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Modeling the Proton Sponge Hypothesis: Examining Proton Sponge Effectiveness for Enhancing Intracellular Gene Delivery through Multiscale Modeling

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Abstract

Dendrimers have been proposed as therapeutic gene delivery platforms. Their superior transfection efficiency is attributed to their ability to buffer the acidification of the endosome and attach to the nucleic acids. For effective transfection the strategy is to synthesize novel dendrimers that optimize both of these traits, but the prediction of the buffering behavior in the endosome remains elusive. It is suggested that buffering dendrimers induce an osmotic pressure sufficient to rupture the endosome and release nucleic acids, which forms to sequester most internalized exogenous materials. Presented here are the results of a computational study modeling osmotically-driven endosome burst, or the “proton sponge effect.” The approach builds on previous cellular simulation efforts by linking the previous model with a sponge protonation model then observing the impact on endosomal swelling and acidification. Calibrated and validated using reported experimental data, the simulations offer insights into defining the properties of suitable dendrimers for enhancing gene delivery as a function of polymer structure.

Keywords

dendrimer; modeling; drug delivery; gene therapy; proton sponge

1.0 Introduction

Gene delivery through non-viral means is limited by instability of the DNA/vector complexes in blood circulation and extracellular fluids, as well as sequestration of the nucleic acids in cellular organelles [1–4]. Biological activities of exogenous nucleic acids are contingent upon their accumulation in the cytosol and/or nucleus in which molecular targets are located. A common entry pathway of exogenous particulates into cells is through endocytosis. This process encompasses multiple mechanisms, with the common initial step of encasing the nucleic acids within small lipid vesicles, or endosomes. Such vesicle undergoes maturation, and eventually fuses with a lysosome wherein degradation of nucleic acids occurs.

It has been postulated that endosome lysis (or burst) may be induced shortly after endocytosis to allow the release of endocytosed materials prior to fusion with lysosomes [5]. If correct, optimizing this process may be a useful approach tool for improving the

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efficiency of nucleic acid delivery. The “Proton Sponge Effect” [6–8] states that the presence of a weakly basic molecule may cause an endosome to burst. In particular, the proton sponge would be introduced alongside a nucleic acid to affect release of the nucleic acid prior to the onset fusion with lysosomes. Dendrimers complex with DNA molecules through the protonated primary amines, but their superior transfection efficiency has been attributed to the abundance of tertiary amines. These weakly basic tertiary amines become protonated when enclosed in acidic endosomes yet remain unprotonated in the prior stages. Varying the ratios of primary and tertiary amines in dendrimers may optimize both the nucleic acid binding and endosome escape.

Sponges, which typically are polyamines, work as a buffer by absorbing free protons in endosomes (Figure 1). Absorbed protons are not allowed to escape the endosome through ¹ diffusion, thus no longer contribute to the internal pH or the Nernst equilibrium potential of the protons. As the absorbed protons accumulate, they gradually increase the membrane potential past the equilibrium level. Since this equilibrium potential is primarily established by chloride diffusion [9], chloride will then begin to diffuse into the endosome in an attempt to restore the equilibrium potential which raises the osmotic pressure further. This causes the endosome to swell and expand until it passes a critical area strain, rupturing the lipid bilayer membrane and releasing the endosome contents into the cell.

Experimental observations supportive of the proton sponge effect have been reported by Sonawane et al [6]. Their group used hamster ovary cells to track and observe conditions such as concentrations, pH and endosome volume with respect to time and compared the impact of using several different polyamines (2nd generation polyamidoamine (PAMAM), polyethylenimine (PEI), and poly-L-lysine (PLL)). While all three dendrimers bind and condense plasmid DNA, they differ in composition of titratable tertiary amines. They observed that PAMAM and PEI decreased acidification while simultaneously increasing chloride accumulation, causing endosomal swelling. Similar observations have been made of polyethylenimine (PEI) complexed with DNA by Godbey et al. [10], and Forrest and Pack [11]. On the other hand, Funhoff et al. found no evidence of endosome burst for certain polyamines containing titratable amines, suggesting involvement of other undetermined factors [12]. Models describing the effect with predictive power are needed.

The proton sponge hypothesis will be explored in this paper using a previously validated endosome burst computational model [13]. In the current study a computational model developed for cellular behavior is combined with an additional system of equations for proton sponge behavior with the goal of determining suitable sponge properties for enhancing vaccine delivery. The results indicate that the model may be used as a tool to help design the next generation of polyamines for gene delivery.

