A facile method for studying interaction of rhodamine B and bovine serum albumin: Towards physical-binding mediated fluorescence labeling of proteins (Postprint)

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Date: 2023-06-18T00:00:00+00:00

Abstract

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Full Text

Preamble

ChinaXiv Cooperative Journal NUCLEAR SCIENCE AND TECHNIQUES 26, 060502 (2015)

A Facile Method for Studying Interaction of Rhodamine B and Bovine Serum Albumin: Towards Physical-Binding Mediated Fluorescence Labeling of Proteins

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(Received June 8, 2015; accepted in revised form August 2, 2015; published online December 20, 2015)

Strategies for labeling proteins with fluorophores are always important for biotechnology. Here we take a model protein (bovine serum albumin) and a typical fluorophore (rhodamine B) to demonstrate a direct labeling method just by physical adsorption. In combination with size exclusion chromatography and the Scatchard equation, we have developed a facile analysis method for calculating the binding constant and binding sites. The molecular docking method has been used to study the binding site at the amino acid level.

Keywords: Bovine serum albumin, Rhodamine B, Binding sites, Scatchard equation, Size exclusion chromatography

DOI: 10.13538/j.1001-8042/nst.26.060502

Introduction

Fluorescence labeling of proteins, especially antibodies, has been widely used in current biotechnology applications. Generally, there are two requirements for successful bio-labeling: first, both the original functionality of biomolecules and the original fluorescence properties of the markers must remain intact after labeling; and second, the labeling should be robust enough for subsequent applications. Protein labeling kits are commercially available from Invitrogen, Sigma-Aldrich, and other suppliers. The labeling technologies can be classified as chemical conjugation and physical binding. The former includes carbodiimide-mediated crosslinking of carboxylic groups and primary amino groups, as well as azide and alkyne-mediated click chemistry, while the latter includes biomolecular pair-mediated labeling using DNA, biotin-avidin, aptamer-target, protein A/G-antibody, ligand-receptor, and antigen-antibody interactions.

Although fluorescent nanoparticles such as semiconductor quantum dots [1–4], graphene quantum dots [5–7], nanodiamonds [8–10], and fluorescent carbon dots [11, 12] have attracted increasing interest for both labeling/imaging and sensing due to their excellent photostability, organic dyes still play the main labeling role when considering toxicity and size limitations.

Rhodamine B (RB), as a dye or dye laser gain medium [13, 14], is often used as a tracer dye in aqueous solutions to determine flow rates and transport directions. Due to its good water solubility (15 g/L), high quantum yield (about 94%), and the fact that its fluorescence can be easily and inexpensively detected by a fluorometer, RB has been extensively used as a biomarker in biotechnological labeling and imaging for fluorescence microscopy, flow cytometry, fluorescence correlation spectroscopy (FCS), and enzyme-linked immunosorbent assay (ELISA).

Bovine serum albumin (BSA), also known as "fraction V" —a term referring to albumin being the fifth fraction of the original Edwin Cohn purification methodology that utilized differential solubility characteristics of plasma proteins [15]— is a serum albumin protein derived from cows. Due to its stability, low cost, and well-defined structural information, BSA has been used as a model protein for

many research purposes. For example, it can serve as a protein concentration standard in laboratory experiments, and its amphiphilic properties also make it a good carrier for both natural and artificial drugs such as vitamins and paclitaxel. The mature BSA protein contains 583 amino acids (Mw 66,463 Da, 66.5 kDa), which is produced through two enzymatic cleavages from a 607-amino-acid full-length BSA precursor protein [16].

In this paper, we take the conventional fluorophore RB and the model protein BSA as objects for an interaction study towards developing a direct labeling method. With the goal of creating a new facile analysis approach based on size exclusion chromatography (SEC), we have determined the association constant/binding affinity (Ka) and the maximum binding sites of RB to BSA by employing the well-known Scatchard equation, and further studied the detailed interaction information using molecular docking.

Experimental Section

A. Materials

BSA (lyophilized powder >98%), RB (>99%), and all other chemical reagents were purchased from Sigma-Aldrich Corporation. Deionized water (18.2 MΩ · cm) from a Milli-Q system (Millipore, Bedford, MA) was used for all experiments. BSA was dissolved in Milli-Q water to prepare a stock solution with a concentration of 600 µM. RB was dissolved in Milli-Q water with a stock concentration of 600 µM.

B. Spectroscopic Measurements

UV-vis absorption spectra were recorded on a U-2900 UV-vis spectrometer (Hitachi).

C. SEC Assays

The SEC assays were performed on a sephacryl S-300 column equipped with a high performance liquid chromatography (HPLC) system (Hitachi L2000 or Agilent 1260) using SB9 buffer (sodium borate 50 mM, pH = 9) as the mobile phase at a flow rate of 1 mL/min. The spectra were monitored at 280 nm for the UV detector and at excitation (Ex): 555 nm/emission (Em): 575 nm for the fluorescence detector. For the fluorometric titration experiment, 995 µL solutions containing different RB/BSA ratios of 64/1, 32/1, 16/1, 8/1, and 4/1 with a fixed final BSA concentration of 2.29 nM were prepared and incubated at 298 K for 2 h.

