Canadian Journal of Chemistry
Revue canadienne de chimie

**Successive Outermost-to-Core Shell Directionality of the
Protonation of Poly(Propyl Ether Imine) Dendritic Gene
Delivery Vectors**

Journal:	<i>Canadian Journal of Chemistry</i>
Manuscript ID	cjc-2017-0153.R1
Manuscript Type:	Article
Date Submitted by the Author:	01-May-2017
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Is the invited manuscript for consideration in a Special Issue?:	Dendrimers
Keyword:	Buffering capacity, Dendrimers, Gene delivery, Ising model, Polycations

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1 **Successive Outermost-to-Core Shell Directionality of the Protonation of**
2 **Poly(Propyl Ether Imine) Dendritic Gene Delivery Vectors**

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Draft

Abstract. The protonation behaviour of polycationic compounds have direct relevance to their ability to condense and deliver nucleic acids. This report pertains to a study of the protonation behaviour of polycationic poly(propyl ether imine) (PETIM) dendritic gene delivery vectors, that are constituted with tertiary amine core moiety and branch sites, n-propyl ether linkages and primary amine peripheries. The ability of this series of dendrimers to condense nucleic acids and mediate endosomal escape was studied by unravelling the protonation behaviour of the dendrimers aided by pH-metric titrations, ^1H and ^{15}N NMR spectroscopies. The results demonstrate protonation of the primary and tertiary amines of outermost-to-core shells occurring in a successive step-wise fashion, in contrast to other polycationic vectors. Theoretical calculations based on the Ising model rationalize further the finer details of protonation at each shell. The protonation pattern correlates with the endosomal buffering and nucleic acid condensation properties of this PETIM based dendritic gene delivery vectors. The study establishes that the protonation behaviour is a critical and essential parameter in order to assess the gene condensation and delivery vector properties of a polycationic compound.

Keywords: Buffering capacity; Dendrimers; Gene delivery; Ising model; Polycations

1 Introduction

2 Synthetic vectors are important tools in order to achieve successful gene delivery, by
3 overcoming various physiological barriers.¹ Nucleic acid condensation by a vector and
4 subsequent endosomal escape of the complex are tunable through site-specific modifications of
5 the synthetic vector. Few examples are the cyclodextrins,² triazines,³ histidine, imidazole,⁴ poly-
6 lysine⁵ and cations⁶ that facilitate increased nucleic acid condensation and endosomal escape.
7 Determining the effects of chemical modifications of a synthetic vector is optimal and provides
8 invaluable information about the gene transfection properties of a synthetic vector. Identifying
9 the protonation behavior of a cationic vector is important in this context. Whereas theoretical
10 studies based on molecular dynamic simulations have highlighted the importance of protonation
11 behavior,^{7,8} only few reports concerning the development of synthetic polycationic vectors for
12 gene delivery consider this important aspect. Earlier studies established the protonation of
13 polycationic poly(amidoamine) (PAMAM) and poly(propylene imine) (PPI) dendrimers,^{9,10} as
14 well as, linear and branched polyethylene imine (PEI),⁹ chitosan,¹¹ poly(acrylic acid) (PAA) and
15 poly(methylacrylic acid) (PMAA) polymers.¹² The studies demonstrate that protonation of
16 cationic sites influence polymer properties, such as, protein aggregation,¹³ host-guest
17 interactions, gene delivery,^{10,12} catalysis,¹⁴ molecular sensing and controlled drug release.^{15,16}

18 The PETIM series of dendrimers is characterized by tertiary amine branch sites, ether linkages,
19 *n*-propyl spacer and primary amine at their peripheries.^{17,18} Systematic molecular dynamics
20 simulation studies of the structure of PETIM dendrimers illustrated a flexible structure, due to
21 the presence of *n*-propyl spacers with ether linkages.^{19,20} Furthermore, PETIM dendrimers
22 exhibit profound gene vector abilities²¹⁻²³ and hence, a study of the protonation behavior of this
23 series of dendrimers is important. An assessment of the protonation pattern in PETIM

dendrimers was undertaken through the techniques of pH titrations, ^1H and ^{15}N NMR spectroscopies and theoretical calculations. The mechanism of protonation provided insights to the nucleic acid condensation properties of the dendrimers. The study was extended further so as to determine the buffering capacity of the dendrimers relevant to the endosomal pH range of 4-6.