2.0 Methods and materials

2.1 Description of the model

The modeling approach may be calibrated to simultaneously consider any combination of protein transporters, diffusion, and evolution of membrane potential, elastic energy, and internal species concentrations (Figure 2). In all cases, the strength of this modeling approach is its ability to yield time-dependent state predictions of the inclusion being studied.

The primary active components considered includes flow across Na⁺/K⁺ ATPase (pumps), H⁺ ATPase (pumps), Na⁺, K⁺, H⁺, and Cl⁻ channels/diffusion, and osmotic swelling. In

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addition the membrane properties, including bilayer membrane elasticity and rupture, are considered. Concentrations of each of the species present (water, Na^+ , K^+ , H^+ , Cl^-) are tracked in the simulation resulting in predictions of the evolution of internal pH, internal pressure, and volume change. The model is calibrated with experimental parameters available in the literature and subsequently similarly validated through three criteria. A modeling process similar to this approach has been validated for other studies in past research [14–16]. The first criterion satisfied is the development of the pH gradient [17,18]. The second satisfied criterion is the buffering action of the Na^+/K^+ ATPase is in agreement with experimental observation [18–20]. The third and final validation criterion satisfied is the development of the expected membrane potential of 90 mV, which is inverse of the standard potential across the plasma membrane as the endosome interior is the cell exterior and vice versa [9]. Thus, the calibrated and validated methodology is extended in this study to test the proton sponge effect.

The processes considered in this model may be summarized as follows.

- Protein Transporters activate. H^+ ATPase begins acidification process.
- Membrane potential is increased by protein activity and increasing ion concentration gradients across the endosome membrane. These phenomena lead to increases in diffusion currents (H^+ and Cl^- diffusion).
- Incoming H^+ are buffered by a proton sponge. This reduces the acidification and diffusion flow by holding the H^+ ions inside the sponge.
- This causes further increases in the membrane potential, generating additional rectifying inward Cl^- diffusion, increasing the osmotic pressure.
- The osmotic pressure gradient causes swelling, increasing the endosome volume and leading to eventual burst dependent on sponge properties.

The following section briefly describes the cellular model for endosome simulation. Afterwards discussion on the model employed for combining the endosome model with the proton sponge model is provided.

2.2 Model components

I. Hodgkin Huxley Approximation of the evolution of membrane potential [21]:

$$\frac{dv}{dt} = -\frac{1}{C} \left(\sum i_{\text{transport}} \right) \quad (1)$$

Here v is the membrane potential, C is the capacitance of the membrane, and i is the transporter current. The transporter density is assumed to be high enough to allow the model to approximate the membrane as a capacitance circuit. The change in the membrane potential is directly related to the net transport flow.

II. Nernst equilibrium potentials for species S [22]:

$$v_s = \frac{kT}{e} \ln \left(\frac{[S]_e}{[S]_i} \right) \quad (2)$$

The second potential term that must be considered is the Nernst equilibrium potential. v_s is the potential for species s , k is Boltzmann's constant, T is the temperature e is the charge of an electron, and $[S]$ is the external (e) or internal (i) concentrations of species S . As the

concentration gradient of a charged species increases, this potential term will increase as well.

III. Pump ion flow rate [23]:

$$i_{pump} = k_{pump} \tanh \left(\frac{e(v + v_s - v_{ATP})}{2kT} \right) \quad (3)$$

Pumps establish a concentration gradient by breaking down the biochemical fuel ATP. The resulting energy is then used to transport an ionic species across the membrane against its electrochemical gradient. In the endosome case, the dominant transporter is the pump, and it is responsible for the acidification of the endosome. The net flow is a function of the difference between the Nernst equilibrium potentials (v_s) and the membrane potential itself (v), and then boosted by the energy gained from ATP (v_{ATP}) multiplied by charge of a single atom e . The total flow is also a function of the transport coefficient k_{pump} , which is based on the density of the transporters and their rate coefficients.

IV. Channel ion flow rate [23]:

$$i_{channel} = k_{channel} x \sinh \left(\frac{z_s e (v - v_s)}{2kT} \right) \quad (4)$$

Channels allow a selected species to travel along its electrochemical gradient. The equation employed here is similar to the one derived for protein pumps; however it employs a hyperbolic sine rather than a hyperbolic tangent as transporter saturation is not assumed. This function is also multiplied by a voltage gating term x , which determines the probability of the channel being open dependent on the instantaneous membrane potential. Channels allow for a passive diffusion of species to reduce a concentration gradient. The channels considered here are voltage gated, and will remain shut until the membrane potential of the cell passes a threshold value. At this point the channels will open such that the membrane potential might be restored to an equilibrium value.

