Synthesis of a Novel Histidine-Targeting Poly(ethylene glycol) and Modification of Lysozyme

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ABSTRACT: A simple and highly efficient method was developed to prepare a synthetic poly(ethylene glycol) (PEG) derivative, which can be used for conjugation to histidine residue. This derivative reacted with sodium nitrite and the intermediate 4-diazo-N-mPEG-benzamide proved to have a good performance for histidine modification of lysozyme. Then the pegylated lysozyme was separated and its bacteriolytic activities were determined. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 119: 2183–2188, 2011

Key words: poly(ethylene glycol); functionalization of polymers; conjugated polymers; histidine; separation of polymers

INTRODUCTION

The use of proteins and peptides as medicines has become an important part of human therapeutics. However, these biomacromolecules have some shortcomings, including short circulating half-life, immunogenicity, proteolytic degradation, and low solubility. Pegylation is one of the most significant strategies to improve the pharmacokinetic and pharmacodynamic properties of biopharmaceuticals.[1] There have been many marketed pegylation protein drugs, such as poly(ethylene glycol) (PEG)–asparaginase (Oncaspar®), PEG–adenosine deaminase (Adagen®), PEG–interferonα-2a (Pegasys®), and PEG–G-CSF (PEG-filgrastim, Neulasta®) since 1994.[2] PEG derivatives, which are used for drug modification could be classified in several ways. According to the structure of chain they could be divided into linear[3] and branched-chain PEG modifiers,[4] in accordance with the purpose of modification they could be divided into random and site-directed PEG modifiers. Random PEG modifiers are invariably used to modify amino group or thiol group, such as PEG succinimide[5] correspondence amino group, PEG maleimide[6] correspondence thiol group. The random PEG modifiers are generally used in pegylation drugs, and some of them have been already on the market and have simple synthetic methods compared with other complex PEG ramifications. Nevertheless, after modification with random PEG modifiers, the drugs are still mixtures for the reason that there are many sites within the structures of protein and peptide can be linked.[7] The purification of these mixtures is usually quite difficult. So the synthesis of site-directed PEG modifiers is necessary.

Site-directed PEG modifiers target different groups, such as terminal amino group[8], disulfide bridge[9] and glutamines,[10] which compose proteins and peptides. PEG aldehyde is a site-directed modifier, which can specifically link terminal amino group, because the pKa of the terminal amino group is lower than that of ε-amino of lysine. This site-directed method of linking proteins to PEG is presented, which allows for the preparation of essentially homogeneous PEG–protein derivatives with a single PEG chain conjugated to the amine terminus of the protein.[11] Another example of site-directed PEG modifier is site-specific pegylated disulfide bridge. It is a kind of α, β-unsaturated-β′- monosulfone functionalized PEG reagent, which could react with two disulfide reduction released cystine thiols and rearrange to a three-carbon bridge.[12] In addition to the natural amino acids that can be modified, the unnatural amino acids that can also be modified. PEG acetylene is a specific reagent cycloaddicted to an azide, which was selectively incorporated into proteins.[13,14]

In this work, a novel histidine-targeting poly(ethylene glycol) modifier was synthesis. This derivative reacted with sodium nitrite and the intermediate 4-diazo-N-mPEG-benzamide proved to have a good performance for histidine modification of lysozyme.

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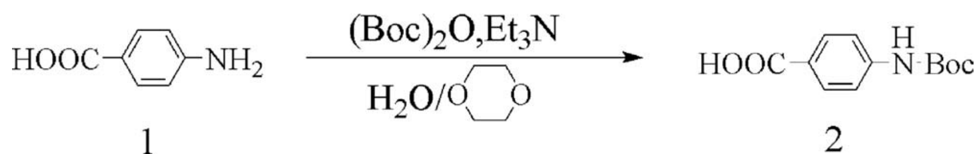


Scheme 1 Preparation of Boc-4-aminobenzoic acid.

