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Investigation of the interaction between poly(ethylene glycol) and protein molecules using low field nuclear magnetic resonance

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ABSTRACT

A comprehensive insight into the interaction between proteins and poly(ethylene glycol) (PEG) is crucial to understand the behavior of PEG, which is widely used in pharmaceutical and medical applications. Although PEG is believed to be an excellent material to resist non-specific protein adsorption, there is a lack of quantitative information about the interactions between proteins and PEG. In this paper the interactions of bovine serum albumin (BSA) and lysozyme (LYZ) with different molecular weight (MW) PEGs were investigated through the T_2 relaxation time of PEGs measured by low field nuclear magnetic resonance spectroscopy. The integrated signal intensity of PEGs was quantified under various conditions from the concentrations and MWs of PEG, and ionic strength of solutions, as well as the molar ratios of PEG to protein. The results show that a large number of PEG molecules could associate with protein molecules with association constants in the range $\sim 10^4$–$10^5\ M^{-1}$. The association constant is insensitive to the ionic strength change in the physiological range and the lowest associate constant occurs at the medium MW PEG with protein. This suggests that the interaction between PEG and protein molecules might not be negligible in investigations of the resistance to non-specific protein adsorption. Long chain PEG coatings might cause modest protein adsorption, which could interfere with any weak specific interaction between ligand and receptor. Thus, it is necessary to reconsider the popular accepted method of protecting nanoparticles (NP) in blood with long chain PEG coatings since these NPs might be surrounded by a layer of weakly adsorbed plasma protein in the circulatory system.

1. Introduction

The stealth property of non-ionic hydrophilic poly(ethylene glycol) (PEG) polymers is applied in lots of PEG conjugates and PEGylated carriers for various drug delivery applications [1–3]. It is well known that PEG segments can mask foreign materials, for example they are less detectable by the immune system, not only via humeral reactions but also at the cell level. The molecular weight and the polydispersity of PEG have been shown to be very important effects in the prevention of protein adsorption [4]. Thus it is vital that we understand the origin of the repulsive force, the degree of resistance to protein adsorption and also the influence of solution conditions. A possible interaction of PEG with biological macromolecules might alter our understanding of the behavior of PEG-protected protein conjugates and nanoscale drug carriers for biological and biomedical engineering [5–7]. In fact, such a possible interaction might induce structural changes or adsorption of proteins due to the physico-chemical nature of the polymer-coated surface, which might affect intrinsic properties of the protein [8,9], as well as induce the accelerated blood clearance phenomenon upon repeated injections of PEG-coated colloidal carriers [10,11]. Moreover, it might also decrease the biological activity of PEGylated enzymes [12] and compromise the specific selectivity of some weak ligand–receptor interactions. For example, the association constants of PEG–protein and weak ligand–receptor pairs are rather comparable. The average association constants [13] for the binding of organic molecules by cyclodextrins and albumins in water are $10^{3.5\pm2.5}\ M^{-1}$. The binding affinities for the complexation of biological antigens by antibodies are about $10^{8\pm2}\ M^{-1}$. For example, the AB_3 isolectin binds α-N-acetylgalactosamine on type A erythrocytes with an association constant of $7.5 \times 10^5\ M^{-1}$. It is obvious that possible inference caused by the PEG chain might not be ignorable. Severe competition might occur if a protein could contact multiple PEG chains. Thus a comprehensive understanding of the interaction between proteins and PEG will lead to improvements in the resistance to protein adsorption by choice of the right form of PEG-containing polymer. It may also improve the design of new non-fouling materials through an understanding of the details of the interactions between synthetic polymers and biomacromolecules, especially proteins [14,15].

To investigate the possible interactions between PEG and proteins two typical model proteins, serum albumin and lysozyme (LYZ), were selected. Serum albumins are the major soluble protein

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constituents of the circulatory system and also have various physiological functions [16]. One of the most important properties of this group of proteins is that they serve as transporters for a number of hydrophobic compounds [17]. Bovine serum albumin (BSA) is one of the most frequently studied proteins in this group due to the structural homology between BSA and human serum albumin (HSA). LYZ is a basic protein belonging to the class of enzymes that lyse the cell walls of bacteria [18,19], as well as being a model small sized, positively charged to evaluate the resistance to non-specific protein adsorption of PEG or other material coated surfaces. LYZ is extensively used in the pharmaceutical and food fields due to its biological functions, including antimicrobacterial, anti-inflammatory, anti-viral, immune modulatory, anti-histaminic and anti-tumor activities [18,20–22]. Thus the differences between the interaction of these two model proteins with PEG will provide more information on the mechanism of PEG resistance to protein adsorption.

A number of studies have been proposed to explain the interactions between PEG and BSA and LYZ, such as ^{1}H NMR spectroscopy [14], circular dichroism (CD) spectroscopy [7,17,23], Fourier transform infrared (FTIR) spectroscopy [17,23], ultraviolet (UV–VIS) spectroscopy [23], differential scanning calorimetry (DSC) [7,24], fluorescence spectroscopy [7,17], light scattering experiments [25], and isothermal titration calorimetry (ITC) [24]. All these investigations indicated that PEG interacts with proteins through the hydrophobic area or via hydrogen bonding. However, there is a lack of detail and quantitative information on the interactions between PEG and BSA and LYZ under physiological conditions, although evidence of these interactions were provided by these studies.