D. Molecular Docking

The three-dimensional (3D) structure of BSA (PDB ID: 4JK4) was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do). The AutoDock Tools 1.5.6 package (http://mgltools.scripps.edu) was employed to

generate the docking input files. The search grid for BSA was identified with center x: 97.127, center y: 24.933, and center z: 20.919, and dimensions size x: 15, size y: 15, and size z: 15. For Vina docking, default parameters were used unless otherwise specified. The best-scoring pose as judged by the Vina docking score was selected and visually analyzed using PyMOL software (http://www.pymol.org/).

Results and Discussion

A. Binding Constant and Binding Sites

A number of methods can be used to calculate or measure binding constants and binding sites. Some researchers prefer using fluorescence quenching [17–19] to study interactions between proteins and other molecules. This method is facile and easily accessible, but it is limited when the molecules are not quenchers of the proteins' fluorescence. Other popular methods include quartz crystal microbalance (QCM)-based [20] and surface plasmon resonance (SPR)-based [21] techniques, but these are quite expensive due to the consumption of specific chips. The raw data obtained with these methods normally require further processing in combination with classical equations such as the Stern-Volmer equation, Scatchard equation, and Hill equation to obtain binding constants, sites, and even cooperativity.

SEC is a powerful tool for both analysis and separation. Because proteins are several times larger than labeling fluorophores, which are normally less than 1 nm, we can readily separate bound fluorophores from free ones, which provides exactly the data required by the Scatchard equation:

$$\nu/C_f(RB) = (n - \nu)K_a$$

where C_f is the concentration of the free ligand, which in our case is the concentration of unbound RB; n is the number of binding sites per protein molecule; K_a is the association/binding constant/affinity of RB for BSA; and ν is the real bound ratio defined as $\nu = (C_t(RB) - C_f(RB))/C_t(BSA)$, where $C_t(RB)$ is the total concentration of RB known before the binding assay, $C_f(RB)$ is the free or unbound RB concentration obtained by SEC measurement, and $C_t(BSA)$ is the total BSA concentration for the binding assay, which is known and constant in the current assay.

We used RB and BSA as an example to justify the use of SEC in combination with the Scatchard equation to calculate binding constants and sites between molecules and proteins. To be more precise, we performed SEC with pure dye to obtain an SEC-based calibration curve of concentration (Fig. 1) by plotting integrated elution peaks against concentrations. From Fig. 2, it can be seen that with the molar ratios of RB/BSA described in Section II C, the RB fluorescence intensity decreases linearly with increasing BSA/RB ratios at a fixed RB molar concentration. The binding constant and binding sites were calculated

to be 2.2×10^5 M^{-1} and 1.3, respectively. Cai et al. reported binding constant and binding sites of 4.8×10^4 M^{-1} and 1.2, respectively [22]. However, their further analysis by time-resolved fluorescence indicated that at least two binding sites existed [22]. Real binding sites can be highly resolved by crystallographic studies of protein-molecule complexes; for example, Sekula B. and colleagues found four binding sites of 3,5-diiodosalicylic acid to BSA [23]. The binding affinity obtained by the current method appears much higher than in Ref. [22]. We believe that different buffers and instrument sensitivities might be responsible, and that the fluorescence quenching method can produce large deviations for binding affinity evaluation [24]. Therefore, it would be helpful to compare different methods under the same conditions.

It is not difficult to find intermolecular interaction studies by searching scientific publication databases, but it may be difficult to determine how those scientists obtained the necessary values for the Scatchard plot. By using SEC assisted with an HPLC system, one can clearly learn how to create this plot step-by-step. With the well-known reproducibility of commercial HPLC systems such as the Agilent used in this paper, one can directly calculate both binding constant and binding sites simply by preparing the mixture solution. Additionally, the concentration calibration curve of free dye alone obtained using the same HPLC configuration parameters—such as column, flow rates, and loading volumes—is necessary because it minimizes measurement error.

B. Molecular Docking of RB and BSA

To study the binding mode of RB to BSA, molecular docking was performed using AutoDock. As shown in Fig. 3, the RB molecule is docked into the binding pocket of BSA. One diethylamino group of RB fits into the bottom of the BSA binding pocket, surrounded by residues Trp213, Arg194, and Arg198, while the other diethylamino group is located at the entrance of the pocket and interacts with hydrophobic residues Val342, Ala341, and Pro446. Importantly, the carboxyl group forms two key hydrogen bonds (2.0 and 3.3 Å) with residue Arg194, which is important for the affinity between RB and BSA.

In summary, the computational approach helps in better understanding inhibitor binding to the protein active site, providing valuable information for further study of the interaction between RB and BSA. Limited by the docking software, which does not support multi-molecular docking, we did not attempt to identify additional binding sites.

Conclusion

Compared with different chemical labeling strategies, the direct labeling method with such high binding affinity holds great promise for future biotechnology applications. The facile method combining SEC and the Scatchard equation has demonstrated feasibility for determining the binding constant and sites of BSA-RB interaction, which may find broader applications with other proteins

and small molecules or any two substances with large size differences. We hope this study provides a good example of the effective combination of experimental measurements and theoretical simulations.

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Note: Figure translations are in progress. See original paper for figures.