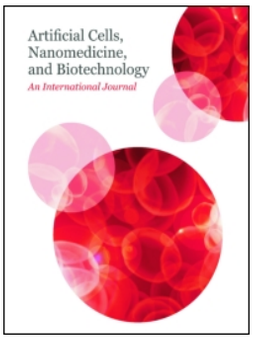

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Characterization of Poly(ethylene glycol)-modified Bovine Hemoglobin By Capillary Zone Electrophoresis

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Abstract: In this study capillary zone electrophoresis (CZE) was used to analyze the poly(ethylene glycol)-modified bovine hemoglobin(PEG-bHb). The results show that CZE separated the subunit of bovine hemoglobin based on the number of PEG conjugating to the protein surface, which makes it possible to evaluate the degree of modification of hemoglobin subunit; meanwhile, it also reflected the stability of PEG attaching to hemoglobin after incubating with hydroxylamine, which makes it successful to detect the distribution of attachment site and evaluate the stability of PEG on the surface of hemoglobin. As a simple, fast and accurate method, CZE is suitable to monitor the production procedures and quality control of the final products of the PEG-bHb.

Keywords: Capillary zone electrophoresis; Characterization; PEG-bHb

INTRODUCTION

There are many concerns over the risks of blood-borne pathogens transmission in transfusion [1,2], which makes the development of

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hemoglobin-based oxygen carriers (HBOC) very attractive [3]. Because of its renal toxicity, short circulation time in body, and high oxygen affinity, hemoglobin itself can not be used for HBOC without modification [4,5,6]. This shortcoming has been overcome by conjugating PEG molecule to the nucleophilic amine acid such as lysine on the surface of hemoglobin [7,8]. The biological properties of hemoglobin is related to the degree of modification by the PEG [9], and therefore the number of PEG on the surface of PEG-bHb, especially the number of PEG on each subunit of PEG-bHb, is a key indicator to evaluate the safety and efficiency of PEG-bHb. However, it is difficult to analyze PEGylated hemoglobin because PEGylated hemoglobin is heterogeneous with respect to the number and position of PEG attached to its surface, and PEG molecule curls randomly in liquid phase and its hydrodynamic radius is equal to those of proteins whose molecular weight are 4–5 times of it [10]. A method has been developed in our lab to determine the number of PEG and its distribution on the surface of hemoglobin by using size-exclusion high-performance liquid chromatography (SE-HPLC) connecting with UV absorbance, light scattering and refractive index [11]. However, this method only detect the number of PEG and its distribution of the tetramer of hemoglobin. The status of the hemoglobin subunit is perplexed and the numbers of PEG on the subunit surface is more important to evaluate the safety and efficiency of PEG.-bHb. As a fast and simple method, the capillary zone electrophoresis (CZE) has been applied in analyzing PEGylated protein [12,13,14,15]. A method using CZE to determine the number of PEG of each PEG-bHb subunit and evaluation the stability of the PEG on the surface of hemoglobin was reported in this paper.

MATERIALS AND METHODS

Preparation of PEG-bHb

Stroma-free hemoglobin of bovine was purified according to literature [16]. The fresh whole bovine blood was washed three times with saline (3000 g, 4°C, 10 min), and the red blood cells were lysed by adding 10 mM sodium phosphate (pH 7.0). The stroma was removed through 0.45 μm membrane cartridges (Pall Technology Corp., USA), and the solution was diafiltered with 100 kDa membrane to remove stroma particles.

Stroma-free hemoglobin was mixed with N-succinimidyl carbonate mPEG (SC-mPEG, 5 kDa) or with N-succinimidyl propionate mPEG (SPA-mPEG, 5 kDa) (NAKTA Technology Corp., USA) at different molar ratio, and stired at 4°C for 30 minutes. The unactive mPEG was removed by 50 kDa ultrafiltration membrane cartridges (Pall Technology

Corp., USA), the SC-PEG-bHb or SPA-PEG-bHb was concentrated to 60 mg/ml and stored at 4°C for use.

