

Chapter 2

Molecular Modeling and Simulation of G-Quadruplexes and Quadruplex-Ligand Complexes

Shozeb Haider and Stephen Neidle

Abstract

Methods for the molecular modeling and simulation of G-quadruplex structures and their drug/ligand complexes are discussed, and a range of protocols is presented for undertaking a variety of tasks including model-building, ligand docking, dynamics simulation, continuum solvent modeling, energetic calculations, principal component analysis, and quantum chemical computations. The scope and limitations of these approaches are discussed.

Key words: G-quadruplex, Molecular modeling, Ligand complexes, Molecular dynamics, Simulations

1. Introduction

Guanine (G)-quadruplexes are built from short lengths of G-tract separated by lengths of general sequence. In the case of intramolecular quadruplexes, at least four G-tracts are required:



In general the G-tracts form the underlying core of quadruplex structures, with sets of four guanines at a time interacting together to form planar hydrogen-bonded G-quartets, which can then stack on top of each other. Quadruplexes can be formed (1, 2) from a single strand (termed unimolecular, or intramolecular quadruplexes), from two strands (bimolecular, or dimeric), or from four separate strands (tetramolecular). All have a requirement for alkali metal ion stabilization with $K^+ > Na^+$; these are coordinated to the O6 guanine atoms at the centre of a G-quartet and form a central ion channel. The N_L sequences link the G-quartets to form loops

and grooves, with variability in the nature of the connections being a major factor in the resultant variety of quadruplex topologies that have been observed (1). There are currently, as of autumn 2009, only 32 crystal structures of quadruplexes in the Protein Data Bank (PDB), and a rather larger number of NMR-derived structures. The former have been recently reviewed (3). There are as yet no general rules governing the folding of these sequences, although a start on their classification has been made (4). Evidence to date indicates that it is not yet possible to reliably predict overall quadruplex topology, although the simple topological rules for short N_L linkers appear to be robust (5). Folding is unpredictable once linkers have > two nucleotides, and especially when they themselves contain guanine residues, as has been shown by the unexpected and unique arrangement formed by a 22-mer sequence from the promoter region of the *c-kit* oncogene (6). The human genome contains over 250,000 distinct nontelomeric putative quadruplex sequences (7, 8) of which those in oncogenic promoter regions have been most studied (9). Quadruplexes formed from human telomeric sequences comprise repeats of the simple sequence d(TTAGGG), whereas non-telomeric sequences generally have no such symmetry.

Small-molecule ligands can promote the formation of quadruplex structures from telomeric DNA, which can then inhibit the telomerase enzyme and destabilize telomere end-capping in cancer cells (10). This finding has led to studies aimed at designing, synthesizing, and evaluating such molecules as anticancer agents [reviewed in, for example, refs 11–14]. A large number of quadruplex-binding ligands have been reported [summarized in ref 15], the majority of which share the common structural feature of a planar aromatic chromophore.

There are remarkably few detailed crystal or NMR structures for ligand–quadruplex complexes (16, 17). Those for bimolecular human telomeric quadruplexes all show a single topology, the parallel fold (18–20), as does the porphyrin complex with a *c-myc* oncogene promoter intramolecular quadruplex (21). The topology of human telomeric intramolecular quadruplexes is more varied, with crystallographic studies on a 22-mer showing the all-parallel fold (18), whereas NMR studies on several related sequences with small changes at 5' and 3' ends show (3+1) antiparallel folds (22–25).

1.1. Quadruplex Modeling – Challenges and Approaches

The structural polymorphism of many quadruplexes is as yet incompletely understood and presents a challenge for molecular simulation studies that to date has not been met. The problems of modeling individual quadruplex structures are similar to those of nucleic acids generally, but with the added complexity of the central ion channel (26). Given the variety of nucleic acid structures and their complexes combined with the inherent flexibility of

nucleic acids, there are many problems to which computational techniques such as molecular dynamics (MD) can contribute. This has been made possible due to the increasingly accurate parameter determination in nucleic acid force fields and algorithmic development (27, 28), inclusion of explicit counter ions and solvent molecules, as well as the use of more complex methods for evaluation of long-range electrostatic effects, which are important in charged systems. The maturity of the field is further indicated by the substantial body of recent literature on application of novel computational methods to a variety of biomolecular systems that contain complex nucleic acid arrangements such as DNA quadruplexes and drug-DNA complexes. Such improvement in methods and more careful comparison with experimental data give us increasing confidence in modeling methods (29).

