J Artif Organs (2002) 5:141–145

ORIGINAL ARTICLE

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Virus removal from hemoglobin solution using Planova Membrane

Abstract Hemoglobin (Hb) vesicles are artificial oxygen carriers that encapsulate concentrated purified Hb with a phospholipid bilayer membrane. They have been confirmed to have sufficient oxygen-transporting ability. Even though strictly inspected outdated red cells are used as a source of Hb, it is necessary to introduce an additional process that inactivates or removes viruses in the process of Hb purification in order to guarantee the utmost safety from infection. In this study, Hb filtration to remove viruses was tested with Planova 35N and 15N (virus removal fitters with a Bemberg microporous membrane). The permeation flux (LMH) and the permeated ratio of Hb solution ([Hb] = 5.6 g/dl) through Planova 35N at 13°C were 36 l/m^2/h and almost 100%, respectively. The values for Planova 15N at 13°C were 15 l/m^2/h and 95%, respectively. The permeation flux increased to 18 l/m^2/h when the temperature was raised to 25°C. Under the same conditions, a high efficiency of removal of a bacteriophage, ϕX174, was confirmed (>7.7 log). These results indicate that Planova 15N is effective for the process of virus removal from Hb solution.

Key words Oxygen carriers · Hemoglobin vesicles · Hb purification · Virus removal · Bemberg microporous membrane

Received: July 23, 2001 / Accepted: December 27, 2001

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Introduction

Hemoglobin (Hb) vesicles (HbV), which encapsulate Hb with a mixed phospholipid bilayer membrane, have been developed as artificial oxygen carriers.[1,2] The Hb as a raw material is purified from donated and outdated human red blood cells and is concentrated to 40 g/dl. We have confirmed its excellent O_2 transporting ability and safety in preclinical studies of resuscitation from hemorrhagic shock with HbV,[3,4] extreme hemodilution,[5] microcirculatory observation in a hamster skin chamber model and perfused rat livers, and metabolism of HbV in the reticuloendothelial system.[6–8] We are currently studying efficient preparation processes for HbV, especially purification of Hb from human-derived red blood cells. Even if inspected red blood cells are used, careful protection against viruses is essential.[9] We have already evaluated heat treatment for denatur-ation of concomitant proteins and virus inactivation.[10–12] However, virus removal filters should also be examined for application to the Hb purification process as a double protection feature.

Planova is a virus removal filter that was developed by Asahi Kasei Corporation and is made of Bemberg microporous membrane (BMM) hollow fiber membranes. Each membrane has a multilayered pore structure, approximately 150 layers thick, consisting of large, bulky void pores connected by fine capillary pores. While proteins pass readily through this structure, viruses are excluded from passing through the capillary pores, and instead are retained in the large, bulky void pores, thus eliminating them from the purification stream. Planova 35N and 15N have nominal mean pore sizes of 35 and 15 nm, respectively. Planova 35N is suitable for removing viruses ranging from 35 to 100 nm, such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), etc., and Planova 15N is effective for removing viruses of less than 35 nm, such as parvoviruses. However, when the pores of the membrane filter are plugged by impurities, Planova 35N is sometimes used as a prefilter for Planova 15N. Nakai et al. reported the use of Planova for the removal of stroma in the purification of Hb from human red blood cells.[13]

In this paper, we studied more concretely the optimization of the filtration process for a concentrated Hb solution (5.6 g/dl). Most previous reports of Planova studied removal of virus from much diluted protein solutions.[14,15] We evaluated Planova for the Hb solution in terms of filtration pressure, permeation flux, and Hb permeation ratio, and in addition, the removal of a bacteriophage (ϕX174) was tested.

Materials and methods

Purification of the Hb solution

Outdated human red blood cells, provided from the Hokkaido Red Cross Blood Center, were centrifuged at 4000 *g* for 20 min, and the precipitate was dispersed into a saline solution. This procedure was repeated twice. The washed red blood cells were hemolyzed by isovolemic dilution with pure water. After the removal of stroma by ultrafiltration (cutoff MW 1000 kDa, Biomax V screen, Millipore, Bedford, MA, USA), carbonylation of the Hb was carried out with an artificial lung (0.8 m^2, CX-II08, Terumo, Tokyo, Japan). The solution was heated at 60°C for 10 h. The denatured and precipitated proteins were filtered out with Milistack (DE50, Millipore), and the obtained Hb was ultrafiltrated (cutoff MW 1000 kDa, Biomax V screen). The low-molecular-weight components, such as electrolytes, were removed with a Biomax V screen (cutoff MW 8 kDa). After concentration, a purified HbCO solution ([Hb] = 5.6 g/dl, [NaCl] < 0.01%) was obtained.