Capillary Zone Electrophoresis

All CZE separations were carried out on a capillary elctropherograph system P/ACE MDQ (Beckman Instruments, USA). The system was equipped with a fused-silica capillary (75 mm inner diameter; 57 cm effective length; Yongnian Optical Fiber Factory, Hebei, China), the UV detection was set at 214 nm, and the temperature of the capillary cartridge was maintained at 25°C using a thermostated coolant (Beckman). The capillary was rinsed with 1 N NaOH for 10 min, and then rinsed with water for 5 min, finally with run buffer for 5 min. The run buffer was consisted of 40 mM phosphoric acid (pH 2.1, adjusts with 1 N NaOH), 0.1 mg/ml PEO (Mn 10.8 kDa) and 4 mg/ml Jeffamine ED-600). The PEG-bHb was diluted to 4 mg/ml with run buffer and hydrodynamically injected into capillary for 5 s. The separation was performed at +25 KV for 15 min.

RESULTS AND DISCUSSION

Separation Mono-, Di- and Tri-PEGylated Subunit of SC-PEG-bHb

The electropherogram of SC-PEG-bHb is shown in Figure 1. Four almost baseline resolved peaks, whose migration times were 6.35, 7.67,

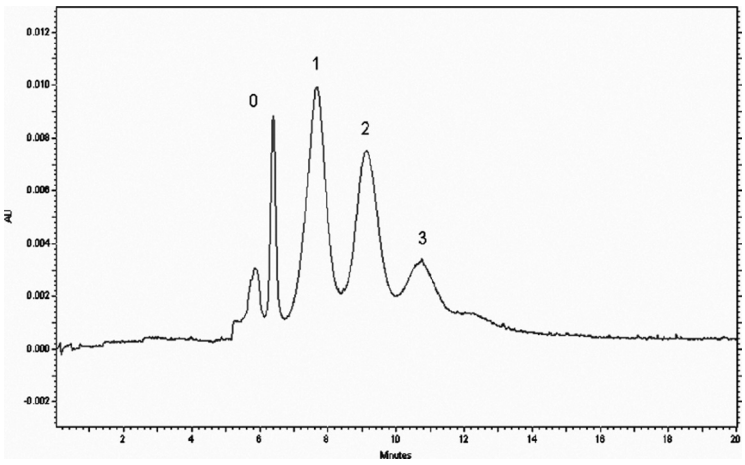

Figure 1. The electropherogram of SC-PEG-bHb (the molar ratio of hemoglobin to SC-PEG was 1 : 8) by capillary zone electrophoresis.

9.16 and 10.73 minutes, respectively, were observed (named as peak 0, peak 1, peak 2, peak 3, accordingly). We deduced that the peak 0 was the native subunit of hemoglobin, and peak 1, peak 2 and peak 3 were the subunits attached by one, two and three molecules of PEG. To identify this hypothesis, we disassociated the hemoglobin tetramer into dimmer by using 1 M $MgCl_2$ [17], and separated the disassociated sample with the GF-250 column (25 cm × 9.4 mm ID., Agilent Technology Corp., USA); the peaks eluting at 8.36 min (named as fraction D), 8.71 (fraction C) min, 9.29 min (fraction B) and 10.31 min (fraction A) (Fig. 2) were collected and then characterized by SDS-PAGE as well as capillary zone electrophoresis. The result of SDS-PAGE (Fig. 3) showed that apparent molecular weight of the fraction A was about 15 kDa (Lane1), the same molecular weight of the native subunit of hemoglobin. And its migration time was 6.34 min (Fig. 4, Graph A), the same migration time as peak 0 in Figure 1. This result implied that the peak 0 in Figure 1 was the native subunit. The result of SDS-PAGE (Fig. 4, Lane 2) showed that except the native subunit, there existed a band whose apparent molecular weight was 24.3 kDa in fraction B, representing one PEG molecule attached to the subunit of hemoglobin. Similar to the result of SDS-PAGE, two peaks were observed in the fraction B by CZE (Fig. 4, Graph B), their migration times were 6.4 min and 7.68 min respectively, and the later component was almost overlapped with peak 1 in Figure 1. So we deduced that the peak 1 was the subunit