V. Diffusion of ions directly through membrane [24]:

$$i_{diff} = -AP_s F \left(\frac{zFv}{RT} \right) \left[\frac{[S]_i - [S]_e e^{\left(-\frac{zFv}{RT}\right)}}{1 - e^{\left(-\frac{zFv}{RT}\right)}} \right] \quad (5)$$

The final method of transport is passive diffusion. The membrane is considered permeable to various ions, and this passive diffusion may occur as a function of the concentration gradient and the instantaneous membrane potential. This diffusion property has been observed to play a key role in the acidification of endosomes. A is the surface area of the membrane, P_s is the permeability of the membrane to species s , F is Faraday's constant, R is the gas constant, and z is the charge of the species. This equation is responsible for the pH plateau illustrated in the following results.

VI. Internal Rate of Species Concentration Changes:

$$\frac{d[S]}{dt} = \frac{\sum i_s}{Vol * F} \quad (6a)$$

$$\frac{dpH}{dt} = \frac{\sum i_{H^+}}{Vol * F * C_{buff}} \quad (6b)$$

The above transport terms may be directly translated into change in internal concentrations. 6b refers to the change in internal pH, which is directly related to the rate of proton uptake (influenced by H^+ ATPase currents, diffusion/channel currents, and dendrimer uptake). The change in pH is directly related to the change in H^+ (mM) by a buffering constant C_{buff} with units of mM/(pH).

VII. Osmotic Pressure Generation [25]:

$$\pi = RT\theta \sum ([S]_i - [S]_e) - C\theta \quad (7)$$

The changes in internal concentrations will then generate an osmotic pressure, based on the difference in external and internal concentrations. This is the driving force behind endosome rupture. θ is the osmotic constant (taken to be 0.73 [16]) and $C\theta$ is a constant used to ensure that the initial osmotic pressure is zero.

VIII. Volume Change [26]:

$$\frac{dVol}{dt} = KA \left(\sum_s \pi_s - P_r \right) + \sum_s \bar{V}_s \frac{d}{dt} (n_s)_i \quad (8)$$

The rate of volume change may be expressed as a combination of the osmotic pressure (first term) and the molar volume of the incoming species (second term). In this study, the osmotic pressure portion overwhelms the transport portion, which may be shown by halting the simulation to compare the contribution from either term. The volume of the species transported across the membrane is minimal in comparison to the osmotically driven fluid. K is the hydraulic diffusivity, and P_r is the internal pressure generated due to deformation (Equation (10)).

IX. Membrane Diffusivity [27]:

$$K = \frac{P_{os} V_w}{RT} \quad (9)$$

The hydraulic diffusivity required for the previous equation may be obtained as a function of the osmotic permeability P_{os} and the molar volume of water V_w .

X. Internal Pressure Generated [28]:

$$P_r(\lambda) = \frac{2\mu_0}{r_0} \left[1 - \frac{1}{\lambda^6} \right] e^{\gamma(2\lambda^2 - \lambda^{-4} - 3)} \quad \lambda = \frac{r}{r_0} \quad (10)$$

As the endosome expands, the membrane will resist the expansion generating an internal pressure which may be calculated as a function of the membrane shear modulus of elasticity μ , the instantaneous deformation λ , the initial endosome radius r_0 , and a material characteristic γ . γ is assumed to be 0.067, which corresponds to the point at which $dp/d\lambda$

≥ 0 for all λ [28]. This ensures that the values remain stable throughout the simulation while allowing the greatest degree of deformation. The shear modulus was assumed to be $2.5 \mu\text{N}$ [29], or the shear modulus for human red blood cells. It should be noted that for most of the cases considered the deformation remains within the linear deformation region; however when comparing the model predictions to experimental data with large deformation values the nonlinear elasticity is necessary.

XI. Protonation:

For evaluating the use of various proton sponges, a secondary system of equations must be generated for simulating their ability to buffer incoming protons and generate an osmotic pressure for burst.