Experimental Section

Amine functionalized PETIM dendrimers of generations 0, 1, 2 and 3 were synthesized according to procedures reported previously.^{17,18,22} Aqueous stock solutions (4 mM) of the amine terminated dendrimers of each generation were prepared by dissolving required amounts **G0, G1, G2** and **G3** dendrimers in 10 mL MilliQ water. Aq. NaCl (1M) stock solution and aq. solution of HCl (0.3 M) were also prepared in MilliQ water. Solutions for titration, at a final concentration of 16 mM of amine in the presence of 150 mM NaCl, were prepared to a final volume of 5 mL by diluting the required volumes of dendrimer and NaCl in MilliQ water.

pH titrations. The pH titration of the dendrimer solutions with aq. HCl was carried out using a glass electrode, Ag/AgCl reference electrode with a salt bridge. Prior to the titration experiments, the instrument was calibrated using standard buffer solutions (pH 4.008, 50.0 mM K_2HPO_4 ; pH 10.012, 25.0 mM Na_2CO_3 plus 25.0 mM NaHCO_3). All the titrations were carried out at 25 ± 0.2 °C. Aq. dendrimer solutions at a final concentration of 16 mM, in the presence of 150 mM NaCl, were prepared and the initial pH of the solution was recorded. The solutions were then titrated with aq. HCl solution (10 μL) and pH was recorded after 60 s equilibration of the solutions. The titration was continued until pH 3. The readings obtained were analysed using the software Origin 8. All the titrations were carried out in triplicates and back titrations were performed, in order to validate the reproducibility of the pH measurements.

NMR titrations. For NMR titrations, a solution of each dendrimer (16 mM) was prepared in D₂O (450 μ L) and ¹H NMR spectra were recorded at 400 MHz with 1 s delay and accumulation of 4 scans per sample. The dendrimer solutions were then titrated DCI (10 N), such that the incremental acid concentration was 0.03 moles per titration. The solutions were homogenized and equilibrated for 60 s prior to recording of ¹H NMR spectra. ¹⁵N NMR spectra were recorded at 40.5 MHz using a 45 s delay time. Due to the low abundance of ¹⁵N nuclei (<0.3%), 10000 scans for **G3(NH₂)₂₄** and 3000 scans for **G1(NH₂)₆** were accumulated for each sample. CH₃NO₂ was used as an external reference for calibration of the ¹⁵N NMR spectra, which was inserted as a sealed sample in the NMR tube. Chemical shift of the nitrogen atom of CH₃NO₂ (δ = 380.23 ppm) was used to calibrate the spectra.²⁴

Results

pH Titrations. PETIM dendrimers, constituted with primary amine functionalities at the peripheries, were synthesized by two iterative Michael addition reactions and two iterative functional group reductions, as described in **Scheme 1**.²² A pH titration of the amine functionalized dendrimers **G0(NH₂)₃**, **G1(NH₂)₆**, **G2(NH₂)₁₂** and **G3(NH₂)₂₄** (**Figure 1a**) was undertaken to determine the pH required for complete protonation of the dendrimer amines. Aqueous solutions of the dendrimer (16 mM) in aq. NaCl (150 mM) were titrated with aq. HCl (0.3 M) and the pH of the solution was measured after 60 s equilibration (**Figure 1b**). A first order analysis of the titration curves indicated the equivalence points to be 6.2, 6.0, 6.5 and 5.8 for dendrimers of generations 0, 1, 2, and 3, respectively. The observed changes in pH of the dendrimer solution, are reversed fully by performing a back-titration with aq. NaOH (0.3 M) and

thus the protonation-deprotonation profiles are fully reversible (Supporting Information Figure S1). The results indicated that PETIM dendrimers are completely protonated at a pH of ~6.

