Maturation of siRNA by strand separation: Steered molecular dynamics study

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Communicated by Ramaswamy H. Sarma

ABSTRACT
RNA interference, particularly siRNA induced gene silencing is becoming an important avenue of modern therapeutics. The siRNA is delivered to the cells as short double helical RNA which becomes single stranded for forming the RISC complex. Significant experimental evidence is available for most of the steps except the process of the separation of the two strands. We have attempted to understand the pathway for double stranded siRNA (dsRNA) to single stranded (ssRNA) molecules using steered molecular dynamics simulations. As the process is completely unexplored we have applied force from all possible directions restraining all possible residues to convert dsRNA to ssRNA. We found pulling one strand along the helical axis direction restraining the far end of the other strand demands excessive force for ssRNA formation. Pulling a central residue of one strand, in a direction perpendicular to the helix axis, while keeping the base paired residue fixed requires intermediate force for strand separation. Moreover, we found that in this process the force requirement is quite high for the first bubble formation (nucleation energy) and the bubble propagation energies are quite small. We believe the success rate of the design of siRNA sequences for gene silencing may increase if this mechanistic knowledge is utilized for such a design process.

1. Introduction
Ribonucleic acid (RNA) interference (RNAi) was initially discovered in plants, but it was not noted in animals until Fire and Mello demonstrated that double-stranded RNA (dsRNA) can cause greater suppression of gene expression than single-stranded RNA (ssRNA) in Caenorhabditis elegans (Fire et al., 1998). Due to the excellent gene silencing potential of RNAi, it has attracted broad attention to exploit its capabilities. In recent years, RNAi has become more and more important in gene silencing and drug development because of its high specificity, significant effect, minor side effects, and ease of synthesis (Dogini et al., 2014). When dsRNA enters the cell, it is first cleaved into short double stranded fragments of 20–23 nucleotide silencing RNAs. These cleaved products have been recognized as the small interfering RNAs (siRNAs) in the form of double stranded helices. They are generally named passenger strand and guide strand. The process of separation of two strands of the double helical siRNA for the formation of matured RISC complex with only the guide strand attracted the attention of the number of scientific groups. Various modes of action for the maturation process were studied, namely wedging method, rubber-band mechanism, and chaperone induced mechanism (Kobayashi & Tomari, 2016). Although siRNA treatment has become very fruitful for different diseases, and several clinical trials are in process. Yet the process of separation of the two strands remained questionable (Filipowicz, 2005). In the RISC, the guide strand of siRNA pairs with a complementary sequence in a messenger RNA (mRNA) molecule and induces cleavage of mRNA by enzyme Argonaute. Thus, the process of mRNA translation can be interrupted by siRNA (Li et al., 2016; Martinez et al., 2002; Meister & Tuschl, 2004; Sashital & Doudna, 2010). Since the rational design of siRNA can specifically inhibit endogenous and heterologous genes, it can modulate any disease-related gene expression. Following this strategic revelation, several synthetic siRNA is being designed with desirable sequences to inhibit any target gene expression (Davidson & McCray, 2011; Fellmann & Lowe, 2014; Wu et al., 2014). The siRNAs undergo further processing inside the cell, where, one strand or part of one (guided strand) gets separated from the other strand (passenger strand). In fact, many cellular processes are driven by mechanical forces (Cook, 1999; Guo & Lee, 2007; Neuwald et al., 1999). The naturally coded microRNA (miRNA) also goes through similar steps for their action. Thus, the structure and force involved in the separation of the strands become one of the important aspects to deal with the efficiency of siRNA.

Double-helical deoxyribonucleic acid (dsDNA) has been widely studied with respect to strand separation based on experiments and theory (Bockelmann et al., 1997; Cocco et al., 2001; Danilowicz et al., 2004; 2009; Essevaz-Roulet et al., 1997; Hatch et al., 2008; Kühner et al., 2007; Kumar
et al., 2007; Kumar & Li, 2010; Schumakovitch et al., 2002; Strunz et al., 1999), where researchers have studied the effect of mechanical force on the structural changes of dsDNA. It has been shown that differences in the chemical structure of dsDNA and dsRNA molecules affects the intra-strand distances (Marin-Gonzalez et al., 2017). Recardo et al. (Herrero-Galán et al., 2013) have studied the effect of force using optical and magnetic tweezers on the stretching of dsRNA and compared it with dsDNA results. Lipfert et al. (2014) have studied the effects of force and torque on the structural changes of the long stretch of dsRNA and pointed out striking differences between dsRNA and dsDNA. The unfolding of the compact structure of RNA was also studied recently by various groups using experiment and simulation (Chandra et al., 2017; Gupta & Bansal, 2016; Mandal et al., 2004; Savinov et al., 2014). These studies provide the path to explore the structural changes due to the application of force. Now these days, siRNA has evolved as one of the unprecedented molecules that require a broad study of structural changes under the application of mechanical force.