Permeation test of Hb solution

Planova 35N or 15N (0.01 m^2) was connected to a silicone tube to form a dead-end-type circuit (Fig. 1). The Hb solution was circulated through the Planova module with a peristaltic pump (Econo Pump, Bio-Rad Laboratories, Hercules, CA, USA), and the solution pressure at the entrance was measured with a pressure gauge (Millipore). The permeated solution was received in a volumetric cylinder, and the water level was monitored with a video recorder to calculate the permeation flux (l/m^2/h). The sample was pipetted from the cylinder to measure the Hb concentration with a UV-vis spectrophotometer (cyanomethemoglobin method). The permeation ratio was calculated from the Hb concentrations of the permeate and the original solution. The temperatures in the clean room and the laboratory were maintained at 13° and 25°C, respectively.

Leakage test

According to the procedure in the manufacturer's manual (Asahi Kasei Corporation, Tokyo, Japan), a leakage test was performed. After the permeation test, the Hb solution in Planova 15N was replaced with pure water, and the tube at the exit side was pinched with forceps. The module was compressed with N_2 gas up to 98 kPa for the leakage test to determine whether bubbling occurred.

Virus removal test

A 70-ml bacteriophage (ϕX174) suspension was added to 700 ml of the HbCO solution (5 g/dl). The HbCO solution in an 1-l bottle was connected to Planova 15N and passed through it by a N_2 gas pressure of 80 kPa at 25°C. The permeation flux was measured at the initial (0–3 min) and final (17–19 min) stages, and samples were obtained for virus infection tests at the initial (3–5 min) and final (15–17 min) stages. Titers of ϕX174 bacteriophage were determined by a standard top agar overlay method using *Escherichia coli C* as a host strain. The above tests were performed for three Planova 15N modules.

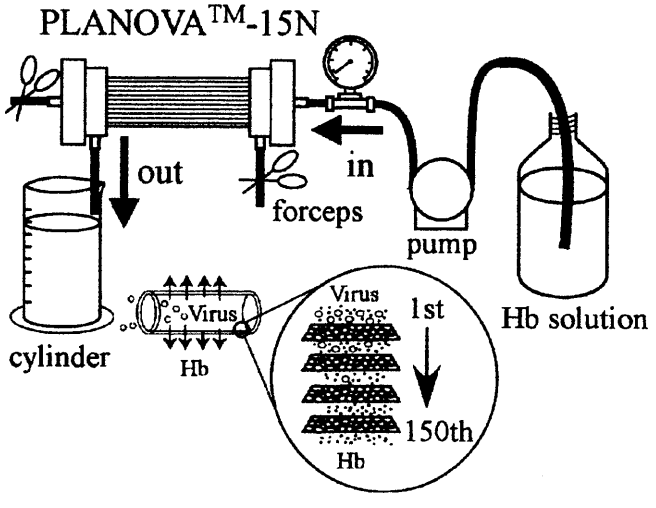

Fig. 1. Schematic representation of the process of virus removal from Hb solution using Planova 15N

Results and discussion

Permeation of the Hb solution for Planova 35N

The relationship between the pumping rate and the filtration pressure at the entrance was obtained using pure water. The upper limit of the pumping rate (6.0 ml/min) resulted in a filtration pressure of 60 kPa, which was less than 98 kPa, the upper limit of the Planova module. Therefore, we determined the pumping rate to be 6.0 ml/min. Figure 2a and b shows the time courses of the permeation flux and the permeation ratio of the 5.6 g/dl Hb solution, respectively. The permeation flux and the permeation ratio were constant at 36 l/m^2/h and 100% for 180 min during the experiment. These results indicate that there is no problem with the use of Planova 35N for filtration of the Hb solution, because the average pore size of Planova 35N is 35 nm, which is significantly larger than