NMR titrations. The protonation of primary and tertiary amine sites were probed further through NMR experiments, for which the methylene protons adjacent to the amine moieties and nitrogen nuclei were monitored by ^1H and ^{15}N NMR spectroscopies, respectively.

A D_2O solution of amine functionalized dendrimer (16 mM) was titrated with DCl and protons of the methylene groups adjacent to the primary and tertiary amines, labelled 'a' and 'b' respectively, were monitored. The solvent residual peak was used as the internal standard. Resulting spectra were plotted as a stack plot and the pH values were assigned from the corresponding acid mole fraction in the pH titrations. NMR titration profiles of dendrimers **G0(NH₂)₃** and **G1(NH₂)₆** are shown in (**Figures 2a** and **3a**). Changes in chemical shifts (δ) of methylene protons adjacent to nitrogens upon addition of DCl are shown as a plot of δ vs the moles of DCl added (**Figures 2b** and **3b**). ^1H NMR titrations of **G2(NH₂)₁₂** and **G3(NH₂)₂₄** and the plot of δ vs moles of DCl added are shown in **Figures 4** and **5**. A sharp singlet peak at δ 3.19 ppm corresponded to residual methanol and remained unaffected during the study. Although methylene protons adjacent to oxygens and internal methylene protons of the propyl spacer group also shifted down-field, major changes were observed prominently in 'a' and 'b' protons, and thus are discussed further herein.

In general, the methylene proton resonances underwent down-field shift of ~0.45–0.5 ppm upon addition of DCl. Analysis of the chemical shifts showed that methylene protons adjacent to the primary amine moiety, namely 'a', resonating at δ ~2.6 ppm, underwent down-field shift immediately upon addition of DCl, followed by shift of protons adjacent to the tertiary amine

1 sites, namely, protons 'b', which appeared initially at $\delta \sim 2.4$ ppm, in all the dendrimer
2 generations. From these analyses, we infer that tertiary amine protonation is initiated at a pH at
3 which the primary amines are completely protonated.

4 ^1H NMR titrations of the corresponding hydroxyl group terminated dendrimers **G0(OH)₃**,
5 **G1(OH)₆**, **G2(OH)₁₂** and **G3(OH)₂₄**, were also performed (**Figure 6a**). These dendrimers do not
6 possess primary amine moieties at their peripheries, unlike amine-terminated dendrimers,
7 whereas the tertiary amine sites are intact in hydroxyl group terminated dendrimers as in the case
8 of **G0(NH₂)₃**, **G1(NH₂)₆**, **G2(NH₂)₁₂** and **G3(NH₂)₂₄**. Since the hydroxyl group terminated
9 dendrimers contain only the tertiary amines, changes in the resonances correspond to the
10 protonation of the tertiary amines only. Accordingly, dendrimer solutions in D₂O (16 mM) were
11 titrated with DCl (10 N) and the resonances of ^1H nucleus was monitored. A representative stack
12 plot of **G1(OH)₆** is shown in **Figure 6b**. The ^1H NMR stack plot spectra of **G0(OH)₃**, **G2(OH)₁₂**
13 and **G3(OH)₂₄** are given in the Supporting Information.

