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Review

Molecular modeling of polynucleotide complexes

Deniz Meneksedag-Erol [a,c], Tian Tang [a,b,**], Hasan Uludağ [a,c,d,*]

[a] Department of Biomedical Engineering, University of Alberta, Edmonton, Canada
[b] Department of Mechanical Engineering, University of Alberta, Edmonton, Canada
[c] Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Canada
[d] Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada

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ABSTRACT

Delivery of polynucleotides into patient cells is a promising strategy for treatment of genetic disorders. Gene therapy aims to either synthesize desired proteins (DNA delivery) or suppress expression of endogenous genes (siRNA delivery). Carriers constitute an important part of gene therapeutics due to limitations arising from the pharmacokinetics of polynucleotides. Non-viral carriers such as polymers and lipids protect polynucleotides from intra and extracellular threats and facilitate formation of cell-permeable nanoparticles through shielding and/or bridging multiple polynucleotide molecules. Formation of nanoparticulate systems with optimal features, their cellular uptake and intracellular trafficking are crucial steps for an effective gene therapy. Despite the great amount of experimental work pursued, critical features of the nanoparticles as well as their processing mechanisms are still under debate due to the lack of instrumentation at atomic resolution. Molecular modeling based computational approaches can shed light onto the atomic level details of gene delivery systems, thus provide valuable input that cannot be readily obtained with experimental techniques. Here, we review the molecular modeling research pursued on critical gene therapy steps, highlight the knowledge gaps in the field and providing future perspectives. Existing modeling studies revealed several important aspects of gene delivery, such as nanoparticle formation dynamics with various carriers, effect of carrier properties on complexation, carrier conformations in endosomal stages, and release of polynucleotides from carriers. Rate-limiting steps related to cellular events (i.e. internalization, endosomal escape, and nuclear uptake) are now beginning to be addressed by computational approaches. Limitations arising from current computational power and accuracy of modeling have been hindering the development of more realistic models. With the help of rapidly-growing computational power, the critical aspects of gene therapy are expected to be better investigated and direct comparison between more realistic molecular modeling and experiments may open the path for design of next generation gene therapeutics.

1. Introduction

Gene therapy aims to treat a wide range of disorders by altering gene expression with the delivery of genetic materials (polynucleotides). The initial impetus behind gene therapy was the desire to synthesize therapeutic proteins *in situ* with functional DNA expression vectors. Exogenous DNA has to reach cell nucleus and produce mRNAs for desired proteins by recruiting the appropriate transcription factors. With the discovery of RNA interference (RNAi) [1] the scope of gene therapy was expanded. In RNAi, relatively long double-stranded RNAs (dsRNAs) are cleaved by the enzyme Dicer into short (21–22 nucleotide) RNAs. The guide strand in truncated dsRNAs, after dissociation, gets incorporated into RNA-induced silencing complex (RISC) to identify complementary sequence in mRNAs, leading to mRNA cleavage. The therapeutic use of RNAi relies on short interfering RNAs (siRNAs), synthetic 22 nucleotide dsRNAs. The challenges in the delivery of polynucleotides, however, have dampened the great interest in DNA and siRNA therapeutics.

The anionic polynucleotides cannot efficiently cross hydrophobic and anionic lipid bilayers of cell membranes. This limitation

* Corresponding author. Department of Chemical and Materials Engineering, University of Alberta, Edmonton, Canada.
** Corresponding author. Department of Biomedical Engineering, University of Alberta, Edmonton, Canada.
E-mail addresses: tian.tang@ualberta.ca (T. Tang), huludag@ualberta.ca (H. Uludağ).

