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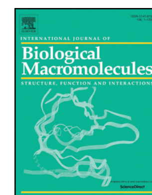
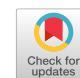


Conformational change of lysozyme on the interaction with gene carrier polyethyleneimine



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ABSTRACT

Polyethyleneimine (PEI) has been considered as "golden standard" for polymeric gene delivery carriers, however also induces cytotoxicity. To reveal the molecular basis of PEI cytotoxicity, absorption, resonance Rayleigh scattering, fluorescence, circular dichroism and isothermal titration calorimetry were conducted to investigate the interaction between PEI (average molecular weight 25,000 Da) and lysozyme and its influence on the conformation of the enzyme. PEI is an amphiphilic polymer with strong hydrogen bonding capability and weak hydrophobicity due to dense amine groups and methyl groups. However, the hydropathic characteristics that hydrophobic methyl groups and hydrophilic amine groups distributed alternatively along the polymer chain made it difficult to penetrate into the hydrophobic core, but subject to binding onto the surface of lysozyme via hydrophobic interaction and hydrogen bond, leading to a more compact conformation and an increased surface hydrophobicity of the enzyme.

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1. Introduction

Gene therapy is one of the most advanced topics in the field of modern biomedicine due to its potential application in the treatment or prevention of inherited or acquired human diseases [1]. However, progress in human gene therapy is hindered by the lack of suitable vectors. Toxicity, transfection efficiency and biocompatibility are major considerations in the design of new delivery systems [2–5]. Polyethyleneimine (PEI) is a polymer with dense amine groups prone to protonation and has been considered as "golden standard" for polymeric gene delivery systems due to its high charge density and buffering capacity [6–9]. Unfortunately, PEI also induced cytotoxicity [10–12]. Many efforts have been made to develop PEI-based gene vector with reduced cytotoxicity and promoted transfection efficiency [6–9]. However, cytotoxicity tests usually used, such as MTT assay, were based on assessing the in vitro metabolic activity of cells at 4–8 h following treatment with cationic macromolecules and may not provide a true representation of polymer safety in gene therapy trials, as the tests were not indicative of apoptosis which occurred at the later stage long after PEI-treatment [12]. Up to date, the molecular basis of PEI cytotoxicity is still poorly understood. Knowledge about the influence of PEI on the structure and function of biomacromolecules and its mechanism is limited.

Due to the high charge density, electrostatic interaction was suggested to dominate the complex formation of PEI with proteins and thus had little influence on conformation and function of basic ones [13]. However, PEI was found to increase the catalytic activity and stability of both muscle lactate dehydrogenase (pI ~ 8.2) [14] and glucose dehydrogenase (pI ~ 6.0) [15], suggesting other mechanisms in the interaction between PEI and proteins. Previous work from our group confirmed that PEI bound onto the surface of horseradish peroxidase (HRP, pI ~ 7.0) predominantly via hydrophobic interaction and hydrogen bond, inducing a more compact and hydrophobic conformation and an enhancement of catalytic efficiency of the enzyme [16].

One question is logically raised that whether complex formation can occur between PEI and basic proteins via hydrophobic interaction, hydrogen bond or van der Waals interaction. Lysozyme (Lys) is a basic monomeric globular protein (pI ~ 11 [13]), often found in tears, saliva, mucous, blood, lymphatic tissues and milk. Lysozyme can cleave β 1,4 glycosidic bond followed by disruption of cell wall with diminished bacterial functions and thereby protecting against bacterial infections [17,18]. Due to the abundance, small size, high stability and capacity of transporting drugs and functional organic compounds, lysozyme has been purposely chosen to explore the nature of interaction between proteins and exogenous ligands [19,20]. In this work, a combinational study, including UV–vis absorption, resonance Rayleigh scattering (RRS), fluorescence, circular dichroism (CD) and isothermal titration calorimetry (ITC), was conducted to reveal the interaction between hen egg white lysozyme and PEI and its influence on the conformation of the enzyme. The results from this work and previous report [16] were expected to provide useful information about the mechanism of PEI-protein interaction and PEI cytotoxicity.

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2. Materials and methods

2.1. Materials

Branched PEI (average molecular weight 25,000 Da, labeled as PEI25k), lysozyme (EC 3.2.1.17, from hen egg white, lyophilized powder, ≥100,000 $U \cdot mg^{-1}$), and 8 anilino 1 naphthalenesulfonic acid (ANS, ≥97%, HPLC) were obtained from Sigma-Aldrich (USA) and used as received. All of the solutions were prepared in 10 mM phosphate buffered solution (PBS, pH 7.4).

Stock solutions of PEI25k (10.0 $mg \cdot ml^{-1}$), ANS and lysozyme were prepared by dissolving the products in PBS buffer and adjusted to pH 7.4 using HCl and NaOH solution. Concentration of ANS and lysozyme was determined based on the absorbance at 350 and 280 nm, using an extinction coefficient of 6300 $M^{-1} \cdot cm^{-1}$ [21] and 37,470 $M^{-1} \cdot cm^{-1}$ [22], respectively. The stock solutions were stored at 0–5 °C and diluted to the desired concentration when used. Solutions for spectra analysis were prepared by diluting the stock solution into PBS buffer containing PEI25k of various concentration (0–1.0 $mg \cdot ml^{-1}$) and incubated in a water bath thermostated to 25 °C for 30 min before performance.

2.2. Methods

2.2.1. UV–vis absorption

Absorption spectra of lysozyme were recorded on an UV-2501PC UV–vis spectrophotometer (Shimadzu, Japan), using a quartz cell of 1 cm path length in a jacketed holder connecting to a water bath thermostated to 25 °C. PBS buffer containing PEI of the same concentration was used as reference. The concentration of lysozyme was 5 μM and the absorption spectra were recorded from 200 to 500 nm.

