Immobilization of Erythrocytes by Radiation Polymerization of Glass-Forming Monomers at Low Temperatures

Fumio Yoshii, Isao Kaetsu, and Akio Yamada *

Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Watanuki-machi, Takasaki, Gunma-ken, 370-12, Japan

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The immobilization of erythrocyte as the whole cell without hemolysis was studied. It found that erythrocyte could be treated and immobilized stably by radiation polymerization of specific monomers having high viscous and long oxyethylene units chain such as methoxypolyethyleneglycol methacrylate (M-23G) and polyethyleneglycol dimethacrylate (14G). Irradiation dose without hemolysis was limited less than 1×10^5 r and a comonomer system consisting of M-23G-14G, 1:1 and small quantity of glutaraldehyde (GA) was the optimum carrier composition. The functional properties of the immobilized erythrocyte was also investigated. It was found that the immobilized cell could be carried out carbon monoxide-oxygen gas exchange effectively and reversibly so as in the intact cell. The immobilized erythrocyte also showed the catalase activity just as in the intact cell. The stability of erythrocyte increased greatly by the immobilization for standing at low and room temperatures and hardly hemolyzed in non-isotonic medium such as pure water. It was observed in scanning electron microscope that the immobilized erythrocyte had a hollow disk shape same as in intact cell and covered with a thin polymer layer.

Introduction

It is well known that erythrocyte is very labile and easily destroyed to cause so-called hemolysis. Recently, capsulation of erythrocytes by polymer have been studied by some workers for the purpose of utilization as a artificial red blood cells [1, 2]. On the other hand, the immobilized of isolated hemoglobins with 2-hydroxyethyl methacrylate polymer by radiation polymerization was also studied, showing that the immobilized hemoglobins acted as an oxygen carrier so as in the intact hemoglobins [3]. Furthermore, poly(phthaloyl L-lysine) microcapsule including sheep red blood cell hemolysate were also reported by Arakawa and Kondo [4]. However, erythrocytes exists stably only in the presence of isotonic buffer and otherwise resulted in hemolysis. Therefore, it is very difficult to treat erythrocytes stably for immobilization in a whole cell state without causing hemolysis.

The authors found in the previous works that bioactive components such as microbial cell [5, 6] and tissue cell such as chloroplasts [7, 8] could be immobilized stably and effectively by radiation polymerization of glass-forming monomers in a supercooled state at low termperature and immobilized bioactive components increased stability by covering with polymer after immobilization. Especially, the spinach chloroplasts was stably immobilized with the specific long chain monomers at low temperatures and retained the activity about 30% of the original value after 40 days by immobilization, while activity of intact chloroplasts disappeared completely after 2–3 days. It is expected that whole cell erythrocytes might be immobilized without hemolysis and without loosing the original functions by the same immobilization method.

In this report, immobilization of erythrocyte was studied by radiation polymerization of some glass-forming monomers as well as properties of the immobilized erythrocyte.

Materials and Methods

Immobilization of erythrocytes

Erythrocyte was isolated from blood by centrifugation at 3000 rpm for 5 min. The precipitated erythrocyte was suspended in Hanks buffer and then oxyhemoglobin (hemoglobin absorbing oxygen) in erythrocyte was completely converted to carboxy-

* Present address: Institute of Medical Engineering, Tokyo Women's Medical College, Shinjuku, Tokyo, 162, Japan.

Reprint requests to J. Kaetsu.

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hemoglobin by introducing carbon monoxide gas for 10 min. Methoxypolyethyleneglycol methacrylate (M-23 G), polyethyleneglycol dimethacrylate (14 G) or glutaraldehyde, GA (50% in water) as a monomer was dissolved in Hanks buffer of 2 ml. 2.5 ml of the buffer-monomer solution was mixed with 2.5 ml of carboxy-type erythrocyte suspension. For immobilization, the mixture was irradiated by γ-ray from a 100000 ^{60}Co source at 0–4 °C. After immobilization, erythrocyte was cut down to fine piecies and stored polyhydrogel including in buffer at 4 °C.

Gas exchange reaction of oxygen and carbon monoxide of hemoglobin in erythrocytes

Carboxyhemoglobin in intact and immobilized erythrocytes were prepared by carbon monoxide bubbling into buffer containing intact and immobilized erythrocytes of oxyhemoglobin. On the contrary, intact and immobilized erythrocytes of oxyhemoglobin were obtained by oxygen bubbling under illumination of white light into buffer including intact and immobilized erythrocytes of carboxyhemoglobin.