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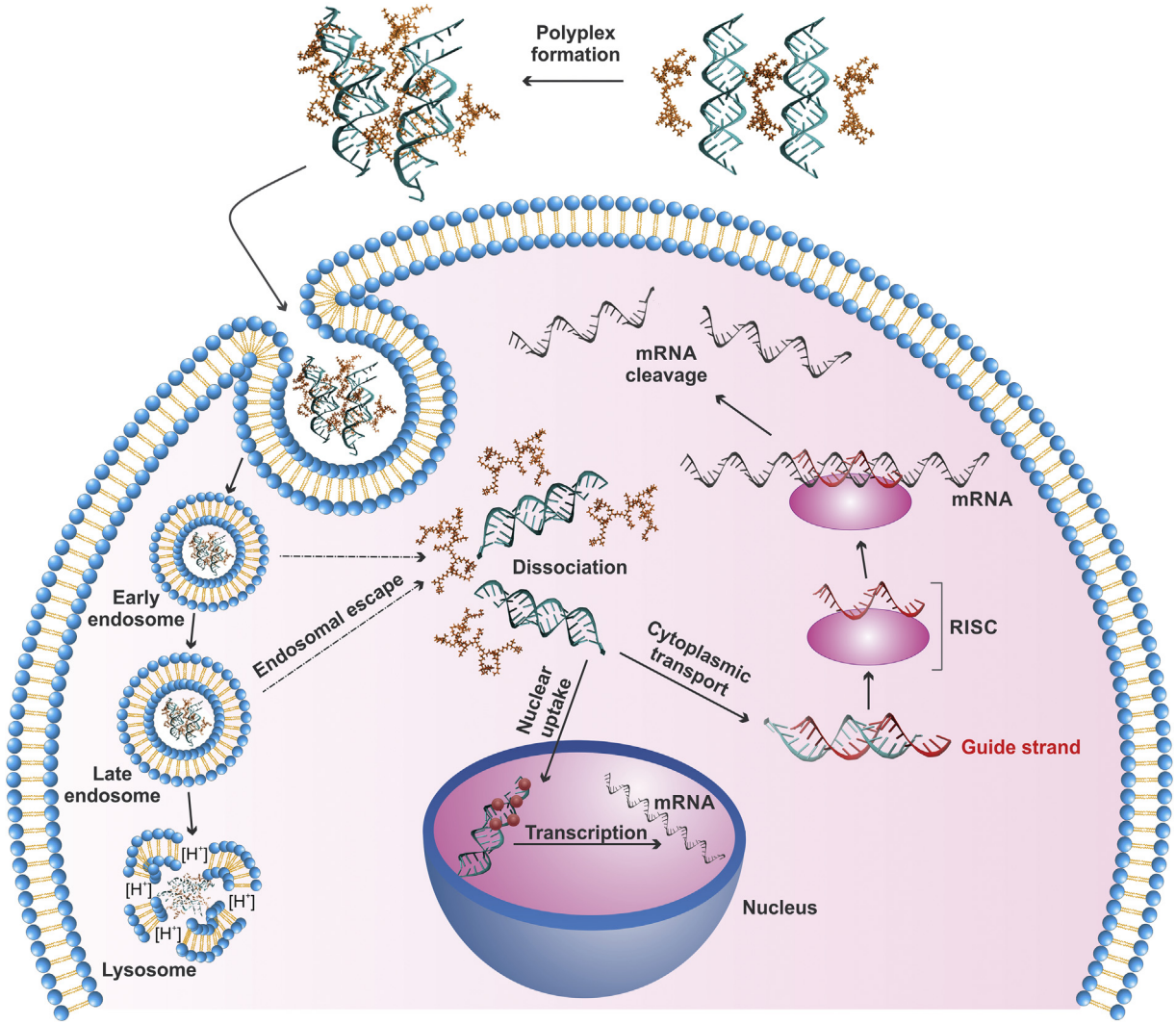

Fig. 1. Main steps involved in gene delivery. While siRNA (in red) gets incorporated into RISC in cytosol, DNA gets trafficked to nucleus to recruit transcription factors (represented as red spheres) to produce desired mRNAs. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

stimulated design of delivery systems (also known as carriers) to neutralize and compact the polynucleotides. Polynucleotides complexed with cationic polymers and lipids are known as 'polyplexes' and 'lipoplexes' (Fig. 1), respectively [2]. Binding of complexes to cell surface is governed by electrostatic interactions between cationic carriers and anionic membrane proteins and/or cell-surface receptors. Endocytosis follows via a variety of mechanisms, such as clathrin- and caveolin-1 independent, clathrin-mediated (CME), caveolae/raft-mediated (CvME) and macropinocytosis [3]. Uptake of the complexes depends on many factors and there have been some contradictory proposals on most effective endocytosis mechanism(s). While some studies proposed CvME to be the most conducive, others suggested CME as well as macropinocytosis for larger complexes that cannot be trafficked with CvME or CME [4].

Intracellular trafficking of complexes starts in early endosomes, which generally fuse into late endosomes (pH ~ 5–6) and lysosomes (pH ~ 4.5). Complexes must efficiently escape into cytosol before lysosomal degradation (Fig. 1). It is possible to facilitate endosomal escape by combining polynucleotides with fusogenic ligands, pH-sensitive carriers, and photosensitive agents [5]. Endosomal escape is also possible through 'proton-sponge effect' if the carriers possess H-buffering properties, such as polyethylenimine (PEI) [6]. Upon release into cytoplasm, polynucleotide dissociation takes place and anionic molecules such as cytoplasmic RNA and heparin-like glycosaminoglaycans are thought to be involved in this process [7]. After release, DNA has to be trafficked to nucleus for transcription and siRNA has to get incorporated into RISC in the cytoplasm to give mRNA cleavage for gene silencing (Fig. 1).

Many issues related to the mechanism(s) of action of carriers cannot be directly addressed due to instrumental limitations at atomic resolution. Molecular modeling is beginning to be employed to overcome some of these limitations. Via computer simulations, motions of individual or groups of atoms are obtained, and physical properties can be extracted from time average of equilibrated systems. Since the first simulation on a biological macromolecule in 1977 [8], molecular modeling has become a unique tool for analyzing complex biosystems. Features of complexes and critical mechanisms in delivery have been explored, placing experimental observations in a better context. An overview of molecular modeling techniques will be first given, followed by a review on modeling of polyplexes and lipoplexes.

