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Base Invaders. Coupling Experiments and Multiscale Modeling of Dendrimer-Based siRNA Delivery Agents

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Abstract. Injected, nano-scale drug delivery systems, or *nanovectors*, are ideal candidates to provide breakthrough solutions to the time-honored problem of optimizing therapeutic index for a treatment. Even modest amounts of progress towards this goal have historically engendered substantial benefits across multiple fields of medicine, with the translability, for example, from oncology to infectious diseases being granted by the fact that the progresses had a single common denominator in the underlying technological platform. In this work we combine multiscale molecular modeling and experimental approaches to define the mode and the molecular requirements of the interaction of oligonucleotide-based therapeutics (e.g., small interfering (si)RNA) and dendrimeric delivery reagents. In details, by mimicking *in silico* the experiments performed *in vitro*, information at the molecular level (e.g., interaction forces, mechanisms, structures, free energies of binding, self-assembly, etc.), which cannot be accessed by other experimental techniques, are obtained. Thus, critical molecular parameters for optimizing and *de novo* designing nanocargos for tissues and tumor specific uptake can be determined. This would provide valuable information to devise optimal delivery modalities that would increase the efficacy of siRNA therapeutics in cells and laboratory animals and move them toward clinical applications.

Introduction

When viruses infect eukaryotic cells, or when transposons and transgenes are randomly integrated into host genomes, double-strand (ds)RNA is frequently produced from the foreign genes. Most eukaryotes, including humans, possess an innate cellular immune surveillance system that specifically responds to the presence of dsRNA and activate processes that act post-transcriptionally

to silence the expression of the interloping genes [1,2]. This mechanism is now commonly referred to as *RNA interference* (RNAi) [3]. During RNAi, long transcripts of dsRNA are rapidly processed into *small interfering RNAs* (siRNAs), which represent RNA duplexes of specific length and structure that finally guide sequence-specific degradation of mRNAs homologous in sequence to the siRNA. The transfection of siRNAs into animal cells results in the potent, long-lasting post-transcriptional silencing of specific genes [4]; indeed, they are highly effective at lowering the amount of targeted RNA – and by extension proteins – frequently to undetectable levels. Furthermore, the silencing effect is extraordinarily specific, because one nucleotide mismatch between the target RNA and the central region of the siRNA is frequently sufficient to prevent silencing. Therefore, similarly to antisense oligonucleotide technology, the use of siRNAs holds great promises for the application of gene-specific therapies in treating viral infections, cancer and many other diseases.

However, the challenge for successful development of RNAi for clinical applications is the *in vivo* delivery of siRNAs into target cells [5], as several extracellular and intracellular barriers have to be overcome. Accordingly, appropriate carriers that efficiently deliver nucleic acids to a desired population of cells are required to bring out maximum therapeutic effects [6]. Virus-derived carriers have been predominantly employed in clinical trials, since viruses have evolved to efficiently deliver their genetic material to host cells by hiding from host surveillance and by taking advantage of intracellular trafficking machineries. However, several drawbacks, including the lack of specificity toward target cells, high cost of production, and safety concerns such as risk of potential immunogenicity and chromosomal insertion of viral genome, limit the practical use of viral vectors. Injected, nano-scale molecular drug delivery systems, or `nanovectors', are ideal alternatives to provide breakthrough solutions to the time-honored problem of optimizing therapeutic index for a treatment, as they possess wide drug loading capacity, well-defined physico-chemical properties, and high degree of molecular diversity that allows extensive modifications to overcome the extra/intracellular hurdles of gene delivery. Linear, branched and dendritic cationic polymers containing several amine groups in their backbone have been used extensively to these purposes. The interaction between the positively charged polymer backbone and negatively charged nucleic acids leads to the spontaneous formation of nano-size complexes (polyplexes) in aqueous milieu. The compact structure of the charge neutralized polyplex core efficiently prevents the access of nucleases to the enclosed nucleic acid drugs. The polyplexes should maintain their stability before cellular uptake by endocytosis. Once localized inside the cells, the polyplexes should escape from the endosomal compartment and unload the nucleic acid drug either in the cytoplasm or leave it ready to be transported within the nucleus. Understanding the structure-property relationships of these carrier/cargo assemblies is a prerequisite step for their rational design. Based on that information, non-viral gene delivery systems can be carefully designed and fine-tuned to achieve optimal transfection efficiency along with the desired clinical significance.