Measurement of UV spectrum

Carrier matrix were destroyed by manual homogenizer, after immobilized erythrocytes were done exchange reaction of carbon monoxide and oxygen gas, which the erythrocytes in carrier occurred hemolysis. The spectra of hemoglobins in hemolysis were observed using UV spectrometer.

Measurement of O_2 uptake

Oxygen concentration in Hanks buffer were prepared to 20 ppm by oxygen gas bubbling. Then, the intact and immobilized erythrocytes were added into oxygen saturated buffer, and oxygen uptake due to hemoglobin were estimated by determining reduction amounts of oxygen in buffer using oxygen electrode under white light illumination from projector.

Measurement of catalase activity

Catalase activity were estimated by determing the amounts of hydrogen peroxide decomposed by the action of the catalase in the intact and immobilized erythrocytes at 4 °C.

Results and Discussion

Survey on condition for stable immobilization erythrocytes

As erythrocyte is very easily hemolyzed by contacting with organic compounds, and suitable monomer as carrier for immobilization was screened. Effect of addition of various monomers on hemolysis was shown in Table I. Hemolysis occurred in case of all hydrophobic monomers addition. However it was found that in the case of hydrophilic monomers such as 14 G and M-23 G no hemolysis occurred. Hemolysis of erythrocytes in buffer occurred hardly even at the increased concentration of M-23 G monomer. But erythrocytes hemolyzed at higher concentration of same monomer than 60%. Moreover, it was found that addition of non-polymerizable materials such as polyethyleneglycol of long chain and high molecular weight (about 1000) caused hardly hemolysis in buffer. In the case of

Table I. Effect of addition by various glass-forming monomers on to erythrocytes in buffer. Monomer concentration in buffer including erythrocytes used for test, 10%. (+) hemolysis occurred, (–) no hemolysis occurred.

Hydrophilic monomers	
2-hydroxyethyl methacrylate (HEMA)	+
2-hydroxyethyl acrylate (HEA)	+
polyethyleneglycol dimethacrylate (9 G)	+
polyethyleneglycol diacrylate (A-9 G)	+
polyethyleneglycol dimethacrylate (14 G)	–
methoxydiethyleneglycol methacrylate (M-4 G)	+
methoxypolyethyleneglycol methacrylate (M-9 G)	+
methoxypolyethyleneglycol methacrylate (M-23 G)	–
N-vinyl-2-pyrrolidone	+
2-hydroxypropyl methacrylate	+
2-hydroxypropyl acrylate	+
acrylic acid	+
Hydrophobic monomers	
diethyleneglycol dimethacrylate (2 G)	+
diethyleneglycol diacrylate (A-2 G)	+
tetraethyleneglycol dimethacrylate (4 G)	+
tetraethyleneglycol diacrylate (A-4 G)	+
glycidyl methacrylate (GMA)	+
glycidyl acrylate (GA)	+
hexanediol dimethacrylate	+
hexanediol diacrylate	+
hexanediol monomethacrylate	+
methyl methacrylate	+
ethyl acrylate	+
ethyl methacrylate	+
neopentylglycol diacrylate	+
trimethylolpropane trimethacrylate	+
trimethylolpropane triacrylate	+
trimethylolethane trimethacrylate	+

chloroplast treatment, it inactivation was remarkably retarded in the presence of buffer including viscous solvents and long chain monomers such as M-23 G, glycerine and polyethyleneglycol (PEG) as protectant [8]. From these results, it was deduced that compounds having relatively high viscosity prevents hemolysis of erythrocytes and the suitable for carrier materials.