14 The hydroxyl group terminated dendrimers showed one resonance for the methylene groups
15 adjacent to the tertiary amine moieties, namely 'b', at $\delta \sim 2.45$ ppm. Upon the first addition of
16 DCl, an additional peak emerged, accompanied by a net down-field shift of ~ 0.6 ppm. This
17 change continued with further additions of DCl until the peak at $\delta \sim 2.45$ ppm disappeared. This
18 indicated emergence of two types of tertiary amine groups upon protonation. Integration of
19 peaks corresponded to deuteration of peripheral shell tertiary amines (b1) initially, followed by
20 the deuteration of inner shell tertiary amines (b2) (**Figure 6b**). Plots of moles of DCl vs the
21 chemical shift of methylene protons b1 and b2 of **G0(OH)₃**, **G1(OH)₆**, **G2(OH)₁₂** and **G3(OH)₂₄**
22 dendrimers are shown in **Figure 7**, from which sharper sigmoidal transition in the case of inner
23 core tertiary amine moieties than that at the peripheries was observed.

^{15}N NMR titrations. The NMR studies were undertaken further by assessing the ^{15}N chemical shifts of the dendrimers. ^{15}N NMR spectra of the dendrimers in D_2O were recorded in CH_3NO_2 ($\delta=380$ ppm) as the internal standard.²⁴ The dendrimer solutions were titrated with DCl as described previously for the ^1H NMR titration study. Initial addition of 0.01 moles of DCl corresponded to the molar equivalent with respect to the molar equivalent of primary amines, whereas subsequent additions of 0.1 mole of DCl, initiated deuteration of the interior tertiary amine sites. ^{15}N NMR spectra of **G1(NH₂)₆** and **G3(NH₂)₂₄** were recorded and two distinct resonances, corresponding to primary and tertiary amines at ~ 21.5 ppm and 41 ppm, respectively, were followed (**Figure 8**). These chemical shifts were assigned on the basis of the corresponding spectra of hydroxyl group functionalized dendrimers that have only the tertiary amine sites, thereby providing only one resonance at ~ 41.5 ppm. The first addition resulted in a down-field shift in the peak of the primary amines by $\sim 4 - 5$ ppm, with no observable effect on the resonance corresponding to tertiary nitrogens. Subsequent additions of DCl resulted in $\sim 3 - 4$ ppm down-field shift in the tertiary nitrogen resonance, without affecting further shifts in the primary nitrogen nuclei. These observations from ^{15}N NMR experiments thus reiterated a similar protonation behaviour, as assessed by the ^1H NMR titrations, which showed that primary amines undergo protonation initially, followed by protonation of the tertiary amines.

Determination of the degree of protonation. Degree of protonation (θ) was calculated from the experimental pH data using eq. 1.²⁵

$$\theta = \frac{C_x - C_{\text{H}^+} + C_{\text{HO}^-}}{C_N} \quad (1)$$

1 where, C_x , C_{H^+} and C_{OH^-} are the concentrations of added HCl, free H^+ and free OH^- ions in
 2 $molL^{-1}$ calculated at a given pH, respectively. C_N is the total dendrimer amine concentration (16
 3 mM). A plot of θ vs pH for each dendrimer is shown in **Figure 9**. Analyses of results provided
 4 in the figure show that transitions pertaining to G2 and G3 dendrimers are sharper than that for
 5 G0 and G1 dendrimers. In addition to prominent changes at θ values of 0.5 – 0.6, much sharper
 6 transitions occur at θ of 0.6 - 0.85 to G2 and G3 generations, within a pH change of ~ 0.5 unit,
 7 than that for the remaining two lower generation dendrimers, where θ values required broader pH
 8 change of ~ 1 unit. Such a sharp change in pH as a function of θ would involve tertiary amine
 9 sites that are many more for higher generation dendrimers than that for lower generations. Sharp
 10 pH changes with respect to θ values prompted us to account tertiary amine sites in **Figure 9** for
 11 G2 and G3 generation dendrimers.

12 **Determination of pK values.** In order to account for the observed pH transitions and θ values,
 13 pK values of the dendrimer amines were calculated from the experimental data and theoretical
 14 simulations as described below.

