PEGylation of Bovine Serum Albumin Using Click Chemistry for the Application as Drug Carriers

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Monomethyl poly(ethylene glycol) (mPEG)-modified bovine serum albumin (BSA) conjugates (BSA-mPEG) were obtained by the mild Cu(I)-mediated cycloaddition reaction of azided BSA (BSA-N_3) and alkyne-terminated mPEG. The structure and characteristics of BSA-mPEG conjugates were thoroughly investigated. There were about two PEG chains conjugated onto each BSA molecule as determined by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis. The intrinsic nonspecific binding ability of BSA was used for adsorption and sustained release of both rifampicn and 5-fluorouracil (5-FU). The helical structures of BSA were preserved to a large extent after modification and drug adsorption on BSA was confirmed via circular dichroism spectroscopy. Drugs adsorbed onto the conjugated formulation to a lesser extent than on BSA due to mPEG modification. The in vitro release of both rifampicin and 5-FU, however, indicated that BSA-mPEG can function as a drug carrier. Overall, the click reaction provided a convenient tool for the pegylation of BSA. The biological activity of the BSA-mPEG conjugates, including the drug transportation capacity and biocompatibility, were largely retained.
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Keywords: bovine serum albumin, mPEG, click chemistry, rifampicin, 5-fluorouracil, drug carriers

Introduction

Proteins are responsible for the majority of functional attributes in all living organisms. They act as building blocks of the cell structure, catalysts in the metabolism of cells, and essential transport carriers that bind a variety of metabolites and organic compounds. However, free proteins present drawbacks such as toxicity, immunogenicity, enzyme degradation, and other limitations. Many efforts have been made to circumvent these disadvantages without sacrificing the excellent properties of proteins. Among the different strategies, judicious combination of protein with synthetic polymers is an interesting approach to synergistically integrate the properties of these different classes of materials.

Poly(ethylene glycol) (PEG) has been used in a wide range of biomedical applications.[1] It is one of the few synthetic degradable polymers with FDA approval for internal consumption and injection in a variety of foods, cosmetics,

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personal care products, and pharmaceuticals. In the 1970s, Davis and Abuchowski found that covalent attachment of PEG to bovine serum albumin (BSA) and bovine liver catalase was a useful strategy to reduce or eliminate the immunogenicity of these proteins and increase their blood circulation time.[2,3] PEGylation has also improved the targeting and tissue penetration of protein pharmaceuticals.[4–8] By a coacervation method and crosslinked with glutaraldehyde, Wu et al. prepared surface-modified human serum albumin (HSA)-PEG nanoparticles with a size of ~150 nm in diameter, which were able to reduce plasma protein adsorption and the RES (reticuloendothelial system) uptake.[9] Recently, Huang et al.[10] has modified HSA-heme by maleimide- or succinimide-terminated PEG for the stabilization of oxygen carriers and long-term circulation of heme in rats. Some PEGylated proteins have entered clinical evaluation, such as PEG-antibody fragment angiogenesis inhibitor (CDP791), PEG-interferon-alpha conjugates, PEG-granulocyte colony-stimulating factor, and PEG-megakaryocyte growth and development factor.[11–14] The aim of these PEGylated proteins is to control or ameliorate the patient's condition after a classic chemotherapy protocol.[15]

Various synthetic pathways utilized to specifically couple proteins with polymers have been reported. For example, the Michael addition reaction uses maleimide groups to react with Cys-34 of BSA.[16] The click chemistry or Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) has recently proved to be a powerful synthetic tool in numerous areas of chemistry, including protein–polymer conjugation.[17,18] The click process can be conducted in aqueous or phosphate buffer solution, without the disturbance of an organic solvent, which enables the preservation of a protein's conformation and characteristics. In this article, BSA, the most abundant protein in blood, was selected as a model protein. We aimed to achieve a suitable combination of biological and pharmacokinetic properties following the specific modification provided by the click reaction to generate a promising lead for the development of BSA-monomethyl poly(ethylene glycol) (mPEG) conjugates as drug carriers (Scheme 1).