EXPERIMENTAL

Characterization

1H-NMR analysis curves were obtained with a Bruker 500AV nuclear magnetic resonance (NMR) spectrometer. The PEG derivative samples were dissolved in deuterated trichlormethane ($CDCl_3$). The operating frequency was 500 MHz, and the temperature was 303 K. Chemical shifts were recorded in ppm units with reference to the internal tetramethylsilane.

Materials

mPEG-NH_2 (20 kDa) was purchased form JenKem Technology. 4-Dimethylaminopyridine (DMAP) were obtained from Sigma-Aldrich, Steinheim, Germany. Dicyclohexylcarbodiimide (DCC) was purchased from Sinopharm Chemical Reagent, China. Organic solvents were obtained from Nanjing Chemical Reagent, China. Lysozyme (Egg White) was purchased from Sigma-Aldrich, Steinheim, Germany. CM Sepharose FF ion exchange media were obtained from GE Healthcare. Millipore-YM-30 30KD purchased from Millipore. Sephacryl S-100 prepacked colum GE 16/60 were obtained from GE Healthcare. Micrococcus lysodeikticus ATCC was purchased from Sigma-Aldrich.

Synthesis of Boc-4-aminobenzoic acid (Compound 2)

A total of 6.8 g of 4-aminobenzoic acid (0.05 mol) and 10 g of TEA (0.10 mol) was added into 200 mL mixed solution of 1, 4-dioxane and H_2O (volume ratio = 1 : 1), and cooled to 10°C. A total of 10.8 g of $(Boc)_2O$ (0.05 mol) dropped into the solution, kept the temperature below 10°C. The reaction was carried out for 12 h under stirring at room temperature. After reaction finished by TLC testing, 1,4-dioxane was removed by vacuum distillation. The water solution was washed with ether, adjusted to pH 3.0 with 1 mol/L hydrochloric acid, and extracted with acetic ether twice. The extraction was washed with brine twice, dried with $MgSO_4$. After vacuum distillation 9.6 g Boc-4-aminobenzoic acid was ready (yield 71%).[15]

1H-NMR ($CDCl_3$, 500 MHz) analysis of Boc-4-aminobenzoic acid: σ = 1.538 (s, 9H, —$C(CH_3)_3$), σ = 6.832 (s, 1H, —NH—O—$C(CH_3)_3$), σ = 7.448–7.477 (d, 2H, —Ph—O—$C(CH_3)_3$), σ = 8.03–8.06 (d, 2H, —HOOC—Ph—).

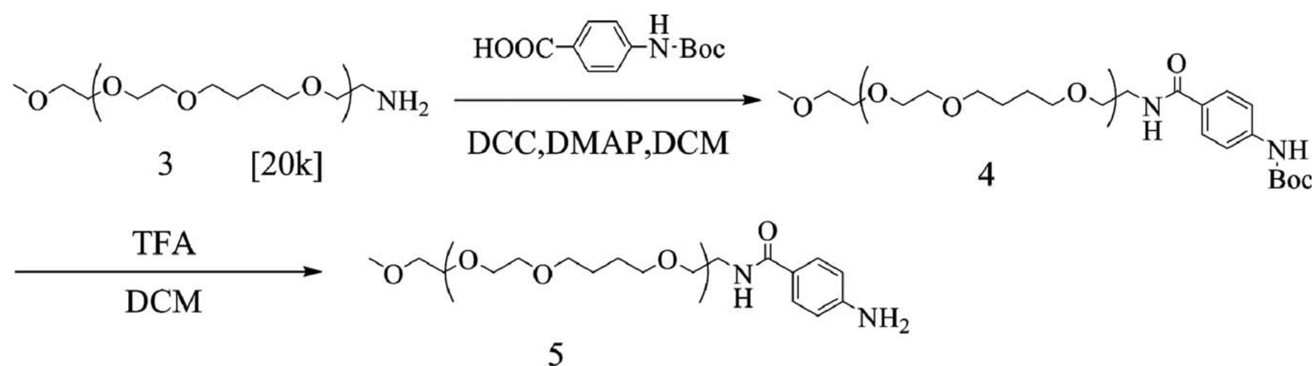
Synthesis of mPEG-Boc-4-aminobenzoic (Compound 4)

mPEG-NH_2 (M = 20000, 0.5 g, 0.025 mmol) dissolved in 20.0 mL of DCM and Boc-4-aminobenzoic acid (0.06 g, 0.25 mmol), DCC (0.05 g, 0.25 mmol), DMAP (0.03 g, 0.25 mmol) added. The reaction was carried out first in ice bath for 0.5 h, then at room temperature for 32 h. After filter the pipettes, the mPEG-Boc-4-aminobenzoic was concentrate to small volume and recovered by precipitation in cold diethyl ether and dried under vacuum (yield 95%).[16]

