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From sequence to 3D structure of hyperbranched molecules: application to surface modified PAMAM dendrimers

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Abstract The molecular modeling of hyperbranched molecules is currently constrained by difficulties in model building, due partly to lack of parameterization of their building blocks. We have addressed this problem with specific relevance to a class of hyperbranched macromolecules known as dendrimers by describing a new concept and developing a method that translates monomeric linear sequences into a full atomistic model of a hyperbranched molecule. Such molecular-modeling-based advances will enable modeling studies of important biological interactions between naturally occurring macromolecules and synthetic macromolecules. Our results also suggest that it should be possible to apply this sequence-based methodology to generate hyperbranched structures of other dendrimeric structures and of linear polymers.

Keywords Hyperbranched molecule · Dendrimer · Molecular modeling · Molecular dynamics

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Introduction

Dendrimers constitute an exciting class of macromolecules that continue to be studied as part of the nanotechnology revolution [1]. They are an architectural class of hyperbranched synthetic nano-molecules that can be made by sequential reactions that are repeated to give well defined macromolecular structures. Dendrimers are prepared from a starting core; a sequence of 1 to 3 reactions then produces an incremental "generation". Each time the reaction sequence is conducted, the generation increases by one, resulting in a doubling of the number of end groups and an increase in the molecular weight of the dendrimer. Since dendrimers are hyperbranched molecules, the end groups of each branch define the molecular surface of the dendrimer.

Studies using anionic dendrimers have confirmed that (1) their physico-chemical properties are similar to conventional small molecule drugs; (2) they can be modified to exist as zwitterions at physiological pH; and (3) they have considerable buffering capacity, making them physico-chemically "similar" to blood proteins (e.g., albumin), and therefore biocompatible. However, unlike proteins, they do not undergo proteolytic degradation in plasma; are not immunogenic; are not toxic after repeated intravenous administration; can be optimized for their circulation time; and show preferential accumulation in tissues containing inflammatory cells compared to healthy tissue at a ratio of 50:1 [2, 3]. Despite these remarkable protein-like properties, their pharmacological development has been restricted to drug delivery and imaging agents [4].

Unlike proteins, molecular modeling of dendrimers is currently limited because of the lack of modeling tools that are applicable to building and optimizing hyperbranched molecules. Although the morphology of these dendritic structures can be seen as a sequence of interconnected

monomers (Scheme 1), for which a nomenclature has been proposed [5], there are currently no methods to generate 3D structures from their sequence. In this study, we describe a new concept and then develop a method to translate the linear sequence of monomers into a full atomistic model of a hyperbranched molecule.

Our long-term aim is to develop novel methods to computationally model the interaction of dendrimers with biologically important proteins. In the present study, we have used monosaccharide modified polyamidoamine (PAMAM) dendrimers to illustrate both our concept and our methods. These molecules have been reported to have important immuno-modulatory and anti-angiogenic properties [2]. To further understand this biological interaction, it is now necessary to generate 3D models.

The hyperbranched structure of PAMAM dendrimers is derived from a diaminobutane core. Repeating polyamidoamine units form branches that originate from a core. These branches are then capped at the surface (i.e., end groups) with carboxylates. Each of these units can be represented as a monomer in an analogous way to the amino acid residues in proteins (Scheme 1). These dendrimer monomers can then be used to build the macromolecular structure of the dendrimer by starting with the core followed by a step sequence of addition of the repeat units to build up successive generations of the dendrimer. Once the dendrimer generation required has been achieved, the branches can be terminated with the end group monomers.

Topology and parameter data for these monomers were calculated, and XPLOR-NIH [6, 7] was utilized to generate the 3D structures. The preliminary molecular dynamics simulations reported here demonstrate the correctness of these structures. Further extensive simulations of these glycosylated dendrimers were successfully carried out to study their molecular properties and dynamic behavior [8].

This approach can be used more widely to develop parameters for other dendrimers that are derived from different types of units. Such advances in moving from sequence to 3D structure will greatly facilitate the molecular modeling studies of hyperbranched macromolecules. This becomes particularly important for dendrimer molecules with biological properties.