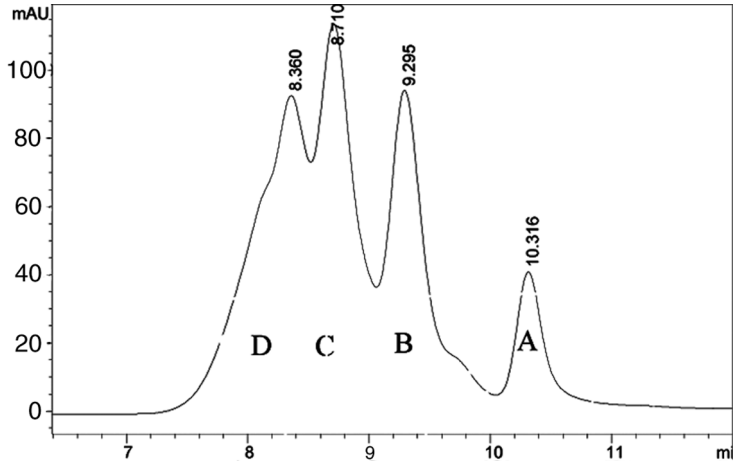

Figure 2. The SE-HPLC profile of disassociated SC-PEG-bHb. (mobile: 5 mM sodium phosphate, 150 mM NaCl, 1 M $MgCl_2$, pH 7.0; UV : 280 nm; Flow rate: 1 ml/min).

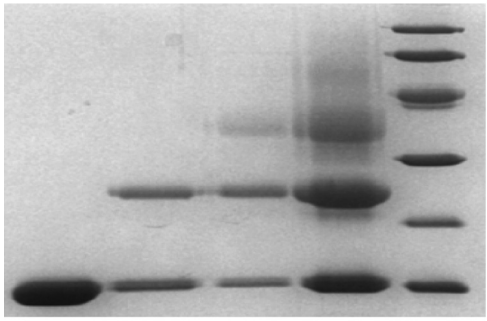

Figure 3. SDS-PAGE analysis of each fraction of SE-HPLC stained with Coomassie blue. (Lane1, 2, 3, and 4 represent the fractions A, B, C, and D in Fig. 2, and Lane 5 is the molecular mark).

of hemoglobin modified by one PEG molecule. Because only one PEG chain conjugated to the surface of one dimmer of the hemoglobin, there were two bands or two peaks in the result of SDS-PAGE or CZE; one was native α or β subunit, while the other was α or β subunit attached by one PEG chain. For the fraction eluting at 8.71 min, comparing to lane 2, SDS-PAGE (Lane 3) showed a new band whose molecular weight was 35.6 kDa, suggesting that the subunit was modified by two PEG molecules appeared. Similar to the result of SDS-PAGE, the electropherogram of CZE of fraction 3 (Fig. 4, Graph C) showed that there existed another new peak, whose migration time was 9.16 min, which was overlapped completely with peak 2. According to these results, peak 2

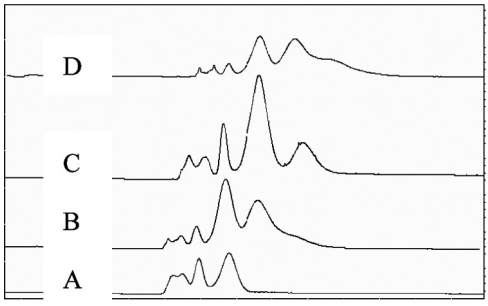

Figure 4. The electropherogram of fractions A, B, C, and D of SE-HPLC by capillary zone electrophoresis.

