Published in final edited form as:

J Comput Theor Nanosci. 2009 July 1; 6(7): 1430–1436. doi:10.1166/jctn.2009.1189.

Surface interaction and behavior of poly(amidoamine) dendrimers: deformability and lipid bilayer disruption

Istvan J. Majoros^{*}, Christopher R. Williams, Andrew C. Becker, and James R. Baker Jr.
Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan,
Ann Arbor, MI 48109-0533, www.nano.med.umich.edu

Abstract

Dendrimers are synthetically built macromolecules that, through the conjugation of various functional moieties, have become the basis of the emerging field of nanomedicine. However, research is beginning to show that the dynamic interactions between PAMAM dendrimers and cellular lipid membranes can stimulate membrane hole formation and expansion. These membrane disruptions are not unique to dendrimers and are the observed functions of natural proteins such as MSI-78 (pexiganan) and Trans-Activator of Transcription protein (TAT). Membrane interactions can also affect the dendrimers, causing structural deformations and encapsulation within a lipid bilayer vesicle. Acetamide capping of the positively charged PAMAM terminal end groups neutralizes the dendrimer, and many of these effects can be minimized or eliminated. Knowledge gained from these studies will indeed have an impact on the future designs of dendrimer-based nanodevices.

Introduction

Recent work in nanomedicine has been aimed at developing and improving imaging, diagnosis, and drug delivery methods. An integral component in many of these research advances has been poly(amidoamine) (PAMAM) dendrimers—synthesized, precise, branched macromolecules which become more interesting through the attachment of biological substances¹. Imaging contrast agents, cell-specific targeting molecules, and chemotherapy drugs are just three categories of the many molecules that have been experimentally attached to dendrimers. The cell-targeting agents are especially fascinating in that they allow the targeting of the dendrimer to very specific cell types. Through membrane receptor interactions, these targeting molecules signal the cell to begin an endocytotic pathway through which they ingest the targeting moiety and all other molecules to which it may be conjugated.

The construction of a poly(amidoamine) dendrimer results in a very accurate and carefully planned macromolecule, unique in its structure and properties². Dendrimers are typically grown in layers, and the addition of each new layer produces a higher-generation dendrimer. That is, the dendrimer branches, called dendrons, begin with a single functional group attached to an ethylenediamine (EDA) core, forming a Generation 1 dendrimer, or G1. Further addition of functional groups in “layers” creates new generations which have an increase in circumference, surface density, and terminal end groups. This is done through a repetition of the same two-step sequence, growth and activation. The growth phase is a Michael addition and produces a half-generation dendrimer with ester terminal groups. Thereafter, excessive amidation adds a new layer and results in a full-generation, amine-

^{*}Corresponding author: majoros@umich.edu.

terminated dendrimer. Most biological research utilizes either G5 or G7 dendrimers. Both G5 and G7 dendrimers are considered to be roughly spherically shaped molecules with a constant surface density of terminal amine groups to which other molecules are able to conjugate. A larger dendrimer will yield more terminal groups and, therefore, more molecules can form stable bonds with the dendrimer's surface.

Recently, it has been observed that PAMAM dendrimers interact with not only the cell membrane receptors through conjugated targeting moieties, but also with the lipid bilayer of the cell as well. Depending on its size, the dendrimer can either puncture holes in the cell membrane or attach itself to existing disruptions in the bilayer, forming expanded holes in a previously intact membrane layer. This review describes the current studies in dendrimer bilayer disruption and the general significance of these interactions in relation to understanding the various possibilities of material transport across the cell membrane.

Lipid bilayer disruption by PAMAM dendrimers

PAMAM dendrimers' ability to disrupt cellular membranes is dependent on physical and chemical properties, specifically their size and the composition of end groups⁴. The resulting holes can be related to intracellular transport and the passive diffusion of large particles that usually must be actively transported into and out of the cell.

There are three hypotheses regarding dendrimer-stimulated membrane disruptions and their effects. The first hypothesis states that the presence of holes can be determined by the relative amounts of certain large molecules inside and outside of the respective cell membrane. The second is that positively charged, amine-terminated, whole-generation PAMAM dendrimers induce holes in the lipid bilayer of cellular membranes. Finally, the third and possibly most important hypothesis is that neutrally charged acetamide-terminated dendrimers, as opposed to positively charged amine-bound end groups, do not interact with the membrane molecules and therefore do not create holes⁶.