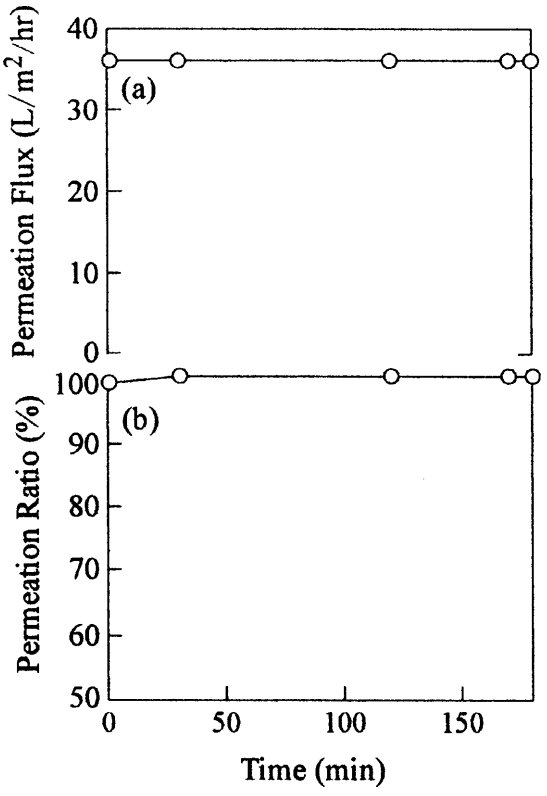

Fig. 2. Time courses of (**a**) permeation flux and (**b**) permeation ratio of the Hb solution ([Hb] = 5.6 g/dl) through Planova 35N

the size of the Hb molecule (5 nm). This also indicates the high purity of the Hb solution and the high uniformity of the pore size of the filter.

Permeation test of Hb solution for Planova 15N

In this test, the temperature was maintained at 13°C, and the Hb solution that had permeated through Planova 35N was used. The permeation flux increased with increasing filtration pressure in the cases of both pure water and the Hb solution (5.6 g/dl), as shown in Fig. 3. A linear relationship was obtained between the solution pressure and the permeation flux, indicating that the microstructure of the BMM membrane does not change over the applied pressure range. Furthermore, the slope of the relation of the Hb solution was half that of water. This might be due to the resistance of the 5-nm Hb molecules to penetration through the 15-nm pore of the BMM membrane, in other words, the higher solution viscosity of the Hb solution. The solution pressure was set at 80 kPa in the following experiments.

Figure 4a and b shows the permeation flux and the permeation ratio of Planova 15N when the 5.6 g/dl Hb solution was used. The permeation flux was constant at 15 l/m^2/h during the experiment. The permeation ratio of Hb was 86% at the beginning but became 95% after 1 h. This can be explained by the remaining water in the module, which should dilute the Hb solution at the beginning before reaching a constant concentration. The permeation ratio of 95% was a good value for the high-Hb solution (5.6 g/dl), because

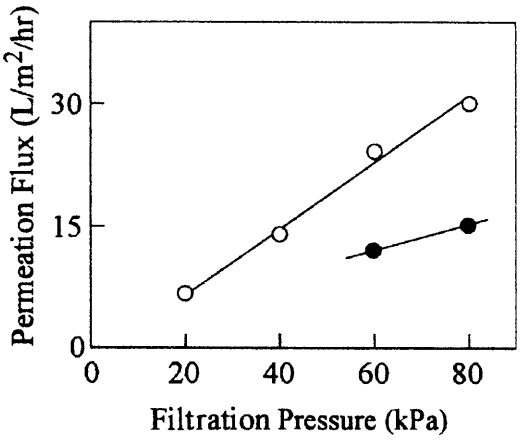

Fig. 3. Relationship between filtration pressure and permeation flux when water for infusion (○) or the Hb solution ([Hb] = 5.6 g/dl) (●) was permeated through Planova 15N

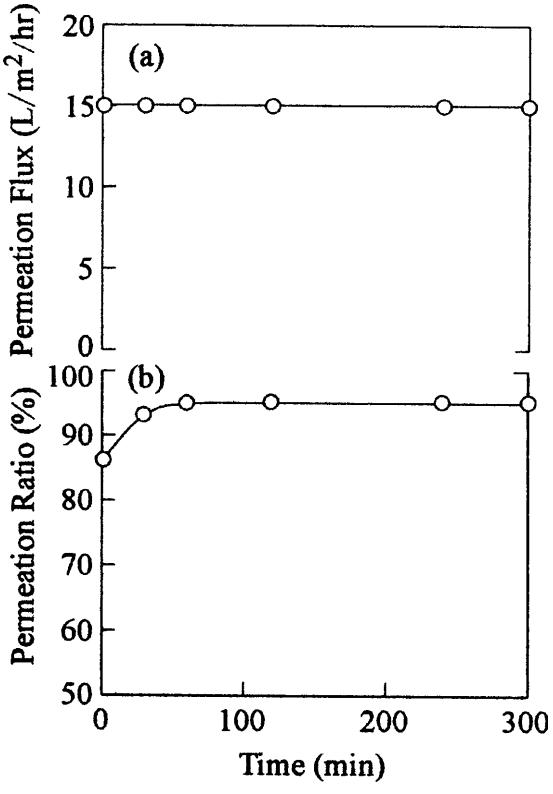

Fig. 4. Time courses of (**a**) permeation flux and (**b**) permeation ratio of the Hb solution ([Hb] = 5.6 g/dl) through Planova 15N

