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Anti-complement Effects of Anion-Substituted Poly(vinyl alcohol) Membranes

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Abstract: In a continuation of our previous studies on blood compatibility profiles of anion-substituted poly(vinyl alcohol) (PVA) membranes, in which hydroxyl groups have been replaced with carboxymethyl (C-PVA) and sulfonyl groups (S-PVA), we have studied the activation of complement components and the changes in white cell and platelet count *in vitro* and compared them with those of unmodified PVA, Cuprophane, and low-density polyethylene. Complement activation of fluid phase components, C3a, Bb, iC3b, and SC5b-9, and of bound phases, C3c, C3d, and SC5b-9, were assessed by enzyme-linked immunosorbent assay (ELISA) and immunoblot, respectively. The changes in the number of white cells and platelets following complement activation were counted using a Coulter counter. C-PVA and S-PVA activated C3 to a lesser extent than did PVA, which we attribute to the diminished level of surface nucleophiles of the samples. In addition, C- and S-PVA exhibit increased inhibition of Bb production, resulting in a decrease in the extent of C5 activation. Consequently, because of the reduced activation of C3 and C5, C- and S-PVA samples cause marked decreases in the SC5b-9 levels in plasma. We also found that the negatively charged sulfonate and carboxylate groups of the samples cause a greater extent of adsorbtion of the positively charged anaphylatoxins, C3a and C5a, because of strong electrostatic attraction, which in turn provides an inhibition of chemotaxis and activation of leukocytes. The ability to inhibit complement production, together with the binding ability of anaphylatoxins of the C- and S-PVA samples, leads to a prominent decrease in lysis of leukocytes as well as activation of platelets.

Keywords: carboxymethylated poly(vinyl alcohol), sulfonated poly(vinyl alcohol), hemodialysis membrane, complement activation.

Introduction

Renal replacement therapy relies upon the use of the semi-permeable hemodialysis membranes, which vary in chemical composition, transport properties, and biocompatibility.[1] Hemodialysis membranes are fabricated from three classes of materials: regenerated cellulose, modified cellulose, and synthetics.[2] The principal advantage of the regenerated cellulose is low unit cost, complemented by the strength of the highly crystalline cellulose, which allows polymer membranes to be made very thin.[3] In addition, regenerated cellulose has an extremely hydrophilic structure, which enables it to form a true hydrogel when in contact with aqueous media.[4] The highly swollen amorphous regions of regenerated cellulose provide effective small-solute transport in relatively small exchange devices.[1] The drawback of the regenerated cellulose is the presence of the hydroxyl groups that trigger the complement activation and transient leukopenia.[2] In order to minimize a poor blood compatibility profile, numerous approaches have focused on the alteration of the materials structure by the substitution of hydroxyl groups on cellulose to other functional groups.[5,6] Hemophan and cellulose acetate are the typical examples of those synthetically modified cellulose that exhibits improved blood compatibility.

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Taking the membrane performances into account, a cross-linked poly(vinyl alcohol) membrane can be a suitable candidate for the hemodialysis membrane. As a polyhydroxyl compound, poly(vinyl alcohol) membrane provides possible analogies with those of cellulose.[7] Even though, the hydroxyl groups in poly(vinyl alcohol) may activate the whole complement systems in plasma, replacing hydroxyl groups with other types of functional groups can reduce the degree of the complement activation. Our previous studies revealed that anion substituted poly(vinyl alcohol) membranes, carboxymethylated and sulfonated poly(vinyl alcohol) membranes, possess improved anti-coagulation activities, solute permeabilities, and inhibition properties of plasma protein adsorption due to substitutions.[7-10] The aim of this study is to examine the effects of these substitutions on complement activation. The present paper reports the blood compatibility profile of the anion substituted cross-linked poly(vinyl alcohol) membranes characterized by the activation of complement together with the changes in white cell and platelet counts.

Experimental

Preparation of Modified PVA Membrane. The anion-substituted PVA membranes, carboxymethylated PVA (C-PVA) and sulfonated PVA (S-PVA) membranes, with maximum substitution ratio of 25%, were prepared as described in previous works.[8-10] The sheet type Cuprophane (250-PM, Enka, Germany) and low-density polyethylene (LDPE, Abiomed Inc., Danvers, Massachusetts) were used as a positive and negative control, respectively.

