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Chimia 51 (1997) 34–36

ISSN 0009–4293

The Proton Sponge: a Trick to Enter Cells the Viruses Did Not Exploit

Jean-Paul Behr*

Abstract. Several non-permanent polycations possessing substantial buffering capacity below physiological pH, such as lipopolyamines and polyethylenimines, are efficient transfection agents *per se*, *i.e.* without the addition of lysosomotropic bases, or cell targeting, or membrane disruption agents. These vectors have been shown to deliver genes as well as oligonucleotides both *in vitro* and *in vivo*. Our hypothesis is that their efficiency relies on extensive endosome swelling and rupture that provides an escape mechanism for the polycation/DNA particles.

Introduction

Gene transfer is the weak link of gene therapy because DNA is a pro-drug rather than the therapeutic effector molecule itself. The cascade of events leading to the synthesis of a large number of therapeutic protein molecules from a single gene begins in the nucleus. Therefore, vector systems are required to carry the exogenous DNA through the plasma and nuclear membranes. Most often, recombinant viral vectors are used for this task [1], since viruses have evolved sophisticated break-in ways [2] that can now be exploited. These include efficient cell-membrane rupture mechanisms and nuclear targeting. Membrane rupture can occur either directly at the cell surface or after endocytosis. In any case, the viral fusogenic protein becomes 'informed' of the cell proximity and undergoes a major conformational change induced either by binding to a cell surface receptor or by the acidic nature of the endosomal compartment. Chemists examining such complex molecular sequences may well be daunted. Yet synthetic nonviral gene transfer systems, however basic, will be of great potential to the gene therapy field just as soon as they show sufficient *in vivo* transfection capacities. We insist on the term *sufficient*, – the same adjective is apparently used to describe the Rolls-Royce engine power in the technical notice accompanying the car! – as it suggests an adequate performance. However, as emphasized by the recent British and American attempts to apply gene therapy to cystic fibrosis or melanoma patients, we know that this criterion is far from being satisfied [1b].

Chemistry is not constrained by the need for replication characteristic of a biological system and can therefore explore and exploit a much wider spectrum of candidate molecules for a given task. With some imaginative leads and a great deal of 'evolutionary' trial and error, two classes of synthetic vectors have been developed over the last decade. These compounds, whether lipids [3] or polymers [4], are all cationic like their classical predecessors used for *in vitro* transfection (calcium phosphate, DEAE-dextran). On complexing with DNA, they cause several plasmid molecules to condense together into submicrometric particles.

In vitro Transfection with Cationic Lipids [5]

When a cationic lipid is used at an excess ratio of cationic charges to nucleic-acid phosphates, the resulting nucleolipid particles will fix to the cell surface. Indeed, electrostatic interactions between the positively charged DNA/lipid complexes and anionic heparan sulfate proteoglycans of the cell membranes are enhanced by increasing the overall charge of the complexes, which is in turn achieved by increasing the ratio of lipid to DNA. This interaction between the particle and the cell membrane is spontaneously followed by endocytosis. Cationic lipids give variable transfection efficiencies that depend both on the chemical structure of the vector and on the cell type. Even so, irrespective of the cell type, the lipopolyamines constitute one of most efficient vector classes [3][6][7]. This general efficiency is an intrisic property of the charged head-group, and the addition of neither fusogenic lipids or of nuclear localization signals can increase it, suggesting that the polyamine head group may carry in itself these multifunctional properties [8]. Moreover, when the potentiometric protonation states of the amines were measured, it was found that at physiological pH only three of the four nitrogens in the spermine head were cationic (*Fig. 1*). The pK_a of the last amine is 5.5, halfway between the extracellular and intralysosomial pH values, a clue to a possible buffering property that could well be exploited, and a point we shall return to later.

In vitro Transfection with Cationic Polymers

Compared to the lipopolyamines, most members of the other class of cationic

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molecules – the cationic polymers such as poly-L-lysine – are relatively inefficient unless conjugated to a ligand that can provoke endocytosis when bound to its cell-surface-located receptor [4]. Even then, transfection levels only reach those obtained with lipopolyamines when the polymer is conjugated to adenoviral particles that lyse endosomes [9]. However, the use of the nonspecific adenovirus components counteracts the cell targeting function obtained with the ligand. Recently, an exemplary class member of the cationic polymers has emerged with the description of the transfection properties of the polyamidoamine dendrimers [10]. These quasispherical macromolecules bear a large number of amino groups on their surface and again, as for the lipopolyamines, not all of these amino groups are protonated at physiological pH.

So by observing two completely different cationic vectors, a lipid and a polymer, we are led to the same question: whether there is a causal relation between the overall buffering capacity of a vector under physiological conditions and its transfection possibilities [11]. Accordingly, a number of macromolecular compounds bearing high amino-group densities were considered for synthesis. Such cationic compounds would still be able to compact DNA, but owing to the repulsion predicted between like charges at close proximity, they would not be fully protonated at physiological pH.