15 The pK values were calculated from the experimentally determined θ values using equations
 16 2 and 3.²⁶

$$17 \quad \theta = \frac{1}{N} \frac{\sum_{n=0}^N n \bar{K}_n z^n}{\sum_{n=0}^N \bar{K}_n z^n} \quad \text{for } n = 1, 2, \dots, N \quad (2)$$

$$18 \quad pK_n = \log(\bar{K}_n / \bar{K}_{n-1}) \text{ for } n = 1, 2, \dots, N \quad (3)$$

where, z is the proton activity given by $pH = -\log z$, N is the number of amine moieties in the dendrimer and $\bar{K}_n$ and $\bar{K}_{n-1}$ are the dissociation constants.

For theoretical determination of pK values, the protonation pattern is considered to be regulated by the interaction of the protonation site with the neighbouring groups, in accordance with the Ising model.²⁵ Protonation process leads to amine sites being positively charged, subsequent protonation of remaining amine sites requires consideration of the already protonated sites. This consideration is defined by the pair-interaction parameter (ε) and is based on the site-binding model for polyamine systems, as described by Koper and Borkovec. The parameters are illustrated in **Figure 10**. In the present study, the ε values are used on the basis of the nearest neighbour interaction analysis formulated by Koper and Borkovec for the polyamine systems, including polyamine dendrimers, with the aid of theoretical simulations.²⁵⁻²⁸ Interaction between the outer shell primary amine and the next shell tertiary amine was assigned a value $\varepsilon_1 = 1.05$, whereas the interactions between all the tertiary amines was assigned $\varepsilon_2 = 1.21$, similar to the assignments made to PPI dendrimers reported previously.²⁸ We also included an interaction of dendrimer amines with the linker oxygen²⁹ as $\varepsilon_3 = 0.18$.²⁶ The through-space interactions between the peripheral amine and tertiary amines was assigned a value $\varepsilon_4 = 0.14$, as reported for the PAMAM dendrimers. The pK values using the pair-interaction parameters was determined using equation 4.²⁵

$$pK = pK_0 - \log \frac{N+1-n}{n} - \sum \varepsilon \quad (4)$$

where N is the total number of amines in dendrimer, $n = 1$ to N and ε is the pair-interaction parameter. The pK values for **G0(NH₂)₃** and **G1(NH₂)₆**, having four and ten nitrogens,

respectively, were determined using the pair-interaction parameters. The experimentally derived pK_n values according to equation 3 and that derived theoretically through equation 4 are given in **Table 1**. The experimental and theoretical assessments show that the values are in agreement with each other, with which we infer that the protonations occur stepwise, from outer-to-inner shell of the dendrimer structure. This inference is discussed in detail in a section below.

Determination of buffering capacities. The protonation behaviour of dendrimers provides valuable information their buffering capacities (BC) and ability to effect endosomal escape, relevant to efficient gene delivery.³⁰ The BC were thus derived from the pH titration data of the amine dendrimers using equation 5, as described by Buschmann and co-workers.³¹

$$BC = -\frac{dn_{HCl}}{dpH} \times \frac{1}{n_N} \quad (4)$$

where, n_{HCl} and n_N are the number of moles of HCl added and the total moles of amine in solution, respectively.

A plot of the buffering capacity of the dendrimer vs pH is shown in **Figure 11**. A major observation is that the buffering capacity of the dendrimers increases with increasing dendrimer generation. A high BC indicates that larger amount of acid is required in order to protonate amines at a given pH. At physiological pH, a high BC assumes importance, as it implies the phenomenon of proton-sponge mechanism for endosomal escape.³²

Discussion

The influence of pH-variation on the dendrimer structure, exemplified by phenomena such as ‘swelling-shrinking’ and ‘back-folding’ demonstrate the importance of acid-base properties of

dendrimers in drug encapsulation, delivery and molecular sensing.³³⁻³⁵ Thus, a study of protonation behaviour of polycationic vectors, and hence PETIM dendrimers assumes importance.

