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Immobilization of Heparin onto Microporous Polyvinyl Alcohol Hollow-Fiber Membranes for Improved Antithrombogenicity

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Microporous polyvinyl alcohol(PVA) hollow-fiber(HF) membranes crosslinked by glutaraldehyde have been modified by several cationic amine polymers such as chitosan or polyethyleneimine to introduce amino groups into the membrane *via* reductive amination utilizing a residual monoreacted dialdehyde in the membrane, and subsequently ionic and covalent immobilization of heparin have been performed for achieving improved antithrombogenicity. Of amine polymers examined, a water-soluble chitosan was found the most suitable modifying agent for heparin immobilization in terms of bioactivity of the immobilized heparin, nontoxicity and biocompatibility. Both ionically immobilized heparin and covalently immobilized heparin obtained by a coupling of aldehyde-terminated heparin with chitosan modified membranes exhibited the highest anticoagulant activities (anti-factor Xa, activated partial thromboplastin time (APTT), and prothrombin time), and even the ionically heparinized membranes retained significant anticoagulant activity with less heparin elution after a long-term incubation of 30 days in 0.9% NaCl aqueous solution. The observed anticoagulant activity of covalently heparinized membranes suggested that antithrombin III was actually bound to covalently immobilized heparin on the membrane, thus leading to an inactivation of thrombin and activated coagulation factor X (FXa). Interestingly, the anticoagulant activity (APTT) of covalently heparinized membranes after washing with 20% NaCl solution increased significantly during the extended incubation in 0.9% NaCl for more than a month without loss of immobilized heparin content, being quite sensitive to an ionic strength in NaCl solutions. The covalently heparinized membranes showed relatively low plasma protein adsorption, and the least platelet activation as determined by the release of β-thromboglobulin and platelet factor 4 after contacting with human whole blood containing 15% acid-citrate- dextrose(ACD) solution. From the results of the *in vitro* tests of anticoagulant property and blood compatibility, it was concluded that the heparinized membranes actually showed a high bioactivity in the suppression of blood coagulation. In addition, permeability of water and 0.9% NaCl solution across the heparinized membrane was not affected by the heparin-chitosan immobilization. Therefore, it is expected that heparinized PVA HF would find potential application in blood contacting devices such as an intravenous hyperalimentation catheter as well as plasma separators.

1. Introduction

Microporous polyvinyl alcohol (PVA) hollow-fiber (HF) membranes are currently used as a microfiltration membrane not only in industrial fields but also in medical treatments such as extracorporeal plasmapheresis. The PVA membrane is characterized by its hydrophilicity, thus showing protein-resistant property and acceptable biocompatibility, compared to those of hydrophobic polymers such as polysulfone or polyethylene. The membrane structure is highly crosslinked by acetal linkages with dialdehydes, so that there are small amounts of the

monoreacted dialdehydes remaining on pore surfaces after crosslinking formation, which allow various kinds of chemical modification of membrane. As a practical approach to improve its blood compatibility we have modified the membrane by introducing amine groups utilizing the residual aldehyde groups *via* reductive amination, and attempted to immobilize bioactive agents like heparin onto the aminated membranes (1).

In this study, we have investigatd the chemical modification of PVA HF membranes with several polycationic amine polymers, such as polyethyleneimine or chitosan, and the subsequent immobilization of heparin onto the modified membranes for achieving improved anti-thrombogenicity. Consequently, of amine polymers examined, a water-soluble chitosan, a (1,4)-linked 2-amino-2-deoxy-β-D-glucan was found the most suitable as a heparin immobilizing agent because of the observed highest bioactivity of the immobilized heparin, as well as its non-toxicity and biocompatibility (2,3). Heparin is a common anticoagulant used to prevent blood clotting during surgery and in the treatment of postoperative thrombosis and embolism. It is generally accepted that heparin can enhance the inactivation of clotting proteases such as FXa and IIa (thrombin) by antithrombin III (ATIII) through the formation of a heparin-ATIII complex (4,5). Immobilization of heparin has been frequently used to improve the blood compatibility of biomaterials. Heparin can either be ionically or covalently immobilized on blood-contacting surfaces. Covalent immobilization of heparin is more permanent and therefore more suitable for long-term applications. The bioactivity of immobilized heparin is quite dependent on immobilization methods, and is usually reduced as compared to the activity of free heparin in solution. The activity loss is considered to be due to reduced flexibility of the immobilized heparin chain and/or by a decreased accessibility of the heparin for ATIII and clotting proteases as a result of steric hindrance.