1H-NMR ($CDCl_3$, 500 MHz) analysis of mPEG-Boc-4-aminobenzoic: σ = 1.52 (s, 9H, —$C(CH_3)_3$), σ = 3.22 (s, 3H, —O—CH_3),σ = 3.47–3.78 (m, 1816H, —(OCH_2CH_2)n—), σ = 4.40 (s, 1H, —NH—O—$C(CH_3)_3$), σ = 7.55–7.60 (d, 2H, —Ph—NH—CH_2—CH_2—O—), σ = 7. 90–8.00(d, 2H, —CO—Ph—), σ = 8.80 (t, 1H, —NH—CH_2—CH_2—O—).

Synthesis of mPEG-4-aminobenzoic (Compound 5)

mPEG-Boc-4-aminobenzoic (0.4 g) was treated with 5.0 mL mixed solution of trifluoroacetic acid (TFA)



Scheme 2 Preparation of mPEG-4-aminobenzoic.

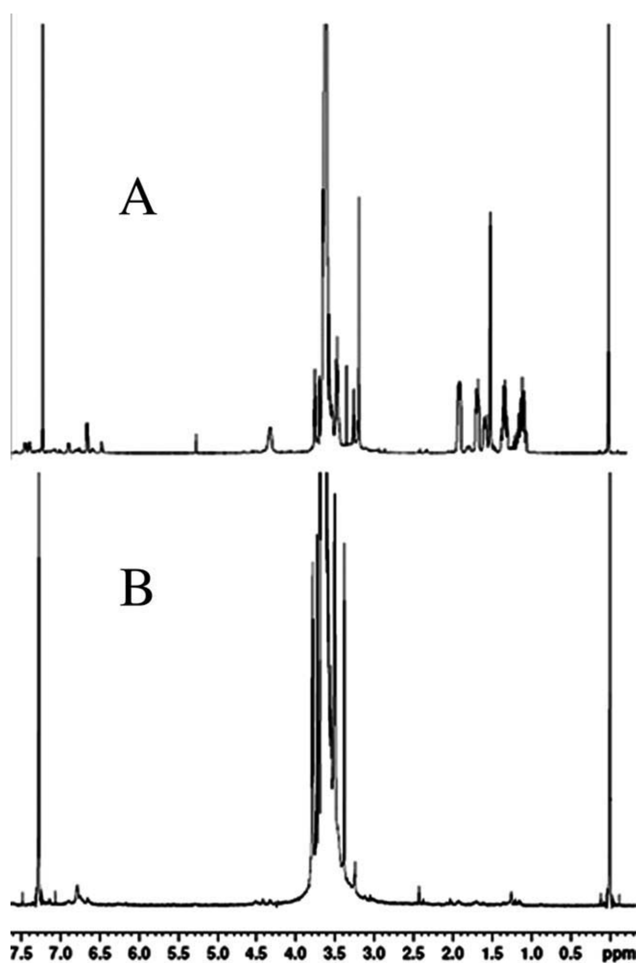


Figure 1 **A**: 1H-NMR spectra of mPEG-Boc-4-aminobenzoic. **B**: 1H-NMR spectra of mPEG-4-aminobenzoic.

and dichlormethane (volume ratio = 1 : 1) for 2 h. After vacuum distillation, the mPEG-4-aminobenzoic was recovered by precipitation in cold diethyl ether and collected (yield 86%).