Electron Spectroscopy for Chemical Analysis (ESCA).[11-13] The substitution was confirmed using a Physical Electronic PHI 558 ESCA spectrometer. Wide scan (0-800 eV) and high-resolution scans (20 eV wide) for C1s were taken using the source of a 10 kV-30 mA monochromatized X-ray beam from a magnesium anode. In addition, the substitution ratio of C- and S-PVA samples were determined by acid-base titration and sulfur analysis as described in our previous studies.[14]

Hemodialysis. Five units of fresh whole blood were donated from 5 healthy volunteers, and the blood was collected into the 400 mL of blood bag (Green Cross Co. Ltd., Korea) containing 56 mL of Citrate Phosphate Dextrose Adenine anticoagulant solution (CPDA-1). The collected blood was dialyzed by using a laboratory scale continuous flow dialysis apparatus, which contains the flat type two chamber dialysis cell as described in a previous study.[9] The blood passed through the one compartment for 4 hrs at a flow rate of 1.5 mL min^{-1} using SciLog® Peristaltic Pump with ACCU® FM-40 Metering Head Pump.

Changes in White Cell and Platelet. Blood passed the dialyser was collected in a 50 mL of Falcon® tubes at intervals of 15 min, and the number of white cell and platelet was counted at three times by Coulter counter (Coulter®, Model STKS, USA).

Preparation of Plasma Samples. The complement activation on the materials surface was measured on the dialyzed samples drawn at times corresponding to those for white cell change. 400 μL of whole blood was sampled to Eppendorf® tube and centrifuged (3000$\times$ g) for 5 min at 4 °C. The plasma fraction was pooled and stored at -70 °C until assay. For ELISA, frozen samples were thawed quickly at 37 °C and transferred to ice immediately.

Immunoassays of Complement Activation. Complement components of fluid phase were analyzed by using commercially available ELISA kits (C3a, Bb, iC3b, and CS5b-9; C3a EIA, Bb fragment EIA, iC3b EIA, and SC5b-9 EIA; Quidel, USA, respectively, and C5a; EIA C5a micro; Behring Diagnostics, Germany). Complement components bound to the surface were evaluated by immunoblot analysis of eluted complements using monoclonal antibody for SC5b-9 (Quidel, USA) and polyclonal antibodies for C3c and C3d (Dako, Denmark). Detailed procedures for elution of bound proteins and immunoblot were described in a previous study.[10]

Results and Discussion

ESCA Studies. Since the aim of this study is to examine the effects of substituted anions to PVA on the activation of complement components, the confirmation of substitution of test samples is a matter of importance. In this study, the presence of the anionic substitution of the modified PVAs was confirmed by using ESCA. Figure 1 shows the ESCA survey scan spectra of PVA, S-PVA, and C-PVA. In Figure 1(A), PVA exhibited C1s and O1s spectra at 286 and 532 eV, respectively.[15] In Figure 1(B) and (C), S-PVA reveals the additional peaks that confirm the existence of $-SO_3$ groups on the surface. The peaks at 180 and 228 eV might be

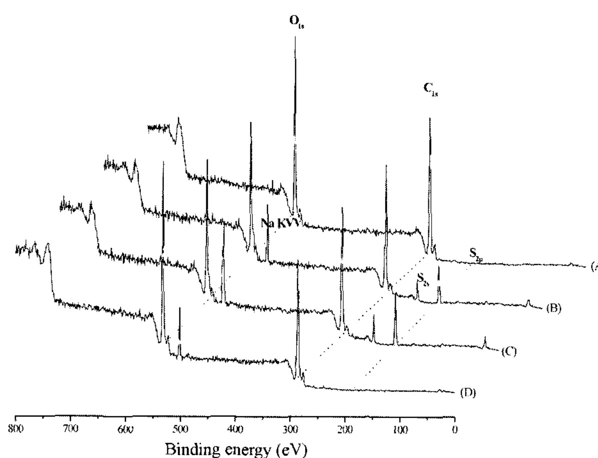

Figure 1. ESCA survey scan spectra of (A) PVA, (B) S-PVA low (20% of substitution ratio), (C) S-PVA high (30% of substitution ratio), and (D) C-PVA.

related to S 2p and S 2s spectra of $-SO_3$ groups, respectively, and these peaks increased with increase in the substitution efficiency of S-PVA. In addition, Na KVV spectra due to neutralization of $-SO_3$ and -COO groups were also found in ESCA spectra of S- and C-PVA samples. Figure 2 represents the C1s core spectra of PVA and C-PVA samples. The C1s core spectrum of PVA (Figure 2(A)) reveals the peaks at 285.00 and 286.47 eV corresponding to C-C and C-O groups, respectively. The C1s core spectrum of C-PVA shows the additional peak of O-C=O group at 289.17 eV, confirming that the carboxyl group was successfully substituted to the hydroxyl groups of PVA.