2. Methodology and Force Fields

The most common modeling method is that of molecular dynamics (MD). It is based on solving Newton's laws of motion for all atoms in the system. The force on each atom is calculated from the derivative of the sum of potential energy terms for Coulombic, van der Waals, bond length, bond angle, and dihedral angle contributions. The acceleration on an atom can be calculated from the force and integrated to calculate velocities, which in turn can be integrated to find atomic position vectors. The time course of these position vectors forms the trajectory. The integration time step is adjusted depending upon the highest frequency vibrations in the system e.g. bond stretching along C–H and O–H bonds. The trajectories usually employ the NPT statistical ensemble that is generated if the number of atoms, pressure, and temperature are kept constant during the simulation. Cheap computational power means that simulations can now be carried out using explicitly solvated systems. In such a system, the solute is immersed in a large box of explicitly solvated water molecules and counter-ions. The box is replicated in all directions to satisfy periodic boundary conditions. The molecules are described by simple pair-additive atomistic potentials known as force fields that treat atoms as Lennard-Jones van der Waals spheres with partial constant point charges localized at the individual atomic centers, linked by harmonic springs supplemented by valence angle and torsion profiles mimicking the covalent structures.

The explicitly solvated simulations employ the particle-mesh Ewald (PME) method (30) or atom-based force shift approaches (31, 32) for taking into account the long-range electrostatic effects in an efficient manner. These effects have been shown to be significant in nucleic acid systems because of the charge on the

phosphate backbone and counter-ions and are even more important for quadruplex DNAs with their multi-faceted electrostatics features. Such complications resulted in the expulsion of the cations from the central electronegative channel in the quadruplex core, leading to the collapse of the structure in the first MD simulation of a quadruplex structure (33). Introduction of the atom-based force-shift truncation method using a 12 Å cutoff and PME treatment of electrostatics (34, 35) produced stable and very similar nanosecond MD simulations of nucleic acids. The CPU time requirements are similar for optimized cutoff radius and convergence parameters for PME summation, but the periodic boundary conditions necessary with standard implementations of PME make it slower than a spherical cutoff in a non-periodic geometry adapted to the shape of the system being studied. The pros and cons of the Ewald summation method and the periodicity it imposes on the system have been studied in detail and the results suggest that the artifacts of the method are small for biomolecular systems when comparing to errors arising from sampling and force field limitations (36).

Progress in force field development in recent years has made stable multi-nanosecond molecular simulations routine, although challenges in adequately simulating loop regions remain to be fully overcome (71). Several force field parameter sets such as in the AMBER package (parm99SB) (<http://amber.scripps.edu>) (27), CHARMM27 (37), and the latest GROMOS (38) force field have all yielded reasonable results for the simulation of conventional B-DNA conformations. Implementation of CHARMM for the simulation of unusual nucleic acid structures such as quadruplex DNA has not yet been extensively reported. The CHARMM force field contains similar functional forms including bond stretching, angle bending, torsion angle, and nonbonded interaction, but they are all derived differently (37). However, use of CHARMM27 to simulate folded RNAs has resulted in unstable trajectories (39). The GROMOS force field is yet to be tested and published independently for nucleic acids. A recent 10 ns benchmarking simulation using this force field by the authors on quadruplex DNA resulted in a complete loss of four-stranded structure. One should avoid using force fields that have not been explicitly parameterized for nucleic acids and tested for quadruplex structures.

An improved version of the AMBER parm99 force field (parmbsc0) for nucleic acids has been reported recently (40). It emphasizes the correct representation of α/γ concerted rotation in the nucleic acid backbone. The force field has been derived by fitting to high-level quantum mechanical data, verified by comparison with very high-level quantum mechanical calculations, and by a very extensive comparison between simulation and experimental data (40). The total simulation time used to validate the force field includes 1 μ s molecular simulations in aqueous solution.

In addition to the improvement of force fields, one of the main computational challenges is to simulate large systems over longer time scales. The time scale of events happening in real biological time is much longer than what can be simulated with the computational power available today. The result is limited sampling of conformational space. Faster computers would improve sampling but at the same time would also result in accumulation of force field deficiencies that can have detrimental effects over time. Enhanced sampling of conformational space can be approached by running multiple simulations using a rational approach of multiple starting structures or by using enhanced sampling methods. It must be kept in mind that the force fields being used to simulate biomolecular systems are over-simplified representations that are unable to accurately capture all energy contributions simultaneously.

