Nonviral gene delivery: Towards artificial viruses

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Abstract

Several limitations to nonviral gene delivery have been overcome. Small nanometric particles have been obtained by condensation of DNA with a polymerizable cation followed by DNA template-directed homopolymerization of the vector. Targeting of specific cell types has been achieved by using polyethylenimine (PEI), a cationic polymer, coupled to cell ligands such as galactose or small RGD peptide that allows cell entry by a receptor-mediated endocytosis pathway. Escape of the DNA complexes from the endosomes is favored by the 'proton sponge' effect as a consequence of the high buffering capacity of PEI. The last barrier to gene delivery, i.e. the nuclear membrane, can be crossed at the level of the nuclear pore complexes, by using nuclear localization signal DNA molecule conjugates.

Abbreviations: NLS, nuclear localization signal; PEI, polyethylenimine; RGD, Arg-Gly-Asp peptide sequence

Introduction

Oligonucleotides and genes have shown considerable therapeutic potential in model systems. Comparable clinical success is delayed essentially because of their poor bioavailability, of immune responses against viral vectors or of ineffective intracellular trafficking of synthetic vectors. Artificial vectors being neither limited to the molecules nor to the solutions found by evolution to carry viral genomes, cell-mediated immune response against the vector can essentially be avoided. Moreover, advances in the mechanism of nonviral gene delivery (Hagstrom et al., 1997; Labat-Moleur et al., 1996; Pollard et al., 1998; Xu et Szoka, 1996; Zabner et al., 1995) provide new ideas for the design of improved modular systems.

In vivo diffusion of the cationic vector/anionic DNA complexes is size-dependent. Complex size can be decreased by condensing each gene molecule individually into a neutral particle. This has been achieved by reversible DNA condensation with a polymerizable cation followed by DNA template-directed homopolymerization of the vector (Blessing et al., 1998a,

b) (Figure 1). As an example, a designed cationic cysteine-based detergent was used to condense plasmid DNA into 23 nm particles, which were then 'frozen' by spontaneous aerobic dimerization of the cysteine-detergent into a cystine-lipid on the template DNA.

Monomolecular collapse was confirmed by measuring the sizes of particles resulting from the detergent-mediated condensation of DNA of various length (Figure 2). Supercoiled pCMVLuc (5.6 Kb) plasmid as well as linear phage λ (48 Kb) and phage T4 DNAs were successfully condensed into monodisperse population of spheres of increasing size. As size varies as the cubic root of volume, a plot of observed size vs. the cubic root of number of DNA base pairs fitted with a straight line crossing the origin.

For the next step, i.e., targeting, hepatocytes or mitotic endothelial cells have been chosen as they can be physically reached following systemic delivery. Preliminary *in vitro* results show that neutral DNA particles bearing galactose (Zanta et al., 1997) or small RGD peptide residues (Erbacher et al., 1999) enter

Figure 1. (a) Reversible condensation of DNA is induced by cations with low binding cooperativity. The resulting particles are stabilized by template oligomerization of the monomeric cationic species. (b) Transmission electron microscopy of an unfiltered solution of cationic lipid/DNA particles showing spheres with a diameter of ≈ 23 nm. Bar = 100 nm.

Figure 2. Particle size varies as the cubic root of the DNA size. Particle size was determined by dynamic laser light scattering.

specifically their target cells *via* asialoglycoprotein receptor- or integrin-mediated endocytosis.

In both examples, the cationic polymer polyethylenimine (PEI) was used for DNA complexation. Introduction of the galactose ligand was obtained *via* imine formation between lactose and the amine functions of PEI, followed by *in situ* reduction (Figure 3). Using the luciferase reporter gene, transfection of murine and human hepatocytes was shown to be ca. 10^4 -fold more efficient with galactose-derivatized PEI than with the glucose derivative. Competition with excess asialofetuin (a natural ligand for the receptor) inhibited receptor-mediated transfection.

PEI-RGD peptide derivatives were prepared by conjugation of a cysteine-containing RGD peptide to a thiol-derivatized PEI through a disulfide bridge. Short synthetic RGD-containing peptides were shown to bind $\alpha v \beta 3$ and $\alpha v \beta 5$ integrins present at the surface of epithelial and endothelial cells. Gene transfer of DNA/RGD-peptide particles *via* receptor-mediated endocytosis was demonstrated by transfection experi-

Figure 3. Scheme for the conjugation of galactose to PEI via imine formation with lactose, and subsequent condensation of PEI-gal with plasmid DNA into neutral galactose-bearing complexes.

Figure 4. Integrin-mediated transfection efficiency increases with the amount of grafted peptide. Transfection experiments were performed in HeLa cells.

Figure 5. (a) Buffering capacity and viscosity properties of PEI. (b) Schematic representation of the 'proton sponge' effect.

ments performed on HeLa epithelial cells (Figure 4). Both selectivity and gene transfer efficiency increased with the number of RGD residues present in the complexes, up to hundred fold. No such effect was observed with the mutated RGE-peptide where aspartic acid was replaced by glutamic acid.

Once inside the cell, most of the DNA particles seem to remain sequestered in endocytic vesicles. For PEI, however, escape of the complexes from endosomes is favored by the 'proton sponge' effect (Boussif et al., 1995). Indeed, PEI possesses substantial amine buffering capacity between pH 7 and 5 (Figure 5). The vesicular ATPase induces massive proton accumulation in these endosomes, with concomitant passive chloride diffusion (Figure 5). Increased ionicity will cause osmotic swelling and subsequent endosome disruption, thus allowing escape of the endocytosed material. This hypothesis is supported by the dramatic transfection decrease observed in the presence of bafilomycin A, a selective vesicular ATPase inhibitor.

Yet, the largest barrier to gene delivery remains the nuclear membrane. Like DNA viruses, we took advantage of the cellular nuclear import machinery. For

Figure 6. Luciferase expression levels obtained with NLS and mutated NLS-DNA constructions. Transfection experiments were performed with PEI.

Figure 7. Hypothetical scheme of NLS-DNA entering the nucleus. According to the mean interpore distance in active cells ($10 \text{ pores } \mu\text{m}^{-2}$), calculation shows that two NLS peptides grafted to the ends of a 1 Kbp DNA fragment would lead to the unfavorable situation depicted.

this purpose, a single nuclear localization signal peptide (NLS) was covalently bound to a reporter gene. This construction was obtained by ligation of a pair of hairpin oligonucleotides to unique cohesive termini on the reporter gene fragment. A cysteine-containing NLS peptide was conjugated to a derivatized thymine base in one of the hairpins via thio-ether bond formation.

Transfection of cells using this tagged gene remained effective down to nanogram amounts of DNA (Zanta et al., 1999) (Figure 6). Transfection enhancements of up to thousandfold were observed irrespective of the cationic vector used. A single lysine to threonine mutation in the signal peptide, that was shown to abolish nuclear import, also abolished the transfection enhancement, strongly suggesting the involvement of importin-mediated translocation

(Figure 6). Our hypothesis is that the 3 nm-wide DNA molecules present in the cytoplasm are initially docked to and translocated through a nuclear pore by the nuclear import machinery. As DNA enters the nucleus, it is condensed into a chromatin-like structure, which provides the mechanism for threading the remaining worm-like molecule through the pore (Figure 7).

Solutions to overcome individual barriers to gene delivery can thus be found. Making the pieces of the puzzle fit together to create an artificial virus is still another challenge.

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