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SYNTHESIS OF MONOMETHOXYPOLYOXYETHYLENE-BOUND HAEMOGLOBINS

M. LEONARD, J. NEEL and E. DELLACHERIE*
Laboratoire de Chimie-Physique Macromoléculaire, ERA-CNRS No. 23-1, rue Grandville-54042, Nancy Cedex, France

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Abstract—Monomethoxypolyoxyethylene ($\bar{M}_w \simeq 5000$) was chemically modified and activated in order to react with the amino-groups of human haemoglobin. The best active polymeric derivatives were obtained by substituting a carboxymethoxyl group for the hydroxyl function of the polymer and by converting the carboxyl function into a N-hydroxysuccinimide active ester. When such modified polyoxyethylene derivatives were allowed to react with haemoglobin, soluble conjugates were formed with relatively low molecular weights. Some of the polymer-bound haemoglobins exhibit good oxygen-binding properties relative to unbound haemoglobin and could be suitable for use as erythrocyte substitutes.

Solutions of stroma-free haemoglobin, the blood's natural oxygen carrier, cannot be satisfactorily used as an erythrocyte substitute because the protein is rapidly excreted through the kidneys. To overcome the strong haemoglobinuria which follows administration of stroma-free haemoglobin and, consequently to increase the half-life of the protein in plasma,[1] its hydrodynamic volume must be increased and various attempts have been made in this way. Haemoglobin has been polymerized[2] or crosslinked by means of glutaraldehyde[3] and other polyfunctional reagents,[4] or coupled with dextran,[5-7] hydroxyethylstarch[6] and polyethyleneglycol.[8]

When haemoglobin is linked to polyfunctional polymers such as dextran or hydroxyethylstarch,[5-7] its oxygen binding properties are often strongly altered: its oxygen affinity is increased whereas the subunits' cooperativity is decreased[5,7] or sometimes suppressed.[6]

Concerning the modification of haemoglobin with polyethyleneglycol activated by cyanuric chloride, the conjugates obtained have high molecular weights[8] because of the possibility of crosslinking, and their oxygen dissociation curves are no longer sigmoid.

We now report another way of binding polyethyleneglycol to haemoglobin in order to prepare low-molecular-weight conjugates. The polymeric derivative used is the monomethoxypolyoxyethylene, and it was coupled to the protein amino-groups by several methods. The oxygen binding properties of the modified haemoglobin were studied as a function of the conditions used in the condensation reaction.

MATERIALS AND METHODS

Stroma-free hemoglobin was prepared according to the usual method, from outdated human blood.[7]

Monomethoxypolyoxyethylene (MeO–POE, $\bar{M}_w \simeq 5000$) was obtained from Aldrich (Belgium) and Ultrogel from IBF (France). Activated MeO–POE derivatives (1–3) were prepared following the reactions described in Fig. 1.

Derivative **1** was synthesised by refluxing MeO–POE (5 g; 1 mmol) and cyanuric chloride (1.85 g; 10 mmol) in toluene for 48 h, according to Monnerie *et al.*[9]

Derivatives **2** and **3** were prepared following methods described by Bückmann *et al.*[10] The carboxylic polymeric compound **6** was obtained by reacting the amino-derivative **5** (5 g; 1 mmol) with succinic anhydride (1.1 g; 1.1 mmol) in water for 4 h. During the reaction, pH was maintained between 9 and 9.5 by adding small amounts of 0.2N NaOH. The solution was then acidified with 1N HCl down to pH 2.5 and the polymer was taken up with chloroform. After several washings with water, the organic layer was dried over $MgSO_4$ then treated with charcoal. The polymer was precipitated with dry ether, filtered and dried under vacuum. This run of operations was repeated until the potentiometric titration gave a constant value for the quantity of fixed COOH.

To prepare derivative **7**, MeO–POE (20 g; 4 mmol) was dissolved in tetrahydrofuran (300 ml) and treated with naphthalenesodium under nitrogen at room temperature for 3 h. Then $BrCH_2COOC_2H_5$ (1.4 ml; 12 mmol) was added dropwise with stirring. After 4 h of reaction the ester obtained was precipitated with ether, dried, dissolved in water and saponified with 0.1N NaOH at 55° for 24 h. The polymeric carboxylate was acidified and treated as described above.

