Biochimica et Biophysica Acta 1828 (2013) 2215–2222

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Polystyrene nanoparticle exposure induces ion-selective pores in lipid bilayers

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ARTICLE INFO

Article history:
Received 22 February 2013
Received in revised form 22 May 2013
Accepted 27 May 2013
Available online 5 June 2013

Keywords:
Biomembrane
Engineered nanomaterials
Pore
Ion selectivity
Electrophysiology
Lipid bilayer

ABSTRACT

A diverse range of molecular interactions can occur between engineered nanomaterials (ENM) and biomembranes, some of which could lead to toxic outcomes following human exposure to ENM. In this study, we adapted electrophysiology methods to investigate the ability of 20 nm polystyrene nanoparticles (PNP) to induce pores in model bilayer lipid membranes (BLM) that mimic biomembranes. PNP charge was varied using PNP decorated with either positive (amidine) groups or negative (carboxyl) groups, and BLM charge was varied using dioleoyl phospholipids having cationic (ethylphosphocholine), zwitterionic (phosphocholine), or anionic (phosphatidic acid) headgroups. Both positive and negative PNP induced BLM pores for all lipid compositions studied, as evidenced by current spikes and integral conductance. Stable PNP-induced pores exhibited ion selectivity, with the highest selectivity for K^+ (P_K/P_{Cl} ~ 8.3) observed when both the PNP and lipids were negatively charged, and the highest selectivity for Cl^- (P_K/P_{Cl} ~ 0.2) observed when both the PNP and lipids were positively charged. This trend is consistent with the finding that selectivity for an ion in channel proteins is imparted by oppositely charged functional groups within the channel's filter region. The P_K/P_{Cl} value was unaffected by the voltage-ramp method, the pore conductance, or the side of the BLM to which the PNP were applied. These results demonstrate for the first time that PNP can induce ion-selective pores in BLM, and that the degree of ion selectivity is influenced synergistically by the charges of both the lipid headgroups and functional groups on the PNP.

1. Introduction

Engineered nanomaterials (ENM) exhibit desirable properties that make them useful for a wide range of applications such as drug delivery systems, gene carriers [1,2], biosensors [3,4], imaging reagents [5,6] and consumer products [7]. The increasingly widespread use of ENM raises questions about possible toxic effects. ENM toxicity has traditionally been investigated using animal (*in vivo*) or cell (*in vitro*) platforms that measure parameters such as lactic dehydrogenase (LDH) release and immune response [8,9]. Moreover, it has been shown that inhaled ambient ultrafine particles can be found in the heart, bone marrow, blood vessels and other organs [10–12]. These observations suggested that the particles could penetrate through the alveolar epithelium. Mechanisms by which ENM interact with, injure, and are transported across the alveolar epithelium are important in understanding health effects related to ENM [13]. However, for ENM to exhibit toxic effects, they must first interact with cell membranes, which are composed of a bilayer lipid membrane (BLM) with a variety of biomolecules that impart desired molecular functions [14]. Consequently, *ex vivo* assays that directly measure molecular interactions between ENM and model BLM can also provide important insights into ENM translocation across epithelial cell barriers and toxicity [15].

A principal role of cell membranes is to provide a selectively permeable barrier that defines cell boundaries and maintains the essential environment for cytoplasm and organelles. ENM have been shown to penetrate biomembranes dependent in part on ENM properties [16–18]. For example, more efficient cellular uptake has been reported for negatively charged nanoparticles as compared with positively charged nanoparticles [16] and negatively charged nanoparticles displayed a less efficient rate of endocytosis than positively charged nanoparticles [17]. A recent review of the influence of surface properties of ENM-cell interactions has indicated that, in general, uncharged ENM interact less aggressively with cells and positively charged ENM are most effective

Abbreviations: ENM, engineered nanomaterials; BLM, bilayer lipid membranes; LDH, lactic dehydrogenase; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOEPC, 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine; DOPA, 1,2-dioleoyl-*sn*-glycero-3-phosphate; PNP, polystyrene nanoparticles; amidine-PNP, amidine-terminated polystyrene nanoparticles; COOH-PNP, carboxyl-terminated PNP; PC, phosphocholine; EPC, ethylphosphocholine; PA, phosphatidic acid; E_{rev}, reversal potential

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0005-2736/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbamem.2013.05.029

in crossing cell membranes [18]. Consistent with this trend, Yacobi et al. [19,20] observed that transcellular trafficking of positively charged, amidine-terminated polystyrene nanoparticles (amidine-PNP) across primary rat alveolar epithelial cell monolayers was 20–40 times faster than that of negatively charged, carboxyl-terminated PNP (COOH-PNP), and that trafficking did not occur *via* endocytic pathways, suggesting that such translocation may take place by diffusion of PNP through the lipid bilayer of cell plasma membranes.

A second role of cell membranes is to regulate transport of specific ions and molecules. Ion-selective transport generates ion gradients, which in turn play essential roles in energy generation, signaling and other cell functions [21]. Ion-selective transport may involve membrane protein 'channels' with a specific sequence of amino acids, and extensive research has been conducted to identify molecular mechanisms that impart selectivity [22–26]. Besides this class of highly selective channels, there are also channels with mild selectivity. For example, members of the BCL-2 protein family that were reconstituted into BLM formed partially selective channels. A mild cation selectivity was observed for antiapoptotic BCL-2 ($P_K/P_{Cl} = 2.4$) and mild anion selectivity was found for proapoptotic molecule BAX ($P_K/P_{Cl} = 0.3$). These selectivities may reflect the positively charged residues of BAX and negatively charged residues of BCL-2 [27]. The permeability ratio calculated for voltage dependent anion-selective channel (VDAC) from outer mitochondrial membrane is usually quoted as 2:1 Cl^-:K^+ [28]. It has also been found that VDAC has a cation selective open state [29]. Although the mechanism of switching selectivity is unclear, charged side chains that face the lumen of the channel must influence the selectivity [28]. Moreover, evidence exists that highly polar complexes of polyphosphate and polyhydroxybutyrate may impart ion selectivity in certain bacterial channels [30]. In general, selectivity for cations may involve local negative charge, and selectivity for anions may involve local positive charge, within the channel. Such local charge characteristics are likely generated by specific amino acids as well as inorganic compounds (polyphosphate).

