The Proton Sponge Hypothesis

An evaluation of the evidence

Stefan G.M. Broeren, BSc

ABSTRACT

In 1995, Boussif et al. showed that polyethylenimine (PEI) is an efficient transfection vector. Their hypothesis was that amine groups in polymers buffer the acidification of vesicles. This would 1) prevent DNA degradation, since nucleases are less effective at higher pH; 2) increase proton pump activity and increase influx of H^+, Cl^- and water into the vesicle; 3) lead to expansion of the polymeric network due to internal charge repulsion. The latter two effects would increase the volume of the vesicle until rupture would ensue. High vesicular escape and low DNA degradation should make PEI an efficient transfection vector. In this article we evaluate the evidence regarding the proton sponge hypothesis. We focus on PEI.

We found that PEI particles generally reach lysosomes within 3 to 6 hours after transfection. We found little evidence that identified the specific type of vesicle PEI particles escape from. Reports agree on the importance of vesicular acidification for transfection efficiency of PEI. The literature is not unequivocal about how chemical structure of polymers relates to their transfection efficiency. Two groups found a negative relationship between buffering capacity and transfection efficiency. One group found that maximally N-alkylating PEI led to an decreased vesicular pH environment and to a loss of transfection efficiency. Increasing the buffering capacity of PDMAEMA, a similar polymeric transfection vector with buffering capacity in the pH range 7.5 to 5, did not lead to increased transfection efficiency. Many studies measured intracellular pH environment of particles. Most studies found that the pH environment for PEI particles was roughly 1 point higher than for PLL particles. One study found the opposite. A high pH environment can be caused by buffering, but also by escape into the cytosol. No buffering of lysosomes was found.

From the accumulated evidence we cannot conclude that a proton sponge mechanism is the important factor responsible for high vesicular escape of PEI particles.

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1. INTRODUCTION

1.1 Gene therapy

Gene therapy is the insertion, alteration or removal of genetic material from an individual's cells in order to treat diseases caused by defective genes. The potential for gene therapy to treat diseases is great, yet no satisfying technique has been devised.

Viruses have the natural ability to insert genetic material into cells and therefore can be used as a vector for gene therapy. This been done with very limited success. In 2000, a French group cured three people with severe combined

immunodeficiency-X1 using gene therapy (Cavazzana-Calvo 2000). Unfortunately three years later two of these patients developed leukaemia (Hacein-Bey-Abina 2003). In 2008, in London three young adults were treated with gene therapy for Leber's congenital amaurosis, an eye disease (Bainbridge 2008). One of them showed improvements in visual functioning. Although viruses are highly efficient at delivering DNA, there are drawbacks: inducing mutations, inciting an immune response, and limitations with respect to scale-up procedures (Mintzer 2009).

Non-viral methods for gene therapy have been investigated for over two decades. The term used for non–viral in vitro alteration of genetic material in eukaryotic cells is "transfection". Low transfection efficiency, compared to viral vectors, is the major problem with non-viral vectors. Viruses are complex protein assemblages that have evolved over millions of year to be able to insert genetic material into cells. In contrast, most non-viral vectors are simple molecules that have to do the same complex job. Yet their relative safety and potential for large-scale production makes them attractive for gene therapy (Mintzer 2009).

1.2 Polyplexes

There are many types of non-viral transfection vectors and one of these are polyplexes. A polyplex is a complex of polymer and DNA. Polymers are chemical compounds consisting of repeating structural units usually with large molecular mass. (For example, DNA is a polymer of nucleotides.) Polymers used for transfection are positively charged and have a molecular mass between 1,000 and 100,000 Da. Both cationic polymers and anionic DNA are large spread-out molecules, but when added together the electrostatic interaction between the two, makes them condense into a much smaller, roughly spherical particle.

Positive charges in transfection polymers are generally carried by nitrogen atoms; negative charges in DNA are carried by phosphate groups. The ratio in which polymer and DNA are added together to create a polyplex is sometimes expressed as a nitrogen to phosphate ratio (N:P ratio) and sometimes as a polymer:DNA weight ratio.

1.3 In vitro barriers to transfection

This article will only concern itself with barriers that are relevant to in vitro transfection. First, polyplexes have to bind cell membranes. Positively charged polyplexes can be made by combining polymer with DNA in a ratio at which the positive charges outweigh the negative charges. The attraction between the cationic particles and anionic glycoaminoglycans on the exterior of cell membranes binds particles to the plasma membrane (Mintzer 2009).

Secondly, polyplexes have to gain access to the cell. This generally occurs by endocytosis: the invagination and pinching-off of endocytic vesicles from the plasma membrane. There are different endocytic entry routes into the cell. Some of these routes are specifically triggered events (phagocytosis), while others are continuously occurring processes (pinocytosis).

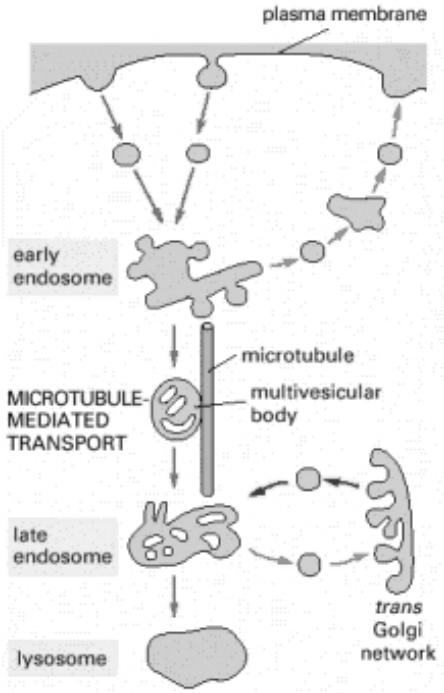

Figure 1, Alberts 2002: The endolysosomal system.

The third barrier is escaping the endolysosomal system (Figure 1). Endocytic vesicles fuse with the first intracellular sorting station, the early endosome, a mildly acidic vesicle with a pH of ~6 (Mellman 1986). From here most membrane proteins are returned to the plasma membrane via recycling vesicles, whereas membrane proteins destined for destruction are accumulated in internal vesicles. Through these vesicle fusion and fission events, early endosomes transform into late endosomes (with multivesicular bodies as an intermediate). During maturation, endosomes are acidified by the action of H^+ ATPases. Eventually, late endosomes fuse with lysosomes, strongly acidic vesicles with a pH of ~5 (Mellman 1986) filled with degradative enzymes (among which nucleases). Here the contents of the

endosomes are degraded (Van Meel 2008, Alberts 2002). At some point polyplexes have to escape the endolysosomal system into the cytosol in order not to be degraded.

Finally, to get access to the transcriptional machinery the DNA has to get inside the nucleus. (There are forms of gene therapy, e.g. with siRNA, that do not require nuclear entry.) Diffusion of free DNA in the cytosol is negligible, thus polyplexes have to aid in transporting the DNA to the nucleus. There are two ways for DNA to get into the nucleus: through the nuclear pore complex or after mitosis, when the nuclear envelop is reconstructed around the chromosomes. Whether the later is a viable option depends on the division rate of the cell. (Mintzer 2009).

1.4 Polyethylenimine and the proton sponge hypothesis

In 1995, Boussif et al. suggested polyethylenimine (PEI) as a transfection vector. They had noticed that two cationic polymers (polyamidoamine and lipopolyamines) were effective vectors and that, although very different in structure, the two shared a feature: both contain amine groups which are not protonated at physiological pH. This means both have buffering capacity below physiological pH. Boussif et al. wondered whether there was a causal relation between the buffering capacity and transfection efficiency. In a chemical catalogue they found an ideal candidate to test this hypothesis, innocuous and with a high buffering potential: polyethylenimine. They showed, both in vitro and in vivo, that PEI is an effective transfection agent.

Boussif et al. formulated a hypothesis that would explain for the high transfection efficiency of polyplexes with buffering capacity. Unprotonated nitrogen atoms in the polymer would buffer the acidification of endosomes by proton pumps. This buffering should have the following effects. 1) Lysosomal nucleases are less effective at higher pH values. 2) Less acidification should lead to continued activity of the proton pumps. Water and chloride ions follow the protons into the endosomes to keep osmotic and charge balance. 3) The positive charges accumulating in the polymeric network would lead to internal charge repulsion and expansion of the polymeric network (Kokofuta 1974). The first effect would keep the genetic material safe from digestion. The latter two effects would both increase the volume inside the endosomes leading to rupture of the endosomes. Efficient endosomal escape would lead to high transfection efficiency. (Boussif 1995, Behr 1997, Demeneix 1997). Because such a polymer would suck up protons and swell in the act, the hypothesis was dubbed the proton sponge hypothesis.

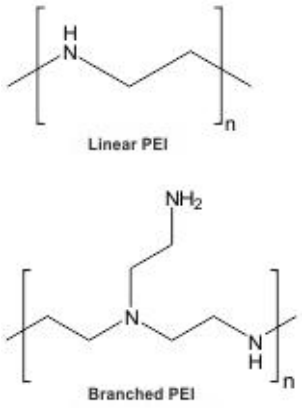

Figure 2: Chemical structure of linear and branched PEI.

PEI exists in two forms: a branched and a linear form (Figure 2). Branched PEI (bPEI) is the most used PEI variant and the only one discussed in this article.

1.5 Buffering properties of polyethylenimine

Branched PEI contains three types of reactive functional groups: primary (1°), secondary (2°) and tertiary (3°) amine groups (Figure 3). The different classes of amines are named after the number of side chains, that is non-hydrogen atoms, attached to the nitrogen atom.

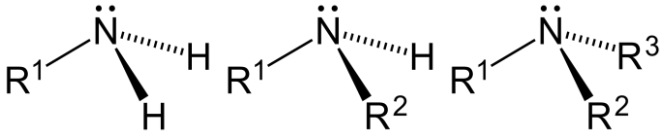

Figure 3: Structure of different classes of amines. Side chains are indicated with R^x. From left to right: a primary, a secondary, a tertiary amine group.

The two dots above the nitrogen atom symbolize a free electron pair. A free electron pair can bind a proton and therefore amine groups are weak bases. The ease with which the different classes of amine groups bind protons (their basicity), is reflected in the pK_a value of their conjugate acid. The pK_a is the pH value at with 50% of the nitrogen atoms has bound a proton. With increasing proton concentration, that is decreasing pH (because pH = -log[H^+]), more amine groups will have a proton bound. The higher the pK_a value (of the conjugate acid) the stronger the base.

The pK_a is important for the buffer capacity at a particular pH. At a pH much higher than the pK_a no free electron pair will bind a proton; at a pH much lower than the pK_a all free electron pairs will

have a proton bound. The pK_a value is the pH value at which the buffering capacity is to be expected to be highest. For amine groups with only hydrocarbon side chains in water applies: the pK_a of primary and secondary amines is ~10.5 (secondary amines are slightly stronger bases) and that of isolated tertiary amines is ~9.5 (Bruice 2006). In PEI molecules, the side chains are not solely hydrocarbon; in close vicinity to each amine groups is another amine group. The protonation state of one amine group can influence the pK_a of its neighbours.

While originally the ratio of 1°:2°:3° amine groups was thought to be 1:2:1, Von Harpe et al. (2000) showed by NMR that the ratio in commercially available bPEI is closer to 1:1:1. The bPEI they synthesized themselves did have a 1:2:1 ratio. From titration curves they calculated buffer capacity (Figure 4).

