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# Effects of the gene carrier polyethyleneimines on structure and function of blood components

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ABSTRACT

As a synthetic polycation, polyethylenimine (PEI) is currently one of the most effective non-viral gene carriers. For *in vivo* applications, PEI will enter systemic circulation and interact with various blood components and then affect their individual bio-functions. Up to now, overall and systematic investigation on the interaction of PEI with multiple blood components at cellular, membrane, and molecular levels is lacking, even though it is critically important for the *in vivo* safety of PEI. To learn a structure–activity relationship, we investigated the effects of PEI with different molecular weight (MW) and shape (branched or linear) on key blood components and function, specifically, on RBC aggregation and morphological change, platelet activation, conformation change of albumin (as a representative of plasma proteins), and blood coagulation process. Additionally, more proteins from plasma were screened and identified to have associations with PEI by a proteomic analysis. It was found that, the PEIs have severe impact on RBC membrane structure, albumin conformation, and blood coagulation process, but do not significantly activate platelets at low concentrations. Furthermore, 41 plasma proteins were identified to have some interaction with PEI. This indicates that, besides albumin, PEI does interact with a variety of blood plasma proteins, and could have unexplored effects on their structures and bio-functions. The results provide good insight into the molecular design and blood safety of PEI and other polycations for *in vivo* applications.



## 1. Introduction

In biomedical fields, a variety of natural or synthetic polymers with interesting properties are exponentially developed to improve human health. The biopolymers are rapidly revolutionizing many areas of medicine and technology [1,2]. For biomedical applications, the *in vivo* 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 bio-macromolecules, cells, tissues and organs in the living system. In the laboratory level, these biomedical polymers are intensively studied and most of them have been proven to be "biocompatible". Despite the numerous laboratory studies [3,4], their overall, long-term impact *in vivo* is barely known. For this reason, the "clinical" applications of the biomedical polymers are much lagged. Further development of the biomedical polymers for clinical applications urgently requires clarifying the mutual effects between the polymers and biological components at cellular and molecular levels.

In practical applications, the biopolymers are often introduced into the living system by various routes. In view of the abundant and far-reaching blood vessel network, the biopolymers or their degradation products would more or less enter the systemic circulation sooner or later, directly or indirectly, regardless of any administration route [5]. This is also applicable to biodegradable bulk polymeric materials such as tissue-engineering scaffolds, since the degradation fragments could enter blood circulation. Let alone intravenous administration, the mostly used route for systemic therapy or disease diagnosis. In many cases (for example, drug/gene delivery, bioimaging, and blood substitutes, etc.), these polymers are used in blood-soluble/suspended state but not as formed

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materials, as they are small enough to enter almost all areas of the body and exert their efficacy at molecular level.

In this sense, it is almost unavoidable for any biopolymers implanted *in vivo* to contact with blood tissue to a different extent. Even so, relatively little is known about the complicated interactions of the biopolymers with blood, and this is now a serious bottleneck in further development of the biopolymers from the lab to bedside [6]. Once the biopolymers enter blood circulation, they instantaneously become associated with numerous blood cells and abundant plasma proteins, which may alter cell membrane structure and protein conformation, and then perturb blood functions and eventually impair the whole organism [6]. In spite of well-established hemocompatibility evaluation methods, the overall influences of the polymers on blood are actually little known due to the complex composition and functions of blood tissue. On the other hand, the interaction with blood cells and/or plasma proteins also defines the biological identity, and alters the *in vivo* pharmacokinetic behaviors, *in vivo* distribution, plasma half-life, delivery route and final clearance of the biopolymers. Thus, knowledge of the interaction of the biopolymers with blood components is critical to understand the *in vivo* safety and efficacy of the biopolymers [5,7].