The present paper deals with the *in vitro* study on the anticoagulant properties and water permeability performance of the heparinized PVA HF membranes mainly modified by chitosan.

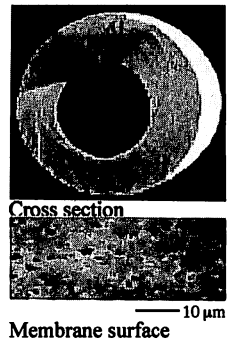
2. Materials and Methods

2. 1. Materials

All the chemicals were of analytical grade or higher and were used as received. The sources of chemicals were as follows: heparin sodium salt (208U/mg), nacalai tesque (Tokyo); chitosan (water-soluble, degree of *N*-deacetylation of >90%, <MW> 13,000), sodium borohydride, 2-mercaptoethylamine hydrochloride, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 2,4,6,-trinitro-benzenesulfonic acid (TNBS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), ethylene diamine (EDA) and sodium nitrite, $NaNO_2$, Wako Pure Chemical Ind. (Osaka); polyethyleneimine (PEI) (branched, <MW> 10,000), Polyscience (Warrington, USA); polyallylamine (PAA)(<MW> 10,000), Nitto Boseki (Tokyo); Acid Orange 7, Tokyo Kasei(Tokyo); sodium triacetoxyborohydride, $NaBH(OAc)_3$, Aldrich (Milwaukee, WI).

2. 2. PVA hollow fibers and determination of residual aldehyde groups

The microporous hollow fibers which were highly crosslinked by glutaraldehyde, having outer dia. of ca. 1mm with a nominal pore size of 0.2 μm were kindly supplied by Kuraray Co., Ltd., Kurashiki, Japan. The HFs were washed with methanol before use. The concentration of residual free aldehyde groups was determined to be 48 μmol/gHF (ca. 0.2mol%) according to the DTNB method (6) after conversion of the aldehyde to $-SH$ group with 2-mercaptoethylamine hydrochloride. Pictures of a microporous PVA HF membrane and its chemical structure are shown in Figure 1.



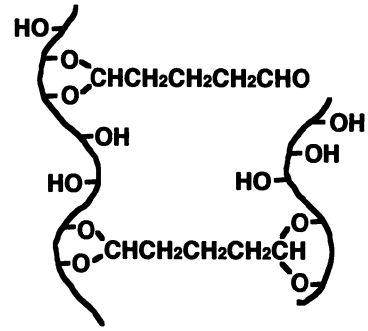


Fig.1. Pictures of PVA HF membrane and its chemical structure.

2. 3. Chemical modification of HF membranes with amine polymers

The chemical modification of HF membranes was performed using cationic amine polymers, that is, water-soluble chitosan, PEI, and PAA as follows: for example, HF membranes were treated in 1% aqueous solution of chitosan containing 50mM $NaBH(OAc)_3$ as a reducing agent at 30℃ for 48h, and then in 2.5% $NaBH_4$ solution for 3h. Quaternization of amino groups of the immobilized chitosan was performed by reacting with methyl iodide in the alkaline medium according to Domard *et al.*(7). These quaternized chitosan immobilized membranes were rinsed with 0.2M HCl solution, and then washed thoroughly with ultrapure water.

2. 4. Immobilization of heparin

The heparin immobilization onto the aminated membranes was carried out in the two different ways, ionic and covalent bonding. Typical examples for heparin immobilization are illustrated in Figure 2 for chitosan modified membranes. Ionically heparinized membrane was prepared by immersing chitosan- or quaternized chitosan-modified membranes in aqueous solution of 0.3% heparin sodium salt (208IU/mg) at pH7.4 for 48h at 30℃, followed by rinsing with 0.9% saline. Secondly, the covalent immobilization was carried out in a similar manner according to a procedure by Larm *et al.*(8). Briefly, 0.3% heparin fragments bearing a terminal reactive aldehyde group(bioactivity ≧80%), obtained by the deaminative cleavage of heparin with nitrous acid was coupled to the primary amino-groups of the modified membrane at pH 3.5 for 48h at 50℃ with 1.5mM $NaBH(OAc)_3$ as a reducing agent, and then the heparinized membrane was washed thoroughly by perfusing aqueous solution of 20% NaCl through the pores to remove ionically bonded heparin. For a comparative experiment, covalent coupling of heparin to chitosan modified membrane was made *via* EDC-mediated amide bond formation according to the conventional technique (9).