Derivative **6** (or **7**) (5 g; 1 mmol) was dissolved in dry ethylacetate (60 ml) and activated by N-hydroxysuccinimide (0.15 g; 1.25 mmol) and dicyclohexylcarbodi-imide (0.26 g, 1.25 mmol) at 30° for 15 h. Urea was removed by filtration and the polymer precipitated with dry ether was taken up with chloroform and crystallized from this solution by dropwise addition of ether at 0°. This procedure was repeated several times until the spectrophotometric analysis of succinimidyl groups[15] gave a constant value.

Coupling of haemoglobin to compounds **1**, **2** and **3** was performed at 5°C as follows: 1.5 ml of a 10% haemoglobin solution (2.3 μmol) was diluted with 2 ml of the appropriate buffer and 300 mg (60 μmol) of activated polymer were then added under stirring. The reaction mixtures were analysed at various times by gel permeation on Ultrogel AcA 34 (linear fractionation range 20,000–350,000, exclusion limit 750,000 daltons) in 0.05M sodium phosphate buffer, pH 7.2 at 6°. The reaction was considered complete when there was no significant peak left at the elution volume of free haemoglobin on the chromatogram.

Oxygen equilibrium curves were determined manually with a tonometer according to a spectrophotometric method first described by Benesch *et al.*[11] and modified by Labie and Byckova.[12] The optical density of the solutions (0.1M sodium phosphate buffer, pH 7.2, 25°) was measured at 560 and 578 nm in a 320 Hitachi spectrophotometer.

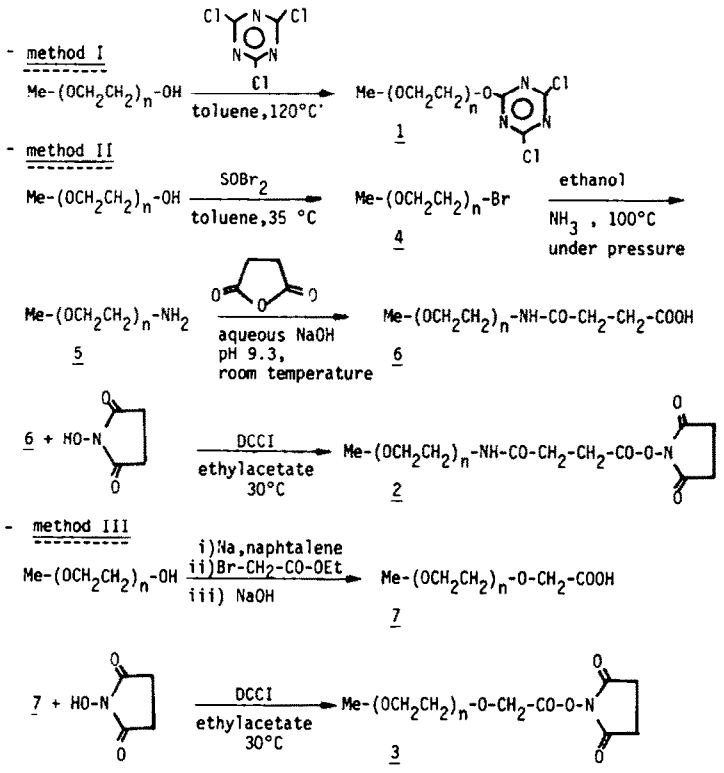

Fig. 1. Methods of activation of methoxypolyoxyethylene (Me = methyl, Et = ethyl, DCCI = dicyclohexylcarbodiimide).

RESULTS AND DISCUSSION

In this study, MeO–POE was chosen in order to avoid any intermolecular crosslinking of haemoglobin molecules. MeO–POE was activated using different methods (derivatives **1**, **2** and **3**; Fig. 1) and the resulting derivatives were allowed to react with the free haemoglobin amino-groups.