Of all synthetic polycations, polyethyleneimine (PEI) is one of the most effective non-viral gene carriers *in vitro* and *vivo* because of its unique combination of high charge density and enhanced "proton spong effect" in endolysosome [8], which is regarded as the "golden" standard for gene delivery. Despite the wide applications, the detailed and overall studies on the interaction of PEI with blood are sparsely found. Here, we investigated the effects of PEI on important blood components and coagulation function. Due to the complicated biofunctions of leucocytes, their interaction with PEI would later be studied based on this work. The PEIs with different molecular weight (MW, 0.6, 1.8 or 25 kDa) and shape (branched or linear) were chosen in order to clarify the structure–activity relationship. This study will facilitate deep understanding on the interaction mechanisms between polycations and blood from five aspects: the effect of the PEIs on RBC membrane structure, the effect on platelet activation, the effect on the molecular structure and conformation of a key plasma protein albumin (as a representative), the effect on principal blood function coagulation, and finally screening all those plasma proteins associated with the PEIs.

## 2. Experimental

### 2.1. Materials

PEIs with different MW and shape (0.6 kDa and branched, 1.8 kDa and branched, 25 kDa and branched, 25 kDa and linear) were used in this study, as labeled as BPEI-0.6k, BPEI-1.8k, BPEI-25k and LPEI-25k, respectively. BPEI-0.6k and 1.8k were purchased from Alfa Aesar (USA). BPEI-25k and LPEI-25k were purchased from Sigma–Aldrich (USA) and Polysciences, Inc. (USA), respectively. All of the PEIs and BSA were dissolved in 10 mM PBS (pH7.4) buffer solution before use. BSA was purchased from Roche Molecular Biochemicals (Canada). Magnetic beads coated with carboxylic acid (Dynabeads® MyOne™, Carboxylic Acid, 1 μm in diameter, 10 mg/ml aqueous suspension) were purchased from Invitrogen Dynal AS Oslo Company (Norway). N-hydroxysuccinimide (NHS), N-ethyl-N′-(3-dimethylaminopropyl) carbodiimide (EDC), and 2-[N-morpholino] ethane sulfonic acid (MES) were purchased from Aladdin Regent Inc. (Shanghai, China).

Blood from healthy consented volunteers was collected in sodium citrate tube with a blood/anticoagulant ratio of 9:1. Platelet-poor plasma (PPP) and platelet-rich plasma (PRP) were prepared by centrifuging the citrated whole blood at 3000 *g* for 15 min or at 800 *g* for 15 min respectively and used below, unless otherwise stated. Reagents for conventional coagulation assays APTT, PT and FT were provided by the First Affiliated Hospital, Jinan University.

### 2.2. Effect of the PEIs on the aggregation and morphology of RBCs

The citrated whole blood was centrifuged at 1000 *g* for 5 min. After removing plasma and buffy coat layer, the RBCs were washed with PBS. The washed RBCs were incubated with various PEI solutions for 10 min, washed with PBS, and then fixed with 4% paraformaldehyde overnight. The fixed RBCs were deposited on glass slides, dehydrated with 70, 85, 95, 100% (v/v) ethanol for 10 min respectively, and then dried in air. The dried RBCs were coated with gold and observed with scanning electron microscope (SEM, Philips XL-30, Holand).

### 2.3. Effect of the PEIs on the aggregation and activation of platelets

#### 2.3.1. Effect of the PEIs on the aggregation of platelets

Effect of BPEI-25k on the aggregation of platelets was studied by using SEM observation according to previous report [9]. Briefly, the whole blood was centrifuged at 80 *g* for 8 min to obtain PRP. The PRP (0.9 ml) was incubated with 0.1 ml of BPEI-25k solution in PBS for 10 min. At the end of incubation, the platelets were fixed with 2% paraformaldehyde for 15 min. The suspension was centrifuged at 1000 *g* for 10 min. The resulting precipitate was re-suspended with 0.5 ml of PBS and deposited on glass slides and re-fixed with 4% paraformaldehyde for 60 min. Then, the platelets were dehydrated with 70, 85, 95, 100% (v/v) ethanol respectively and dried in air. The dried platelets were coated with gold and observed with the SEM.

