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Characterization of the binding of shikonin to human immunoglobulin using scanning electron microscope, molecular modeling and multi-spectroscopic methods

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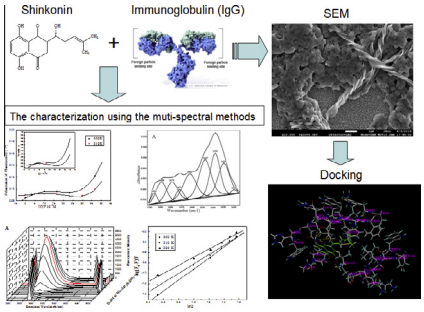
HIGHLIGHTS

- The ultrastructure on the interaction of shikonin and HIgG was visually displayed.
- The present of shinkonin induced the change of conformation and secondary structure of HIgG.
- The binding and thermodynamic parameters illustrated the acting mechanism of shinkonin and HIgG at molecule level.

GRAPHICAL ABSTRACT



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ABSTRACT

Shikonin, one of the active components isolated from the root of *Arnebia euchroma* (Royle) Johnst, have anti-tumor, anti-bacterial and anti-inflammatory activities and has been used clinically in phlebitis and vascular purpura. In the present work, the interaction of human immunoglobulin (HIg) with shikonin has been investigated by using scanning electron microscope (SEM), Fourier transform infrared (FT-IR) spectroscopy, fluorescence polarization, synchronous and 3D fluorescence spectroscopy in combination with molecular modeling techniques under physiological conditions with drug concentrations of 3.33–36.67 μM. The results of SEM exhibited visually the special effect on aggregation behavior of the complex formed between HIg and shikonin. The fluorescence polarization values indicated that shikonin molecules were found in a motionally unrestricted environment introduced by HIg. Molecular docking showed the shikonin moiety bound to the hydrophobic cavity of HIg, and there are four hydrogen-bonding interactions between shikonin and the residues of protein. The synchronous and 3D fluorescence spectra confirmed that shikonin could quench the intrinsic fluorescence of HIg and has an effect on the microenvironment around HIg in aqueous solution. The changes in the secondary structure of HIg were estimated by qualitative and quantitative FT-IR spectroscopic analysis. The binding constants and thermodynamic parameters for shikonin–HIg systems were obtained under different temperatures (300 K, 310 K and 320 K). The above results revealed the binding mechanism of shikonin and HIg at the ultrastructure and molecular level.

Abbreviations: HIg, human immunoglobulin; SEM, scanning electron microscope; FT-IR, Fourier transform infrared; FP, fluorescence polarization; AIR, attenuated total reflection; H, heavy chains; L, light chains; Fab, fragment of antigen binding; Fc, crystallizable fragments regions; CDR, complement-determining region; FR, framework region.

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Introduction

A number of biochemical and molecular biological investigations have revealed that proteins are frequently the 'targets' for therapeutically active drugs of both natural and synthetic origin. Especially, binding of small-molecule to proteins is an important biochemical and biological process and is used as a basis for drug design [1]. Such binding can cause conformational changes in proteins, and such changes have been observed in many experimentally determined three-dimensional structures [2]. As cell surface receptor of B lymphocyte, immunoglobulin also plays critical roles in human immune response and many cell actions. Especially, human immune gammaglobulin is present itself in the blood of adults at 9.5–12.5 mg/mL, and as one of human plasma proteins, it is capable of binding an extraordinarily diverse range of metabolites, drugs, organic compounds and relevant antigens [3]. The globular protein human immunoglobulin (HIg), which consists of 670 amino acids in two identical polypeptide chain groups with molecular weight of 150 kDa, is commonly used as a model protein in the study of such interactions [4–6]. The binding of drugs to HIg also has an important role in therapeutic drug monitoring as the binding may be influenced by a number of drug and patient-associated factors, resulting in altered free drug concentration and thus drug efficacy and toxicity may be altered. On the other hand, the interaction between protein and small molecule may cause the change of secondary structure, which is extremely important to elucidate biological function and mechanism of action of protein. However, more studies have been focused on the specificity of antibody–antigen interactions about HIg by various molecular biological methods, and fewer studies on the binding interaction between HIg and active component of plant medicine by chemical analysis methods were reported.