Oligomeric siRNA, having 20–22 base-pairs and UU overhang in both the strands requires special attention in its structural changes during unzipping. It may be mentioned that natural microRNAs (miRNA) also require unzipping after they are processed by DICER protein, which may require some assistance from proteins. We approach this novel problem of siRNA strand separation by focusing our study on the opening of both the strand of siRNA under the application of external mechanical force. Furthermore, during interaction with proteins, such as Argonaute, however it is not clearly characterized (Kwik & Tomari, 2012), whether the passenger strand is released intact by the process of strand separation. It may be noted that partial strand separation of dsDNA also takes place during transcription initiation. It was found that the sigma-factor of RNA polymerase is responsible for that (Mallick Gupta et al., 2017; Saecker et al., 2011; Zuo & Steitz, 2015). Thus, the separation of strands of double helical nucleic acid chains is an important aspect for understanding several biochemical pathways. In this report, we present the effects of the pulling with the constant velocity steered molecular dynamics (SMD) simulations under different protocols as shown in Figure 1, broadly classifying them as axial pulling and unzipping. We provided a comparative study using all atom MD simulation, which may help to characterize the structural changes that occur during the pulling of double-stranded siRNA. We address the problem with extensive studies of structural parameters, hydrogen bonds (H-bonds) disruption, and stacking interactions.

The present studies, which are involved in the calculation of path dependent force and disruption of H-bonds by various protocols may shed the light to provide a future perspective of binding proteins with siRNA for gene silencing. The analyses may also be important for designing more effective siRNA sequences.

2. Model and method

2.1. Equilibrium MD simulation

Starting system: Well studied siRNA crystal structure with PDB ID 2F8S (Yuan et al., 2006) is taken for all the simulations. The structure is comprised of 22 nucleotides on each strand of the duplex with characteristic UU overhang in both the 3′-ends. The self-complementary sequence is, 5′(AGACAGCAUAUAUGCUGUCUUU)2. The dimension of the duplex is ≈ 8.0 nm in length in normal double helical form.

Protocol: All molecular dynamics simulations were carried out using GROMACS 5.1 package (Abraham et al., 2015) and CHARMM36 Force Field (Best et al., 2012). The complete siRNA double helical structure, without the Argonaute protein interacting through one end of the RNA, was considered including the 5′-terminal phosphate groups. The siRNA is solvated with a TIP3P water model in a cubic box with sufficient dimension (10 nm × 10 nm × 10 nm) and neutralized with 44 Na+ ions. The system is then subjected to energy minimization by the steepest descent method to eliminate initial stress. For initial equilibration, standard protocol of 100 ps each of NVT and NPT simulations were done (Biswas et al., 2019). Position restraints were applied to the RNA atoms during equilibration of the system for both NVT and NPT processes. The siRNA and non siRNA atoms were coupled to separate temperature coupling baths, maintaining 300 K using Berendsen weak coupling method (Berendsen et al., 1984). The final 100 ns NPT production MD run was conducted in absence of any restraints. In the production run, the Nosé-Hoover thermostat (Hoover, 1985; Nosé, 1984) was used to maintain temperature, and the Parrinello-Rahman barostat (Hoover, 1985; Nosé, 1984; Nosé & Klein, 1983; Parrinello & Rahman, 1981) was used to isotropically regulate pressure. Periodic boundary conditions (PBC) were
employed for all simulations and the particle mesh Ewald (PME) method (Darden et al., 1993) was used for long-range electrostatic interactions. The simulation time step was set to 2 fs with the LINKS algorithm to maintain bond lengths involving hydrogen atoms. The final structure from the end of the 100 ns equilibrium trajectory was used as starting configurations for pulling simulations.