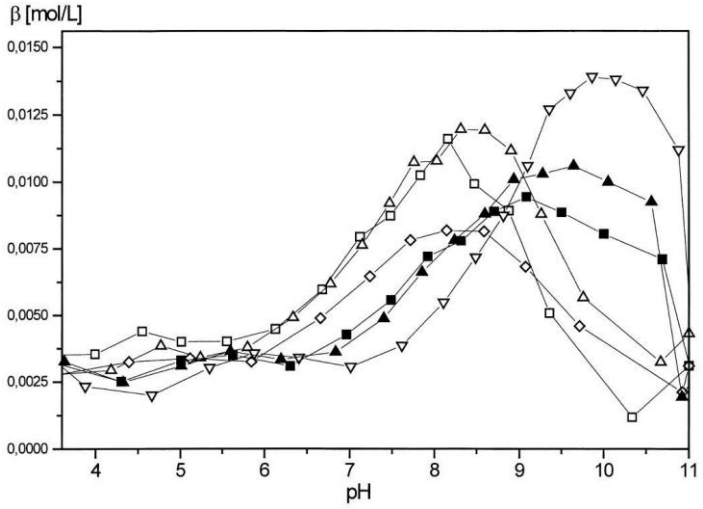

Figure 4, Von Harpe 2000: Buffer capacities (β) of different PEIs in solution (0.1165 mol N/l) as function of pH. PEI 1 (□), PEI 2 (◇) and PEI 3 (△) are commercially available and have an approximated 1°:2°:3° ratio of 1:1:1. PEI 4 (▽) is also commercially available, but has a ratio of approximately 4:3:3. PEI 5 (■) and PEI 6 (▲) are synthesized by Von Harpe et al. and have an approximated 1°:2°:3° ratio of 1:2:1. Buffer capacities were calculated from potentiometric titration curves.

Von Harpe et al. show that for all PEIs, the area of high buffer capacity lies above the physiological pH range, typically between 8 and 9.5 and the differences between the PEIs in the pH range from 7.5 to 4.5 are relatively small. But comparing the PEIs with a 1:1:1 ratio of 1°:2°:3° amine groups, with PEIs with a 1:2:1 ratio, there is a difference in buffering in the range from 7.5 to 6 (Figure 4). The PEIs with relatively more secondary amines have less buffer capacity in this region. The PEI tested with an abnormally high amount of primary amine groups (marked ▽ in Figure 4) has a particularly high pK_a. From this one might carefully conclude that, in accordance with the theory of basicity of amine groups, the tertiary amine groups in PEI have the highest buffering capacity at physiological pH.

1.6 Goal of this article

Since Boussif et al. formulated the proton sponge hypothesis, significant progress has been made in the field of non-viral gene therapy, but no review article has been devoted to evaluating the evidence regarding the proton sponge hypothesis accumulated since 1995. The proton sponge hypothesis should hold true for all polymers with buffering capacity below physiological pH. In this article we will mainly look at PEI.

The most important issue we hope to solve is whether the buffering capacity of PEI is the most important factor in endosomal escape of PEI particles. Interwoven with this question is the question from which specific vesicular compartment the escape takes place: from early endosomes, late endosomes or lysosomes. And that is where we will start.

2. TRACKING PEI PARTICLES INTRACELLULARLY

3.1 Confocal microscopy

To follow the intracellular path of 25kDa bPEI particles Godbey et al. (1999) transfected EA.hy 926 cells (hybrids between human lung carcinoma cells and human umbilical vein endothelial cells) and followed the intracellular movement of the particles by confocal microscopy in live cells. Either PEI was labelled with Oregon Green 488 or the plasmid with rhodamine or both. Incubation time was 2 hours.

In all cases they saw the same things happening (Figure 5): "By 30 minutes after transfection, PEI/DNA complexes began to attach to cell surfaces and form aggregates. Endocytosis of the complexes was common at 2-3 hours posttransfection, and nuclear localization of PEI or PEI/DNA complexes was relatively common by 3.5-4.5 hours after administration to cells. The earliest localization of complexes within nuclei preceded the earliest observed transgene expression by 1 hour."

Godbey et al. (1999) saw some DNA separating from its carrier, but predominantly DNA was colocalized with PEI. Occasionally some of the endocytic vesicles showed signs of disruption beginning 4 hours posttransfection. The particles then could freely disperse relatively homogeneously in the cytoplasm (Figure 5d, Figure 6c).

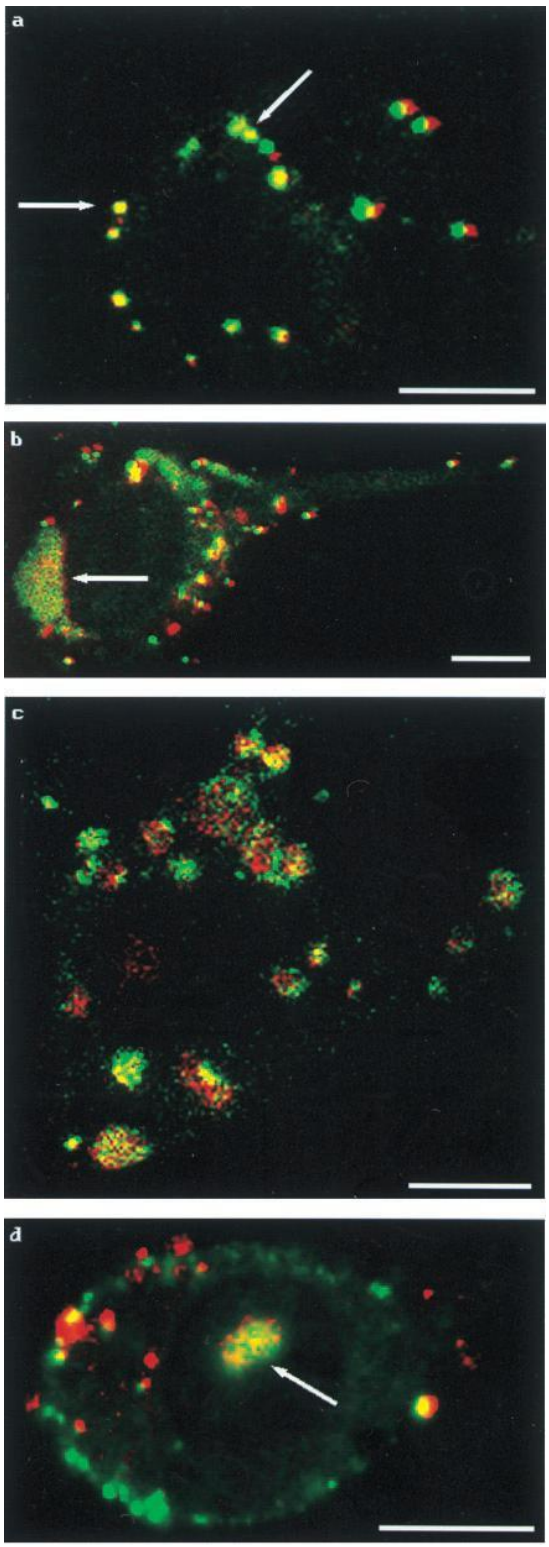

Figure 5, Godbey 1999: Tracking of double-labelled PEI/DNA complexes (PEI, green; DNA, red). A: 2 hours posttransfection, visible complexes appear as clumps on the cell's exterior, as indicated by arrows. B: 3 hours posttransfection, both surface aggregation and endosomes are visible. The arrow indicates endosomal formation. C: 4 hours posttransfection, endosomes containing both PEI and DNA are visible throughout the cell cytoplasm. D: At 4.5 hours posttransfection, fluorescent structures containing both PEI and DNA inside the cell nucleus are present, as indicated by the arrow. (Bar = 10 μm.)

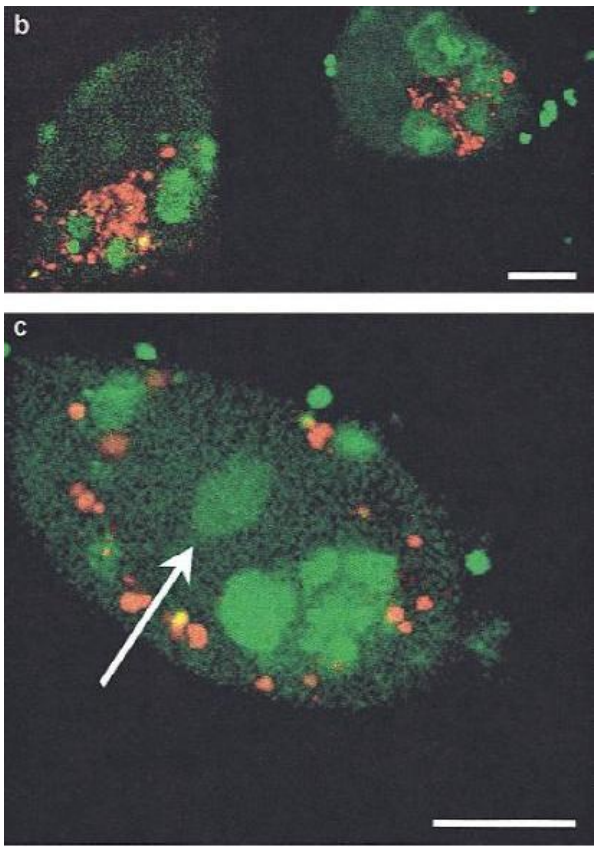

Figure 6, Godbey 2000 (edited): Tracking of labelled PEI/DNA complexes (green) in cells with stained lysosomes (red). Yellow colour indicates close proximity of the green and red fluorophores and therefore complex/lysosome interaction. Each bar indicates 10μm. B: 3 hours posttransfection, PEI/DNA complexes are located in endocytic vesicles and are somewhat surrounded by lysosomes. There is no overlap of PEI and lysosomal probes; C: 5 hours posttransfection, PEI/DNA complexes have entered the nucleus (shown by arrow) while cytoplasmic vesicles containing PEI/DNA complexes still have not fused with lysosomes.

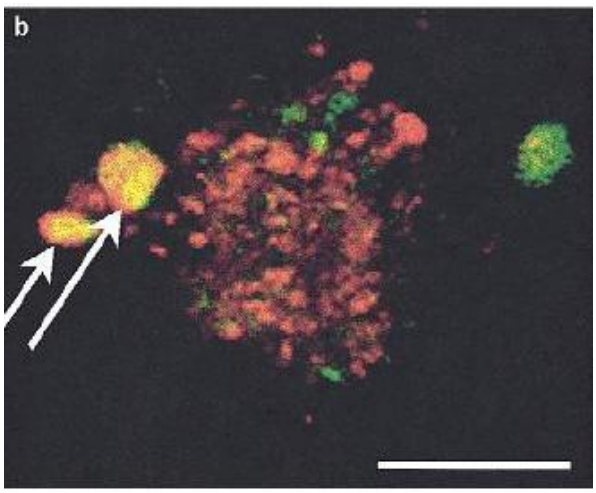

Figure 7, Godbey 2000 (edited): Tracking of labelled PLL/DNA complexes (green) in cells with stained lysosomes (red). Otherwise similar to Figure 6. 4 hours posttransfection, after the PLL/DNA complexes enter the cytoplasm, they are seen to meet up with lysosomes. These endolysosomes are visible as yellow and are denoted by arrows.

In their follow-up study, Godbey et al. (2000) tested whether PEI containing vesicles fuse with lysosomes in live EA.hy 926 cells. Lysosomes were stained with LysoTracker Red, a lysosomotropic fluorophore. Lysosomotropic agents accumulate in acidic compartments (late endosomes and lysosomes), because the charges they accumulate at low pH prevent them from crossing the membrane (Mellman 1986). PEI was labelled with Oregon Green 488. No fusion between PEI-containing endosomes and lysosomes was observed (Figure 6). In contrast, poly-L-lysine (PLL, a non-protonable polymer) particles were seen to fuse with lysosomes 4 to 5 hours after transfection (Figure 7).