Under these perspectives, in this work we combined multiscale molecular modeling and experimental approaches to define the mode and the molecular requirements of the interaction of siRNA therapeutics and dendrimeric/polymeric delivery reagents. In details, by performing *in silico* molecular simulations, information at the molecular level (e.g., interaction forces, mechanisms, structures, free energies of binding, self-assembly, etc.), which cannot be accessed by other experimental techniques, were obtained, and the critical molecular parameters for optimizing/de novo designing nanocargos for tissues and tumor specific uptake can be determined.

Materials and methods

Computational Details. Atomistic models of poly(amidoamine) (PAMAM) dendrimers up to generation 6 were built and, after adding the suitable number of counterions and water molecules, the ionic strength was adjusted and each global system geometry was optimized using the *Sander* modulus of Amber 9.0 [7]. Similar procedures were employed to build the siRNA molecules considered. The GL3 siRNA, which is directed to a complementary sequence in the firefly luciferase mRNA, was used as standard siRNA in the modeling experiments with 19-mer dsRNA

portion and 3'-UU overhangs at both ends of the duplex. The individual systems were gradually heated to 300K by performing 50 ps of molecular dynamics (MD) simulations in the canonical (constant volume-constant temperature, or NVT) ensemble, followed by further 50 ps MD simulations under constant pressure-constant temperature (NPT) conditions at atmospheric pressure to optimize system density. Complex systems were built and optimized using analogous recipes.

Systems were equilibrated by 4 ns NPT MD simulations, followed by production runs of 2 ns under NVT conditions. All MD calculations were run on the Tartaglia cluster at University of Trieste using 32 processors for each simulation. Each system featured periodic boundary conditions; a time step of 2 fs was employed to integrate the equations of motions using the SHAKE algorithm. The molecular mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method [8] was exploited to derive the free energy of binding, ΔG_{bind}, between the dendrimeric molecules and the siRNAs. According to this approach, ΔG_{bind} can be calculated from computational analysis of a single simulation of each siRNA-bound dendrimer, and includes explicit computed components corresponding to 'gas-phase' nanocarrier/cargo interactions (ΔE_{MM}), conformational entropy ($T\Delta S$), and solvation contributions (ΔG_{solv}):

$$\Delta G_{bind} = \Delta E_{MM} + \Delta G_{SOLV} - T\Delta S. \quad (1)$$

The term ΔE_{MM} in Eq. (1) can be further split into contributions from internal (ΔE_{int}), electrostatic (ΔE_{elec}), and van der Waals (ΔE_{vdW}) energies:

$$\Delta E_{MM} = \Delta E_{int} + \Delta E_{elec} + \Delta E_{vdW}. \quad (2)$$

The solvation free energy, ΔG_{solv}, can in turn be expressed as the sum of an electrostatic component, ΔG_P, and a nonpolar contribution, ΔG_{NP}:

$$\Delta G_{solv} = \Delta G_P + \Delta G_{NP}. \quad (3)$$

The first term in Eq. (3) can be calculated by solving the finite-difference Poisson-Boltzmann equation, whilst the nonpolar component can be obtained from the Solvent Accessible Surface Area (SASA) using the following relationship $\Delta G_{NP} = \gamma SASA + \beta$, in which $\gamma = 0.00542$ kcal/(mol Å^2), and $\beta = 0.92$ kcal/mol. Finally, normal mode analysis can be used to estimate the entropic variation upon binding.

For the mesoscopic simulation of the self-assembly of dendrimers, we developed an innovative multiscale hierarchical procedure [9]. In particular, a hybrid method (Self-Consistent Field/Brownian Dynamics) [10] was applied for exploring the mesoscopic behavior of hydrophobically modified PAMAM dendrimers up to G6. Briefly, the computational recipe consists of a hierarchy of independent models, where information from one (lower) level is passed to the next (higher) level in a sort of "message-passing" procedure. As a consequence, all necessary input parameters for the mesoscopic models are estimated from atomistic calculations by a step-by-step procedure involving: (a) the matching of the atomistic and mesoscopic pair correlation functions to determine the most effective mesoscopic topology for a single dendrimer, (b) the matching of the atomistic and mesoscopic dendrimer density profiles to calculate the bead-field coupling parameters, and (c) the matching of atomistic and mesoscopic radius of gyration to obtain the interaction parameters. All mesoscopic hybrid simulations were performed with the Culgi software package [11].