2. Molecular modeling employed in gene delivery[1]

Molecular modeling aims at studying the behavior of molecules through model building and numerical simulation over a range of length and time scales (Fig. 2). The established approaches include quantum mechanical (QM) methods, all atom molecular mechanics (MM), hybrid QM/MM methods, Monte-Carlo (MC) and coarse-grained (CG) simulations. QM methods, such as *ab initio*, density functional theory (DFT) and semi-empirical approaches, determine the detailed electron distribution. It is often used when electron transfer and chemical reaction is important, an issue not generally applicable for gene delivery. All-atom (AA) molecular dynamics (MD) is based on MM, where intra and intermolecular interactions are described by a force field (FF) that specifies the potential energy in terms of geometrical variables, e.g., atom distances and bond angles [9]. Parameters in the FF are obtained through QM calculations or comparison to experimental data. Force on each atom is calculated from the potential energy and numerically integrating equations of motion provides time trajectory of the system [9]. One way to increase time efficiency is to constrain some intramolecular interactions, such as bond lengths and angles. Constraints in MD can also be applied when the system has a high tendency to be trapped in free energy wells, resulting in poor sampling. Umbrella sampling (US) method [9] is one such approach, where certain degrees of freedom are restrained by applying a biasing potential. US generally requires performing a series of simulations with different biasing potentials. Unbiased results are then obtained with statistical methods like weighted histogram analysis method (WHAM) [10]. Current state-of-the-art in AA–MD allows simulating systems involving $<10^6$ atoms for less than one μs, which is much smaller than experimental systems. Another challenge is the accuracy of FFs [11], but the fast growing computing power and extensive studies on more reliable FFs are facilitating MD simulations at realistic time and length scales.

Hybrid QM/MM approach provides a compromise between maintaining a reasonable size of simulated system and accurately describing certain chemically active regions: the reactive part is described by QM and the rest by a FF. An effective potential energy function describes the interactions within each region and on the QM–MM interface. Since its introduction [12], QM/MM method has been widely used in modeling biomolecular systems [13]. Because of the high computational cost associated with QM calculations, current QM/MM simulations can only be performed for hundreds of ps at *ab initio* or DFT levels, although this can be 100 times larger with semi-empirical approaches. Furthermore, most QM/MM simulations have been performed for structural optimization rather than unrestrained dynamics [13].

To overcome the computational limits, approaches that can bridge atomistic and mesoscopic scales have been developed. CG is one such approach where a number of atoms are clustered into beads, namely CG sites. Interactions among CG sites are described by parameterized potential energy functions and time trajectory can be obtained by solving equations of motions [14]. Dissipative particle dynamics (DPD) is an example of CG simulations where the force acting on each CG site ('DPD particle') is a sum of conservative, dissipative and stochastic forces. The reduced degrees of freedom has allowed CG simulations to reach ms range, facilitating direct comparison to experiments [15]. Treating several atoms as a group, however, causes loss of atomistic information, and may lead to inaccuracy [15].

Unlike deterministic MD, stochastic MC approach samples the phase space by using a random number generator to obtain new coordinates and performing trial moves [11]. These trial moves are either accepted or rejected according to probability distribution for the specific ensemble under consideration. Many MC simulations have been performed for biomolecules and the majority of them are coupled with CG approach.

3. Modeling on complexation of polynucleotides with carriers

Most molecular simulations performed on gene delivery focused on complexation of polynucleotides with cationic carriers and their aggregation into larger structures, while other steps of the delivery process are only now beginning to be addressed (Fig. 3).

3.1. Complexation of carriers with polynucleotides

Significant efforts have been spent on understanding how carriers bind to individual polynucleotides. DNA binding of small oligoamines, such as putrescine (2^+), spermidine (3^+) and spermine (4^+), which stabilize DNA in cells, was investigated in a series of MD studies. These oligoamines were shown to bind DNA through the backbone O1P and O2P atoms [16–20]. Similarly, different groups reported the main interactions of PEI with DNA and siRNA to be between PEI amines and backbone phosphates in polynucleotides [21–24]. First study on DNA-lipid systems probed the interactions of dimyristoyltrimethylammonium propane (DMTAP) and dimyristoylphosphatidylcholine (DMPC) bilayers with DNA and showed that zwitterionic phosphatidylcholine (PC) head groups competed with cationic trimethylammonium moieties for screening of DNA charges [25]. Simulations of DMPC monolayers with DNA showed that although DNA was able to conserve its double helical structure, its base pairing was affected by the interactions with lipid head groups [26]. More systematic studies have been conducted with polycationic carriers employed for experimental gene delivery.

3.1.1. Effect of pH, H_2O and salt (NaCl)

Dendrimers with variable extent of protonation were simulated to better understand pH ($[H^+]$) effects during delivery. In line with experiments, a more favorable attraction between dendrimers and siRNA was present under low pH (~5) compared to neutral pH (~7), as indicated by Molecular Mechanics Poisson-Boltzmann Surface Area (MM-PBSA) analysis [27–29]. In our simulation, a decrease in PEI's protonation ratio caused formation of less stable

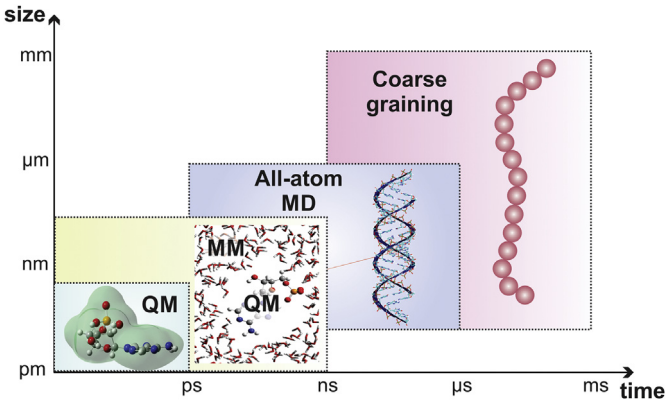

Fig. 2. Time and length scales typically used by different modeling approaches.