Complement Assay. It is believed that the alternative pathway is responsible for most of the complement activation produced by biomaterials, although the classical pathway may also be involved to some degree.[16] The actions of proteins of this pathway can be conceptually divided into three phases: initiation, amplification, and regulation. In fluid phase, activation occurs spontaneously at a slow rate and is amplified by the presence of a foreign surface. A spontaneous hydrolysis of the internal thioester of C3 takes place and generates a $C3(H_2O)$ molecule that can bind and activate Factor B, and this $C3(H_2O){\cdot}Bb$ fluid convertase cleaves C3 into C3a and C3b.[17] Figure 3 shows the changes in C3a complement generations (initiation level) due to the contact of the samples with treatment duration. The amount C3a increased in the order of LDPE, S-PVA, C-PVA, PVA, and Cuprophane. Since the realization of a complement activation capacity by artificial surfaces upon contact with blood, a common belief has evolved that nucleophilic surface groups such as hydroxyl and amine react with and eventually bind to the internal thioester group in C3, that is, initiation is facilitated by nucleophilies.[18] Therefore, it can be presumed that C- and S-PVA, and/or LDPE having lowered or diminished level of surface nucleophiles, rightly activate less extent of C3 than PVA and Cuprophane. Amplification follows the initiation, if the metastable C3b binds to a surface. Attachment of C3b to an activating surface favors the binding of Factor B to C3b, and thus the alternative convertase C3b·Bb is formed and cleaves more C3 molecules into C3a and C3b. If C3b binds to a non-activating surface, it is assumed that binding to the regulatory proteins, Factor H and I, is favored: C3b is inactivated by Factor I and becomes iC3b. This iC3b is further degraded to C3c and C3d (regulation stage).[16,17] Figures 4 and 5 show the content of Bb and iC3b fragments, due to the amplification and regulation stages, respectively, in human plasma. Starting at 15 min, the production of Bb was detectable for all samples except LDPE. For LDPE, the extent of Bb remained almost constant up to 60 min, and start increasing. The production of iC3b fragments was also observed at 15 min with a significant increase for all samples. After 90 min, a plateau was reached for LDPE, C-, and S-PVA, while iC3b for PVA and Cuprophane continued its increase. Anyhow, both Bb and iC3b showed the similar

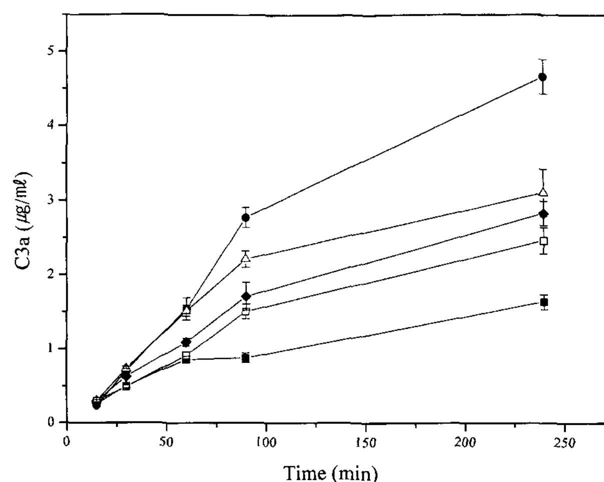

Figure 3. Quantification of C3a in human plasma during hemodialysis using an ELISA: ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

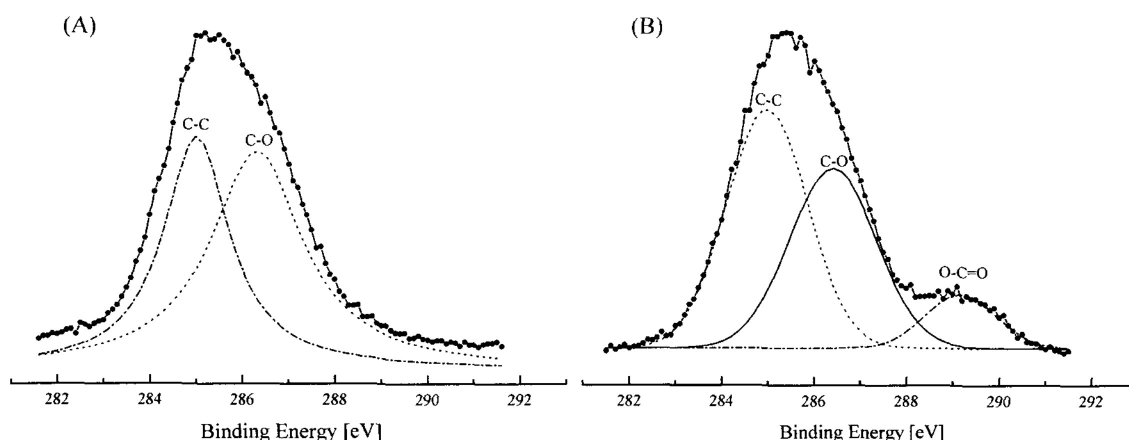

Figure 2. C1s core spectra of (A) PVA and (B) C-PVA.