This experiment by Godbey et al. (2000) has been repeated by other groups with identical setups (25kDa bPEI, DNA labelled with Oregon Green 488 and lysosomes stained with Lysotracker), but with different results. Colocalization of DNA with lysosomes 1 to 2 hours after transfection was reported in SW-13 cells (human adrenal cortex carcinoma) and NIH:OVCAR-3 cells (human ovary carcinoma) by Merdan et al. (2002), in COS-1 cells (monkey kidney fibroblasts) by Dubruel et al. (2004), in COS-7 cells by Suh et al. (2012), and in a primary neuron cell culture by Suk et al. (2007). On the other hand, Bieber et al. (2002) reported the absence of colocalization in PaTu 8902 cells (human pancreatic carcinoma) (Figure 8A).

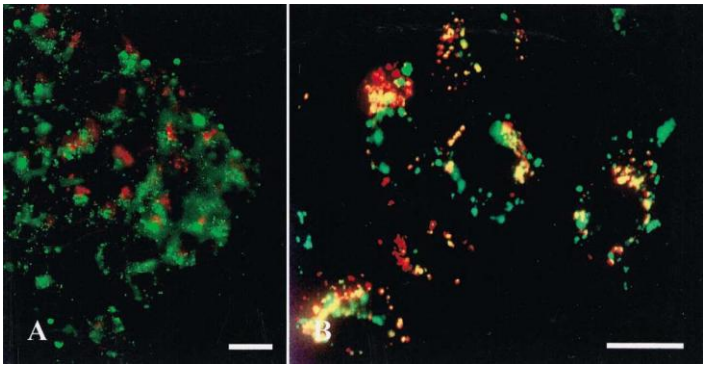

Figure 8, Bieber 2002: Intracellular localization of PEI–DNA complexes 18 h after transfection of PaTu 8902 cells. A: When cells were counterstained before fixation with the lysosomotropic agent LysoTracker Red (red), the PEI–DNA complexes (green) did not colocalize with the lysosomal marker. Bar: 25 mm. B: In contrast, lysosomal staining using an antibody against LAMP1 (red) led to a significant colocalization with the labelled PEI (green). Bar: 15 µm.

Bieber et al. suggested that buffering by PEI could negate the lysosomotropic properties of Lysotracker, thereby leading to false negative results. Bieber et al. (2002) tried another technique to test colocalization of PEI particles and lysosomes. PaTu 8902 cells were incubated for 6 hours with particles of Oregon Green labelled 25 kDa bPEI complexed to a green fluorescent protein expression plasmid (pGFP). After 18 hours incubation the cells were fixated with paraformaldehyde and after permeabilization counterstained with an antibody against LAMP1, lysosomal-associated membrane protein 1, which can found in late endosomes and lysosoems. Bieber et al. did find colocalization of PEI and LAMP1 (Figure 8B).

Suh et al. (2012) investigated the localization of PEI particles after transfection. Goat bone marrow mesenchymal stem cells were pretransfected by electroporation with one of two plasmids: 1) early endosome antigen 1 fused to green fluorescent protein (EEA1-GFP); or 2) Niemann-Pick disease (type C1) protein fused to GFP (NPC1-GFP). EEA1 is a marker for early endosomes. NPC1 has been shown to localize to late endosomes and lysosomes and to colocalize with Lysotracker and LAMP1. Particles were made of 25kDa bPEI labelled with Oregon Green 488 and salmon DNA. Suh et al. quantized the colocalization with a pixel analysis. Their results clearly showed that PEI/DNA particles are first associated with early endosomes and later with late endosomes and lysosomes (Figure 9).

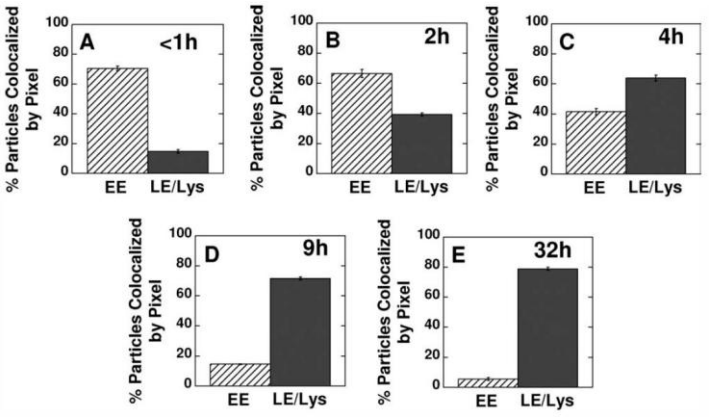

Figure 9, Suh 2012: Per pixel co-localization of PEI/DNA complexes with either early endosomes (EE) or late endosomes/lysosomes (LE/Lys) at various times post-transfection in mesenchymal stem cells. Cells were pre-transfected with GFP fusion genes of EEA1 for EE or NPC1 for LE/Lys. Five cells from each condition were imaged.

3.2 Electron microscopy

Bieber et al. (2002) showed electron dense particles in lysosomes of PaTu 8902 cells after transfection with 25kDa bPEI particles (Figure 10). Furthermore they saw ruptured lysosomes in the same cells.

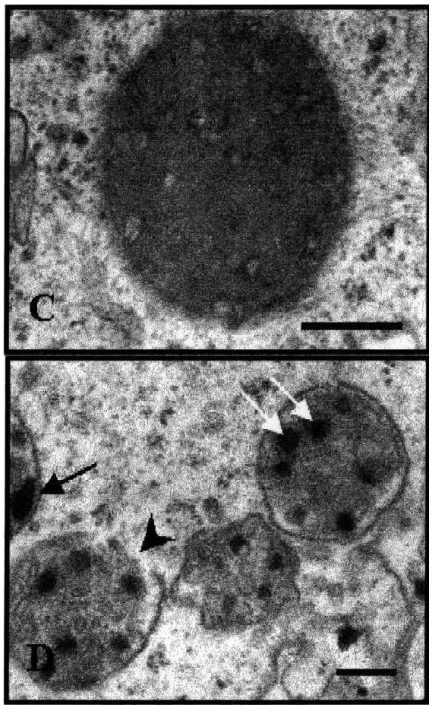

Figure 10, Bieber 2002: D: electron dense material can be observed in lamellar type lysosomes (white arrows) of PaTu 8902 cells after treated with PEI–DNA complexes. Some PEI aggregates were attached to the inner side of the lysosomal membranes (black arrow). Most lysosomes containing PEI exhibited membrane disruptions (arrowhead). C: Mock-transfected cells lacked electron dense spots and were delineated by an intact membrane.

3.3 With centrifuge techniques

Bieber et al. (2002) fractionated cells by equilibrium centrifugation in the presence of monodansylpentane. Monodansylpentane is a lysosomotropic fluorophore that increases its yield in a hydrophobic environment. The whirl-like membrane structure of lamellar bodies provides the necessary hydrophobicity to increase dansyl fluorescent. Monodansylpentane is thus a fluorescent marker for lamellar bodies (Nieman 2001). Bieber el al. transfected PaTu 8902 cells with 25 kDa bPEI particles for 6 hours.

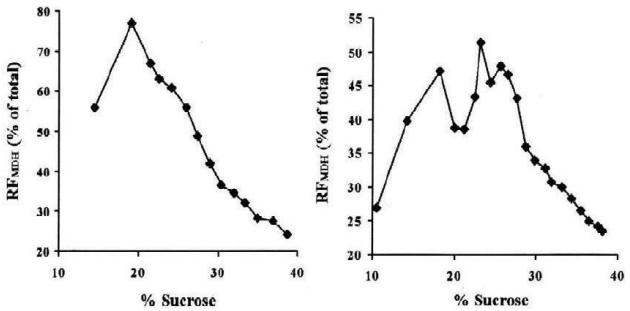

Figure 11, Bieber 2002: Cells were transfected for 6 h with PEI–DNA and lamellar type lysosomes were labelled using MDP. Cell fractionation analysis showed a partial shift of lysosomes to higher sucrose concentrations (B), compared with untransfected cells (A).

Their results show that after transfection the dansyl fluorescence was present in a higher sucrose density (Figure 11). This means that transfection with PEI made lamellar bodies heavier.

Lecocq et al. (2000) injected rats intravenously with ^{125}I-tyramine cellobiose-PEI (^{125}I-TC-PEI) without a DNA component. They sacrificed the rats at different time points after injection; homogenized the livers (the organ with the highest PEI concentration) and separated cell fractions by differential centrifugation. At 5 minutes after transfection they recovered the radioactivity largely in the microsomal fraction, like alkaline phosphodiesterase a protein found in the plasma membrane and in pinocytic vesicles. At 4 hours the radioactivity was localized in the mitochondrial fraction, like the lysosomal enzyme cathepsin C (Figure 12). The distribution did not change significantly after 4 hours.

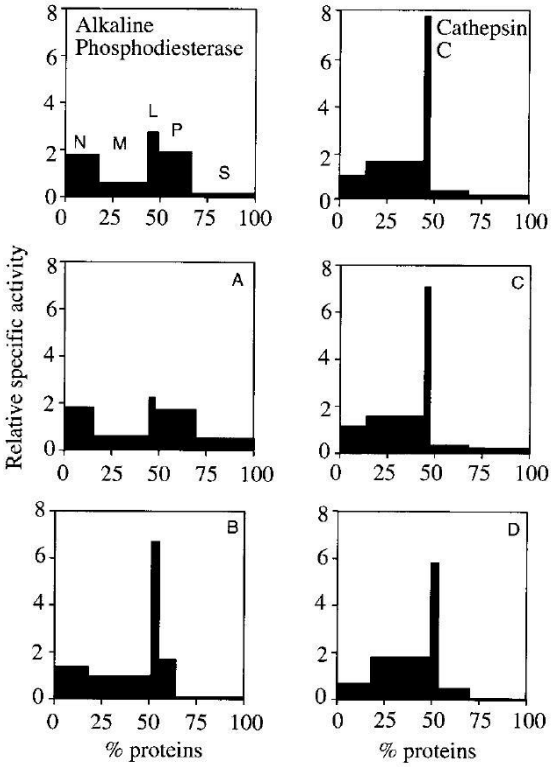

Figure 12, Lecocq 2000: Distribution of radioactivity after differential centrifugation. The distributions were obtained from livers of rats killed 5 min (A), 4 h (B), 18 h (C) and 4 days (D) after ^{125}I-TC-PEI injection. For the sake of comparison, representative distributions of a plasma membrane enzyme, alkaline phosphodiesterase, and of a lysosomal enzyme, cathepsin C are given. N: nuclear fraction; M: heavy mitochondrial fraction; L: light mitochondrial fraction; P: microsomal fraction; S: soluble fraction. Ordinate: relative specific activity (percentage of total recovered radioactivity/percentage of total recovered proteins); abscissa: relative protein content of fractions (cumulatively from left to right).

To prove that PEI was localized in lysosomes they centrifuged the total mitochondrial fraction in a density gradient both with and without Triton WR1339. Triton is a non-ionic detergent of low density that is able to specifically decrease the density of lysosomes. Without the Triton WR1339 treatment, the radioactivity and the cathepsin C were found in the same density region of the gradient. With treatment both the radioactivity and the cathepsin C shifted to a lower density (Figure 13).

