J. Biochem. Mol. Biol. Vol. 29, No. 3 pp. 261~265 May 31, 1996

Chemical Modification of Sheep Hemoglobin with Methoxy-Polyethylene Glycol

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(Received February 22, 1996)

Abstract: Sheep hemoglobin (SHb) was modified with methoxy-polyethylene glycol (mPEG) to develop a potential blood substitute. mPEG has been used to decrease antigenicity and immunogenicity of foreign proteins. When the mPEG was attached to SHb, the modified hemoglobins showed decreased electrophoretic mobility on SDS-PAGE and decreased free amino groups. When the remaining free amino groups of mPEG modified SHb were determined by TNBS free amino group titration methods, about 34% of total free amino groups were modified with mPEG. This mPEG-SHb conjugate of 34% amino groups modified showed no precipitation by double immunodiffusion with polyclonal antibodies against SHb. This modified hemoglobin still has oxygen transport activity. So this antigenicity decreased hemoglobin may be used in humans as a potential blood substitute.

Key words: blood substitute, chemical modification, methoxy-polyethylene glycol, sheep hemoglobin.

Chemical modification of proteins was initially developed to clarify protein structure, especially the states of amino acid residues in proteins, using various kinds of modifiers which interact with specific amino acids (Vallee *et al.*, 1969). Thereafter, study on the modification of proteins with synthetic polymers such as polyethylene glycol and polyaspartic acid derivative has been used to alter immunoreactivity or immunogenicity of antigen proteins (Matsushima *et al.*, 1980) or suppress IgE production of allergenic proteins (Lee *et al.*, 1977). Attachment of methoxy-polyethylene glycol (mPEG) to proteins has found wide applications for biomedical and biotechnological purposes. mPEG modified proteins show both reduced antigenic and immunogenic properties and extended blood-circulating lifetimes, features which allow the use of such proteins as drugs (Abuchowski *et al.*, 1977). Perhaps the most promising use of mPEG is in making blood substitutes. The protein hemoglobin is the part of blood that carries oxygen from lungs to peripheral tissues. But hemoglobin molecules that are not encased in red blood cells are quickly removed from the blood by the body. Antigenicity and immunogenicity may be the problem inhibiting the use of animal hemoglobin as blood source (Fronticelli *et al.*, 1986). There are several reasons for exploring the usage of sheep hemoglobin in the quest for hemoglobin-based oxygen carriers. Sheep blood is available in practically unlimited amounts and may constitute an indispensable alternative in case of catastrophic events involving large numbers of individuals. Fluids containing SHb derivatives could be conveniently used in veterinary medicine and surgery. A variety of other applications can be envisaged where the utilization of human hemoglobin may be both unnecessary and too expensive; such as animal experimentation, organ perfusion and organ storage. With regard to the potential antigenicity of sheep hemoglobin, it is relevant to stress that the development of a hemoglobin-based oxygen carrier requires some modifications of hemoglobin molecules. The mPEG modification technique may be useful to SHb for blood substitute development. The polymers selected for attachment were the methoxy-polyethylene glycols, with the following general structure $CH_3(OCH_2CH_2)_nOH$. These nonimmunogenic compounds are linear, uncharged, and available in various sizes. They are water soluble by virtue of hydrogen bonding of three water molecules per ethylene oxide unit (Abuchowski *et al.*, 1977). The single terminal hydroxyl group is available for coupling reactions. The hydroxyl groups of mPEG may be activated by cyanuric chloride ($C_3N_3Cl_3$) and several other reagents and then coupled to lysine ε-amino groups of proteins (Beauchamp *et al.*, 1983).

In this study, we tried to overcome the problems,

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mainly antigenicity and immunogenicity, by chemical modification with mPEG when animal hemoglobin was used as a blood substitute.

Materials and Methods

Materials

Sheep Red Blood Cell (SRBC) used in this experiments was prepared from Sheep blood which was purchased from Dong-Seong Medical Co. (Han-Gook Media-Co. Korea). Poly(ethylene)glycol methyl ether (mPEG), with an average molecular weight of 5000, and cyanuric chloride were purchased from Aldrich Chemical Co. (Milwaukee, USA). Benzene and petroleum ether (b.p. 36.6~56.5℃) were purchased from Allied Fisher Chemical Co. (Pittsburgh, USA). SDS (sodium dodecyl sulfate), Tris-base, TEMED (N,N,N',N'-tetramethylethylenediamine), acrylamide, bis-acrylamide were from Sigma Chemical Co. (St. Louis, USA). Other chemicals were of analytical reagent grade.