As it turned out, there was no need to start synthesizing candidates, as the ideal molecule was already available. In the commercially available polymer polyethylenimine (PEI, *Fig. 2*), in every third position is an amino group and the overall protonation level increases from 20 to 45% between pH 7 and 5. Moreover, the compound was described over 50 years ago and its innocuousness demonstrated by its intensive and various uses: in water purification, ore extraction and even in shampoos. We tested the transfection efficiency of this polymer, comparing it to lipopolyamines on a large variety of cell lines and primary cultures [12]. The results are most promising, showing efficiencies at least as high as the best currently available synthetic vectors (*Fig. 3*) indicating an entirely new function for this simple molecule.

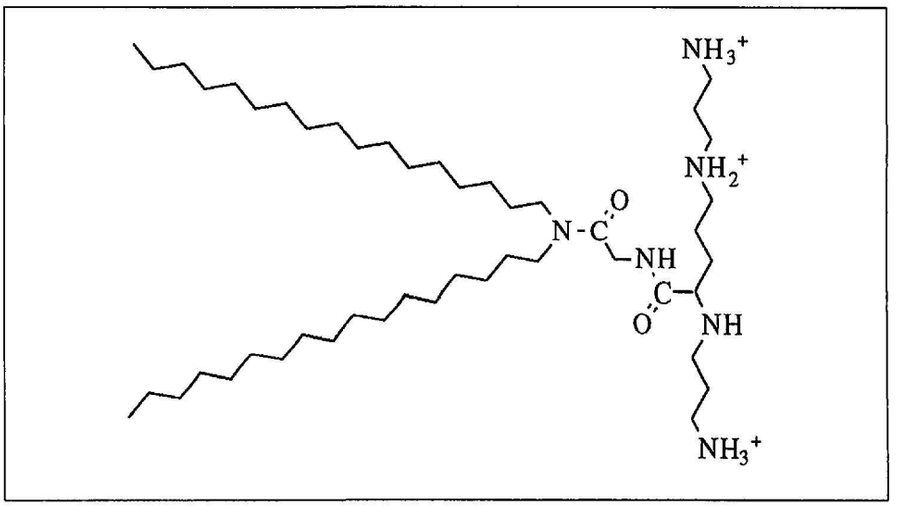

Fig. 1. *Chemical structure of the lipopolyamine* Transfectam® *at neutral pH*

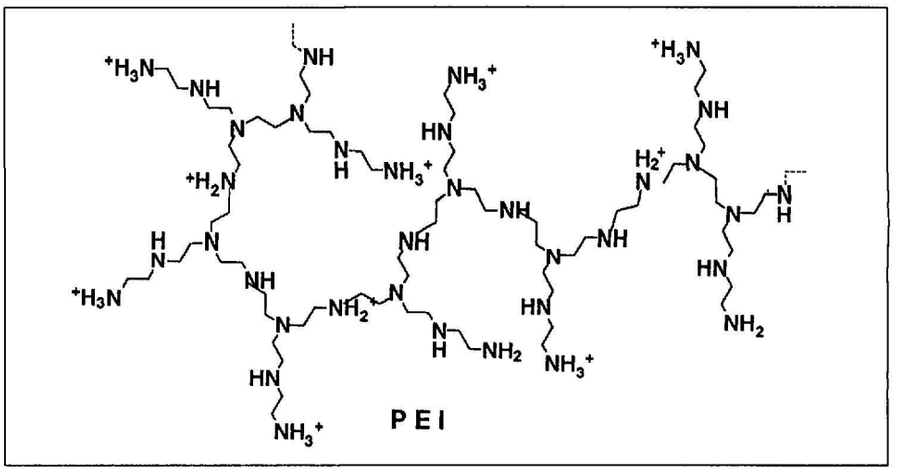

Fig. 2. *Chemical structure of polyethylenimine at neutral pH: only every fifth amino function is protonated, which leaves most N-atoms for the proton-sponge effect*