Materials and methods

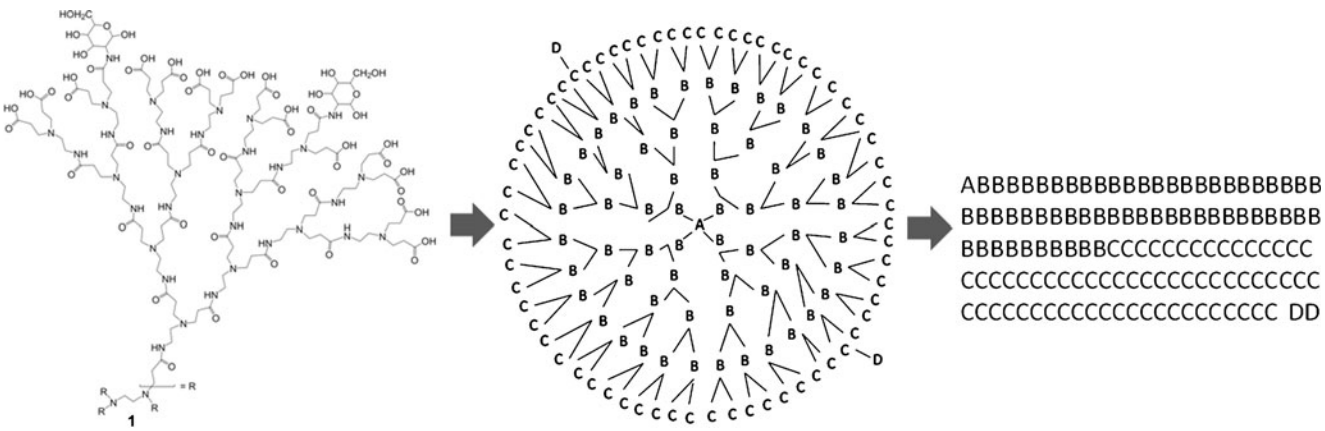
The development of a method to generate 3D models of a dendrimer from a sequence was required in order to be able to study the conformation and properties of glycosylated dendrimers. The parameter, topology and XPLOR input files are presented in the electronic supplementary material and will be available from the author's website.

Monomer assignment

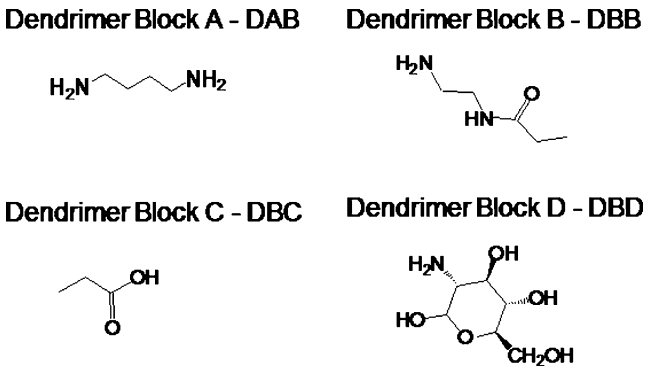
The hyper-branched structure of Starburst PAMAM dendrimers is derived from a diaminobutane core. Three main monomers (Scheme 2) were defined to build the dendrimer structure (Scheme 1): one diaminobutane core monomer (DAB; A), a monomer constituting the repetitive branch subunit (DBB; B), a monomer representing the carboxylated end group at the surface (DBC; C). A fourth monomer, which can be conjugated to some of the carboxylic acid end groups, was also introduced. This monomer was a saccharide selected from glucosamine (DBD; D), N-acetyl-glucosamine (DBE; E) and chitobiose (DBF; F).

Topology of monomers and connectivity

Topology information about building block units was stored in a file that contained the description of the atom types, the connectivity between atoms (where each atom was given a name, type and charge), and the bonds, angles and torsional angles between all atoms in a monomer. The patch defined



Scheme 1 Schematic representation of the dendrimer glucosamine structure. **a** Hyperbranched structure of a quarter segment of the dendrimer; **b** one letter code representation of the subunits; **c** corresponding linear sequence used for 3D model generation



Scheme 2 Monomers assigned for the dendrimer glucosamine used as a model for the development of 3D dendrimer structures. Additional blocks can be found in the electronic supplementary material

how the monomers were linked in the sequence and defined the changes on atom types, deleted bonds and angles, and new bonds and angles formed as monomers were added to the growing dendrimer. Most of the building blocks had at least three places where conjugation between two monomers could occur.