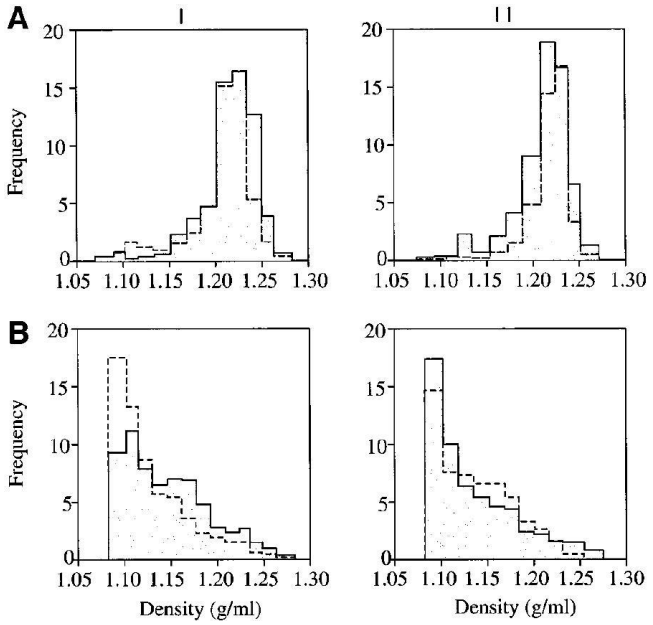

Figure 13, Lecocq 2000: Density distribution histograms of radioactivity (unbroken line) and of cathepsin C (broken line) after isopycnic centrifugation of a total mitochondrial fraction (M+L) in a sucrose gradient. The mitochondrial fractions were isolated 4 h (I) and 18 h (II) after ^{125}I-TC-PEI injection. The animals in A were or in B were not injected intraperitoneally with Triton WR-1339 4 days before receiving ^{125}I-TC-PEI injection.

3.4 Discussion & conclusions

The intracellular fate of PEI particles has been extensively studied with a variety of techniques in a variety of cells. Colocalization op PEI particles with the lysosomotropic dye LysoTracker is frequently repeated with nearly identical setup, but with different results. Some studies found colocalization (Merdan 2002, Suh 2012, Suk 2007, Dubruel 2004) and others did not (Godbey 2000, Bieber 2002). The cell types that did not show Lysotracker colocalization of PEI particles were epithelioid, but so were many of the cell lines that did show colocalization.

Bieber et al. did not find colocalization of PEI particles with LysoTracker in live PaTu 8902 cells, but did find colocalization of PEI particles with LAMP1 in fixated cells, while LysoTracker and LAMP1 are reported to colocalize themselves. Based on these results Bieber et al. suggested that buffering by PEI could prevent lysosomotropic agents from acquiring charges, thereby negate the lysosomotropic property of Lysotracker and lead to false negative results.

Colocalization with LysoTracker, LAMP1 or NPC1 does not differentiate between late endosomes and lysosomes. The electron microscopic images of Bieber et al. (2002) showed PEI particles inside lysosomes, but also disrupted lysosomal membranes. The centrifuge experiments by Bieber et al. (2002) and Lecocq et al. (2000) place PEI particles specifically in lysosomes aswell.

PEI particles can have different intracellular fates in different cell types. Endosomes containing PEI particles can mature to lysosomes in one cell line, but can be transcytosed in another. In general PEI particles seem to reach lysosomes within 3 to 6 hours after transfection. This does not prove that PEI particles escape from lysosomes. Aside from the electron microscopy photos by Bieber et al. (2002) that show ruptured lysosomes, we found no evidence that identified the specific type of vesicle PEI particles escape from.

3. EFFECT OF VACUOLAR-ATPASE INHIBITORS ON TRANSFECTION EFFICIENCY

An experiment first done by Kichler et al. (2001) and later repeated by other groups with consistent results, is testing transfection efficiency of bPEI in the presence of bafilomycine A_1, a proton pump inhibitor specific for vacuolar-ATPase (V-ATPases). (Bowman 1988). V-ATPases are located in Golgi systems, endosomes, lysosomes and granules and in a number of more specific places in specific tissues. V-ATPases are responsible for the acidification of endosomes and lysosomes after endocytosis (Nelson 1991, Mellman 1986).

Hep G2 cells (human liver carcinoma) were incubated for three hours with particles in the presence of different concentrations of bafilomycin A_1. The particles were either 25kDa bPEI complexed with a luciferase expression plasmid at a N/P-ratio of 12.5 or DOTAP complexed with the same plasmid at a N/P-ratio of 4. DOTAP is a monocationic lipid with a protonation state that is not significantly altered in the pH range 5 to 7.5. Luciferase fluorescence was measured 30 to 48 hours after transfection.

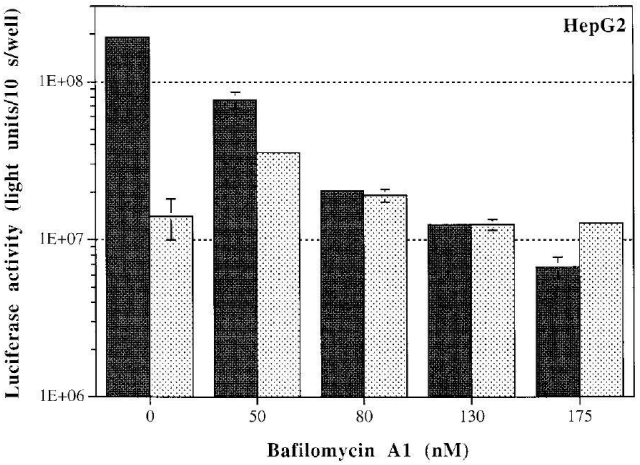

Figure 14, Kichler 2001: Effect of bafilomycin A_1 on PEI-mediated transfection efficiency. Hep G2 cells were transfected either with PEI (solid bars) or with DOTAP (stippled bars) in the presence of increasing concentrations of bafilomycin A1. Incubation lasted for 3 hours. The luciferase activity was measured 30–48 h later. The reported transfection efficiency is the mean of the duplicates.

Transfection with PEI particles became progressively less effective (up to 30 times) with increasing concentrations of bafilomycin A_1 (Figure 14). The inhibitory effect of 175 nM bafilomycin A_1 on DOTAP transfection was only 2-fold. Kichler et al. repeated this experiment with different cell lines (Rb1, rabbit aorta smooth muscle, and Hela 229, human cervix carcinoma) and another inhibitor of V-ATPases (Concanamycine A), with comparable results. Addition of bafilomycin A_1 at a later time after transfection hindered transfection substantially less: 64% reduced efficiency when adding after 2 hours and only 23% reduction when adding after 4 hours.

Merdan et al. (2002) and Akinc et al. (2005) later reproduced similar results using a similar luciferase transfection assay, comparable concentrations of bafilomycin A_1 and 25kDa bPEI, but different cell lines (SW-13 and COS-7 respectively). They both found a 200-fold reduction in transfection efficiency of PEI particles. Akinc et al. found that transfection with PLL particles decreased only 4 to 5-fold with bafilomycin A_1.

Kichler et al. tested whether bafilomycin A_1 influenced the uptake of PEI particles. They incubated Hep G2 cells for 4 hours with fluorescently labelled polyplexes, in which either the PEI (with FITC) or the DNA (with YOYO-1) was labelled, in both the presence and absence of bafilomycin A_1. Flow cytometry was used to measure the mean fluorescence. Bafilomycin A_1 did not reduce the uptake of PEI particles.

Discussion & conclusions

Different groups (Kichler 2001, Merdan 2002, Akinc 2005) proved in five different cell lines that V-ATPase inhibitors reduces the transfection efficiency of PEI with one or two orders of magnitude. Since cellular uptake of PEI particles was not decreased by bafilomycin A1, the inhibitory effect is likely to be downstream relative to cellular uptake (Kichler 2001). V-ATPase inhibitors reduced the efficiency of transfection vectors with limited buffer capacity (DOTAP and PLL) significantly less.

These results show that vesicular acidification is very important for the transfection efficiency of PEI; more so than for non-buffering transfection vectors. From these results only we cannot infer an escape mechanism. Nonetheless, these results are in line with the proton sponge hypothesis.

4. RELATIONSHIP BETWEEN CHEMICAL STRUCTURE AND TRANSFECTION EFFICIENCY OF PEI

In 2002 Thomas et al. modified the chemical structure of 25kDa bPEI to deduce a relationship between structure and transfection efficiency. First they maximally alkylated all amine-groups in PEI, that is, alkylating until the maximum of four side chains per nitrogen was reached. A nitrogen with four side chains is not an amine, but a quaternary ammonium cation (Figure 15). It has no free electron pair and thus cannot buffer.

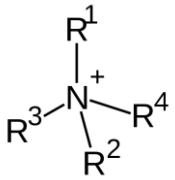

Figure 15: Quaternary ammonium cation. Side chains are indicated with Rx.

The transfection efficiency of regular PEI was compared with quaternized PEI in COS-7 cells using a β-galactosidase reporter system. Polyplexes were made by complexing PEI and N-quaternized PEI to a β-galactosidase expression plasmid at optimal N/P ratio. Cells were exposed to the polyplexes for 6 hours. After another 42 hours of incubation the amount of galactosidase fluorescence was determined.

N-quaternization reduced PEI's transfection efficiency more than 20-fold (Figure 16; compare c with d and e). Similar result using a luciferase reporter system were produced by the same group

in 2005 (Akinc 2005): maximally alkylating of PEI led to a 50-fold reduction in luciferase fluorescence.

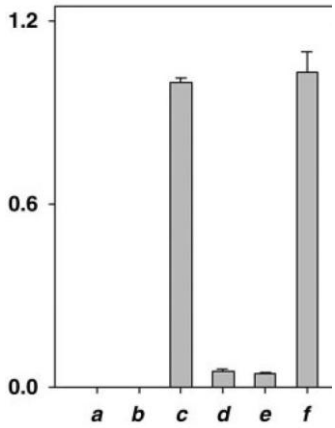

Figure 16, Thomas 2002 (edited): Expression of β-galactosidase gene in COS-7 cell after transfection with by bPEI and its derivatives in the absence of serum. A: nontransfected cells; b: cells transfected with the plasmid but without PEI; c: underivatized PEI; d: maximally methylated PEI, e: maximally ethylated PEI; f: PEI alkylated with choline. The N/P ratio was 10. All measurements were done in triplicate; the bar height represents the mean value with the SD shown.

Because the reduced transfection efficiency of N-quaternized PEI could result from the presence of quaternary ammonium cations, rather than the lack of protonable amine groups, Thomas et al. alkylated amine groups of PEI with choline under conditions which only allowed the formation of secondary and tertiary amine groups. Choline itself contains a quaternary ammonium cation. This cholinated PEI has the same amount of protonable amine groups as unmodified PEI, although only secondary and tertiary protonable amine groups. The number of quaternary ammonium cations in cholinated PEI is comparable to maximally alkylated PEI. This cholinated PEI retained its transfection efficiency (Figure 16, compare f with c, d and e), thus proving that the alkylated PEI is not less effective than regular bPEI because of the presence of quaternary ammonium cation, but because of the lack of protonable amine groups

To confirm that the decreased transfection efficiency of N-quaternized PEI particles is a result of decreased endosomal escape and not a result of an upstream effect (eg. binding to the cell membrane or internalization), Akinc at el. (2005) measured the amount of DNA internalized during a transfection assay. DNA was labelled with Cy5 and Cy5 fluorescence was measured with flow cytometry. They showed that N-quaternized PEI particles are better internalized than regular bPEI particles. Decreased internalization was not the cause of the decreased transfection efficiency of N-quaternized PEI particles.