Rifampicin and 5-fluorouraicl (5-FU) were used as hydrophobic models to comprehensively study their adsorption on the conjugates and subsequent release behavior in vitro. Herein, hydrophobic force and electrostatic interactions drove the entrapment of the drugs to BSA and its conjugates. This loading process utilizes physical forces, which eliminates covalent bonds and thus lessens the concern for reducing the therapeutic efficacy. Both continuous and discontinuous release systems were investigated.

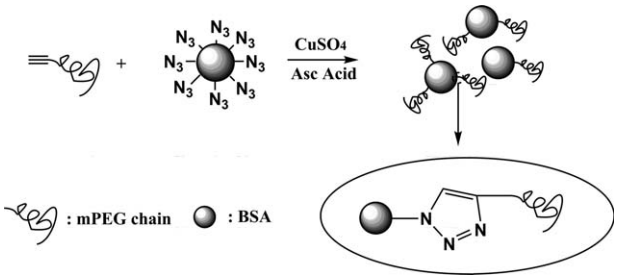
Materials and Methods

Materials

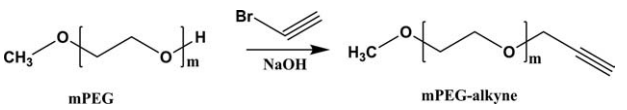
BSA (fraction V, minimum 98%), ascorbic acid, and mPEG (99% purity) were purchased from Sigma-Aldrich (St. Louis, MO). Rifampicin was imported from Fluka and packaged by Beijing Solarbio Science and Technology (Beijing, China). Propargyl bromide was purchased from Aladdin Biotech (Shanghai, China). All other organic solvents and reagents were purchased from Beijing Chemical Factory and were used as received.

General measurements

^{1}H NMR spectra were measured using a Unity-400 NMR spectrometer at room temperature, with $CDCl_3$ as solvent and tetramethylsilane as internal reference. FTIR spectra were recorded on a Bruker Vertex70 Win-IR instrument. MALDI-TOF spectra were measured by Quattro Premier (Waters) and the results were analyzed by Bruker Daltonics flex analysis. Circular dichroism (CD) was recorded by JASCO CD spectrometer, model J-820 using a 0.1-cm path length quartz cell over the wavelength range 200–250 nm. The surface electrical charge analyses were completed using a zeta potential analyzer from Brookhaven by electrophoretic light scattering method. The measurements were performed in triplicate at a temperature of 25°C with field strength of 10 V cm^{-1}. UV–vis spectroscopic measurements were carried out on a Shimadazu UV 2450 spectrometer.



Scheme 1.



Scheme 2.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast 12% polyacrylamide gels. Protein solution was mixed with SDS-PAGE sample buffer and heated in boiling water for 5 min. The slab gel was composed of 2 cm of concentrating gel and 6 cm of separating gel with 1 mm thickness. Slab gel electrophoresis was performed by JY3000 electrophoresis meter (Beijing Junyi-Dongfang Electrophoresis Equipment, China) for about 120 min with maximum voltage of 120 V. Gels were incubated in staining solution of Coomassie blue overnight and incubated in water with gentle shaking to remove background.[19]

Synthesis of alkyne-terminated mPEG

Introduction of the terminal alkyne group to PEG (Scheme 2) was achieved by phase-transfer catalysis with NaOH powder and propargyl bromide as described by Teodorescu et al.[20] Briefly, one equivalence of mPEG was reacted with an excess of propargyl bromide (20 equiv.) and 20 equiv. of NaOH powder in toluene for 15 h at 50°C. The mixture was filtered, and the solution was concentrated under reduced pressure. The residue was dissolved in water which contains a small portion of sodium chloride and then extracted with dichloromethane until the pH was neutral. After drying with sodium sulfate, the solvent was evaporated, and the final product was obtained in a yield of 84%.

^{1}H NMR ($CDCl_3$): δ = 4.20 (d, —O—C**H$_2$**—C—CH), 3.46–3.70 (m, —C**H$_2$**—C**H$_2$**—O—), 2.44 (s, —O—CH$_2$—C—C**H**).

