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Studies on the interaction of heparin with lysozyme by multi-spectroscopic techniques and atomic force microscopy

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ABSTRACT

The interaction between heparin (Hep) and lysozyme (Lyso) in vitro was studied by fluorescence, UV–vis, circular dichroism (CD), resonance Rayleigh scattering (RRS) spectroscopy and atomic force microscopy (AFM) under normal physiological conditions. UV–vis spectra of Lyso showed the absorbance was significantly increased with the addition of Hep. Fluorescence studies revealed that the emission quenching of Lyso with Hep was initiated by static quenching mechanism. CD spectral studies showed that Hep induced conformational changes in the secondary structure of Lyso. RRS spectra of Lyso showed the intensity of scattering was significantly increased with the addition of Hep and the enhanced RRS intensities were proportional to the concentration of Hep in a certain range. Thus, a new RRS method using Lyso as a probe could be used for the determination of Hep. The detection limit for Hep was 3.9 ng mL^{-1}. In addition, the shape of the complex was characterized by AFM. The possible reaction mechanism and the reasons for the enhancement of RRS intensity had been discussed through experimental results.

1. Indroduction

Heparin (Hep), a linear polysaccharide, consists of repeating units of pyranosyluronic acid and glucosamine residues. Due to the ionization of acid groups, such as the carboxylate or sulphate residues, the Hep molecule is highly negatively charged in water solution. As a member of the family of glycosaminoglycan, Hep has many important biological functions and medicinal values. It is involved in many essential processes such as blood coagulation, inflammation, immune defense, lipid transport and metabolism [1,2]. In clinics, Hep has been widely used as an anticoagulant or antithrombotic agent during medical therapies. However, the overdose of Hep could cause some seriously side effects, such as hemorrhages and thrombocytopenia, arthritis and so on. So, it is quite important to monitor the amount of Hep exactly.

Up to now, many methods have been used for the determination of Hep, such as capillary electrophoresis [3,4], high performance liquid chromatography [5,6], electrochemical method [7–10], spectrophotometry [11–14], spectrofluorimetry [15–20] and RRS method using dyes or nanoparticles as probe [21–24]. However, every method has its own advantage and disadvantage. Some methods need complex pretreatment, others suffer from poor sensitivity or high cost. Thus, a sensitive, easy and reliable detection method for Hep is still expected. RRS, a special elastic scattering produced when the incident beam is located at or close to molecular absorption bands, can provide a high signal level and good selectivity [25,26]. It has been widely used to the study and determination of proteins [27,28], nucleic acids [29], saccharides [30,31], food [32,33], drugs [34,35] and environmental pollutants [36,37]. Tang [21] developed a RRS determination of Hep based on the aggregation of polyethyleneimine-capped Ag nanoclusters, Wu [22] determined Hep using Co(II)/5-Cl-PADAB complex as a RRS probe. However, the RRS determination of Hep using bio-macromolecule as a probe has scarcely been reported.

Lysozyme (Lyso) is a basic monomeric globular protein. It can damage bacterial cell wall and thereby acting as a mild antiseptic. In addition, Lyso is regarded as an important defense molecule of the innate immune system because it can protect higher organisms from the infection of microorganisms [38]. Therefore, studying the interaction of Lyso with Hep may not only provide some valuable information for understanding the molecular basis for Lyso–Hep interaction, but also may aid the development of potent detection methods for Hep or Lyso. In this work, we observed the significant enhancement of RRS with the addition of Hep to Lyso under normal physiological conditions. Due to the enhanced RRS intensities proportional to the concentration of Hep in a certain range, a new RRS method using Lyso as a probe could be established for the determination of Hep. Furthermore, UV–vis, fluorescence, CD, AFM were employed to further investigated the possible reaction mechanisms of Lyso with Hep. Thus, such a study may not only provide a new way for the determination of trace of Hep, but also give some new information for understanding the interaction of Hep with Lyso.

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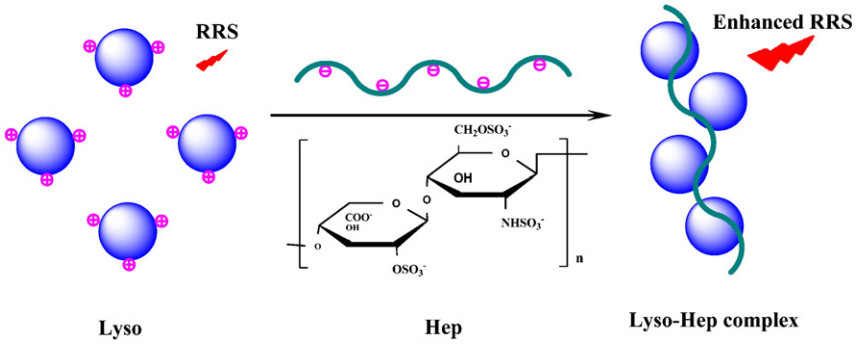

Fig. 1. The scheme for RRS detection of Hep utilizing interaction between positively charged Lyso and negatively charged Hep.

