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Investigation of Transport and Unpacking Mechanisms of Polyplexes for Transfection Efficacy on Different Cell Lines

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Gene therapy is based on correcting the mechanisms underlying diseases and/or their causes through the delivery and subsequent expression of exogenous DNA molecules encoding the missing or damaged gene products. In recent years, polyplexes (nonviral vectors in the form of DNA complexes with polycations) are more and more often used for DNA delivery [1]. The goal of this work was to study the characteristics of polyplexes based on polyethylenimine (PEI) that affect their transfecting ability and the intracellular mechanisms that influence the transfection of various cell lines.

PEI with a molecular weight of 25 kDa (Polysciences, United States) was used as a DNA-condensing polycation. To reduce the excess positive charge and increase the hydrophilicity of the complex, PEI was conjugated with heterobifunctional polyethylene glycol (Quanta BioDesign, United States). To facilitate penetration into cells, the TAT peptide (GRKKRRQRC, Rusbiolink, Russia) was attached to the block-copolymer PEI-PEG.

The stability of polyplexes was determined by the degree of their unpacking after the addition of the polyanion heparin (0.2 to 0.5 IU; Sintez, Russia), which is commonly used [2] as a standard model of the polyplex unpacking under conditions of polyanion exchange. Heparin competes with DNA for binding to the polycation, which simulates the situation in transfected cells when a polyplex can be unpacked by other negatively charged molecules. This may reduce the transfection efficacy. It was found that the polyplexes based on PEI-PEG-TAT are more resistant to unpacking than the polyplexes with unmodified PEI, because their unpacking requires higher heparin concentrations (Fig. 1a).

On its way to the target cells as well as during endocytosis, DNA in polyplexes can be cleaved by nucleases [3]. To determine the resistance to DNase, we studied polyplexes with different PEI nitrogen to DNA phosphate (N/P) ratios (N/P = 10, 20, 30, and 40). At first, polyplexes were treated for 30 min with DNase I (3 IU, Silex, Russia). Then, a stop solution was added to completely inactivate DNase, after which heparin (2 IU) was added to release DNA from the complex. It was found that DNA in the PEI-PEG-TAT-based polyplex was more resistant to cleavage than DNA in the PEI-based polyplex (Fig. 1, b). It was also shown that the resistance to DNase increased as the N/P ratio increased.

Thus, we studied the unpacking of polyplexes based on PEI-PEG-TAT and on PEI alone in a simple model system in vitro and found that polyplexes based on PEI-PEG-TAT more efficiently ensure condensation of DNA and better protect it from enzymatic degradation. Next, we attempted to assess the effect of factors related to intracellular transport and unpacking of polyplexes on transfection of different cell lines with the same polyplexes. For

this purpose, we used polyplexes consisting of PEI-PEG-TAT block-copolymers and the pEGFP-N3 plasmid (Clontech, United States) carrying the gene for the green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter (CMV). We have selected the conditions of transfection [2] that most effectively ensured transfection in different cell lines (Table).

Differences in the maximum transfection percentage may be determined by at least two causes: (1) differences in the EGFP expression under the control of the CMV promoter or (2) differences in the processes of transport and unpacking of polyplexes on different cell lines. To test the first hypothesis, we studied cell lines transfected with the reporter gene under the control of another strong and nonspecific promoter CAGGS [4], which was kindly provided by A.A. Buzdin (Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences). For the CAGGS promoter, the same series of values for transfection was obtained as for the CMV promoter ($r = 0.962$, $p < 0.0001$). For this reason, we tested the second assumption.

The mechanisms of transport and unpacking of polyplexes on different cell lines were studied by the Förster resonance energy transfer (FRET) method [5]. Components of polyplexes—DNA and the carrier conjugate—were labeled with two fluorophores: (1) biotinylated DNA, with quantum dots streptavidin conjugate (Qdot605) and (2) block-copolymer PEI-PEG-TAT, with the fluorophore AlexaFluor647. Both fluorophores were purchased from Invitrogen/Molecular Probes, United States. Preliminary measurements in suspensions of labeled polyplexes showed that energy transfer was observed in more than 90% of polyplexes. In cells, unpacked polyplexes we determined the same way (by AlexaFluor647 fluorescence emitted upon the excitation of quantum dots, which was possibly due to FRET). If the excitation of quantum dots led to fluorescence of quantum dots alone whereas fluorescence in the channel for AlexaFluor647 was absent (which takes place when the donor and acceptor are located at distances more than 10 nm from each other), we assumed that the polyplex was unpacked.