Hemolysis occurred considerably by irradiation of relatively higher irradiation dose than 2×10^5 r. Authors [7] found previously that inactivation of the chloroplast by irradiation was prevented at low temperatures such as -78 °C and -24 °C and dose up to 2×10^6 r. Erythrocyte was more sensitive for monomer contact and irradiation, though chloroplast was very unstable for these factors in comparison with microbial cells and enzymes. In all case, erythrocytes changed its color from red to black according to the occurrence of hemolysis. Consequently, erythrocytes could be immobilized without hemolysis using specific long chain monomers such as M-23 G and 14 G and with relatively lower irradiation dose. In the case of chloroplast, viscous and long chain compound were effective for the protection against freezing at low temperatures. Erythrocytes in buffer was readily hemolyzed by freezing at temperatures below 0 °C. This hemolysis of erythrocytes was not prevented completely even in the presence of protectant such as PEG or glycerine. Therefore, the erythrocytes was immobilized by radiation polymerization at 0–4 °C with lower dose than 1×10^5 r without hemolysis. However, the polymerization of M-23 G or 14 G monomers in natural erythrocytes suspension was not completed in low irradiation dose, such as 1×10^5 r and sometimes erythrocytes was leaked out from polymer matrix. This fact suggested that oxygen absorbed in erythrocytes acted as polymerization inhibitor for M-23 G or 14 G monomers. Therefore, oxyhemoglobin including erythrocyte was converted to carboxyhemoglobin including one by carbon monoxide bubbling. It was found that M-23 G or 14 G were easily polymerized in the presence of carboxyhemoglobin including erythrocyte without hemolysis with irradiation dose of 1×10^5 r at 0 °C. The M-23 G polymer swelled in buffer and became very brittle, while the 14 G polymer swelled in buffer was rather hard. So, the immobilization by copolymerization of M-23 G and 14 G comonomers were investigated. Optimum comonomer composition was 10% of M-23 G-14 G (1:1 in volume) and 90% buffer. However, the immobilized erythrocyte had a tendency to leak out slightly from the swelled copolymer after storage for a long period. Then, glutaraldehyde (GA) was further added at a concentration of 0.5–0.1% in a comonomer composition in order to increase the crosslinked structure. Consequently, the optimum carrier composition for stable immobilization of erythrocyte whole cell without hemolysis was 50–60% M-23 G, 40% 14 G and 5–10% GA. For erythrocytes, GA hardly caused hemolysis at low concentration below 2%. But, color of erythrocytes changed in concentration above 3% of GA.

Oxygen-Carbon monoxide exchange activity in immobilized erythrocytes

It is well-known that oxyhemoglobin and carboxyhemoglobin have characteristic absorption in UV spectra. UV spectra of oxyhemoglobin and carboxyhemoglobin in the immobilized erythrocytes were shown in Fig. 1. Spectrum was measured using the sliced sample of block form immobilized erythrocyte as polymerized. In this case, definitive absorption do not appeared in the spectrum. This fact was attributed to that prevention of transmission of UV light by polymer carrier. The same result was obtained for hemoglobin in ghost-free hemolysis microcapsulated within poly(phthaloyl L-lysine) [9]. Therefore, spectra were measured again using the crushed sample of immobilized erythrocytes by manual homogenizer, after carbon monoxide-oxygen gas exchange reaction in erythrocytes were carried out.

These spectra were 2–5 in the Fig. 1. Spectra 2 and 4 were measured after immediately immobilization. This spectra corresponds to carboxyhemoglobin and was completely same as that before immobilization. Spectra 3 and 5 were obtained after oxygen gas bubbling for 10 min under illumination of white light to the samples (spectra 2 and 4) after immediately immobilization. These spectra corresponds to oxyhemoglobin. The oxyhemoglobin within immobilized erythrocytes in buffer could be converted to carboxyhemoglobin by carbon monoxide bubbling. These facts proved that oxygen and carbon monoxide gas exchange reaction of hemoglobin in erythrocyte occurred reversibly in the immobilized whole cell just in intact erythrocyte.

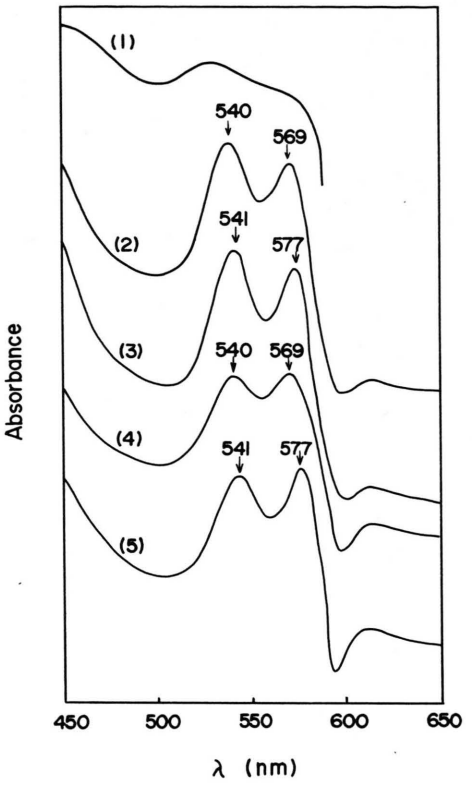

Fig. 1. UV absorption spectra of hemoglobin in immobilized erythrocytes. Sample of spectrum (1) sliced the immobilized erythrocyte. Sample of spectra (2)–(5) homogenized by manual homogenizer the immobilized erythrocyte. Monomer composition: (1) M-23G 5%-14G 5%, (2) M-23G 5%-14G 5%, carboxyhemoglobin, (3) M-23G 5%-14G 5%, oxyhemoglobin, (4) M-23G 5%-14G 4.5%-GA 0.5%, carboxyhemoglobin, (5) M-23G 5%-14G 4.5%-GA 0.5%, oxyhemoglobin. Irradiation dose, 1×10^5 r at 4 °C.

