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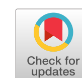
Effects of interaction with gene carrier polyethyleneimines on conformation and enzymatic activity of pig heart lactate dehydrogenase



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A B S T R A C T

Polyethyleneimine (PEI) has long been considered as "golden standard" for polymeric gene delivery carrier, however also induces cytotoxicity. To make a further insight into the molecular basis of PEI cytotoxicity, fluorescence, absorption and circular dichroism spectroscopy were conducted to investigate the influence of PEI (average molecular weight 25,000 and 1800 Da) on the conformation of pig heart lactate dehydrogenase (LDH) and its catalytic efficiency. Zeta-potential measurement and isothermal titration calorimetry were used to reveal the interaction between PEI and LDH. PEI was found to bind onto the surface of LDH predominantly via hydrophobic interaction, inducing a more compact conformation and an increased surface hydrophobicity of the enzyme. The conformational change of LDH induced by PEI binding had little influence on the complex formation between LDH and reduced nicotinamide adenine dinucleotide (NADH, the co-enzyme). However, the nonspecific binding of PEI on the surface of LDH retarded the turnover of the enzyme. Meanwhile, the large quantity of amine groups on the polymer chain made PEI subject to form complexes with NADH and pyruvate (the substrate) via hydrogen bond and electrostatic interaction, which greatly reduced the binding efficient of LDH. The polymer size played an important role in PEI-LDH interaction. The smaller size of lower molecular weight PEI facilitated the close contact with LDH and consequential reduction of the turnover number of the enzyme. However, higher molecular weight PEI was more favorable for competitive binding with NADH and pyruvate and generally decreased the catalytic efficient of LDH.

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1. Introduction

In biomedical fields, a variety of biopolymers with interesting properties are developed to improve human health and rapidly revolutionizing many areas of medicine and technology. For biomedical applications, the safety of the polymers is the most important issue and must be strictly evaluated, as they are often used *in vivo* to exert their efficacy and will inevitably contact various biomacromolecules, cells, tissues and organs in the living system [1].

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 [2]. A major barrier for progress in human gene therapy is the lack of safe and effective vectors for gene transfer [3–6]. Polyethyleneimine (PEI), a cationic polymer with high charge density and buffering capacity, has long been considered as "golden standard" for polymeric gene delivery systems [7–10]. Unfortunately, PEI can induce cytotoxicity [11, 12]. Many efforts have been made to reduce the cytotoxicity and to promote the transfection efficiency of PEI [7–10]. However, the molecular basis of PEI cytotoxicity is still poorly understood. Knowledge about the influence of PEI on the structure and functions of proteins and its mechanism is limited. Due to the high charge density, electrostatic interaction was proposed to dominate the complex formation of PEI with proteins and 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. In our previous work, PEI was found to bind onto the surface of horseradish peroxidase (HRP, pI~7.0) predominantly via hydrophobic interaction and hydrogen bond. The complex formation between HRP and PEI induced a more compact and hydrophobic conformation and an enhancement of catalytic efficiency of the enzyme [16].

A question was logically raised that whether hydrophobic interaction, hydrogen bond or van der Waals interaction also contribute to the interaction of PEI with acidic proteins. In this work, fluorescence, UV–vis absorption and circular dichroism (CD) spectroscopy was conducted to investigate the influence of PEI on the conformation of pig heart *L*-lactate dehydrogenase (LDH), an acidic protein of pI value of 5.6 [13]. Zeta-potential measurement and isothermal titration calorimetry (ITC) were used to reveal the mechanism of the interaction between PEI and LDH. Catalytic activity of LDH was evaluated by

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monitoring the reduction of sodium pyruvate to lactate by reduced nicotinamide adenine dinucleotide (NADH). Steady-state kinetic studies were carried out to get a further insight into the mechanism of functional alteration of LDH upon the binding with PEI. PEI of typical high molecular weight (MW 25,000 Da, labeled as PEI25k) and low molecular weight (MW 1800 Da, labeled as PEI1.8k) was chosen in order to clarify the structure-activity relationship, which would facilitate the design of degradable PEI-based polymeric matrix.

2. Experimental

2.1. Materials

Branched PEI of average molecular weight 25,000 and 1800 Da (labeled as PEI25k and PEI1.8k, respectively.), LDH (EC 1.1.1.27, from pig heart, ammonium sulfate suspension, ≥200 $U\cdot mg^{-1}$), sodium pyruvate, NADH (>98%) and 8-anilino-1-naphthalenesulfonic acid (ANS, ≥97%, HPLC) were obtained from Sigma-Aldrich (USA). All of the solutions were prepared in 10 mM phosphate buffered solution (PBS) (pH 7.4).

Stock solutions of PEI (10.0 $mg\cdot mL^{-1}$), sodium pyruvate, NADH and ANS, prepared by dissolving the products in PBS buffer and adjusted to pH 7.4 using HCl and NaOH solution, were stored at 0–5 °C before used. For the sake of comparison to display the role of polymer size in the interaction with protein, concentration of PEI was presented in mass volume concentration rather in molarity. Concentration of NADH and ANS were determined based on the absorbance at 340 and 350 nm, using an extinction coefficient of 6220 $M^{-1}\cdot cm^{-1}$ [17] and 6300 $M^{-1}\cdot cm^{-1}$ [18], respectively.

