The proton sponge hypothesis:

Fable or Fact?

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ABSTRACT

In non-viral gene therapy, cationic polymers and lipids are frequently used to encapsulate macromolecular therapeutics into nanoparticles. During their journey to deliver the cargo to the intended intracellular target, many biological barriers need to be overcome. One of the major bottlenecks for efficient transfection is the endosomal barrier since nanoparticles often remain entrapped inside endosomes and are trafficked towards the lysosomes where the cargo is degraded. For cationic polymers, the proton sponge hypothesis was introduced in the late '90s as a way to explain their endosomal escape properties. However, to date, no consensus has been reached in the scientific community about the validity of this hypothesis due to many contradictory reports. Here we review the sometimes conflicting reports that have been published on the proton sponge hypothesis. We also discuss membrane destabilization and polymer swelling as additional factors that might influence endosomal escape of polyplexes. Based on the key publications on this subject, we aim to launch a consensus on the role of the proton sponge hypothesis in endosomal escape.

KEYWORDS

Nanomedicine; Non-viral gene therapy; Cationic polymers; Endosomal escape; Proton sponge hypothesis

INTRODUCTION

First conceptualized in the early 1970's[1], gene therapy aims at delivering nucleic acids such as pDNA (and by extension mRNA or siRNA) to the intracellular environment in order to adjust dysregulated protein expression.[2] Gene delivery vectors are used to deliver therapeutic macromolecules to the desired intracellular target. Based on their origin, gene delivery vectors are generally subdivided into viral vectors and non-viral vectors. Both viral and non-viral vectors are able to incorporate therapeutic macromolecules to form nanomedicines. Non-viral vectors hold great promise since they are easier to scale up and far less immunogenic than their viral counterparts. However, viral vectors have the key advantage of a high transfection potential, which can be explained by the fact that viruses have evolved over millions of years to become highly efficient in evading the cellular barriers.[3,4] This becomes evident when comparing the relatively high amount of viral vectors that go into clinical trials as opposed to the few non-viral ones that have reached this stage (>70% of clinical trials concerns viral vectors).[5] To become as efficient as their viral competitors, non-viral vectors should improve their ability to conquer the many cellular barriers that are currently preventing them from reaching their full potential.[6]

Non-viral nanoparticles are generally subdivided into liposomes (using a lipid carrier) and polyplexes (using a polymeric carrier).[7] When these nanomedicines reach their target cell, they are mainly internalized through endocytosis.[8] Although endocytosis is an efficient way to gain entrance to the intracellular environment, the vast majority of nanoparticles remains subsequently entrapped inside the endosomes. During this entrapment, nanoparticles are trafficked towards the lysosomes, where lysosomal digestive enzymes may cause degradation of the macromolecular therapeutic cargo. In order to avoid enzymatic degradation, nanoparticles should find a way to induce endosomal escape.[9,10] Since only a very limited amount of nanoparticles are able to efficiently evade the endosomal barrier, endosomal escape is still considered the major hurdle for gene therapy.[11–13] Several strategies have been explored to promote endosomal escape of non-viral nanoparticles.[14,15] The most well-known and intensively studied strategy for endosomal escape of NPs based on cationic polymers is the so-called 'proton sponge effect',[16] which will be the subject of this review. First discovered by Behr in the '90s,[17] the proton sponge hypothesis has ruffled a few feathers over the years with both supporters and opponents. We will discuss the discovery and the principle of the proton sponge hypothesis and we will reflect on the often conflicting reports that have been published on this subject over the years. Based

on this reflection, we will conclude on the role of the proton sponge hypothesis related to endosomal escape of NPs based on buffering polymers.

