Vol. 97, No. 3, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
December 16, 1980 Pages 1076-1081

MODIFICATION OF HUMAN HEMOGLOBIN WITH POLYETHYLENE GLYCOL : A NEW CANDIDATE FOR BLOOD SUBSTITUTE

Katsumi Ajisaka and Yuji Iwashita

Ajinomoto Co., Inc., Central Research Laboratories

1-1 Suzuki-cho, Kawasaki-ku, Kawasaki-shi, 210 Japan

Received October 27,1980

Human hemoglobin was modified with polyethylene glycols. The conjugates exhibited P_{50} values of 10-15 mmHg, those are enough to deliver oxygen from the lungs to tissues. The most remarkable characteristic is their long half disappearance time from the circulation. The longest half disappearance time of these derivatives is about 180 minutes in contrast to 45 minutes of free hemoglobin. The half disappearance time shows a good corelation not to molecular weight but to the effective molecular size, which is determined by the elution time of HPLC on a gel permeation column.

In many approaches to artificial blood, the use of the stroma free hemoglobin seems most promising (1,2). However the infused hemoglobin is known to disappear from the circulation in a short time and so many researchers have tried to extend the residence time in the circulation by increasing the bulkiness of hemoglobin; for example they prepared the polymerized hemoglobin with the help of glutaraldehyde (3) and the dextran-hemoglobin conjugates (4-6). But the need for more biocompatible oxygen carriers still remains because some compounds of them show high viscosity and some have too tight oxygen affinity to supply it to tissues.

Recently Abuchowski et al (7) reported that conjugation of polyethylene glycol to albumin reduces its antigenicity and extend its residence time in the circulation (8). We modified the hemoglobin with polyethylene glycols of different molecular weight and tried to see the effect of molecular size on the disappearance time from the circulation. We would like to report in this

0006-291X/80/231076-06$01.00/0

communication the characteristics of the polyethylene glycol modified hemoglobin as a potential candidate for the blood substitute.

MATERIALS AND METHOD

A stroma free hemoglobin solution was prepared according to the method of Savitzky et al (9), from outdated human blood. The reaction of polyethylene glycol with hemoglobin was followed by the procedure of Abuchowski et al (7). The reaction mixture was analyzed with a JASCO Trirotar HPLC apparatus equipped with a TSK G 3,000 SW column (7.6x600 mm). The eluates were monitored with a Uvidec 100 II W visible spectrometer and a Shodex SE-11 refractive index detecter. The modified hemoglobin solution was treated with an Amicon UM 30 menbrane filter untill the unreacted polyethylene glycol or hemoglobin is no more detected. The number of the coupled polyethylene glycol was determined by the following two methods; one is by comparison of peak areas of carbonyl carbons of the hemoglobin with methylene carbons of the polyethylene glycol in carbon-13 NMR spectrum measured by a Varian XL-100 NMR spectrometer and the other is by measuring of the net weight after freeze-drying the modified hemoglobin solution of the known concentration. The absorption spectra were measured with a Hitachi 320 spectrometer and oxygen dissociation curve (and also P_{50} value) was obtained by an Aminco Hem-O-Scan. The disappearance time from the circulation was measured as follows; male Spaque Dawley rats (200-300 g) were anesthetized by ip. injection of 400 mg/Kg pentobarbital. After phlebotomy of 5 ml/Kg blood through a polyethylene catheter inserted in femoral vein, the same volume of the hemoglobin or polyethylene glycol-hemoglobin conjugate (4-6 % concentration) was infused through the same catheter. Aliquots (0.5 ml) of the blood were collected at 5, 10, 30, 60, 90, 120 minutes after infusion and the concentration of the injected samples in the plasma was determined by the cyanomethemoglobin method after centrifugation. The half disappearance time was obtained from semilogarithmic plots of concentration against the time after the infusion.

RESULTS AND DISCUSSION

UV and visible spectra of the modified hemoglobin were the same as those of intact hemoglobin regardless of the kind or the number of the coupled polyethylene glycols (Figure 1). The molecular weight of the conjugates was plotted against the retention volume of a HPLC equipped with a TSK G 3,000 SW column (Figure 2). Subunits of hemoglobin and its derivatives seem to dissociate into monomers in this column (10). The semilogarithmic plot of molecular weight of the polyethylene glycol-hemoglobin conjugates against the retention volume is not the same as that of globular proteins. Furthermore it is noteworthy that the gradient in Figure 2 is dependent on the molecular weight of polyethylene glycol used for the modification. A series of modified hemoglobins from smaller polyethylene glycol exhibited shorter elution time than