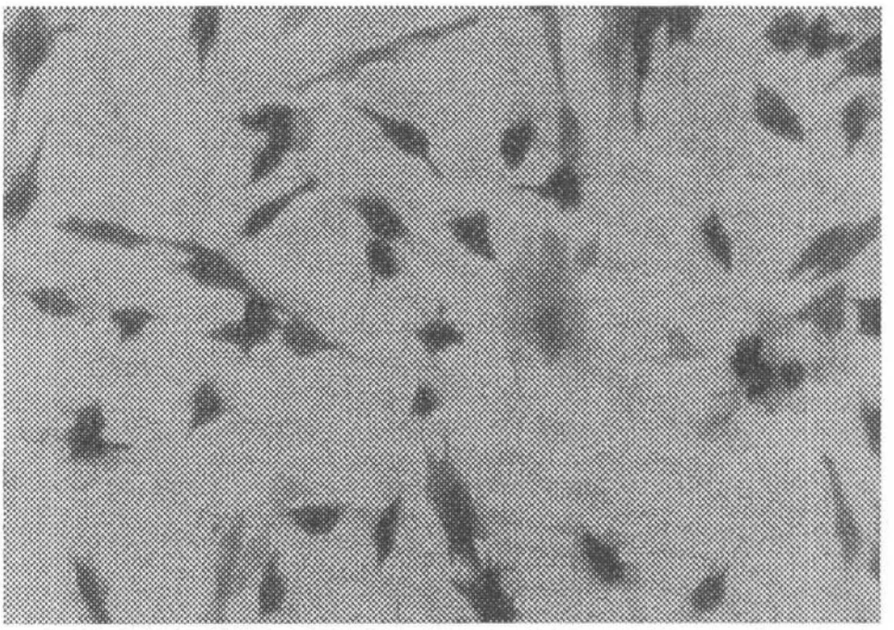

Fig. 3. *Expression of β-galactosidase* (dark blue cells) *following transfection* (2 μg CMV-βgal transgene) *with PEI in the murine fibroblast 3T3 cell line.* Over 95% of the cells are histochemically stained.

Gene Transfer Mechanism [5]

The sequence of events that we hypothesize to account for the remarkable transfection properties of PEI are summa-

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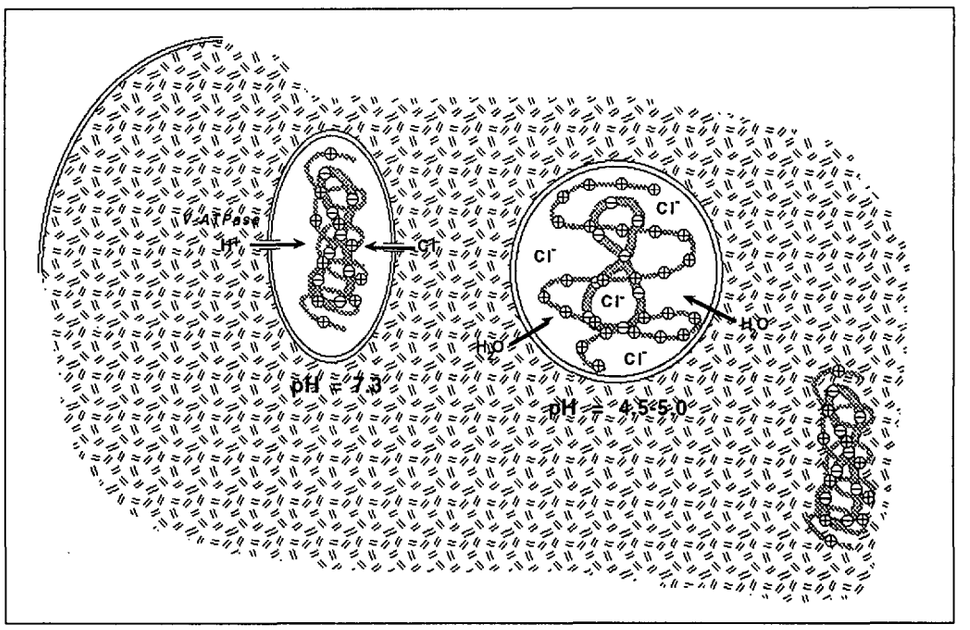

Fig. 4. *The proton-sponge hypothesis: H^+ and Cl^- entry into the endosome leads to osmotic swelling and finally to endosome rupture*

rized in *Fig. 4*. The polycation/DNA complexes enter the cell by spontaneous endocytosis. A complex wholly covered in positive charges interacting with the cell membrane will produce a high local concentration of PEI in the endosome. During the intracellular trafficking the buffering capacity of PEI will not only tend to inhibit the action of the lysosomal nucleases that have an acid optimal pH, but will also alter the osmolarity of the vesicle. The accumulation of protons brought in by the endosomal ATPase is coupled to an influx of chloride anions [13]. In the presence of PEI there will be a large increase in the ionic concentration within the endosome resulting in osmotic swelling of the endosome. Moreover, PEI protonation will also expand its polymeric network by internal charge repulsion [14]. With the two phenomena occuring simultaneously it is likely that endosomal life expectancy is sorely reduced! Taking into account the protonation profile of PEI we can expect that about a third of the N-atoms in the molecule participate in the swelling action, making the molecule a virtual proton sponge. For gene therapy the interesting aspect of this mechanism (which is somewhat 'primitive' compared to the mechanisms developed by viruses) is that it will lead to enhanced gene transfer, as the DNA introduced with PEI will be rapidly liberated from the damaging endosomal environment. Thus, this molecule constitutes, *per se*, a promising vector for gene therapy and an ideal structural base for constructing more sophisticated vectors that could include supplementary functions such as cell-specific targeting ligands.

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