The determination of amino groups was performed by Acid Orange 7 and /or TNBS method (10,11) and the concentration of immobilized heparin was measured with spectrophotometric method based on indole reaction in HCl aqueous solutions(12).

2. 5. Bioactivity measurement

Bioactivity of heparinized membranes was measured with a factor Xa inhibition assay using the chromogenic substrate S-2222 (Daiichi Pure Chemicals' reagent kit) according to the manufactures' description. The anticoagulant activity was also evaluated by measuring the coagulation times, i.e., APTT, and prothrombin time (PT) for the plasma solutions preincubated with heparinized membranes at 37℃ for 1 hr using an automatic coagulimeter, Koagulab M J (Ortho Diagnostic System). Briefly, a heparinized HF sample (5mm) was incubated with 300 μl

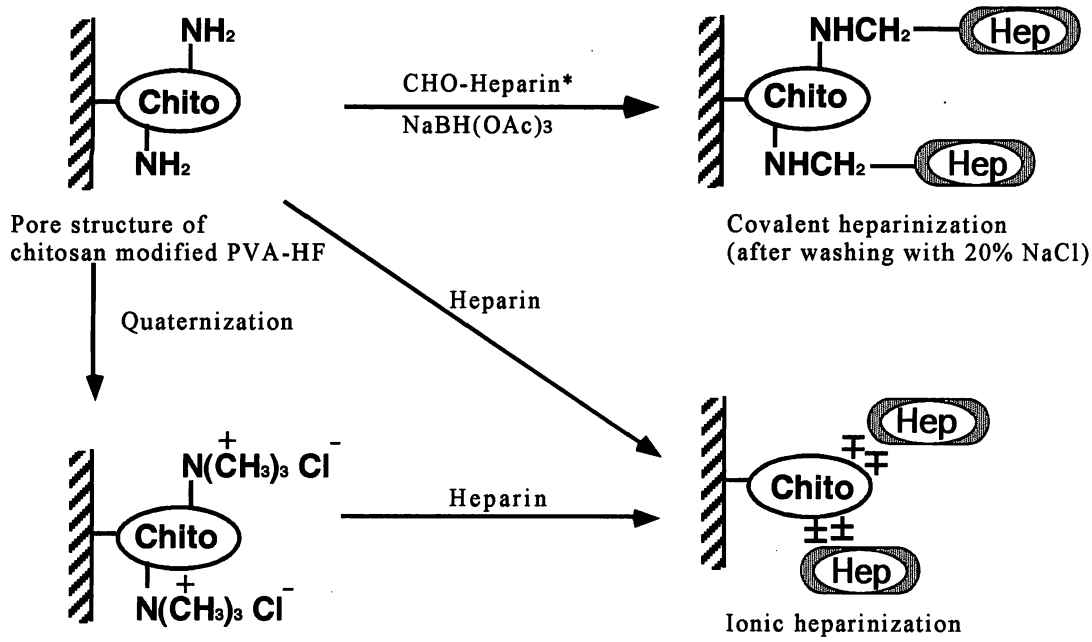


Fig.2. Schematic diagram showing immobilization of heparin onto chitosan or quaternized chitosan modified membranes with ionic and/or covalent bonding.
*Partially degraded heparin bearing a terminal aldehyde group obtained by deaminative cleavage with nitrous acid.

of human plasma dilutions in a polypropylene micro test-tube at 37°C for 1hr. After removal of HF sample, 100 μl of the Ortho APTT reagent (cephalin from bovine brain/ellagic acid) was added to 100 μl of the incubated plasma dilutions, followed by 5 min. incubation, and then APTT was measured after adding 100 μl of 20mM CaCl2 aqueous solution. The PT measurement was performed in a similar manner with the addition of Ortho rabbit brain thromboplastin. Further details of bioassay procedures were described elsewhere (13).