By studying the flow of lactate dehydrogenase (LDH) and luciferase (LUC) out of cellular membranes, Hong, Bielinska, *et al* have tested and supported the three previously stated hypotheses. LDH and LUC, two cytoplasmic enzymes of the KBpLuc and Rat2pLuc cells used in their research, are not normally excreted from their cellular source. The extracellular presence of LDH was quantified using an LDH assay kit and a spectrophotometer to perform ELISA tests. Extracellular LUC was detected through a chemiluminescence assay. In their study, the diffusion of LDH and LUC out of the cellular membrane followed the same general trends of dendrimer concentration-dependent and temperature-dependent release.

Quantifying the percentage of cellular LDH and LUC release led to conclusions about the cell membrane hole formation's dependence on dendrimer generation, concentration, and terminal group, as well as its dependence on temperature. To obtain confidence in these findings it is important to understand that PAMAM G5-NH₂ and G5-Ac are not cytotoxic up to a 500 nM concentration (>90% cell viability); therefore, the release of said enzymes is not due to the lysing of dead cells. This study resulted in some very interesting and conclusive data relating hole formation to terminal end group (-NH₂ vs. -Ac), ambient culture temperature (6°C vs. 37°C), dendrimer concentration (0 – 500 nM), and experimental cell type (KBpLuc vs. Rat2pLuc). All of this data is clearly illustrated in Figure 1.

The data in Figure 1 offer a plethora of information in support of the second and third hypotheses mentioned above. The most conclusive data can be seen by examining the difference between LDH release due to the positively charged G5-NH₂ dendrimers and the neutral G5-Ac dendrimers. At 37°C, both KBpLuc and Rat2pLuc cells showed a remarkable

difference in LDH release between the two dendrimer types; climaxing at a concentration of 500 nM when the G5-NH₂ dendrimers induced an 8- to 12-fold increase in LDH release over the G5-Ac dendrimers. At 6°C, a conclusive difference in LDH release was seen in the KBpLuc cells but not in the Rat2pLuc cells; for both cell types the release of LDH was much less at 6°C than at 37°C. Remarkably, under no circumstance did the G5-Ac dendrimers cause a sizeable increase in LDH release over the baseline release percentage. The percent release of Luc, data not shown, corresponded very tightly with that of LDH under all circumstances.

To confirm that G5-NH₂ was entering the cell through membrane hole formation and not through endocytosis, a cell staining experiment was performed using certain common dyes. The interior of the cells was stained with FITC to examine the release of cytoplasmic contents; the exterior of the cell was immersed in a solution containing Propidium iodide (PI). Positively charged PI is repelled by the polycationic G5-NH₂ and thereby is not expected to enter the cell along with the dendrimer via endocytosis. The data showed a cellular increase in G5-NH₂ concentration and PI intensity and a decrease in FITC intensity. This information is consistent with the hypothesis that the dendrimers formed holes in the membrane, allowing the dyes to diffuse down their concentration gradients⁶.

It has been shown that larger dendrimers have a greater capacity for affecting cell membranes by forming new holes or expanding existing holes. In these studies, supported 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) lipid bilayers were immersed in solutions containing G7, G5, or G3 dendrimers capped by either amine, acetamide, or carboxyl groups^{4,7}. The G7 amine- and carboxyl-capped dendrimers were able to create new holes in the lipid bilayer within a matter of minutes⁴. Even the G7-Ac-capped dendrimers punctured new and expanded existing holes in the DMPC bilayer; effects not observed in lower-generation acetamide-capped dendrimers⁷. As stated earlier in this review, the G5-NH₂ dendrimer expanded existing holes, while the G5-Ac dendrimers exhibited no significant effect on the bilayer. Interestingly, the G3-NH₂ was not able to expand the existing holes in the DMPC bilayer but did absorb around the periphery of the existing holes. The G3 dendrimers formed a layer about 1.5 nm high around the boundaries of the lipid bilayer⁷.

The reversibility of the damaged membranes is fairly acceptable. When G5-NH₂ dendrimers are removed from the solution surrounding a cell, the cell's membrane deformities revert and "reseal" to normal membrane integrity and a normal state. This is consistent with the acceptance that G5-NH₂ dendrimers are not cytotoxic up to 500nM. The toxicity of other variations of the dendrimer functional groups and generation sizes is yet to be heavily investigated.