of electropherogram of CZE may be the subunit whose surface attached by two PEG molecules. Since there were two PEG molecules on the surface of dimmer, PEG may attach to the surface of dimmer of PEG-bHb in two possible ways: each subunit of dimmer attached by one PEG molecular, or one subunit of dimmer attached by two PEG molecules, but one subunit was native, so there were three kinds of bands or peaks in the result of SDS-PAGE or CZE. With the same method, we proved that peak 3 of electropherogram of CZE (Fig. 4, Graph D) was the subunit attached by three PEG molecules, and its apparent molecular weight was 63.9 kDa (Lane 4). From these results described above it was obvious that we had successfully found a way to separate mono-, di- and tri-PEGylated subunits of PEG-bHb. This method was also useful to determine the percent of each component in PEG-bHb to calculate the distribution of PEG chains on the subunits of PEG-bHb.

Comparison SC-mPEG-bHb Produced by Different Ratio of Hemoglobin to PEG

The number of PEG attaching to hemoglobin is critical to guarantee the safety and efficiency of PEG-bHb. It can be controlled by the reaction ratio of the actived mPEG to hemoglobin. Figure 5 shows the electropherogram

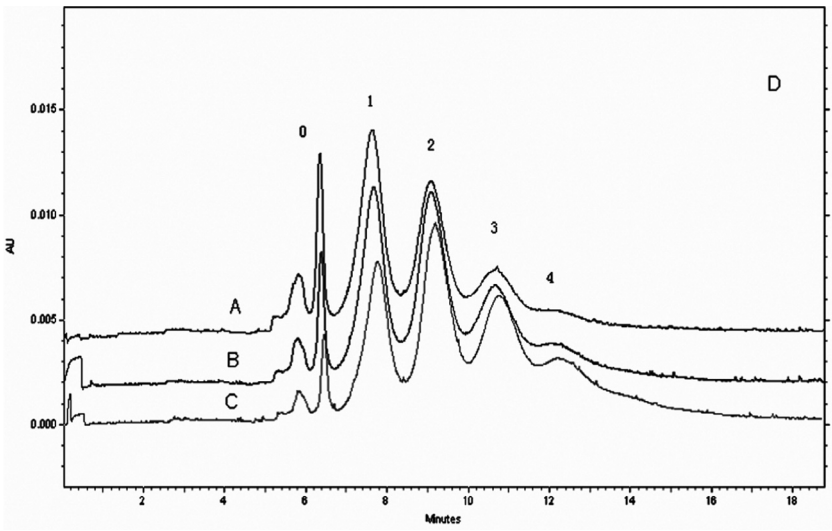

Figure 5. Electropherogram of PEG-bHb produced by different reaction ratios of hemoglobin to PEG (A,1:8; B,1:10; C,1:12).

of PEG-bHb whose molar ratios of hemoglobin to the actived SC-mPEG were 1:8, 1:10 and 1:12, respectively. The results demonstrated that the peaks of native and mono-PEGylated subunits of PEG-bHb decrease as the actived SC-mPEG increased in the reaction system, and the peaks of di- and tri- PEGylated subunit were increased. When the molar ratios were 1:12, the native subunit even disappeared and a new peak, which may be the tetra-pegylated subunit (migration time was 12.5 min), appeared. These results suggested that the reaction conditions might be effectively monitored by using capillary zone electrophoresis during the process to produce the desired PEG-bHb.

