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AFM visualization of sub-50 nm polyplex disposition to the nuclear pore complex without compromising the integrity of the nuclear envelope

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ABSTRACT

It has been questioned as to whether polyplexes in the cytoplasm can reach the nuclear compartment and if so in what form. By applying atomic force microscopy (AFM) to the nuclear envelope and the nuclear pore complexes, we demonstrate that disposition of polyethylenimine (PEI)/DNA polyplexes that were microinjected into the oocytes of *Xenopus laevis*, as an example of a non-dividing cell, is exclusive to the nuclear pore complex (NPC). AFM images show NPCs clogged only with sub-50 nm polyplexes. This mode of disposition neither altered the morphology/integrity of the nuclear membrane nor the NPC. AFM images further show polyplexes on the nucleoplasmic side of the envelope, presumably indicating species in transit. Transmission electron microscopy studies of ruptured nuclei from transfected human cell lines demonstrate the presence of sub-50 nm particles resembling polyplexes in morphology compared with control preparations.

1. Introduction

Synthetic polycations of different chemical make-up and architecture are receiving increasing attention in transfection protocols by virtue of their capacity to compact nucleic acids, forming complexes known as polyplexes [1,2]. This not only results in protection of the nucleic acids from degradation, but polyplexes can be taken up by a wide range of mammalian cells through integrated mechanisms involving plasma membrane destabilization as well as clathrin- and/or caveolae-mediated pathways [1,2]. The most prominent and widely investigated polycationic macromolecule in gene delivery is polyethylenimine (PEI) [3]. PEI exists in a wide range of sizes and in both linear and branched architectures [1,4]. While the mode of PEI/DNA polyplex entry into the cells and mechanisms of PEI-induced multifaceted cell-death processes have been widely investigated [5–8], not much is known as to whether PEI and PEI/DNA polyplexes can enter the nuclear compartment and if so, how? A number of reports have suggested that DNA may enter the nucleus while complexed to PEI [9,10] with some hypothesis speculating a role for anionic phospholipids mediating fusion between polyplexes and the nuclear envelope [11]. Contrary to these observations, others have shown that in some transfected mammalian cells PEI-based polyplexes were excluded from the nucleus [12]. Alternatively, others have suggested that on transfection the nuclear entry of plasmid DNA and polyplexes may be restricted to mitosis, where cytoplasmic mixing occurs and the nuclear envelope is broken [13,14]. The translocation of unpacked DNA from the cytoplasm to the nucleus is also debatable; it is expected to be slow and inefficient, since DNA is rapidly degraded in the cytoplasm with an apparent half-life of 50–90 min [15].

Nuclear entry, however, is a selective process tightly modulated by the nuclear pore complex (NPC) [16,17]. NPCs are supramolecular assemblies of nucleoporins displaying a ≤39 nm wide transport channel that mediates passive diffusion of molecules up to 40 kDa in size as well as facilitating selective transport of larger molecules bound to soluble transport receptors that shuttle between the cytoplasm and nucleus in eukaryotic cells [18,19]. To address the abovementioned discrepancies in polyplex disposition to the nucleus, and bearing the evidence-based size-dependent transportation through NPCs, we have now monitored and visualized polyplex interaction with the nuclear envelope and NPCs by atomic force microscopy (AFM) following polyplex microinjection into the oocytes of *Xenopus laevis* [20,21]. Oocytes do not undergo cell division. Accordingly, due to lack of mitosis the nuclear envelope remains intact. This makes oocytes as appropriate examples for examining the routes of polyplex entry across the nuclear envelope, where we show a polyplex size-dependent mode of entry across NPC. In addition to these, we also used transmission electron microscopy (TEM) to show exclusive presence of small-sized polyplexes in the

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ruptured nuclei fractions from polyplex transfected human H1299 cell line, which undergo cell division.

2. Materials and methods

2.1. Polyplex formation

25 kDa branched PEI (Sigma-Aldrich, Denmark) and 10 kDa linear PEI (Polysciences, Germany) was complexed with mammalian DNA or the plasmid DNA pcDNA3-EGFP (579 base pair; a kind gift from Dr. F. Rahbarizadeh, University of Copenhagen) as previously described [22]. Briefly, PEI and DNA were diluted to the desired concentration in sterile 150 mM NaCl. PEI was added to DNA during vortexing and following 10 min incubation polyplexes were used for biological experiments. For all polyplexes N/P = 7.

2.2. Nanoparticle tracking analysis

Polyplex size distribution was followed by Nanoparticle Tracking Analysis (NTA) using a NanoSight LM10 (Malvern, UK) equipped with a sample chamber and a 405 nm blue laser and a Viton fluoroelastomerO-ring [23]. All measurements were performed at room temperature and repeated at least three times and with different preparations. EMCCD camera and NTA software (version 2.3) was used for size measurement. Freshly prepared polyplexes were diluted in physiological saline to appropriate concentrations prior to analysis.

2.3. Transmission electron microscopy

Polyplexes were adhered to carbon filter and negatively stained with 2% (w/v) phosphotungstic acid before transfer to the grid, dried and imaged. Imaging was carried out on a Phillips CM 100 TWIN transmission electron microscope (Phillips, Eindhoven, The Netherlands) equipped with a side-mounted Olympus Megaview-2 camera.

