Newly Developed Biocompatible Membrane and Effects of its Smoother Surface on Antithrombogenicity

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ABSTRACT: The new biocompatible cellulosic membrane (AM-BC-F[AM-BIO-HX], Asahi-Medical Co. Ltd., Tokyo, Japan) has been developed, which has a higher flux than conventional membranes and more excellent antithrombogenicity because of its smoother membrane surface. The roughness of the inner surface of the AM-BC-F membrane was smaller than that of conventional membranes, as observed by Atomic Force Microscopy, because it was produced by the newly developed spinning method of cuprammonium cellulose solution, which has a different composition from that of a conventional cuprammonium cellulose solution. The degree of platelet adhesion (number of platelets adhered) on the membrane surface was evaluated *in vitro* by the measurement of the amount of the LDH released from the adhered platelets on the membrane surface after contact with fresh blood of Japanese male white rabbits weighing 2.5–3.0 kg. The number of platelets adhered of AM-BC-F was far smaller than that of conventional membranes. It was deduced from the smoother surface of the membrane. It can be expected that AM-BC-F will have an excellent antithrombogenicity on a dynamic state during actual dialysis treatments, because it is considered that the shearing stress of blood on the inner surface and the interaction between platelets and the membrane surface are less than that of conventional membrane2s. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 72: 1249–1256, 1999

Key words: biocompatibility; smoother surface; antithrombogenicity; platelet adhesion; cuprammonium cellulose solution

INTRODUCTION

Complement activation during hemodialysis using cellulosic hollow-fiber dialysis membranes has been widely discussed and reported.[1,2] The modification methods of cellulosic membrane surfaces were developed and industrialized to reduce complement activation. Biocompatibility is dominated primarily by interaction between blood and dialysis of the membrane surface. Surface modification of dialysis membranes is an essential method to improve biocompatibility. Among a variety of surface modification methods, grafting of water-soluble polymer chains to the surface of the biomaterial has been considered to be a promising method to improve biocompatibility,[3–5] and in the case of a cellulosic membrane, the reactivity of a glucose hydroxyl group is utilized in those methods.

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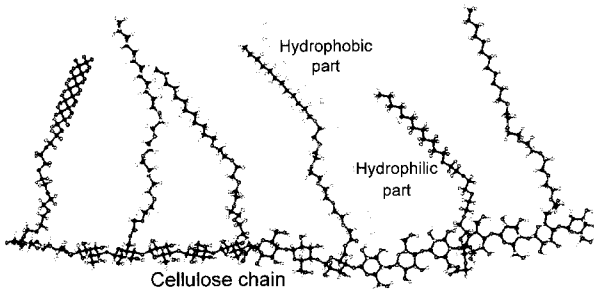


Figure 1 Initial structure for molecular dynamics calculation—alkyl ether chain-grafted cellulose.

Alkyl ether carboxylic acid (PEG acid) grafted on cellulosic membranes (AM-PC[AM-BIO] and AM-BC-P[AM-BIO-UP], Asahi-Medical Co. Ltd., Tokyo, Japan) were also developed and industrialized to reduce complement activation during the hemodialysis using a cuprammonium cellulosic membrane.[5] It had been shown that these types of membranes effectively and significantly reduced complement activation and thrombogenicity *in vivo*.[7,8]

This modification method has been applied to higher flux cellulosic membranes. It has been concluded that the amount of PEG acid grafted on higher flux cellulosic membranes had to be larger than that of conventional membranes (AM-PC and AM-BC-P) to reduce complement activation as well as conventional membranes. This is because the intrinsic surface area influencing the complement components was larger than that of conventional membranes due to many larger pores inside the membrane wall.[9] In addition, it was considered that higher flux cellulosic membranes enhanced thrombogenicity because of their rough membrane surface.