Evaluation of the Stability of SC-mPEG-bHb and SPA-mPEG-bHb

SC-mPEG-bHb and SPA-mPEG-bHb were dilution to 4 mg/ml with running buffer, composed of 10 mM hydroxylamine, and were incubated at 37°C for 30 min. The electropherogram is shown in Figure 6. The results of SC-mPEG-bHb showed that the peaks of native subunit and mono-mPEG subunit were increased but the peak of tri-mPEG subunit was decreased after incubation with hydroxylamine, suggesting that SC-mPEG broke off from the surface of SC-mPEG-bHb subunit during these procedures. We also compared the electropherogram of the SC-PEG-bHb treated with hydroxylamine to the electropherogram of the same batch of SC-mPEG-bHb that incubated at 37°C for 10 hours, the two electropherogram were almost overlapping exactly (date not shown), indicating that SC-PEG-bHb incubation with hydroxylamine may

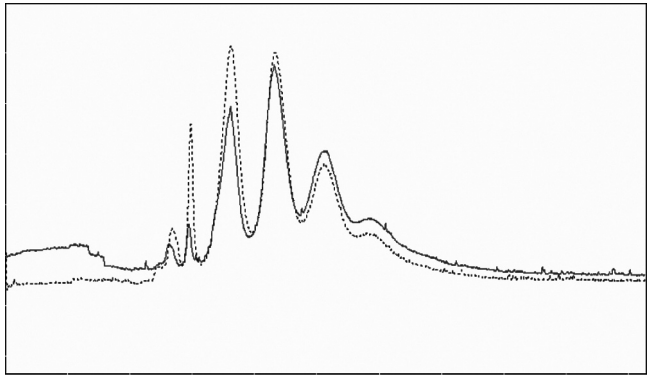

Figure 6. The electropherogram of SC-PEG-bHb before (solid line) and after (dot line) incubating with 10 mM hydroxylamine (molar ratio 1:12).

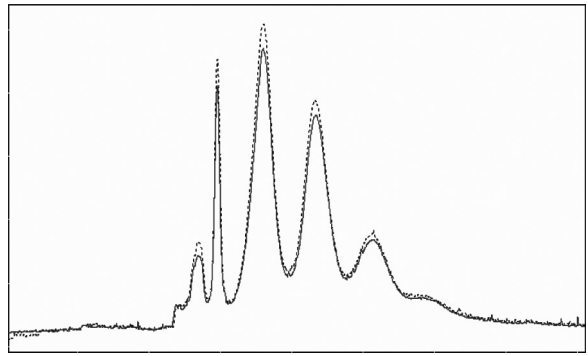

Figure 7. The electropherogram of SPA-PEG-bHb before (solid line) and after (dot line) incubating with 10 mM hydroxylamine (molar ratio 1:12).

simulate its depegylation during the period of preservation. Since the nucleophilic amino acids such as lysine and histidine located on the surface of protein could react with active mPEG, the covalent bond between mPEG and lysine was stable, but the bond between mPEG and histidine was unstable and the mPEG was liable to break off from the PEGylated protein in the presence of hydroxylamine [18]. It could be deduced that the SC-mPEG breaking off from the bHb in the presence of hydroxylamine was the component that conjugated to the histidine and this kind of SC-mPEG broke off slowly too during preservation. It was also suggested that there may be at least two kinds of sites, which could be modified by SC-mPEG on the surface of hemoglobin subunit and the distribution of modification site could be determined in the presence of hydroxylamine. In opposition to the results of SC-mPEG-bHb, each peak area of the SPA-mPEG-bHb was identical despite the presence of hydroxylamine, suggesting that the active SPA-mPEG attached mainly to the lysine during modification. The result also demonstrated that SPA-mPEG-bHb was supper to SC-mPEG-bHb in term of stability mPEG conjugating to the surface of hemoglobin subunits.

CONCLUSION

CZE is a powerful method to characterize PEG-bHb. It can separate the subunit of PEG-bHb conjugated with different numbers of PEG, evaluate the stability of PEG attaching to hemoglobin, while it is helpful to determine the distribution of the PEG on the surface of hemoglobin. As a simple and fast method, CZE is suitable to monitor the production procedures of PEG-bHb and quality control of its final products.

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