THE DISCOVERY OF THE PROTON SPONGE HYPOTHESIS

Cationic polymers are able to form polyplexes with nucleic acids through electrostatic interactions and are being explored for many years to transfer nucleic acids to the cell's interior.[18,19] One of the first cationic polymers explored for nucleic acid delivery was polylysine (PLL) (**Fig. 1A**). However, since it failed to transfect cells on its own, it was quickly realized that the addition of other compounds would be required to induce endosomal release (*e.g.* chloroquine or fusogenic peptides that cause endosome disruption).[16,20] During the early '90s, it was discovered that several cationic polymers with substantial buffering capacity below physiological pH (e.g. lipopolyamines (**Fig. 1B**) and polyamidoamines) were able to mediate high transfection efficiencies without the need of adding such membrane-disruptive agents.[21,22] This observation inspired Boussif *et al.* in 1995 to test the gene delivery potential of polyethylenimine (PEI; structure shown in **Fig. 1C-D**), a synthetic cationic polymer with high amine density and high buffer capacity. Although the cellular mechanisms underlying this relationship were not understood, several hypotheses were proposed as possible explanations: endosome buffering could i) protect DNA from lysosomal nucleases; ii) alter endosomal trafficking and iii) alter osmolarity, which could lead to osmotic swelling and subsequent endosome disruption.[23] The latter hypothesis is currently known to be an essential part of the proton sponge hypothesis.

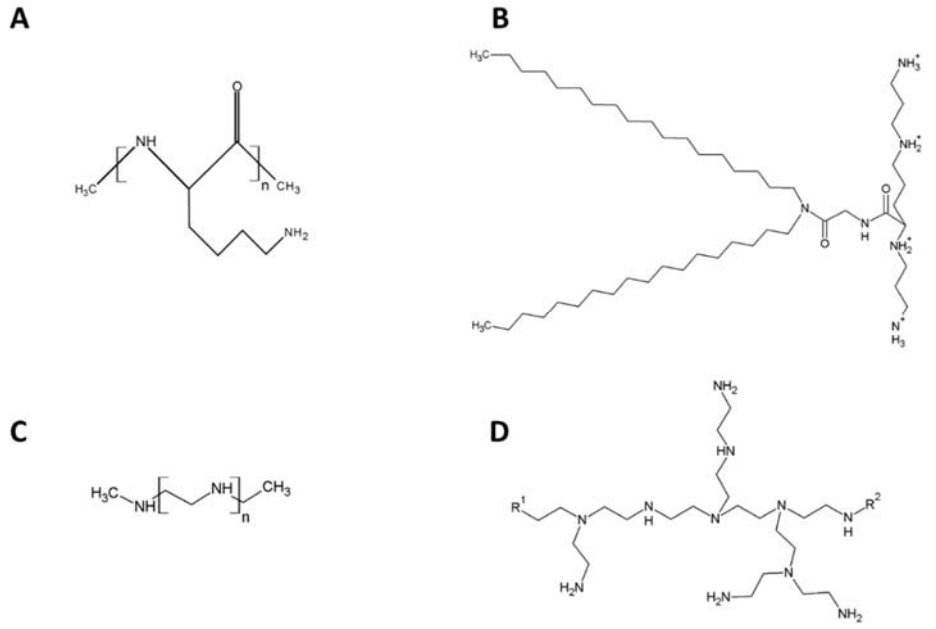

Figure 1 Chemical structures of cationic polymers used for mediating transfection efficiency. (A) Poly-L-lysine (PLL) (B) the lipopolyamine DOGS (C) linear polyethylenimine (PEI) (D) branched PEI

Indeed, some years later, in 1997, Behr and colleagues summarized the essence of the proton sponge hypothesis as follows: "*The accumulation of protons brought in by the endosomal ATPase is coupled to an influx of chloride anions. 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. With the two phenomena occurring 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.*"[17] A schematic representation of the proton sponge hypothesis, as proposed by Behr, is depicted in **Figure 2**. Over the years, several cationic polymers (usually containing protonable secondary and/or tertiary amine groups with a pKa close to endosomal/lysosomal pH) were found to exhibit high transfection efficiencies, a quality that was generally attributed to the proton sponge phenomenon.[16]