To determine the effect of polyplex unpacking on the transfection efficacy, we selected three cell lines that significantly differed in the maximum transfection percentage: A549, Calu-1, and M3 (17.7, 63.2, and 98.0%, respectively). To visualize cellular compartments, we used specific dyes: (1) wheat germ agglutinin labeled with Cy3 (Biodye, Russia) was used to stain the plasma membrane, (2) LysoTracker Green DND-26 (Invitrogen/Molecular Probes, United States) was used to stain endosomes and lysosomes, and (3) Hoechst 33258 (Sigma-Aldrich, United States) was used to stain nuclei. We used an LSM-510 Meta NLO confocal microscope (Carl Zeiss, Germany) to obtain optical sections and three-dimensional images of living transfected cells at different time intervals after the addition of polyplexes to cells (from 30 min to 16 h).

Based on the experimental data, we developed a model of transport and unpacking of polyplexes (Fig. 2), which is described by the following system of differential equations:

$$\begin{aligned} \frac{dL_{u1}}{dt} &= k_{unL} L_p, \\ \frac{dL_{u2}}{dt} &= v_1 e^{-k_1 t} - k_{u2} L_{u2}, \\ \frac{dL_p}{dt} &= v_0 e^{-k_0 t} - k_{unL} L_p - k_{p2} L_p, \\ \frac{d(C_u + N)}{dt} &= k_{unC} C_p + k_{u2} L_{u2}, \\ \frac{dC_p}{dt} &= k_{p2} L_p - k_{unC} C_p, \\ \frac{dN}{dt} &= k_{u3} C_u. \end{aligned}$$

The number of polyplexes is characterized by the voxel volume of quantum dots for which FRET was not observed (in the nucleus (N), cytosol (C_u), and endo/lysosomes (L_{u1} and L_{u2})) as well as by the voxel volume of quantum dots for which FRET was observed (in cytosol (C_p) and endo/lysosomes (L_p)) calculated per cell. We assessed the following parameters: v_0 , the initial rate of cell entry of packed polyplexes (FRET was observed); k_0 , the rate constant of decrease in entry of packed quantum dots; v_1 , the initial rate of cell entry of unpacked quantum dots (FRET was not observed); k_1 , the rate constant of decrease in entry of unpacked quantum dots; k_{unL} , the rate constant of unpacking in endo/lysosomes; k_{unC} , the rate constant of unpacking in the cytosol; k_{p2} and k_{u2} , the rate constants of exit of packed and unpacked quantum dots from endo/lysosomes, respectively; and k_{u3} , the rate constant of entry into the nuclei.

The developed model describes well the experimental data for the used cell lines A549 ($r = 0.975$), Calu-1 ($r = 0.833$), and M3 ($r = 0.941$). It allowed us to reveal a negative correlation between the reciprocal rate of polyplex entry into the cell and the maximum transfection efficacy on different cell lines ($r = -1$, $p = 0.0016$). We also found a negative correlation between the rate constant of unpacking in endosomes/lysosomes and the maximum transfection efficacy in different cell lines ($r = -0.997$, $p = 0.05$).

Thus, in this work we have created and studied highly efficient polyplex systems for delivery of genetic information to cells. We suggest that data obtained for the limiting stages of unpacking and transport of polyplexes in the cell can be used in designing nonviral vectors by modification of the block-copolymer.

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Fig. 2. General model of transport and unpacking of polyplexes in the cell (for designations of model parameters, see text).

Table

Maximum transfection efficacy of different cell lines with polyplexes

Cell line	Maximum transfection percentage
Human	
Human-epithelial cervical cancer cells (HeLa)	up to 100
Human embryonic kidney (HEK) 293 cells	96
Human lung carcinoma cells Calu-1	63
Mucoepidermoid pulmonary carcinoma cells H292	59
Human epidermoid carcinoma cells A431	50
Human lung carcinoma cells H358	46
Human lung carcinoma cells A549	18
BT-474 human breast cancer cells	4.4
Mouse	
Murine Cloudman S91 melanoma (clone M3)	98
Murine melanoma cells B16F1	80
Murine Lewis lung carcinoma cells LLC-1	34