Dendrimer-filled vesicles

Generation 7 PAMAM dendrimers create holes in lipid bilayer membranes by forming stable bonds with hydrophilic headgroups and forming dendrimer-filled vesicles⁷. These self-assembling vesicles are formed due to several unique characteristics specific to G7-NH₂ dendrimers. G7 dendrimers have the optimal diameter to minimize the free energy of a bilayer lipid vesicle. Mecke *et al.* have mathematically shown that a vesicle, internally stabilized by a G7 dendrimer, is most likely to have an outer diameter of 18 nm. Considering the diameter of the G7 dendrimer, 8 nm, and the thickness of the lipid vesicle bilayer, 4.5 nm⁹, the theoretical density of this structure would be 17 nm. These two estimates of vesicle size correlate nicely with the diameter of observed vesicles; especially considering that the diameter of small unilamellar vesicles (SUV's) normally range from 25–30 nm, well above that of dendrimer-stabilized models. Additionally, The 512 positively charged amine groups

which are packed onto the surface of the G7 dendrimer form a dense layer able to attract and form stabilizing bonds with the hydrophilic headgroups of membrane lipids. In fact, by examining the size and density of the lipid headgroups to the 512 amine groups of the G7 dendrimer, these models show a favorable 3:1 head group-to-amine ratio⁷.

Qualities of the G7-NH₂ dendrimer that facilitate the formation of dendrimer-filled vesicles do not all apply to other commonly used dendrimers. G5 dendrimers are considerably smaller and, with only 128 terminal amine groups, are much less able to form the stabilizing bonds needed to create vesicles. Additionally, acetimide-terminated dendrimers have a lesser charge and are thereby less able to form the stabilizing bonds with the hydrophilic lipid headgroups. These shortcomings of the G5 and the acetimide-terminated dendrimers also explain why they are unable to form holes in the dendrimer bilayer, while the G7-NH₂ dendrimer can.

Membrane thinning and disruption by proteins and smaller molecules

The described interactions between PAMAM-NH₂ dendrimers and lipid bilayers have also been observed while studying other cationic macromolecules. Several macromolecules, including the antimicrobial-protein MSI-78 (pexiganan), Trans-Activator of Transcription protein (TAT), and two inorganic nanoparticles (Au-NH₂ and SiO₂-NH₂) disrupt the membrane of the cells to various degrees¹¹. The effects of these nanoparticles range from areas of reduced membrane thickness to relatively large holes in the phospholipids bilayer³. A common theme amongst these various cases is that membrane thinning and hole formation occur primarily with membrane domains in the liquid-like L_α phase as opposed to the gel-like L_β⁸. Through atomic force microscopy, the composition and integrity of a bilayer lipid membrane as well as the effects of said macromolecules can be visualized.

Antimicrobial peptides, such as MSI-78, are important immune-defense mechanisms of plants and animals. When exposed to a solution containing MSI-78, DMPC bilayers, supported by a mica-surface, exhibit familiar patterns of deformation. The helical peptide components of MSI-78 actually replace the outer layer of lipids in the bilayer, thereby causing a change in the membrane thickness. The pattern of membrane thinning begins as depression-like channels and expands into a network formation until only isolated plateaus of the original membrane thickness remain³. This expansion of existing defects reflects the pattern of expanding hole-formation caused by PAMAM-NH₂ dendrimers.

TAT, a much larger protein than MSI-78, also induces membrane holes and at a much lower concentration than that observed by MSI-78. Simple electrostatic interactions are the source of TAT's high affinity for the negatively charged phospholipids and glycosaminoglycans of the cell membrane. It has been hypothesized that through these interactions the TAT is able to enter and exit the cell and by this process, rearrange the membrane phospholipids. This rearrangement of phospholipids causes not only membrane thinning and hole-formation but also phospholipid inversions. The latest phenomenon was observed by tracking the inner membrane lipid phosphatidylserine (PS) and confirming its presence on the outside of the cell after TAT exposure¹².

PAMAM polymers will also exhibit very similar characteristics to the aforementioned natural proteins when exposed to the lipid membranes. Specifically, G7 PAMAM dendrimers will create holes only in the L_α phase and will have very little to no effect in the L_β phase bilayers. Even the smaller G5-NH₂ dendrimers are able to disrupt the lipid bilayer through interactions that echo those of natural proteins. These observations are due to the electrostatic interactions between the positively charged surface of the dendrimer and the negatively charged components of lipid bilayers. The behavior of these charged natural

proteins further supports the proposed explanations for dendrimer bilayer interactions and explains why acetylated dendrimers do not react with the membranes.

PAMAM deformability at substrate contact

A possible contribution to membrane-dendrimer interaction and some subsequent removal of lipids to form dendrimer-filled vesicles is the deformability property of dendrimers. These molecules are very large and, therefore, their shape can be readily manipulated by interactions with the environment. Additionally the chemical composition of the branch ends has a profound effect on the shape and mechanical behavior of the PAMAM dendrimers. Results from mathematical simulations and experiments with Atomic Force Microscopy (AFM) support each other with the following conclusions.

