DNA translocation through vertically stacked 2D layers of graphene & hexagonal Boron Nitride heterostructure nanopore

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ABSTRACT: Cost effective, fast and reliable DNA sequencing can be enabled by advances in nanopore based methods, such as the use of atomically thin graphene membranes. However, strong interaction of DNA bases with graphene leads to undesirable effects such as sticking of DNA strands to the membrane surface. While surface functionalization is one way to counter this problem, here we present another solution based on a heterostructure nanopore system, consisting of a monolayer of graphene and hexagonal Boron Nitride (hBN) each. Molecular dynamics studies of DNA translocation through this heterostructure nanopore revealed a surprising and crucial influence of heterostructure layer order in controlling the base specific signal variability. Specifically, the heterostructure with graphene on top of hBN had nearly 3-10x lower signal variability than the one with hBN on top of graphene. Simulations point to the role of differential underside sticking of DNA bases as a possible reason for the observed influence of layer order. Our studies can guide the development of experimental systems to study and exploit DNA translocation through two-dimensional heterostructure nanopores for single molecule sequencing and sensing applications.

Introduction:

Rapid, affordable and reliable DNA detection and sequencing technique is a challenging and imperative objective in the field of personalized medicine.[1,2] There is a strong association between DNA sequence alteration and the progression and development of a disease.[3] Analysing the DNA sequence not only helps in detecting diseases, but has also become essential in the fields of forensic sciences, biological systematics, metagenomics and virology.[4–9] Sequencing technologies which were used for human genome project[10] suffer from limitations such as low throughput, high cost and are time consuming[11,12] and may not be sensitive enough for base modifications such as methylation.[13,14] Nanopore based single molecule sequencing techniques have emerged as a promising alternative in this regard.[15–18] Here, the DNA strand is electrophoretically driven through a nanopore whose pore dimensions are of the order of 2-5 nanometer (nm) by applying a potential difference typically in the ranges of 0.1V – 1V. The motion of the DNA strand across the nanopore is referred to as translocation. The nanopore resides on a membrane which separates two reservoirs containing electrolytes and between which there exists an ionic current. The translocation of the DNA strand through the nanopore results in modulation of the ionic current which is used for base identification.[19,20] Existing nanopores fall into two categories: biological nanopores, which consist of pore forming proteins such as α-hemolysin or Mspa embedded in a lipid-bilayer,[21–26] and solid-state nanopores, which consist of a pore fabricated on an inorganic thin membrane such as Silicon Nitride or Graphene. DNA detection and sequencing using biological nanopores have been successfully demonstrated and even commercial systems are available now.[27–30] However, the inherent weaknesses of biological nanopores, such as high sensitivity to pH, temperature, salt concentration and lack of high mechanical strength[31] have motivated various groups to develop solid-state nanopores even though solid-state nanopore based sequencing significantly lags behind biological nanopore based sequencing.[32–34] An additional advantage of solid-state

nanopores is the possibility of cost-effective mass-manufacturing by leveraging the use of existing semi-conductor foundries.[35–37] For controlling translocation times, the use of mechanical force for spontaneous encapsulation of DNA and RNA inside nanopores,[38,39] integration of DNA origami[40,41] and functionalisation of nanopores[42] have also been reported

Ultra-thin nanopores made from two-dimensional materials such as Graphene, MoS_2, hexagonal Boron Nitride (hBN) have emerged as highly promising solid-state membranes due to the potentially high base-resolution.[43,44] For instance, the thickness of a single graphene layer is same as the gap between successive bases in a DNA strand. Nanopore sensors using Graphene has been extensively studies in simulations and experiments.[45–47] DNA translocation through graphene nanopores however, suffers from drawbacks such as DNA sticking to graphene due to its hydrophobicity, resulting in pore clogging and non-consistent translocation times.[41,48–50] The hydrophilic nature of hBN, another two-dimensional material with similar mechanical properties as that of graphene, makes it a potentially better alternative substrate for nanopore based sensors[51]. Previous studies of DNA translocation through hBN nanopores have revealed that though the translocation events were devoid of sticking, the translocation velocity was not significant enough to differentiate among base-pairs.[52,53]