#### 2.3.2. Platelet activation by the PEIs

Platelet activation by the PEIs was studied by measuring activated platelet surface glycoproteins GPIIb-IIIa and P-Selectin with a commercially available ELISA assay (Shanghai Shensu Biotech Company, China). In brief, 20 μL of PBS (as a control) or PEI solutions was incubated with 180 μL of the PPP for 1 h at room temperature, and then measured according to the specifications provided by the supplier.

### 2.4. Interaction studies between the PEIs and BSA

#### 2.4.1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Direct *in vitro* interaction of BSA with PEI was detected by SDS–PAGE using a 12% acrylamide separating and a 5% acrylamide stacking slab gel. An MW reference marker (MW 25–175 kDa), BPEI-25k-contaning BSA solutions or pure BSA solution (final concentration 0.33 mg/ml BSA) were loaded onto acrylamide gel and run using a Bio-Rad Mini Protean II Electrophoresis System at 100V. After electrophoresis, the gel was stained with Coomassie Brilliant Blue for 2 h. The image of the gel was taken with a gel scanning device.

#### 2.4.2. UV absorption spectroscopy

UV absorption measurements were performed for pure BSA (0.33 mg/ml) and various PEIs-containing BSA solutions in the PBS buffer at room temperature on a UV-2550 spectrophotometer (Shimadzu Corporation, Japan). Quartz cuvettes of 1 cm were used and the absorption spectra were recorded from 200 to 400 nm.

#### 2.4.3. Fluorescence spectroscopy

Fluorometric experiments were carried out for pure BSA (0.33 mg/ml) and various PEIs-containing BSA solutions in the PBS buffer on a Hitachi F-7000 fluorescence spectrophotometer (Hitachi High-Technologies Corp., Tokyo, Japan). The fluorescence spectra were recorded at $\lambda_{exc}$ 295 nm and $\lambda_{em}$ from 300 to 500 nm using quartz cells ($4 \times 1 \times 1$ cm). The excitation and emission slit widths (each 5 nm) and scan rate (1200 nm/min) were set for all the experiments [10].

#### 2.4.4. Circular dichroism (CD) measurements

CD spectroscopy analysis was carried out in an Applied PhotophysicsTM Chirascan CD spectrometer (Applied Photophysics Ltd, Leatherhead, UK). The CD spectra were recorded in a cuvette of 1 cm path length at 25 °C in a nitrogen atmosphere with the PBS solution as a running buffer. The final BSA concentration was kept at 0.132 mg/ml with varying PEI concentrations. All spectral data presented are a raw data of average molar ellipticity ($\theta$) of three scans recorded from 200 to 260 nm with a scan rate of 60 nm/min. The mean molar ellipticity per residue ($\theta_{mrd}$) (at 208 nm) can be obtained using the equation $\theta_{mrd} = \theta/(10nlC)$, where $n$ is the number of amino acid residues, $l$ is the path length, and $C$ is the BSA concentration [11]. Then, the α-helix content of BSA was calculated according to the following equation [12]: %α-helix content $= (\theta_{mrd} - 4000)/(33000 - 4000)$.

### 2.5. Effect of PEIs on blood coagulation

#### 2.5.1. Activated partial thromboplastin time (APTT), prothrombin time (PT) and fibrinogen time (FT) assays

The assays were performed on an SF-8000 automatic coagulation analyzer (Beijing Succeeder Company, Beijing, China) with corresponding reagents. The PPP (180 μL) was mixed with the PBS (as a control) or PEI solution (20 μL) and used for APTT, PT and FT analysis at 37°C. Each experiment was repeated three times. In each measurement, the samples were detected for clot formation for up to 100s according to the setting of the analyzer.

#### 2.5.2. Thromboelastography (TEG)

Whole blood, mixed with different PEI solution, was subjected to a coagulation study using a Thromboelastograph Hemostasis System 5000 (TEG) from Haemoscope Corporation at 37°C. Briefly, citrated whole blood (900 μL) was mixed with 100 μL of PEI solution in PBS in a tube containing kaolin. The same volume of PBS solution was used as