The biocompatible cellulosic membrane (AM-BC-F[AM-BIO-HX], Asahi-Medical Co. Ltd., Tokyo, Japan) has been developed, which has a higher flux than conventional membranes and more excellent antithrombogenicity with a smoother membrane surface.[10]

In this article the authors have focused on the roughness of the membrane's inner surface and platelet adhesion representing the antithrombogenicity. The newly developed biocompatible membrane (AM-BC-F[AM-BIO-HX]) has been compared with conventional cellulosic membranes with respect to the roughness of the membrane's inner surface measured by atomic force microscopy, the mechanism of cuprammonium cellulose solution coagulation during the spinning, and the degree of platelet adhesion (number of platelets adhered) on the membrane surface was evaluated *in vitro* by the measurement of the amount of the LDH released from the adhered platelets on the membrane surface after contact with the fresh blood of Japanese male white rabbits weighing 2.5–3.0 kg.[11]

MATERIALS AND METHODS

Hemodialysis Membranes

Low-flux nonmodified cellulosic membrane (AM-SD), high-flux nonmodified cellulosic membrane (AM-GP), low-flux and high-flux PEG acid-grafted cellulosic membranes (AM-PC[AM-BIO], AM-BC-P[AM-BIO-UP]) and the newly developed higher flux PEG acid-grafted cellulosic membranes (AM-BC-F[AM-BIO-HX]), which are gamma sterilized, were used for this study (all the membranes, Asahi-Medical Co. Ltd., Tokyo, Japan). Alkyl ether carboxylic acid (PEG acid), in which the poly(ethylene glycol) chain bore an alkyl ether group on one side and carboxyl group on the other side of the chain, was grafted onto the cellulosic membrane through an esterification reaction between the hydroxyl group on the cellulose and the terminal carboxyl group of PEG acid according to the methods of Kishida et al.[5] PEG acid-grafted cellulosic membranes were immersed in a solution of PEG acid, *N,N*-dicyclohexylcarbodiimide and dimethylaminopyridine at room temperature. Grafting amounts were controlled by the concentration of PEG acid in the solution. The structure is schematically shown in Figure 1. PEG acid-grafted cellulosic membranes have the so-called diffusive layer (hydrogel layer made of

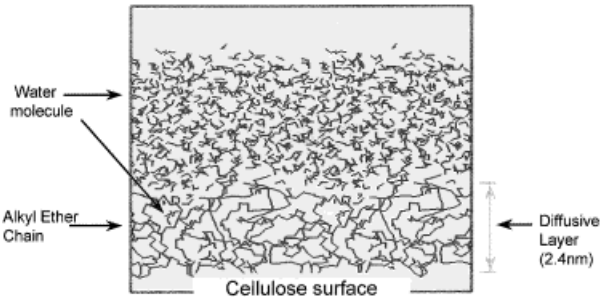


Figure 2 Diffusive layer for molecular dynamics calculation—alkyl ether chain-grafted cellulose.

Table I Specification of Cuprammonium Cellulosic Membranes

Products	Membrane	Ultra Filtration Rate [ml/m^{2}h mmHg]	Cuprammonium Cellulose Solution (CCS)
Sample A	Cellulose	4.9	I
Sample B	Cellulose	21.4	I
Sample C	PEG-graftedcellulose	5.5	I
Sample D	PEG-graftedcellulose	15.2	I
Sample E	PEG-graftedcellulose	11.9	II
Sample F	PEG-graftedcellulose	18.7	II

PEG acid, thickness: 2.4 nm), which may act as buffer layer between the blood ingredients and the membrane surface shown in Figure 2. The formation of the diffusive layer is dependent on the balance between hydrophobicity of alkyl ether groups and hydrophilicity of PEG chains.

The newly developed biocompatible cellulosic membrane (AM-BC-F[AM-BIO-HX]) has a higher flux than that of the conventional membranes, and it is expected to have an excellent antithrombogenicity with a smoother membrane surface because it is produced by the new spinning method of a hollow-fiber dialysis membrane determining the membrane structure, by controlling the composition of cuprammonium cellulose solution (CCS) and the coagulant of CCS.

Specifications of each membrane are shown in Table I. Samples A, B, C, D, E, and F correspond to AM-SD, AM-GP, AM-PC[AM-BIO], AM-BC-P[AM-BIO-UP], prototype, and AM-BC-F[AM-BIO-HX], respectively. Samples E and F were prepared by the spinning of using CCS II. The inner diameter and membrane thickness of each dry membrane were 180 and 15 μm, respectively. They were measured by an optical microscope at room temperature. The ultra filtration rate (UFR) of Sample F was 18.7 ml/m^2 h mmHg, and it corresponded the reduction rate of β_2 microglobulin was 25–30%, and Sample F was expected to be a mild-flux dialysis membrane.