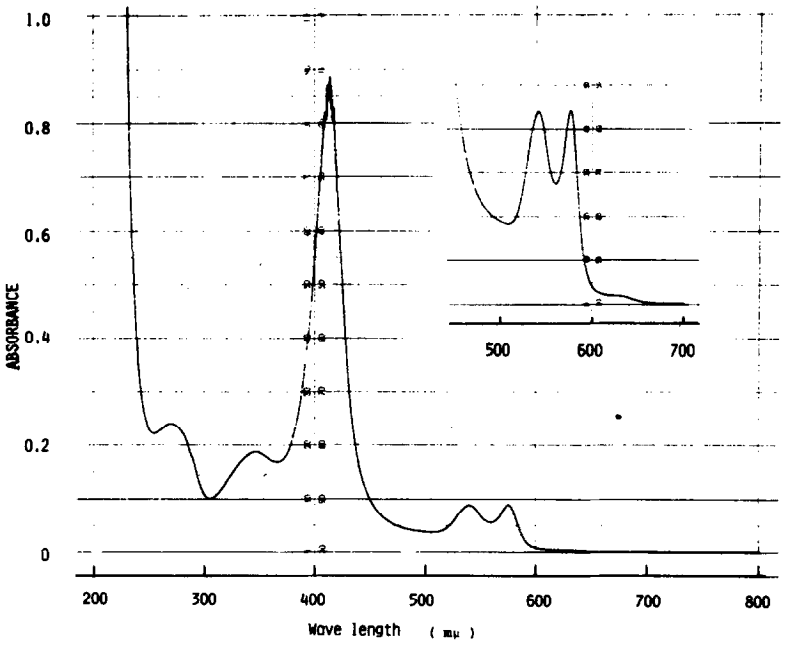
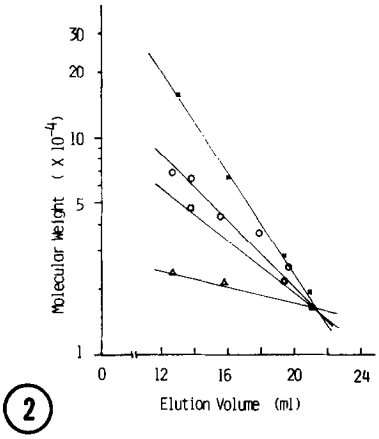


Figure 1. Absorption spectrum of the polyethylene glycol (4,000 dalton)-hemoglobin conjugate.

that from larger polyethylene glycol, even though both have the same molecular weight. The result means that, between the modified hemoglobins of the same molecular weight, the effective size of the conjugates from smaller polyethylene glycols is bulkier than that from larger polyethylene glycols, and it is the reason why the former elutes faster.

Figure 3 represents the relation between the half disappearance time and the retention volume in HPLC. The half disappearance time has better corelationship with the effective size of the conjugates than with the molecular weight. For example, two modified hemoglobins of molecular weight 70,000 obtained from the polyethylene glycol 4,000, and of 48,000 obtained from the polyethylene glycol 1,900, which eluted at 13.5-14 ml of retention volume, and showed the almost equal half disappearance time (95-110 minutes). From Figure 2, the molecular size of these two modified hemoglobins correspond to a globular protein with molecular weight of about 100,000. As it is known widely that the upper limit of the glomerular filtration is about 100,000 for



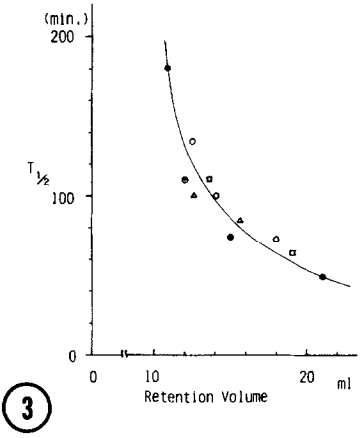


Figure 2. Plots of the retention volume in HPLC versus the molecular weights of the hemoglobin (•), the globular protein (×——×), and the modified hemoglobin with polyethylene glycols [4,000 dalton (○——○), 1,900 dalton (□——□), 750 dalton (△——△)]. TSK G 3,000 SW column was used with 0.05 M phosphate buffer (pH 7.0) at 1 ml/min.

Figure 3. Plots of the retention volume versus half disappearance time ($T_{1/2}$) of hemoglobin (•) and the modified hemoglobin with polyethylene glycols [5,000 dalton (◎), 4,000 dalton (○), 1,900 dalton (□), 750 dalton (△)]. The condition in HPLC is same as in Figure 2.

globular proteins, these two modified hemoglobins described above may not be cleared through the kidneys into urine.

The half disappearance time in the plasma of rats of modified hemoglobins is summarized in Table 1. The half disappearance time of the stroma free hemoglobin of our experiment (45 minutes) is cosiderably short compared with 3.5 hours reported by DeVenuto et al (11). As reported by Mok et al (12) that the half disappearance time depends strongly on the amount of the infused solution, the difference, pointed out above, may be ascribed to the difference of the infused volume, namely we infused only 10 percent of the total blood volume, while they exchange-transfused 91-93 percent. Consequently, the half disappearance time of the polyethylene glycol-hemoglobin conjugate will survive longer if a larger amount of the blood is exchange-transfused.