2. Materials and methods

2.1. Materials

Lyso and Hep sodium were purchased from Sigma-Aldrich and used without any further purification. All other reagents were of analytical grade, and doubly distilled water was used throughout the experiments. Tris–HCl (pH 7.4) buffer solution.

2.2. Apparatus and measurements

2.2.1. RRS measurement

RRS spectra of Lyso in the absence and presence of Hep were measured with a F-2500 spectrofluorometer (Hitachi Ltd., Japan) equipped with a 1 cm quartz cell at room temperature, using 10 nm/10 nm slit widths. The RRS spectra were obtained by scanning synchronously the excitation and emission monochromators of the spectrofluorometer from 200 to 700 nm with $\Delta\lambda = 0$.

2.2.2. UV–vis absorption measurement

Absorption spectra of Lyso in the absence and presence of Hep were determined in the range of 210–360 nm using a 2450 UV–visible spectrophotometer (Shimadzu, Japan) equipped with a 1 cm quartz cell at room temperature.

2.2.3. Fluorescence emission measurement

Fluorescence spectra of Lyso in the absence and presence of Hep were measured with a F-2500 spectrofluorometer (Hitachi Ltd., Japan) equipped with a 1 cm quartz cell at 283 K, 293 K and 303 K, respectively, using 5 nm/5 nm slit widths. The excitation wavelength was 280 nm, and the emission was read at 300–500 nm.

2.2.4. CD measurement

CD measurement was carried out on a J-810 spectropolarimeter (JASCO Co., Japan) with a 1 cm quartz cell at room temperature. CD measurements of Lyso in the absence and presence of Hep were recorded in the range of 200–300 nm.

2.2.5. AFM measurement

AFM images were obtained on an atomic force microscopy (Veeco Metrology Group, NY). Mica slips were used to prepare the AFM slides for different samples. Samples of Lyso, Hep and Hep–Lyso complex solution was dropped on the carefully cleaned surfaces of the mica and was allowed to air dry, respectively.

3. Results and discussion

3.1. RRS spectral characteristics

Since Hep is a highly negative charged linear polysaccharide and Lyso with abundant basic amino groups are highly positive charged, the electrostatic attraction is consequently expected (Fig. 1). Fig. 2 shows the RRS spectra of Hep–Lyso system. It can be seen that the RRS intensity of free Lyso is very low. However, with the gradually increasing addition of Hep, the RRS intensity is gradually enhanced and the maximum peak is at about 335 nm. According to the Mie scattering theory, the RRS properties have close relationship with the molecular volume and morphology of the particles [28,39]. The bigger the molecular volume, the higher the RRS intensity can be obtained. The presence of Hep can unfold and induce aggregation of Lyso by electrostatic forces and results in increasing the size of scatters [40–42]. Therefore, the RRS intensity is enhanced. Furthermore, the enhanced RRS intensities present a good linear relationship with the concentration of Hep, which suggests that the RRS spectra could be used for the quantitative determination of Hep.

3.2. Optimization of the experimental conditions

3.2.1. Effect of pH and buffer

The influence of pH on the RRS intensity of the system was investigated. In this work, the Tris–HCl buffer solution was selected to control the acidity of analytical system. The results showed that the optimum ranges of acidity were pH 7.0–9.0 and pH 7.4 was chosen as the optimal reaction acidity for the analytical system.

3.2.2. Effect of Lyso concentration

The effect of Lyso concentration on the RRS intensity of system was studied. The results showed that the optimum ranges of Lyso concentration were 30–50 μg mL^{-1} and 40 μg mL^{-1} of Lyso was used for the assay of Hep.

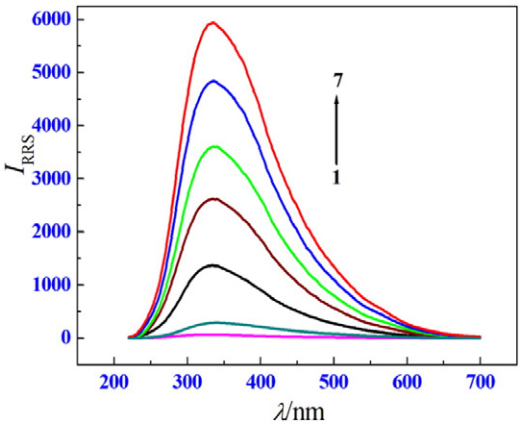

Fig. 2. RRS spectra of Hep, Lyso and Hep–Lyso complex. 1: Hep; 2: Lyso; 3–7: Hep–Lyso. Hep/(μg mL^{-1}): (3)1.0, (4)2.0, (5)3.0, (6)4.0, (7)5.0; Lyso: 40.0 μg mL^{-1}; pH 7.4.