Internal coordinates (IC) for all monomeric units were also defined to aid the generation of coordinates without any additional input. The existing information about dendrimer glucosamine monomeric units [9] was added. The topology information is saved as a topdend22.top (electronic supplementary material).

Atom type definition

The atom types necessary to describe dendrimer monomers were defined by comparing the nature of the atoms in our structure and its environment with those already present in CHARMM22 and CHARMM27 force field [10]. Appropriate names were given to the atom types to add to the force field parameters. However, not all atoms present in the dendrimer structure could be assigned based on similarity, and in these cases, new atom types were defined.

Parameter data generation

This file contained values of all bond lengths, angles, dihedrals, improper dihedrals and non-bonded parameters, and the constants associated with these were defined in terms of atom type rather than atom name. The values were grouped according to atom types and the average value determined and registered in the parameter file. The constants for bond length, bond angle and dihedrals were assigned based on similarity with previous development as well as the non-bonded parameter for each atom type. The files were written according to charmm22 and charmm27 syntax.

The determination of bond length, bond angles and torsion angles involved generating a generation 2.5 dendrimer glucosamine using a Monte Carlo conformational search. An initial structure of the generation 2.5 dendrimer glucosamine was built in Maestro [11]. The first step was to determine the protonation states of ionizable groups with the target pH set to 7 using Ligprep 2.2 [11] and OPLS-2005 force field [12]. Macromodel 9.6 [13] was used for conformational searching with OPLS_2005 force field and an implicitly defined model for water as solvent. The option to distinguish enantiomers was used to preserve defined stereochemistry. The cut-off values were 8 Å for van der Waals interactions, 20 Å for electrostatics and 4 Å for H-bonds. Additionally, 2,500 iteration steps were used to optimize 1,000 generated conformations. Different conformations were saved within an energy window of 5 kJ mol^{-1}.

The output files were saved as files with .mae extension and imported to Chem Bio3D (ChemBioOffice 2008, CambrigeSoft, Cambridge) where a table containing all the bond lengths, bond angles and torsion angles was generated. This table was then exported to Microsoft Excel (Microsoft Office; http://www.microsoft.com) and saved for further analysis. Dimeric combinations of the monomeric units defined for dendrimer glucosamine were built in Maestro [11]. The protonation state was determined using Ligprep 2.2 [11] and a conformational search was performed with Macromodel 9.6, as described above. The output files were saved as .mae for further calculations.

Further structure optimization at higher theory levels was performed with Jaguar 7.5 [11] using the HF/6-31G* method, and selecting water as a solvent with the SM6/PBF algorithm. The properties established were ESP charges and surfaces, and number of orbitals to be visualized were set as HOMO and LUMO +2 and −2, respectively. The output files were saved as .mae and the charges analyzed manually with a Maestro measuring tool menu.

Initially, the dihedral values used for the monomeric units of dendrimer glucosamine were determined using the same method as for bond length or angles. However, these values did not correspond to the syntax used by the force field chosen. The new values were assigned according to Table 1.

Sequence-based generation of 3D dendrimer structures and molecular dynamics simulation

The 3D structure generation was conducted using Xplor-NIH [6, 7] 2.18. The generate.inp was used to generate psf files utilizing the topology file and the sequence of monomers. A reference number for the patch statement was attributed to each monomer of the sequence. The

Table 1 CHARMM dihedral parameters used for dihedrals assignments of the dendrimer monomers (http://www.ks.uiuc.edu/Training/Tutorials/science/forcefield-tutorial/forcefield-html/node6.html)

Dihedral Parameters in CHARMM format			
Multiplicity, n	Phase δ	Location of minima	Notes
1	0	180	Yields trans conformation
1	180	0	Yields cis conformation
2	0	90, 270	
2	180	0, 180	Useful for enforcing planarity
3	0	60, 180, 300	Emphasises staggered conformation of sp3 carbons
3	180	0, 120, 240	Emphasises eclipsed conformation of sp3 carbons

generated psf file was then used as an input for a random.inp file, where random coordinates for all atoms were generated based on the previously defined parameters and a coordinate file (.pdb) was saved. Initial optimization of atom coordinates was achieved by a loop of 500 simulation steps with increasing contributions of van der Waals, and decreasing the temperature from 1,500K to 300K. This set of simulations was followed by a 5,000 step minimization. Resulting pdb and psf files can be utilized for further simulation using XPLOR-NIH [6, 7] or NAMD [14]. Additionally, these output files can be either visualized or converted into other file formats by VMD [15] or OpenBabel [16]. Bond orders or atom types have to be checked after conversion.