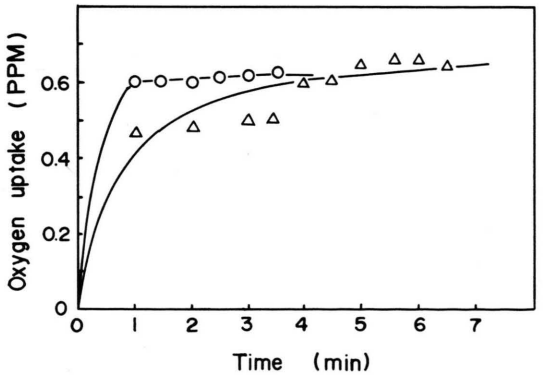

Fig. 2. Oxygen uptake of hemoglobin in intact and immobilized erythrocytes. (○) intact erythrocyte, (△) immobilized erythrocyte. Monomer composition, M-23G 5%-14G 4.5%-GA 0.5%. Irradiation dose, 1×10^5 r at 4 °C.

The UV spectra absorption almost unchanged by the polymer composition as the carrier. The oxygen uptake function in the immobilized erythrocyte in buffer was shown in Fig. 2. Intact and immobilized erythrocytes added into oxygen saturated buffer under illumination of white light. According to this result, absorption of oxygen in the immobilized erythrocytes were clearly recognized as well as in the intact one. This fact supported that the absorbed carbon monoxide in the immobilized erythrocyte as the whole cell covered with polymer was effectively replaced by oxygen.

Catalase activity in immobilized erythrocytes

It is that the erythrocyte contain eleven kinds of enzymes in the cell. Catalase was one of the enzyme. The catalase activity was tested as one of the function in the immobilized cell. Fig. 3 showed change of hydrogen peroxide decomposition using the immobilized erythrocyte. According to this result, decomposition of the hydrogen peroxide by catalase increased with increasing time. On the other hand, in the intact erythrocyte, hemolysis occurred rapidly during idone titration for catalase activity assay and an end point or titration was hardly determined. The intact erythrocyte was indicated qualitatively a little higher catalase activity than that of immobilized one. However, enzyme hardly released out in the immobilized erythrocyte due to hemolysis and could be used for a long time

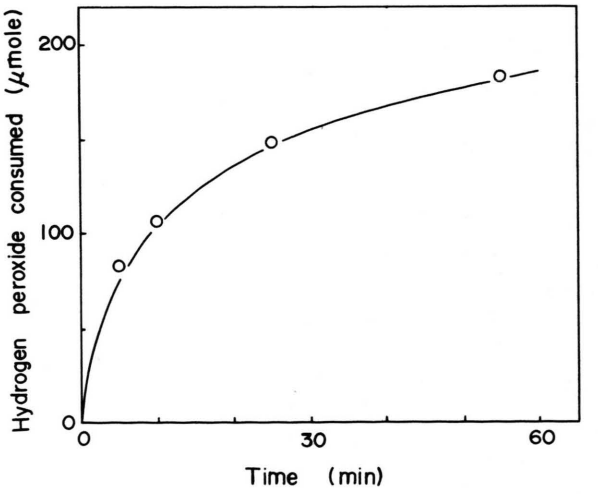

Fig. 3. Hydrogen peroxide decomposition by catalase in immobilized erythrocytes. Monomer composition, M-23G 5%-14 G 4.5%-GA 0.5%. Irradiation dose, 1×10^5 r at 4 °C.

repeated decomposition of hydrogen peroxide, while the life of intact erythrocyte was short. The results were shown in Fig. 4. Catalase activity was almost similar in early stage to several time repetition in the immobilized erythrocyte.

Scanning electron microscope observation of intact and immobilized erythrocytes

Fig. 5 shows the picture in scanning electrophotographs of intact and immobilized erythrocytes. It is known that intact erythrocyte has a disk-like circle shape of about 5–10 μm diameter having a hollow in the center. According to this microphotographs, diameter of the used intact erythrocyte was 7 μm, indicating typical disk shape of erythrocyte. It was observed that the immobilized erythrocyte also showed the circle shape just as in the intact cell. The most erythrocytes were covered with thin polymer layer and some of them showed clearly the hollow circle form. This fact certified again that erythrocyte was stably immobilized in a whole cell state covered and protected by the thin polymer layer without losing the original functions.

