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Interactive Design Strategy for a Multi-Functional PAMAM Dendrimer-Based Nano-Therapeutic Using Computational Models and Experimental Analysis

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Abstract

Molecular dynamics simulations of nano-therapeutics as a final product and of all intermediates in the process of generating a multi-functional nano-therapeutic based on a poly(amidoamine) (PAMAM) dendrimer were performed along with chemical analyses of each of them. The actual structures of the dendrimers were predicted, based on potentiometric titration, gel permeation chromatography, and NMR. The chemical analyses determined the numbers of functional molecules, based on the actual structure of the dendrimer. Molecular dynamics simulations calculated the configurations of the intermediates and the radial distributions of functional molecules, based on their numbers. This interactive process between the simulation results and the chemical analyses provided a further strategy to design the next reaction steps and to gain insight into the products at each chemical reaction step.

Keywords

Molecular Dynamics Simulations; Poly(amidoamine) Dendrimers; Multi-Functional Nano-Therapeutics

1. INTRODUCTION

Poly(amidoamine) (PAMAM) dendrimers are highly-branched biocompatible macromolecules with many functional groups on the terminals. Due to the large number of functional groups and the accessibility of the internal branches, PAMAM dendrimers have been utilized as drug-delivery carriers either by conjugating the drugs to the terminal¹ or entrapping them in the interior.² Positively charged amine groups at neutral pH in PAMAM dendrimers have also enabled complex formation with nucleotides, resulting in successful gene deliveries in cells.^{3–6} Recently, our group reported PAMAM dendrimer-based nano-therapeutics the aim of which is use as cancer therapeutics.⁷ Excessive cationic devices, however, have shown little targeting efficiency, mostly because of their nonselective interaction with anionic cell surfaces. Biological experiments showed that optimal folic acid

(FA) targeting was achieved by acetylation, in accordance with computational predictions based on molecular dynamics (MD) simulations.^{7–10} Further systematic chemical studies of the acetylation of PAMAM generation 5 (G5) dendrimers have been performed with several experimental methods.¹¹ Dendrimers have been investigated using various theoretical models and computer simulations, including selfconsistent-field theory,¹² Monte Carlo simulations,^{13–15} coarse-grain MD simulation,^{13–16–17} and atomistic MD simulations.^{18–20} These models and simulations have provided valuable insights into dendrimers which could not have been achieved by experiments alone. Atomistic MD simulations can provide specific molecular information but require considerably more computational time, especially when solvent atoms are explicitly included. MD simulations on PAMAM dendrimers were carried out extensively to find the appropriate parameters for simulating an aqueous condition without explicit water molecules, thereby extending the size of practical simulations.²⁰ However, none of the simulations dealt with actual chemical structures that include the defects encountered in the experimental processes. In this study, we present MD simulations of the intermediate products during chemical synthesis as well as the final products in the process of generating a multi-functional nano-therapeutic based on generation 5 (G5) PAMAM dendrimers in correlation with the chemical analyses in each step. We used PAMAM dendrimer-appropriate parameters shown from the previous study for a fast turn-around time. Functions include fluorescence isothiocyanate (FITC) as an imaging molecule, folic acid (FA) as a targeting molecule for cancer cells, and methotrexate (MTX) as a drug. In order to obtain a realistic number of each functional molecule, the characteristics (the number of primary amines and the molecular weight) of the PAMAM dendrimer were precisely calculated, based on potentiometric titration and gel permeation chromatography (GPC) results. A possible chemical structure was suggested from these experiments. The theoretical and actual structures were compared, based on MD simulations, and a theoretical model was chosen for further simulations, based on the comparison. All MD simulations were performed to the point of obtaining equilibrium-state structures. These “practical” MD simulations provided the interactive design strategy for the next chemical reaction step. The number of each functional molecule was calculated after chemical analyses, based on the actual structure, and was used in the practical MD simulations of the theoretical structure. Density profiles of all functional molecules and primary amine locations for each chemical synthetic process provided the structural changes at different chemical conditions and the availability for the next conjugation steps.

2. MATERIALS AND METHODS

2.1. Materials

All chemicals were purchased from Aldrich Co. and used as received. All solvents were HPLC grade. The PAMAM generation 5 dendrimers were synthesized as described previously²¹ at the Michigan Nanotechnology Institute for Medicine and Biologic Sciences, University of Michigan.