Sheep hemoglobin preparation

SRBC was prepared from whole blood according to Dellacheri *et al.* (Dellacherie *et al.*, 1983). SHb was prepared from SRBC as described (Barksdale *et al.*, 1975). The preparation of hemolysates from mammalian red cells is particularly easy because nuclei are absent. The oxyhemoglobin concentration was determined spectrophotometrically, with a molar extinction coefficient of 5.6×10^4 at 540 nm and 5.9×10^4 at 576 nm. Solutions were discarded when the ratio of extinctions at 576/540 nm fell below 1.05.

Synthesis of mPEG-Sheep hemoglobin conjugates

mPEG activation and mPEG coupling were carried out as described earlier (Jackson *et al.*, 1987). The mPEG intermediate was added in varying amounts to the sheep hemoglobin in sodium tetraborate buffer (10 mg/ml). Coupling was carried out in ice water bath for 30 min and room temperature for 30 min with slow stirring. Reaction was stopped by dialyzing against PBS (phosphate buffered saline, pH 7.4).

Determination of degree of modification

The number of free amino groups of modified SHb was determined as previously described (Habeeb *et al.*, 1966). Modified hemoglobin solutions were adjusted to the same concentration using a spectrophotometer (Ab. 540 nm) and serially diluted. 25 ml of aqueous 0.03 M 2,4,6-trinitrobenzene sulfonic acid (TNBS) is added to 1 ml of sample contained in a test tube, agitated to ensure complete mixing and allowed to stand for 30 min at room temperature. The reagent blank consisted of 25 ml of 0.03 M TNBS in 1 ml of 0.1 M sodium tetraborate solution (pH 9.4). Absorbance was read at 420 nm. Unmodified free amino groups were calculated from the following equation:

$$\%\ \text{Free amino groups} = \frac{\text{Slope A}}{\text{Slope B}} \times 100$$

Slope A = slope of modified SHb and Slope B = slope of unmodified SHb.

Separation of mPEG-SHb conjugates by PAGE

The heterogeneity of mPEG modified SHb was analyzed on dissociating and non-dissociating discontinuous polyacrylamide gel electrophoretic systems. In non-dissociating electrophoresis, the discontinuous buffer system was used as described (Reifeld *et al.*, 1962). In dissociating electrophoresis, the SDS-discontinuous buffer system was used as described (Laemmli, 1970) was used. The resolving gel was prepared from a stock of 30% acrylamide and 0.8% N,N'-bisacrylamide to a final concentration of 15% acrylamide in SDS-PAGE and 6% acrylamide in native-PAGE. The stacking gel was made to a final concentration of 4% in both systems.

Double immunodiffusion test

The procedure for preparation of antiserum from rats was performed as described (Johnstone *et al.*, 1982). Prepared 0.5% agarose in water was poured into the slide glass and well dried at 50℃ dry oven. The center well was loaded with 20 μl antiserum and surrounding wells were filled with modified SHb solutions. Then the plate was incubated at 37℃ in the humid chamber until precipitin line was visible and agarose gel was stained with coomassie brilliant blue R.

Oxygen dissociation curve analysis

Oxygen dissociation curve was determined as described earlier (Rossie-Fanelli *et al.*, 1958). A solution of oxyhemoglobin is placed in a tonometer which consists of modified Thunberg tube with the lower part sealed to a optical cuvette. Then the system is deoxygenated under vacuum. After 10~15 min, achievement of complete deoxygenation is checked spectrophotometrically and the level of the liquid in the cuvette is measured to ascertain the actual solution concentration. Subsequently the vessel is placed in a thermostatted bath at 37℃ and equilibrated by slow rotation. The spectral properties of the solution, which should correspond to those of the fully deoxygenated derivative, are then recorded over the desired wavelength range (500~600 nm). Oxygenation achieved by introducing small and known amounts of air at atmospheric pressure in the tonometer. Finally air at 1 atm is introduced into the

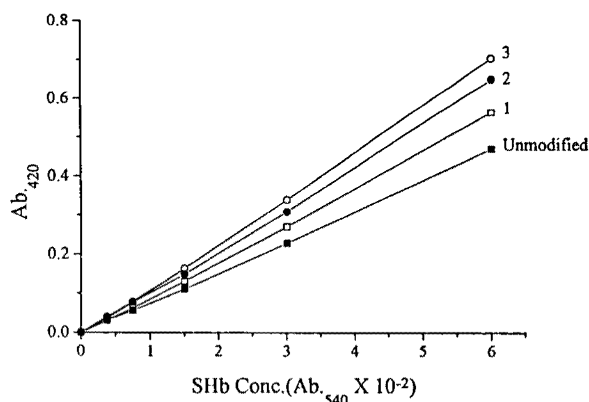

Fig. 1. Remained free amino groups at mPEG-SHb conjugates. 1: 50 mg mPEG added, 2: 100 mg mPEG added, 3: 200 mg mPEG added SHb.

vessel in order to obtain the optical density for complete oxygenation. The average value Yi were calculated at several wavelengths (540 nm, 560 nm and 576 nm), and the oxygen pressures (Pi) were calculated from the following equation:

$$Yi = \frac{(Ab.)obs. - (Ab.)Hb}{(Ab.)HbO_2 - (Ab.)Hb}$$

$$Pi = (Po + Pi)/Vt$$

Vi=amounts of air introduced, Po=partial oxygen pressure in the air and Vt=volume of the gas phase in the vessel.