Stabilization of immobilized erythrocytes

Intact erythrocyte was stable only in isotonic buffer and rapidly hemolyzed in unisotonic solution such as pure water or pure water including sodium chloride and ethanol of low concentration (0.1–0.5%). On the other hand, the immobilized erythrocyte was hardly hemolyzed even in the unisotonic mediums. Moreover, intact erythrocyte was hemolyzed completely in standing at 25 °C for one day and at 4 °C for 7 days. On the other hand, the immobilized erythrocyte was hardly hemolyzed even after 5 days at 25 °C and 14 days at 4 °C, though red color of the immobilized erythrocyte was decolorized slightly after 15 days at 4 °C. It was obvious that erythrocyte was considerably stabilized by immobilization. Further, study for stabilization and utilization as a artificial cell by immobilization technique would be done in the near future.

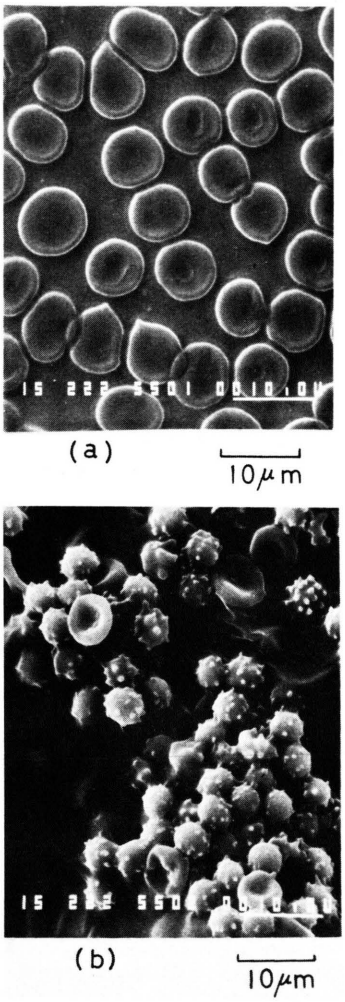

Fig. 5 Scanning electron micrograph of intact and immobilized erythrocytes. (a) intact erythrocyte, (b) immobilized erythrocyte. Irradiation dose, 1×10^5 r at 4 °C.

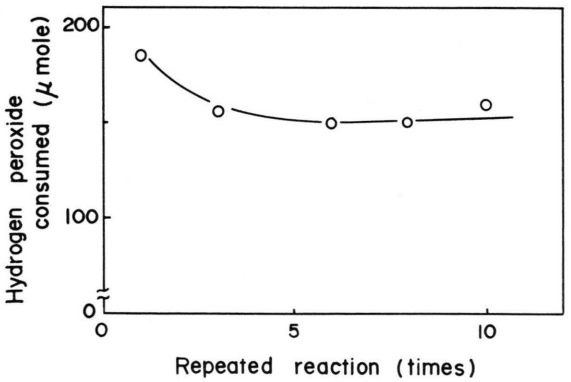

Fig. 4. Catalase activity in immobilized erythrocytes. Monomer composition, M-23G 5%-14G 4.5%-GA 0.5%. Irradiation dose, 1×10^5 r at 4 °C.

Acknowledgement

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[1] M. Kitajima, W. Sekiguchi, and A. Kondo, Bull. Chem. Soc. Jap. **44,** 139 (1971).
[2] G. Pilwat, P. Washausen, J. Llein, and U. Zimmermann, Z. Naturforsch. **35 c,** 352 (1980).
[3] M. Miki and K. Hayashi, Polymer Preprints, Japan, **28,** 1234 (1979).
[4] M. Arakawa and T. Kondo, Can. J. Physiol. Pharmacol. **55,** 1378 (1977).
[5] M. Kumakura, M. Yoshida, and I. Kaetsu, European J. Appl. Microbial. Biotechnol. **6,** 13 (1978).
[6] M. Kumakura, M. Yoshida, and I. Kaetsu, Applied Environmental Microbiology **37,** 310 (1979).
[7] T. Fujimura, F. Yoshii, I. Kaetsu, Y. Inoue, and K. Shibata, Z. Naturforsch. **35 c,** 477 (1980).
[8] F. Yoshii, T. Fujimura, and I. Kaetsu, Biotechnol. Bioeng. **23,** 833 (1981).
[9] T. Kondo, Gendaikagaku, December, 32 (1979).