Results and discussion

Xplor is a software package designed for computational structural biology. It focuses on 3D structure determination of macromolecules using NMR and X-Ray crystallography data as experimental constraints. This software uses an energy function where arbitrary combinations of empirical, geometric and effective energy terms that describe experimental data can be used [6, 10]. The combined energy function can be minimized by a variety of gradient descent, simulated annealing, and conformational search procedures [6].

One of its main features is the ability to generate a 3D structure from a sequence of monomers defined in the force field topology file. The main advantage of this software for this work is the possibility to apply several patches (i.e., connections) between two or more residues. Although it has traditionally been used to generate structures from NMR and crystallography data, it also allows for the generation of macromolecules where the only constraints imposed are taken from the force field parameter file [6].

Initially, monomeric units for the dendrimers were defined for the glucosamine modified dendrimers (Scheme 2) rather than non-modified dendrimers (i.e., without glucosamine). The geometry and electronic properties of the monomers within the dendrimer are not known because of the absence of experimental structural data on a similar dendrimer type. A set of ab initio and molecular mechanics calculations were conducted on various fragments of the dendrimers to evaluate the geometric parameters of a 3D structure. The resulting values were used for the development of the different force field parameters. CHARMM22 and CHARMM27 force fields were used as reference for the atom type assignment since previous work provides a great diversity of atom types and parameters that are helpful for the development of novel monomers in macromolecules (http://mackerell.umaryland.edu/MacKerell_Lab.html).

Topology and parameter files development

Most force field topology and parameter files found in the literature have been developed for proteins using amino acids as the monomeric units. More recently, additional work has been done with nucleic acids [17], sugars [18] and lipids [19]. The starting point for atom type assignment was to look for similarities between the new dendrimer monomers and those parameters previously developed for proteins, nucleic acids and lipids.

When analyzing the dendrimer's monomeric structures, it is possible to identify some protein-like atom types (Scheme 2); for example, the carbonyl carbon resembles the carbonyl C present in some amino acids such as aspartic acid, asparagine or glutamine. In the sugar monomer (DBD), the nitrogen resembles the peptide nitrogen. All other atoms in this monomer are equivalent to those found online in a sugar parameters file [9].

These parameters were developed for the purpose of creating a flexible set of tools that allowed the generation of 3D models for dendrimers. Thus different cores were tested and the parameters for these were assigned so that, independent of the size of the chain, the same atom types could be used. Using a diaminobutane core as a model, the atoms in the chain were assigned different atom types according to their distance from the terminal nitrogen. Those closest to the NH_3 were assigned as generic aliphatic sp3 for CH_2. All others were assigned as described in a file,

found online, that is specific for lipid chains [9]. Looking at the topology file, there is no significant difference in their charges. However, in the parameter files, this change allows for the definition of different dihedrals.

Nevertheless, some of the atoms, particularly after patching the monomers, present a unique environment; thus, defining novel atom types for these cases became necessary (Scheme 2, block A). This is the case for all terminal nitrogens from the core and branch monomers (DAB, DBB). Scheme 2 block C shows that the nitrogen atom acquires an unusual tetrahedral conformation. In this case, it was assigned as an amide nitrogen, although a different name was given (from the generic charmm22 force field) to allow the attribution of different bond lengths, angles and dihedrals to this case. Another example of this was the oxygen atom on the surface monomer (DBC). Although this is represented as protonated, when Ligprep was run establishing 7 as the target pH, the oxygen atom was deprotonated in solution. After patch, this atom acquired a carboxylate oxygen type configuration. This situation is not found in any of the reference files of Chamm22. Thus, a new atom type was established and fully parameterized using the procedure described below.

Ab initio methods are based on quantum chemistry principles and could be used for the calculation of charges for the force field. The Hartree Fock theory and 6-31G* basis set were used in this study as it was used for the charge determination in the original development of the parameter set. Calculations of individual monomers and conjugated monomers were used to assess the values before and after conjugation. Combinations of the blocks representing fragments of the dendrimer structure were designed in Maestro and a conformational search followed by optimization with Jaguar was performed (Fig. 1).