Dendrimers are introduced to the model through a combination of protonation site interaction equations and the Henderson-Hasselbalch equations. These polyamines contain primary and tertiary amines. In the extracellular environment ($\text{pH} \sim 7.4$), the primary amines ($\text{pK}_a \sim 9$) located on the surface of the polymer are protonated while the tertiary amines remain largely as free base. The superior transfection efficiency of dendrimers compared to polymers containing solely primary amines has been attributed to the abundance of tertiary amines. Because dendrimers occupy definable volumes, the tertiary amines can be differentiated as “interior” and “exterior” protonation sites with approximated pK_a of 4–6 and 6–7, respectively. It has been proposed that protonation of the exterior tertiary amines is crucial for endosome buffering and subsequent bursting [30], due to overlap of its pK_a with pH in early endosomes. The interior tertiary amines remain un-protonated in the life span of an early endosome. The current computational model is applied to simulate and differentiate the events driven by the putative functions of the tertiary amines.

Protons entering the endosome are taken up by the enclosed polyamine. This reaction is modeled through the Henderson-Hasselbalch approach, where the rate of uptake is a function of the polymer pK_a and the internal pH of the endosome. Free protons are tracked and attached through Equation (11).

$$\frac{d[H^+]_{\text{sponge}}}{dt} = k_{\text{prot}}[X]_{\text{free}} - k_{\text{loss}}[X]_{\text{prot}} \quad (11)$$

Here $[X]_{\text{free}}$ is the number of available protonation sites and the k values are the reaction rates for protonation and dissociation. The constants k_{prot} and k_{loss} are taken directly from Henderson-Hasselbalch.

$$k_{\text{prot}} = k * \frac{10^{\text{pK}_a - \text{pH}}}{1 + 10^{\text{pK}_a - \text{pH}}} \quad (12a)$$

$$k_{\text{loss}} = k * \frac{10^{\text{pH} - \text{pK}_a}}{1 + 10^{\text{pH} - \text{pK}_a}} \quad (12b)$$

While this is suitable for a sponge with a single protonation site, these equations do not account for site-interactions. Dendrimers contain many protonation sites with varying pK_a s, and as these sites protonate the positively charged sites will directly affect the pK_a s of their neighboring sites. This interaction behavior is accounted for through a site-interaction model derived by Koper [31].

In this model the change in pKa for a specified site is related to the initial unmodified pKa and the interaction coefficients with neighboring sites. This is simulated through Equation (13) [31].

$$pK_i = pK_{i0} - \sum_{i \neq j} \varepsilon_{ij} \langle x \rangle_j \quad (13)$$

Here pK_i is the current pKa of the protonation site, pK_{i0} is the original pKa value prior to protonation, and the summation term loops over all nearby neighbors and their chance of protonation. Because the model is able to track the level of protonation this method is ideal for determining changes in the sponge pKas as acidification occurs. The interaction coefficients ε_{ij} of Equation (13) are calculated through Equations (14)–(17). The interaction coefficients are functions of the interatomic energy function W divided by the Boltzmann constant k_B and the temperature T .

$$\varepsilon_{ij} = \frac{W(r_{ij})}{k_B T \ln 10} \quad (14)$$

The interatomic energy function is calculated as a function of the distance between neighboring protonation sites r , the permittivity of the medium $\varepsilon_0 D_w$, and the Debye length κ .

$$W(r) = \frac{e^2}{4\pi\varepsilon_0 D_w} \cdot \frac{e^{-\kappa r}}{r} \quad (15)$$

The Debye length in an electrolyte is calculated as a function of the permittivity and the ionic strength I .

$$\kappa^{-1} = \sqrt{\frac{D_w \varepsilon_0 k_B T}{2 N_A e^2 I}} \quad (16)$$

The ionic strength is calculated through the current instantaneous concentrations of charged species through Equation (17).

$$I = \frac{1}{2} \sum [X]_i z_i^2 \quad (17)$$

Through this method, dendrimer pKas are constantly updated as a function of their level of protonation.

The dendrimers considered in this paper typically contain over a hundred titrable amines. For simplicity in calculating the interaction equations, several assumptions are applied. The dendrimer is assumed to be roughly spherical in shape. This allows the polymer to be divided into three shells as depicted in the cross sectional schematic (Figure 3). The outer shell contains the exterior primary amines, which are typically fully protonated at extracellular pH levels (pKa $\approx$ 9–11). The two internal shells contain the titrable tertiary amines, which aid in buffering the endosome (pKa $\approx$ 6–7) and generating the required osmotic pressure. The numbers of these amine types for each proton sponge considered are listed in Table 1, which contains all of the data required for the simulation of each sponge in

the model. The volume of each shell is calculated and average distances between sites inside the shell are calculated. Distances from the middle of each shell to the neighboring shells are also calculated. Rather than model each protonation site individually, all of the sites in each shell are assumed to share the same general conditions such as %protonation, pKa value, distance between neighboring sites, etc. Since the interaction potentials are distance dependent and decrease rapidly as the sites move further away, only the nearest neighbors are considered for protonation site interactions. Finally, it has been shown that dendrimers expand in volume when protonated. Molecular dynamics simulations of PAMAM molecules show that the expected change in radius from low protonation to full protonation is roughly 50% [32]. The radius is scaled accordingly as a function of protonation level and the original sponge radius throughout the simulation. This allows for an estimation of the dendrimer's changes in geometry as it protonates.