Akinc et al. (2005) also excluded downstream effects. Decreased transfection of quaternized-PEI particles could result from impaired vector unpackaging. N-quaternized PEI is stronger positively charged and a stronger electrostatic interaction with negatively charged DNA could lead to more stable particles. A salt titration, progressively adding more salt to a solution of the different particles, in the presence of PicoGreen was done. PicoGreen is fluorophore that increase its fluorescence upon intercalating with DNA. Condensation of DNA with polycations results in exclusion of PicoGreen and hence to reduced fluorescence. Adding salt neutralizes the attraction between the opposite charges of PEI and DNA, inhibits condensation of DNA and increases PicoGreen fluorescence. The DNA condensation of N-quaternized PEI was inhibited at a lower salt concentration than that of regular bPEI. Thus, increased particle stability due to strong electrostatic interaction, could not account for the decreased transfection efficiency of N-quaternized PEI particles.

In two articles by the same group (Forrest 2004 and Gabrielson 2006) the buffering capacity of 25kDa bPEI is abolished by acetylating the amine groups. Reacting PEI with acetic anhydride in different molar ratios converts part of the amine groups to amide groups. Compared to amines, amides are very weak bases. The conjugate acid of an amide has a pKa around -0.5 (Bruice 2006). Therefore acetylated PEI is expected to have a lower buffering capacity in the physiological pH range. PEI derivatives were referred to as PEI-Ac_x, where x is the percentage of primary amines converted to amides. In Forrest 2004 the group uses PEI-Ac_{15}, PEI-Ac_{27} and PEI-Ac_{43}. In Gabrielson 2006 the group uses more extensively acetylated PEI derivatives (PEI-Ac_{34}, PEI-Ac_{57}, PEI-Ac_{76}, PEI-Ac_{96} and PEI-Ac_{100}). In the later paper the group probes the mechanism between observed relationship between acetylation of PEI and transfection efficiency of PEI. The results of these two studies are well in line with each other and we will discuss them simultaneously.

In Forrest 2004 buffering capacity of polymers is determined by titration and expressed as a "physiological buffering capacity", defined as the change in protonation per amine between pH 7.5 and 4.5. PEI exhibited a physiological buffering capacity of 0.29 mol H^+/mol N. The acetylated derivatives, PEI-Ac_{15}, PEI-Ac_{27}, and PEI-Ac_{43} exhibited a physiological buffering capacity of 0.17, 0.12 and 0.09 mol H^+/mol N, respectively.

In Gabrielson 2006 the buffering capacity is also determined by titration, but expressed as the reciprocal slope of the titration plots over the pH range from 4 to 10. PEI exhibited a slope of 6.7 µL of 1 M HCl/pH unit. An increase in the degree of acetylation resulted in a reduction of the buffering capacity. Slopes of 4.3, 3.8, 3.1, 2.2 and 1.8 µL of 1 M HCl/pH unit were measured for PEI-Ac_{34}, PEI-Ac_{57}, PEI-Ac_{76}, PEI-Ac_{96} and PEI-Ac_{100}, respectively (Figure 17).

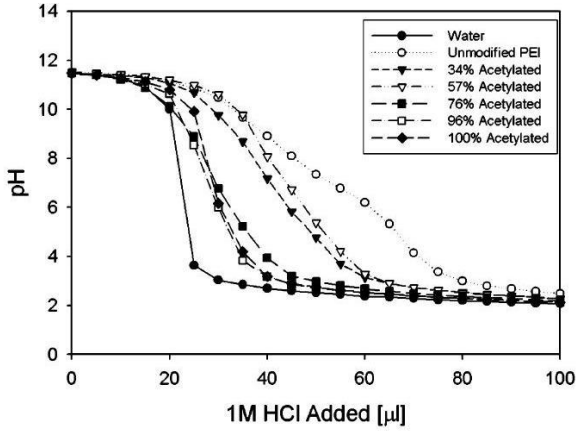

Figure 17, Gabrielson 2006: Titration of aqueous polymer solutions (0.4 mg/mL) with 1 M HCl from pH 11.5 to 2. Solutions were adjusted to pH 11.5 with 1 M NaOH, and 5 µL aliquots of 1 M HCl were subsequently added and the pH measured.

Polyplexes were made by complexing polymer to the luciferase plasmid (pGL3) in weight ratios varying from 1:1 to 6:1. Luciferase activity was measured 24 hours after transfection and normalized to total cell protein. Each transfection experiment was repeated at least 3 times. Three cell lines were used: C2C12 (mouse myoblasts), MDA-MB-231 (human mammary carcinoma) and HEK-293 (human embryonic kidney).

In all three cell lines, efficiency of gene delivery increased with the degree of acetylation up to 57% acetylation, but decreased with further acetylation (Figure 19 and Figure 18, note that only the results of C2C12 cells are shown here). Unmodified PEI was most efficient at a 2:1 PEI:DNA weight ratio. The derivatives PEI-Ac_{15} to PEI-Ac_{57} were most effective at 3:1 ratio. The transfection efficiency of PEI-Ac_{57} (3:1) was approximately 30-, 47- and 58-fold higher than unmodified PEI (2:1) in C2C12, MDA-MB-231 and HEK-293 cells, respectively. At higher degrees of acetylation, gene delivery efficiency decreased, but the optimal polymer/DNA ratio increased.

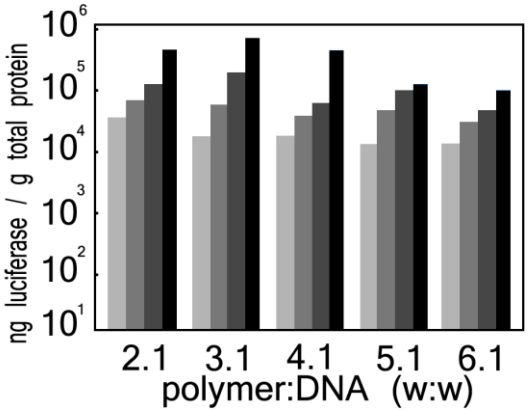

Figure 18, Forrest 2004 (redrawn): In vitro transfection efficiency. Polyplexes of plasmid DNA (pGL3) with unmodified or acetylated PEI were used to transfect C2C12 cells. Luciferase activity in the cell lysates is normalized by the mass of total protein in the lysate. Bars represent from left to right: unmodified 25-kDa PEI, PEI-Ac_{15}, PEI-Ac_{27} and PEIAc_{43}. ($n \geq 3$, error bars represent standard deviation).

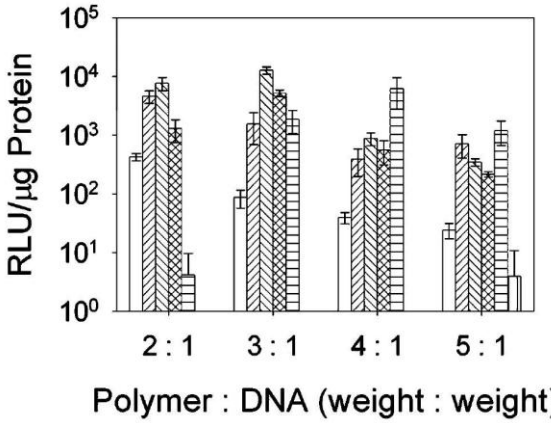

Figure 19, Gabrielson 2006: Identical to Figure 18. Bars represent from left to right: unmodified 25kDa-PEI, PEI-Ac_{34}, PEI-Ac_{57}, PEI-Ac_{76}, PEI-Ac_{96} and PEI-Ac_{100}.

In order to be expressed, DNA has to dissociate from its vector intracellular. This is called particle unpackaging. DNA bound to PEI can be displaced by adding heparan sulphate. The amount of heparan sulfate required to displace the DNA depends on the binding strength between DNA and PEI and is used as an estimate of the ease of intracellular particle unpacking. Gabrielson et al. (2006) tested the different PEI derivatives at polymer:DNA ratio optimal for transfection and found that as the degree of acetylation increases, less heparan sulfate is required to displace the DNA from PEI.

This result suggests better particle unpacking might increase the transfection efficiency of acetylated PEI. To test this Gabrielson et al. use fluorescence resonance energy transfer (FRET). A donor fluorophore, in this case Alexa Fluor 488 (AF488), in excited state can transfer energy to an acceptor fluorophore, here 5,6-

tetramethylrhodamine (TAMRA), causing the acceptor to emit light instead of the donor. The efficiency of the energy transfer is inversely proportional to the sixth power of the distance between donor and acceptor. The FRET signal (AF488:TAMRA ratio) is positively related to the distance between the two fluorophores. Two batches of PEI are labelled with either AF488 or TAMRA and subsequently mixed. Free in solution, individual chains will repel each other, there will be little energy transfer and a high FRET signal. When multiple chains are complexed in a particle, there will be more energy transfer and a low FRET signal.

MDA-MB-231 cells were transfected with double labelled PEI and the FRET signal was determined from confocal fluorescence micrographs at 0.5, 1, 2 and 4 hours posttransfection. The FRET signals were normalized to the value measured at 30 min.

Within the 4 hour measuring frame, unmodified PEI polyplexes show no increase in FRET signal, suggesting no significant unpacking. PEI-Ac_{57} and PEI-Ac_{76} particles show significant increase in FRET signal. The maximum difference in FRET signal registered was 1 hour posttransfection, the ratio for PEI-Ac_{76} was a factor 4 higher than that of unmodified PEI. There might be a relation between the degree of PEI acetylation and the relative rate and extent of unpackaging inside cells accounting for the difference in transfection efficiency.

Secondly, the uptake of different PEI derivatives was quantified by flow cytometry (Gabrielson 2006). DNA was fluorescently labelled with YOYO-1. For each cell line, unmodified PEI is endocytosed less than its acetylated counterparts. Thus increased cellular uptake is partially responsible for the increased transfection efficiency of acetylated PEI. Uptake of acetylated PEI compared to unmodified PEI increases by a factor 3 at most, whereas transfection efficiency increases between 30- and 58-fold.

Fischer et al. (2002) synthesized a series of five PEI derivatives by copolymerization of aziridine and N-(2-hydroxyethyl)-aziridine. Polymerization of pure aziridine leads to bPEI with a ratio of 1°:2°:3° amine groups of approximately 1:2:1. Polymerization of pure N-(2-hydroxyethyl)-aziridine produces poly(N-(2-hydroxyethyl)-ethylene imine) (PHEEI), a molecule with only 3°-amine groups. Co-polymerization of the two in five different molar ratios gives five poly[(ethylene imine)-co-N-(2-hydroxyethyl-ethylene imine)] (PEIHEEI) polymers with different ratios of 1°:2°:3° amine groups. Fischer et al. used this series to determine the influence of polymer structure on transfection efficiency.

Buffering capacity was measured in triplicate by titration of HCl. Results show that polymers with a high amount of EI residues buffer maximally at pH 9.5; polymers with a high amount of HEEI residues buffer maximally at pH 8 (Figure 20). This is to be expected because HEEI residues contain only tertiary amines with a lower pK_a.

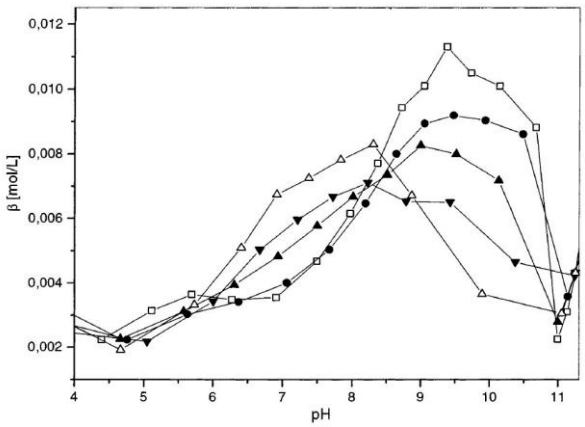

Figure 20, Fischer 2002: Buffer capacities (β) of PEI and PEIHEEI copolymers in solution as a function of pH (△ LMW-HEEI 0/100; ▼ PEIHEEI 7/93; ▲ PEIHEEI 44/56; ● PEIHEEI 76/24; □ PEI 100/0). The titration analysis showed an increase of pKa value and buffer capacity with increasing EI content.