Cuprammonium Cellulose Solution (CCS)

Cotton linters supplied by Buckeye Cellulose Co., Ltd., were dissolved in a cuprammonium solution by the method of Gibson et al.[11] with minor modifications. Their viscosities were determined on a standard viscometer (Mettler Rheomat 115). Both CCSs were coagulated in the same coagulant solution, and it was reported that the pore structure of the hollow-fiber dialysis membranes was influenced by the interfacial potential and the ion flux between CCS and coagulant solutions.[12]

Observation of the Membrane Inner Surface by Atomic Force Microscopy and Evaluation of Its Roughness

The dried samples of hollow-fiber dialysis membranes were cut into oblique shapes, and their inner surfaces were subjected to atomic force microscopic observation (AFM, model NanoScope IIIa, Digital Instruments Co., Ltd., USA). Observations were conducted under the following conditions: mode, tapping mode; resonance frequency, 200–400 kHz; using the probe made of silicon single crystal, of which the length of cantilever was 125 μm. The roughness (PV, peak-to-valley) was measured using the atomic force microscopy photographs by the AFM analyzing system.

Observation of the Cross-Section of the Membrane Wall by Scanning Electron Microscopy and Evaluation of the Cellulosic Particle Size (Equivalent Diameter of the Cellulosic Particle)

The wet samples of hollow-fiber dialysis membranes were dehydrated by 50, 70, 80, 90, 95, and 99% ethyl alcohol aqueous solution successively for 30 min each, and a cross-section containing ethyl alcohol was frozen and cracked. In addition, the ethyl alcohol contained in the samples was substituted by 100% ethyl alcohol for 30 min (two times), *t*-butanol/ethyl alcohol (1 : 1) for 30 min (one time) and *t*-butanol for 30 min (two times) successively, then they were freeze dried for 2 days.

The freeze-dried samples were coated by Pt-Pd, and subjected to a scanning electron microscopic observation at an accelerating voltage of 1.0 kV (UHR-SEM, model S-5000, Hitachi Seisakusyo Co., Ltd., Japan).

The inner surface of the membranes for the electron microphotographs obtained above was analyzed by a high-resolution image analyzing system (model IP-1000PC, Asahi Chemical Ind. Co., Ltd., Japan) to evaluate the size (equivalent diameter) of cellulosic particles approximated as spherical particle from the area of the particle, by picking 50 cellulosic particles up per a membrane inner surface arbitrarily. The distribution of sizes of 50 cellulosic particles was determined; in addition, the average and the standard deviation of sizes of 50 cellulosic particles were calculated.

Evaluation of Platelet Adhesion

A sample module that contained 300 hollow fiber membranes was used for the experiments. The total length of the module was 8 cm; and both edges of the fibers were potted with silicone, one of which was equipped with a cap to allow centrifugation. Modules were checked for hydraulic leakage and for uniform perfusion of all fibers before use.

Blood from Japanese male white rabbits weighing 2.5–3.0 kg were used for the platelet adhesion experiment.[13] Blood was collected from an carotid artery. Nine volumes of blood were mixed with one volume of 3.8% sodium citrate solution. Platelet-rich plasma (PRP) was prepared by centrifugation for 10 min at 200 $\times$ g. Heparin was adjusted to a concentration of 2.0 U/mL. To maintain the effects of divalent cation, $CaCl_2$ was added to the platelet solution to a concentration of 5 mM of free Ca^{++} ion. The chelation of Ca^{++} ions by citrate was also calculated. The platelet concentration in PRP was assessed with a Coulter counter and adjusted by dilution with platelet-poor plasma to a concentration of 5 $\times$ 10^8 platelets/mL.

The module was washed for 10 min with a physiological saline solution, and was centrifuged for 5 min at 400 $\times$ g. A total volume of 0.5 mL of platelet suspension was placed into the inner part of the hollow fiber membranes and allowed to stand for a period of 60 min at 37°C under a static condition. After incubation, the module was centrifuged for 5 min at 400 $\times$ g, and was immersed in physiological saline solution for 10 min to remove nonadherent platelets and plasma protein. The fibers were cut into about 1-mm length to count the adhered platelets.

To determine the number of adhered platelets, 1 mL of lysis solution [0.5% Triton X-100 in PBS(−)] was added to the fibers in a test tube and kept for 1 h at room temperature. The LDH activity of lysate was measured according to the method of Stolzenbach,[14] excluding the reaction time of 60 min. The LDH calibration curve was obtained by measuring the enzymatic activity with the known concentration of washed platelet.