2.1.1. Steered MD simulation

Four different steered molecular dynamics simulations (SMD) were carried out with equilibrated conformations of siRNA along with solvents at 300 K. Henceforth we would refer to them as: (i) Axial Rupture, (ii) Axial Stretch, (iii) Terminal Unzip, and (iv) Central Unzip. In the case of Axial Rupture, shown in Figure 1a, force is applied at the 3’-terminal residue of one chain keeping the 3’-terminal residue of its complementary strand fixed (immobile) to their original position. For Axial Stretch (Figure 1b), we have fixed the 5’-terminal residue of one strand and applied force on the 3’-terminal residue of the same strand along the helical direction of the system. In the case of Terminal Unzip, shown in Figure 1c, we have fixed the 3’-terminal residue of one strand, and force is applied on the 5’-end of its complementary strand along a direction perpendicular to the helical axis. Here both the 3’- and 5’-terminal residues are on the same side of the double helix. In the Central Unzip case (Figure 1d) the force has been applied in the central point (residue 11) of one strand keeping the central residue of the complementary strand fixed. This is also the case, where force is applied perpendicular to the helical direction. In the case of pulling simulations, big rectangular boxes with dimensions sufficient to satisfy minimum image convention for complete separation of siRNA were generated. This provided space for the nearly elongated single stranded RNA along the Z-axis for the rupture and along Y-axis (perpendicular to the Z-axis) for the unzipping. We have adopted the boxes of the size 15 nm × 15 nm × 54 nm, 15 nm × 15 nm × 54 nm, 15 nm × 40 nm × 15 nm, and 15 nm × 40 nm × 15 nm, for Axial Rupture, Axial Stretch, Terminal Unzip, and Central Unzip respectively. These boxes were filled with a TIP3P model of explicit water with adequate Na⁺- counterions to neutralize the systems. Equilibration was performed for 200 ps NVT and 10 ns NPT simulations, using the same methodology described above before SMD simulations.

2.1.2. Protocol

SMD is based on applying external forces to particles in a selected direction by adding a spring-like restraint, thus imitating directly the basic idea of an AFM experiment through optical or magnetic tweezers. The SMD simulations with constant velocity (CV) stretching (SMD-CV protocol) were carried out by fixing one of the residues and applying external forces to the dummy atoms attached to the center of mass of another residue (SMD residue) with a virtual spring. We adopted a spring constant value of 1000 kJ mol⁻¹ nm⁻² and a pulling rate of 0.0008 nm ns⁻¹ (Huang et al., 2012; Lemkul & Bevan, 2010; Singh et al., 2013). To evaluate the pulling for multiple systems is always computationally expensive. Resultantly, the computational value of stretching velocity is always very high compared to the experimental value, hence the velocity of stretch has been taken faster. The force experienced by the pulled terminal residue, F is defined as $F(t) = k(vt - x)$ where, $x$ is the displacement of the pulled atom from its original position, $v$ is the pulling velocity, and $k$ is the spring constant. The direction of pulling was such that the end-to-end distance always increased, i.e. the SMD residue was pulled away from the fixed residues. The important thing is that pulling of all model systems is depends on their end state hence the duration of pulling is different for each case. In the Axial Rupture case time of simulation is around 24 ns, in the case of Axial Stretch time of simulation is around 10 ns, in the case of Terminal Unzip it is around 24 ns, and 18 ns is the simulation time of Central Unzip. It is noted that single seed simulation is statistically not very much reliable hence always prefer simulations for some more seeds (Knapp et al., 2018). For a large system with a huge box, it is not possible to carry the SMD simulation for many different seeds. Hence, here we have done this simulation for 2 different seeds, which gives qualitatively similar results for all model systems. From the all figures shown in the supplementary material, it is clear that the qualitative behavior is the same across all types of analysis like force variation, H-bond breaking variations, and stacking parameter variations.

2.1.3. Analysis

Analysis of the trajectories, including finding the number of H-bonds was done by GROMACS 5.1 (Abraham et al., 2015). Base-pair orientation parameters and stacking geometry were analysed by NUPARM (Bansal et al., 1995; Mukherjee et al., 2006; Pingali et al., 2014).