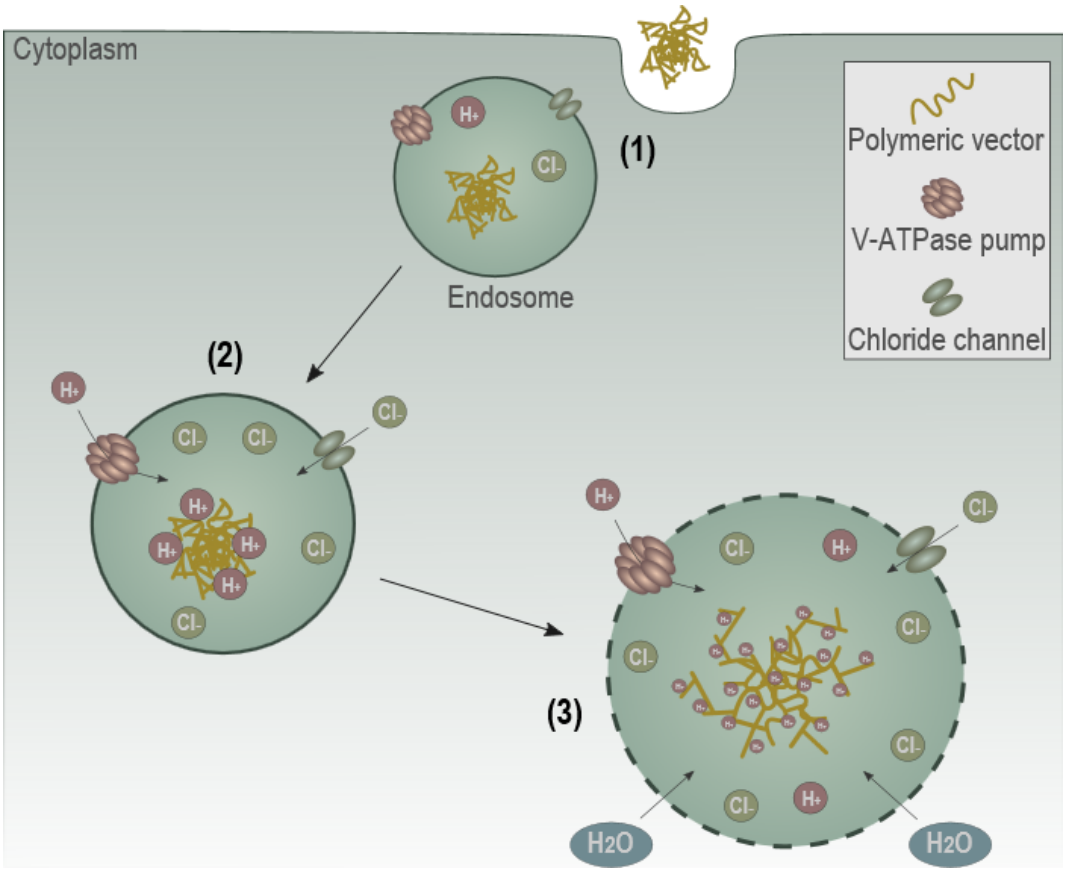

Figure 2. The proton sponge hypothesis according to Behr and colleagues.[17] (1) When polyplexes enter the cells through endocytosis, they reside inside endosomal vesicles. (2) Upon maturation, the membrane-bound V-ATPase proton pumps actively translocate protons into the endosomal lumen. Since the polymers used in the proton sponge hypothesis have a high buffer capacity, they are able to bind these protons, thereby limiting the acidification of the endosome. (3) As a result, the proton pumps will translocate even more protons to the endosomal compartment in an attempt to lower the pH. The translocation of protons is accompanied by entry of chloride ions

(to maintain the charge balance) which will lead to an increase in ionic concentration and influx of water to maintain osmolarity. The influx of water molecules generates an osmotic pressure that makes the endosome swell and, combined with swelling of the polymer due to internal charge repulsion, eventually causes endosomal rupture with release of the endosomal content into the cytosol.