Preparation of mPEG-modified BSA

At 37°C, 20 mg of BSA powder was reacted with 10-fold (molar ratio) of NaN_3 in phosphate buffer solution (PBS; pH

7.2, 0.1 M) for 24 h. The BSA-N_3 solution could be directly used without further separation and purification. The alkyne-terminated mPEG (1.2 equiv. to the azido group) was added into the BSA-N_3 solution. The catalyst solution was prepared from $CuSO_4{\cdot}5H_2O$ (5.0 mg) and ascorbic acid (17.6 mg) in degassed phosphate buffer (PB 5 mL, pH 7.2, 0.1 M, containing 1 mM EDTA). About 250 μL of the catalyst was added to the solution containing alkyne-terminated mPEG and BSA-N_3, and the mixture was kept stirring for 24 h at 37°C. After that, the solution was washed for more than three times using a Millipore ultracentrifuge tube (Molecular weight cutoff 10 kDa) to remove excess amount of the small molecules until it became light yellow. Finally, the aqueous solution was lyophilized, and the product was obtained as a light yellow powder (yield: 55%).

Drug loading

Different amounts of rifampicin in ethanol (1 mg mL^{-1}) were added dropwise to the aqueous solution of BSA-mPEG conjugate. After incubation for 2 h, the precipitant in the solution was removed by centrifugation (12,000 rpm, 3 min). The unloaded rifampicin was separated out by ultrafiltration (cutoff molecular weight of 10 kDa; MicroconYM-30, Millipore). The loaded rifampicin concentration was determined by its absorbance at 475 nm according to the working curve measured using standard rifampicin solutions. The measurement of the adsorption of 5-FU to BSA was conducted in the same way.

Rifampicin release in continuous flow dialysis system

The drug release profile was determined by using a dialysis technique adapted from Kostanski and DeLuca.[21] Under the room temperature, the lyophilized drug-loaded sample was dissolved in PBS (pH 7.4, 0.1 M) and poured into a dialysis tube which was immersed into 40 mL of PBS (pH 7.4, 0.1 M). The dialysis buffer was refreshed at the rate of 2.0 mL min^{-1} and the volume was kept constant. At selected time intervals, a certain amount of the dialyzed buffer was sampled and assayed at 475 nm.

5-FU release in discontinuous dialysis system

The solution of 5-FU loaded BSA was sealed into a dialysis bag (MWCO 7 kDa) and dialyzed against 50-mL PBS at 37°C. Periodically, 3 mL of drug-releasing medium was withdrawn at predetermined time intervals and assayed at 266 nm, and then the same volume of fresh PBS was added to keep the volume of the dialysis solution constant. Similar trials had been conducted by 5-Fu loaded BSA-$mPEG_{2000}$ and BSA-$mPEG_{5000}$ solutions, which were calculated to the same initial drug concentration as the 5-FU loaded BSA system. The release experiments were repeated three times and average data are reported.

Results and Discussion

Preparation and characterization of BSA-mPEG conjugates

It is well known that azido group-N_3 is a nucleophilic group and can exchange with —OH, —NH_2 and especially —X (halogen Br, Cl, or I) under certain conditions.[22] Commonly, protein was first modified with a halogen-containing molecule, and then the halogen group was exchanged to azido group using sodium azide to achieve the final azidation of protein.[23] However, the involved complex steps would probably raise the risk of organic contamination for protein. In this study, BSA was azidized by simply stirring with NaN_3 in aqueous solution for 1 day. Because the content of azido groups was very low in comparison with the abundant N element in the protein, it was difficult to directly determine the degree of azidation. For example, the IR peak corresponding to azido group around 2100 cm^{-1} was not apparent (data not shown). On the other hand, zeta potential measurement, which is usually used in the determination of isoelectric point (IEP) and the investigation of the charged surface of biomacromolecules, was applied to make sure of the azidaiton.[24,25] With an acidic IEP, BSA is negatively charged in pure water, exhibiting the values from −33.53 to −20.40 mV. While for the resultant BSA-N_3 system, its zeta potential sharply increased to almost 0 mV, which demonstrated the successful azidation consumed the negative charge on the surface of BSA. After further PEGylation, the value remained unchanged (still around 0 mV). It is in good agreement with the fact that PEG is neutrally charged and would result in a reduction in absolute values.[26] The neutral zeta potential will not facilitate the recognition of nanoparticles by the macrophages of the mononuclear phagocyte system and thus enable long circulation in blood.[27]