Transfection experiments were done in triplicate on NIH/3T3 cells (mouse embryonic fibroblast). Particles were formed by complexing polymers to pGL3 plasmid. Efficiency was determined by measuring luciferase activity. Result show that a higher amount of EI residues correlates with a higher transfection efficiency (Figure 21).

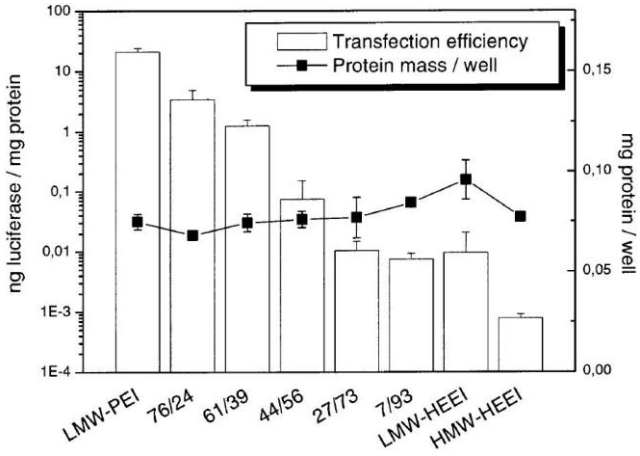

Figure 21, Fischer 2002: Transfection efficiency in and protein concentration in cell lysates. Gene expression decreased with increasing HEEI content of the polymers. Protein concentration in cell lysates was very similar for all polymers tested.

These experiments show that an increase in buffer capacity in the range 5 to 7.5 (the pH range within the endolysosomal system) is not associated with an increase in transfection efficiency.

Discussion & conclusions

Thomas et al. (2002) found that maximally alkylating the amine groups in PEI, reduced its transfection efficiency 20-fold. They also proved that incorporation of quaternary ammonium cations in the polymer does not by itself hinder transfection. They did not measure the buffering capacity of N-quaternized PEI. Chemical analysis of their N-quaternized PEI showed that the quaternization was incomplete (77% and 88%). Their N-quaternized PEI therefore should have some buffer capacity left. In what pH range it can buffer depends on the nature of the remaining amine groups. In Akinc et al. (2005) the pH environment of N-quaternized PEI particles was measured and these results suggest that the remaining buffering is relevant (see below).

Akinc et al. (2005) proved that neither decreased internalization nor increased DNA binding strength caused the decreased transfection efficiency of N-quaternized PEI. Akinc et al. did not measure endosomal escape in a direct way. In

Forrest et al. (2004) and Gabrielson et al. (2006) found in three cell lines that progressively increasing the degree of acetylation of PEI decreased buffer capacity, but increased transfection efficiency. The transfection efficiency increased up to the degree of acetylation where 57% of the primary amine groups were converted to amide groups. Further acetylation decreased transfection efficiency. Depending on the cell line, PEI-Ac57 was 30 to 60-fold more efficient than unmodified PEI.

Gabrielson et al. (2006) investigated which step in the transfection process was positively influenced by the acetylation. They found that internalization (up to 3 times) and intracellular unpackaging (up to 4 times) were increased in acetylated PEI compared to unmodified PEI. Both might be partial responsible for the increase in transfection efficiency. Gabrielson et al. did make an unsubstantiated assumption in the FRET analysis used to determine intracellular unpackaging. They normalized the FRET signals to the values 30 minutes posttransfection, assuming that no significant unpackaging had occurred before that. A small error in the normalization might grossly interfere with the results.

Fischer et al. (2002) progressively increased the relative amount of tertiary amine groups in PEIHEEI, a compound very closely related to PEI. They proved that this increased the buffer capacity of PEIHEEI but reduced transfection efficiency.

We feel that these experiments that relate chemical structure of PEI to its transfection efficiency are crucial. If it is a proton sponge mechanism that makes PEI an effective transfection vector, then decreasing its buffering capacity should lead to an decrease in efficiency. Unfortunately, reports on this matter contradict each other. Three studies (Fischer 2002, Forrest 2004, Gabrielson 2006) found a negative relationship between buffering capacity and transfection efficiency, while two studies (Thomas 2002, Akinc 2005) found that maximally alkylating PEI led to a loss of transfection efficiency. It would be worthwhile to confirm the findings of Akinc et al. and Thomas et al. in at least one other cell line and to confirm that N-quaternized PEI indeed has a reduced buffering capacity.

5. RELATIONSHIP BETWEEN STRUCTURE AND EFFICIENCY OF A SIMILAR POLYMER, PDMAEMA

Every non-viral vector has a pKa and thus possible buffering capacity. Correlating the buffer capacity and transfection efficiency of all non-viral vectors goes beyond the scope of this review. Here, we briefly discuss two reports regarding poly[2-(dimethylaminoethyl)methacrylate] (PDMAEMA). PDMAEMA is polymeric transfection vector with an efficiency comparable to PEI (Dubruel 2003, Figure 24). Each of its monomer residues contains a single tertiary amine group (Figure 22). It has buffering capacity between pH 8 and 6. (Dubruel 2003, Funhoff 2004).

Funhoff et al. (2004) created a polyamine with two tertiary amine groups per monomeric unit, poly(2-methyl-acrylic acid 2-[(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester) (PDAMA, Figure 22).

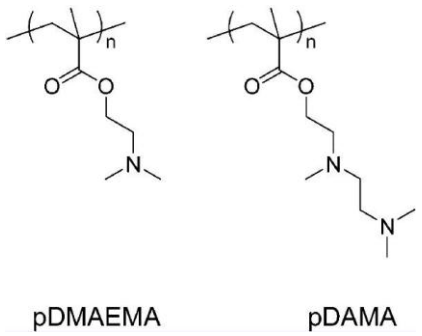

Figure 22: Chemical structures of PDMAEMA and PDAMA.

Funhoff et al. compared buffering capacity with transfection efficiency for PDMAEMA, PDAMA, and PEI. From a NaOH titration they calculated that, going from pH 7.4 to 5.0, pDMAEMA, pDAMA, and pEI can bind 0.35, 0.38, and 0.50 mol H^+ per mol monomeric unit, respectively. Thus, PDAMA is structurally very similar to PDMAEMA, but has a slightly larger buffering capacity in endolysosomal pH range. Next they tested transfection efficiency of PDAMA using a β-galactosidase assay in COS-7 cells. They found that PDAMA with a M_w was most effective but still only had 5% the efficiency of PDMAEMA.

Funhoff et al. did a PicoGreen exclusion assay to test the binding strength of PDAMA to DNA. At a N/P ratio of 4, PDAMA polyplexes displayed 4 times less fluorescence than PDMAEMA polyplexes, suggesting that PDAMA binds DNA much stronger than PDMAEMA. Co-polymerization of DAMA and methacrylic acid (MA), decreased binding strength, but did not increase transfection efficiency.

Adding chloroquine to the transfection did not increase the efficiency. Coating the polyplexes with INF-7, a synthetic protein derived from influenza virus capable of disrupting endosomes of living cells, increased transfection activity about 10-fold.

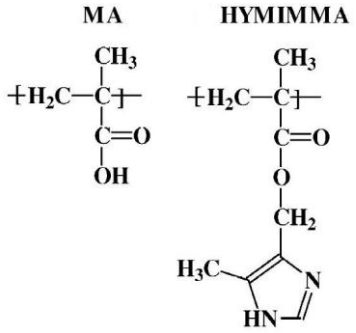

Figure 23: Chemical structure of MA and HYMIMMA residues.

Polymer	M_w (kDa)	N:P ratio	
		2/1	4/1
PDMAEMA	93	0.02	0.36
PDMAEMA	201	5.78	19.57
P($DMAEMA_{0.82}$-co-$MA_{0.18}$)	108	0.04	0.04
P($DMAEMA_{0.65}$-co-$MA_{0.35}$)	316	0.02	0.01
P($DMAEMA_{0.94}$-co-$HYMIMMA_{0.06}$)	99.5	0.23	0.12
P($DMAEMA_{0.88}$-co-$HYMIMMA_{0.12}$)	72	0.06	0.01
P($DMAEMA_{0.81}$-co-$HYMIMMA_{0.19}$)	54	0.03	1.12
bPEI	25	22.34	9.25

Figure 24, Dubruel 2003 (redrawn): Transfection efficiency of various polymers, expressed as β-galactosidase mass (ng/ml). The index figures represent the fraction of the polymer consisting of the respective monomer residue.

Dubruel et al. made copolymers of DMAEMA and MA and of DMAEMA and 4-methyl-5-imidazoyl methyl methacrylate (HYMIMMA). MA contains a carboxylic acid group and HYMIMMA a imidazol group (Figure 23). They showed that both copolymers had a higher buffer capacity than PDMAEMA (Dubruel 2000). Using a β-galactosidase reporter assay in COS-1 cells they showed that these copolymers hardly had any transfection efficiency. (Dubruel 2003, Figure 24).

Discussion & conclusions

Funhoff et al. (2004) and Dubruel et al. (2000 & 2003) both found a way to create a polymer with increased the buffering capacity compared to PDMAEMA. In both studies this did not cause an increase in transfection efficiency.

Dubruel et al. (2003) found that even if only 6% of the monomer residues was HYMIMMA, the polymer hardly had any transfection efficiency. This is a flawed comparison though, because Dubruel et al. also found that the molar mass is an important parameter for the efficiency of PDMAEMA: at a M_w of ~200 kDa PDMAEMA is effective, at ~100 kDa it is not. Most of the copolymers Dubruel et al. constructed had a low molar mass. Repeating this experiments with copolymers with a M_w of ~200 kDa would be insightful.

Funhoff et al. found that coating their PDAMA particles with INF-7 increased its transfection efficiency about 10-fold. This give reason to believe that escape from the endolysosomal system is the limiting factor in PDAMA efficiency.

6. MEASURING ENDOLYSOSOMAL BUFFERING BY PEI

Godbey et al. (2000) first tried to measure possible buffering effects of PEI in vesicles. EA.hy 926 cells were incubated with PEI particles for 3 hours; 45 minutes prior to measurements LysoSensor Yellow/Blue DND-160 was added, a lysosomotropic fluorescent pH indicator. The fluorescent signal was compared to a calibration curve made in buffers of known pH.

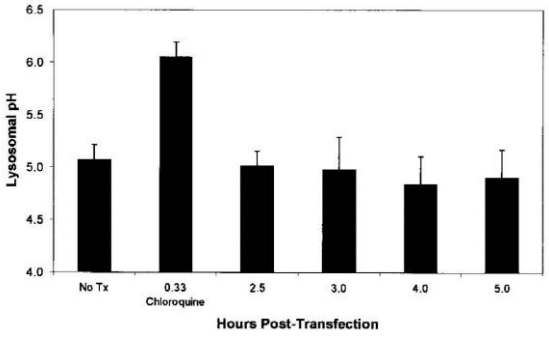

Figure 25, Godbey 2000 (edited): Lysosomal pH at various times post-transfection. Error bars represent 1 standard deviation ($n \geq 9$).

Measurements 2.5 to 5 hours after transfection showed a constant pH of ~5 (Figure 25), a pH value typical of late lysosomes (Mellman 1986).