2. 6. Plasma protein adsorption

Plasma protein adsorption onto the membrane was evaluated by the following method. Bovine plasma solutions(1%) in 10mM phosphate-buffered saline (PBS) (pH 7.4) were circulated across a covalently heparinized membrane through pores at a flow rate of about 3ml/min at 37 °C for 15min. After membranes were rinsed with PBS (pH 7.4), the remaining adsorbed proteins were removed with 1% TritonX-100 and 1% SDS in 0.01M NaOH with shaking for 1h, and then the concentration of proteins was determined by micro BCA protein assay kit based on bicinchoninic reagent (Pierce, Rockford, IL).

2. 7. Release of β-thromboglobulin and platelet factor 4

The release of β-thromboglobulin(β-TG) and platelet factor 4 (PF-4) into plasma after incubation of the membranes with human ACD blood was determined as a measure of the extent of platelet activation. Briefly, the membrane samples (6 pieces, each of 2.3cm in length) were incubated with fresh human whole blood containing 15% ACD of 1.2ml in a polypropylene microtube for 30min. The blood was then collected into tubes containing ice-cold citrate buffer supplemented with theophylline, adenosine and dipyridamole, and then centrifuged at 3000

rpm at 4℃ and the platelet poor plasma was collected, followed by freezing at−30℃. The amount of the released β-TG and PF-4 in plasma was assayed by ELISA at SRL, Inc., Tokyo.

2. 8. Permeability measurement of HF membranes

The permeability measurement of heparinized HF membranes was performed in the following manner. Pure water or 0.9% NaCl solution was permeated across the HF membrane from the inside to the outside through its pores at a certain flow rate for a blind-ended HF in 10cm length using an experimental apparatus as shown in Figure 3, and the relationship between pressure drop and flow rate was obtained.

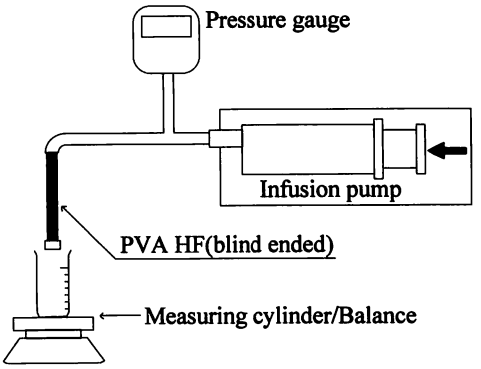


Fig.3. Schematic drawing of experimental flow apparatus.

3. Results and Discussion

3. 1. Anticoagulant activity of heparin immobilized membranes

In Table 1 are represented the results of anti-FXa activity, APTT (intrinsic coagulation) and PT (extrinsic coagulation) for various heparinized HF membranes. As expected, the heparinized membranes could prolong clotting time to considerable extent, and showed extremely high anti-FXa activity, while the chitosan or PEI modified membranes without immobilized heparin showed no anticoagulant activity. The elongation of APTT was much more pronounced than that of PT. It was demonstrated that the chitosan modified membrane generally gave much higher anticoagulant activity of immobilized heparin than PEI or PAA modified membranes, irrespective of the amount of immobilized heparin.

As for ionic immobilization of heparin onto chitosan, several studies have already been reported in order to develop antithrombotic biomaterials. For example, the blood compatibility of chitosan-heparin coated polyethylene shunt tubings was studied by Lambrecht *et al.* as earlier as 1981(14). Also, ionically heparinized chitosan membranes for hemodialysis(15), and the heparin-quaternized chitosan polyionic complex membrane for ultrafiltration(16) were investigated to improve the antithrombogenicity.