At present, very few hybrid models exist. The reason for this lies in a difficulty in combining forces and energies from different types of model. The center of the mesoscopic simulations in our approach is based on the self-consistent field (SCF) theory. In particular, the dynamic density functional theory (DDFT) for 2D and 3D pattern formation in complex amphiphilic systems. DDFT provides a general framework to calculate the dynamics of mesoscale pattern formation, on a coarse-grained scale 10^{-9} m to 10^{-6} m, in a variety of polymer liquids, including charged systems

and systems containing hard particles such as colloids or surfaces. Into this model we have incorporated bead models, such as dissipative particle dynamics (DPD) and Brownian dynamics. The bead-model is absorbed in such a way that it is possible to perform particle- and field-based calculations simultaneously in one simulation box [10]. This means that specified parts of a system can be modeled as particles, whereas the rest of the system acts as fields.

Results and Discussion

Atomistic molecular dynamics simulations. The application of the MM/PBSA methodology allowed us to calculate the free energy of binding between G4-G6 PAMAM nanocarriers and their siRNA cargos. Figure 1 illustrates a snapshot of the MD simulation of the complex between PAMAM G4 and GL3 siRNA.

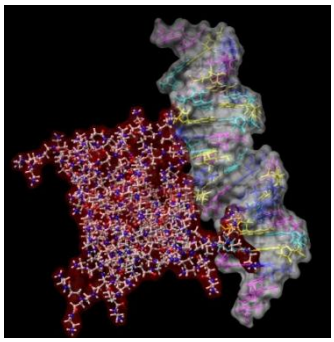

Figure 1. Equilibrated MD snapshot of the complex between PAMAM G4 and GL3 siRNA. Water molecules and counterions have been omitted for clarity.

Quantitative information about the forces involved in nanocarrier/cargo binding can be obtained by analyzing the values of the free energy of binding ΔG_{bind} and its components, which are listed in Table 1 for the G4 PAMAM/GL3 siRNA complex of Fig. 1 as an example. As we can see from Table 1, both the intermolecular van der Waals and the electrostatics are important contributions to the binding. However, as expected, the association G4 and GL3 is mainly driven by more favorable polar (i.e., electrostatic) interactions in the complex than in solution. When examining the role of the electrostatics in a complex formation, however, it is fundamental to consider the electrostatic component of the molecular mechanical energy (ΔE_{elec}) together with the electrostatic contribution to solvation ΔG_P. In fact, electrostatics generally disfavor the binding process because the unfavorable change in the electrostatics of solvation is often mostly, but not fully, compensated by the favorable electrostatics within the resulting complex.

Component	*Mean [kcal/mol]*	*Standard deviation [kcal/mol]*
ΔE_{elec}	- 27187	210
ΔE_{vdW}	- 44.58	5.93
ΔE_{int}	0.0	0.0
ΔE_{MM}	- 27231	210
ΔG_{NP}	- 11.12	0.57
ΔG_P	26870	203
ΔG_{solv}	26859	203
ΔH	- 371.9	13.7
$-T\Delta S$	64.78	3.64
ΔG_{bind}	**- 307.9**	

Table 1. Free energy components and total free energy of binding the PAMAM G4/GL3 siRNA complex.

Interestingly, in this case, given the highly positively charged nature of the nanovector and the highly negatively charged nature of the siRNA, a reverse behavior is observed, because of a less positive total electrostatic term in which the penalty paid by the electrostatics of solvation is better

compensated by favorable electrostatic interactions within the complex. Thus, even though solvation tends to counteract complex formation, it is the optimized balance of opposing electrostatic contributions that leads to a very tight binding between the PAMAM nanovector and its genetic material cargo.