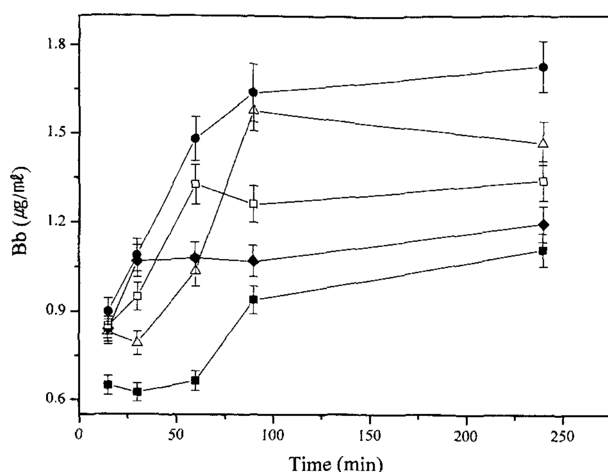

Figure 4. Quantification of Bb in human plasma during hemodialysis using an ELISA: ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

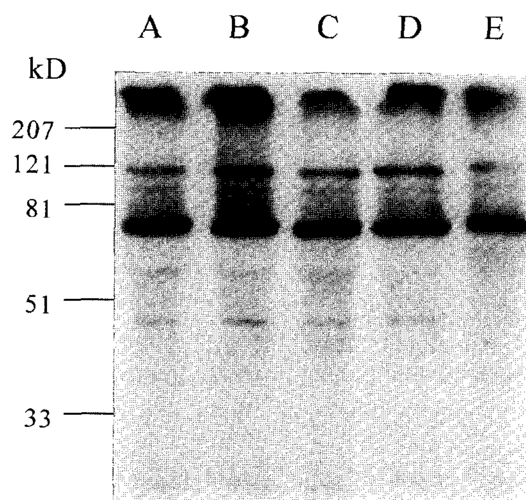

Figure 6. Immunoblot of C3 fragments present on the surface of samples after 1.5 h of dialysis: (A) Cuprophane, (B) PVA, (C) C-PVA, (D) S-PVA, and (E) LDPE.

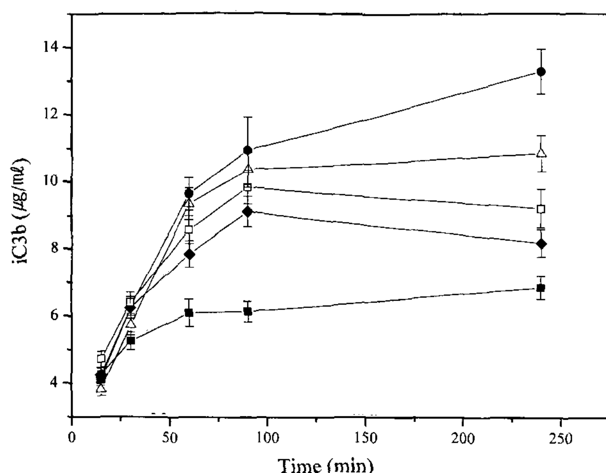

Figure 5. Quantification of iC3b in human plasma during hemodialysis using an ELISA: ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

increasing patterns, that is, the production of Bb and iC3b increased in the order of LDPE, C-, S-PVA, PVA, and Cuprophane. Based on the known mechanism of complement activation in the presence of a surface, the values obtained from iC3b measurements (Figure 5) were questionable, which could be attributed that some iC3b from the conversion of the C3b bound to the surface may have remained bound, and thus would not be assessed through plasma analysis.[19] The remained bound proteins, C3c and C3d, were analyzed by immunoblot analysis. Figure 6 represents the immunoblots of the bound C3 fragments on the surface of samples. The band of strong intensity for C3c was visible around a molecular mass of 75 kDa. The C3d also showed the strong band at 120 kDa and weak band at 29 kDa, respectively. The intensities of those bands for Cuprophane and PVA decreased compared to those of C-and S-PVA. These provide the evidence that C3c and C3d adsorbed to the surface of samples, and the amount of C3c and C3d bound to C-and S-PVA is greater than that of PVA and Cuprophane. However, the intensities of bands for LDPE were found to be lowest. Sefton *et al.* had reported the similar result to this, that is, they had found that LDPE showed the least amount of Bb as well as iC3b production among the samples.[19] This may be ascribed the fact that LDPE activated the least amount of C3. In other words, LDPE produced the least amount of C3a and C3b, whether produced C3b bound to Bb or converted into iC3b. Therefore, the result that LDPE produced the least amount of iC3b among samples is of little importance, but the fact that it produced much lower amount of Bb than iC3b is of great importance. Another interesting aspect of samples on C3 activation is that C-PVA exhibited increased inhibition activity of Bb compared to S-PVA, though both C- and S-PVA samples have a diminished level of nucleophiles due to substitution. This may be attributed the fact that the compounds possessing carboxyl group, such as K76COOH (K76 monocarboxylic acid), induce suppression of Factors B and D, leading to depression of Bb production.[20] On the other hands, compounds possessing a sulfonate group potently minimize the activity of Factor B, but inhibit Factor D activity only marginally. FUT-175 (Futhan, 6-amindino-2-naphthyl *p*-guanidinobenzoate dimethanesulfonate), a synthetic inhibitor of serine protease, is the typical example of the above mentioned compounds with a sulfonate group.[20] Figure 7 represents the C5a activation due to the contact of the samples with treatment duration. The amount of C5a increased in the order of LDPE, S-PVA, C-PVA, PVA, and Cuprophane. Since Cuprophane and PVA gener-

