we have focused our attention on water structure and investigated the structures in PMEA and other polymers. On the basis of these results the mechanism of the expression of the excellent blood compatibility was discussed.

In **Chapter 2**, we reported the excellent blood compatibility of PMEA by estimating the early stage reactions of platelet (platelet adhesion test and β-TG), leukocyte, complement system, and coagulation system which were induced by the contact of PMEA surface with human blood for 30-60 min in vitro. We showed that PMEA had a possibility of applying PMEA to clinical usage. That is, they coated an oxygenator with PMEA and evaluated the blood compatibility under the circulation of human blood for 6 h in terms of the activation of platelet (retention of the number and β-TG), coagulation system (bradykinin and TAT), and leukocyte (retention of the number, and PMN-elastase). After the circulation, they analyzed the adsorbed proteins on the oxygenator and concluded that the surface of the oxygenator had significantly small amount of proteins compared with the non-coated one.

In **Chapter 3**, to clarify the reason for the suppression of platelet adhesion and

spreading of PMEA, the relationship among the amount of the plasma protein adsorbed onto PMEA, its secondary structure and platelet adhesion was investigated. Poly(2-hydroxyethylmethacrylate) (PHEMA) and polyacrylate analogous were used as references. The amount of protein adsorbed onto PMEA was very low and similar to that absorbed onto PHEMA. Circular dichroism (CD) spectroscopy was applied to examine changes in the secondary structure of the proteins after adsorption onto the polymer surface. The conformation of the proteins adsorbed onto PHEMA changed considerably, but that of proteins adsorbed onto PMEA differed only a little from the native one. These results suggest that low platelet adhesion and spreading are closely related to the low degree of the denaturation of the protein adsorbed onto PMEA.

In **Chapter 4**, the adsorption behaviors of bovine serum albumin (BSA) and human fibrinogen onto the surfaces of PMEA, poly(2-hydroxyethyl methacrylate) (PHEMA) and polypropylene (PP) were investigated by using a quartz crystal microbalance (QCM), Both proteins were observed to adsorb onto the all polymer surfaces according to the Langmuir's adsorption isotherm. The maximum adsorption amounts and the apparent association constants of the proteins for PMEA obtained from the isotherm were lower than those for PHEMA and PP. These results suggest that the interaction between PMEA and the proteins is weaker than the interaction appearing on PP and PHEMA. The characteristic of PMEA is that its detachment rate constant k_{-1} was higher than those from poly(2-hydroxyethylmethacrylate) (PHEMA) and polypropylene (PP) which were used as references. The degree of the conformational changes of the proteins decreases in the following order; PP>PHEMA>>PMEA. This was strongly related to the difference of the desorption rate constant k_{-1}.

In **Chapter 5**, the water structures in PMEA and PMEA analogous polymers were

compared on the basis of results of contact angle, equilibrium water content (EWC), and DSC to clarify the reasons for the excellent blood compatibility of PMEA. The water structure in PMEA defined by DSC was quite different from those of other polymers. PMEA had three types of water: free water, freezing bound water, and non-freezing water while the analogous polymers had just two types: free and non-freezing waters regardless of the chemical structure of the side chain. The amount ratios of these waters in the polymers did not depend on only the chemical structure of the side ester chain. It was found that the contact angle related to the content of the bound water (non-freezing water + freezing bound water), which indicated that the surface property was strongly affected by the amount of the bound water. The platelet compatibility of the polymer in *in vitro* test did not relate to free water or the bound water. We conclude that the excellent compatibility is expressed by freezing bound water, which prevents the biocomponents from contacting the polymer surface or non-freezing water on the polymer surface.

Chapter 6 is conclusions. These data are promising for the design of new biomaterial surfaces. In addition, further knowledge of how protein and cell interact with biomaterials will stimulate the development of better implants to avoid biomaterial-associated infections. Finally, systematic interdisciplinary research by physicians and biomaterial scientists is essential for the successful development of new materials with improved biocompatibility.

Acknowledgments

The present investigation was carried out under the Research and Development Center, Terumo Corporation. I would like to extend my thanks to all members in Terumo Corporation for accepting my study of basic biomaterial science in the research and development center. We shared precious period for even good or bad. I will never forget their gratitude to Dr. Akira Mochizuki, who has provided enduring support for many years.

I would like to express my deepest gratitude to Professor Masatsugu Shimomura, who has been my adviser at the Research Institute for Electronic Science (RIES), Hokkaido University in the last two and a half years of my study of interdisciplinary fields. I also would like to express my deepest appreciation to Associate Professor Kuniharu Ijiro in RIES, who showed a how to be a researcher in my graduate days, which is until now my basis to be a researcher.

In preparing this thesis, I have benefited from questions, suggestions, criticism, and advice from Professor Kohei Uosaki and Professor Michio Yazawa (both in Hokkaido University) and Yoshio Okahata (in Tokyo Institute of Technology). We would like to Yoshio Okahata thank for their help in the QCM experiments. His comments for my study inspired my research and lead to a right solution.

We are grateful to Professor Haruma Kawaguchi and Associate Professor Keiji Fujimoto (both in Keio University) for helpful discussion and support in the use of the CD system. We are very grateful to Professor Emeritus Teiji Tsuruta (in Tokyo University) and Professor Tatsuko Hatakeyama (in Otsuma Women's University) for their valuable advice. I wish to thank all member of Shimomura Laboratory for their warm assistance and cooperation.

Finally, I would like to express my special thanks to my parents, brother and my wife for their constant supports and encouragement.

May, 2003

Masaru Tanaka