[1] The Nobel Prize in Chemistry 2013 was awarded jointly to Martin Karplus, Michael Levitt and Arieh Warshel for "*the development of multiscale models for complex chemical systems*". Their discoveries have laid the foundation for the application of molecular simulations bridging different scales to biological systems, the theme of this review.

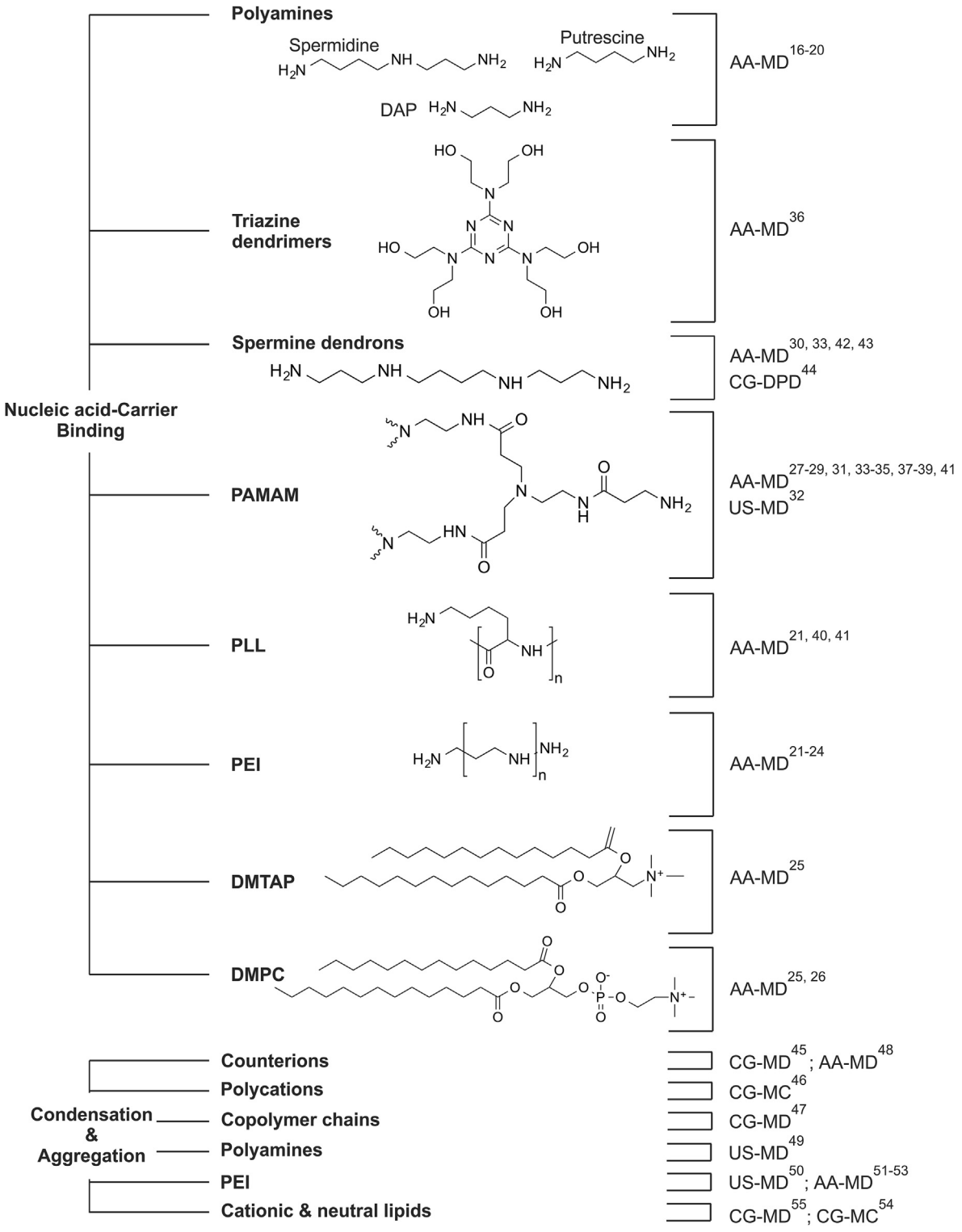

Fig. 3. Summary of the reviewed concepts, carriers and approaches used in their modeling.