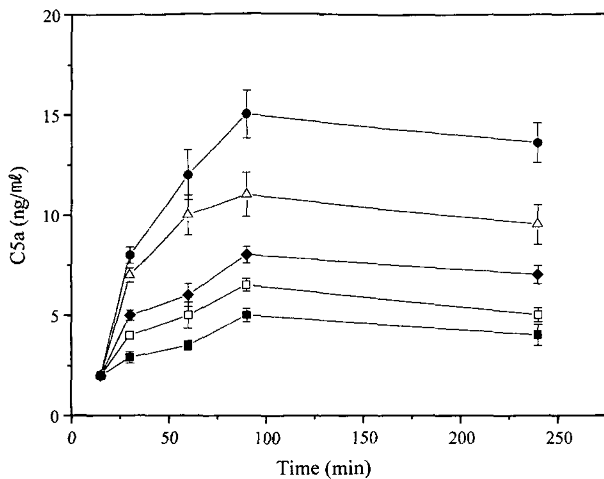

Figure 7. Quantification of C5a in human plasma during hemodialysis using an ELISA: ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

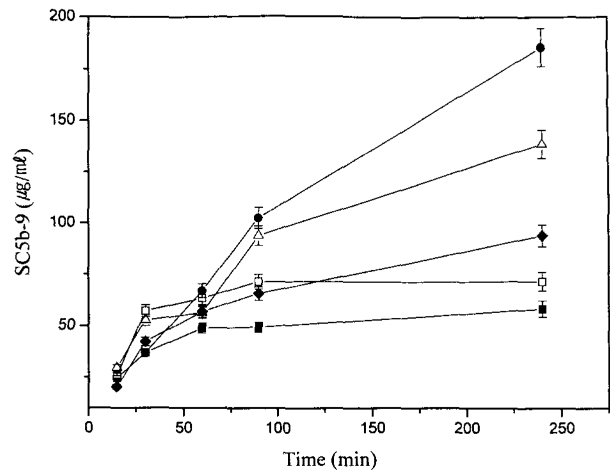

Figure 8. Quantification of SC5b-9 in human plasma during hemodialysis using an ELISA: ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

ated increased amounts of C3a and Bb, the fact that these two samples produced more amount of C5a that the others is of little doubt. However, it has to be discussed that S-PVA activated less extent of C5a than C-PVA. Mauzac *et al.* had found that dextran derivatives extensively modified with sulfonate groups diminished complement activation by facilitated inhibition of C5 conversion.[21] This inhibition of C5a conversion is due to the binding capacity of C5a to a sulfonate group. The anaphylatoxins (C3a and C5a) generated upon extracorporeal circulation are highly basic proteins. This indicates that negatively charged surfaces could preferentially adsorb these basic proteins from plasma. Though both C- and S-PVA possess negative charges, S-PVA adsorbed increased amount of C5a compared to C-PVA. In a previous study, surface potentials of samples were examined by ζ-potential measurements.[10] As expected, C- and S-PVA exhibited negatively large zeta potentials, because the substituted chains have an electric charge by themselves. Interestingly, zeta potential generated on the surface of S-PVA was 1.6-fold greater than that of C-PVA. This is attributed that ionization ability of a sulfonate group exceeds to that of a carboxylate group, resulting in increased amounts of C5a adsorption by S-PVA. The reason that dextran sulfonate (DS) column has been being favored for immunoadsorption treatment to remove anaphylatoxins from blood of the patients with systemic lupus erythematosus (SLE)[22] can be explained by above reason.

Removal of C5a is of great importance in the clinical point of view. Production of C5a leads to receptor-dependent white cell activation. This results in profound neutropenia, increased concentrations of degradative enzymes, and reactive oxygen species that ultimately may lead to tissue damage and dysfunction of the important immune cells.[16] Consequently, strong electrostatic attraction between negatively charged sulfonate groups and positively charged C5a may limit C5a exposure, in turn, leads to marginate clinical consequences of complement activation. The cleavage of C5 also release C5b, which can ultimately lead to formation of the terminal complement complex. The terminal complement complex is a general term for membrane-associated C5b-9 and fluid phase SC5b-9.