EVIDENCE PRO AND CON THE PROTON SPONGE EFFECT

Ever since the proposition of the proton sponge effect as a gene transfer mechanism there have been supporters of the hypothesis on the one side and critics on the other side. Indeed, there is a substantial amount of evidence to support both parties. Although the proton sponge effect used to be linked predominantly to the buffering capacity of the polymer, recent findings indicate that membrane destabilization might play a substantial role in this process as well. In this section, we will comment on the data that has been collected over the years regarding the essential components that govern the proton sponge hypothesis. These components include the buffering effect of polymers, the acidification of endosomes and endosomal swelling. In the next section, we will contemplate on the added value of polymer swelling and membrane destabilization to the osmotic forces that are at the basis of the proton sponge hypothesis.

Buffering effect of polymers

Since the buffer capacity of polymers is at the basis of the proton sponge hypothesis, it seems reasonable to test its validity by investigating the relation between buffer capacity of the polymer and the amount of transfection efficiency it can induce. The most well-known example is the comparison between PLL and PEI. PLL, with low buffer capacity at endo-lysosomal pH, was unable to induce cell transfection, whereas PEI, with high buffer capacity at endo-lysosomal pH, produced high transfection efficiency.[24] Singh *et al.* synthetized glycerol-crosslinked PEIs in order to produce polymers with different buffer capacities but similar uptake, DNA binding and unpacking. They confirmed that decreasing the buffer capacity in the endolysosomal pH range also decreased transfection efficiency.[25] The importance of the buffering moieties was further confirmed by removing the buffer capacity of PEI through N-quaternization, a manipulation which again resulted in a substantial reduction of transfection efficiency.[26,27] Pack *et al*. developed complexes of pDNA with transferrin-conjugated PLL and gluconic acid-modified polyhistidine. Transferrin-conjugated PLL was used to maximize DNA condensation and to provide a ligand for endocytosis. Gluconic acid-modified polyhistidine, containing imidazole groups with pKa of 6.15, was added to the complex to provide buffer capacity. In accordance with the proton sponge hypothesis, the authors showed that the addition of polyhistidine greatly enhanced the level of

transfection.[28] Similar evidence was provided by Midoux *et al*. who found that partially substituting PLL with histidyl residues increased transfection efficiency.[29]

At the same time evidence arose that pointed against the proton sponge effect. Funhoff *et al.* added an extra amine group with pKa 5 to pDMAEMA (poly(2-dimethylamino ethyl)-methacrylate) in order to increase the buffer capacity of the polymer. Surprisingly they found that these polymers exhibited lower transfection efficiencies than the original pDMAEMA. After addition of a membrane disruptive peptide, the transfection efficiency was restored, suggesting that the decrease in transfection was due to limited endosomal escape.[30] Forrest *et al.* generated PEI derivatives by acetylation of primary amines; a modification that resulted in a decreased buffer capacity. They observed a 21-fold increase in transfection efficiency compared to unmodified PEI. However, as pointed out by the authors it could not be excluded that increased transfection was the consequence of altered vector unpacking, endocytic trafficking or increased lipophilicity of the polymers.[31] This is indeed a point of crucial importance: polymer modifications might alter the carrier's performance at the level of intracellular barriers preceding or following endosomal escape and looking at the endpoint of transfection might not be the best approach to evaluate the proton sponge effect.