most of the previous studies of Planova used diluted protein solutions.[14,15] This indicates that Planova 15N was applicable to large-scale removal of virus from the Hb solution. In this case, the Hb solution, which had been passed through Planova 35N, was used. However, the second experiment, in which the Hb solution was used without treatment with Planova 35N, showed the same permeation flux and a permeation ratio of 92%. The slightly smaller value should not influence the actual use of Planova 15N. After permeation of the Hb solution through Planova 15N, the removal ratio

Table 1. Removal of virus from Hb solutions by Planova 15N

Experiment no.	Measurement stage[a]	Concentration of Hb solution (g/dl)	Permeation flux ($l/m^2/h$)	Permeation ratio (%)	Virus removal efficiency
1	Initial	4.39	19.8	90.5	>7.7 log
	Final	4.62	20.4	95.3	>7.7 log
2	Initial	4.35	18.8	89.6	>7.7 log
	Final	4.86	18.0	100	>7.7 log
3	Initial	4.25	19.8	87.6	>7.7 log
	Final	4.69	18.6	96.7	>7.7 log

[a] Initial (0–5 min), final (15–19 min)

of the choline-type phospholipid, which is the main stromal lipid component of the red blood cell, was improved to 99.99% (above the detection limit) from 99.70% just after ultrafiltration (cut off Mw: 1000 kDa). This indicates that the molecular assemblies, such as liposomes or micelles of the remaining phospholipids, were removed by Planova 15N.

When the same permeation test was performed at 25°C instead of 13°C, the permeation flux and the permeation ratio were constant at 18 $l/m^2/h$ and 96%, respectively, at 78.5 kPa. Such a difference can be explained in terms of the difference in the viscosity of the Hb solutions: the viscosity was 1.10 cP at 25°C and 1.39 cP at 13°C. It is obvious that the more viscous solution should result in a lower flux.

Virus removal test with Planova 15N

The virus removal test for the 5.6 g/dl Hb solution was carried out three times at 25°C and 80 kPa, and the permeation flux ranging from 18 to 20 $l/m^2/h$ was confirmed to be reproducible. Constant Hb permeation ratios of more than 95% were attained, though some of the initial ratios were less than 90%, probably due to the remaining water at the beginning. As summarized in Table 1, the virus removal efficiency of the bacteriophage (ϕX174) was higher than 7.7 log in all series of dilution of the filtrates. It has been reported that Planova 15N has a removal efficiency for poliovirus of more than 7.6 log.[15] A similar removal efficiency represents the effectiveness of Planova 15N in the removal of the nonenveloped viruses, as well as the enveloped viruses such as HIV. Because we use inspected red blood cells as a raw material, the safety is already high. However, in order to increase the safety with respect to nonspecified viruses, Planova 15N is applicable to the purified Hb solution. Before this process, the Hb solution has already been heated at 60°C for 10 h in a purification process with a virus inactivation efficiency of 6.0 log. Therefore, the safety of the Hb vesicles as an infusion should be increased to an acceptable level (ca. 14 log) in combination with those two processes.

Conclusion

We confirmed that Planova 15N showed an efficiency of removal of a virus (ϕX174) from the Hb solution (5.6 g/dl) of more than 7.7 log while the Hb permeation flux was kept at more than 18 $l/m^2/h$ and the permeation ratio at more than 95%. Quite recently, it was reported that Planova 15N can significantly reduce pathogenic scrapie agents, which threaten transfusion medicine.[16] Therefore, filtration of Hb solution through Planova 15N contributes to the utmost safety from infection.

Acknowledgments The authors would like to thank Mr. M. Yokogi, Mr. G. Ishikawa, and Mr. N. Nakano of the Science and Technology Planova division in Asahi Kasei Corporation for providing Planova filters and for their kind instruction. This work was supported in part by Health Science Research Grants (Research on Advanced Medical Technology, Artificial Blood Project), Ministry of Health, Labour and Welfare, Japan, and Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B-12558112) and the Mukai Science and Technology Foundation.

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