The protonation pattern of PAMAM and PPI dendrimers and branched polymers were reported previously.^{26,36} In these studies, the pH or potentiometric titrations were combined further with theoretical treatment using the Ising model. It was concluded that PAMAM dendrimer would undergo a stepwise protonation, in which the first protonation step involved protonation of all terminal primary amine groups ($pK_a \sim 9$), between pH of 7 and 8. This was followed by protonation at pH 3 – 5, of all the internal tertiary amines (pK_a 5.8), with the exception of the core nitrogen. The central core nitrogen was protonated only at very low pH and was calculated to have a pK_a value of 3.5. On the other hand, a detailed study of PPI dendrimers by ^{15}N NMR spectroscopy, potentiometric titrations and simulations showed that all the odd shells protonated in the first instance, followed by protonation of the even shells.³⁶ This protonation behaviour, described as “onion shell” like behaviour, showed that primary and tertiary amines in alternate shells of PPI dendrimer have similar pK_a values. Two-thirds of the nitrogens were protonated at pH 10 and the remaining nitrogens, with pK_a of 6, were protonated at pH ~ 5 .³⁷

The titration of amine moieties of PETIM dendrimers with an acid followed a sigmoidal transition, that confirmed to the characteristic sigmoidal transitions obtained upon protonation of polyelectrolyte solutions, such as, PEI, chitosan and dendrimers.^{6,31} Further, the fully reversible nature of the titration curves, as verified by the back-titration experiments, indicated that dendrimer-dendrimer interactions are negligible.

Having established the protonation of dendrimer nitrogens with acid by pH titration, NMR titrations studies were performed in order to gain insights into the protonation pattern at a molecular level. NMR chemical shifts are sensitive to changes in chemical environment and thus provide a method to assess the protonation pattern. The ^{15}N NMR chemical shifts of PPI dendrimers of generations 1 to 3 were studied earlier to deduce protonation pattern of this class of dendrimer, which showed distinct peaks for nitrogens of each shell. An alternate shell protonation model for PPI dendrimers was proposed on the basis of ^{15}N NMR chemical shifts.³⁶ ^{15}N NMR spectrum of PETIM dendrimers studied herein showed only two peaks, corresponding to the primary amine nitrogens at ~ 21 ppm and tertiary amine nitrogens at 41 ppm. These two chemical shifts indicated two distinct chemical environments, one at the periphery and the second at the dendrimer interior. Each shell nitrogen underwent distinct shifts upon the addition of acid (**Figure 8**). From the observed chemical shifts, it is inferred that outer-shell primary amines are protonated first followed by the protonation of the inner-shell tertiary amines, at pH values as given in **Figure 12**.

Upon increasing the proton concentration of an aq. dendrimer solution, the outer shell primary amines are protonated initially. The protonation state in which all the peripheral primary amines are protonated leads to the formation of a stable species, which is reflected in the plateau at degree of protonation, $\theta \sim 0.6 - 0.7$ in the titration curve (**Figure 9**). This species is found to exist at pH ~ 9 . Protonation of primary amines is followed by the protonation of penultimate shell tertiary amines. The core nitrogen is the last to undergo protonation at acidic pH of $\sim 4 - 4.5$. This protonation pattern was further verified by calculating the pK values from experimental data, as well as, Ising model equations. Calculation of pK values using Ising model required the use of different pair interaction parameters (ϵ). In addition to the interactions

1 between the primary amine and tertiary amines, the ether-amine interactions and through-space
2 interactions between the peripheral amines and tertiary amines were also included to account for
3 the flexibility and back-folding phenomena observed in PETIM dendrimer.¹⁹

4 From the above studies, the protonation behaviour of PETIM dendrimers is summarized as
5 follows: (i) the degree of protonation exhibits a major transition at $\theta \sim 0.45 - 0.6$, indicating a
6 differing protonation behaviour of primary and tertiary amines; (ii) minor transitions at $\theta \sim 0.8$
7 and 0.9 indicate differential protonation of tertiary amines; (iii) protonation of interior amines
8 prior to complete protonation of the preceding shell was unfavourable due to the larger pair
9 interaction energies; (iv) pK values of the dendrimer amines, calculated using the Ising model,
10 are in good agreement with the experimental values and showed a distinct pK value range for
11 nitrogens in each shell.