The resulting combination of the dendrimer buffering equations allows for the simulation of a combination of titratable amines. This provides the framework for testing the buffering effect in endosomes. As summarized in more detail below, the foregoing method was employed for several dendrimer cases. However, for the PAM-DET sponge discussed in the validation studies, a simple modification must be made. The primary amines of this sponge exhibit a double protonation effect, with two distinct pKa values. When the pH approaches the second pKa value, the amine is able to doubly protonate. For this purpose, Equation (13) is calculated twice for all neighboring protonation sites (once for the initial level of protonation and once for the double protonation) and both levels of protonation are stored as separate values (initial protonation and secondary protonation) within the model.

3.0 Results and discussion

Before the model may be employed to project the viability of the proton sponge hypothesis the system of governing equations must be calibrated and the resulting predictions validated. This section is therefore broken into Calibration, Validation, and Projection subsections.

3.1 Calibration/validation

The model of the endosome behavior alone has been previously validated [13], leaving the sponge buffering behavior and sponge-endosome interaction as points of interest. Sponge buffering, which corresponds with the first step of the proton sponge effect (Figure 1), will be validated through comparison with Jin et al.'s studies on sponge buffering [30]. The interaction between the sponge and the endosome, which correspond closely with the Cl⁻ accumulation and osmotic swelling steps (Figure 1), will be validated through comparison to Sonawanes' studies on chloride flux and volume expansion [7].

Several sponges will be considered during this process. A full list may be seen in Table 1, including information on the reason for their recreation in the model. The sponges selected for consideration cover a broad range of mixtures of primary and tertiary amines, with a few additional validation cases taken from the literature for comparison. In a few cases complete calibration information on the sponge utilized was not available in the literature, and values were extrapolated from other data sets. For PLL, a repeating dendrimer structure does not exist so the sponge was modeled as a concentration of titratable amines per volume with a similar density to the PAMAM case. The first step for buffering capacity validation was completed through comparison to the research of Jin et al. [30]. The validated sponge behavior was then carried over to the endosome model where data from Sonawane was used to explore the incoming chloride flux and the resulting osmotic swelling.

3.1.1 Validation step 1 - buffering capacity—The assumptions and equations in the interaction site model (Equations (11)–(17)) were validated using dendrimers tested by Jin et

al. [30]. The model conditions are set to account only for incoming protons to simulate a simple buffering phenomenon – protons are introduced to the system at a gradual and constant rate and the relation between the internal pH and the total incoming protons is tracked. The rate of introduction is slow enough to ensure quasi-equilibrium, resulting in a time-independent pH-proton relationship. The concentration of polyamines is estimated through varying the mass of PAM-DET until the intercept at an internal pH value of four is roughly equivalent, which corresponds to the lowest value recorded by Jin et al [30]. All subsequent cases for the buffering validation utilize the same mass of dendrimer (grams/mm³) that was obtained from matching the intercepts at pH = 4.0.

Because the purpose of Equations (11)–(17) is to project dendrimer buffering, this effect needs to be distinguished from any buffering inherent to the endosomal fluid. For this validation study, the inherent fluid buffering coefficient was taken from the initial slope of the PAM-OH curve. This is because PAM-OH does not contain primary amines, which ensures that the slope of the line at the onset of acidification for the PAM-OH case as reported by Jin et al. [30] matches the innate buffering capacity of the fluid (value C_{buff} in Equation (6b)).

The predicted buffering behavior of the model is a near-exact match of the experimental data reported by Jin et al. [30] (Figure 4). Moreover, inclusion of double protonation in the model for the PAM-DET sponge produces a marked increase in buffering ability, suggesting that the postulated double-protonation effect discussed by Jin et al. [30] is the phenomena responsible for the increase in buffering capacity and the resulting increase in transfection efficiency.

