# The study of terminated PEG maleimide synthesis and modification of hemoglobin

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**Abstract.**There is a worldwide increased demand for blood transfusions. In Third World countries is not sufficient due to the spreading of infections, such as HIV. The current generation of so-called hemoglobin-based oxygen carriers (HBOCs) comprise hemoglobins from different species (human and bovine) that have been chemically modified to improve structural stability. However, some adverse effects such as hypertension, myocardial infarction and acute kidney failure have been found in several clinical trials. The exact factors leading to the symptoms are still uncertain, but high molecular weight was believed to play an important role. Therefore, Maleimide polyethylene glycol 5000 monomethyl ether(mPEG-MAL) as Hemoglobin(Hb) modifier can change the Molecular weight of Human placenta hemoglobin effectively. In addition, hemoglobin weight could be severely controlled by changing the amount of the sulfhydryl modifier and mPEG-MAL, so as to achieve the demand of the people of different molecular weight.

## Introduction

Blood transfusion is the key life-saving treatment in many traumatic emergencies, chronic or acute pathologies, and during or upon surgical interventions. Over the years, several attempts have been made to transfuse humans with either human or animal. Results were obviously negative. Indeed, transfusions are exposed to a series of limitations that can lead to potential health risks.The research on blood substitutes has been regularly reviewed. Over the years, several attempts have been made to transfuse humanswith either human or animal whole blood, red cells lysate, or evenwine, beer and milk. Results were obviously negative. It is only in the sixties, and more vigorously from the eighties, that the research for hemoglobin-based oxygen carriers (HbOCs) began taking into account the vast knowledge that was emerging on the oxygen transport physiology and Hb structure and function . Several strategies aimed at the development of a Hb derivative that exhibits, when free in the plasma, the same oxygen affinity of Hb within the red cell, and does not cause the main adverse effects of cell-free Hb, vasoactivity and renal failure. The different approaches that make use of haemoglobin chemical modifications are reported. They are based on reagents, such as glutaraldehyde, that cross-link tetramers intra- or inter-molecularly, generating stable tetramers or polymers, or on compounds that decorate the surface of haemoglobin with organic molecules, such as polyethylene glycol (PEG), thus increasing protein molecular weight and actual size. The PEGylated bovine Hb developed by Enzon Inc. is one of the early PEG-Hbs and carries ten copies of PEG-5K chain per tetramer on amino groups. The decoration of hemoglobin with PEGylation to increase the molecular size in order to use it as blood substitute is our goal.

## Experimental

### Synthesis of mPEG5000-MAL.

**a)mPEG-tosylate**

mPEG5000-OH (10g,2mmol), TEA 2ml and 4-toluene sulfonyl chloride (1.9g,10mmol) were dissolved in acetone(30ml), and then stirred for 4h at room temperature. The reaction mixture was

filtered, and the filtrate was washed with saturated saline (3×50 mL). The organic layer was vaporated under vacuum,and recrystallization from $Et_2O$ (20 mL) gave 9.2 g of mPEG-tosylate (yield, 89.2%) as a white solid.

**b)mPEG-phthalimide**

mPEG-tosylate (5.2g ,1mmol)was dissolved in 20mL DMF, and then potassium phthalimide (0.93 g, 5 mmol) was added. The reaction mixture was stirred at 120℃ under N2 for 4h. DMF was removed under reduced pressure, and the resulting yellow oil was dissolved in 35mL of ethanol and 2ml diamid hydrate. The reaction mixture was refluxed for 4h.Solvent was removed under reduced pressure .The solution was filtered and recrystallization from $Et_2O$ gave 5.8 g (yield, 90.3%) of mPEG-phthalimide as a white solid.

**c)mPEG-$NH_2$**

mPEG-phthalimide (2.5g,1.25mmol) and hydrazine hydrate (8.1 mmol) were dissolved in ethanol (20 mL), and the solution was refluxed for 2 h. The reaction mixture was evaporated under vacuum, and the thick oil was crystallized from the mixture of Et2O and DCM to give mPEG-$NH_2$ (2.8 g, 56%)as a white solid.

**d)mPEG-maleic acid**

mPEG-$NH_2$ (2.5g, 1.25 mmol) in dioxane (10 mL) was treated with maleic acid anhydride (0.49 g, 5mmol) and DMAP (0.01 g) at 70℃ for 1 h. The reaction solution was cooled to room temperature and precipitated with cold ethyl ether. The solid was collected by filtration and washed with cold $Et_2O$ to give 2.3g of mPEG-maleic acid yield 88%.

**e)mPEG-MAL**

mPEG-maleic acid (2.3 g, 1.1 mmol) was dissolved in $Ac_2O$ (10 mL),and then sodium acetate (0.18 g, 2.2 mmol) was added. The reaction mixture was stirred at 80 ℃ for 1.5h and then evaporated under vacuum. The resulting thick oil was precipitated with cold ethyl ether and recrystallized twice from the mixed solvent of $Et_2O$ and DCM to give mPEG-MAL(1.25 g, 54%) as a white solid.

**Preparation of the PEG-Hbs.**

Human adult hemoglobin (HbA) was purified from human erythrocytes as previously described. Briefly, HbA at 1.0 mM (tetramer) was reacted with 10mM,15mM and 20mM 2-iminothiolane in PBS buffer (pH 7.4) at 4 ℃for 4 h. The resultant products are reacted with 5mM,10mM and 15mM mPEG-MAL in PBS buffer (pH 7.4) at 4 ℃for 12 h. Glycine and acetylcysteine (1.5mM) were added and the retentate was stored at -80℃.