12 The unique shell-wise protonation pattern of PETIM dendrimers was extended to its
13 applicability as gene delivery vectors. Study of the protonation behaviour of PAMAM
14 dendrimers showed that only the primary amines are protonated at physiological pH. The
15 tertiary amines which undergo protonation at acidic pH were shown to affect the endosomal
16 escape *via* the proton-sponge mechanism.⁹ On the other hand, both primary and tertiary amines
17 in PPI dendrimers participated in nucleic acid condensation due to the alternate-shell protonation
18 pattern.³⁸ The difference in protonation pattern was evident in the gene transfection efficiencies
19 of these dendrimers. Complexes formed with PAMAM dendrimers through interactions with
20 primary amines were easily accessible to transcription factors and resulted in higher gene
21 transfections, in comparison to the tight complexes formed by PPI dendrimer.³⁹ The importance
22 of dendrimer flexibility to the formation of stable complexes was also demonstrated using lower
23 generation modified PAMAM dendrimers for siRNA delivery.⁴⁰

The PETIM dendrimers, **G2(NH₂)₁₂** and **G3(NH₂)₂₄**, showed a good buffering at the pH range of 4 - 6 in the presence of 150 mM NaCl. This observation indicated that amine moieties in PETIM dendrimer of higher generations could efficiently aid endosomal escape, through the protonation of the inner shell tertiary amines. The effective endosomal escape properties of the dendrimer are evident from previous studies, wherein, the addition of the lysomotropic agent, chloroquine did not lead to enhanced transfection.²² It was postulated that the dendrimer amines are sufficient to enable endosomal escape and did not require an additional reagent. The studies of buffering capacity of **G3(NH₂)₂₄** presented in this work help to ascertain this hypothesis. Further, the protonation studies showed that the primary amines, as well as, the penultimate shell tertiary amines of the dendrimer are protonated at the physiological pH and can participate in nucleic acid condensation *via* electrostatic interactions. The increased number of ionic sites would, in turn, require the use of lesser amounts of dendrimer for nucleic acid condensation, in comparison to PAMAM dendrimers with similar number of peripheral primary amine sites.

Conclusion

The influence of protonation of amine moieties of the polycationic vectors in modulating critical properties related to their application in biology is relevant, yet underexplored. The study of the protonation behaviour is a critical parameter to develop a synthetic vector as a suitable gene delivery agent. Whereas theoretical simulations and experimental details of protonation behaviour of synthetic polycationic vectors were studied earlier,^{6,41,42} there are only very few reports that document the direct applicability of the protonation behaviour of dendritic macromolecular gene delivery vectors. In this context, the newly developed poly(propyl ether imine) (PETIM) dendritic gene delivery vectors of generations **G0 – G3** were evaluated herein

for their protonation behaviour. Initial pH titrations show that at pH of ~ 10 , the outermost shell primary amines are protonated. As the pH is lowered, the inner shell tertiary amines are protonated periodically, in a shell-wise fashion. The protonation constant for each of the inner shell tertiary amines decreases progressively between pH 9.5 and 6. The central core amine is the last to protonate, at a pH ~ 4.5 –5. This protonation pattern, where the primary amines are protonated faster, followed by the sequential shell-wise protonation of tertiary amines, is studied in detail subsequently by ^1H , ^{15}N NMR spectroscopies and theoretical calculation of the pK values using the Ising model. The protonation pattern shows a high buffering capacity of higher generation dendrimer, in the pH range of 4 – 6. This high buffering capacity of the higher generation dendrimer, in turn, enables more efficient gene delivery vector properties. The studies presented herein demonstrate the applicability of PETIM dendritic vectors to the nucleic acid condensation and endosomal escape properties at the physiological pH range of 4–8, relevant to the gene transfection protocols.