According to a previous study of the hydration of PEG using NMR the observed PEG peak in the T_2 inversion spectra of PEG–water mixture can provide direct information on the structural and dynamic changes in PEGs caused by interactions with biomacromolecules [26]. Thus it would be of interest to use NMR to investigate the interaction between PEG and proteins such as BSA and LYZ to characterize the polymer–protein complexes. In this work we quantified the integrated signal intensity of PEGs under various conditions with respect to the concentration and molecular weight (MW) of PEG and the ionic strength of the solutions, as well as the mass ratios of PEG to BSA or LYZ, which reveal quantitative data about the non-specific interactions between BSA or LYZ and PEG polymers in aqueous solution.

2. Materials and methods

2.1. Materials

2 kDa PEG polymer (MW PEG 2 K) and PEG 6 K were purchased from Aladdin Reagents (China). PEG 10 K and PEG 200 K were purchased from J&K Chemicals. BSA and LYZ were purchased from Sangon Biotech (Shanghai) Co. Ltd. Water used in the experiments was purified using a Millipore water purification system with a minimum resistivity of 18.0 MΩ cm. Sodium chloride (NaCl) was purchased from Aladdin Reagents (China).

2.2. Methods

2.2.1. Sample preparation

Protein–polymer solutions with different protein/polymer ratios, concentrations and ionic strengths were prepared by a similar procedure. In brief, 0.01, 0.02, 0.04, 0.06 or 0.08 g of BSA and 0.2 g of PEG of different MW (2 K, 6 K, 10 K and 200 K) were dissolved in 2 ml of water in a glass tube (diameter 15 mm) to investigate the effect of MW on the PEG–BSA interaction. Diluted PEG solutions at 3/4, 1/2 and 1/4 of the initial concentrations were also used to investigate the effect of concentration. To determine the differences between different proteins LYZ was used as well as BSA. NaCl was added to adjust the final salt concentration to 0.15 M to compare the possible effects of ionic strength.

2.2.2. NMR measurement

NMR relaxation measurements were performed using the same method as used to detect the hydration behavior with PEG [26] on a Niumag bench-top pulsed NMR Analyzer PQ001 (Niumag Electric Corp., Shang, China) operating at a resonance frequency for protons of 21 MHz (0.5 T). Samples were inserted in the NMR probe. The spin–spin relaxation time T_2 was measured using the Carr–Purcell–Meiboom–Gill (CPMG) sequence [27]. The maximum point of every second echo was accumulated using a 90° pulse of 17 μs and a 180° pulse of 34 μs. The delay between the 180° pulses τ was 500 μs. The amplitude of every second echo was measured, a total of 18,000 points collected, and scans coded using a repetition time T_R of 3 s. The relaxation measurements were performed at 32 °C.

2.2.3. Spin–spin T_2 relaxation time inversion spectra

The rapid development of low field nuclear magnetic resonance (LF-NMR) spectroscopy and the T_2 inversion method for analyzing multi-component T_2 relaxation times using the CPMG sequence has allowed the development of a simple way to quantify the number of small molecules in different environments or states. In these CPMG experiments echo heights are usually recorded at discrete time points and decay of the echo heights characterized by the time constant T_2, with the time-dependent echo signal being represented as [27]:

$$M(t) = \sum_i P_i \exp\left(\frac{-t}{T_{2i}}\right) \quad (1)$$

where P_i is the signal intensity of the ith component whose spin–spin relaxation time is T_{2i}.

When the sample under investigation has only one species in a homogeneous environment the echo height would obey a mono-exponential decay pattern. However, in most systems there are several different species in multiple environments. All these "species–environment" combinations will, in principle, have characteristic T_2 values for their decaying echos. In our experiment the continuous distribution exponentials related to the water and PEG protons in the NMR T_2 relaxation data were analyzed using the CONTIN algorithm [28] for all the CPMG curves. "T_2 inversion spectrum" analysis gives a plot of the relaxation component intensity with the distributed relaxation time.

2.2.4. Adsorption binding constant

The absorbance titrations were performed by keeping the concentration of PEG constant while varying the concentration of BSA. The binding constant K values were obtained according to the method described by Zhong et al. [29]. Assuming that there is only one type of interaction between polymer and protein in aqueous solution gives Eqs. (2) and (3):

$$\mathrm{BSA} + \mathrm{polymer} \leftrightarrow \mathrm{BSA-polymer} \mathrm{BSA} + \mathrm{polymer} \Longleftrightarrow \mathrm{BSA-Polymer} \quad (2)$$

$$K = [\mathrm{BSA-polymer}]/[\mathrm{BSA}][\mathrm{polymer}] K = \frac{[\mathrm{BSA-polymer}]}{[\mathrm{BSA}][\mathrm{polymer}]} \quad (3)$$

where K is the binding constant and polymer is PEG. In our experiment the recorded integrated signal intensity A is related to the polymer concentration of free and loosely adsorbed PEG at different BSA concentrations. A_0 is the initial integrated signal intensity of free PEG and A is the recorded integrated signal intensity at