3. Results analysis

We first look at the stability of the equilibrium MD simulation. Previous equilibrium molecular dynamics studies by several groups had observed that separation of the strands of siRNA took place during interaction with graphene or carbon nanotube (Ghosh & Chakrabarti, 2016; Jung et al., 2010; Landry et al., 2015; Santosh et al., 2012a, 2012b). It was also demonstrated that such separation of strands did not happen in the case of double stranded DNA (Santosh et al., 2012a, 2012b), thus attributed this siRNA separation to the specific interaction between carbon nanomaterial and siRNA. However, In Our recent study, it has been observed that the siRNA remains in the double helical form in the physiological environment (Biswas et al., 2019). This indicates that external force is possibly needed to compel the separation of strands in siRNA. The results compliments our equilibrium simulations, which also demonstrate only moderate RNA breathing in equilibrium MD throughout the 100 ns production run. We have taken the final equilibrated structure of siRNA duplex from the equilibrium MD simulation for further force induced SMD simulations. In SMD or center of mass (COM) pulling
the system is biased to demonstrate the behavior toward a particular phenomenon. Application of an external force to cause displacement in the simulated system allows for the calculation of work, a path-dependent quantity. For the opening of the strands of the siRNA we adopted two standard protocols as described in the method section (Figure 1), one is to apply force along the helical direction of the system and the other is to apply the force perpendicular to the helical direction of the system, the later can be generalised as unzipping.

We have measured the forces experienced by the siRNA, which vary with time for the different model systems (Figure 2). Figure 2a shows the variation of the force with time for the Axial Rupture model. We observe a nearly linear variation of extension with simulation time (Figure SI 1), hence the force vs. extension curves also look very similar. In the case of Axial Rupture, one strand is pulled along the helical axis direction keeping the far end of the other strand constrained. During the initial phase of pulling, the variation of force with time (Figure 2d) as compared to other systems. After the initial phase, the force reduces to zero when the strands dissociate completely (Figure 3a, blue curve). After about 10 ns, the number of H-bonds does not reduce significantly with time, possibly indicating most of the phase transitions took place by 10 ns. However, the number of H-bonds in the double helix does not reduce significantly with time, possibly indicating that both take place simultaneously during this phase. To understand the mechanism, we have analysed the succession of base-pairs also may get affected during this phase of simulation with a large force. The third option is that both take place simultaneously during this phase. To understand the mechanism, we have analysed the number of H-bonds present in the system at each time frame (Figure 3). The total number of H-bonds continues to decrease with time in the first phase, i.e. up to 10 ns (Figure 3a, blue curve). After about 10 ns, the number of H-bonds does not reduce significantly with time, possibly indicating most of the phase transitions took place by 10 ns. However, the number of H-bonds in the double helix does not reduce to zero value within this time. As seen in Figure 2a, the force increases at this point of time when the number of H-bonds between the two strands appears to increase slightly. After this phase transition, the number of H-bonds slowly reduces to zero when the strands dissociate completely (Figure 3a, blue line). But after this critical interaction, the system breaks, and the requirement of an additional force starts to decrease. In this time duration, force reduction from maximum to minimum reveals the structural change of the system from the bound double helical state to the completely unbound ssRNA state. Both the strands become almost separated, where most of the base-pairs are broken. It may be noted that the unfolded single stranded chains can form intra strand H-bonds and hence, the blue line does not reach zero value. This can be visualised by the various snapshots shown in the first row of Figure 4.