The square planar arrangement of guanines in a G-quartet results in the carbonyl oxygens pointing towards the helical axis within the central core of the structure. Repeats of stacked G-quartets result in the formation of a central channel that is lined by carbonyl oxygen atoms, and thus the central channel running along the helical axis is highly electronegative in character. To avoid electrostatic repulsion, quadruplexes are stabilized by cations (preferably monovalent) that are embedded within the channel. Depending upon the size of the cation, they can be sandwiched symmetrically between two planes of the four G-quartets, each forming a square anti-prismatic arrangement in which the square plane of oxygen atoms above the ion is rotated with respect to the plane below, as observed in the crystalline state with K^+ ions (41). Two K^+ ions very rarely occur with a separation of $<3.5 \text{ \AA}$ in order to avoid an unstable electrostatic configuration. However, K^+ ions in principle may occupy adjacent sites and form stable complexes in which the cation is encapsulated, sandwiched, and coordinated, as observed in potassium-coordinated crown ethers (42). By contrast, sodium ions have a smaller ionic radius which can fit into the in-plane site and have been observed to lie close to each quartet plane (43, 44). The channel cations impart stability to the structure and have been observed in all quadruplex crystal structures solved to date, as well as in NMR structures. These cations are mobile and can readily exchange with the bulk solvent and have been observed experimentally on a millisecond timescale (45). However, once the cations are removed the rigidity of the structure is immediately lost (46). The solvent can move freely within the quadruplex core but is unable to stabilize the structure. At no point are the quadruplex G-quartets left vacant by cations and their stability is dependent on the cations associated with them. The present Amber ff99SB and parmbsc0 force field parameter sets are capable of accommodating monovalent cations in the simulations and have been shown to stabilize G-quadruplexes

(47, 48). However, the radii of K^+ and Na^+ ions are over-sized. Studies by Sponer and coworkers have shown that the positions for in-plane Na^+ ion are under-populated and K^+ ions can readily move out of the channel (49). Reducing the cation radii increases the sampling of ions inside the channel (49, 50).

Simulating a sugar-phosphate backbone in nucleic acids has always been a challenge for force fields because of both its flexible nature and the anionic electrostatic potential generated by the phosphate groups. The contributions from the complex electronic structure of the backbone change with solvation and conformational dynamics are not taken into account by nonpolarizable atom-atom pair additive force fields. A new and improved version of the parm94 (51) and parm99 (52) force fields has been introduced (parmbse0) that is able to accommodate the α/γ angles in the backbone of nucleic acids including G-quadruplexes (40).

3. Methods

3.1. Model Preparation for Molecular Modeling

1. The appropriate coordinates of the structure (x-ray or NMR) can be obtained from the Nucleic Acid Database (NDB) or Protein Data Bank (PDB). This forms the initial coordinates for molecular modeling.
2. The initial starting coordinates are subjected to molecular mechanics energy minimization.
3. The first round of minimization involves 1,000 steps of steepest descent with line searching.
4. The second round of minimization involves 1,000 steps of Polak-Ribiere conjugate gradient with a derivative convergence of $0.05 \text{ kJ}\text{\AA}^{-1} \text{ mol}^{-1}$.
5. This is followed by a short run of MD (50 ps at 300 K) with a 2.0 fs time step.
6. A final 1,000 steps of molecular mechanics energy minimization (Polak-Ribiere conjugate gradient) of the time-averaged MD structure is employed to obtain a low-energy final model for further studies.

3.2. Ligand-Quadruplex Modeling

Quantitative molecular modeling can be performed in order to visualize and describe in energetic terms how small molecules interact with and stabilize G-quadruplex structures. An appropriate binding site for the ligand can be chosen depending on the model being chosen for the study, for example, within a loop in the human intramolecular G-quadruplex NMR structure (PDB code 143D) or an external binding site in the human intramolecular

quadruplex crystal structure (PDB code 1KF1). As the solution and the crystal structure of the intramolecular G-quadruplex formed by four repeats of the human sequence are different, there are at least two plausible ways by which a ligand can bind to these systems. If there is no loop or the loops are positioned laterally as in the crystal structure (PDB code 1KF1), then the molecule can sit externally on the quartets. The other alternative is that the ligand is intercalated between the diagonal loop and the quartets, which is the most plausible model for ligand binding to the Na⁺ solution structure of the human 22-mer intramolecular G-quadruplex, or to any quadruplex with a loop positioned above the G-quartets.

To create a pseudo-intercalation ligand binding site in the human intramolecular G-quadruplex NMR structure (PDB code 143D) the following steps are employed:

1. The binding site is to be created between the diagonal TTA loop and the G-quartet segment of the structure (at the 5' AG step).
2. Two phosphate backbones are broken at the 5' AG step.
3. The two halves of the structure are separated so that the separation of the A:A base pair and the G-quartet is increased from 3.4 to 6.8 Å.
4. The sugar-phosphate backbones are reconnected.
5. Positional restraints are placed on the structure except for residues contributing to the pseudo-intercalation binding site which includes the reconnected sugar-phosphate backbone.
6. The first round of minimization involves 1,000 steps of steepest descent with line searching.
7. The second round of minimization involves 1,000 steps of Polak–Ribiere conjugate gradient with a derivative convergence of 0.05 kJ Å⁻¹ mol⁻¹.
8. A final 1,000 steps of molecular mechanics energy minimization (Polak–Ribiere conjugate gradient) is employed to relieve any resulting steric distortion while retaining the intercalation geometry between the G-quartet and the loop motifs.

