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Unique concentration dependence on the fusion of anionic liposomes induced by polyethyleneimine

Kazuma Yasuhara*, Manami Tsukamoto, Yasutaka Tsuji, Jun-ichi Kikuchi*

Graduate School of Materials Science, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan

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ABSTRACT

We investigated the interaction of branched polyethyleneimine (PEI) with anionic vesicles formed with 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) by spectroscopic measurements and microscopic observations. PEI induced the fusion of the vesicles only over a specific concentration range, which was found to be below the transition of vesicular zeta-potential from negative to positive value. Above the concentration window of membrane fusion, charge inversion of DOPS vesicles due to the further adsorption of PEI onto the surface of vesicle, inhibited the membrane fusion events. The PEI with low molecular weight induced the membrane fusion in a wider range of concentration than high molecular weight PEIs. Our results suggest that the mechanism of PEI interaction with the lipids depends on the molecular weight and stoichiometry of the polycation to negatively charged lipids.

1. Introduction

Currently, a variety of cationic polymers have been designed and synthesized for the development of functional materials. In particular, various biological and medicinal applications of cationic polymers have been developed, including non-viral gene carriers [1,2] and disinfectants [3,4]. Polyethyleneimine (PEI) is a classic polycation with dense amino groups on the main chain of the polymer under physiological conditions [5]. Many biological applications of PEI have been suggested such as cell adhesion [6,7] and gene transfection [8,9]. However, the cytotoxicity of PEI significantly limits its practical use [10,11]. Some classes of cationic polymers including PEI are believed to disrupt cell membranes through strong interaction and are therefore toxic [12].

In general, most cell membranes display a negatively charged surface due to the presence of acidic lipids in contrast to the fact that cationic membranes are rarely found in nature. For example, the membrane of *Escherichia coli* is enriched in phosphatidylglycerol [13] whereas the membranes of erythrocytes include phosphatidylserine as part of the inner leaflet [14]. Thus, it is reasonable to assume that the membrane interacting activity of cationic polymers partly relies on the electrostatic interaction between the polymer and the negatively charged surface of the membrane. Liposomes or phospholipid vesicles are excellent model systems that mimic cell membranes for physicochemical studies examining the interaction of membrane-active agents with lipid bilayers [15]. Previously, the action mechanisms of several cationic polymers or polypeptides have been investigated using a liposomal system. Such polycations were found to display various effects on the structure or property of lipid membranes including the flip-flop rates of lipids [16,17], membrane fusion [18–21], domain formation in the lipid membrane [22–24] and pore formation [25,26]. The interaction of polymers or polypeptides with the lipid membranes listed above consequently results in various biological functions of the polycations. Thus, a physicochemical study using liposomes should provide valuable information that describes the interaction mechanism of biologically active polymers with lipid membranes.

In this report, the interaction of branched PEI with negatively charged vesicles was investigated from a physicochemical viewpoint. An anionic lipid, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), was used for the preparation of the vesicles as model cell membranes. The following series of techniques were combined to clarify the action mechanism of the PEI on a lipid membrane: dynamic light scattering (DLS), a lipid mixing assay by fluorescence measurements and zeta-potential measurements. Additionally, cryogenic transmission electron microscopy (cryo-TEM) was used for the direct visualization of vesicular morphology. Finally, we discuss the unique effect of the concentration and molecular weight of PEIs on the interaction mode with DOPS membranes. This discussion provides insight into the mechanism of action of PEIs on biomembranes.

* Corresponding authors. Tel.: +81 743 72 6093; fax: +81 743 72 6099.
E-mail addresses: yasuhara@ms.naist.jp (K. Yasuhara), jkikuchi@ms.naist.jp (J.-i. Kikuchi).

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2. Experimental

2.1. Materials

1,2-Dioleoyl-*sn*-glycero-3-phospho-L-serine, sodium salt (DOPS) was purchased from NOF Co. (Tokyo, Japan). *N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phoshoethanolamine, triethylammonium salt (NBD-PE) and *N*-(Lissamine rhodamine B)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rh-PE) were purchased from Molecular Probes (OR, USA). All other chemicals, including branched polyethyleneimine (PEI), were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All reagents used in this study were used without further purification.

2.2. Potentiometric titration of PEIs

Ionization behavior of PEIs was evaluated by potentiometric titration according to the procedure from the literature [27]. First, approximately 0.5 mg of PEI was dissolved in 10 mL of aqueous saline ([NaCl] = 100 mM) to obtain the polymer solution, then 200 μL of 0.1 N hydrochloric acid was added to the polymer solution. Aliquots (10 μL) of 0.1 N sodium hydroxide solution were added with rapid stirring. For each addition of sodium hydroxide solution, pH of the polymer solution was recorded when the pH became stable. The degree of ionization of the PEIs was calculated from the charge neutrality condition as follows:

$$\alpha = \frac{[\text{amines H}^+]}{[\text{amines}]_{\text{total}}} = \frac{[\text{Cl}^-] + [\text{OH}^-] - [\text{Na}^+] - [\text{H}^+]}{[\text{amines}]_{\text{total}}} \quad (1)$$

where α is the fraction of amine group in solution that are protonated. Total molar concentration of the amines, $[\text{amines}]_{\text{total}}$ was estimated from the mass concentration (g L^{-1}) of PEIs divided by the molecular weight of monomer unit (C_2H_5N, 43.07 g mol^{-1}). The apparent pK_a of each PEI was defined as the pH at the half-neutralization point ($\alpha = 0.5$) on the titration curve.