The oxygen dissociation curve of the modified hemoglobin in Figure 4 scarcely showed sigmoidicity or the Bohr effect when pH of the solution was changed. The same phenomenon was observed for all the polyethylene glycol-

Table 1

Characteristics of the polyethylene glycol-hemoglobin conjugates.

mol. wt. of polyethylene glycol	retention[a] volume in HPLC	number of[b] polyethylene glycol	mol. wt.[b]	P_{50}	half disappearance time
750	12.6 ml	8.8	24,000	11.5 mmHg	100 min.
	15.6	6.6	22,000	13.0	85
1,900	13.5	15.5	48,000	11.5	110
	19.0	3.6	23,000	14.7	65
4,000	12.4	16.0	85,000	11.5	135
	14.0	12.6	70,000	9.5	100
5,000	10.9	14.7	91,000	10.5	180
	12.0	12.0	78,000	10.5	110
	15.0	6.6	50,000	12.0	75
hemoglobin	21.0	----	16,100	15.5	45

a) In HPLC, TSK G 3,000 SW column was used with 0.05 M phosphate buffer (pH 7.0) at 1 ml/min.
b) Values for one subunit of hemoglobin.

hemoglobin conjugates. So it may be suggested that the polyethylene glycols attach preferentially to the amino groups of α - 1 Val, β - 82 Lys, and/or β - 40 Lys, which are recognized to have the strong contribution to the cooperativity and the alkaline Bohr effect (13). The P_{50} values in Table 1, though it seems a little smaller than that of the intact hemoglobin, would be enough

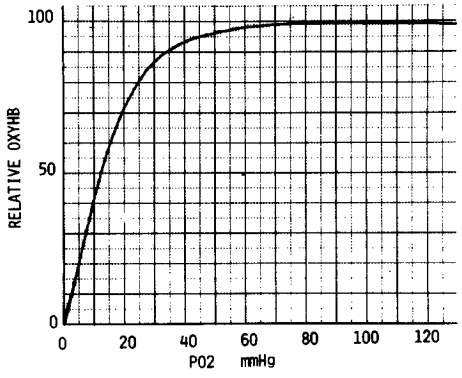


Figure 4. Oxygen dissociation curve of the polyethylene glycol (4,000 dalton)-hemoglobin conjugate in 0.1 M NaCl solution at pH 7.0.

values for oxygen delivery to tissues, for Tam et al (6) reported that dogs continued to survive when 95 percent of the total blood volume was exchange-transfused with dextran-hemoglobin conjugate which showed 2.5 fold greater oxygen affinity than the free hemoglobin.

From the viewpoint of blood substitute, the modified hemoglobin with smaller polyethylene glycol seems superior to that from the larger polyethylene glycol, but the former has a disadvantage that a large number of polyethylene glycol must be linked in order to get the enough molecular size, and also such modification makes the conjugates unstable to irreversible oxidation. Consequently, the modification by the polyethylene glycol of 4,000-5,000 daltons seems practical preparation for the blood sbstitute.

REFERENCES

1. Palani, C. K., Dewoskin, R., and Moss, G. S., (1977) Surg. Clin. North. Am. 55, 3-10.
2. DeVenuto, F., Friedman, H. I., Neville, J. R., and Peck, C. C. (1979) Surgery 149, 417-436.
3. Payne, J. W. (1973) Biochem. J. 135, 867-873.
4. Tam, S. C., Blumenstein, J., and Wong, J. T. F. (1976) Proc. Natl. Acad. Sci. USA. 73, 2128-2131.
5. Chang, J. E. and Wong, J. T. F. (1977) Can. J. Biochem. 55, 398-403.
6. Tam, S. C., Blumenstein, J., and Wong, J. T. F. (1978) Can. J. Biochem. 56, 981-984.
7. Abuchowski, a., MoCoy, J. R., Palczuk, N. C., Es, T. V., and Davis, F. F. (1977). J. Biol. Chem. 252, 3582-3586.
8. Savoca, K. V., Abuchowski, A., Es, T. V., Davis, F. F., and Palczuk, N. C. (1979) Biochem. Biophys. Acta. 578, 47-53.
9. Savitzky, J. P., Doczi, J., Black, J., and Arnold, J. D. (1978) Clin. Pharmacol. Ther. 23, 73-80.
10. Hashimoto, T., Sasaki, H., Aiura, M., and Kato, Y. (1978) J. Chromatogr. 160, 301-305.
11. Devenuto, F. Moores, W. Y., Zegna, A. I., and Zuck, T. F. (1977) Transfusion 17, 555-562.
12. Mok, W., Chen, D. E., and Mazur, A. (1975) Fed. Proc. 34, 1458-1460.
13. Peruz, M. F., Kilmartin, J. V., Nishikura, K., Fogg, J. H., Butler, P. J. G., and Rollema, H. S. (1980) J. Mol. Biol. 138, 649-670.