2.2. Potentiometric Titration

Titration was manually carried out using a Mettler Toledo MP230 pH meter and a MicroComb pH electrode at room temperature. A 10 mL solution of 0.1 N NaCl was added to a precisely weighted 100 mg portion of PAMAM dendrimer to shield amine group interactions. Titration was performed with 0.1028 N HCl, and 0.1009 N NaOH was used for back-titration. The numbers of primary and tertiary amines were determined by back-titration.

2.3. Gel Permeation Chromatography

GPC experiments were performed on an Alliance Waters 2690 separation module equipped with 2487 dual wavelength UV absorbance detectors (Waters Co.), a Wyatt Dawn DSP laser photometer, an Optilab DSP interferometric refractometer (Wyatt Technology Co.), and

TosoHaas TSK-Gel Guard PHW 06762 (75 mm×7.5 mm, 12 μm), G 2000 PW 05761 (300 mm×7.5 mm, 10 μm), G3000 PW 05762 (300 mm 7.5 mm, 10 μm), and G 4000 PW (300 mm×7.5 mm, 17 μm) columns. Column temperature was maintained at 25±0.1 °C with a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025 wt% sodium azide, pH 2.72 at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL with an injection volume of 100 μL. The weight average molecular weight, M_w , has been accurately determined by GPC, and the number average molecular weight, M_n , was calculated with Astra 4.7 software (Wyatt Technology Co.) based on the molecular weight distribution.

2.4. Simulation Conditions

Generation 5 PAMAM dendrimers of theoretical and defected structures were built on an Onyx workstation (Silicon Graphics, Inc.; Mountain View, CA) using the Insight II software package (Accelrys, Inc.; San Diego, CA). All primary amines were protonated to simulate pH 7 conditions. After the steepest descent minimization process for 5000 steps, MD simulations were performed at 1000 K for 5 ps followed next by 50 ps runs at 295 K, using a consistent valence force field (CVFF) in Insight II software. The potential energies stabilized much earlier than 50 ps. The total potential energy function for MD calculations is described as

$$U_{total} = U_{bonded} + U_{nonbonded}$$

$$U_{nonbonded} = U_{Lennard-Jones} + U_{Coulomb} = \frac{1}{2} \sum_i \sum_j \{ \epsilon [(\frac{\sigma}{r})^{12} - 2(\frac{\sigma}{r})^6] + \frac{q_i q_j}{D r} \}$$
(1)

where ϵ is the minimum energy of the Lennard-Jones potential, σ is the distance yielding the minimum Lennard-Jones potential, q is the partial charge on the atom, D is the dielectric constant (1 for vacuum), r is the distance between i and j , and i and j are nonbonded atom pairs. We used the dielectric constant D to be r , so that it varies with the distance r (a previous study determined there is no longrange interaction cut-off 20). All the models were performed under these conditions.

Figure 1 describes the overall scheme of the generating multi-functional device. The blue spheres represent the G5 PAMAM dendrimer, showing the terminal groups for emphasis. Starting from the amine-terminated G5 PAMAM dendrimer, partial acetylation (Ac) followed. The remaining amine groups were used for further conjugation of FITC and FA. Two types of conjugation of MTX were used: (1) direct conjugation using an amine group and (2) after hydroxylation, MTX conjugation using a hydroxyl group. Eighty percent acetylated PAMAM dendrimers (G5-Ac(80)) were modeled, using the final configurations of the theoretical and defected models following the simulations. Acetylation sites were randomly selected among 80% of the primary amines and then connected to acetamide to prepare acetylated terminals (Fig. 2(B)). All the models subsequent to this process were similarly prepared, based on the final configuration of the theoretical model; the reaction sites were randomly selected from the easily accessible primary amines of the final configurations of the earlier-step models. The precise chemical structures of the terminal groups of a dendrimer are shown in Figure 2(A–C) and all the chemicals used in the models are shown in Figure 2(D–F).

3. RESULTS AND DISCUSSION

The theoretical structure of a G5 PAMAM dendrimer with an ethylenediamine (EDA) core is shown in Figure 3(A), with 128 primary amine groups on the terminal. However, potentiometric titration and GPC data showed that G5 dendrimers in the real experiments deviated slightly from the theoretical model. As shown in Table I, the number of primary and tertiary amines calculated from the experiments were 110 and 108, respectively. There are

several possible models to account for these “missing arms.” Considering a molecular weight (MW) of 27530 based on GPC measurement, we propose a realistic model in Figure 3(B). This model assumes twelve missing arms corresponding to one generation (Figure 3(C)) and three missing arms corresponding to two generations (Figure 3(D)). The missing arm in Figure 3(C) will result in losing 113 MW, one primary and one tertiary amine, and the missing arm in Figure 3(D) will contribute to 341 MW, two primary and two tertiary amines. This approach provides 2382 MW, 18 primary and 18 tertiary amines less than the theoretical structure.