Our experimental results were almost consistent with those previous reports. However, it is noteworthy that ionically immobilized heparins were found not to be easily eluted from the heparin-polyamine immobilized membranes, retaining the initial content of heparin and anticoagulant activity even after the long-term incubation of 30 days in 0.9% NaCl with shaking at 30℃, as is seen in Figure 4. In a circulation experiment of 0.9% NaCl across the membrane at a flow rate of about 20ml/h, ionicallly heparinized chitosan-modified membranes still retained

Table 1. Anticoagulant activity of various heparinized HF membranes

Hollow fiber (HF)	Anti-FXa (IU/gHF)	APTT (sec)	PT (sec)	Immobilized heparin(mg/gHF)
None	0	28.0	14.2	0
Unmodified	0	28.4	14.4	0
Chitosan, Q-chitosan	0	28.1～28.4	14.3～14.4	0
PEI, PAA	0	28.1～28.4	14.1～14.2	0
Heparin/chitosan(ionic)	≫27	≫999	≫999	6.5
Heparin/Q-chitosan(ionic)	≫27	≫999	≫999	9.2
Heparin*/chitosan(ionic+covalent)	≫27	≫999	120.9	6.4
Heparin*/chitosan(covalent)	≫27	50.9[a],159.1[b]	14.5[a],18.6[b]	4.5[b]
Heparin/chitosan(EDC,covalent)**	0	28.2	14.3[a]	2.9
Heparin/PEI(ionic)	≫27	154.7	19.1	27.5
Heparin*/PEI(ionic+covalent)	≫27	≫999	28.2	29.8
Heparin*/PEI(covalent)	24.3	34.2[a]	15.3[a]	5.2[a]
Heparin/PAA(ionic)	≫27	6.26	15.3	12.1
Heparin*/PAA(ionic+covalent)	≫27	106.1	18.7	11.6
Heparin*/PAA(covalent)	1.54	28.5[a]	13.5[a]	2.6[a]
Heparin*/EDA(ionic+covalent)	≫27	≫999	26.1	5.8
Heparin*/EDA(covalent)	23.4	40.3[a]	14.4[a]	5.1[a]

HF, 5mm in length(1.25mg); Q-chitosan, quaternized chitosan; Heparin*, CHO-terminated heparin (partially depolymerized by HNO_2); ** Heparin/chitosan immobilized onto HF by EDC-mediated amide bond formation. Ethylene diamine(EDA) was used as a modifier for comparison.

a) Incubated for 3days in 0.9% NaCl at 37℃ after washing with 20% NaCl solution.

b) Incubated for 20days under the same condition as above.

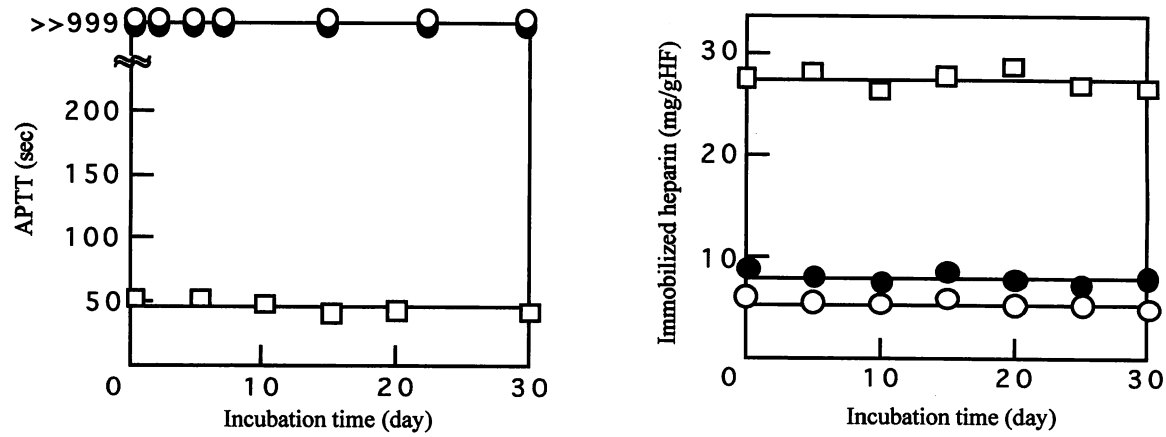


Fig.4. Time course of anticoagulant activity of ionically heparinized HF membranes incubated in an excess of 0.9%NaCl with shaking at 37℃.