polyplexes due to the loss of direct H-bonding to DNA [22]. While increased salt concentrations did not affect the binding affinity of a 2nd generation (G2) spermine dendrimer to DNA, this was not the case for G1 [30]. A reduced affinity was also found when salt was introduced to a G5 polyamidoamine (PAMAM)-DNA system [31]. Therefore, screening from salt seems to become important for small or rigid carriers that have limited interaction with polynucleotides. H_2O molecules were also critical in binding dynamics; in a DNA-G3 PAMAM system, US–MD simulations revealed that ordered H_2O was capable of bridging DNA and a highly charged dendrimer [32]. Our own work indicated the release of H_2O from the hydration shell surrounding either DNA or PEIs, as polyplexes evolve from individual molecules [23]. Water molecules can also affect the structures of carriers themselves; PAMAM (G3 to G5) was reported to

swell due to H_2O penetration, which was proportional to dendrimer generation [31].

3.1.2. Effect of carrier architecture

Flexibility of carriers is a critical issue for polyplex/lipoplex formation. For PAMAM dendrimers, rigidity was reported to increase with an increase in size/generation [33–35]. Among the G4 to G6 dendrimers, G4 showed higher binding affinity to siRNA due to its flexibility, while the affinity was lost with G6 due to its rigidity [33]. A comparison among G1, G4 and G7 PAMAM dendrimers showed that the increased rigidity associated with larger size caused higher entropic cost in binding to siRNA [35]. However, Pavan and coworkers published a contradictory report on the effect of flexibility. Flexible G2 triazine dendrimers designed with ethyleneglycol chains could not establish efficient interactions either with siRNA or DNA, due to their compact structure formed by the collapse of flexible linkers in salt environment, whereas rigid dendrimers with piperazine rings resulted in more favorable binding [36]. G5 dendrimers showed a transition from a flexible to a rigid structure with a change in protonation; flexibility observed at neutral pH (pH ~ 7.4) was lost with a decrease in pH to <5, causing a lower affinity towards siRNA [33]. Binding affinity varied with dendrimer generation. Some studies reported reduced affinity with an increase in generation, possibly due to increased rigidity, while high generation PAMAMs were found to bind more favorably to single-stranded DNA [37], dsDNA [31] and siRNA [29,38,39]. The latter should be attributed to increased number of positive charges, and not to the context of carrier flexibility/rigidity.

Our group focused on the influence of PEI molecular weight and structure on its complexation with DNA and siRNA. Degree of branching in 570 Da PEI did not cause any significant changes in binding dynamics [22], but modifications in 2 kDa PEI's architecture resulted in different binding patterns. Flexible linear PEI was observed to bind like cords and covering DNA's surface efficiently, whereas branched PEIs bound like beads, thus allowed bridging multiple DNAs [23]. In addition, DNA charges were screened more efficiently with 2 kDa PEI [23], in comparison with 570 Da PEI [22]. Zheng and coworkers studied a high molecular weight (25 kDa) PEI-DNA system, and reported that one PEI molecule was not sufficient for a stable DNA polyplex to form [24].

In comparison with linear poly-L-lysine (PLL)s, graft PLLs (where lysines are located on the branches) showed less favorable binding to DNA due to the steric hindrance caused by their hydrophobic backbone [40]. If the charge densities of the carriers are equivalent, binding pattern was found to be similar for linear PLL and PAMAM dendrimers [38,41], which indicated electrostatic interactions to be the main driving force rather than molecular architecture of the carriers.

3.1.3. Effect of functional groups

Functional groups can be incorporated into carriers for controlling binding to polynucleotides. MM-PBSA analysis on DNA binding to G1 and G2 dendrons functionalized with spermine, 1,3-diaminopropane (DAP) and N,N-di-(3-aminopropyl)-N-(methyl) amine (DAPMA) showed that surface charges participated in enthalpic interactions. Spermine (9^+ in G1 and 27^+ in G2) had the most favorable interaction due to its highest surface charges. G2 DAPMA (18^+) was found to be similar to G1 and G2 spermine in terms of binding affinity toward DNA. This observation, however, was attributed to favorable enthalpic interactions arising from dendrimer backfolding and DNA bending, rather than surface charges [42]. Functionalization of dendrons with photolabile linkers using UV-degradable spermine resulted in branched structures. This led to uniform vibrational behavior of dendron amines and DNA phosphates, which reduced the entropic cost upon binding compared to non-degradable dendrons, resulting in more stable interactions [43]. CG-DPD simulations showed that modification of spermine dendrons with hydrophobic cholesterol caused self-assembly of G1 dendrons and consequently higher charge density compared to G2, thus enabling more effective DNA binding [44].

3.1.4. Nature of polynucleotide

The siRNA was able to establish stronger interactions with carriers due to its more flexible structure compared to DNA, as reported in several studies [24,36,43]. This observation was concurrent to previous results seen with PAMAMs, where the rigidity of the carrier reduced the binding strength.

3.2. Condensation and aggregation

Condensing agents (i.e., gene carriers) can lead to formation of self-assembled (ordered) or aggregated (disordered) structures as a result of dramatic change in volume. Under practical conditions, these structures could end up in ~100 nm ('characteristic' length) nanoparticles. Condensation is crucial for efficient DNA delivery, while siRNA delivery relies on aggregation; both have been probed by molecular modeling.