The remaining dendrimers considered in this report (Table 1) are assumed to be appropriately characterized by Equations (11)–(17). These equations assume that the dendrimer geometry is roughly spherical with evenly spaced titration sites, where the primary amines are located along the outer shell and the tertiary amines are contained at the interior shells. This is based in part on the validation established for the PAM-DET, PAMAM G4, and the PAM-OH cases. For the PAMAM and PLL cases considered for the endosome-sponge interaction, it is assumed that the molecular weight to protonation site relation is equivalent to the dendrimers taken from the studies of Jin et al [30]. This assumption is also one of necessity as experimental parameters for these cases are elusive.

3.1.2 Validation step 2 – pH changes, chloride flux and endosome expansion

—Next the interaction between the dendrimer (Equations (11)–(17)) and the endosome (Equations (1)–(10)) must be examined and validated against experimental data. Validation of this aspect of model predictive capability is considered in comparison to the experimental report of Sonawane et al [6]. Table 2 summarizes the calibration of the model, where parameters derive largely from earlier theoretical studies of endosomes [13]. The values presented in this table have been taken from experimental studies where cited, and the variable values are constrained to ranges that match experimental data. No physically unreasonable values have been employed during the calibration process. For the following studies the modified Cl^- concentration (35 mM) will be employed to match the experiments performed by Sonawane et al.

Calculations were performed to simulate time-dependent changes of protonation of polyamines, pH, chloride accumulation, and volume expansion in endosomes simultaneously. This allowed for direction comparison to the three expected stages of the proton sponge hypothesis (Figure 1). Two dendrimers were considered for these simulations: PAMAM (second generation) and poly-L-lysine (PLL) with inputs taken from Table 1. It is noteworthy that the results are reported in *percent* of total time. This enables

comparative inspection of appropriate trends, especially as they relate to later figures. In particular, the Sonawane et al. experimental conditions result in an expanded test time of ~75 minute test which is ~3X (or more) longer than time to endosomal liposome fusion.

Overall the model's predictions mirror the proton sponge effect steps (Figure 1). Experimental and model-predicted changes in pH, $[Cl^-]$ and volume are summarized in Figures 5–7. Initially PAMAM (second generation) provides a buffering capacity by absorbing incoming protons. This leads to an increase in the membrane potential, which in turn causes an inwards diffusion of chloride. The accumulation of trapped protons and chloride then leads to an expansion through osmotic pressure. While the PLL case does not include considerable sponge protonation due to the lack of tertiary amines, chloride will continue to diffuse into the endosome as the pumps work to acidify the contents. This is most likely an over estimation, as the high chloride concentration gradient offsets the membrane potential in an actual endosome.

While it appears that the PLL case has a faster increase in internal chloride, it must be noted that the measurement is in mM, and is therefore volume dependent. The overall number of chloride ions for PAMAM is much higher in comparison.

As an aside, the total volume measured during the experiments of Sonawane et al is much higher than the expected values for endosomal burst which may be due to non-spherical geometries in the experimental case. A burst criterion of 5% areal strain will be enforced for the following projections.

3.2 Projections of validated model

With the validation steps completed, the model may be used to examine the cases of interest mentioned earlier; specifically the seemingly contradictory reports of Funhoff – with some instances of no proton sponge effect, and of Jin et al. – with apparent strong support of this effect for the double protonating PAM-DET. For these projection studies the values shown in Table 2 will be employed to closely simulate *in vitro* conditions, and the internal chloride concentration will be set to the unmodified value of 116 mM.

Because endosome burst is a necessary outcome of the proton sponge effect a means to estimate the likelihood of burst is required. Literature defines the critical area strain for membranes to be 5% [37], which suggests a more or less fixed burst criterion. While the cells observed by Sonawane et al.'s exhibited considerably higher strain before rupture, the relative sponge performance will still offer insights into their respective abilities. Thus, in reality the point of failure may vary considerably with 5% offering a representative median value. In this simulation, while still reported in % of total time to enable comparison, the total timescale will be shortened to 15 minutes, which is a more realistic time frame in intracellular events compared to the longer period (e.g. 75 minutes) from the experimental work[6].