Acidification of endosomes

Rather than by polymer modifications, others have challenged the validity of the proton sponge hypothesis by looking into endosomal acidification. The proton sponge hypothesis states that an intraluminal influx of protons (and consequently chloride ions and water) is needed to increase the osmotic pressure inside the endosome, eventually leading to the bursting of the endosome. Rehman *et al*. evaluated the necessity of endosomal acidification on the induction of endosomal escape by pre-incubating HeLa cells with Bafilomycin A1, which prevents endosome acidification by blocking the V-ATPase pump. Rather than looking at the final transfection efficiency, they used an assay that evaluated endosomal escape frequency directly *via* co-incorporation of fluorescently labeled oligonucleotides (ONs) into the polyplexes. Upon endosomal escape, the ONs spread towards the cytoplasm and eventually accumulate into the nucleus. The authors found that in control cells, treated with PEI polyplexes, virtually all cells showed ON accumulation inside the nucleus, indicative of endosomal escape, while in Bafilomycin A1-treated cells, the ONs remained entrapped within the endosomes.[32] Consequently, treatment with Bafilomycin A1 inhibited transfection efficiency of PEI polyplexes, an observation also reported by several others before.[26,29,32–34] These findings clearly illustrate that the

endosomal acidification process is essential for PEI-mediated transfection, as proposed by the proton sponge hypothesis. In a second set of experiments, researchers evaluated the effect of buffering polymers on the actual pH inside the endosomes. Several reports showed that endosomal acidification slows down after administration of buffering polymers, whereas the pH of endosomes containing a non-buffering polymer decreases more rapidly.[24,26]

These observations are contradicted by others, who found that buffering polymers are unable to increase endolysosomal pH, potentially disproving the proton sponge effect.[35,36] For instance, Godbey *et al*. measured lysosomal pH (using LysoSensor Yellow/Blue) 2.5 – 5 h after transfection with PEI and did not see lysosomal buffering. However, it should be noted that the authors also stated that pDNA/PEI polyplexes did not interact with lysosomes, stained with LysoTracker Red, which makes the conclusions rather confounding.[37] Further adding to the debate, the lack of colocalization with LysoTracker in microscopy images was proposed by several researchers to be a confirmation of the proton sponge hypothesis since the buffering effect of the polymer inhibits staining with acidotropic dyes such as LysoTracker.[38,39] Moreover, the successful colocalization between polymer and LysoTracker does not necessarily implicate that buffering polymers did not buffer the endosome. Indeed, an increased flux of protons into the endosome could allow acidification of the endosome once the buffering polymer is fully protonated. Thus, even when polymers do exert a buffering effect in endosomes, this is no guarantee that the eventual pH of the vesicle remains increased. [35,40,41]

Chloride accumulation and endosomal swelling

According to the proton sponge hypothesis, chloride ions migrate towards the endosomal interior following the influx of protons, for reasons of charge neutralization. As such, the proton sponge hypothesis has been tested by evaluating the concentration of chloride ions inside the endosomes with and without buffering polymer. Sonawane *et al*. developed a fluorescent Cl^- indicator that enabled the measurement of endosomal chloride concentrations. They found that the addition of Bafilomycin A1 not only inhibited acidification, but also hindered the increase in endosomal chloride concentration, providing evidence that the influx of protons in endosomes is indeed accompanied by an influx of chloride ions.[42] Next, they used this probe to examine the endosomal chloride concentration after administration of PLL and PEI. Results showed an enhanced chloride accumulation for PEI polyplexes (115 mM at 60min) as compared to PLL polyplexes (80 mM at 60min), providing direct evidence that these polymers provoke an influx of chloride ions.[24] The influx of chloride ions is believed to be

accompanied by entry of water molecules, creating an osmotic pressure, which induces swelling and eventually endosomal rupture. This was investigated by Sonawane *et al.* via light microscopy who confirmed that PEI polyplexes induced a 140% increase of endosomal volume, whereas this was only 20% for PLL polyplexes.[24] Likewise, Merdan *et al.* observed an increase in vesicle size after administration of PEI through confocal microscopy, which they attributed to osmotic swelling or fusion with other PEI-containing vesicles.[33]