Recent studies have shown that dendrimers flatten when they come in contact with a solid surface, in this case mica. AFM experiments have shown that the dendrimers lose their optimum spherical form and adopt a condensed, more disc-like morphology⁵. The absorption of the dendrimer onto the mica leads to many of the branches' attaching to the mica surface through hydrogen bonding and Vander Walls forces. Amine-terminated dendrimers have positively charged terminal groups which repel each other and the core of the dendrimer. These forces act together to pull the dendrimer toward the mica surface and expand its area of contact. Observations of acetamide-terminated PAMAM dendrimers showed a similar pattern of flattening and expansion but to a much less extreme. The neutral charge of the acetamide terminal groups does not cause intramolecular repulsion, as charged amine groups do; the branch ends are able to fold in on themselves when they come in contact with the solid surface⁵.

Variations on this experiment have been conducted to examine how environmental factors can affect the deformability of the dendrimers. For example, a reduction in dendrimer-substrate interactions occurs when the substrate is hydrophobic. This resulted in large dendrimer aggregates, rather than individual dendrimers, being observed on the surface¹⁴. The solution in which the surface is immersed will also affect the deformability of the attached dendrimers. For example more spreading of the amine-terminated dendrimers was observed on a dry mica surface as opposed to one immersed in aqueous solution⁵. Additionally an acidic solution protonates the dendrimers, making them stiff and less able to deform and aggregate¹⁴.

Some studies are already applying the understanding of dendrimer deformation to help explain how dendrimer structure can affect function. N. Tomczak and G. Vansco went one step further in examining the deformability of dendrimers by quantifying the Young's modulus of various PAMAM constructs. Using compression mode AFM they were able to determine that a single G5 PAMAM dendrimer has a modulus around 700 MPa. Interestingly, aggregates of G5 PAMAM dendrimers show a much lower modulus of only 150 MPa. The source of this large variation in Young's modulus was not reported and may not be completely understood¹³. The binding avidity of a Generation 5-folic acid (G5-FA) nanomolecule to high affinity folic acid receptors (FAR) on a cell membrane surface has been related to its deformability and FA and FAR densities¹⁵. These theoretical studies have indicated that a G5 PAMAM dendrimer can deform to a disc-like structure with a radius of 4.8 nm and a surface area of 72 nm²¹⁵. This information combined with the density of FAR receptors on a cell's surface has helped to explain the binding efficacy of folate targeted PAMAM dendrimers in ways that are outside the scope of this paper¹⁵.

Conclusion

Recent studies have begun to show that dendrimer membrane interactions are very dynamic and more complicated than previously understood. Images obtained using AFM show that positively charged PAMAM dendrimers can create new and expand existing holes in bilipid membranes. These data coupled with the observation of macromolecule transmembrane diffusion as well as dendrimer-filled vesicles have shed light on the possible advantages and disadvantages of dendrimer-based therapeutics. Certainly an uninhibited disruption of cellular membranes could limit the value of PAMAM dendrimers in medical applications. However, it has been observed that the aforementioned membrane disruptions are naturally and spontaneously reversible and can be limited by neutralizing the dendrimer terminal groups with acetamide.

Understanding the physical and chemical origins of these interactions will advance the ability of researchers to manipulate dendrimer constructs to meet the needs of their specific applications. Several controllable dendrimer parameters such as generation and terminal group composition can be manipulated toward the achievement of desirable membrane interaction characteristics. Additionally, these same parameters can be used to spawn limited adjustments in dendrimer deformability. When designing dendrimer-based nanomachines, these functional properties should be taken into consideration and optimized for each unique application. The field of nanomedicine is relatively young and the research described here is invaluable towards its progression into the next generation of mainstream diagnostics and therapeutics.

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Figure 1.

The dose-dependent LDH release from (a) KBpLuc and (b) Rat2pLuc cells supports the hypotheses that G5-NH₂ dendrimers form holes in cellular membranes while G5-Ac dendrimers do not⁶.

Figure 2. AFM height images of a DMPC bilayer (a) prior to dendrimer exposure, (b) 3 minutes post-G7-Ac exposure and (c) 17 minutes post-G7-Ac exposure illustrate the membrane hole formation and expansion due to G7-Ac, G7-NH₂, and G5-NH₂ exposure⁷.

Figure 3. Schematic generated cross-section of a dendrimer-filled vesicle. Diameter of the G7 dendrimer ~ 8 nm; thickness of lipid double layer ~ 4.5 nm⁴.

Figure 4.

Computer-generated images demonstrate how G2–G5 (a) acetamide-terminated PAMAM dendrimers can fold in on themselves, roughly retaining a spherical form while (b) amine-terminated PAMAM dendrimers spread and flatten due to surface absorption⁵.