3.3. Modeling Ligands and Docking in G-Quadruplex Structures

1. Molecular models of the ligands are created in the builder package of the Insight suite of programs.
2. The CFF force field is used to parameterize the ligand.
3. The partial charges are added semi-empirically using the MOPAC package.
4. The ligand is minimized using 1,000 steps of steepest descent with line searching.

5. The ligand is then docked in the binding site using a multi-phased docking protocol employing the grid docking method available within the Affinity program in the Insight suite of packages. An advantage of this module is that it can explore molecular orientations while interactively monitoring changes in the electrostatic and van der Waals ligand G-quadruplex interactions.
6. The ligand is randomly oriented 200 times and is centered on the more polar face of the quadruplex.
7. The van der Waals radius is set to 10% of the full value ($\times 0.1$).
8. The charges are not considered and the nonbonded cut-off value is set to 8.0 Å.
9. The system is minimized for 300 steps using the conjugate gradient method.
10. The maximum allowable change for succeeding structures (energy tolerance) is set to 10,000 kcal/mol and the energy range is set to 30–40 kcal/mol.
11. The 75 lowest energy structures are used for the second phase of docking.
12. Simulated annealing is used to further refine the initial placement for the 75 structures.
13. During the second phase, the van der Waals radius value is adjusted back to its full value ($\times 1.0$).
14. Nonbonded cut-off is set to 12 Å.
15. The system is minimized for 300 steps of conjugate gradient.
16. This is followed by a short burst of MD where the starting temperature is set at 500 K and the system is cooled to 300 K over 7.5–10 ps.
17. The resulting structures are minimized for 800 steps of conjugate gradient.
18. Twenty-five structures with the lowest total energy are used for further evaluation.
19. Many different positions in three-dimensional space are evaluated in this procedure. The selection of low energy conformation is made on the basis of maximization of π -electron overlap between the G-quartet, ligand chromophore, and the nucleobases that sit over the ligand.
20. A typical ligand chromophore may be positioned directly over the centre of the adjacent G-quartet while its substituent side chains protrude out through to the sugar-phosphate backbone.
21. Quantitatively, the docked molecules are selected on the basis of three criteria: (a) total energy of the system, (b) energy of

the ligand and the binding pocket, and (c) the total number of intermolecular hydrogen bonds.

22. The final confirmation of the ligand complex is then subjected to a further 500 steps of molecular mechanics energy minimization employing the conjugate gradient algorithm.

3.4. Simulating Ligand-G-Quadruplex Complexes

1. The ligand is docked onto the G-quadruplex using the docking protocol mentioned above.
2. The docked ligand-G-quadruplex complex is then transferred to the AMBER9.0 or 10.0 package.
3. The force field parameters for the ligand are calculated employing the new general amber force field (GAFF) for small organic molecules in the *antechamber* program.
4. The parameters are then extrapolated onto the ligand in the *leap* program.
5. The system is then set up in the *leap* program using protocols mentioned below.
6. MD simulations are carried out using the *sander* program within AMBER.
7. The trajectory is analyzed using the program *ptraj* within AMBER.

3.5. Multimeric G-Quadruplex Model Building

1. The crystal structure of the 22-mer human telomeric DNA d[AG₃(T₂AG₃)₃] (PDB code 1KF1) is taken as a primary unit for the construction of the higher-order model. It consists of three stacks of G-quartets connected by TTA loops.
2. The adenine nucleobase at the extreme 5' end is removed to generate a 21-mer.
3. Two 21-mer units are taken and positioned end-to-end (3'→5').
4. The two units are rotated 30° relative to each other. The 30° angle is taken from the original crystallographic analysis and is the twist angle found between two consecutive stacked G-quartets.
5. The rise and rotation of G-quartets between the two units are kept at values observed in the primary unit.
6. The rise is positioned at an optimal separation of 3.5 Å.
7. A TTA loop is extracted from the crystal structure.
8. The two units are joined by the extracted TTA loop.
9. The final 45 nucleotide multimer is subjected to three rounds of molecular mechanics energy minimization and a short burst of MD (described above) to relieve any steric clashes within the model.