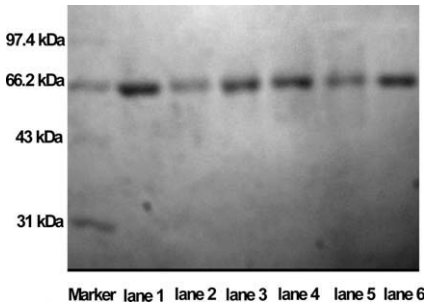


Figure 1. SDS–PAGE analysis of BSA modified with different amounts of alkyne-$mPEG_{2000}$.

Lane 1: BSA; lanes 2–6 (different feed molar ratio of mPEG to BSA): 50:1, 40:1, 30:1, 20:1, 10:1. Molecular masses are expressed in kDa.

Different molar ratios of azide groups to BSA were used in an attempt to optimize the reaction conditions. As shown in the SDS-PAGE image (Figure 1), when the ratio of azide to BSA was 20:1, the lane reached the highest level. This ratio was finally selected as the optimality for our further experiments. Nevertheless, the difference among the lanes was not quite obvious. This is probably due to the abundance of amino group and complicated 3D structures of BSA, which are different from the planar surface.

Typical conjugation methods between polymers primarily consist of grafting-from and grafting-to strategies.[28] In our work, we applied a facile preparation of polymer–protein conjugates by highly efficient CuAAC reactions. The alkyne-terminated mPEG was synthesized according to Teodorescu et al.[20] The presence of chemical shift at 2.4 ppm in the NMR spectra confirmed the successful modification of hydroxyl group with alkynyl group (Supporting Information Figure S1). The cycloaddition reaction between alkyne-terminated mPEG and BSA-N_3 was conducted under the presence of $CuSO_4$ and ascorbic acid at room temperature. The FTIR results clearly indicated the successful conjugation between BSA and PEG (Figure 2). As shown in the spectra,

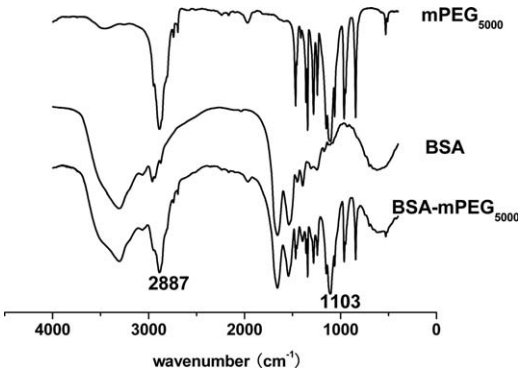


Figure 2. FTIR spectra of $mPEG_{5000}$, BSA, and BSA-$mPEG_{5000}$.

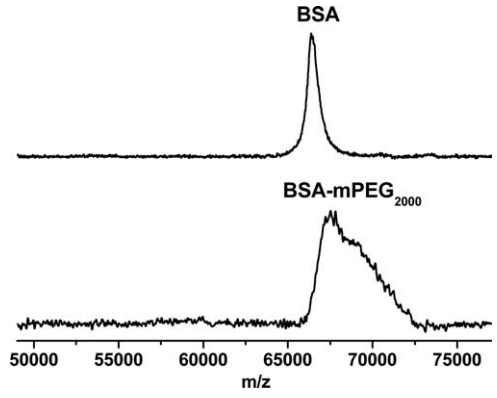


Figure 3. MALDI-TOF MS spectra of BSA and BSA-$mPEG_{2000}$ conjugates.

besides the original typical absorbance of BSA, the BSA-mPEG conjugate also possessed the absorption peaks at the 1103, 2887, and 960 cm^{-1}, which were assigned to the stretching vibration of C—O, —CH_2, and crystal peak of PEG, respectively. Samples of BSA-$mPEG_{2000}$, as prepared under the condition of NaN_3/BSA = 20/1 (mol mol^{-1}), were further analyzed by MALDI-TOF MS, which offers the possibility to determine the precise molar mass of polymers and polymer–protein conjugates. As shown in Figure 3, after PEG conjugation, well-resolved mass peaks were obtained and a shift to higher molecular masses with broader distribution could be clearly observed. With the maximum value reaching at 73 kDa, the number of PEG chains on each BSA molecule was calculated to be one to three (MW of BSA is 66 kDa).