Akinc et al. (2002) double labelled plasmid with both fluorescein and Cy5. Fluorescein is a pH sensitive fluorophore; Cy5 is pH insensitive. The fluorescein:Cy5 fluorescence ratio is a indicator of pH. Polyplexes were made by complexing this double labelled plasmid with either bPEI or PLL, using DNA:polymer weight ratios with an good balance between transfection efficiency and cytotoxicity. NIH/3T3 cells were incubated with the particles for 30 minutes. At 5 time points after transfection they measured fluorescence by flow cytometry. In each batch of the six samples, in four samples the intracellular pH in was clamped to a specific pH value. The fluorescence was measured for all six samples with a flow cytometer. The four intracellular pH clamped samples were used to generate a linear pH calibration curve, which related the fluorescein:Cy5 fluorescence ratio to pH. Using the calibration curve the pH in the other two samples could be determined, yielding a duplicate measurement. (In this article we will refer to a calibration curve made in this manner as an "in situ" calibration curve.)

For PLL Akinc et al. (2002) found pH values ranging from 4 to 4.7. This value is typical for lysosomes (Mellman 1986). For PEI particles they measured pH values between of 5.5 to 6, a value halfway between that of a lysosome and cytosol (Figure 26).

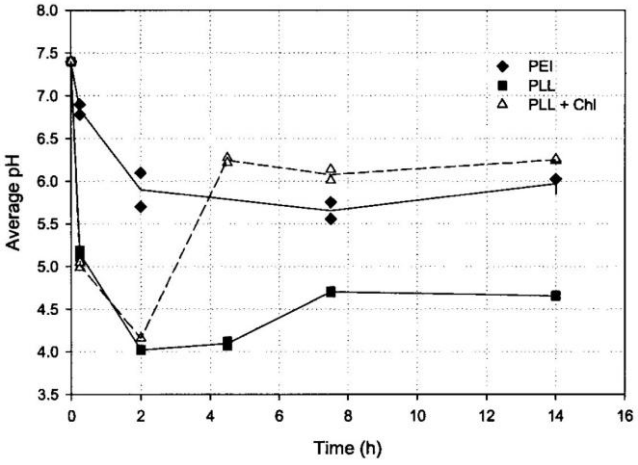

Figure 26, Akinc 2002: Average pH environment of delivered plasmid DNA for PEI, PLL and PLL + 100 μM chloroquine polyplexes as a function of time. Data was acquired in duplicate.

In another study by the same group (Akinc 2005) this experiment was repeated in COS-7 cells with similar results. In this study N-quaternized PEI particles were included and tested. N-quaternized PEI particles were found to resided in a pH environment of about 5.2 (Figure 27).

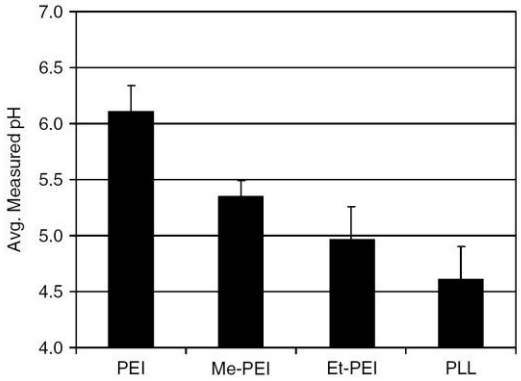

Figure 27, Akinc 2005: Measured average pH environment of DNA delivered using PEI, Me-PEI, Et-PEI, and PLL (n = 3, error bars represent standard deviation)

Forrest et al. (2002) used a very similar approach to measure pH microenvironment of PEI particles, but double labelled polymer, instead of plasmid, with a pH sensitive (Flu-X) and a pH insensitive fluorophore (Cy5). Polyplexes were made of polymers (25kDa bPEI and PLL) and pGL3 at N/P ratios optimal for transfection. Cells from three cell lines (HEK-293, C2C12 and Hep G2) were incubated with polyplexes for 1 hours. Fluorescence was measured with flow cytometry. A 7-point in situ calibration curve was constructed for each experiment. Luciferase activity was used to measure transfection efficiency.

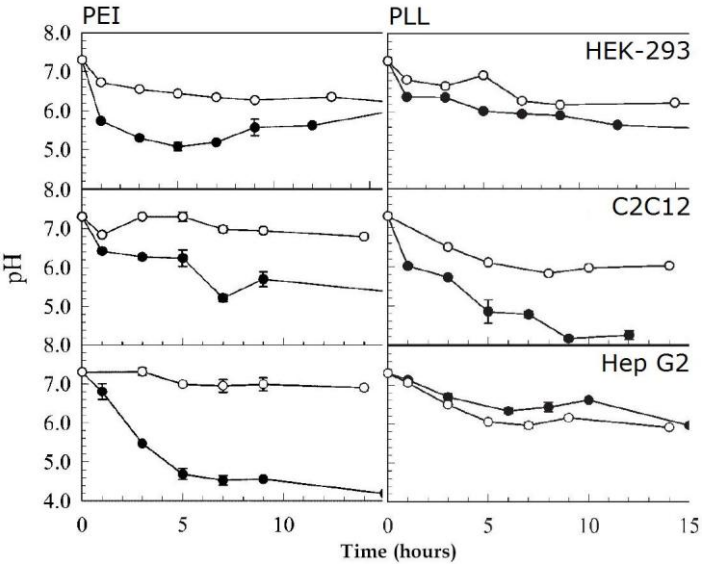

Figure 28, Forrest 2002 (edited): pH microenvironment of PEI and PLL particles as a function of time post-transfection in different cell lines without chloroquine (filled symbols) or with 100 μM chloroquine (open symbols). Symbols represent the mean (n = 3) and error bars represent the standard error (error bars not visible are smaller than the symbol). Each cell line/polymer combination was examined on at least two days, and the same trends were observed.

Depending on the cell line they reported a drop in pH to 5 or as low as 4 for PEI particles (Figure 28). The pH micro-environment of PLL was in general less acidic (Figure 28). Both the micro-environment of PEI and of PLL increased in pH after adding chloroquine. They found no correlation between pH and transfection efficiency (Figure 29)

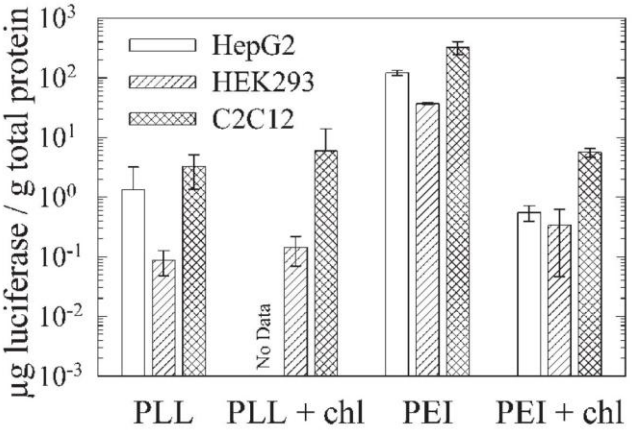

Figure 29, Forrest 2002: Comparison of luciferase expression mediated by PLL- and PEI-DNA polyplexes in the absence or presence of 100 M chloroquine (chl). Gene expression is plotted as mean ± standard deviation (n = 3).

Sonawane et al. (2003) improved upon the experiments described above to measure pH changes in multiple ways. First, they use a temperature jump protocol get all cells to start endocytosis at the same time. Cells were incubated with particles in a cold (4 °C) buffer. Then in a perfusion chamber, perfusion of the cells with warm (37 °C) buffer was started. Secondly, they use confocal microscopy to measure fluorescence, which makes it possible to track individual particles. Lastly, they added another fluorescent marker to the experiment, one that is dependent on the chloride ion concentration: 10,10'-bis[3-carboxypropyl]-9,9'-biacridinium dinitrate (BAC).

Two types of constructs were made: 1) BAC-dextran-polyamine-TMR and 2) FITC-polyamine-TMR. Three polyamines were used: PEI, PAM (polyamidoamine) and PLL. PAM contains primary and tertiary amine-groups; PLL primary amines. BAC is a fluorophore that is quenched by chloride ions and its fluorescence thus negatively relates to $[Cl^-]$. FITC fluorescence positively relates to $[H^+]$. TMR is a pH and $[Cl^-]$ insensitive fluorophore. By measuring the ratio of BAC:TMR fluorescence and FITC:TMR fluorescence with a confocal microscope and comparing these ratios to a in situ calibration curve the $[Cl^-]$ and pH can be calculated. CHO-K1 cells (hamster ovary epithelial-like) were used. Measurements were performed up to 75 minutes after transfection.

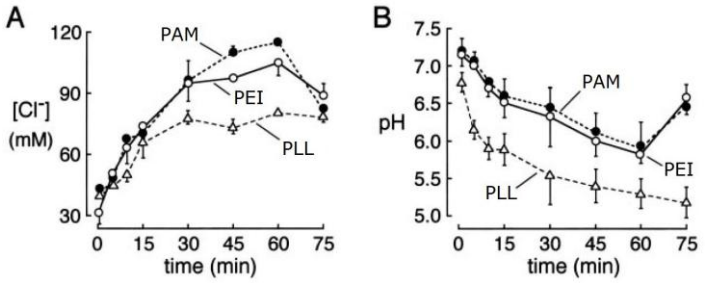

Figure 30, Sonawane 2003 (edited): Chloride accumulation (A) and acidification (B) in polyplex-containing endosomes. (S.E., n = 4)

The average pH decreased to ~6 after 60 min for PAM and PEI containing endosomes/lysosomes and rose to ~6.5 at minute 75. The average pH in POL containing particles decreased rapidly in the first 15 minutes to ~6 and then more slowly below 5.5. There was no sign of rising pH after 60 min. (Figure 30B)

Furthermore in PAM or PEI containing endosomes/lysosomes the $[Cl^-]$ concentration rose about 50% higher in the first hour compared to endosomes/lysosomes containing POL. After 60 min the $[Cl^-]$ in PAM or PEI containing endosomes/lysosomes started to decrease. (Figure 30A)

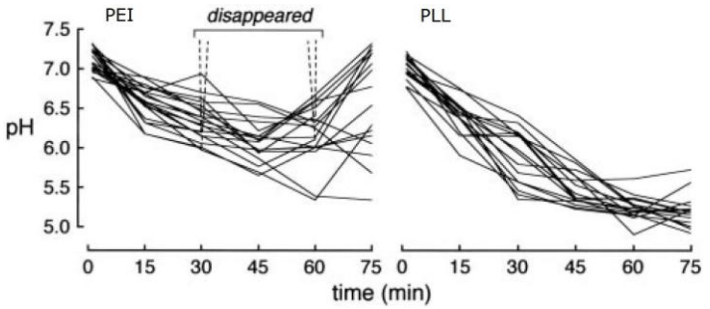

Figure 31, Sonawane 2003 (edited): time course of pH in individual endosomes showing alkalinization and disappearance of a subset of endosomes containing PEI (left) but not PLL (right) polyplexes.

Results for individual particles (Figure 31) showed that after an initial decline in pH, the pH environment of part of the PEI particles started to rise 45 minutes after transfection. This was not observed in PLL endosomes. The pH environment of PLL particles gradually decreased to ~5 during 75 minutes after transfection.

Sonewane et al. hypothesized that if PEI or PAM loaded endosomes swell and become "stretched" due to the proton sponge effect of PEI or PAM, these endosomes would become susceptible to hypotonic stress. This hypothesis was put to the test. After 45 minutes incubation with FITC/TMR labelled polyplexes, endosomes were extracted from the cells by cell homogenization

followed by centrifugation. The intact endosomes are exposed to a hypotonic medium. After 2 minutes NaOH was added and immediately thereafter FITC fluorescence was measured. The sudden increase in alkalinity would have changed the FITC fluorescence of polyplexes outside of endosomes instantaneously, but not of polyplexes inside endosomes. Then, triton X-100 was added, which lyses all the endosomes and FITC fluorescence was measured again. The ratio between FITC fluorescence immediately after NaOH addition and after Triton X-100 addition was a measure for the amount of endosomes that have survived the osmotic stress. Sonewane et al. found that endosomes with PAM inside are less resilient to osmotic stress than PLL endosomes.