HF sample, 2mm in length (0.5mg);

○ heparin/chitosan immobilized HF; ● heparin/quaternized chitosan immobilized HF;

□ heparin/PEI immobilized HF.

*Determined by indole-HCl method.

*ca.*80% of the initial content of heparin after the 0.9% saline perfusion for 20 days. The slow release of heparin may be considered as due to a relatively stable polyionic complexation of chitosan-heparin arising from the structural similarity. Nonetheless,as heparin elution seems to be dependent on a flow rate and ionic strength of the medium, further detailed study is required.

As for covalent heparinizations, the heparinized membrane obtained by an end-point attachment of CHO-heparin to chitosan modified membrane exhibited the highest anti-FXa activity of ≧ 27IU/gHF, and much longer APTT(50~160sec), as compared to those obtained from PEI or PAA modified membranes. On the other hand, a heparinized membrane with multi-point attachment of heparin molecule, which was obtained by EDC-mediated amide bond formation showed no anti-FXa activity, as is seen in Table 1. This result indicates that the heparin chain immobilized by the end point attachment could has an adequate flexibility to bind to ATIII, thus leading to a suppresion of thrombin activity. As is seen in Figure 5, it is interesting that the APTT of covalently immobilized heparin onto chitosan modified membranes increasd significantly during the extended incubation in 0.9% saline for more than a month. The concentration of the immobilized heparin remained almost unaltered (4.5mg/gHF) over a month and the anti-FXa activity increased from 19 up to ≫27, exceeding the measurable limit after one day incubation in 0.9% NaCl at 37℃. However, a free heparin molecule which had been incubated with 20% NaCl restore the original bioactivity immediately by the incubation with 0.9% saline at 37℃. It may be suggested that the highly prolonged APTT is attributed to either possible heparin leakage or the removal of clotting factor proteins involved in the intrinsic coagulation cascade during incubation of heparinized membranes with plasma dilutions. However, the heparin leakage during one hour incubation at 37℃ was confirmed to be negligible by anti-FXa assay. Further study is now under way to clarify this unique phenomenon.

3. 2. Plasma protein adsorption

The plasma protein adsorption on the heparinized membrane was studied as a possible indicator of blood compatibility. Figure 6 shows the result of the plasma protein adsorption onto the covalently heparinized membrane (immobilized by EDC) after contacting with 1% bovine plasma in 10mM PBS (pH7.4), together with two control membranes. The heparinized

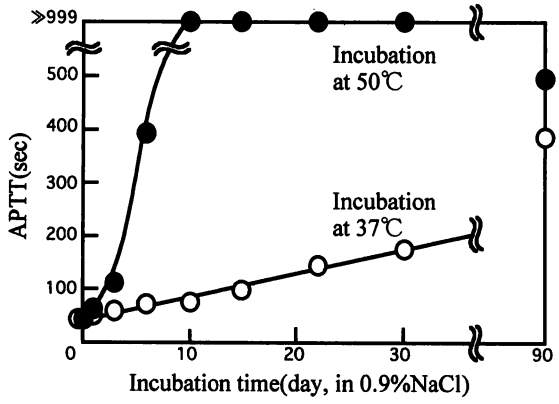


Fig.5. Time course of anticoagulant activity of covalently immobilized heparin after washing heparinized membranes with 20%NaCl. CHO-heparin/chitosan immobilized HF, 5mm.

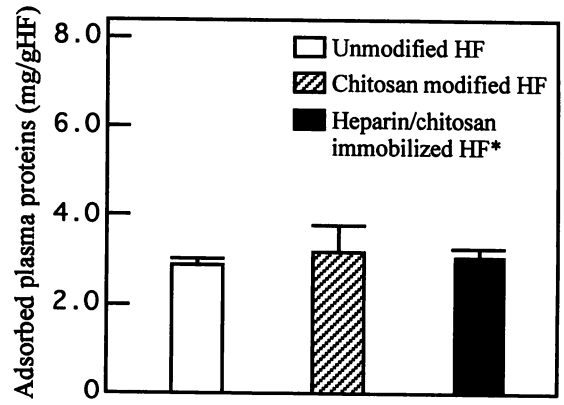


Fig.6. Amount of adsorbed plasma proteins onto three different membranes.
* Heparin was covalently immobilized by EDC method.

membranes showed relatively low protein adsorption, almost similar to the unmodified PVA membrane, probably due to an electrostatic repulsion between anionic heparin and negatively charged plasma proteins.