3.2.1. Condensation

CG–MD simulations on di, tri and tetravalent counterion-DNA systems showed the effect of valence on DNA condensation. While DNA was effectively condensed with tri and tetravalent counterions, divalent counterions lacked this ability [45]. Increasing the numbers of either polycations or polycation charges was reported to be beneficial in polyanion condensation and collapse. While condensation occurred when the carrier to polynucleotide charge ratio >1, only small deformations on polyanion chains were observed with charge ratio <1 [46]. In the case of copolymers, effect of charge was probed by varying the length of copolymers containing both neutral and poycationic parts. Effective condensation was visible with copolymer chains that were longer than four cationic blocks [47].

3.2.2. Aggregation

DNA aggregation by counterions, polyamines and polymers was investigated in several studies. In a system involving two DNAs and salt (KCl and NaCl), DNA charges were screened more effectively with Na^+ than K^+, indicated by the steeper DNA repulsion profile in the presence of KCl [48]. Effect of valence was probed for DNA-oligoamine (putrescine, spermidine and spermine) systems. Concurrent with previous observations on counterions-induced condensation [45], increasing valence of oligoamines was reported to provide stronger DNA–DNA attraction [49,56]. Compared to oligoamines, we reported better DNA–DNA attraction with 568 Da PEI, indicated by a larger depth in the potential of mean force (PMF) curves for DNA interactions. In addition, we reported formation of more stable aggregates with an increase either in N/P (ratio of PEI Ns to DNA Ps) or PEI protonation ratio [50]. In a series of recent publications [51–53], we focused on PEI mediated aggregation of multiple (>2) DNA and siRNA molecules. With 568 Da PEI, aggregation occurred via efficient screening of DNA charges and polyion bridging (Fig. 4a) between DNAs [51]. Oleic acid (OA, C18) modification on PEI, which resulted in 831 Da PEI, brought up an additional aggregation pattern, the association of lipid tails (Fig. 4a) [52]. In addition to oleic acid, we also simulated substitutions on 1874 Da PEI with caprylic acid (CA, C8) and linoleic acid (LA, C18). More stable lipid association was achieved with LAs than CAs, due to the longer length of LA. Level of lipid substitution per PEI was important; while at one lipid substitution per PEI, a fraction of PEIs

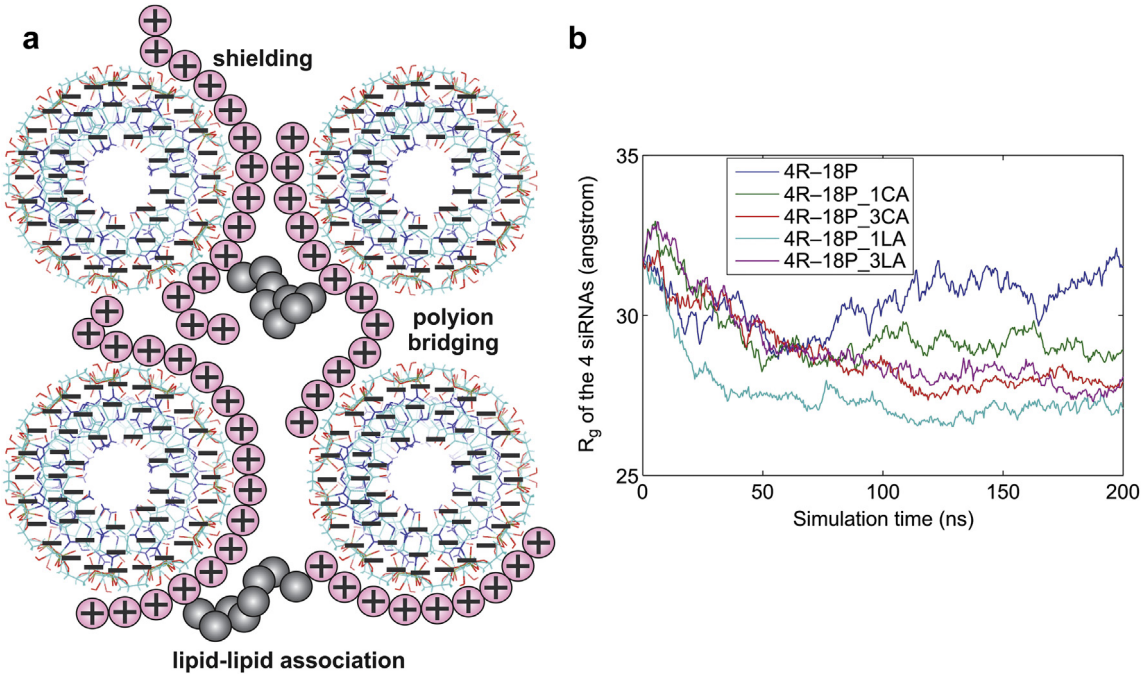

Fig. 4. a. Mechanisms of polynucleotide aggregation by polycationic carriers. b. Radius of gyration of four siRNAs in complex with native and lipid substituted (CA and LA) PEIs. Reprinted from: Biomaterials, Vol. 34 (11), Sun, C., Tang, T., Uludag, H., A Molecular Dynamics Simulation Study on the Effect of Lipid Substitution on Polyethylenimine Mediated siRNA Complexation, Pages: 2822–2833, Copyright (2013), with permission from Elsevier.

stayed in solution rather than approaching and binding to siRNA; all PEIs contributed to polyplex formation at three lipid substitutions per PEI. Compared with native PEI systems, more compact and stable siRNA aggregates were obtained as a result of lipid substitution (Fig. 4b) [53].