Results

Determination of modification degrees with TNBS titration

Reaction mixtures were prepared by mixing 50 mg, 100 mg and 200 mg of activated mPEG to 10 ml of SHb solutions (10 mg/ml). Molar ratios were 6, 12 and 24, and molar ratios of reaction groups were 0.15, 0.3 and 0.6, because SHb has about 40 lysine residues. The modification degrees of SHb by mPEG were calculated form the linear graph of absorbance at 420 nm versus concentration of sample as shown in Fig. 1. TNBS titration results were 93.7%, 80.5% and 66% of titrable amino groups as compared to the unmodified proteins by 50 mg, 100 mg and 200 mg of mPEG respectively. From this the modification degrees are 6.3 %, 19.5% and 34.0% respectively.

Separation of mPEG-SHb conjugates by PAGE

The heterogeneity of mPEG-SHb conjugates was analyzed by dissociating and non-dissociating discontinuous polyacrylamide gel electrophoretic system. At first, native-PAGE was carried out in a 6% resolving gel and

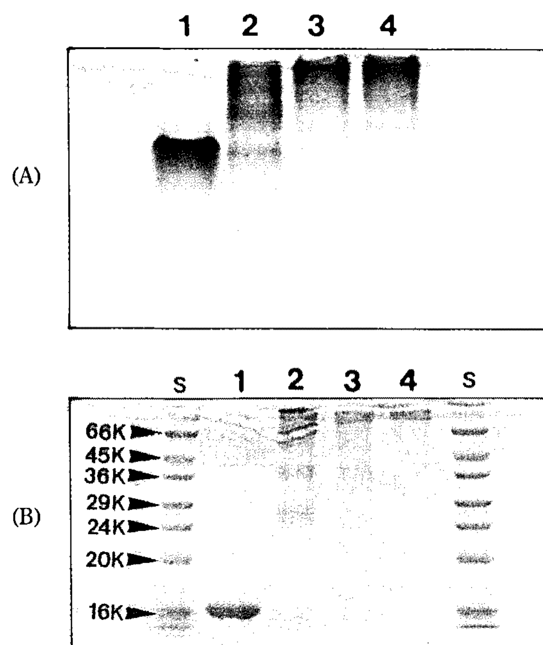

Fig. 2. Electrophoresis of mPEG-SHb conjugates. Performed at (A) nondenaturing condition and (B) denaturing condition. S: protein size marker, 1: unmodified SHb, 2: 6.3% modified mPEG-SHb conjugate, 3: 19.5% modified mPEG-SHb conjugate, 4: 34% modified mPEG-SHb conjugate.

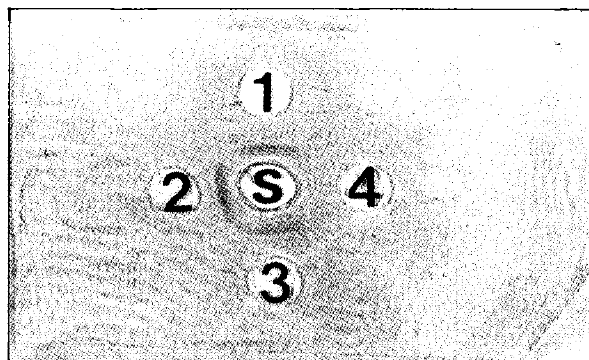

Fig. 3. Double immunodiffusion of mPEG-SHb conjugates. It was performed against antiserum to SHb. S: polyclonal antiserum to SHb, 1: unmodified SHb, 2: 6.3% modified mPEG-SHb conjugate, 3: 19.5% modified mPEG-SHb conjugate, 4: 34% modified mPEG-SHb conjugate.

the results are as shown in Fig. 2(A). Lane 1 is for hemoglobin and lanes 2, 3 and 4 are for mPEG conjugated hemoglobin derivatives. SDS-PAGE was carried out in a 15% resolving gel and the results are as shown in Fig. 2(B). Lane 1 is for dissociated hemoglobin subunits, lanes 2, 3 and 4 are for mPEG conjugated hemoglobin derivatives.