Typically, C5b-9 is diverted to the fluid phase by reacting with S protein (vitronectin) to form a soluble lytically inactive stable complex called SC5b-9. Therefore, assessment of SC5b-9 is an excellent means to quantify terminal complement activation.[17] Figure 8 shows the changes in SC5b-9 levels due to the contact of samples. Owing to reduced activation of C3 and C5, C- and S-PVA samples exhibited marked decrease in SC5b-9 levels in plasma. Recently, the importance of bound SC5b-9 to biomaterials has been being emphasized, because the substantial levels of adsorbed SC5b-9 support the hypothesis that materials induced complement activation that could mediate cellular reactivity via an opsonization or via direct cell activation/lysis at the blood and materials interface by the membrane attack complex.[17]

So far, the effects of substitution of carboxylate and sulfonate groups on activation of various complement components have been discussed. Besides these, the effect of degree of substitution on the activation of complement has to be studied, since the substitution degree of sample can be another important factor that may influence the degree of complement activation. Figure 9 shows the changes in the activation of fluid phase complement components, C3a, C5a, and SC5b-9. This figure provides the evidence that complement-activating capacity of polymer samples was closely related to substitution efficiency. Both C- and S-PVA shows dramatic decrease in complement-activating

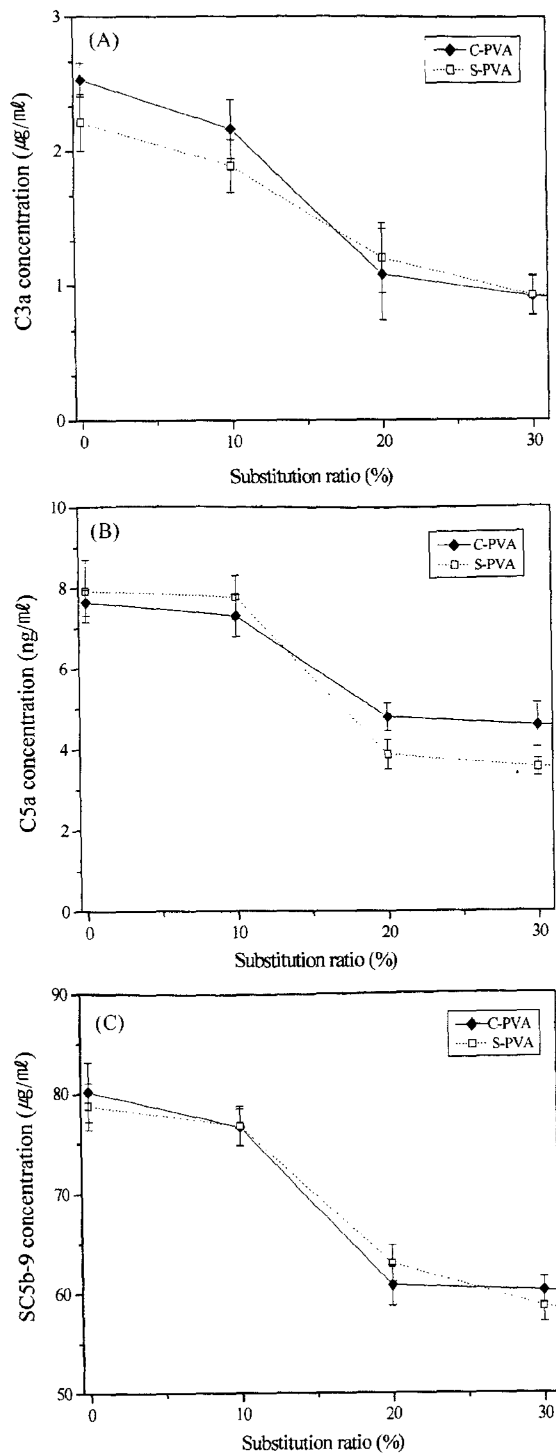

Figure 9. Changes in activation of fluid phase complements with respect to the substitution ratio. (A) C3a, (B) C5a, and (c) SC5b-9 (Substitution ratio of samples were double confirmed by acid-base titration and sulfur analysis).

capacities for samples substituted at 20% and above.