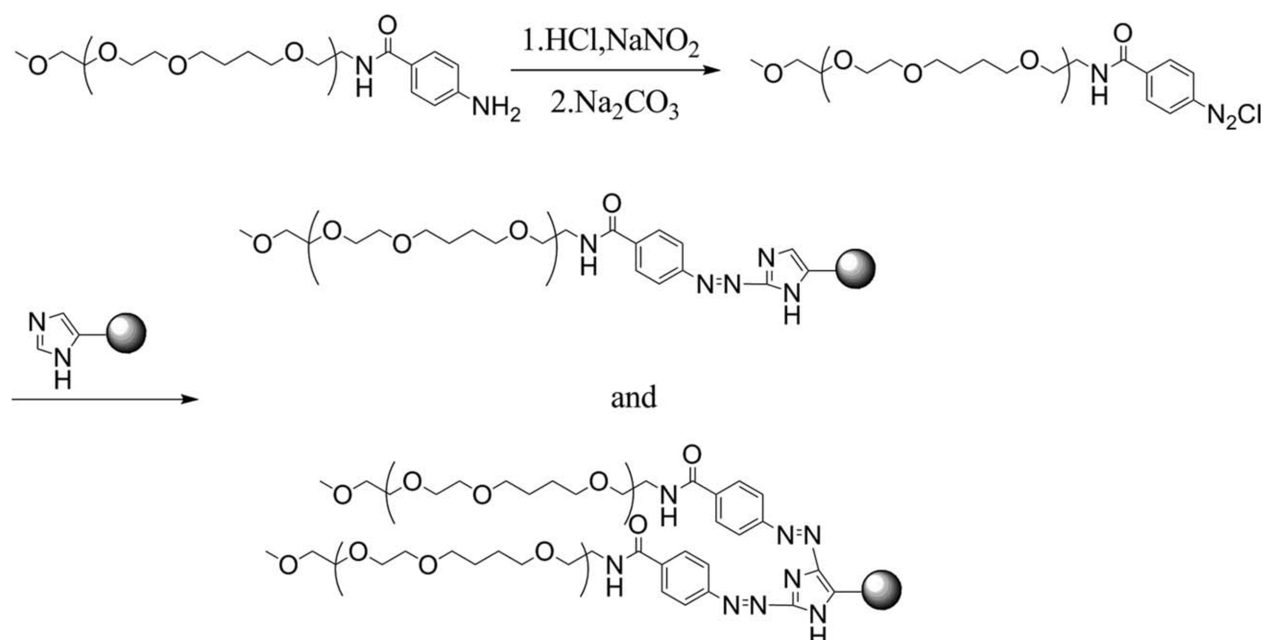
1H-NMR (CDCl3, 500 MHz) analysis of mPEG-4-aminobenzoic: σ = 3.38 (s, 3H, —O—CH_3), σ = 3.51–3.79 (m, 1816 H, — (OCH_2CH_2)n—), σ = 6.78 (s, 2H, —NH_2), σ = 7.70–7.80 (d, 2H, —Ph—NH—CH_2—CH_2—O—), σ = 7.85–7.90(d, 2H, —CO—Ph—), σ = 8.10 (t, 1H, —NH—CH_2—CH_2—O—).

PEGylation of lysozyme

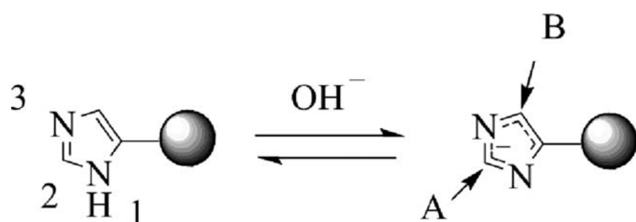
Compound 5 (56 mg) was dissolved in 2 mL water and 4 μL 1 mol/L hydrochloric acid was added. After stirring for 5 minutes, 6.8 μL 5% $NaNO_2$ was added into the reaction system, which continued to stir for 10 minutes. The Compound 6 was formed and should be used within half an hour. Then we adjusted the pH value to 8.0 with 5% Na_2CO_3, added lysozyme (40 mg), and stirred for 3 h. All the operations were under 4°C. We took samples at 30 minutes, 1 h, 2 h, 3 h of the reaction and added loading buffer to terminate it. These samples were tested by SDS-polyacrylamide gel electrophoresis (PAGE).