However, values in Table I reflect a collection of many PAMAM populations; one representative structure may not be enough to provide all generalized features, and it can bias some features. Therefore, we compared the configurations of the theoretical model and a realistic one using MD simulations.

Figure 4 shows the final configurations of theoretical models of a G5 PAMAM dendrimer (A) and G5-Ac(80) (B) after 50 ps MD simulations. Without explicit water molecules, G5-Ac(80) is much more compact than a 100% primary amine terminated PAMAM dendrimer. In terms of chemical synthesis, the distributions of the terminal groups are of importance. We measured the radial distance from the center of the mass of a dendrimer to the terminal nitrogen atom location for all branches. The terminal nitrogen atom density profiles of Figure 4 are shown as blue lines in Figure 5. The atom density profiles of the realistic models of the G5 PAMAM dendrimer and the G5-Ac(80) after 50 ps MD simulations are represented together in Figure 5 as pink lines. Both the realistic models of G5 dendrimer and the G5-Ac(80) show reduced atom density in the outer range of dendrimers. Except for the additional reduction in the radius, 20–30 Å, of G5 PAMAM dendrimer in a realistic model, however, the general shape of the density profiles of the theoretical and realistic models are similar. We decided to use the theoretical model as a base model for G5-Ac(80) to avoid the intrinsic asymmetry in the base model. On the other hand, the number of functional groups attached to the dendrimers after chemical synthesis were obtained based on the realistic model. They are described in Table II and were used in building the model in Figure 1; therefore, x, y, and z are based on the theoretical structure and i, j, and k are based on the experimental data.

The average number of FITC moieties attached to G5-Ac(80) was five (Table II). Figure 6(A) shows the final configuration of G5-Ac(80) after 50 ps MD simulations with the primary amines indicated in blue and red. Red indicates randomly chosen sites for FITC attachment. Figure 6(B) represents the final configuration of FITC attached G5-Ac(80) (G5-Ac(80)-FITC) after 50 ps MD simulations. FITC molecules seem to interact with PAMAM dendrimer, resulting in a more condensed configuration than G5-Ac(80). Among the primary amine sites (blue and red in Figure 6(B)) of G5-Ac(80)-FITC, we selected three red sites for FA attachment. The final configuration of FA-attached G5-Ac(80)-FITC (G5-Ac(80)-FITC-FA) after 50 ps MD simulations is shown in Figure 6(C). FA is available for interaction with receptors. We used two methods for MTX conjugation: amide bonds and ester bonds. MTX conjugation through an amide bond can be achieved directly from G5-Ac(80)-FITC-FA, while that through an ester bond requires one more step of chemical reaction with glycidol to generate hydroxyl groups. Direct MTX conjugation through an amide bond to G5-Ac(80)-FITC-FA (G5-Ac(80)-FITCFA- MTXa) turned out to have five MTX per G5-Ac(80)-FITC-FA. Figure 6(D) shows the final configuration of G5-Ac(80)-FITC-FA-MTXa after 50 ps MD simulations. MTX and FA seem to compete with each other, reducing the accessibility of FA to the outside.

However, after the hydroxyl groups were introduced (Figure 6(E)), the FA moieties seem to expand further outside. A smaller number of MTX (three) than G5-Ac(80)-FITC-FA-MTXa was conjugated to G5-Ac(80)-FITC-FA after this stage. This MTX conjugation through ester bonding to G5-Ac(80)-FITC-FA (G5-Ac(80)-FITC-FA-MTXe) represents a more spherical formation (Figure 6(F)). FA availability to the receptor seems to remain.