In order to elucidate whether the osmotic stress, produced by the proton sponge effect, can by itself induce endosomal membrane rupture, Benjaminsen *et al.* measured lysosomal PEI concentrations and used these concentrations to calculate the critical size of the lysosomes at which they might rupture. Since they calculated that the majority (± 63%) of lysosomes needs to swell to a diameter above 1.6 μm to let them burst, they concluded that only a small fraction of the lysosomes will burst because of osmotic swelling and that it is uncertain that this is the dominant effect of endosomal rupture. However, they also acknowledged that a very limited amount of bursts could already be sufficient to induce transfection.[35] Won *et al*. calculated the osmotic pressure which may rise in endosomal vesicles with a diameter of 100-150 nm upon lowering the pH of the endosome from 7.4 to 5.0. They found that the osmotic pressure, originating from a single polyplex that consists of 5 pDNA strands with 5000 base pairs, will expand the vesicle membrane by 2.3%. Since lipid vesicles are able to withstand surface expansion up to 2-5%, the authors claim that the osmotic pressure build-up is probably insufficient to cause endosome disruption. However, they do not exclude that it is likely to be a significant contributing factor to the eventual disruption of the endosomal membrane.[43] It must be noted that it is very well possible for endosomes to contain more than a single polyplex and that the amount of polymer in a polyplex may vary. These are two factors that can greatly influence the effective proton sponge capacity. As recently shown by our group, a third factor that should be reckoned with is endosomal size, a cell type-dependent property. Cell types that contain small endosomes would need to accumulate less polyplexes compared to cell types that have larger endosomes in order to induce efficient endosomal bursting *via* the proton sponge effect.[44]

As discussed above, experimental evidence which supports a proton sponge effect to occur as a consequence of buffering polymers clearly exists. However, to which extent this mechanism is able to introduce endosomal escape is still a matter of debate. The above-mentioned mathematical models, that describe the osmotic swelling resulting from buffering polymers, make us believe that the osmotic

effect alone is perhaps insufficient to induce endosomal bursting and hint towards the involvement of additional factors that contribute to effective endosomal escape.

BEYOND THE BUFFER CAPACITY OF POLYMERS: ADDITIONAL FACTORS THAT INFLUENCE THE PROTON SPONGE HYPOTHESIS

Polymer swelling

In 1997, the expansion of the polymeric network was first added as an extension of the proton sponge hypothesis.[17] The ability of polymers to unfold into an extended conformation after protonation increases the volume and space taken up by the polymer, as can be seen from **Figure 3**.[45] Indeed, it has been shown that upon protonation of PEI, the polymer chain elongates due to electrostatic repulsion. This has been demonstrated by measuring the distance between two amine groups with varying protonation states. Singly protonated ethylenediamine displayed an average distance of 2.9 Å while for doubly protonated molecules, the average distance increased to around 3.5 Å.[46] Tang *et al.* first demonstrated that polymer expansion could indeed contribute to increased transfection. They used intact and fractured PAM dendrimers to vary the degree of flexibility and their ability to expand in response to a decreasing pH. A superior transfection efficiency was found after administration of fractured dendrimers with optimal flexibility compared to intact dendrimers with sterical constraints.[47] Based on these results, Szoka proposed to refer to the volumetric expansion of polymers upon protonation as the 'umbrella hypothesis'.[45] It must be noted, however, that a higher degree of vector unpacking in flexible polymers could provide an alternative explanation for the increased transfection instead of a better endosomal escape efficiency.