Cyanuric chloride, already used to bind biological molecules to polymers[13,14] affords a quantitative activation of the hydroxylic function of MeO–POE and leads to non cross-linked derivatives with 2 mol Cl per mol MeO–POE. The reaction between derivative **1** and haemoglobin is fast but the equivalent molecular weight of the adducts so obtained increases very quickly (up to 750,000) with the reaction time as well as with the pH (Fig. 2). The reason why the molecular weight of the conjugates was so high is not clear, except if one assumes that 2 haemoglobin molecules react with the 2 free Cl atoms of derivative **1** in spite of the steric hindrance. This is confirmed by the fact that the reaction of 2-methoxy-4,6 dichloro-s-triazine with haemoglobin also leads to high molecular weight cross-linked products.

For all the conjugates obtained with derivative **1**, the oxygen affinity is increased and the subunits cooperativity is strongly altered since the Hill coefficient(n) decreases from 2.8 (free haemoglobin) to 1.9 for a conjugate obtained after 1 h of reaction at pH 8.25 in a borate buffer (Fig. 3).

The activated polymers **2** and **3** were synthesised respectively from derivatives **6** and **7** containing about 0.95 mol COOH per mol MeO–POE. The concentration of succinimidyl groups of compounds **2** and **3**, determined by a spectrophotometric method,[15] was found between 0.92 and 0.95 mol per mol MeO–POE.

A second series of polymeric haemoglobins was then prepared by reacting derivatives **2** and **3** with free haemoglobin under the same conditions as for derivative **1** (borate buffers). The final conjugates obtained at various pH from these two derivatives

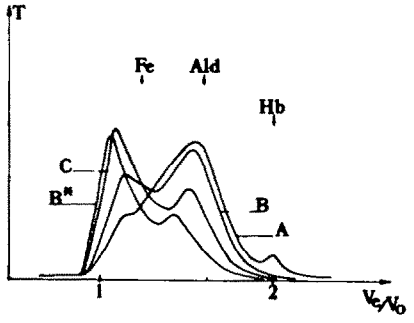

Fig. 2. Gel filtration on Ultrogel AcA 34 of the reaction mixture containing derivative **1** and haemoglobin after 1 h of reaction in borate buffers (12.5 mM $Na_2B_4O_7$) at various pH: A, pH 8.15; B, pH 9.05; B*, pH 9.05 (24 h of reaction); C, pH 10.0. V_e is the elution volume, V_0 the void volume of the column (500 ml bed volume; elution buffer, 0.05M sodium phosphate pH 7.2, 6°C; injection 0.4 ml; flow rate 30 ml · h^{-1}), detection at 280 nm. The arrows correspond to the normal elution volume of haemoglobin (Hb), aldolase (Ald,M = 158,000) and ferritin (Fe,M = 440,000).

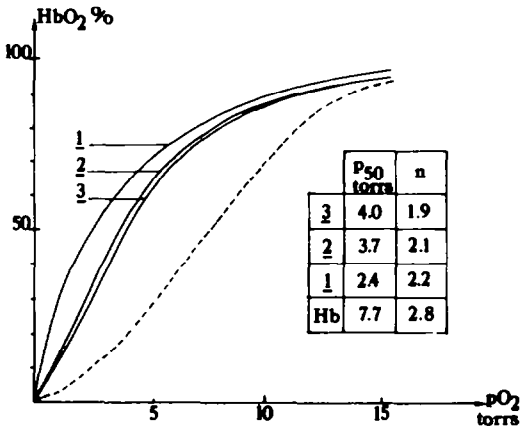

Fig. 3. Oxygen binding curves of the MeO–POE-linked haemoglobin samples obtained with derivatives **1**, **2** and **3** after 1 h of reaction in a borate buffer (12.5 mM $Na_2B_4O_7$), pH 8.25. ---affinity curve of free haemoglobin.

exhibit nearly the same distribution of molecular weights with a major component between 160,000 and 200,000 (Fig. 4). This value is the molecular weight of a globular protein having the same elution volume as that of the polymeric conjugate.