Figures 10 and 11 represent proteins that were specific to the antibody and their optical densities of SC5b-9 bound to samples, respectively. The interesting findings have to be discussed are that S-PVA bound greater amount of SC5b-9 than C-PVA, and PVA bound greater than Cuprophane. The results in Figures 10 and 11 seemed to have no correlation to the amount of fluid phase SC5b-9 in Figure 8. So far, there are no proven mechanisms that explain the interaction between materials surface and SC5b-9. It is anticipated that continued exploitation may lead to findings regarding the impact of this particular complement components on leukocytes at the blood and biomaterials interface. The only significance in this situation is that anion substituted samples, C- and S-PVA, bound decreased extents of SC5b-9, compared to PVA and Cuprophane. In parallel with the changes in complement fractions, C3a, C5a, and fluid phase SC5b-9, C- and S-PVA samples exhibited decreased fall in white cells and platelets compared to PVA (Figures 12 and 13).

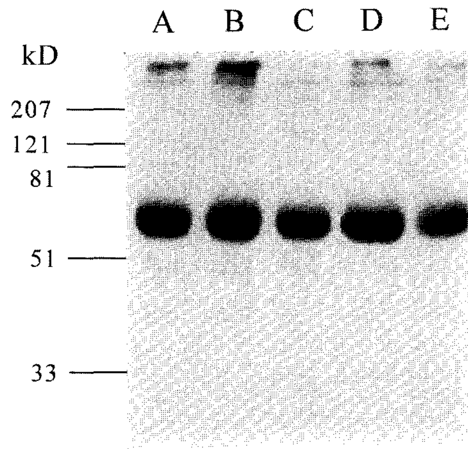

Figure 10. Immunoblot of SC5b-9 fragments present on the surface of samples after 1.5 hrs of dialysis: (A) Cuprophane, (B) PVA, (C) C-PVA, (D) S-PVA, and (E) LDPE.

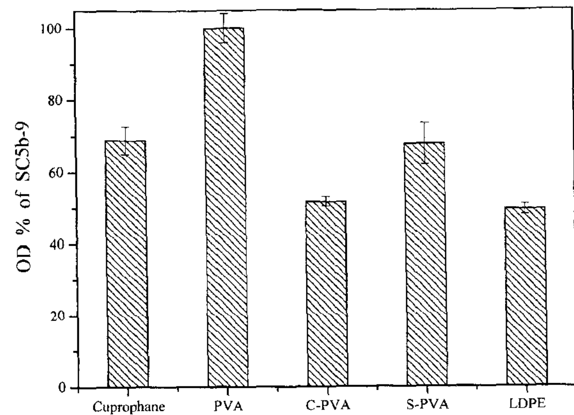

Figure 11. Optical densities of SC5b-9 fragments present on the surface of samples after 1.5 hrs of dialysis. OD values are expressed as the percentage of OD value of PVA (means ± SD, n=3).

In conclusion, the substitution of hydroxyl groups with anion lead to a marked decrease in complement activation. The diminished level of surface nucleophiles of C- and S-PVA activate less extent of C3 than PVA. C-PVA exhibited increased inhibition activity of Bb due to suppression of Factors B and D, lead to decreased production of C5 convertase. Owing to reduced activation of C3 and C5, C- and S-PVA samples exhibited eminent decrease in SC5b-9 levels in plasma. It was also found that negatively charged sulfonate and carboxylate groups of the samples adsorbed large extents of positively charged anaphylatoxins, C3a and C5a, due to strong electrostatic attraction, leads to inhibition of chemotaxis and activation of leukocytes. The inhibition ability of complement production together with binding ability of anaphylatoxins of C- and S-PVA samples lead to a prominent decrease in lysis of leukocytes.

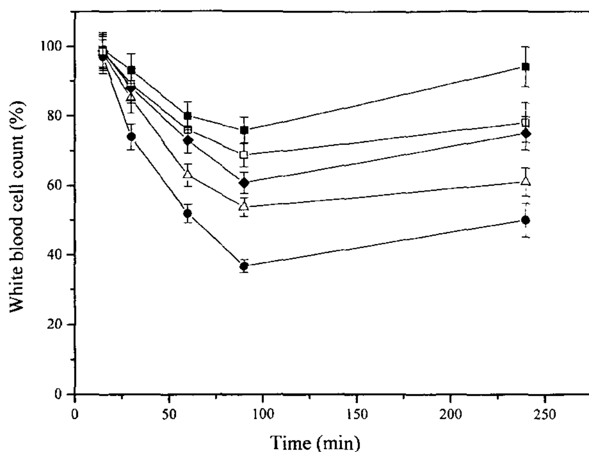

Figure 12. Changes in white blood cell count during hemodialysis with samples. ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE (means ± SD, n = 3).