Purification of pegylated lysozyme

The mixture of PEG derivatives, pegylated lysozyme and lysozyme was purified by CM Sepharose FF[17] a kind of ion exchange resin, which was packed in a column with 1.6 cm diameter to a bed height of 25 cm.



Scheme 3 Modification of lysozyme.



Scheme 4 Principle of modification.

The column was equilibrated with 20 mM phosphate buffer, pH 6.5 (buffer A). The PEG-lysozyme reaction mixture was diluted to 0.5 mg/mL using the equilibration buffer A and the pH was adjusted to 6.5 with phosphoric acid. The pH adjusted reaction mixture was applied to the column at 100 cm/h with AKTA explorer. Following the load, the column was washed with 4 column volumes of equilibration buffer A at 100 cm/h. The protein was eluted with a gradient 0 to 40% 20 mM phosphate buffer, 1*M* NaCl, pH 6.5 at 100 cm/h. The protein elution profile was monitored by UV absorbance at 280 nm and collected in test tube every 3 mL. Fractions were analyzed by SDS-PAGE.

The fractions containing pegylated lysozyme were collected and concentrated using Millipore-YM-30 30KD. Pegylated lysozyme was purified by Sephacryl S-100 prepacked colum GE 16/60. The Pegylated lysozyme was applied to the column at 18 cm/h with AKTA explorer. Following the load, the column was washed with four column volumes of equilibration buffer A at 18 cm/h. The protein was eluted with buffer A. The protein elution profile was monitored by UV absorbance at 280 nm and collected.

Activity of pegylated lysozyme

We dissolved 30 mg micrococcus lysodeikticus freeze-dried powder, 0.6 g agar and 0.06 g sodium azide into 30 mL 50 mM phosphate buffer, pH 6.5. Then we poured out the mixture to an 8 cm diameter Petri dish and used 4 mm punching bear to punch holes every 15 mm. 20 μL test substance was placed into a hole, and the Petri dish was incubated for 24 h at 37°C.[18] Clear zones were produced around the holes and the diameters, which are a logarithmic function of the enzyme concentration were measured. We tested serial concentrations from 3.75 to 500 μg/mL.

Electrophoresis

SDS-PAGE was performed according to the method of Laemmli on a slab gel containing 12% (w/v) polyacrylamide running gel and 4% (w/v) stacking gel.

Stain

The protein bands were stained with coomassie brilliant blue. The PEG derivatives were stained based on the formation of barium iodide complex with PEG. After electrophoresis, we put the gel in 5% barium chloride solution and then add 0.1*M* iodine solution after pour away the barium chloride solution. Decolorizated with water until clear bands appeared.

RESULTS AND DISCUSSION

mPEG-4-aminobenzoic was successfully synthesized, Scheme 1 shows the synthesis route of Boc-4-aminobenzoic acid, the amino of 4-aminobenzoic acid 1 was protected by $(Boc)_2O$ in water-dioxane solution with triethylamine as a base. Scheme 2 shows the synthesis route of mPEG-4-aminobenzoic. Compound 3 reacted with Boc-4-aminobenzoic acid 2 in the presence of DCC and DMAP. Finally, the