Kulkarni et al. (2005) also used confocal microscopy to measure pH environment of individual particles. SNARF-4F, a fluorescent pH indicator, was attached to the 5' end of 21-base RNA oligonucleotides. The SNARF-4F-RNA conjugates were complexed to a variety of vectors: 25kDa bPEI, PLL, β-cyclodextrin-containing polymer (CDP) and CDP conjugated to imidazole (CDP-imid). CDP is a polymer with no buffering capacity. Imidazol moieties do have buffering capacity. All experiments were done in HeLa cells. For the pH measurements the cells were incubated with the particles for 2 hours and measurements were done 2 to 24 hours after transfection. The calibration curve was not constructed in situ. Transfection efficiency was determined by incubating cells with polyplexes for 4 hours and measuring luciferase activity after 48 hours.

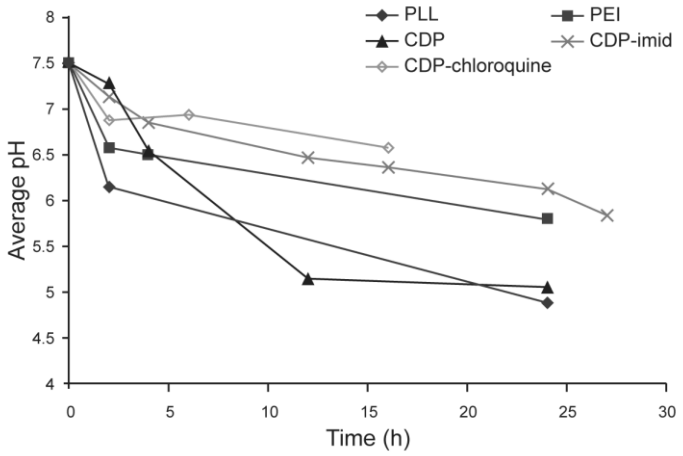

Figure 32, Kulkarni 2005 (redrawn): Plots of average pH of all cells and regions as a function of time.

The average pH in which particles with no buffer capacity (CDP, PLL) resided was much lower than that of particles with buffer capacity (PEI, CDP-imid, CDP-chloroquine) (Figure 32). There was no correlation between observed pH and transfection efficiency (Figure 33).

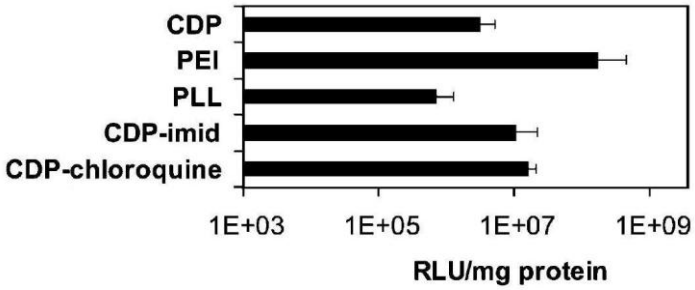

Figure 33, Kulkarni 2005 (edited): Gene delivery efficiency for particles used in this study. The delivery vectors were complexed with plasmid DNA encoding luciferase, and used to transfect HeLa cells.

Discussion & conclusions

We have two major concerns when evaluating the results of the studies above. 1) Results we discussed in this review suggest that PEI particles can escape their vesicles within 4 hours (Godbey 1999, Kichler 2001). This begs the question how relevant pH measurements up to 24 hours (Forrest 2002, Akinc 2002, Kulkarni 2005) are. 2) A high pH environment of an individual particle can be explained by buffering of the vesicles or by the particles escaping into the cytosol. Buffering could be the cause of the vesicular escape; reaching cytosolic pH is the result of vesicular escape.

Akinc et al. (2002) and Forrest et al. (2002) used very similar techniques, only Forrest et al. labelled polymer and Akinc et al. labelled plasmid. Both techniques measure the pH environment of polyplexes. If polyplex unpackaging occurs these techniques will also measure the pH environment of free polymer or free plasmid. Godbey et al. (1999) found that PEI and DNA are colocalized nearly all the time after internalization. Thus, likely the separation of PEI and DNA is of minor influence to these pH measurements. Yet Akinc et al. and Forrest et al. report very different average pH values for both PEI and PLL and both report very small SDs. Forrest et al. also report very different findings between different cell lines.

As we discussed earlier, Thomas et al. (2002) and Akinc et al. (2005) did not measure buffering capacity of N-quaternized PEI explicitly. Akinc et al. did show that N-quaternized PEI particles reside pH environment in a lower than that of PEI particles, but higher than that of PLL, which is consistent with remaining buffering capacity due to incomplete quaternization.

Sonawane et al. (2003) found a higher pH and a higher [Cl^-] in the environment of PEI

particles, compared to PLL particles. PEI particles had a sudden rise in average pH environment and a drop in average [Cl^-] environment 60 minutes after transfection. Results for individual particles reflect this. About half of the PEI particles reach a pH between 7 and 7.5, which can only indicate vesicular escape. Kulkarni et al. (2005), who also use confocal microscopy, unfortunately do not show results for individual particles. Sonawane et al. showed that PEI loaded vesicle are less resistance to a hypotonic stress. This is interesting because this result can only be explained as a cause for vesicle rupture.

Kulkarni et al. (2005) and Forrest et al. (2002) measured both pH environment and transfection efficiency. Both did not find a relationship between them.

pH environment measurements are not in line with each other. Forrest et al. (2002) found predominantly low pH environments for PEI particles and higher values for PLL. Other studies that included both PEI and PLL (Akinc et al. 2002 & 2005, Sonawane 2003, Kulkarni 2005) showed that the pH environment for PEI particles was roughly 1 point higher than for PLL particles. A high pH environment can be explained by buffering of the vesicles or escape into the cytosol. Godbey et al. (2000) found no buffering of lysosomes. This makes escape into the cytosol a more likely cause of the high pH environment. Forrest et al. showed very substantial differences in pH environment for PEI particles between cell lines. Sonawane et al. showed that within a single cell the pH environment for PEI particles can be very different. Sonawane et al. showed that PEI loaded vesicle are less resistance to a hypotonic stress. No relationship between pH environment and transfection efficiency was found.

7. CONCLUSION

PEI particles generally reach lysosomes within 3 to 6 hours after transfection (Merdan 2002, Dubruel 2004, Suh 2012, Suk 2007, Bieber 2002, Lecocq 2000). We found little evidence that identified the specific type of vesicle PEI particles escape from. Vesicular acidification is very important for the transfection efficiency of PEI (Merdan 2002, Kichler 2001, Akinc 2005).

We feel that experiments that relate chemical structure to transfection efficiency are crucial. Unfortunately, reports contradict each other. Two groups found a negative relationship between buffering capacity and transfection efficiency (Forrest 2004, Gabrielson 2006, Fischer 2002). One group found that maximally alkylating PEI led to an decreased vesicular pH environment and to a loss of transfection efficiency (Akinc 2005, Thomas 20002). It would be worthwhile to measure the buffering capacity of N-quaternized PEI. This would facilitate a more direct comparison of their results with those of other. Increasing the buffering capacity of PDMAEMA did not lead to an increased transfection efficiency (Funhoff 2004, Dubruel 2000 & 2003).

One study (Forrest 2002) found predominantly low pH environments for PEI particles and higher values for PLL, while other studies (Akinc et al. 2002 & 2005, Sonawane 2003, Kulkarni 2005) showed that the pH environment for PEI particles was roughly 1 point higher than for PLL particles. A high pH environment can be explained by buffering of the vesicles or escape into the cytosol. No buffering of lysosomes was found (Godbey 2000). This makes escape into the cytosol a more likely cause of the high pH environment. There is evidence that suggests very substantial differences in pH environment for PEI particles between cell lines (Forrest 2002) and within a single cell (Sonawane 2003). No relationship between pH environment and transfection efficiency was found (Forrest 2002, Kulkarni 2005). Isolated vesicle loaded with PEI are less resistance to a hypotonic stress (Sonawane 2003).

We found little evidence that a polymer with chemical structure that allows buffering between pH 7.5 and 5 is a more efficient transfection vector. The pH environment of PEI particles was generally less acidic but it is not proven that this is either cause or consequence of vesicular escape. From this evidence we cannot conclude that a proton sponge mechanism is the most important factor responsible for endolysosomal escape of PEI particles.

8. LIST OF ABBREVIATIONS

^{125}I-TC-PEI: ^{125}I-tyramine cellobiose-PEI;
AF488: Alexa Fluor 488, a fluorophore;
BAC: 10,10′-bis[3-carboxypropyl]-9,9′-biacridinium dinitrate, a fluorophore quenched by chloride ions;
bPEI: branched PEI;
C2C12: mouse myoblast;
CDP: β-cyclodextrin-containing polymers;
CDP-imid: β-cyclodextrin-containing polymers possessing imidazole termini;
CHO-K1: hamster ovary epithelial-like cells;
COS-1: Transformed (with SV40) African green monkey kidney fibroblast-like cells;
COS-7: very similar to COS-1;
Cy5: a fluorophore;

DOTAP: 1,2-Dioleoyl-3-trimethylammonium-propane, non-protonable mono-cationic lipid;
EA.hy926: fusion of the human lung carcinoma cells with human umbilical vein endothelial cells;
EEA1: early endosome antigen 1;
FITC: fluorescein isothiocyanate, pH sensitive fluorophore;
Flu-X: Fluorescein 5-EX succinimidyl ester, a pH sensitive fluorophore;
FRET: fluorescence resonance energy transfer;
GFP: green fluorescent protein;
HEK-293: human embryonic kidney cells;
HeLa 229: Transformed (with human papilloma virus) epithelioid human cervix adenocarcinoma cells;
Hep G2: human liver carcinoma cells;
HYMIMMA: 4-methyl-5-imidazoyl methyl methacrylate;
INF-7: a influenza virus-derived fusogenic protein;
LAMP1: lysosomal-associated membrane protein 1;
MA: methacrylic acid, a monomer;
MDA-MB-231: human mammary gland adenocarcinoma cells;
NIH:OVCAR-3: human ovary adenocarcinoma;
NIH/3T3: mouse embryonic fibroblast;
N/P ratio: polymer nitrogen to DNA phosphate ratio, i.e. charge ratio;
NPC1: Niemann-Pick disease, type C1 protein;
PAM: polyamidoamine;
PaTu 8902: human pancreatic adenocarcinoma;
PDAMA: poly(2-methyl-acrylic acid 2-[(2-(dimethylamino)-ethyl)-methyl-amino]-ethyl ester);
PDMAEMA: poly[2-(dimethylaminoethyl)methacrylate];
PEI: polyethylenimine;
PEIHEEI: poly[(ethylene imine)-co-N-(2-hydroxyethyl-ethylene imine)];
PHEEI: poly(N-(2-hydroxyethyl)-ethylene imine);
pGFP: expression plasmid containing GFP;
pGL3: expression plasmid containing luciferase gene and a SV40 promotor and enhancer;
PLL: poly-L-lysine;
Rb1: rabbit aorta smooth muscle cells;
SNARF-4F: seminaphthorhodafluors, a fluorescent pH indicator;
SV40: Simian virus 40;
SW-13: human adrenal cortex small cell carcinoma;
TAMRA: 5,6-tetramethylrhodamine;
TMR: tetramethylrhodamine;
YOYO-1: fluorophore, increase fluorescence upon intercalating with dsDNA;

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