Next, we observe the unique results on the Central Unzip model system (Figure 1d), where force is also applied perpendicular to the helical direction of the RNA but in this case at the central residue (11th residue) of one of the strands keeping the paired residue of the complementary strand immobile. Here, also the variation of force experienced by the system with time is qualitatively similar to that of the terminal-unzip model system. However, it is notable that the measured force is significantly larger (nearly 300 kJ mol$^{-1}$ nm$^{-1}$) in the initial phase (Figure 2d) as compared to the other systems. After the initial phase, the force reduces to a smaller magnitude around 100 kJ mol$^{-1}$ nm$^{-1}$ and even smaller values. This initial increase of force can be explained as the initiation of base-pair opening and the latter as the propagation of base pair opening to both sides of the central one. This is equivalent to nucleation energy for the
cooperative transition from helix to coil state. The nucleation energy is quite high, as it would disrupt a base pair (at least two hydrogen bonds) and two stacking interactions between the pulled base pair and its two neighboring base-pairs on both sides. The second type of force is supposedly stronger than the base pairing energy and it is doubled also (Yakovchuk et al., 2006). Once the H-bonds of the central base-pair break and the stacking between the central base-pair and its neighboring base-pair is disrupted, the neighbouring base-pairs can have fraying effects. In other words, these neighbouring base-pairs come to contact with solvent water. Hence, these bases can form H-bond with the complementary bases or with solvent water molecules in a competitive manner. The trajectory of this model system can be visualised by the snapshots shown in the second row of Figure 5. Furthermore, the propagation stage is quite faster as compared to the other SMD results, as two base-pair break together, i.e. \( C^+1 \) and \( C-1 \) after base pair \( C \) breaks (where \( C \) is the central base-pair) then \( C+2 \) and \( C-2 \) break and so on. Hence, they can now easily become single
stranded breaking the Watson-Crick base pairing, and stacking interactions, requiring a small amount of force.

4. Structural transition

As indicated above, the H-bonds between the complementary bases in the base-pairs breaks during force induced SMD simulations. Thus the bases do not remain coplanar to each other and the other degrees of freedom of the bases also increase beyond their regular values. Quantitative analysis of these degrees of freedom of the bases with respect to paired ones can be done by the six IUPAC-IUB recommended intra base-pair parameters (Olson et al., 2001). We have therefore looked at the shear values, which are related to the H-bonding features. Similarly, relative orientations of a base-pairs with respect to their neighbouring stacked ones also change significantly when the stacking interactions are disrupted. These can be analysed by tilt, roll, etc., inter base-pair local parameters. The Effect of all these inter base-pair parameters can also be analysed by a composite parameter, namely stacking overlap, and we have analysed that also. Variation of shear, base-pair overlap, and twist, as representative parameters, are shown in Figures 6 and 7 to compare all the systems. The shear parameter gives information about the relative movement of the bases with respect to the paired ones, indicating disruption of hydrogen bonds in a base pair, overlap provides information about stacking between two base-pairs and twist indicates ladder like structure formation and hence is often related to stretching (Marin-Gonzalez et al., 2017). These are illustrated as three dimensional plots. It may be noted that shear values of good Watson-Crick base-pairs are around zero (Mukherjee et al., 2006), twist value of A-RNA stretches are around 33° (Needle et al., 2009), and stacking overlap between successive base-pairs in RNA double helices are around 45 to 50 Å² (Pingali et al., 2014) depending on base sequence. We have also analysed the variation of these parameters during equilibrium MD simulations of the siRNA to understand the force induced effects on the double helical structure (Figure 6a, b & c). From the figures, it is clear that the structure maintains almost the same value of shear, overlap, and twist value throughout the simulations, indicating that no separation of the strands took place in absence of any force or molecules like graphene or carbon nanotube. Furthermore, terminal fraying is also found to be minimum as compared to other simulations using CHARMM force-field (Kundu et al., 2017), possibly due to somewhat capping effect by the two single stranded residues at the two ends of the double helix.

The changes in the parameters for the Axial Rupture system are shown in Figure 6d, e & f. Figure 6d illustrates that initially up to 4 ns shear values of all the 20 base-pairs are near 0 (sky blue color), indicating no disruption of any base-pair. After that minor disruption of base pairing can be seen with minor fluctuation of shear values until 10 ns. Here, the shear values of the 5th, 6th, and 7th base-pairs become more negative and can be seen to be more affected by the force. Base-pairs of 5'-terminal residue adopt large positive shear and all the base-pairing near 3'-terminal assume large negative shear. All the shear values start changing because of structural transition after 12 ns and afterward huge...
Figure 6. Variation of base pair parameters during equilibrium and axial pulling simulations. Residue numbers are shown in the x-axis, time in the y-axis, and the graded color for values of (a) shear (Å), (b) overlap (Å²), and (c) twist (°), for equilibrium simulation. The shear, overlap, and twist for the Axial Rupture model are shown in (d), (e) & (f) while (g), (h) & (i) are the same for Axial Stretch.