Models of higher order (four quadruplex unit repeats) multimeric structures can be generated by taking two 45-mer units and positioning and joining them by a TTA loop in a manner analogous to that described above. The resultant model is then subjected to similar protocols of molecular mechanics energy minimization and dynamics procedures to relieve structural stress.

A pseudo-intercalation ligand binding site can also be generated in the 45-mer human telomeric DNA between the two 21-mer units.

1. The sugar-phosphate backbone is broken between the TTA loop and the joining 21-mer G-quadruplex units.
2. The two 21-mer units are separated and the distance between the two units is increased from 3.5 to 7.0 Å.
3. The sugar-phosphate backbone is reconnected.
4. Positional restraints are placed on the structure except for residues contributing to the pseudo-intercalation binding site, which include the reconnected sugar-phosphate backbone.
5. The first round of minimization involving 1,000 steps of steepest descent with line searching.
6. The second round of minimization involves 1,000 steps of Polak-Ribiere conjugate gradient with a derivative convergence of $0.05 \text{ kJ Å}^{-1} \text{ mol}^{-1}$.
7. A final 1,000 steps of molecular mechanics energy minimization (Polak-Ribiere conjugate gradient) is carried out to relieve any resulting steric clashes arising from the generation of the intercalation site between the two units.

3.6. Molecular Dynamics Simulations

1. The initial starting structure is taken from a structural database (NDB or PDB) and imported in the AMBER package using the *leap* program.
2. The x-ray structures of G-quadruplexes have revealed a vertical alignment of cations along the helical axis within the central core of the structure. The ions are retained in their positions as observed in the crystal structure. However, in the case of NMR derived solution structures, the ions need to be placed at appropriate positions in the structure. The cations are positioned between the G-quartets in case of K^+ ions and within or close to the plane of an individual G-quartet for Na^+ ions.
3. The positions of the water molecules observed in the crystal structure are retained. The structure is then solvated in a periodic box containing explicit TIP3P water. The dimensions of the box are such that its boundaries extend at least 10 Å from any solute atom.

4. Additional positively charged K^+ counter-ions are included in the system to neutralize the charge on the DNA backbone. This also maintains the consistency with the crystallization conditions and prepares the simulation to run in a uniform K^+ ionic environment.
5. The counter-ions can be placed automatically by the *leap* program throughout the water box at grid points of negative Coulombic potential.
6. The final net charge on the system should be zero.
7. MD simulations are carried out using the *sander* module in the AMBER9.0 simulations package.
8. Positional restraints of 500 kcal/mol are placed on the entire G-quadruplex structure (*ntr*=1). This ensures that only the water molecules and the counter-ions move during the first round of minimization.
9. The system is then equilibrated with explicit TIP3P water by 3,000 steps (*maxcyc*=3000) of molecular mechanics energy minimization which employs 500 steps of steepest descent followed by conjugate gradient minimization for the remainder of the steps (*ncyc*=500). The other parameter for the minimization is the use of periodic boundary conditions (*ntb*=1, constant volume) with a non-bonded Lennard-Jones cutoff of 10 Å (*cut*=10.0).
10. The nonbonded pair list is updated every 50 steps (*ntpr*=50).
11. A second round of energy minimization is carried out using the same parameters as in the first round. The only difference is that there are no restraints on the DNA (*ntr*=0).
12. Restrained MD is carried out for twenty picoseconds (*nstlim*=10,000) with positional restraints of 50 kcal/mol being placed on G-quadruplex DNA (*ntr*=1).
13. The time step is set at 2 fs (*dt*=0.002).
14. The coordinates are read in with no initial velocity information (*ntx*=1, *irest*=0).
15. Temperature scaling is switched on (*ntt*=3) with the temperature being increased from 0 K (*tempi*=0) to 300 K (*temp0*=300) using a Langevin temperature equilibration scheme.
16. The periodic boundary conditions are switched on (*ntb*=2) with constant pressure. The system needs to be equilibrated at constant pressure to get proper density and to avoid box edge effects (*ntb*=2, *ntp*>0).
17. Constant pressure dynamics is carried out by setting *pres0*=1. This is the reference pressure (units in bar, 1 bar = ~1 atm) at which the system is maintained. Pressure regulations only apply when constant pressure periodic boundary conditions are used (*ntb*=2).