Randomly distributed lipids around DNAs gave a self-assembled lipid bilayer comprising DNAs sandwiched in the middle [54]. A larger system involving 32 DNA molecules and >1000 neutral and cationic lipids was studied, where the charge density of the system (ϕ_c, % cationic lipids) and the membrane stiffness (κ_s) were systematically varied. An optimal self-assembly threshold was evident in terms of these parameters. There were two main self-assembled phases of interest: inverted hexagonal phase where DNAs were packed inside cylindrical lipid micelles, and lamellar phase where DNA monolayers were sandwiched between lipid bilayers. Transitions between the two phases were observed at different ϕ_c and κ_s [55].

4. Modeling of intracellular trafficking

There have been only a few modeling works that studied intracellular trafficking of gene delivery. The very first model of polyplex attachment to cell membrane studied a simple dendrimer-DNA system using CG-MC simulations. Increasing charge density of membrane or decreasing Debye length of the solution resulted in poor attachment to cell membrane. Dendrimers were observed to dissociate from DNA with increased membrane charge densities, indicating the destabilizing effect of membrane on polyplexes under certain conditions [56].

Endocytosis of complexes was recently investigated by a CG model for a DNA particle coated with polymers containing protonable charges and surface ligands. While ligand–receptor interactions dominated endocytosis patterns, increasing charge density of polymeric carrier led to partial to full endocytosis. DNA length and concentration, as well as the anionic charge of the membrane were shown to significantly influence the process [57]. For a target membrane, these molecular parameters have to be optimized to achieve optimal endocytosis.

Dinh et al. employed a stochastic simulation for 25 kDa PEI–DNA polyplexes and studied the transition between distinct states (membrane binding to unpacking). The probability of a successful DNA delivery was 5% when the polyplexes escaped in the supranuclear region, compared to 1% for escape in cytoplasm. In the supranuclear region, later escape led to lower probability, most likely due to lysosomal degradation. Cell geometry was shown to be critical; greater delivery efficiency was observed for elongated and smaller cells, since such cells had relatively larger perinuclear space and escape location could be closer to the nucleus [58,66].

Finally, decompaction of polyanion/polycation complexes was investigated through CG-MC simulations. Decompaction was shown to be possible with heparin-like molecules and it was of higher degree in the presence of Fe(III), which might indicate the importance of Fe(III) in facilitating polynucleotide release [54,59].

5. Future perspectives

The time and length scales in atomistic simulation have been limited by the current computational power. Under practical conditions, nanoparticles of ~100 nm is typical [60] while largest size of our own atomistic simulation was ~12 nm [53]. Several parameters were defined to characterize nanoparticles (Fig. 5), but new parameters might be needed for larger nanoparticles. CG approaches can accommodate larger sizes; improvement in the accuracies of CG models will therefore allow systems with realistic scales to be simulated.

Nanoparticle endocytosis has been poorly studied; there were only a small number of CG simulations [56,57] and a few atomistic simulations on the uptake of individual carriers [61] and naked polynucleotides [62,63]. In addition, to mimic the effect of pH in the endosomal environment, most studies adopted the same approach where the protonation state of the polycationic carriers was specified at a given pH and it remained unchanged during the