Supporting Information

Supporting Information is available in ESI format

Acknowledgements

We are grateful to the Department of Science and Technology, New Delhi, India, for the financial support. Council for Scientific and Industrial Research (CSIR), New Delhi, is acknowledged for a research fellowship to AL.

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Scheme 1. Reagents and conditions: (i) t-Butyl acrylate, MeOH, 36 h; (ii) LiAlH₄, THF, 0 °C – rt, 4 h; (iii) Acrylonitrile, aq. NaOH, 48 h; (iv) Raney Co, H₂, H₂O, 70 °C, 2 h, quantitative.^{17,18}

Figure 1. (a) Molecular structures of PETIM dendrimers of generations 0 to 3; (b) change in pH of the aq. dendrimer solution (16 mM) upon addition of aq. HCl (0.3 M).

1
2 **Figure 2.** (a) Stack plot of ^1H NMR (400 MHz, D_2O) spectra and (b) plot of δ vs the moles of
3 DCl added, along with sigmoidal fit (solid lines) for $\text{G0}(\text{NH}_2)_3$.

4
5 **Figure 3.** (a) Stack plot of ^1H NMR (400 MHz, D_2O) spectra and (b) plot of δ vs the moles of
6 DCl added, along with sigmoidal fit (solid lines) for $\text{G1}(\text{NH}_2)_6$.

Figure 4. (a) Stack plot of ^1H NMR (400 MHz, D_2O) and (b) plot of δ vs the moles of DCl added, along with sigmoidal fit (solid lines) for $\text{G2}(\text{NH}_2)_{12}$.

Figure 5. (a) Stack plot of ^1H NMR (400 MHz, D_2O) spectra and (b) plot of δ vs the moles of DCl added, along with sigmoidal fit (solid lines) for $\text{G3}(\text{NH}_2)_{24}$.

Figure 6. (a) Molecular structures of PETIM dendrimers of generations 0 to 3: **G0(OH)₃**; **G1(OH)₆**; **G2(OH)₁₂** and **G3(OH)₂₄**. (b) Stack plot of ¹H NMR (400 MHz, D₂O) spectra of **G1(OH)₆**.

1
2 **Figure 7.** Plots of moles of DCl vs chemical shifts of protons 'b' in (a) **G0(OH)₃**; (b) **G1(OH)₆**;
3 (c) **G2(OH)₁₂** and (d) **G3(OH)₂₄**.

Figure 8. ^{15}N NMR spectra of (a) $\text{G1}(\text{NH}_2)_6$ and (b) $\text{G3}(\text{NH}_2)_{24}$ in D_2O (40.5 MHz): (i) dendrimer without addition of the acid, pH~11; (ii) and (iii) dendrimer sample upon titration with DCl , pH ~ 9 and 4, respectively.

Figure 9. Plots of degree of protonation vs pH.

Figure 10. Pair-interaction parameters used to compute pK values.

Table 1. pK values of $G0(NH_2)_3$ and $G1(NH_2)_6$, obtained experimentally and calculated using the Ising model.

n^*	pK _n values of $G0(NH_2)_3$		pK _n values of $G1(NH_2)_6$	
	Experimental	Ising model	Experimental	Ising model
1	10.14	10.14	10.76	10.77
2	10.10	9.8	10.30	10.28
3	9.26	9.3	10.05	10.02
4	6.96	6.7	9.84	9.87
5	-	-	9.72	9.71
6	-	-	9.61	9.56
7	-	-	8.01	7.96
8	-	-	7.48	7.64
9	-	-	7.00	7.27
10	-	-	5.13	5.02

* $n = 1$ to N , where N = total number of amines in dendrimer

Figure 11. Buffering capacity of PETIM dendrimers of generations G0 to G3.

Figure 12. Protonation mechanism in (a) G0(NH₂)₃; (b) G1(NH₂)₆.

Graphical Abstract