Assessment as drug carriers

BSA, being the major plasma protein component in bloodstream, has the capacity to nonspecifically capture many endogenous and exogenous compounds via hydrogen bonding, hydrophobic forces, van der Waals, and electronic interaction, namely noncovalent bonds.[29–31] The crystal structure revealed that BSA possesses three major domains, each with two subdomains. The major binding sites, namely, sites I and II, are located at subdomains IIA and IIIA, respectively.[32] Many hydrophobic drugs have been found to bind to the hydrophobic cavity through hydrophobic or electrostatic forces.[33,34]

In this study, the nonspecific adsorption to BSA conjugates was analyzed using rifampicin and 5-FU as the model hydrophobic drugs. As shown in Figure 4, the rifampicin adsorption efficiency of the BSA-mPEG conjugate was lower

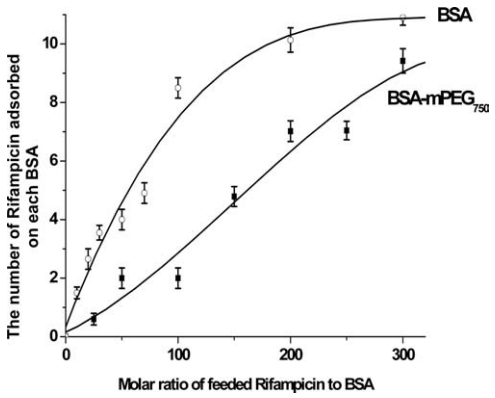


Figure 4. Adsorption profile of rifampicin onto BSA and BSA-mPEG.

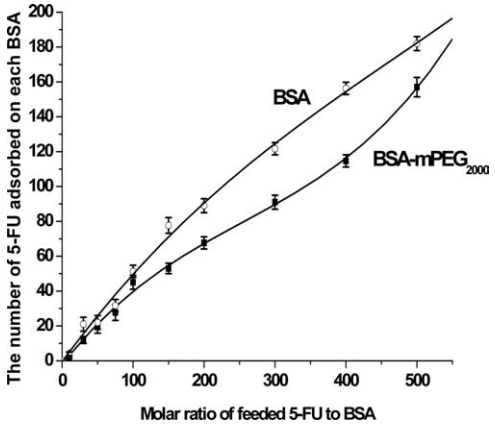


Figure 5. Adsorption profile of 5-FU onto BSA and BSA-mPEG.

than that of the pure BSA. Increasing the rifampicin amount would decrease the adsorption difference between BSA and BSA-mPEG conjugate. The PEG shielding may lessen the tendency for rifampicin to interact with the inner BSA molecule. Alternately, the lower loading efficiency could be due to less drug-protein binding sites are available after PEG modification.[35] Thus, more added rifampicin was required to reach a plateau on the BSA adsorption profile. As expected, the freeze-dried powder could be dissolved easily in aqueous solution. The outcome was consistent with the report that BSA could improve the solubility of hydrophobic drug, which may reduce the side effects caused by an organic assistant after injection.

In the case of 5-FU loading, Figure 5 indicates that the average amount of entrapped drug increased with added drug concentration, which concurs with the trend that Santhi et al. observed when they increased the drug to protein ratio.[36] Similar to rifampicin adsorption, BSA bound more 5-FU than the BSA-mPEG conjugate due to the mPEG modification.

The rifampicin release behavior was evaluated by using a continuous flow dialysis system.[21] The results showed that there was a burst release of rifampicin from BSA, producing a peak-like profile. On the contrary, BSA-mPEG conjugates released rifampicin at a constant rate lasting for more than 24 h and the release speed was about 2.5 times lower than that of drug-loaded BSA during the initial 3 h (Figure 6). Finally, the cumulative degree of the released rifampicin from BSA and the BSA-mPEG conjugates were about 62

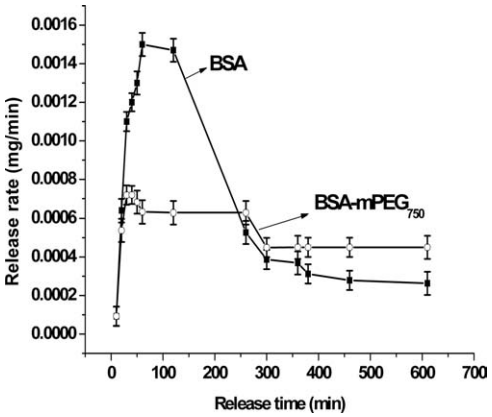


Figure 6. Rifampicin released from BSA and BSA-mPEG conjugates.