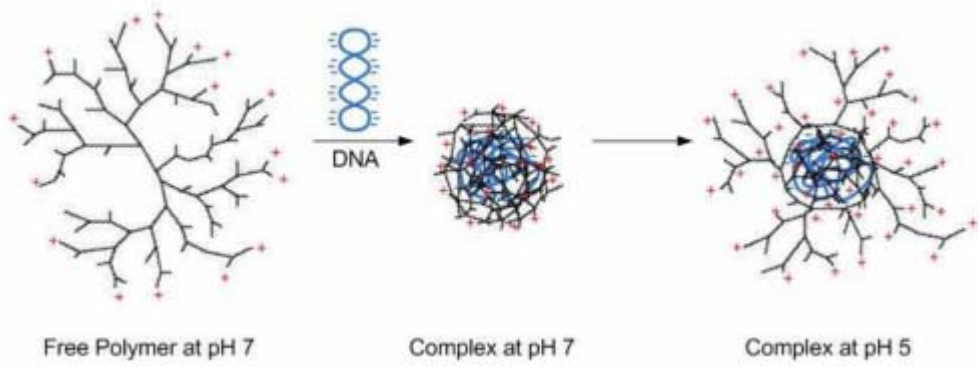

Figure 3. Schematic representation of the umbrella hypothesis. Cationic polymers condense negatively charged nucleic acids into compact nanoparticles. Upon acidification of the endosomes, amine groups of the polymer are protonated, leading to the elongation of the polymer chain due to electrostatic repulsion. The terminal

branches of the polymer unfold from a collapsed state into an extended conformation. Image reprinted with permission of [45].

Membrane destabilization

Recently, it was determined *via* molecular dynamics simulations that elongated PEI chains can interact with the endosomal membrane, leading to the formation of hydrophilic pores in the lipid bilayer. These interactions can cause a local lipid bilayer destabilization, further contributing to the release of endosomal content.[48] Already in 2002, Thomas and Klibanov looked into the structure-activity relationship of various chemically modified PEIs and found that a moderate enhancement of the polymer's hydrophobicity increased transfection efficiency.[27] Rehman *et al*. showed *via* live cell confocal microscopy that endosomal release does not lead to a complete lysis of the endosome but to a release that occurs from one particular region of the endosomal membrane, through which the cargo is jetted into the cytoplasm. They propose a model in which the protonation causes the highly charged polyplex to closely interact with the endosomal membrane. At this interaction site, a local (osmotic or mechanical) initial membrane destabilizing effect leads to rupture of the endosomal membrane due to an increase in membrane tension upon osmotic swelling of the endosome.[32] Additionally, Bieber *et al.* revealed membrane damage in PEI-containing vesicles through electron microscopic analysis, which they attributed to the proton sponge effect or a direct interaction of the polymer with the vesicular membrane.[38] Martens *et al.* agreed that the proton sponge effect is now thought to be assisted by an initial membrane destabilization induced by the cationic charge of the polymer, followed by further destabilization of the membrane as a consequence of the umbrella hypothesis.[11] The current view on proton-sponge based endosomal escape is schematically summarized in **Figure 4**.