Recently a great deal of interest has emerged in exploring 2D heterostructures consisting of vertically stacked 2D layers. Such structures have shown to exhibit better possibility of tuning electronic properties and better structural stability than monolayers when suspended, owing to reduced corrugations. Next generation electronic devices and bio-molecule sensors have been realised with such stacks, consisting of either in-plane lateral heterojunction or vertical stack.[54] Particularly for DNA detection, heterostructures made from Graphene and hBN and in-plane currents have been used to distinguish individual bases .[55,56]

In this paper, using all atom molecular dynamics simulations, we investigate the translocation of double stranded DNA through a nanopore embedded in a graphene-hBN heterostructure. The main focus of this study was to examine whether and how nanopores on 2D heterostructures help mitigate the drawbacks of 2D monolayer based nanopore devices. Our stack heterostructure simulations show that we can distinguish dsDNAs based on their ionic currents and dwell times. The residence times of dsDNA in the pore region, conductivity of the stack pore, mechanism of translocation and ionic current modulations are discussed in detail. The most remarkable observation from this study is the important role of layer ordering of the heterostructure on the reliability of base-specific signal transduction.

Methods:

Design of stacked nanopore system

The graphene and hBN sheets with dimensions of 101 nm x 89 nm were constructed using the inorganic builder plug-in of Visual Molecular Dynamics (VMD).[57] The distance between the adjacent sheets in the vertical stack is 0.34 nm. A nanopore was made in the centre of the sheets in such a way that the pores were aligned. To make the pore, atoms which fulfilled $\sqrt{(x_c - x)^2 + (y_c - y)^2} < r$ were removed, in which x_c, y_c were sheet's centre and x,y represent the atom's co-ordinates. For conductivity studies, the stacked membrane was constructed with the top and bottom layer being graphene and hBN (henceforth referred to as Gr+hBN) respectively. Pores of 1.5 nm, 2.1nm, 3.1 nm, 4.1 nm and 5.1 nm in diameter were made, and simulations were carried out. For translocation studies, the PDB (Protein Data Bank) structure for dsDNA was built using the NAB (Nucleic Acid Builder) program of AMBER14.[58] To get the final dsDNA structure, the NAB generates the base-pairs first and then adds the phosphodiester bonds. Poly(AT) and poly(CG), each of 18 base-pair lengths were generated. In all the translocation simulations, three bases-pairs were inserted into the pore in Gr+hBN to

scale down the time needed for DNA to find the nanopore. Translocation studies were also performed on a pore constructed using hBN and graphene on top and bottom (henceforth referred to as hBN+Gr) respectively. Other details of the system such as the water box-dimensions, no. of atoms and so on have been summarised in Table 1.

Simulation Methodology:

The initially designed structure consisting of the membrane and dsDNA were loaded into the xleap module present in AMBER14,[59] where TIP3P waterbox model was used to solvate the structure. AMBERff99 forcefields with parmbsc0 corrections (OL15)[60,61] were used for describing DNA as it is observed to replicate the arrangements of long DNA structures.[62] The interaction involving graphene carbon were modelled as atom type CA similar to the previous studies.[41,63] The parameters for Boron Nitride were taken from Hilder *et. al*.[64] which has been validated in the study by Kumar *et al*.[65] For modelling the interaction of ions in the system, Joung-Cheatham ion parameters[66] were used. A cut-off of 9 Å was used for short-range interactions. For calculating long-range interactions, particle mesh Ewald (PME)[67] was used. An integration time step of 2 fs was used after using SHAKE algorithm[68] for restraining all the bonds involving hydrogen. Energy minimization, which involved 3000 steps of steepest descent and 1000 steps of conjugate gradient, was performed on the system to reduce bad contacts, during which the membrane and the dsDNA was harmonically restrained to 500 kcal/mol $Å^{-2}$. After this, the system was gradually heated from 0 to 300 K for 40 ps with an integration time step of 1 fs, in which the membrane and dsDNA were harmonically restrained with a force constant of 20 kcal/mol $Å^{-2}$. Following this, the system was equilibrated under constant pressure-temperature (NPT) conditions, that had the following parameters: (a) 1 ns equilibration with harmonic restraint of 1 kcal/mol $Å^{-2}$; (b) 0.5 ns equilibration with harmonic restraint of 0.5 kcal/mol $Å^{-2}$; (c) 25 ps equilibration with no restraint. For temperature and pressure regulation, Berendsen weak coupling method[69] was used with a time constant of 0.5