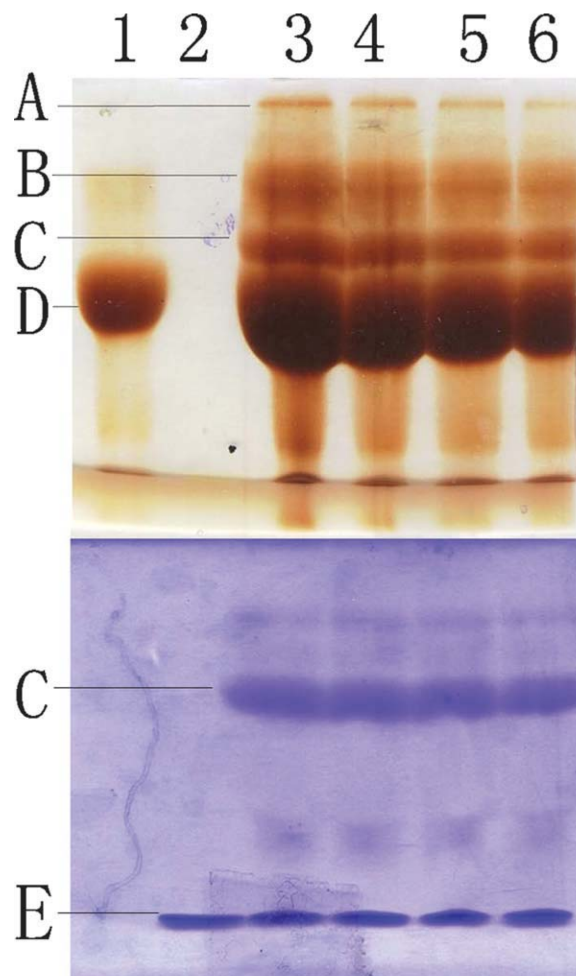
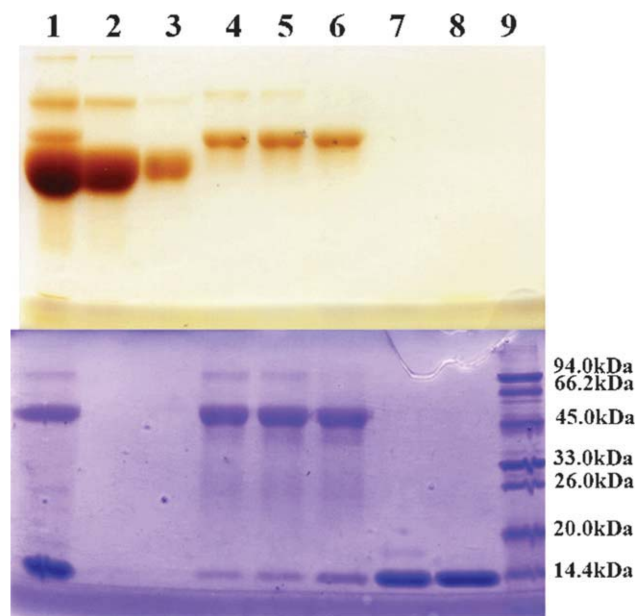


Figure 2 Iodine stained and coomassie stained gel of pegylated lysozyme versus time. Lane 1: Histidine-targeting PEG modifier. Lane 2: lysozyme. Lanes 3–6: 30 minutes, 1, 2, 3 h of the reaction mixture. **A**: PEG (80 kDa) and **B**: PEG (40 kDa) were from the mPEG amino materials; **C**: monomodified product; **D**: mPEG modifier; **E**: lysozyme. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]



product mPEG-4-aminobenzoic 5 was produced by the deprotection of mPEG-Boc-4-aminobenzoic 4 with TFA, in Figure 1 we can find the protons of CH_3 (Boc group) at 1.5 ppm, disappeared when Boc group deprotected.

The Compound 5 reacted with sodium nitrite forming intermediate 4-diazo-N-mPEG-benzamide chloride 6. Compound 6 could site directly modify the histidine of lysozyme (Scheme 3). According to Pauly reaction (Scheme 4),[19] in the imidazole group of lysozyme (only one histidine) dissociated into imidazole anion in alkaline aqueous solution (pH = 8), and the electrons delocalize from Position 1 to Position 3. When reacted with electrophile reagents, the position A will be preferentially attacked. Based on above theory, we modified lysozyme with histidine-targeting PEG modifier. Comparing the samples at 30 minutes, 1 h, 2 h, 3 h of the reaction tested by SDS-PAGE (Fig. 2), the reaction was completed within the first half-hour and the pegylated lysozyme was almost monomodified product. We analyzed component of pegylated lysozyme mixtures through SDS-PAGE.