LDH suspension was extensively dialyzed against PBS and centrifuged to remove insoluble protein. Enzyme concentration was determined spectrophotometrically at 280 nm with an absorption coefficient of 1.96×10^5 $M^{-1}\cdot cm^{-1}$ [19]. The stock solution was diluted into PEI-containing PBS to the desired concentration and incubated at 25 °C for 30 min before spectra analysis and zeta-potential measurement.

2.2. Methods

2.2.1. UV–vis Absorption

UV–vis absorption spectra of LDH 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 molecular weight and of the same concentration was used as reference. The absorption spectra were recorded from 200 to 350 nm.

2.2.2. Circular Dichroism Measurement

CD spectra, from 190 to 260 nm, were collected on a MOS-450 spectropolarimeter (Bio-Logic, France) in nitrogen atmosphere, using a cuvette of 1 mm path length at 25 °C. Each spectrum was the averaging result of 3 scans at 0.5 nm interval with an integration time of 0.1 s. A blank for PEI-containing PBS buffer was measured under the same experimental conditions and subtracted from the data. The secondary structure contents were calculated by using CDSSTR software in the DICHROWEB web server (http:dichroweb.cryst.bbk.ac.uk/html/home.shtml) [20].

2.2.3. Intrinsic Fluorescence

Fluorescence emission of LDH ($\lambda_{ex} = 295$ nm) were performed on a RF6000 spectrofluorimeter (Shimadzu, Japan) using a 1 cm × 1 cm quartz cell at 25 °C. The fluorescence spectra were recorded from 300 to 450 nm. The excitation and emission slit widths were set to 3 nm.

2.2.4. Fluorescence Emission of ANS and NADH

Fluorescence emission of ANS and NADH in PBS buffer containing PEI (0–1.0 $mg\cdot mL^{-1}$), in the absence and presence of LDH, were recorded on a RF6000 spectrofluorimeter (Shimadzu, Japan) using a 1 cm × 1 cm quartz cell thermostated to 25 °C. The final concentration of ANS, NADH and LDH were 4, 4 and 1 μM, respectively. The fluorescence emission for ANS was excited at 350 nm and recorded from 370 to 650 nm. The excitation and emission slit widths were set to 5 nm. Those for NADH were excited at 340 nm and recorded from 370 to 650 nm. The excitation and emission slit widths were set to 3 and 5 nm, respectively.

2.2.5. Zeta-potential Measurement

Zeta-potential of LDH was determined on a Zetasizer Nano-ZS dynamic scattering spectrometer (Malvern, UK) by using disposable folded capillary cells at 25 °C. Zeta-potential was calculated using the Smoluchwski mathematical model. The measurement was performed at least three times.

2.2.6. 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 LDH solution (10 μM) was prepared and adjusted to pH 7.4. The titration was carried out by successive injections of 1 μL of PEI solution from a 40 μL syringe into LDH solution filled in the measurement cell at 25 °C. Interval between injections was 90 s to allow complete equilibration and the agitation speed was set to 600 rpm. Background titrations, consisting in injecting the same PEI solution into PBS and injecting PBS into LDH solution, were subtracted from each experimental titration to account for the dilution effects of PEI and LDH. The heat flow was recorded as a function of time. The concentration of the titrant and the enzyme were used to fit the heat flow per injection to the sequential two-step binding model proposed in the origin software package supplied by Microcal, providing best fit values of apparent binding constant K_a, change in enthalpy ΔH, change in entropy ΔS and free energy ΔG.

2.2.7. Activity Assay

The enzyme activity was assayed by modified Worthington method [21]. The enzyme solution containing 0.01 μM LDH and PEI of predefined concentration (0, 0.1, 0.25, 0.5, 0.75 and 1.0 $mg\cdot mL^{-1}$) and the reaction mixture containing 1.0 mM sodium pyruvate, 0.22 mM NADH and PEI of the same concentration as the enzyme solution were incubated at 25 °C for 30 min. The reaction was started by adding 0.1 mL enzyme solution to 2.9 mL reaction mixture in a 1 cm × 1 cm quartz cell with a water-jacketed holder connecting to a water bath thermostated to 25 °C. The initial rate of consumption of NADH was monitored on a UV-2501PC UV–vis spectrophotometer (Shimadzu, Japan) at 340 nm during 5 min and recorded with time interval 1 s, using a molar extinction coefficient of 6220 $M^{-1}\cdot cm^{-1}$ [17]. One unit of LDH activity was defined as the amount of the enzyme causing the oxidation of 1 μmol of NADH per minute under the assay conditions. The experiment was carried out in triplicates and the relative activity was obtained by using that in the absence of PEI as control.

2.2.8. Catalytic Kinetics

Steady-state kinetic experiments were performed under the same conditions as activity assay, using constant LDH, PEI, sodium pyruvate concentration and varying NADH concentration or constant LDH, PEI, NADH concentration and varying sodium pyruvate concentration. Affinity constant K_m and maximum velocity V_{max} were evaluated by fitting the initial rate of enzymatic reaction, ν, to Michaelis-Menten equation [22],

$$1/\nu = K_m/(V_{max} \cdot [S]) + 1/V_{max} \quad (1)$$

whereas [S] is the concentration of sodium pyruvate or NADH. Turnover number k_{cat} and catalytic efficiency k_{cat}/K_m were further obtained,

$$k_{cat} = V_{max}/[E_0] \quad (2)$$

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