ps. For translocation studies, a harmonic restraint of 5 kcal/mol $Å^{-2}$ was applied to the both the graphene and hBN membrane's atoms present around the pore inside a ring of 2 nm radius and rest of the sheet's atoms were fixed. The entire system was solvated with NaCl solution with a concentration of 1M. Along the Z axis, (the translocation axis), a constant electric field is applied (Figure 1) and is represented in terms of potential difference as $V = -E*L_z$, where L_z is the length of water-box along the translocation axis. The ionic currents through the nanopore was calculated by $I(t) = \frac{\sum_{i=1}^{N} q_i [z_i(t+\Delta t) - z_i(t)]}{\Delta t * L_z}$, in which N is the number of ions passing through length L_z, containing an individual charge of q_i; z_i (t + Δt) and z_i (t) are the z co-ordinates of the corresponding i^{th} ion; The sampling frequency (Δt) of 10ps was used. Production runs were performed using NAMD,[70] using AMBER force field parameters. Visualisation and analysis of data from this study was performed using custom scripts written in VMD and CPPTRAJ.[71]

Results and Discussion:

Pore characteristics:

The conductivities of Gr+hBN stacked nanopore with diameters of 1.5 nm, 2.1 nm, 3.1 nm, 4.1 nm, 5.1 nm were characterised by computing the ionic current corresponding to difference bias voltages applied across the membrane. Electric fields corresponding to biases of -2.5V to +2.5V in steps of 0.5V, were applied to the system for 40 ns. The current values were block averaged over the entire simulation time (i.e.) 40 ns. For these studies, the system was devoid of dsDNA. We observe that the ions pass from one side of the membrane to other only through the nanopore as they are hindered by the graphene and hBN layers on the cis and the trans side respectively. The I-V characteristics for different pore sizes are shown in Figure 2a. The conductance values were computed from the slope of linear fit of the I-V characteristics. As expected, we observe that an increase in pore diameter results in an increase in conductance

owing to a larger region for ions to flow through. For instance, the value of conductance is 2.41 nS, 4.81 nS and 7.96 nS respectively for Gr+hBN stack pores with diameter 2.1 nm, 3.1 nm and 4.1 nm respectively.

Theoretical conductance[72] can be computed from $G = \kappa\left[\frac{4*l_{pore}}{\pi*d^2} + \frac{1}{d}\right]^{-1}$, where κ is the conductivity of the ionic solution (1M NaCl, value obtained from calculations performed by Farimani et al.[40]); l_{pore} and d is the thickness and diameter of the nanopore, respectively. Figure 2b shows the comparison of theoretical and simulated values for different pore sizes. The higher values obtained from theoretical calculations as compared to simulation values can be attributed to the very small thickness of our nanopore system. It has been shown that the theoretical model overestimates the values for thinner nanopore.[73] In order to calculate the resulting transmembrane bias along the direction of applied field (z axis), we calculated the mean total potential over the pore along the x and y axes. Figure 2c shows the comparison of mean bias for different pore diameters. We observe that the potential drop occurs primarily across the nanopore and does not change much along the regions far away from the pore. Also, the drop is very significant for the pores with very less diameter.

Translocation Studies with Gr+hBN nanopore:

Translocation studies on dsDNA were performed under an applied external bias with independent simulations involving poly(AT) and poly(CG). Effectively the translocation data is calculated for 15 base-pairs as three base-pairs are pre-inserted into the pore to reduce the time spent by the DNA strands searching for the pore. High bias voltages in the ranges of 2V to 3V were applied to reduce computational time. The substrate studied here for translocation is Gr+hBN membrane with a pore diameter of 2.1 nm. A base-pair is considered to have translocated if the distance along the axis of applied bias exceeds 3.4 Å from the substrate. For a bias of 2V, poly(CG) has a translocation time ranging from 13 to 24 ns, whereas poly(AT)

relatively longer to translocate with translocation times ranging from 28 ns to 46 ns as evident from Figure 3a. Average residence time of a single base-pair was calculated from translocation simulations of dsDNA under different biases. An overall reduction in translocation time corresponding to an increase in voltage in both A-T and C-G base pairs is observed as expected, shown in Figure 3b. Poly(AT) sequences took longer to translocate than poly(CG) sequences in all the bias conditions studied.