3. 3. Release of β-TG and PF-4 as a measure of platelet activation

Figure 7 shows the release of β-TG and PF-4 after incubation of the heparinized membranes obtained with CHO-heparin in fresh human ACD blood. The release of β-TG from platelet is considered to be closely related to the extent of platelet activation as well as platelet adhesion (17). Therefore, this determination is quite useful for evaluation of blood compatibility of the heparinized membranes. It is noteworthy that the heparinized membranes, particularly the covalently heparinized membranes exhibitd the least release of β-TG and PF-4, compared to original and chitosan modified membranes. Such a reduction of release of β-TG was also observed for heparin-PEI immobilized HF membranes, as previously described (18). These results suggest that the heparinized membranes have an excellent antithrombogenicity with least platelet adhesion and activation.

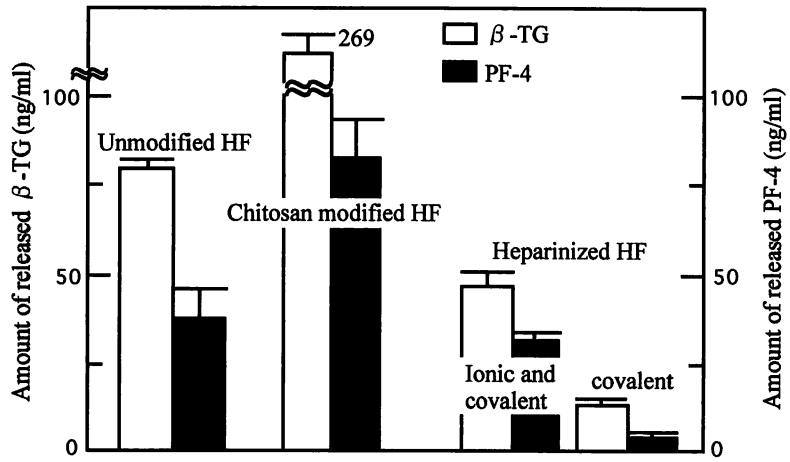


Fig.7. Release of β-TG and PF-4 after incubation of heparinized membranes with ACD blood.
β-TG and PF-4 released were determined by subtracting amounts of polypropylene test tubes (blank), 234 ng/ml and 127 ng/ml, respectively.

3. 4. Permeability of heparinized HF membranes

Permeability of pure water and NaCl solutions through the membranes was briefly investigated. As shown in Figure 8, all the membranes examined exhibited the same linear relationship between pressure drop and flow rate for both water and 0.9% saline, indicating that the water or 0.9% saline permeability across the HF membrane was not affected by chitosan or heparin immobilization. The permeability of plasma proteins across the heparinized membranes is being studied, and will be reported elsewhere. It should be noted that the present methodology of chemical modification has an advantage over usual graft polymerizations of amine monomers onto membranes in that the resulting graft polymers easily induce the clogging of pores to reduce permeability to a significant extent in most cases. Finally, with reference to the effect of NaCl concentration, we used a high ionic strength of 20% NaCl as feed solution, and

observed about twofold pressure drop at the flow rate of 10ml/h, particularly for a covalently heparinized HF membrane, compared to a chitosan modified HF (13). This reduced permeability may possibly be explained as due to the conformational change of heparin chain induced by 20% NaCl, which seemed to be closely related to the observed suppression of anticoagulant activity of the covalently immobilized heparin in 20% NaCl.