In the method described above, experiments were made to determine the concentration of heparin, the incubation time, and the dislodgment force.

The platelet adhesion tests were performed in parallel with the control, which is a low-flux cellulosic membrane. All the data must be discarded, if the platelet adhesion of the low-flux cellulosic membrane is not in the range of 8 $\pm$ 3 $\times$ 10^5 platelets/cm^2. Five modules of each membrane were tested to measure the reproducibility of platelet adhesion. The differences between the value of LDH activity of each membrane and that of the low-flux membrane were presented as the data (n = 5).

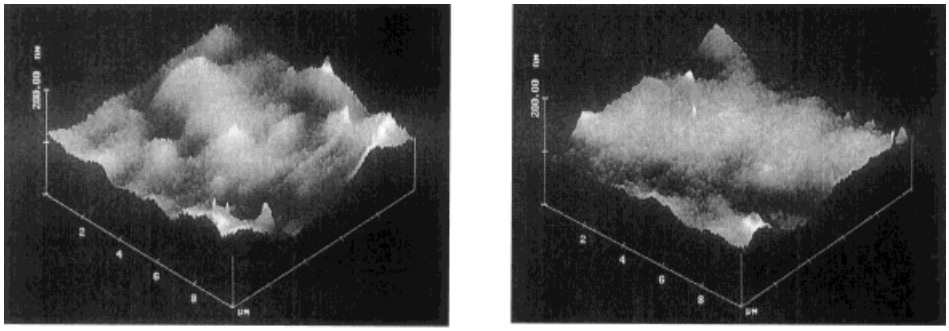
Observation of the Platelets Adhered to the Membrane Surface by Scanning Electron Microscopy

The platelets that adhered to the inner surfaces of the membranes were fixed by glutaric aldehyde, and the samples were dehydrated by 25, 50, 75, 95, and 99% ethyl alcohol aqueous solution successively for 30 min each. Then the ethyl alcohol contained in the samples was substituted by 100% ethyl alcohol for 30 min (two times), isoamyl acetate/ethyl alcohol (1 : 1) for 30 min (1 time), and isoamyl acetate for 30 min (two times) successively, and they were dried at the critical point for 2 days.

Dried samples were coated by Pt-Pd and subjected to scanning electron microscopic observation at accelerating voltage of 8.0 kV (HR-SEM, model S-2400, Hitachi Seisakusyo Co., Ltd., Japan).

RESULTS

Atomic force microphotographs of the inner surface of the membranes for Sample B and Sample F are shown in Figure 3. Here, the scales of vertical axis and width axis are 0.1 and 10 μm, respectively. It is evident that the roughness of



(1) Sample B (2) Sample F

Figure 3 Atomic force microphotographs of the inner surface of the membranes.

the μm scale of the inner surface of Sample F is smaller than that of Sample B.

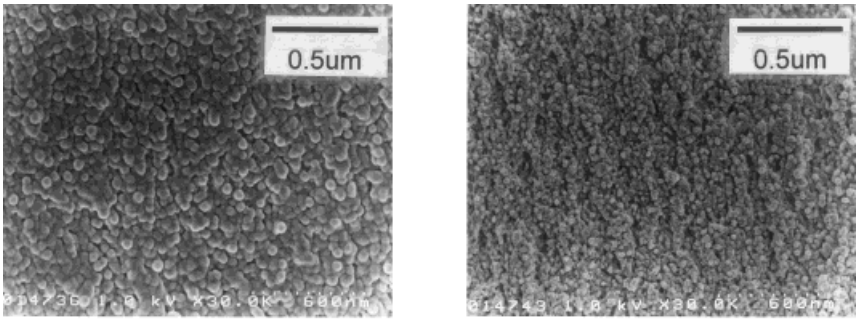
Scanned electron microphotographs of the inner suface of Samples B and F are shown in Figure 4. In addition, the distributions of cellulosic particle sizes (the equivalent diameter of the cellulosic particle is approximated as a circular particle from the area), which were analyzed by a high-resolution image analyzing system, are shown in Figure 5. The average sizes of 50 cellulosic particles of Samples B and F were 0.067 and 0.039 μm, respectively, and the standard deviations were 0.014 and 0.006 μm, respectively.