In a series of study methods concerning the interaction between small molecules and biomacromolecule, spectroscopy techniques are great aids because of their high sensitivity, rapidity and ease of implementation [2]. There are many studies on the binding of small molecules to different proteins by fluorescence spectroscopy [7,8], circular dichroism [9], Fourier-transform infrared (FT-IR) spectroscopy [10], adsorption, and dynamic light scattering [11] etc. Although these spectroscopy methods are useful tools for examining the changes of microenvironment or secondary structure of proteins at the molecular level, more information on the aggregation behavior of the complex formed between small molecules and protein in solution has not been obtained. Fluorescence polarization (FP) is not longer a new technique in theory, but it is a powerful and sensitive technique for the study of intermolecular interactions in aqueous solution. FP is based on the observation of the molecular movement of the fluorescent molecules in solution and does not require physical separation of the excess ligand or acceptor [12,13]. In recent years, some researches have shown that FP constitutes one of the most employed techniques for the routine analysis of small molecules in a variety of application fields including food [14], medicine [15], and environmental areas [16] because of its homogeneous format, speed, accuracy, and automated high-throughput capability. In addition, the scanning electron microscope (SEM) is a versatile instrument for imaging structures with dimensions ranging from nanometres to millimeters. It is favorable to observe the microstructure and ultrastructure of specimen and investigate its potential biological characteristics [17,18]. However, there is less report on the interaction between a small molecule and protein by SEM method. In this article, SEM was used to investigate the binding of shinkonin to HIg for dynamic monitoring as a bright spot.

Shikonin (Fig. 1), isolated from the roots of the traditional herb *Lithospermum erythrorhizon*, is a natural product that has a long history in treating burns, inflammations, wounds, and ulcers in the Far East and Europe [19]. Over the past few decades, its potential as a drug candidate for cancer treatment has evoked a lot of research interest for its high biological activities, such as antibacterial, antiviral, analgesic, angiostatic, anti-inflammatory and anti-tumor properties and has been used clinically [20]. Recently, significant attention has been paid to the mechanism on treating disease of shikonin. Gong et al. investigated the effects of shikonin on human hepatocellular carcinoma *in vitro* and *in vivo* [21]. Duan et al. reported that shikonin interacted with the cytosolic thioredoxin reductase, an important selenocysteine (Sec)-containing antioxidant enzyme with a C-terminal -Gly-Cys-Sec-Gly active site, to induce reactive oxygen species (ROS)-mediated apoptosis in human promyelocytic leukemia HL-60 cells [22]. Zhang et al. found that shikonin could act as a selective estrogen enzyme modulator by down-regulating the steroid sulfatase expression [23]. Ogawa et al. reported that shikonin is a novel contributor to the control of period length in mammalian cells [24]. However, the interaction between shikonin and human immunoglobulin is still unclear. Well understanding of the binding characteristics of shikonin to the protein is very important for evaluation of its efficacy and for revelation of novel biological function and mechanism of action of the protein. Here, the effects of shikonin on the structure of HIg were studied under simulated physiological conditions by SEM, molecular modeling and different spectroscopic methods. These are first spectroscopic results on shikonin–HIg interaction at the ultrastructure and molecular level, which can illustrate the nature of shikonin–protein complications *in vitro* and *in vivo*.

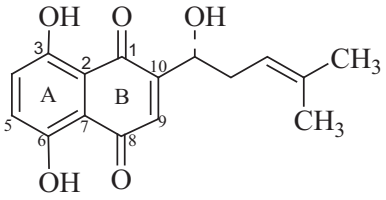

Fig. 1. The chemical structure of shikonin.

Experimental

Materials

Shikonin was of analytical grade, and purchased from the National Institute for Control of Pharmaceutical and Bioproducts, China, and the stock solution was prepared in ethanol. Human immunoglobulin (HIg, 2.5 g/50 mL) purchased from Shanghai Bio Science & Technology Co. Ltd was used without further purification and its molecular weight was deemed to be 150 kDa. Tris (hydroxymethyl) aminomethane of biochemical grade was purchased from Shanghai Chemical Reagent Head Factory (Shanghai, China). Tris–HCl buffer solution (0.05 mol L^{-1}) was prepared and its pH was adjusted to 7.40 using 0.1 M of either hydrochloric acid or sodium hydroxide solution. All HIg solution samples of 3.0×10^{-5} mol L^{-1} were prepared in pH 7.40 Tris–HCl buffer solution. All other reagents were of analytical reagent grade and doubly distilled water was used throughout the experiment.

Sample preparations and SEM experiment

The SEM measurement was performed by using a EVO MA 10/LS 10 thermal field emission scanning electron microscope at room temperature. The sample preparation was carried out in accordance with the following steps. Mixtures of HIg and shikonin were