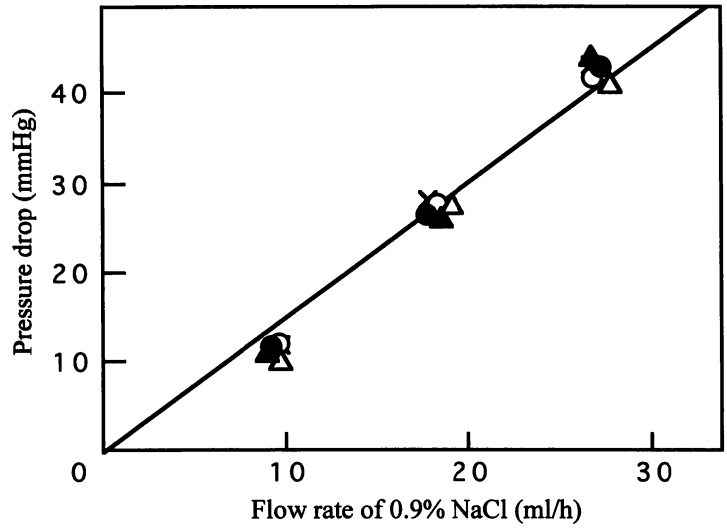


Fig.8. Relationahip between pressure drop and flow rate of 0.9% NaCl aqueous solution.

✕ Unmodified HF; ○ chitosan immobilized HF;
● heparin/chitosan immobilized HF (ionic);
△ heparin/chitosan immobilized HF (ionic+covalent);
▲ heparin/chitosan immobilized HF (covalent).

4. Conclusions

On the basis of the *in vitro* study on the anticoagulant property, blood compatibility and water permeability of the heparinized PVA HF membranes, it was demonstrated that antithrombogenicity of the PVA HF membranes has significantly been improved by heparin-chitosan immobilization without compromising the water permeability. It is expected that the heparinized HF membranes would be useful for applications to blood-contacting devices such as a novel type of intravenous hyperalimentation catheter as proposed by Tsuji *et al.*(19) as well as plasma separators.

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References

1. K.Takakura, H.Hayashi, S.Satoh and N.Hayashi, Preprints of 37th IUPAC International Symposium on Macromolecules, Gold Coast, Australia, July, 12-17, 1998 p.852.
2. S.B.Rao and C.P.Sharma, *J. Biomed. Mater. Res.* **34**, 21 (1997).
3. T.Chandy and C.P.Sharma, *Biomater. Artif. Cell Artif. Org.* **18**, 1 (1990).
4. S.T.Olson, K.R Srinivasan, I. Bjork and J.D.Shore, *J. Biol. Chem.* **256**, 11073 (1981).

5. L.Thunberg, G. Backstrom and U. Lindahl, *Carbohydrate Res.* **100**, 393 (1982).
6. G.L.Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
7. A.Domard, M.Rinaudo and C.Terrassin, *Int. J. Biol. Macromol.* **8**, 105 (1986).
8. O.Larm, R.Larsson, and P.Olsson, *Biomater. Med. Dev. Artif. Organs* **11**, 161 (1983).
9. G.T.Hermason, A.K.Mallia and P.K.Smith,"*Immobilized Affinity Ligand Techniques*", Academic Press, San Diego, 1992, p.81.
10. S.Sano, K.Kato and Y.Ikada, *Biomaterials* **14**, 817 (1993).
11. G.T.Hermason, A.K.Mallia and P.K.Smith, "*Immobilized Affinity Ligand Techniques*", Academic Press, San Diego, 1992, p.286.
12. Z.Dische and E.Borenfreund, *J.Biol.Chem.* **184**, 517 (1950).
13. K.Tsuyutani, Master thesis,Okayama University of Science,Graduate School of Engineering (2000).
14. L.K.Lambrecht, B.R.Young, D.F.Mosher, A.P.Hart, W.J.Hammar and S.L.Cooper, *Trans. Am. Soc. Artif. Intern. Organs* **27**, 380 (1981).
15. M.M.Amiji, *J.Biomater. Sci .Polymer Edn.* **8**, 281 (1996).
16. T.Uragami, H. Mori and Y.Noishiki, *Jpn. J. Artif. Organs* **17**, 511 (1988).
17. I.-K.Kang, Y.Ito, M.Sisido and Y.Imanishi, *J. Biomed. Mater. Res.* **22**, 595 (1988).
18. H.Hayashi, Master thesis, Okayama University of Science, Graduate School of Engineering (1999).
19. F.Mukai, T.Tsuji, T. Fujisato, F.Miyawaki, S. Nakaji and T.Izutsu, *Jpn. J. Artif. Organs* **24**, Suppl. S-108 (1995).