Three sponges were selected for direct comparison. Consider first the implications of introducing in the model a dendrimer akin to that of the Funhoff et al. study. This study reported a minimal increase in transfection efficiency when utilizing a polyamine with pKa values of 9.5 and 5.5 (primary and tertiary amines). Based on this the authors raised concerns about the validity of the proton sponge effect[12]. The sponge was recreated in the model, and the volume plots compared to the other sponges. Multiple variations of similar sponges were considered for Funhoff's study. For our comparison pDAMA5 was selected, with a particle size of 105 nm and an estimated 59 amines based on the reported MW. For comparison the PAMAM (4th Generation) and PAM-DET cases were also considered. PAM-DET is considered for comparison as it provided the highest level of expansion in

Sonawane's comparisons[6], and 4th generation PAMAM is selected as it has been validated within the model against Jin's results [30]. The PAM-DET case is of particular interest, as it demonstrated the highest buffering potential between the pH values of endocytosis, between 7.4 and 5.5; it is therefore selected for establishing a requisite quantity for burst.

The minimal polyamine concentration required to reach 5% critical area strain within 15 minutes was found to be roughly 1.8 mg/ml for the PAM-DET case. Because high concentrations of polycations have been known to be cytotoxic it is subsequently considered appropriate to compare the cases based on this fixed quantity. While this concentration may be alarmingly high, the exposure to the cell is much less, given that the volume of the endosome is much smaller than the volume of the cytoplasm. The predicted volume expansions and pressures (Figures 8, 9) agree with the observations reported by both Funhoff et al. and Sonawane et al. Taking 5% critical areal strain as a mean representation for likelihood of burst, the critical areal strain is represented as a shaded region centered on the 5% area strain discussed previously. While PAMAM is predicted less likely to burst than PAM-DET, it displays a reasonably close approach to critical area strain, and depending on experimental error bars and *in vitro* variations may sometimes display burst. On the other hand, pDAMA5 is predicted to be unlikely to approach critical area strain, which is in agreement with experiment. If the defined 5% threshold is insufficient for burst, the PAM-DET sponge still remains the most suitable candidate for enhancing gene delivery.

These results suggest that the PAM-DET sponge is the most effective sponge for inducing endosome burst out of the group considered as PAM-DET increases transfection efficiency through a sizeable increase in volume expansion and clear probability of burst. This is in agreement with the literature[12, 30], and further these results are supportive of the potential for optimizing dendrimers for buffering as described by the proton sponge effect.

Another point of interest is the geometry of the sponge itself. A case was considered where the radius of the PAM-DET sponge is doubled to imitate a branched system rather than a cluster. For this case the model predicts an increase in volume (Figure 10). While an intriguing prediction, this does not indicate that branched is always better for increasing transfection efficiency. Increasing the dendrimer radius increases the distance between the protonation sites, which results in lower levels of site interaction. In cases where the site pKa values are above the desired range of 7.0-6.0, a more compact structure is preferred as it will increase the site interaction and reduce the pKa values as they protonate. In cases where the initial pKa of the sites are already in the ideal range, it is preferable to avoid interaction through a more branched structure. The internal protonation sites for PAM-DET are already within the desired range with an initial pKa of 6.7, so the increased radius reduces the site interactions and consequently increases the buffering ability. This is in agreement with the observation by Llamas[38] that the buffering potential of the sponge does not directly correlate with the radius. Thus dendrimer geometry is expected to play a role, but how it is manifested is not necessarily straight forward.

The final point of interest is the total level of acidification prior to endosome burst. This is examined through plotting the endosomal pH against the endosomal volume (Figure 11). The shaded region indicates the point at which the areal strain will surpass the defined threshold of 5%, and the predicted pH value at this point is roughly 6.3.

4.0 Conclusions

The model presented here has been calibrated and validated for sponge buffering and subsequently for the interaction of this effect within an endosome. The combined effects were compared against experimental data sets that supported as well as those that challenged the tenets of the proton sponge effect; the predictions agree with both sets of experimental

data, and are simultaneously supportive of the proton sponge effect. Further, the model was used to determine the effectiveness of various dendrimers for gene delivery. The predicted results indicate that among the cases considered – *viz.*, PAM-DET, PAMAM, and pDAMA5 – that PAM-DET is predicted to be most effective for inducing endosome burst.

Specifically, the simulation shows that polymer pKa is a critical parameter when attempting to induce endosome burst. An amine with a high pKa will be protonated prior to endosome entry and will not have a high change in protonation rate as the endosome acidifies, while a sponge with a somewhat lower pKa will be unable to yield significant expansion before the proton diffusion term is equal to the proton pump flow, causing the pH plateau and limiting further expansion. The geometry of the polymer itself does not directly correlate with increased performance. While increasing the polymer radius will in certain cases increase the possible volume expansion, there is not a straight forward relationship. The initial polymer pKas play a major role in determining the ideal volume. The polymer protonation rate does not play a major role as the lower reaction rates appear sufficient to buffer incoming protons via the ATPase pumps. All of this indicates that a polyamine with pKas within the range of 7.25-6.25 (post site interactions) are favorable for enhancing endosome burst. Taken together, these results indicate that effective dendrimers should contain amines with tandem pKas, one between 10 to 8 for binding and condensation of nucleic acids, and another between 7.25 and 6.25 for efficient induction of endosome burst.