The dependence of the number of platelets adhered (the degree of platelet adhesion) on the roughness (PV) of the inner surface of the membranes are shown in Figure 6. The number of adhered platelets decreased with the decreasing roughness. The number of adhered platelets of Sample F was smaller than that of conventional membranes, and the roughness of the inner surface of Sample F was also smaller than that of conventional membranes.

Scanned electron microphotographs of the adhered platelets on the inner surface of the membranes are shown in Figure 7. It was evident that the amount of adhered platelets decreased with a smaller degree of platelet adhesions.

DISCUSSION

The number of adhered platelets of newly developed biocompatible cellulosic membrane (Sample F) was less than that of conventional membranes (Fig. 6), and it was expected to have antithrombogenicity characteristics. It was considered that it could be obtained by the smooth inner surface of



(1) Sample B (2) Sample F

Figure 4 Scanned electron microphotographs of the inner membrane surface.

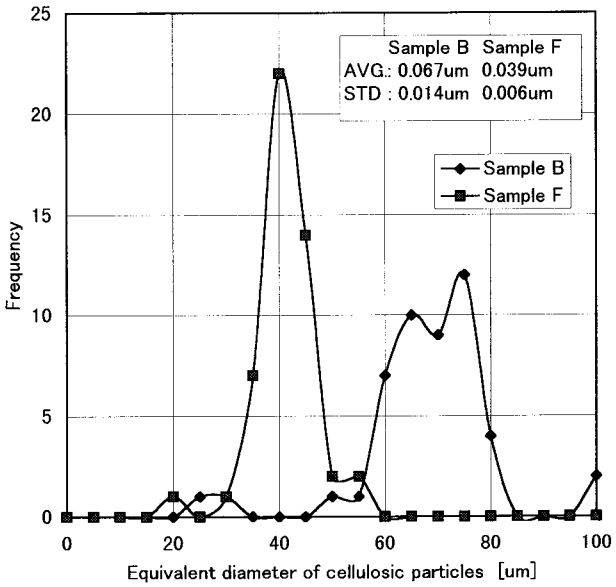


Figure 5 Distribution of the equivalent diameter of cellulosic particles.

Sample F, because it was produced by a new spinning method using CCS II, and the cellulosic particles that constituted a membrane structure and inner surface were smaller and more homogeneous than that of conventional membranes (Figs. 3, 5–7).

The cellulosic particles of Sample F were smaller than that of conventional membranes, because the rate of one cellulosic particle growth was considered to be slow during the process of the formation of membrane structure in the new coagulation method using CCS II.[15]

Because the roughness of the inner surface of the membrane increased with the increasing ultrafiltration rate (Fig. 8) by the spinning method using CCS I, it was estimated that the roughness of the inner surface would become larger in the case of higher flux hollow-fiber dialysis membranes by using CCS I, and it was undesirable from the viewpoint of the antithrombogenicity (Fig. 6), and it had been concluded that a higher flux hollow-fiber dialysis membrane should be produced by using CCS II to decrease the roughness of the inner surface of the membrane compared with that by using CCS I.

It has been reported that the structure of the inner surface of the membrane is considered to be one of the factors enhancing the interaction between blood and membrane.[16] The thrombogenicity should be evaluated not only in a static state, but also in a dynamic state,[16] because the dialysis membranes are used in a dynamic state during dialysis treatments (the wall shear rate of blood is approximately 0.05 to 0.15 1/s), and it is well known that the platelets may be stimulated under high shearing stress on the inner surface of the membrane.[17,18] In this study, the number of adhered platelets of a newly developed biocompatible cellulosic membrane (Sample F) evaluated in the static state was less than that of conventional membranes (Figs. 6 and 7). Moreover, it is expected that Sample F has excellent antithrombogenicity in a dynamic state during the actual dialysis treatments, because it is considered that the shearing stress of blood on the inner surface and the interaction between platelets and the membrane are less than that of conventional membranes.[16]

CONCLUSION

The new biocompatible cellulosic membrane (Sample F) has a higher flux than conventional membranes and excellent antithrombogenicity with a smoother membrane surface, because it was produced by the new spinning method using CCS II, and the inner membrane surface com-