Dendrimer self-assembly at the mesoscopic level. We investigated the influence of the chemical nature of the external superficial groups on the self-assembly behavior of PAMAM dendrimers, having three different surface decorations: anionic, cationic and hydrophobic. In the case of the cationic external moieties, the molecules in solution present a sort of unstable, disordered aggregation involving two or three molecules at time (see Figure 2, left column). In the case of anionic terminal groups, the simulations reveal that after the modification the molecules are well separated in solution and no phenomena of aggregation occur (see Figure 2, middle column). This information is of paramount importance as this surface modification is necessary in order to improve the solubility of PAMAMs in aqueous solution, and to avoid the toxicity and liver accumulation associated with their polycationic surface in solution. Finally, in the case of hydrophobic terminal groups, the self-organization of a single bilayer vesicular aggregate with a thickness T_b of approximately 3÷4 nm can be observed (see Figure 2, right column). These theoretical predictions are in excellent agreement with recent experimental Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) results on different PAMAMs peripherally modified with aromatic moieties [12]. The formation of the bilayer vesicles can be attributed to the amphiphilic structure of the dendrimers, i.e. hydrophilic dendritic branches and hydrophobic periphery. Intuitively, the open and extended molecular architecture of the unsubstituted and the hydrophilically modified dendrimer facilitates and promotes the changes in the shape of the molecules, with the hydrophilic branches tumbling out to occupy more room and, hence, maximizing the contact with water. On the contrary, when the dendrimer surface bears hydrophobic moieties, the molecules shrink and pack together to maximize the hydrophobic interactions and, hence, minimize their energy in water.

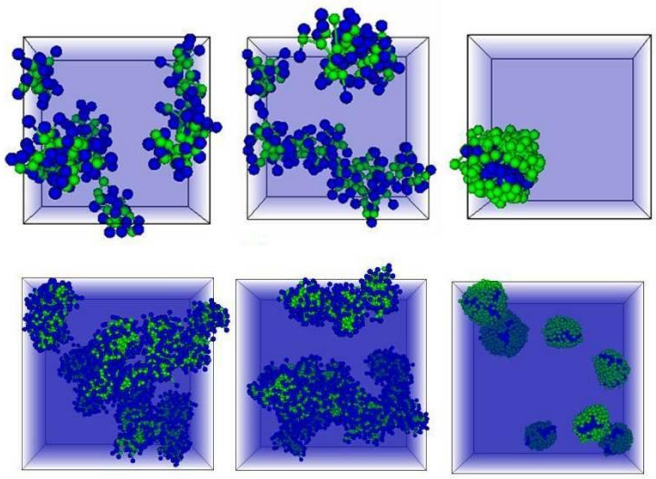

Figure 2. Different states of self-aggregation of concentrated aqueous solutions of ethylenediamine (EDA)-based PAMAM dendrimers G2 (upper panel) and G6 (bottom panel) as a function of surface modification. (left column): hydrophilically modified surface; (middle column) protonated surface; (right column) hydrophobically modified surface. Color legend: blue, surface bead S; light green, branching unit bead U.

Conclusions

We believe that the present study is the first theoretical demonstration of complexation between a siRNA molecule and a dendrimer from a fully atomistic description. The free energy of binding between the genetic material and its nanocarrier, as obtained from the application of the MM/PBSA methodology, is clearly dominated by electrostatic interactions. Further, in this work we presented the derivation and application of a multiscale procedure to simulate complex macromolecules in aqueous solutions, with particular attention to branched systems. This approach relies on a step-by step message-passing technique from the atomistic to the mesoscale level; thus, the two types of simulation are completely integrated, and virtually no experimental data are necessary to characterize the system, at least at a preliminary stage of the analysis. We tested the calculations on different generations of PAMAM dendrimers. The higher generations of these macromolecules are

currently used as drug delivery systems in cancer therapy. We also investigated the aggregation and the self-assembly phenomena in solution with the ultimate purpose, in a work which is currently in progress, of correlating these pieces of information to their eventual drug release properties. The influence and the effect of the external surface modification were also taken into account, modifying anionically and hydrophobically the peripheral groups of the macromolecule. As expected, the chemical nature of the outer shell strongly controls the type and the characteristics of aggregates formation. To the best of our knowledge, this is the first report on a simulation study concerning self-organization of dendritic macromolecules in aqueous solutions. Further elements of novelty are constituted by the development of a multiscale procedure and the use of the hybrid bead-field mesoscale technique. Although further work is still ongoing in our laboratories to expand and confirm these preliminary results, we are confident that the developed modeling approach can acts as a powerful instrument for the design and optimization of synthetic complex macromolecules.

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