Then the mixture of PEG derivatives, pegylated lysozyme, and lysozyme was purified. The SDS-PAGE results show on Figures 3 and 4.

After purifying the reaction mixture, we compared the activity of pegylated lysozyme with lysozyme. The clear zone's diameter of the pegylated lysozyme was 1.2 cm and corresponded with 125 μg/mL enzyme concentration. The protein concentration of pegylated lysozyme was 543 μg/mL, which was measured by UV 280 method. So compared with lysozyme, the pegylated lysozyme remained 23% activity after modified (Figs. 5

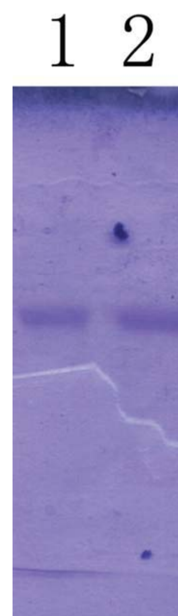


Figure 4 Coomassie stained gel of pegylaed lysozyme purification after size exclusion chromatography. Lane 1: the fraction containing pegylated lysozyme after ion exchange chromatography; Lane 2: the fraction containing pegylated lysozyme after size exclusion chromatography. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 6). As the same method, the activity of unmodified lysozyme remains 90%.

CONCLUSIONS

In summary, an efficient and facile synthesis of histidine- targeting mPEG modifier was successfully

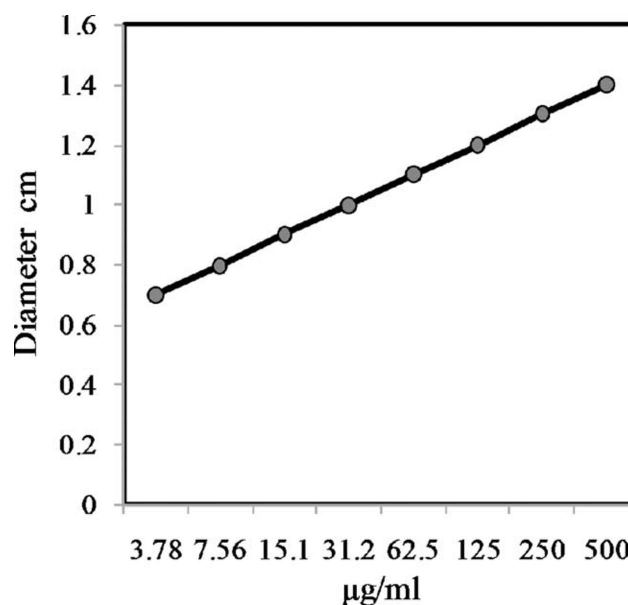


Figure 5 The clear zone's diameter of various concentrations of lysozyme incubated for 24 h at 37°C.

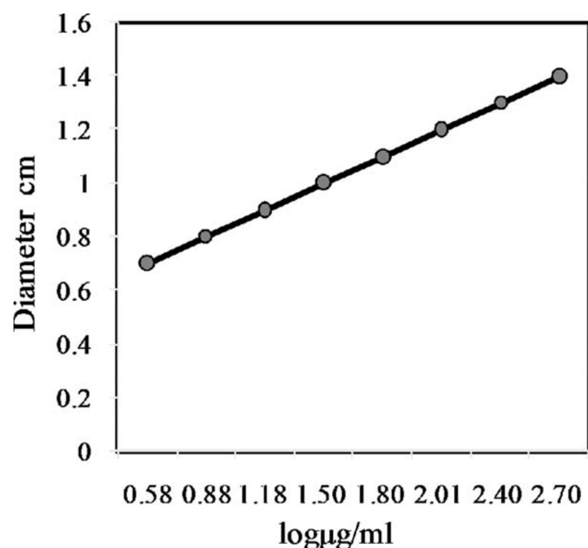


Figure 6 The diameter plotted against the logarithm of the enzyme concentration.

developed. The protein could be modified in short time (half-hour) with few double modified products. After coupling, the protein's activity was still maintained. This method may be used in drug pegylation in the future.

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