Based on recent observations that ENM can induce stable pores in BLM [15], and recognizing that both ENM and phospholipid headgroups of BLM can impart localized changes in charge distribution, we hypothesized that ENM-induced pores could exhibit ion selectivity, and that this selectivity could be influenced by the charge of the ENM and/or the phospholipid headgroup. To test these hypotheses, electrophysiology methods were used to characterize ion currents flowing across BLM exposed to either positively charged amidine-PNP or negatively charged COOH-PNP. The phospholipid headgroup charge was varied by forming BLM from dioleoyl phospholipids having zwitterionic phosphocholine, cationic ethylphosphocholine, and anionic phosphatidic acid headgroups. Our findings establish for the first time that ENM can induce ion-selective pores in BLM and that the ion selectivity can be influenced by the surface charge of both ENM and phospholipid headgroups. These novel findings provide insights into the mechanisms by which PNP may create pores in, and penetrate through, cell plasma membranes.

2. Materials and methods

2.1. Materials

The two types of PNP (20 nm diameter) used in this study were purchased from Invitrogen (Eugene, OR): Fluorescent Yellow-Green amidine-PNP (catalog number C27370) and Fluorescent Red COOH-PNP (catalog number F8786). Phospholipids (with the same lipid tail of 1,2-dioleoyl (DO) but different headgroups) were obtained from Avanti Polar Lipids (Alabaster, AL): 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (DOEPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA). N-decane was purchased from Aldrich (St. Louis, MO).

2.2. Preparation of liposomes

Unilamellar liposomes were obtained by a conventional extrusion technique. Three lipid compositions were used for liposome preparation: DOPC alone, a 3:1 (w/w) mixture of DOPC:DOEPC, and 3:1 mixture of DOPC:DOPA. Dry lipids (1 mg) were hydrated in 1 ml of 10 mM KCl solution for 2 h with periodic stirring in a vortex mixer. After three cycles of freezing/thawing at −10/+30 °C, the suspension of multilamellar liposomes was passed 11 times through a 0.1 μm polycarbonate membrane (Whatman, Buffalo Grove, IL) using Avanti microextruder (Avanti Polar Lipids). All operations, excluding freezing/thawing, were carried out at room temperature. Liposomes were stored at +4 °C during the experiments.

2.3. Hydrodynamic diameter and zeta potential measurements

PNP size (hydrodynamic diameter) and surface charge (zeta potential) were determined at room temperature on a 90 Plus Particle Size Analyzer (Brookhaven Instruments, Holtsville, NY). Particle size distribution was analyzed by dynamic light scattering using 90Plus/BI-MAS software. Electrophoretic mobility of PNP and liposomes was measured with a ZetaPALS (Phase Analysis Light Scattering) software. Data were collected from 10 runs of 10 cycles per run in the presence of 10 mM KCl and either 100 μg/ml PNP or liposomes (with a lipid concentration of 13 μg/ml).

2.4. Characterization of PNP-induced pores in planar bilayer lipid membranes

Planar BLM were formed from a 10 mg/ml lipid solution in n-decane (Aldrich). The solution was painted across the 200 μm aperture of a Delrin cup (Warner Instruments, Hamden, CT). Both *cis* (voltage command side) and *trans* (virtual ground) compartments of the cup contained 10 mM KCl (unless otherwise noted). Either COOH-PNP or amidine-PNP were added to the *cis* side of BLM to a final concentration of 100 μg/ml or 50 μg/ml, respectively. All measurements were performed at room temperature.

The approach commonly used to characterize selectivity of ion channels was adapted for determination of ion selectivity of PNP-induced pores in BLM. The approach entails measuring reversal potentials under the influence of a transmembrane ion gradient. For this purpose, a 10:1 KCl concentration gradient in the *trans*-to-*cis* direction was established by bathing the *cis* compartment with 10 mM KCl and the *trans* compartment with 100 mM KCl. The liquid junction potential offset was compensated prior to membrane painting [31]. PNP were then added to the *cis* compartment in order to minimize electrolyte-induced PNP aggregation.

2.5. Recording and data analysis

Currents flowing across BLM were recorded with an integrating patch-clamp amplifier (Axopatch 200A, Axon Instruments, Sunnyvale, CA). The *cis* compartment was connected to the CV 201A head stage input and the *trans* compartment was held at virtual ground *via* a pair of matched Ag/AgCl electrodes. Currents flowing through voltage-clamped BLM were low-pass-filtered at 10 kHz (−3 dB cutoff) using an eight-pole Bessel filter (902LPF, Frequency Devices, Ottawa, IL) and digitized at 1 kHz using pClamp9 software (Axon Instruments) and recorded after digitization through an analog-to-digital converter (Digidata 1322A, Axon Instruments). Using standard voltage conventions, positive clamping potentials are listed as potentials with respect to ground and positive currents are shown as upward deflections.

Noise in the conductance signal of unmodified BLM had an amplitude of 0.2 pA. Conductance events were identified automatically and analyzed using Clampfit9 software. Total charge transfer through BLM was estimated by integrating area under the current trace over time.