## Result and discussion

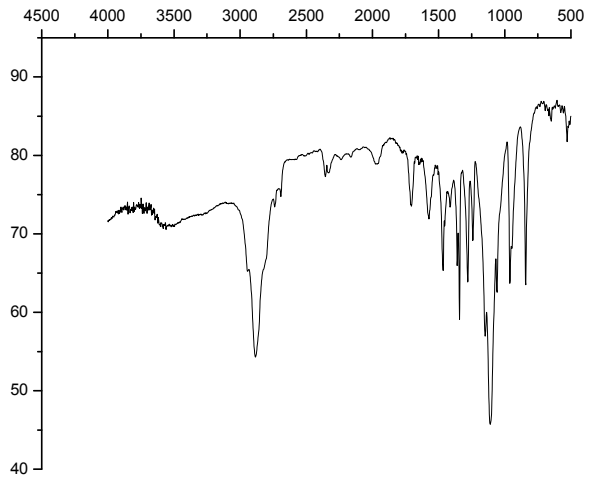


Fig.1 mPEG-MAL

**Infrared spectrum analysis of the mPEG-MAL.**

Fig.1 show the infrared spectrum of the mPEG-MAL, the peak of functional group were appeared at right site. This picture demonstrate that the substance that we wanted is correct.

**HPLC analysis of the PEG-Hbs.**

HPLC analysis of the PEG-Hbs is shown in Figure 2. The samples were loaded on a Vydac C4 column (0.46 ×25 $cm^2$). The columns were equilibrated and eluted with 1M $MgCl_2$ at the flow rate of 0.5mL/min and an excitation wavelength of 280 nm. The HPLC pattern of Hb shows two peaks, however, the other samples show more than two peaks. These new peaks were estimated to correspond to the mono-, di-, and tri-PEGylated globin chains. It should be mentioned that the sum of peak areas in the HPLC pattern of the individual samples are taken as 100% for calculating the level of PEGylation in the respective PEG-Hbs.

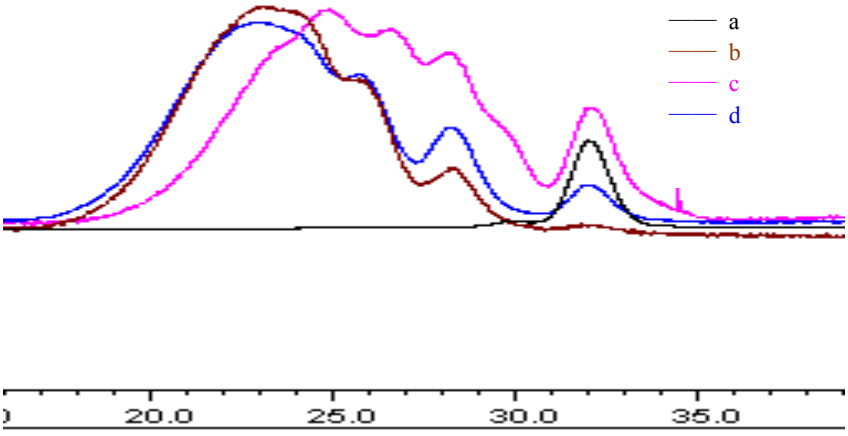


Fig.2 a: Hb; b: 2-IT 5M mPEG-MAL 5M; c:2-IT 5M mPEG-MAL 10M; d:2-IT 5M mPEG-MAL 15M

**SDS-PAGE Analysis of the PEG-Hbs.**

Lanes 3 , the molecular weight markers; lane 2, HbA; lane 1、4、5, the PEG-Hb. Compare to HbA. PEGylation of HbA resulted in the appearance of four bands. It confirms that these bands correspond to one HbA conjugated with 1, 2, 3, and 4 PEG-5K chains. Some smaller additional bands are observed, possibly due to the presence of a small amount of impurity that is not well-separated from Hb.

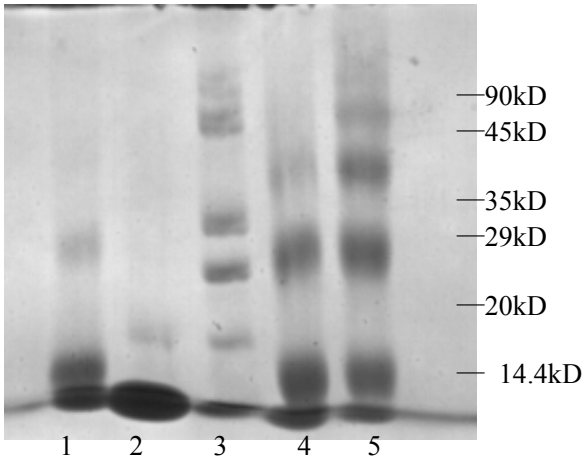


Fig.3 1:2-IT 15M mPEG-MAL 5M 2: HbA 3:Marker 3: 2-IT 15M mPEG-MAL 10M 4:2-IT 15M mPEG-MAL 15M

## Conclusions

PEGylation of Hb was developed to simplify the surface decoration of Hb with the PEG chains

and develop a nonvasoactive Hb derivative. This new PEGylation platform is a cost-effective protocol as exemplified in the development of MP4 by Sangart . In this study, Hb was conjugated with polyethylene glycol (PEG) chains and increasing protein molecular weight and actual size successfully.

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