In order to understand the sequence dependence of translocation times seen in Figure 3b, we studied the dynamics of dsDNA translocation. The Supporting movies S1 and S2 show the translocation of poly(AT) and poly(CG) under an applied bias of 2V, and Figure 3c to 3j show representatives snapshots. The most important observation comparing the translocation of poly(AT) and poly(CG) is that there is significant distortion of the double helix structure during translocation of poly(AT) sequence (See Figures 3c to 3f) while poly(CG) sequence translocates as a nearly intact double helix (See Figures 3g to 3j). This is understandable from the fact that CG base pairing has a higher bond strength compared to AT pairing owing to the presence of an additional hydrogen bond for the CG pair.[74] Due to the higher bond strength, previous studies have shown that poly(CG) is softer[53] and can be stretched compared to poly(AT) under identical stress.[75] Results from our studies also support this fact. For example, for 2V around 62 % of bonds were broken in poly (CG) contrast to around 74 % for poly(AT). (More in supporting information S1). The longer translocation of the poly(AT) sequence is because the DNA-substrate interaction with the graphene distort and break the double helix structure leading to significant lateral diffusion as well as un-correlated motion of the separated double helix strands all of which results in decreased transverse mobility of the poly(AT) sequence and ultimately lead to longer translocation time. In order to quantify this effect, we extracted the center of mass of the translocating DNA around the pore region from each frame of the simulation. Figure 4a and 4b shows that poly(AT) sequence indeed has a much larger

spread around the pore than the poly(CG) sequence. The radial standard deviation σ_r was computed as $\sigma_r^2 = \sigma_x^2 + \sigma_y^2$, and it is 2.03 and 1.15 for poly(AT) and poly(CG) respectively quantitively establishing a larger spread of poly(AT).

In addition to comparing the spread in the center-of-mass of the translocating DNA strands around the pore, we also investigated the area covered by the phosphate backbone in pore region during the translocation by visually comparing frame by frame deviation of backbones between poly(AT) and poly(CG). To do this, the visualisation was set to VDW as in figure 4c and then the visualisation of basepairs were turned off. The system was rotated in such a way that the DNA translocation occurs into the plane of the screen (Figure 4d and 4e). Also, the backbones were made sure it will be displayed only when they reside in a region near to pore. The images were converted to a black and white. Clearly a greater dark region corresponds to a higher spread of backbone. The fraction of dark region was calculated by the following equation. $Fraction\ of\ Dark\ region = \left[\frac{(Number\ of\ dark\ pixels - Number\ of\ white\ pixels)}{Number\ of\ dark\ pixels}\right] * 100.$ Figure 4f shows that the phosphate backbone occupies a larger fraction of the pore during poly(AT) translocation compared to poly(CG) translocation, These observations lead us to conclude that the disruption of the double helix structure during poly(AT) translocation results in greater lateral diffusion of the strands causing reduced transverse mobility across the pore leading to longer translocation times. Throughout the translocation of either type of sequences, we observe that the dsDNA does not stick or get adsorbed to the trans side, owing to the presence of hBN. This is very useful as the underside sticking, which is one of the limiting factors for accurate base identification is eliminated.[41] The non-specific and stochastic nature of underside sticking adds variability to translocation parameters such as ionic current and time taken and reduces the specificity of base-calling. The heterostructure, proposed here, eliminates

underside sticking and therefore will have potentially better capability for accurate base-calling than membranes such as monolayer graphene which suffer from this problem.[41]

The increased lateral diffusivity of the poly(AT) sequences explain the lower ionic current (Figure 5) associated with their translocation compared to poly(CG) sequences. The higher values of poly(CG) is clearly attributed to the lesser spread of the strand in the pore, thereby allowing more ions to pass through when compared to the instance where poly(AT) translocates. In nanopore measurements, the need for having significant difference in dwell time and ionic currents combined enables one to distinguish base-pairs with a better accuracy, which is shown to be possible for dsDNA with a Gr+hBN nanopore system. Thus, the heterostructure proposed here exhibits base-specific translocation times and ionic currents while avoiding problems such as underside sticking associated with monolayer graphene membranes.