Figure 7. Base pair parameters for unzipping SMD simulations. The base-pair numbers are shown in the x-axis, time (ns) in the y-axis and the graded color represents values of (a) shear (Å), (b) overlap (Å²), and (c) twist (°) for the Terminal Unzip model and (c) shear (Å), (d) overlap (Å²) and (e) twist (°) for Central Unzip model.
fluctuation of shear (Yellow color corresponding to values around 10–15 Å or deep blue color for shear values around –15 Å) indicate disruption of initial base pairing. Comparing with disruption of H-bonds (Figure 3a for Axial Rupture model) it can be concluded that after breaking half of the total possible H-bonds (from ≈48 to 25) the disruption is steep linear after around 12 ns. Variation of overlap parameters (Figure 6e) illustrates continuous disruption of stacking for all the base-pair steps within 8 ns, and it is seen to initiate after 2 ns at the central region. However, even at this time (8 ns) most of the bases are paired to their complementary ones, as reflected from the analysis of shear. Thus, base-pairs opening and stacking disruption appear to be independent and unrelated events. For few instances, we found that, after the complete breakdown of the stacking, again overlap value increases due to single stranded helix like structure formation. Such single stranded structure formation leads to stacking between successive bases, instead of stacking between successive base-pairs in double-stranded RNA. From Figure 6f it appears that the twist values of the base-pairs at different helical positions start to fluctuate at diverse time points. The middle base pair steps (9 and 10th) faces twist disruption at as early as 2 ns. Comparing with the two other parameters, shear, and overlap, we found the twist to get disrupted earliest and become most sensitive to the applied force. It is noted that the central base-pairs are getting affected earlier possibly due to weaker base-pairing in the central region (AU rich sequence). These structural transitions can be seen with snapshots throughout the simulation in the first row of Figure 4.

Structural parameter variations for the Axial Stretch model of same strand pulling are presented by Figure 6g, h and i. Interestingly, it can be observed that in this case values of the parameters have a completely different signature of variation as compared to the Axial Rupture model. We found that the regular shear variation is maintained for a much longer time of pulling for all the base-pairs as compared to Axial Rupture model (Figure 6d). However, a somewhat larger fluctuation of shear values is observed for a few base pairs (14–17th) after 4 ns. These base-pairs are also seen to be unstacked with respect to their neighbours from 4 ns (Overlap values reduce to around 20 Å²). As expected twist value of these base-pairs also reduce at the same time. Disruption in stacking overlap is also found less for Axial Stretch model system (Figure 6h) as compared to the Axial Rupture model system (Figure 6e). Even at the end of the Axial Stretch model simulation, significant stacking overlap around 30 Å² is found between successive base-pairs, indicating separation of the strands did not take place. Comparing with the breaking of H-bonds for this case (Figure 3a, maroon line) showing that even after the system is melted the number of H-bonds is still significant (>10 number), which can be observed by different snapshots shown in the second row of Figure 5. We find twist values (Figure 6i) are sensitive to the force as compared to shear and overlap in the Axial Stretch model. However, the fluctuation starts late as compared to the Axial Rupture model. These differences in variations can be related to lesser disruption of H-bonds in Figure 3a for the axial stretch model, indicating the situation that complete force induced rupture is not taking place.

Variations of base pair parameters for the Terminal Unzip model system are shown in Figure 7a, b and c. In this case, the fixed end and the end at which pulling force is applied are on the same terminal side of the duplex and this unzipping mode is prominent in the signature of parameter variations. Variation of shear (Figure 7a) indicates that the disruptions of base-pairs are completely dependent on the base positions. The terminal to the pulling end is first disrupted by the unzipping force, which starts to perturb the system from the pulling end gradually (19th base pair). Similar gradual disruption of overlap values can be observed from Figure 7b. This illustrates that the unzipping effect is progressive and the minimal effect is transmitted to the bases far from the current unzipped base pair. This is almost equivalent to an extension of the fraying effect seen earlier (Kundu et al., 2017).