18. The constant pressure dynamics flag with isotropic position scaling is used ($ntp=1$) and the pressure relaxation time is set to 2.0 ps ($taup=2$).
19. The SHAKE algorithm is enabled for hydrogen atoms ($ntc=2$) with a tolerance of 0.0005 Å and a 2 fs time step. The SHAKE feature constrains the vibrational stretching of hydrogen bond lengths and effectively fixes the bond distance to the equilibrium value.
20. The force evaluation for calculating bond interactions involving hydrogen atoms is omitted ($ntf=2$). If the SHAKE algorithm is being used then it is not necessary to calculate forces for constrained bonds.
21. The energy output frequency is set at 500 steps ($ntpr=500$) in human readable format in the mdout and mdinfo files.
22. The coordinates in the mdcrd trajectory file are updated every 500 steps ($ntwx=500$).
23. The temperature and energy are written to the mden file after every 500 steps ($ntwe=500$).
24. The coordinates are written to the restart file after every 1,000 steps ($ntwr=500$).
25. The system is further subjected to a second round of MD calculations for 200 ps ($nstlim=100,000$) in which the constraints on the G-quadruplex DNA are relaxed to 5.0 kcal/mol.
26. The parameters for MD in the second round are exactly the same as for round one. The only change in parameter $ntx=7$. This option allows the coordinates and the velocities to be read in from the last restart file. The box information is also read if $ntb>0$.
27. The system is then subjected to ten ns ($nstlim=5,000,000$) of production MD in which there are no restraints on the system ($ntr=0$).
28. As the calculation needs to be restarted as a continuation of the previous round of dynamics, velocities in the coordinate input file are required ($irest=1$).
29. The value for ntx is retained at seven, which allows the coordinates and velocities to be read in from the restart file.
30. The rest of the parameters remain the same as in the previous two MD runs.
31. The Particle Mesh Ewalds (PME) summation term is used for all simulations with the charge grid spacing set at 1.0 Å.
32. The trajectories are analyzed using *ptraj* module available in the AMBER suite of programs.
33. The trajectories can be visualized using the VMD program (53).

3.7. Continuum Solvent Modeling

Sometimes simulating explicitly solvated systems can be too computationally expensive. One approach is to employ continuum solvent methods where explicit solvent is replaced with hybrid explicit/implicit (54) or completely implicit models (55). The solvation energies and solvent-dependent conformational changes can be predicted reliably using the Poisson–Boltzmann (PB) approach; however their computational complexity can hinder their use in a MD simulation. The generalized-Born (GB) method is much faster and can be parameterized to yield reasonable solvation energies. Both PB and GB approaches, combined with structural snapshots from explicit solvent MD simulations, have been used in estimating free energies in nucleic acids including G-quadruplex DNA (48, 50, 56). This is carried out by running conventional explicit solvent MD simulations and then postprocessing the trajectory where the explicit solvent and periodicity are removed. The energies are averaged over a sufficient number of snapshots. The MM-PBSA or MM-GBSA free energy methods allow calculation of free energy changes for processes that are not accessible to conventional free energy algorithms. Applications to G-quadruplex DNA require explicit inclusion of ions in the channel.

Continuum solvent modeling can also be applied to calculating ligand binding energies. This can be carried out using two approaches: (a) single trajectory approach where the ΔG s are derived from a single trajectory of the ligand–quadruplex complex and (b) multiple trajectory approach where the free energy difference is evaluated using three separate trajectories of the complex, receptor, and the ligand. It is generally agreed that the single trajectory approach may be more reliable as it cancels sampling errors in the intramolecular terms. These errors can be very significant in separate trajectories.

1. The MM-PBSA method is used to calculate approximate free energies (using a single trajectory approach in the case of ligand binding energies).
2. A conventional MD simulation is carried out using the *sander* program in the AMBER package. The parameters are described above.
3. Snapshots are collected every 20 ps for energetic analysis.
4. The electrostatic contribution to the solvation free energy is calculated using the Delphi II program (57).
5. The hydrophobic contribution to the solvation free energy is determined with solvent accessible surface area dependent terms.
6. Dielectric constants of 1.0 and 80.0 are assigned to the solute and the solvent respectively.
7. A grid spacing of 0.5 Å is chosen, with the longest linear dimension of the molecule occupying 80% of this grid.

8. The AMBER parm99SB charge set and BONDI radii (58) are used.
9. The three K^+ ions are explicitly included within the quadruplex channel.
10. The radius of K^+ ion was determined to be 2.025 Å, by adjusting it until $(\Delta G_{\text{polar}} + \Delta G_{\text{nonpolar}})$ was equal to the experimental $\Delta G_{\text{solvation}}$ of -80.6 kcal/mol.
11. All other energy terms are calculated with programs distributed with AMBER.
12. The solute entropic contribution is estimated with the *nmode* program, using snapshots collected every 200 ps.
13. Each snapshot is minimized in the gas phase, using a distance-dependent dielectric of $\epsilon = 4/r$ before the vibrational mode frequencies are calculated.