Effect of heterostructure layer ordering on DNA translocation

The studies described in the previous section were performed on a heterostructure consisting of a monolayer graphene layer placed on top a monolayer hBN layer, referred to as the Gr+hBN pore. It is interesting to explore the implications of reversing the order of these layers, i.e. monolayer hBN on top of monolayer graphene, which we refer to as hBN+Gr pore. Exactly identical studies as described in the previous section were conducted with this heterostructure as well. Remarkably, reversing the order of the layers forming the heterostructure switched the behaviour of the poly(AT) and poly(CG) translocation. The mean single base residence times (Figure 6i) show that contrary to Gr+hBN system we observe that poly(CG) has a higher residence time at 2V. Figure 6b and figure 6g show that, during translocation with hBN on the top layer, the dsDNA does not appear to interact much. But once it crosses to the trans region, the dsDNA adsorbs heavily to the underside of the membrane containing the graphene. This

effect was observed in the previous studies[41] too where underside sticking was one of the major limiting factors in extracting base-specific translocation signatures. Such a strong adsorption results in a large number of hydrogen bonds being broken and dsDNA almost losing its double helix structure and translocating as two single strands side by side. This is more apparent in the case of poly(CG) as once the hydrogen bonds get broken, the forces involved in differential adsorption of bases takes control. Varghese et al.[76] have shown that the order of DNA bases adsorption to graphene is G>A>C>T. Clearly, Guanine adsorbs more to graphene and thereby has higher dwell time compared to Cytosine in the broken strand. Figure 6h clearly shows this phenomenon where the dsDNA that has been heavily adsorbed, has seen a differential translocation between C and G in poly(CG). At the end of 60 ns, effectively only 1 base of poly(G) has been translocated compared to 5 translocated bases of poly(C). This is also observed to a certain extent in the poly(AT) translocation. The dsDNA losing its double helix structure and the strands translocate at different speeds. In Figure 6c at 35 ns, Thymine bases have totally translocated, yet the Adenine bases have not completely translocated. Such differential adsorption results in higher residence times for poly(CG), compared to poly(AT). More importantly, this differential underside adsorption significantly increases the signal variability in ionic current and dwell-time as described in the next section.

Figure 7a and 7b shows the ionic current distributions for both type of heterostructures. The Gr+hBN nanopore, which avoids the issue of underside sticking, has 2-3x lesser variation (based on standard deviation) in ionic current compared to the hBN+Gr nanopore. The mean value of ionic current in the hBN+Gr pores was higher than the one in the Gr+hBN nanopore. This is because, owing to differential translocation of bases in hBN+Gr pore, there is more space for ions to flow as sometimes one of the strands in dsDNA translocates whilst the other still stays adsorbed. Comparison of per base dwell-time distributions also substantiates (Figure 7c and 7d) the trend of higher signal variability in hBN+Gr pore. Particularly, in the case of

poly(CG), we see that the hBN+Gr pore has nearly an order of magnitude higher signal variability compared to the Gr+hBN pore. However, the hBN+Gr pore only exhibits a 2x higher signal variability in the case of poly(AT) sequence. This behaviour positively correlates with the observation that the variability arises due to underside sticking which is far higher for the poly(CG) sequence than the poly(AT) one. These studies suggest that the Gr+hBN nanopore system will potentially have increased base-pair specificity compared to monolayer graphene or hBN pores as well as hBN+Gr heterostructure pore. Our studies can thus guide experimental studies of translocation with these heterostructure nanopore systems.

Conclusion

The main aim of this study was to understand if it is possible to get distinct residence times and ionic currents, whilst considering the possibility of eliminating the underside adsorption of DNA which was observed to be possible with a Gr+hBN nanopore. Many experimental studies have reported the fabrication of membranes involving graphene and hBN.[77,78] Making such tailormade nanopores is possible by TEM drilling,[72,79] or even by dielectric breakdown of the membrane.[35,80] Prior simulations and experimental studies have shown the ability to detect dsDNA molecules using ultra-thin 2D materials,[18] with certain shortcomings, like higher base-pair velocity (hBN nanopores)[53] or undesirable hydrophobic interactions causing pore clogging[48] (graphene). Using molecular dynamics, we were able to show that our proposed Gr+hBN structure was able to overcome these main drawbacks by demonstrating their ability to have distinguishable dwell times and ionic currents. In summary, the presence of hBN on the trans side resulted in dsDNA translocating almost parallel to the applied field. Such vertical configuration allows for better signal readability and is the key to single molecule sensing. Such a system integrated with electrodes to measure the in-plane sheet currents (tunnelling current), can give an additional level of distinction to the DNA sensor. Also, when coupled

with DNA origami nanosheet these vertically stacked nanopore heterostructures have the potential to resolve single nucleotide as well as modified nucleotides in a genomic sequence.

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