Shear variation for the Central Unzip (Figure 7d), clearly indicates melting of the central base pair occurs just after 1 ns. The base-pairs next to the central pulled one (12th one) acquire large positive shear, while the previous one (10th base pair) gets large negative shear, and this feature is continuous till the end. The base-pairs, which are away from the pulling point, maintain almost the same value (near zero, cyan color) for a much longer time. Similar structural changes can be observed in terms of the stacking overlap value for all base positions as shown in Figure 7e. The overlap values of the 6th residue and 14th residue appear to increase to a value close to 35 Å² after 7 ns, indicating the formation of secondary helix-loop-helix like structure within the separated single strands. This can be visualized by the snapshots (at 9, 12, and 18 ns) in the lower panel of Figure 5. In terms of variation of twist value (Figure 7f) similar trend of separation of the strands starting from central pulling position is observed.

5. Discussion

In this study, we have tried to integrate the structural transition of the system with different protocols of applied force. We found that, when pulling is applied at the axial direction (Axial Rupture) of the system, it experiences the highest force compared to all other protocols. In this case, the highest force (~600 kJ mol⁻¹ nm⁻¹) builds up to a point until certain critical interaction is broken. On the other hand, for the same direction pulling (Axial Stretch) the force continuously increases because the fixed end and pulled end are situated at the same strand. Nevertheless, ultimate disruption of most of the H-bonds, in this case indicates that separation of the strands can also be achieved by this way of steering. In the case of pulling perpendicular to the helix axis, the system unzips in the usual way of strand separation. Here, the opening of base-pairs is progressive and is achieved one by one from the pulling terminal. Due to this, during initial simulation time, force increases and then maintains almost the same value over the pulling time. Among the protocols of pulling, Central Unzipping looks most different as
compared to others. In this case, initially, the system experiences a strong force, because of the opening of the first intact base-pairs and simultaneously unstacking the central base pair from both the sides of pulled nucleotides. But, eventually, the successive opening of progressive H-bonds requires much less force compared to all other cases of pulling. This is also revealed by the measurement of breaking of the number of H-bonds. Hence this mode of opening can be viewed as most feasible as compared to all other possible protocols of siRNA strand separation.

We have also looked at the crystal structures of RNA double helical fragments bound to Argonaute protein from Protein Data Bank (Berman et al., 2000) to evaluate the most interactive structural signature of RNA. We found the protein bound double helical RNA strands are of significant length (10 or more base-pairs) in 3HJF, 3HK2, 3H9M, 4N47, 4NCB, 5AWH, and 5UXO. We have analysed hydrogen bonds between the protein and RNA using PyRHBfind software (Mukherjee et al., 2005) and have focused on the strong ones. The middle portion of the RNA duplex is found to be mostly interacting with protein residues by the formation of very strong H-bonds involving negatively charged phosphate group of RNA and positively charged Lys or Arg residues of Argonaute. In most cases, one of the ends of each strand is also found similarly anchored. e.g. 3HJF.pdb (Wang et al., 2009) and 3HK2.pdb (Wang et al., 2009) have lengths of the double helical regions are 12 and 14, respectively and around 3 to 4 bases of both the strand residing at the middle region of the helix in total forms H-bonds with the Protein. Hence, accepting the idea that more crowding will enable higher grip by protein and subsequently lead to rupture start-point, we can conclude that, central pulling must be the most feasible phenomenon in nature. However, crowding at one of the terminals may indicate that other mechanisms could also take place, though with lesser probability. The complete understanding of anchoring the siRNA at multiple points (center as well as the terminals) may require new methodological development to analyze the system. Again, the role of the UU overhang cannot be detected in the unzipping of siRNA duplex, which might be involved in some other process. Among the helical parameters, we observed that for all the cases of pulling, the twist parameter is most sensitive during the opening of strands of siRNA duplex. Experimental determination of siRNA structure in Protein Data Bank is so far inadequate in number. In a future perspective, it is necessary to study the effects of different sequences and lengths for the system of siRNA and observe the changes associated.

Acknowledgements

We acknowledged CDAC for their computing support. Most of the simulations were performed in the cluster of CAPP-II project of DAE. We thank Prof. Rituparna Sinha Roy of IISER-Kolkata for discussion and useful suggestions.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

We gratefully acknowledge SERB and DST, New Delhi, India, for their financial support through project numbers PDF/2015/00308, and PDF/2017/002110/CS. RKM also thank UGC for D. S. Kothari fellowship with the award number F4-2/2006(BSR)/PHY/18-19/0060. This work was supported by University Grants Commission, India and Science and Engineering Research Board.

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