3.8. Enhanced Sampling Methods

Enhanced sampling methods deal with pronounced sampling of small parts of the molecules such as loops as in the case of G-quadruplex structures. The conformation adopted by the loops can differ and thus in theory MD simulations should be able to show the stability and correctness of one structure over another and whether the different conformations are inter-convertible on an affordable time scale. However, it is quite difficult for conversions to occur between two different conformational states when either of the structures is accurate during the course of a conventional MD simulation. Local enhanced sampling (LES) can be applied to the loops. The selected part of the molecule is split into N copies that are simulated independently, while the rest of the molecule is simulated in the standard manner. The energy barrier height is reduced proportionally to the number of copies being used ($1/N$).

1. The initial model is taken from a database (NDB or PDB).
2. The loop conformational space is searched with simulated annealing procedures in the Discover module of the Insight suite of packages.
3. During the simulated annealing procedures, the G-quartets are kept fixed while the loops are allowed to move.
4. The simulated annealing runs are carried out in implicit solvent using a distance dependent dielectric ($\epsilon = 4/r$) that mimics the solvent.
5. The initial loop conformation is minimized using 1,000 steps of Polak–Ribiere conjugate gradient with a derivative convergence of $0.05 \text{ kJ Å}^{-1} \text{ mol}^{-1}$.
6. During each cycle, the loop is first heated to 1,000 K over 2 ps, simulated at 1,000 K for 2 ps, and eventually cooled to 300 K for 1 ps.

7. The resulting structure is again minimized using 1,000 steps of Polak–Ribiere conjugate gradient with a derivative convergence of $0.05 \text{ kJ } \text{\AA}^{-1} \text{ mol}^{-1}$.
8. The next loop conformation is generated from heating of the latest minimized conformation.
9. The structures obtained from the simulated annealing runs are clustered into conformational families on the basis of the root mean square deviation (rmsd) analysis between all structure pairs.
10. Pairwise rmsds between all structure pairs are calculated.
11. Clustering is then carried out according to the method used by the NMRCLUST program (59).
12. Selected structures from the clusters are subjected to extensive MD simulations in explicit solvent using the AMBER program.
13. The ions are placed in the structure when these are not present in the experimental template.
14. Additional cations are added in order to neutralize the charge on the system.
15. The system is then solvated in a pre-equilibrated TIP3P water box.
16. The box size depends on the system but is always extended at least 10 \AA from the solute in every direction.
17. The equilibration procedure consists of ten steps, beginning with 1,000 steps of molecular mechanics energy minimization and 25 ps of MD where the solvent is only allowed to move.
18. The whole system is then minimized for 1,000 steps followed by 3 ps of dynamics with a restraint of 25 kcal/mol on the DNA.
19. The DNA restraints were lowered by 5 kcal/mol during each of the next five rounds of 1,000 step minimizations.
20. Finally the system is heated to 300 K over 20 ps with no further restraints.
21. The parameters for MD are used as described above in the protocol for MD simulations.
22. The local enhanced sampling (LES) simulations are carried out in a subset of loop conformations which are generated after an initial equilibration period of 1 ns simulations in explicit solvent.
23. Five copies of each loop are generated using the *Addles* module of AMBER9.0 software.
24. Both LES (loops) and non-LES regions (G-quartets) are maintained at 300 K in separate water baths.

25. After LES simulations are finished, the final copies are averaged.
26. Molecular mechanics and the Poisson Boltzmann Solvent Accessibility (MM-PBSA) method are used to calculate the energies and the results are compared to pre-LES energies.
27. The MM-PBSA method to calculate energies is described above.

3.9. Principal Components Analysis (Essential Dynamics)

An important realization in the analysis of a trajectory obtained by MD simulation is that not every aspect of motion is equally important for function. The concept of essential subspace was introduced, which contains large anharmonic motion of atoms and it is these motions that are more biologically relevant than smaller positional fluctuations. The configurational space that contains only a few degrees of freedom in which these anharmonic motions occur can be identified by reducing the dimensionality of the data that is obtained from MD simulations. Principal components analysis (PCA) is a method that takes the trajectory of a MD simulation and extracts the dominant modes in the motion of the molecule. The overall rotation and translation of the structure during the time course of the trajectory are removed by a translation to the average geometrical center of the molecule and by a least squares fit superimposition onto a reference structure. The configurational space is then constructed using a simple linear transformation in Cartesian coordinate space to generate a $3N \times 3N$ covariance matrix. The matrices are summed and averaged over the whole trajectory. The resulting matrix is then diagonalized generating a set of eigenvectors that gives a vectorial description of each component of the motion by indicating the direction of the motion. Each eigenvector describing the motion has a corresponding eigenvalue that represents the energetic contribution of that particular component to the motion. The eigenvalue is the average square displacement of the structure in the direction of the eigenvector. Projection of the trajectory on a particular eigenvector highlights the time dependent motions that the component performs in the particular vibrational mode. The time average of the projection shows the contribution of components of the atomic vibrations to this mode of concerted motion. The eigenvalues are placed in a descending order where the first eigenvector and eigenvalue describe the largest internal motion of the structure. The eigenvalues decline sharply, showing the possibility of separating the dynamics into a small essential space and a relatively large space, containing only small atomic fluctuations. In simpler terms, on average only about 5% of eigenvectors are necessary to describe 90% of the total dynamics.