This research offers a unique mathematical explanation of the behavior of dendrimers, and offers insights into the boundary conditions in designing polyamines intended for enhancing endosomal drug release. This methodology may be employed to construct suitable dendrimers for gene delivery, and inform the user of the dendrimers expected performance.

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Figure 1.

Proton sponge hypothesis. Step 1: Protons are pumped into the endosome and trapped by the sponge. Step 2. Trapped protons raise the membrane potential and cause additional Cl^- to diffuse into the endosome. Step 3. The combination of increased concentrations raises the osmotic pressure and leads to expansion and eventual burst.

Figure 2.
Sketch of the generalized cellular model with the lipid bilayer and embedded protein transporters.

Figure 3. Geometry simplifications. The dendrimer is represented as a series of shells with primary amines on the outer shell and tertiary amines along the interior.

Figure 4. Sponge buffering plots. These results are created using equations 11–17 along with the data from table 1.

Figures 5 – 7.

Comparison of predicted values with experimental values [6]

Figure 8.

Predicted endosome expansion with varying sponge type. The dashed horizontal line corresponds to the 5% critical areal strain.

Figure 9.
Predicted osmotic pressures with varying sponge type.

Figure 10. Predicted volume increases with addition of modified PAM-DET with doubled dendrimer radius. The dashed horizontal line corresponds to the 5% critical areal strain.

Figure 11. Endosomal pH vs. endosomal volume. The dashed region indicates the expected burst threshold.

Table 1

Proton sponge simulation values.

Polymer	Modeling Purpose	Calibration Source	pKa's	MW	Primary Amines	Tertiary Amines	Diameter
PAMAM G4	Mass Calibration, Sponge Validation	[30]	9.2, 6.7	14214	52	128	40
PAM-DET	Double-Protonation Validation	[30]	9.2, 6.7, 6.0	16428	52	178	45
PAM-OH	Fluid Buffering Calibration, Sponge Validation	[30] [*]	6.7	12000	0	78	35
PLL	Endosome-Sponge Validation	[6, 30] [*]	10.53	N/A	N/A	N/A	N/A
PAMAM G2	Endosome-Sponge Validation	[6, 30] [*]	9.2, 6.7	7107 [*]	26 [*]	64 [*]	20 [*]
pDAMA5	Comparison against Funhoff Predictions	[12]	9.5, 5.5	5000	17	42	105

^{*} Extrapolated from text

Table 2

Endosomal simulation values. Internal specifies endosomal, external specifies cellular.

Name	Value	Source
External Ph	7.4 mM	Alberts et al [33]
Internal pH	7.4 mM	Alberts et al [33]
External Cl ⁻	4 mM	Alberts et al [33]
Internal Cl ⁻	116 mM	Alberts et al [33]
Mod Int Cl ⁻	35 mM	Sonawane [6]
External Na ⁺	12 mM	Alberts et al [33]
Internal Na ⁺	145 mM	Alberts et al [33]
External K ⁺	139 mM	Alberts et al [33]
Internal K ⁺	4 mM	Alberts et al [33]
Osmotic Coefficient	0.73	Grabe-Oster [17]
k _{NaKATPase}	30 pA	Grabe-Oster [17]
# of Na ⁺ K ⁺ ATPase	100	Variable
k _{HATPase}	100 pA	Grabe-Oster [17]
# of H ⁺ ATPase	100	Variable
Ini Membrane Potential	90 mV	Fournier [9]
Membrane Thickness	5.2 nm	Standard
Temperature	321 K	Body Temp
Capacitance	0.1 μ F/cm ²	Standard
Shear Modulus	2.5 μ N	Henan [29]
Water Permeability	0.052 cm/sec	Lencer et al [34]
Proton Permeability	0.67E-3 cm/sec	Van Dyke-Belcher [35]
Chlorine Permeability	1.2E-5 cm/sec	Ladinsky et al [36]
Endosome Radius	0.5 μ m	Sonawane [6]
Sonawane Sponge Density	7.85E-02 pg/endosome	Calibration [6]
Extended Sponge Density	1.8 mg/ml	Calibration