2.3. Preparation of liposomes

Appropriate amounts of DOPS were dissolved in chloroform. For the lipid mixing assay by FRET, 0.8 mol% of both fluorescent dye-labeled lipids (NBD-PE and Rh-PE) were also added to the chloroform solution of DOPS. The solvent was evaporated under nitrogen gas flow and the residual trace solvent was completely removed *in vacuo* for 3 h to give a thin film on the wall of a round-bottom flask. Hydration of the obtained lipid film was performed at 40 °C with 10 mM HEPES buffer ([NaCl] = 100 mM, pH = 7.0), followed by five freeze-and-thaw cycles at −196 and 50 °C. The obtained liposome dispersion was extruded 15 times through stacked two polycarbonate membrane filters with 100 nm pores equipped in a LiposoFast miniextruder from Avestin to obtain large unilamellar vesicles (LUVs) [28]. The concentration of phospholipids was determined using a phosphorous assay [29,30].

2.4. DLS and zeta-potential measurements

The hydrodynamic diameter and zeta-potential of the vesicles were measured by a dynamic light scattering spectrometer equipped with a He–Ne laser at 633 nm (Zetasizer Nano-ZS, Malvern). Size distribution of the vesicles in the dispersion was obtained by analyzing a time course of scattering light intensity at an angle of 173° from the incident light using the Cumulant method. Sample dispersion was introduced into a special capillary cell (DTS1060, Malvern). The applied voltage was set to 10 V for zeta potential measurements. The temperature of the samples was maintained at 25 °C by a thermostat temperature controller installed in the spectrometer.

2.5. Lipid mixing assay by FRET

Lipid mixing between vesicles was evaluated by Förster resonance energy transfer (FRET) measurements between NBD-PE and Rh-PE modified vesicles as follows [31,32]. Two different DOPS vesicles with and without a pair of fluorescent dye-labeled lipids were prepared as described in Section 2.3. Dye-labeled vesicles were mixed with unlabeled vesicles at a ratio of 1:9 (v/v). 500 μL of the vesicular mixture was then added to the 1 mL of HEPES buffer solution of polyethyleneimine at different concentrations to obtain samples for the fluorescence measurements. The final concentration of DOPS was 100 μM. The samples were incubated at 25 °C for 30 min prior to the fluorescence measurements. Fluorescence spectra were recorded by excitation at 460 nm using a Hitachi F-4500 spectrofluorometer. Excitation and emission band-passes were set to 10 and 20 nm, respectively. All measurements were carried out at 25 °C using a thermostat water bath.

2.6. Cryo-TEM observations

The specimen for cryogenic TEM (cryo-TEM) was prepared by rapid freezing of a vesicular dispersion. A copper microgrid with 200 mesh was used and pre-treated with the glow-discharger (HDT-400, JEOL) to obtain a hydrophilic surface. An aliquot (3 μL) of a vesicular sample was placed on the mesh and immediately plunged into liquid propane using a specimen preparation machine (EM CPC, Leica). The temperature of the specimen was maintained below −140 °C during the observation using a cryo-transfer holder (Model 626.DH, Gatan). Microscopic observations were carried out using a transmission electron microscope (JEM-3100FEF, JEOL) at an acceleration voltage of 300 kV in zero-loss imaging mode.

3. Results and discussion

3.1. Characterization of PEIs

Prior to studying the interaction of PEI with lipid membrane, we evaluated the characteristics of PEIs in an aqueous solution. Three different branched PEIs with average molecular weights of 600 (PEI600), 1800 (PEI1800) and 10,000 g mol^{-1} (PEI10000) were used in the investigation. In general, branched PEI contains primary, secondary and tertiary amino groups in a ratio of 25%, 50% and 25%, respectively [33]. Since protonation behavior of individual amino groups on PEI is influenced by its local environment due to the electrostatic interaction of neighboring amino groups [34], we estimated the apparent (mean) pK_a values of PEIs by potentiometric titration. Estimated apparent pK_a value and degree of protonation at pH = 7 for each polymer are listed in Table 1. The pK_a values of PEIs were found within the range of 7.2–8.0 and shifted upward with decreasing molecular weight. The obtained trend in pK_a variation depending on the molecular weight was

Table 1
Characterization of branched PEIs used in this study.

Polymer	MW/kg mol^{-1}	pK_a[a]	α at pH = 7[b]/%	D_{hy}[c]/nm
PEI600	0.6	8.0	60	1.29
PEI1800	1.8	7.6	56	2.24
PEI10000	10	7.2	52	4.74

[a] The apparent pK_a value was defined as the pH at the half-neutralization point ($\alpha = 0.5$).
[b] Degree of protonation.
[c] Hydrodynamic diameter evaluated by DLS.