For further investigation, the atom density profiles of each moiety from the center of the mass of all nano-devices were calculated. Each atom location was measured from the center of the mass and normalized for all atoms contributing to FITC, FA, and MTX moieties, shown in Figure 7. Green lines represent FITC, pink FA, and blue MTX. The atom density profile of FITC shifted inward from the G5-Ac(80)-FITC (Fig. 7(A)) to the G5-Ac(80)-FITC-FA (Figure 7(B)), while the FA density profile shows a good portion of FA can be accessible to the outside. Similarly, the FA density profile shifted inward from the G5-Ac(80)-FITC-FA (Figure 7(B)) to the G5-Ac(80)-FITC-FA-MTXa (Figure 7(C)), while a good portion of the MTX density extended outside. Interestingly, a significant portion of the FITC extended outside in the G5-Ac(80)-FITC-FA-MTXa, while a large portion of the FITC shifted more inward from the G5-Ac(80)-FITC-FA to the G5-Ac(80)-FITC-FA-MTXa. On the other hand, density profiles of the FITC, FA, and MTX in the G5-Ac(80)-FITC-FA-MTXe are shaped more coherently, as shown in Figure 7(D). The FITC density profile in the G5-Ac(80)-FITC-FA-MTXe occupies a similar radial range as that of the G5-Ac(80)-FITC and is more symmetric. The range of the FA density profile in the G5-Ac(80)-FITC-FA-MTXe (Figure 7(D)) appears slightly farther into the outer radius than in the G5-Ac(80)-FITC-FA (Figure 7(B)), while preserving the outer density. In the meanwhile, the shape of the FA density becomes dramatically symmetric. The MTX density profile in the G5-Ac(80)-FITC-FA-MTXe has a larger inner radius than the FA profile but shares a similar range with the FA density profile after that.

MTX and FA are chemically very similar to each other, but the affinity of FA to the folate receptor is much higher (about a hundred-fold). While the MTX in the G5-Ac(80)-FITC-FA-MTXa seems to restrict the FA from reaching outside, a significant portion of the FA in the G5-Ac(80)-FITC-FA-MTXe remains in the outer range as in the G5-Ac(80)-FITC-FA. In terms of binding to cells with highly expressed folate receptors, the G5-Ac(80)-FITC-FA-MTXe binds better to those cells than the G5-Ac(80)-FITC-FA-MTXa. In our separate biological study using KB cells (a cell line derived from a human carcinoma of the nasopharynx), dose response binding of the G5-Ac(80)-FITC-FA-MTXe and the G5-Ac(80)-FITC-FA-MTXa at 30 minutes was measured, confirming this prediction. In addition to the difference between the amide and the ester bonds in the MTX conjugation, the whole configurations of the G5-Ac(80)-FITC-FA-MTXa and the G5-Ac(80)-FITC-FA-MTXe are different from each other, yielding different biological outcomes.

4. CONCLUSION

It is very important to understand the real chemical structure of a compound in biological applications. We suggested a possible structure of PAMAM dendrimer and studied the differences with the theoretical PAMAM. We combined a theoretical model and a realistic number of functional moieties for MD simulations to obtain possible configurations. Through relatively simple MD simulations performed in the process of chemical syntheses, we could provide if reaction groups are available and assess the possibility of further chemical synthesis. In addition, careful analysis of the simulation results could predict a biological response, which was confirmed in a separate study.

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Figure 1.
Schematics of synthetic strategy to produce a multi-functional device based on G5 PAMAM dendrimer.

Figure 2. Chemical structures used in the models: (A) amine, (B) acetamide, and (C) hydroxyl terminal groups of a dendrimer and (D) Fluorescein isothiocyanate (FITC), (E) Folic acid (FA), and (F) Methotrexate (MTX).

Figure 3.

Chemical structures of (A) a theoretical G5 PAMAM dendrimer with an EDA core and (B) a realistic model based on GPC and the potentiometric titration results in Table I. Defects: arm missing from the fifth shell (C) and the fourth shell (D).

Figure 4. Final configurations of (A) PAMAM dendrimer and (B) 80% acetylated PAMAM dendrimer of the theoretical structural model after 50 ps molecular dynamics simulations.

Figure 5. Atom density profiles of theoretical and realistic models of (A) PAMAM dendrimer and (B) 80% acetylated PAMAM dendrimer. Blue lines represent the theoretical structure, and the pink lines represent the realistic model structure.

Figure 6.

Step-by-step computational models to generate a multi-functional device based on PAMAM dendrimer. Each configuration is the final stage after 50 ps molecular dynamics simulations. Blue color positions represent primary amines which are available for further conjugation. Fluoresceins, folic acids and methotrexates are shown as green sticks, violet spheres, and red spheres, respectively.

Figure 7. Functional group density profiles at each stage of generating the multi-functional device. Fluoresceins, folic acids and methotrexates are shown as green, pink, and blue lines, respectively.

Table I

Theoretical and experimental characteristics of generation 5 PAMAM dendrimer (EDA core).

	Theoretical	Experimental	
		Value	Method
Primary amine number	128	110	Potentiometric titration
Tertiary amine number	126	108	Potentiometric titration
Molecular weight	28826	27530	GPC

Table II

Number of each functional group after synthesis obtained using potentiometric titration, UV, (NMR) and GPC.

Functional group	Number
Fluorescein	5
Folic acid	3
Methotrexate through amide bonding	5
Methotrexate through ester bonding	3