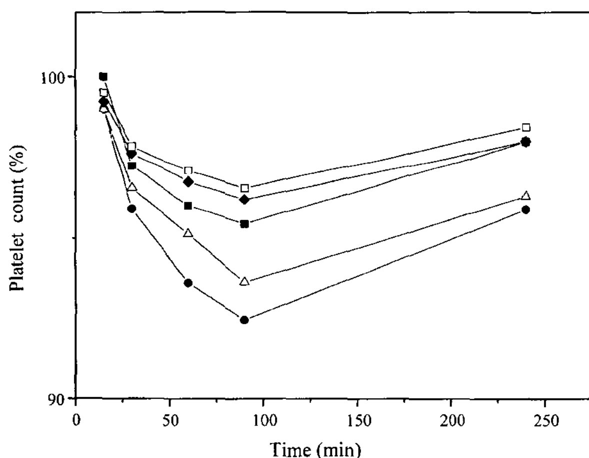

Figure 13. Changes in platelet count during hemodialysis with samples. ● Cuprophane; △ PVA; ◆ C-PVA; □ S-PVA; ■ LDPE ($P < 0.05$).

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References

(1) P. M. Galletti, C. K. Colton, and M. J. Lysaght, *Artificial Kidney in The Biomedical Engineering Handbook*, J.D. Bronzino, Ed., CRC Press Inc., 1995, pp 1898-1924.
(2) R. J. Johnson, M. D. Lelah, T.M. Sutliff, and D. R. Boggs, *Blood Purif.*, **8**, 318 (1990).
(3) P. M. Galletti, P. Aebischer, and M. J. Lysaght, *ASAIO J.*, **41**, 49 (1995).
(4) Y. Ikada, H. Iwata, F. Horii, T. Matsunaga, M. Tanigushi, and M. Suzuki, *J. Biomed. Mater. Res.*, **15**, 697 (1981).
(5) N. A. Hoenich, C. Woffindin, S. Stamp, S. J. Roberts, and J. Turnbull, *Biomaterials*, **18**, 1299 (1997).
(6) J. R. Frautschi, R. C. Eberhart, J. A. Hubbel, B. D. Clark, and J. A. Gelfaud, *J. Biomater. Sci. Polym. Ed.*, **7**, 707 (1996).
(7) A. Finch, *Poly(vinyl alcohol)-Development*, John Wiley & Sons Ltd., New Work, 1992.
(8) H. J. Chun, J. J. Kim, and K. Y. Kim, *Polym. J.*, **22**, 347 (1990).
(9) H. J. Chun, J. J. Kim, S. H. Lee, K. Y. Kim, and U. Y. Kim, *Polym. J.*, **22**, 477 (1990).
(10) K. E. Ryu, H. Rhim, C. W. Park, H. J. Chun, J. J. Kim, and Y. M. Lee, *Macromol. Res.*, **11**, 451 (2003).
(11) T. S. Suh, C. K. Joo, Y. C. Kim, M. S. Lee, H. K. Lee, B. Y. Choe, H. J. Chun, *J. Appl. Polym. Sci.*, **85**, 2361 (2002).
(12) G. S. Khang, J. M. Rhee, J. H. Lee, I. W. Lee, and H. B. Lee, *Korea Polym. J.*, **8**, 276 (2000).
(13) G. S. Khang, M. K.Choi, J. M Rhee, S. J. Lee, H. B. Lee, Y. Iwasaki, N. Nakabayashi, K. Ishihara, *Korea Polym. J.*, **9**, 107 (2001).
(14) S. Y. Nam, H. J. Chun, and Y. M. Lee, *J. Appl. Polym. Sci.*, **72**, 241 (1999).
(15) G. Beamson and D. Briggs, in *High Resolution XPS of Organic Polymers*, John Wiley & Sons Ltd., New Work, 1992.
(16) R. J. Johnson, *Immunology and the Complement System in Biomaterials Science*, B. D. Ratner, A. S. Hoffman, F. R. Schoen, and J. E. Lemons, Eds., Academic Press, San Diego, 1996, pp 173-188.
(17) C. H. Gemmell, *J. Biomed. Mater. Res.*, **37**, 474 (1997).
(18) J. Wettero, A. Askendal, T. Bengtsson, and P. Tengvall, *Biomaterials*, **23**, 981 (2002).
(19) M. Berger, B. Broxup, and M. V. Sefton, *J. Mater. Sci.: Mater. Med.*, **5**, 622 (1994).
(20) S. Miyagawa, R. Shirakura, G. Matsumiya, N. Fukushima, S. Nakata, H. Matsuda, M. Matsumoto, H. Kitamura, and T. Seya, *Transplantation*, **55**, 709 (1993).
(21) M. Mauzac, F. Maillet, J. Jozeforvicz, and M. D. Kazatchkine, *Biomaterials*, **6**, 61 (1985).
(22) Y. Matsuki, K. Suzuki, M. Kawakami, T. Ishizuka, T. Hidaka, and H. Nakamura, *J. Clin. Apheresis*, **13**, 108 (1998).