Bonds length, angles and torsion angles were measured from a generation 2.5 dendrimer glucosamine that had been submitted to a Conformational Search in Maestro (Fig. 2). This is because it was necessary to understand the geometry of monomers and patches within the large molecule environment. Generation 2.5 was used because it was the largest molecule that could be studied by conformational search implemented in Macromodel.

Topology files contain information on the atom types, masses and charges of each atom in a monomer and also the information for applying patches between residues so that a PSF (protein structure) file can be created. In the beginning of the file, a list of atom types and the corresponding masses are listed followed by a description of each monomer. To each atom in the residue, a name, type and charge is given, and the connections between them are described below. Finally, the values of internal coordinates (IC) were described for each pair of four consecutive atoms.

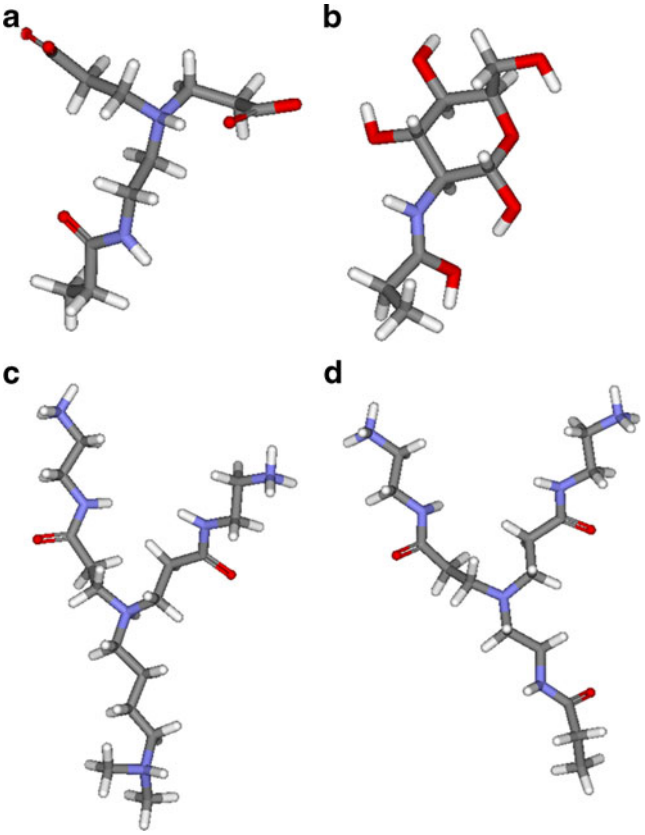


Fig. 1 Monomer combinations submitted to ab initio calculations for atom charge determination. **a** Monomer DBB bound to two DBCs representing the unmodified surface of the dendrimer under study. **b** Monomer DBC bound to DBD representing the glycosylated surface. **c** Monomer DAB bound to two Ch3 groups on one side and two DBB representing the core of the dendrimer. **d** Three monomer DBBs bound together representing the branching ramification of the dendrimer under study

Although these IC values are not essential, they were added to allow calculation of the missing atom coordinates based on coordinates of atoms with known positions. In this study, IC values were assigned based on the conformational search performed on the generation 2.5 dendrimer glucosamine and the constant values based on similarity with the reference files [9]. The importance of adding these values relied on the fact that the structure would be generated by a random method and, since there are no experimental structural data, these constraints were useful to ensure that the correct stereochemistry was achieved.

In addition, information concerning the patch of monomers was also present in this file. All new connections between residues had to be stated for the PSF file as well as those that were removed. Alterations of atom types and charges should also be added so that the correct parameters are given for the PDB (protein data bank) file. The complete topology file can be found in the electronic supplementary material.