The measurements in this work, including absorption, CD, RRS and fluorescence, were performed in triplicates and the spectra were obtained by averaging.

2.2.2. Circular dichroism measurement

CD spectra of lysozyme in PEI-containing PBS were collected on a MOS-450 spectropolarimeter (Bio-Logic, France) in nitrogen atmosphere, using a quartz cell of 1 mm path length at 25 °C. The concentration of lysozyme in the solutions was 15 μM and the spectra were recorded from 190 to 260 nm. A blank for PEI-containing PBS buffer was measured under the same experimental conditions and subtracted from the data. All of the CD spectra were obtained by averaging 3 runs with a step interval 0.5 nm and acquisition duration 0.1 s. The secondary structure contents were calculated by using CDSSTR software in the DICHROWEB web server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [23].

2.2.3. Intrinsic fluorescence and resonance Rayleigh scattering

Fluorescence emission of lysozyme ($\lambda_{ex} = 295$ nm) was performed on a RF6000 spectrofluorimeter (Shimadzu, Japan) equipped with a 1 cm × 1 cm quartz cell at 25 °C. The fluorescence spectra were recorded from 300 to 450 nm.

RRS spectra of PEI solution and PEI-lysozyme solution in PBS were obtained by scanning synchronously the excitation and emission monochromators of the spectrofluorimeter from 200 to 800 nm with $\Delta\lambda = \lambda_{ex} - \lambda_{em} = 0$.

2.2.4. Fluorescence emission of ANS

Fluorescence emission of ANS in PBS buffer containing PEI (0–1.0 $mg \cdot ml^{-1}$), in the absence and presence of lysozyme, was recorded on a RF6000 spectrofluorimeter (Shimadzu, Japan) using a 1 cm × 1 cm quartz cell thermostated to 25 °C. The final concentration of ANS and lysozyme were 15 μM. The fluorescence emission for ANS was excited at 388 nm and recorded from 400 to 700 nm. The excitation and emission slit widths were set to 5 nm.

2.2.5. ITC

The microcalorimetric measurements were carried out using a Microcal ITC_{200} isothermal titration calorimeter (GE, USA). PEI solution (5.0 $mg \cdot ml^{-1}$) and lysozyme solution (50 μM) were prepared and adjusted to pH 7.4 before performance. The titration was carried out by successive injections of 1 μL PEI solution from a 40 μL syringe into lysozyme solution filled in the measurement cell at 25 °C. Interval between injections was 90 s to allow complete equilibration and agitation speed was 600 rpm. Titration profile of injecting PEI solution into PBS was also obtained under the same conditions and subtracted from that of titrating PEI into lysozyme solution to account for the dilution effects of PEI. The heat associated with the interaction between the titrant and the enzyme was fitted to the sequential two-step binding mode proposed in the origin software package supplied by Microcal, providing best fit values of apparent binding constant, change in enthalpy, entropy and free energy.

3. Results and discussion

3.1. UV–vis absorption

To reveal the possible interaction between lysozyme and PEI, UV–vis absorption was performed for lysozyme in PBS containing PEI. Since PEI also displayed absorption in the far-UV region below 275 nm [16], PEI solution in PBS at the same concentration was used as reference in the performance. As shown in Fig. 1, lysozyme exhibited a strong absorption around 206 nm and a weak absorption around 278 nm in PBS, originated from $n \rightarrow \pi^*$ transition of amide group and $\pi \rightarrow \pi^*$ transition of aromatic residues, respectively. The former generally reflects the framework conformation of the protein [24,25] and the latter is usually related to the polarity of the microenvironment around aromatic residues and thus an indication of the change in the tertiary structure of proteins [26]. It can be seen that the addition of PEI in the solution had no significant influence on the absorption of aromatic residues. However, the absorption of amide group decreased and shifted to the longer wavelength regularly along with increasing PEI concentration, indicating a complex formation with PEI and a possible alteration in the secondary structure of the enzyme [27].

3.2. Resonance Rayleigh scattering

To confirm the complex formation between PEI and lysozyme, RRS spectra of lysozyme-PEI solutions were obtained. Since the molecular weight of PEI used in this work is larger than lysozyme, RRS was also performed to PEI solution of the same concentration. As shown in

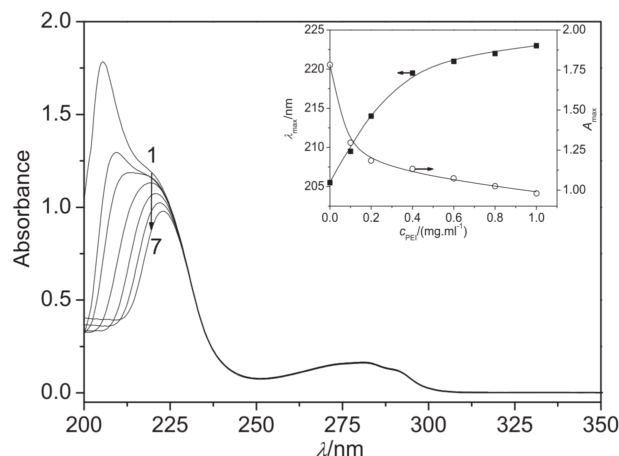

Fig. 1. UV–vis absorption of lysozyme in PBS containing PEI25k at pH 7.4. Inset: PEI concentration dependence of maximum absorption A_{max} and peak position λ_{max} of lysozyme. $c_{Lys} = 5$ μM, c_{PEI}: (1) 0, (2) 0.1, (3) 0.2, (4) 0.4, (5) 0.6, (6) 0.8, (7) 1.0 $mg \cdot ml^{-1}$.

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