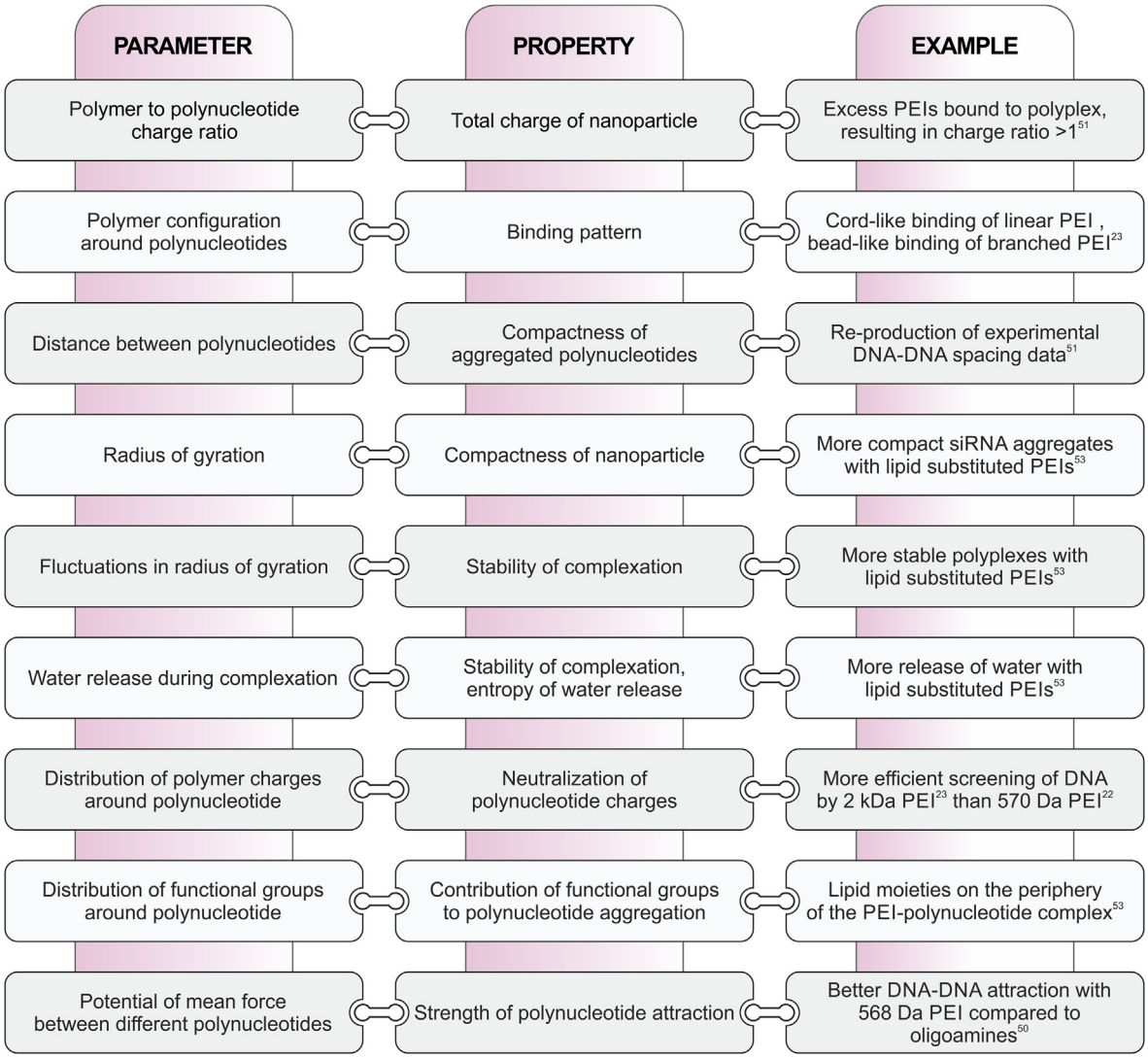

Fig. 5. Parameters defined to capture nanoparticle properties.

simulation [27–29]. A more realistic way is to perform constant pH simulations while allowing the protonation state of the carriers to vary. However, most methods to keep pH constant require the solvent to be implicitly treated [64–66], which cannot capture carrier-polynucleotide H-bonding upon complexation. There have been improvements to treat solvent explicitly [67–69]; application of these methods can help us gain insights into the endosomal stages of delivery.

What triggers the polynucleotide release from the carriers is unclear. One possible explanation is the disruption of nanoparticle integrity due to endosomal pH changes; apart from PEI[6] whether this mechanism holds true for all types of carriers remains unknown. Endogenous molecules might affect nanoparticle integrity; simulating a more cell-like environment by introducing intracellular molecules may better reveal dissociation dynamics of polynucleotides in cytosol.

Like endocytosis and endosomal escape, nuclear uptake is another rate limiting step in gene (DNA) delivery. Exchange of molecules between cytoplasm and nucleus is controlled by nuclear pore complexes (NPC) embedded in nuclear envelope. NPC bears a central channel inside filled with nucleoporins, which are composed of disordered proteins mostly comprising phenylalanine–glycine (FG) repeating sequences [70]. AA and CG–MD simulations revealed the structure of assembled FG nucleoporins and gave insight into nuclear uptake of DNA [71]. Intermolecular interactions of DNA with transport receptors and their interactions with NPC could be simulated to clearly understand the dynamics of nucleocytoplasmic transport.

Finally, coating the carriers with cell-specific ligands would help to reduce off-target effect, hence induce receptor-mediated endocytosis. Recent CG modeling on ligand-coated nanoparticles [72,73] revealed some critical parameters for efficient uptake, e.g., size and shape of nanoparticles, ligand density and rigidity, and membrane surface tension. Specific receptors should be considered in modeling either to improve uptake or target signaling pathways. Epidermal growth factor receptor 2 (ErbB2, also known as HER2) [74], is a member of receptor tyrosine kinases, involved in cell proliferation and differentiation [75]. HER2 is known to be overexpressed in breast cancer [76] and several other cancer types, and associated with aggressive tumor behavior. It can be targeted/blocked with monoclonal antibodies or small molecules [77]; trastuzumab (Herceptin®), pertuzumab (Perjeta®) and lapatinib (Tykerb/Tyverb®). MD and molecular docking studies [78–80] provided information on HER2 activation dynamics and receptor–antibody interactions. Delivery systems capable of incorporating such interactions and specific factors affecting cell selectivity

and endocytosis may lead to next generation therapeutics in HER2-positive cancers.

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