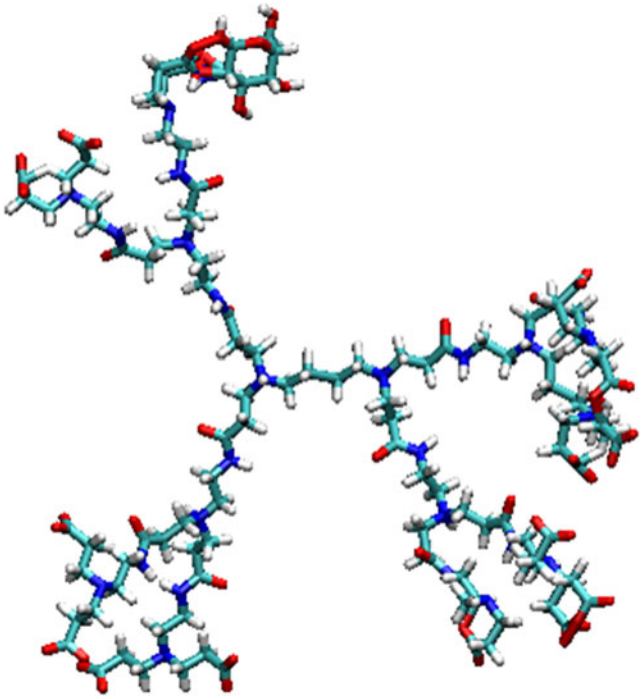


Fig. 2 Dendrimer glucosamine generation 2.5 generated by conformational search using Macromodel

In the parameter file, all descriptors are registered in terms of atom types. For bonds, each entry contains a pair of atoms, a spring constant and an equilibrium length. The formula for the bond potential function is $V = K_b (b - b_0)^2$. For the dendrimer assignments, the value of constants (K_b) was based on the reference files [9] and the equilibrium lengths based on the conformational search performed on the generation 2.5 dendrimer glucosamine.

The second section on the parameter file was bond angles, where all possible combinations of three atom types are registered. Each entry consisted of three atom types, a spring constant, and an equilibrium angle. The potential function for bond angles was defined by the equation $V = K_\theta (\theta - \theta)^2$, where K_θ represented the spring constant and θ the bond angle. The potential functions and spring constants can be calculated accurately by utilizing procedures described elsewhere [20].

The dihedral parameter plays a fundamental role in the structure, being the determinant of the conformations allowing the molecules to be studied. This was one of the most difficult parameters to analyze. Since no structural data were available, it was not possible during the random generation process to add nOe constraints or other experimentally based restrictions; thus the stereochemistry relied to a considerable extent upon the set of dihedral assignments.

The dihedral values were written using Table 1 as a reference (http://www.ks.uiuc.edu/Training/Tutorials/science/forcefield-tutorial/forcefield-html/node6.html), and based on the structure of the generation 2.5 dendrimer glucosamine (Fig. 2).

In the case of the saccharide monomer, this procedure was not followed. A sugar force field file was used as reference and is available online [9]. All dihedrals added were dummy dihedrals. This means that all constant values were set to zero. In this way, the simulation software will not ask for them when generating a molecule and there will be no constraints on setting torsional angles. Additionally, and in the absence of dihedrals, the necessary improper dihedrals were added.

For the core monomer, different atom types were defined so that lipid like dihedrals could be added. In the case of the diaminobutane core, this was not a problem, but when a different core based on dodecane core was generated, problems with the chain conformation were observed. This could be corrected only by the use of parameters for lipids. In specific cases, such as the monomer DBB (Scheme 2), it was necessary to add improper dihedrals (see parameter file in the electronic supplementary material) to maintain the planarity of the O–C–N, which resembles a peptide bond.

The last section of the parameter file concerned the non-bonded interaction terms that are specific for each atom. In this case, the values of the reference files were always used. The complete parameter file can be found in the electronic supplementary material.

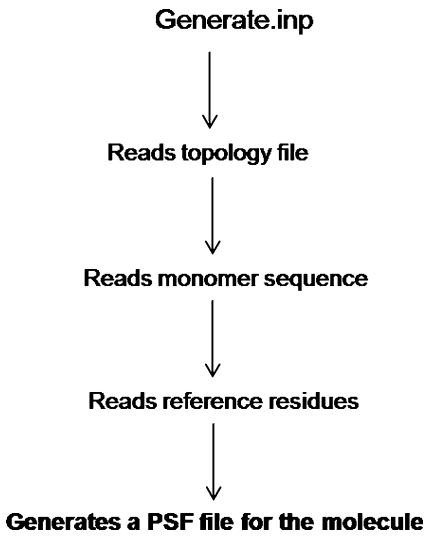
Both parameter and topology files were initially written with the syntax of CHARMM22 force field as this is the force field recognized by Xplor.