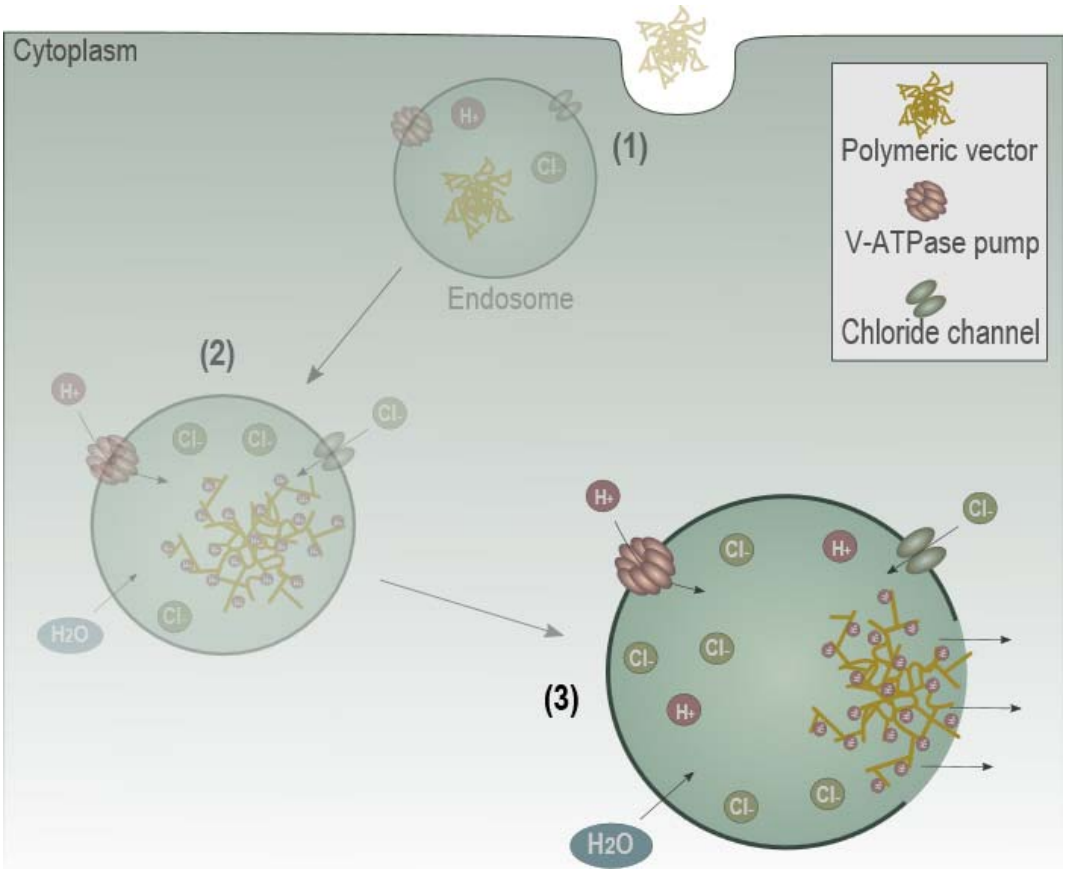

Figure 4. State of the art representation of the proton sponge hypothesis. Endosomal rupture through the proton sponge effect is nowadays considered to be due to a combination of osmotic forces arising from the buffer capacity of the polymer, polymer swelling due to internal charge repulsion upon protonation (as shown in (1) and (2)) and membrane destabilization because of the interaction between the protonated polymer and the endosomal membrane, as shown in (3).

However, inducing membrane destabilization by interaction of the polymer with the endosomal membrane could also be counterproductive. Recently, our group showed that PEI polyplexes can induce leakiness of the endosomal membrane in a cell-type dependent manner. While oligonucleotides (ONs) remained entrapped within the endosomes, small molecules such as water were able to cross the endosomal membrane and reach the cytoplasm. This was visualized by loading endosomes with calcein (as a model for small molecules such as water) and AF647-labeled ONs (cargo molecules). Confocal microscopy confirmed the release of quenched calcein (visualized as a change from punctate to diffuse fluorescent pattern) without the release of AF647-labeled ONs. Since endosomal escape, measured by accumulation of ONs in the nucleus, and transfection efficiency were markedly reduced in cell types in which this leakiness was observed, we hypothesized that endosomal membrane leakiness prevented effective build-up of osmotic pressure by PEI, rendering the proton sponge effect ineffective in leaky endosomes. This clearly indicates that the effectiveness of proton sponge-based endosomal escape is

not only cell type-dependent but also requires exactly the right interplay between osmotic forces and membrane destabilization.[44]

CONCLUSION

Altogether, these results illustrate that achieving effective endosomal escape by the use of proton sponge-based polymers depends on a delicate balance between osmotic pressure, polymer swelling and destabilization of the endosomal membrane. Moderate membrane destabilization due to polyplex interaction with the endosomal membrane likely leads to a locally weakened area where the membrane will rupture by the osmotic forces. However, excessive membrane destabilization should be limited as it may lead to membrane leakiness which is counterproductive, due to the fact that osmotic pressure can no longer be built up. As this is a cell type-dependent phenomenon it will be an interesting area for further research to understand the underlying mechanisms and to find ways to modulate these effects. Furthermore, it must be noted that recently methods were developed that allow to observe and quantify endosomal escape events directly. It should prove useful in the future to quantify endosomal escape efficiency itself, thereby eliminating interference of subsequent intracellular barriers. Obviously, barriers that precede endosomal escape should still be taken into account.

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