2.4. Preparation of nuclear envelopes

Oocytes in stage IV were separated from the ovarian tissue in Hepes Ringer solution [20,21]. Polyplexes (50 nL) were microinjected at equator. The Oocytes were incubated overnight at 17 °C before isolation of the nuclei. The microinjected oocytes were transferred to nuclei isolation medium (NIM; 87 mM NaCl, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, pH 7.4) with 1.5% (w/v) polyvinylpyrrolidone. The oocyte was dissected along the equator with two precision forceps and leftover cytosol was removed. The nuclei were transferred for 5 min to NIM lacking polyvinylpyrrolidone for swelling and afterwards placed onto 15 mm glass coverslips previously coated with 0.1% (w/v) poly-L-lysine (Sigma-Aldrich, Denmark). The chromatin was removed by using two sharp needles and the nuclear envelope was spread out with the cytoplasmic side up. The prepared nuclear envelopes were fixed for 30 min with 1% (w/v) glutaraldehyde.

2.5. Atomic force microscopy (AFM) analysis of nuclear envelopes

Applying AFM to nuclear envelopes has previously been described [20,21]. We used a Bioscope atomic force microscope (NanoScope V controller, Bruker, Santa Barbara, CA) and imaging was carried out at room temperature. Nuclear envelopes were analyzed in contact mode at 1,5 Hz with a V-shaped OTR4 cantilever (Olympus) and with 256 × 256 lines per image.

2.6. Cell culture

The human non-small lung cancer cells, H1299 cells were grown under standard condition in RPMI medium supplemented with 10% (v/v) foetal bovine serum. Cells were kept at 37 °C and 5% CO_2 [23].

2.7. Cell transfection

Two days prior to transfection, H1299 cells were seeded in 12-well plates with confluence of 55–65% on the day of transfection. Polyplexes were added to the cells with final PEI concentration of 5 μg/mL and 7 μg/mL for 25 kDa branched and 10 kDa linear PEI, respectively. The nitrogen to phosphate ratio was always kept at 7. At 24 h post polyplex addition, cells were washed twice in PBS and detached by trypsinization. The percentage of EGFP expressing cells was analyzed by flow cytometry (BD FACS Array Cell Analysis, USA).

2.8. Lactate dehydrogenase assay

Polyplexes and transfection was carried out as described above. LDH release was measured as previously described [8] with the use of CytoTox 96 Non-radioactive Cytotoxicity Assay kit (Promega Biotech AB, Sweden). Briefly, polyplexes were added 24 h before measuring LDH release. Then 50 μL of the medium was transferred to a 96-well plate. Next, 50 μL of reaction reagent was added followed by 30 min incubation before adding 50 μL stop solution. The absorbance was measured at λ = 490 nm using a Multiskan MS Elisa reader (MTX Lab Systems, FL, USA).

2.9. Nuclei fractionation and analysis

H1299 cells are treated with polyplexes for 24 h before washing in PBS. The cells were scraped and pelleted at 1600*g* for 2 min and lysed with cytosolic lysis buffer and nuclei were subsequently harvested by centrifugation. The nuclei were ruptured by treatment with nuclear extraction buffer (10 mM HEPES, 10 mM KCl, 0,1 mM EDTA, 0,1 mM EGTA, 1 mM DTT and 0,1% IGEPAL) and by passing 10 times through a 27 G needle, followed by centrifugation to purify [24]. The purity of the nuclei fraction was determined by Western blotting against lamin-B and actin.

For Western blotting, the nuclei fraction was loaded in each well of a 4–12% BOLT gels (Life Technologies Europe BV, Denmark) or on 4–12% bis tris gels and subjected to electrophoresis in MES buffer depending on the protein of interest. The separated proteins were electrophoretically transferred to PVDF membranes via Iblot Blotting system (Life Technologies Europe BV, Denmark) at 20 V for 7 min. The membranes were blocked for 1 h at room temperature in 5% non-fat dry milk/TBST (137 mM sodium chloride, 20 mM Tris, 0.1% Tween-20, pH 7.6), incubated overnight at 4 °C with appropriate anti-lamin B and anti-actin antibody (Abcam, MA, USA) dilutions in 5% (w/v) BSA/TBS-T. After washing three times for 5 min each time, the membranes were incubated for 1 h at room temperature with appropriate dilutions of HRP-conjugated secondary antibodies in 5% non-fat dry milk/TBST. Membranes were then washed as described above and incubated with ECL Western blot detection reagents (Life Technologies Europe BV, Denmark) and exposed to Hyperfilm-ECL (VWR) to visualize immunoreactive proteins.

Nuclei fractions were prepared as described above. The nuclei were adhered to carbon filter and negatively stained with 2% (w/v) phosphotungstic acid before transfer to the grid, dried and imaged. Imaging was carried out on a Phillips CM 100 TWIN transmission electron microscope (Phillips, Eindhoven, The Netherlands) equipped with a side-mounted Olympus Megaview-2 camera.

2.10. Statistical analysis

All experiments were repeated a minimum of 3 times and statistical analysis was performed using one-way ANOVA. For transfection efficiency experiments Dunnett's Multiple Comparison Test was used.