Generating the structure with Xplor

The second step in the study was the generation of the dendrimer glucosamine which was used as a model for the method development with Xplor-NIH. As previously mentioned, this was a three step process where PSF file generation (Scheme 3) was followed by generating a set of PDB coordinate files using short simulation and minimization (Fig. 3), and finally the structures were refined using a simulated annealing protocol.

As can be seen in Fig. 3, during the generation of the structure the contribution of non-bonded parameters was slowly increased throughout the protocol. This is done so that the atoms can pass through each other during the process and adopt reasonable conformations. A final minimization was performed so that the most stable structures were generated and saved. In this study, ten structures were written and saved for further calculations. Initial structures indicated that some of the branches were folding inwards towards the core of the dendrimer (Fig. 4).

This unfavorable folding was one of the drawbacks of this method because electrostatic forces were ignored



Scheme 3 Scheme representing the sequential actions executed by Xplor-NIH with generate.inp file

during the process of generating the structure. To prevent possible problems with mis-folding of the dendrimer, and to have a similar starting conformation for the different dendrimers that were to be subjected to investigation of their conformational flexibility, a set of non-experimental nOe constraints was introduced during steps 2–3. These constraints were set to position surface groups on opposite

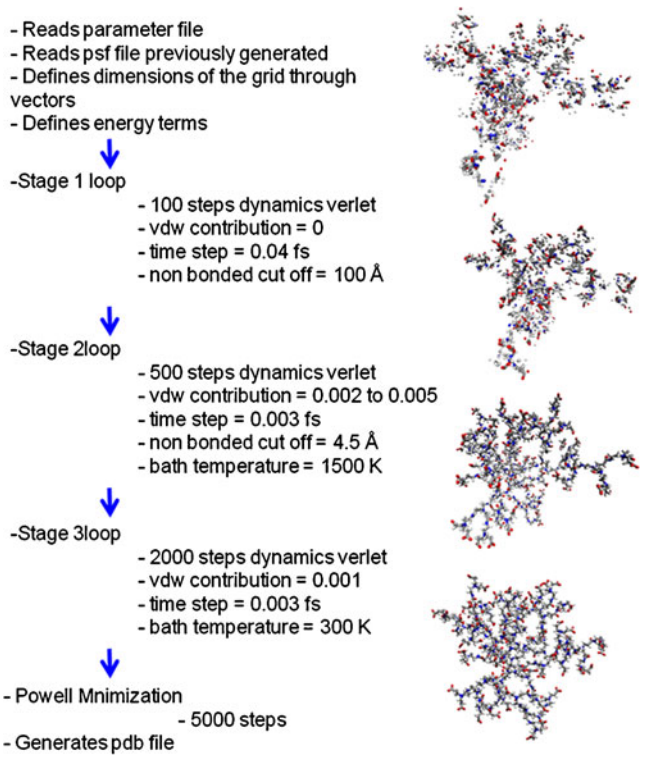


Fig. 3 Scheme representing the sequential generation process executed by Xplor-NIH with random.inp file

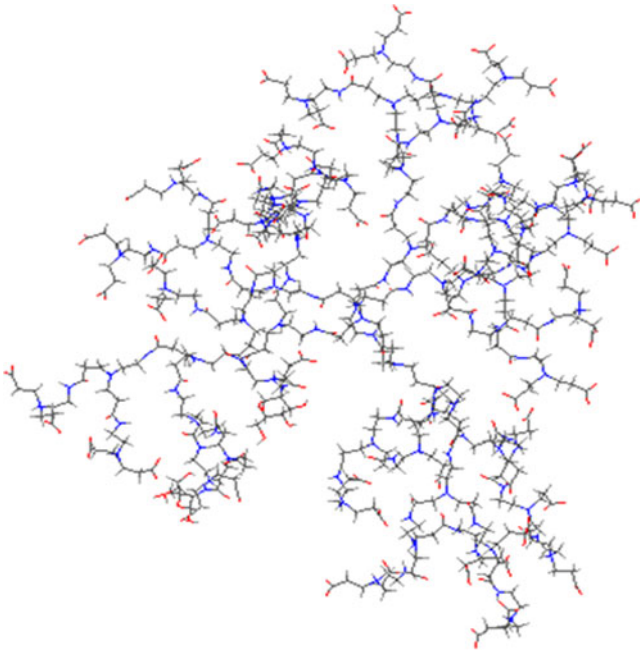


Fig. 4 A 3D structure of a generation 3.5 dendrimer glucosamine generated with random protocol from Xplor-NIH

sides and as far apart from each other as possible. The application of these constraints would produce stretched structures, which would remove an element of randomization of the generated starting structures, and would be suitable as a starting point for further molecular dynamics simulations or other computational studies.