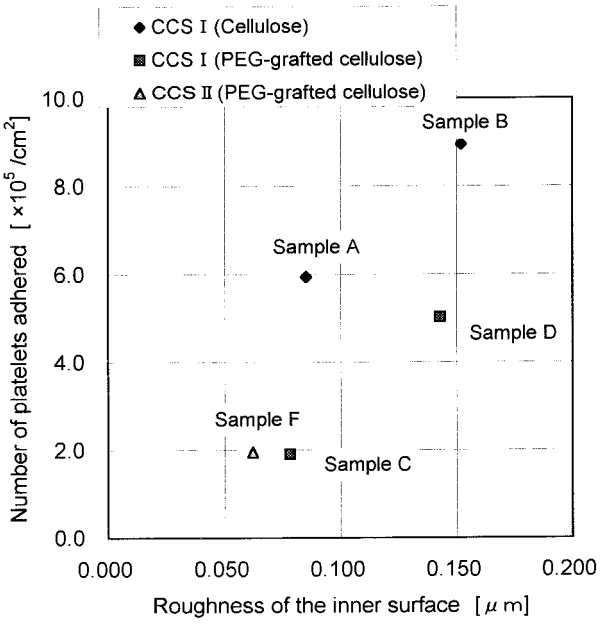


Figure 6 Dependence of the number of platelets adhered on the roughness of the inner surface of the membrane.

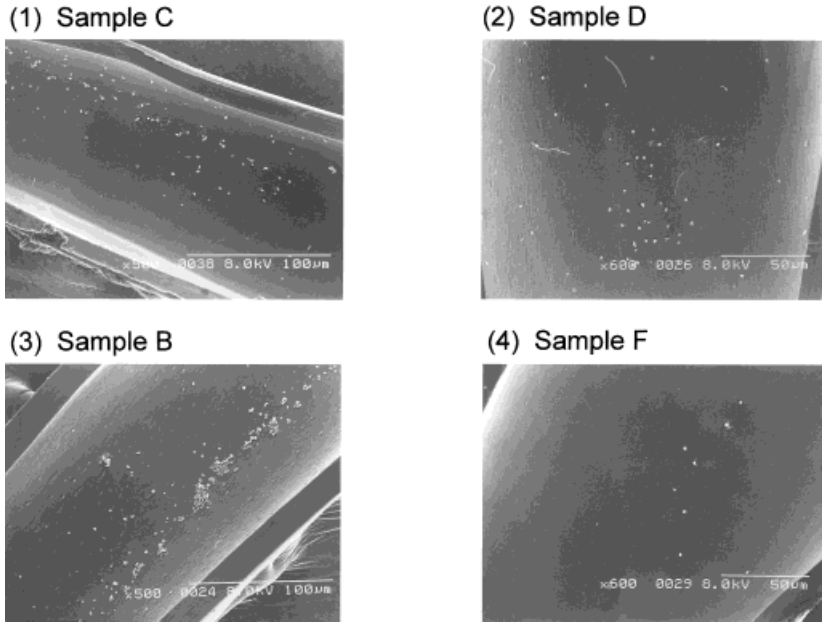


Figure 7 Scanned electron microphotographs of the platelets adhered on the membrane surface.

posed of smaller cellulosic particles was smoother than that of conventional membranes. It is expected that Sample F will have excellent antithrombogenicity in a dynamic state during the dialysis treatments, because it is considered that the shearing stress of blood on the inner surface and the interaction between platelets and the membrane are less than that of conventional membranes.

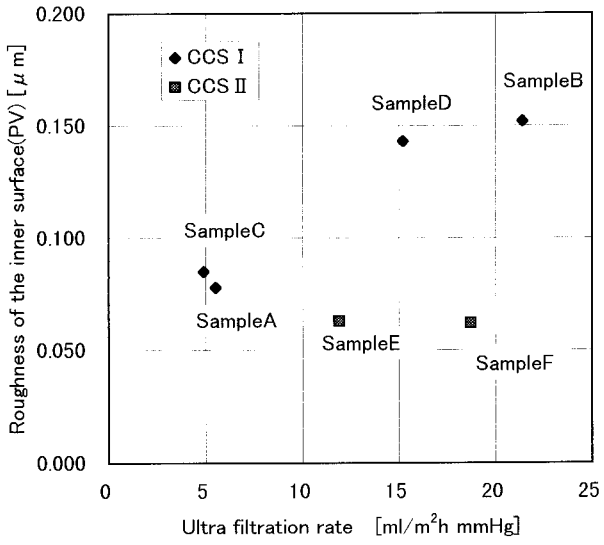


Figure 8 Dependence of the roughness of the inner surface of the membrane on the ultrafiltration rate.

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