1. Conventional MD is carried out on the structure obtained from NDB or PDB, using protocols and parameters described above using the *sander* program in the AMBER package.

2. MD trajectories are extracted using the *ptraj* program in the AMBER package.
3. Principal components analysis on the trajectory is then carried out using the PCAZIP (60) software on the last 5 ns employing 500 frames.

3.10. Quantum Mechanical Calculations on G-quadruplexes

Quantum mechanical calculations (*ab initio*) are more accurate and physically complete than molecular mechanics force field calculations. These calculations, however, do not take into account any forces that arise from long-range electrostatics or salvation effects. QM calculations of multiple quartets can be problematic even while estimating single point energy. The conventional density function theory (DFT) method is much superior to molecular mechanics force fields and can accurately calculate hydrogen bonding patterns within a G-quartet and guanine-cation interactions. DFT however, does not account for base stacking and therefore cannot describe interactions between guanines in different quartets. In order to accurately calculate stacking interactions, one must employ the MP2 method with a large basis set of atomic orbitals or by expanding the basis set limit. This is followed by a cluster correction which scales computer requirements with ~6th power of the number of atoms included, thus making it highly computationally expensive. Gradient optimization of a two G-quartet structure results in a mathematical artifact known as the basis set superposition error (BSSE) that originates from the incompleteness of the basis set of atomic orbitals and causes an artefactual stabilization of complexes. This can be corrected for single-point calculations by employing the standard counterpoise method (61).

3.10.1. Hartree-Fock and Density Function Study of Interactions Between Metal Cations and Hoogsteen Hydrogen Bonded G-Quartets

The Hartree-Fock self-consistent field (HF-SCF) method and the DFT (B3LYP approach) in conjunction with the valence triple-zeta basis set (with *d*- and *p*- like polarization functions) are employed to study the hydrogen bonding pattern within a G-quartet (62).

1. The initial structure of G-quartets can be prepared from the coordinates of a G-quadruplex structure downloaded from PDB or NDB.
2. The bases in the quartets are capped with hydrogen atoms.
3. The C_4 - and S_4 -symmetric G-quartets are studied for comparison with the coplanar complex structure.
4. The metal ions are positioned in the centre of the G-quartet for C_{4h} - and S_4 - symmetry, at a distance of 1.6 Å below the centre for C_{4h} -symmetry.
5. The initial structures used for optimizations consist of four G-monomers with a C_{4h} -symmetric complex geometry except for pyramidal amino groups.

6. The amino hydrogens in the C_4 -symmetric quartet are all on the same side of the base planes, whereas the S_4 -symmetric has hydrogen atoms above and below the base plane in an alternating sequence.
7. The G-quartets are optimized using the B3LYP hybrid density function method (62).
8. The basis sets used are 6-31G(d,p), 6-311G(d,p), and 6-311+G(d,p).
9. The individual bases are also investigated using the MP2/6-31(d,p) method (63, 64).
10. The HF method is employed in order to compare results with the DFT method. This is to ensure that the DFT approach does not overestimate the H-bonding interaction between bases resulting in the hydrogen bond lengths being too short.
11. Force field calculations are carried out using the MMFF94 force field (65) as implemented in the Sybyl 7.0 suite of programs (66).
12. A dielectric constant of 1.0 is used throughout.
13. The optimization is terminated when a gradient of 0.0001 kcal/mol is reached.
14. For metal ions, average relativistic potentials with a large orbital basis and a small core are used (67, 68).
15. A 6-31G(d,p) basis was used for base atoms in complex with metal cations.
16. All calculations are carried out using the GAUSSIAN 03 program (69).
17. The energy minimum structures of the cation-G-quartet complexes are located both at the HF and the B3LYP levels by the analytic gradient techniques.
18. The interaction energy and the frequency of the G-quartets are corrected for the basis set superposition error (BSSE) by the standard counterpoise method (61) implemented in GAUSSIAN 03. In general, this method accounts for the exchange, dispersion, and polarization contributions (70).

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