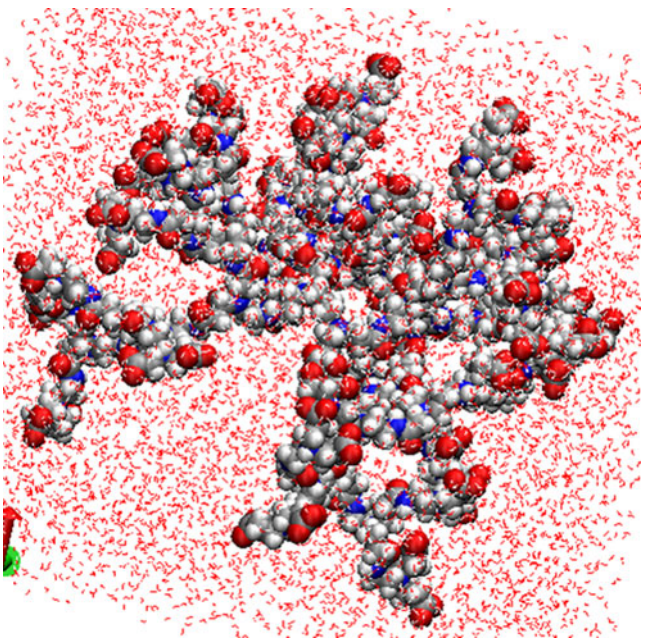


Fig. 5 A snapshot from a molecular dynamics simulation of a fully solvated generation 3.5 dendrimer glucosamine carried out using NAMD and visualized by VMD

The resulting topology, parameter, psf and pdb files can be further used for, but not limited to:

- modeling studies that incorporate experimental restraints by utilizing XPLOR without modifications;
- molecular dynamics simulation of solvated molecules by NAMD; topology and parameter files have to be converted into CHARMM27 format;
- convert pdb files into mol2 files using OpenBabel [21] or Vega ZZ (REF).

Our proposed biological studies of the molecular interaction between dendrimer glucosamine and the immune system's recognition system for lipopolysaccharide (LPS) required us to carry out further simulation studies of a fully solvated system. The structure of a generation 3.5 PAMAM dendrimer and of three conjugated analogues were imported into VMD, solvated and submitted to NAMD to carry out molecular dynamic simulations of the fully solvated dendrimer (Fig. 5), or converted into mol2 file format for use for simulations in explicit solvent using Desmond molecular dynamics software [22, 23] (results not shown).

The simulated structures generated using NAMD and Desmond were analyzed for similarity of structure. Notably, their structural features and their calculated molecular properties along the trajectories were also consistent with the structures produced using Macromodel [13] employing implicit solvation representation by the Generalized Borhn/Surface Area method [24]. We have applied these methods to generate PAMAM dendrimer structures with a range of glucosamine loadings. Molecular dynamics simulations (4.8 ns) were conducted to provide some insight into the surface properties of these derivatized PAMAM dendrimers by varying the amount of glucosamine that was conjugated to the dendrimer end groups [8].

Conclusions

A method to generate 3D models from the sequence of saccharide modified dendrimers has been defined and evaluated in a model test system. The generation of glycosylated PAMAM 3-D structures constitutes a significant incremental advance in the study of the effect of glycosylation of the dendrimer based structures. This will enable a better understanding of biologically important cell surface receptor-ligand interactions. Structures generated by XPLOR can be further optimized by molecular dynamics simulation in an explicit water environment that also includes adequate electrostatic contributions. This was achieved by converting the structures generated into different file formats that were suitable as input for other software packages designed for simulation studies.

It should be possible to apply this method for generating hyperbranched structures from a sequence to the computational study of other dendrimers and of linear polymers. Future studies will require the automation of parameter generation and web user interface development to ensure the wider availability of the method to the scientific community.

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