Double immunodiffusion test

Antiserum was harvested from immunized rats with SHb. It was set up in immunodiffusion plates to test

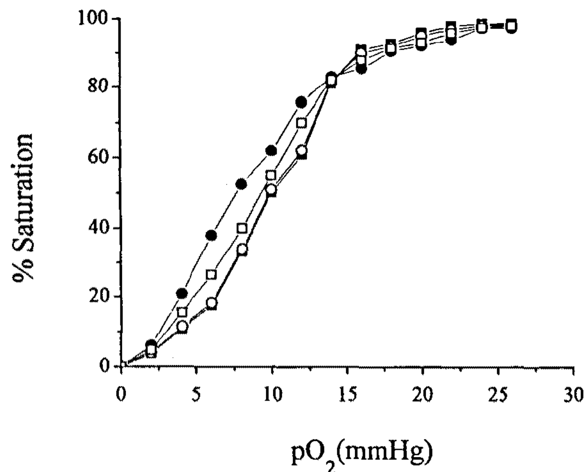

Fig. 4. Oxygen dissociation curves of ummodified and modified mPEG-SHb conjugates. (■-■): Unmodified SHb, (○-○): 6.3% modified mPEG-SHb conjugate, (□-□): 19.5% modified mPEG-SHb conjugate, (●-●): 34% modified mPEG-SHb conjugate.

precipitation activity. Anti-SHb antiserum was tested in immunodiffusion plates to compare with conjugated SHb with increasing amounts of mPEG. Fig. 3 represents the antiserum reaction with mPEG-SHb conjugates of 6.3, 19.5 and 34% modification. However, mPEG-SHb conjugate of 34% modification shows no evidence of reaction with antiserum when compared with unmodified SHb.

Determination of oxygen dissociation curve

The degree of oxygen saturation (Yi) at a given O_2 pressure (Pi) can be easily calculated from the spectra of unliganded (Hb) and fully liganded (HbO_2) protein under extreme conditions. In this experiment, the partial oxygen pressure was 160 mmHg, the amount of air introduced was 0.3 ml and volume of the gas phase in the vessel was 23 ml. Fig. 4 shows oxygen dissociation curves of unmodified SHb and modified SHbs. Modified SHb conjugates show increased oxygen affinity but show delayed saturation according to the added mPEG concentration.

Discussion

The finding (Liu *et al.*, 1969) that each ethylene oxide unit of polyethylene glycol binds approximately 3 molecules of water indicates that protein molecules with substantial amounts of polyethylene glycol attached possess profoundly altered hydrodynamic properties. The altered sedimentation constants, and chromatographic and electrophoretic properties, are consistent with the picture of a protein molecule surrounded by a flexible hydrophilic shell composed of polyethylene glycol and its bound water. Such a shell would cover antigenic determinants and render the protein inert to immune processes (Abuchowski *et al.*, 1977). mPEG-SHb conjugates show altered electrophoretic mobility (Fig. 2 (A), (B)), oxygen carrying capacity (Fig. 4) and antigenicity (Fig. 3). Alteration of electrophoretic mobility can be explained by attachment of mPEG (MW 5000) molecules to SHb. Antigenicity of mPEG-SHb conjugates was determined by double immunodiffusion. Only mPEG-SHb conjugate with 34% amino groups modified showed no evidence of reaction with antiserum against unmodified SHb. As expected, antiserum against unmodified SHb reacted with decreasing effectiveness according to the modification degree. Decreased antigenicity of 34% modified mPEG-SHB conjugate was the result of the mPEG molecules bound to SHb proteins forming a flexible hydrophilic shell which prevented approach of water molecules and antibodies. Therefore it may be possible to transfuse mPEG-SHb conjugates to human without blood grouping and transfusion tests. However, there are many other problems which should be examined such as toxic side effects. These mPEG molecules which were attached to SHb are also responsible for alteration of oxygen carrying capacity. Oxygen carrying capacity was determined tonometrically. mPEG-SHb conjugates with 34% amino groups modified show increased oxygen affinity and increased saturation end point. These results may be explained by interference with approach of oxygen to hemoglobin by mPEG molecules. Putting these results together, mPEG-SHb conjugate with 34% amino groups modified still has oxygen carrying capacity and has decreased antigenicity to antiserum against SHb. For mPEG-SHb conjugate to be a good potential blood substitute, it must have more sufficient oxygen carrying capacity, decreased antigenicity and no toxic side effects. Optimal conditions to satisfy these criteria should be studied further in detail.

Acknowledgement

This work was supported by research grants from Ministry of Health & Welfare in part and KOSEF for SRC (Research center for New Bio-Materials in Agriculture, Seoul National University) in part.

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