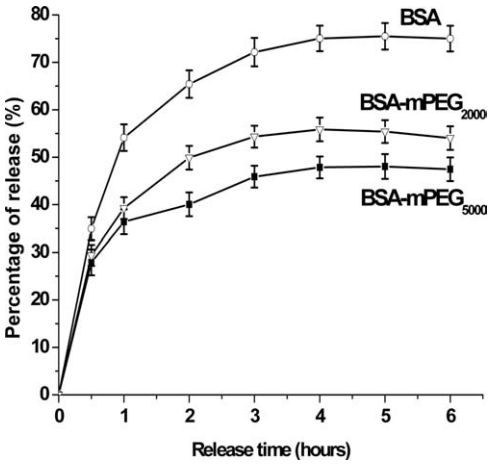


Figure 7. 5-FU released from BSA and BSA-mPEG conjugates.

and 58% of the loaded drug, respectively. These results first indicated that the conjugation of mPEG had little influence on the total drug release amount. What is more, a slow and controlled drug release was successfully achieved by this mPEG modification.

For the in vitro release of 5-FU, the fast clearance of the continuous flow system made it difficult to quantify 5-FU through UV–vis, so a traditional release assessment (i.e., discontinuous system) was conducted. Drug-loaded BSA was used as the negative standard. Compared to the rapid release of 5-FU from the BSA moiety, BSA-mPEG conjugates again slowed down the burst release of 5-FU. Although PEG chains are generally believed to not be involved in the release process, herein, the existence of PEG greatly hindered the diffusion of 5-FU and prolonged the release time. It is obvious that larger MW of PEG would have the heavier shielding effect to extend the release period (Figure 7). Similar results were also obtained for PEG-coated nanospheres, where higher molecular weigh of PEG not only reduced the burst effect but also slowed down drug release.[37] Even for PEG-metaxalone conjugates, the hydrolysis rates of metaxalone decreased with increasing molecular weight of PEG.[38]

CD spectra analyses

CD spectral measurement was used to gain a better understanding of the conformational change of BSA and its conjugates. Two strong negative bands in the UV region at 208 and 222 nm were observed, which are the characteristic bands of the α-helical structure of BSA.[39] The addition of drugs to BSA caused minor change to the α-helical structure of BSA. The more drugs adsorbed to BSA, the more obvious the change became (data not shown). However, denaturation did not occur and BSA could retain most of its helical structure.[40] On the other hand, the conjugation of PEG to BSA also induced some conformational changes in BSA, but the secondary structure of BSA retained its α-helix character predominantly (data not shown). It is assumed that the trace amount of Cu residue would contribute to the minor conformation change of BSA. All these experimental findings demonstrated that the protein–polymer and protein–drug complex retained the secondary structure and helicity of BSA to a large extent to maintain its bioactivity for biomedical applications.[41]

Conclusions

BSA-mPEG conjugates have been prepared through mild "click" reaction under a grafting-to strategy. The mean number of PEG modification was calculated to be about two from the MALDI-TOF spectra. Driven by hydrophobic and electrostatic interactions, rifampicin and 5-FU were loaded on BSA as well as BSA-mPEG conjugates. The water solubility of hydrophobic drugs after adsorption was enhanced. Both the continuous flow dialysis system for Rifampcin and discontinuous system for 5-FU showed that the BSA-mPEG conjugates afforded sustained release in vitro. CD analyses proved that after conjugation with PEG and adsorption of drugs, BSA retained its secondary structure and helicity to a large extent. Overall, these encouraging data indicate that BSA-mPEG conjugates are promising carriers for a variety of drugs.

Acknowledgments

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