The oxygen dissociation curves of some of these adducts are reported in Fig. 3. The curves corresponding to derivatives **2** and **3** are less left-shifted than that related to derivative **1**; the observed p_{50} values for conjugates prepared at pH 8.5 were respectively 2.4(**1**), 3.7(**2**) and 4.0(**3**) torr (free haemoglobin $\simeq$ 7.7 torr) and the Hill coefficient 1.9(**1**), 2.1(**2**) and 2.2(**3**) (free haemoglobin $\simeq$ 2.8).

In a last set of experiments, derivative **3** was reacted with free haemoglobin in phosphate buffers at pH varying from 5.8 to 7.8.

The molecular weights of the final adducts are quite similar to those of the products prepared in borate buffers; no important influence of pH upon their distribution could be seen on the various elution profiles obtained by gel filtration. On the other hand, decreasing the pH from 7.8 to 5.8 significantly improves the oxygen binding properties of the final adducts, as shown in Table 1. It can be assumed that some of the amine groups which form salt bridges in deoxyhaemoglobin become more protonated when the pH is decreased and thus react less easily with the polymer; this results in a reduced oxygen affinity and increased cooperativity of the modified haemoglobin.

The effects of inositolhexaphosphate (IHP), a strong effector of haemoglobin,[16] on the oxygen binding properties of two adducts prepared at pH 5.8 and 7.8 corroborate this hypothesis. Table 2 shows that IHP is more effective on the adduct obtained at the lowest pH, which means that some of the amine groups of the phosphate binding cleft still remain available for interaction with IHP.

In conclusion, monomethoxypolyoxyethylene ($\bar{M}_w \simeq 5000$) carrying one active ester end, can easily react with the hemoglobin amino-groups to yield conjugates whose molecular weights are not excessively high. Obviously, the reaction is not site specific but, contrary to previously reported studies,[6,8] derivative **3** leads to conjugated haemoglobin which can bind oxygen in a manner quite similar to that of the native protein. The p_{50} is reduced very little and the subunits cooperativity barely altered. These results prove that the MeO–POE activated chains do not

Table 1. Oxygen binding properties of MeO–POE-linked haemoglobin samples prepared at various pH (0.1M phosphate buffers, reaction time 2 h)

	Polymer-bound Hb					free Hb
pH of reaction	5.8	6.2	6.6	7.2	7.8	
P_{50} torrs	6.2	5	4.3	4.0	3.8	7.7
n	2.55	2.45	2.2	2.15	2.05	2.8

Table 2. Effect of inositolhexaphosphate (IHP) on the oxygen binding properties of MeO–POE-linked haemoglobin samples prepared at pH 5.8(A) and 7.8(B). Other conditions as in Table 1. Oxygen binding: $[Hb_4] = 15\,\mu M$, $[IHP] = 150\,\mu M$.

	without IHP		with IHP	
	P_{50} torrs	n	P_{50} torrs	n
A	6.2	2.55	14.6	2.1
B	3.8	2.05	6.5	1.2
free Hb	7.7	2.8	28	2.5

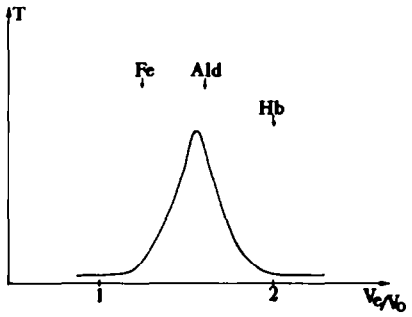

Fig. 4. Gel filtration on Ultrogel AcA 34 of the reaction mixture containing derivative **2** or **3** and haemoglobin after 1 h of reaction at pH 8.25; other conditions as in Fig. 2.

block a great number of the haemoglobin amines among those which are essential for cooperative oxygen binding (amines essential for the salt bridges of the deoxy form and those in the phosphate-binding cleft); these properties make this new polymer-bound haemoglobin a potentially useful oxygen carrier for blood transfusion. Experiments on animals are now being carried out in order to determine the physiological properties of this new erythrocyte substitute.

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