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ACS Appl. Mater. Interfaces, **Just Accepted Manuscript** • Publication Date (Web): 24 Jul 2015

ACS Publications

ACS Applied Materials & Interfaces is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Selectively-Sized Graphene-Based Nanopores for In-situ Single Molecule Sensing

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KEYWORDS – Nanopore, Graphene, DNA, Translocation, Graphene Nanoflake, Size Tuning.

ABSTRACT - The use of nanopore biosensors is set to be extremely important in developing precise single molecule detectors, and providing highly sensitive advanced analysis of biological molecules. The precise tailoring of nanopore size is a significant step toward achieving this, as it would allow for a nanopore to be tuned to a corresponding analyte. The work presented within

details a methodology for selectively opening nanopores in real-time. The tuneable nanopores on a quartz nanopipette platform are fabricated using the electroetching of a graphene-based membrane constructed from individual graphene nanoflakes (ø – 30 nm). The device design allows for in-situ opening of the graphene membrane, from fully closed to fully opened (ø – 25 nm), a feature that has yet to be reported in the literature. The translocation of DNA is studied as the pore size is varied, allowing for sub-features of DNA to be detected with slower DNA translocations at smaller pore sizes, and the ability to observe trends as the pore is opened. This approach opens the door to creating a device that can be target to detect specific analytes.

Introduction

The development of comprehensive and efficient analysis techniques for biological molecules is a rapidly growing area of research.[1–4] A range of methods have recently been reported for the advanced analysis of single biological molecules.[5–8] Plausible outcomes in the future development of devices in this class would be to probe protein-nanopore interactions, protein-protein interactions, and eventually the ability to read the base pairs of DNA molecules by achieving higher special resolution, and requiring only a single molecule to do so.[9,10] One category of these devices are nanopores, which are nanometre-sized holes which have been used in devices aimed towards detecting a range of molecules, including DNA and proteins.[11–14] The general principles of detection are grounded in passing biological molecules through a nanopore (translocation) by application of an electric field, and observing experimental responses, such as the ionic current. Further detection strategies also exist including use of tunnelling currents, fluorescence, and surface-enhanced Raman spectroscopy, which can be carried out independently or employed as a synchronised detection platform.[15–17]

Major milestones for nanopore single molecule detection are the effective analysis of molecular components.[18,19] There are many well-established nanopore systems in the scientific literature are capable of approximating molecular charge, size and conformations.[20–22] The determination of further molecular properties has been reported, including the identification of molecular branching, in addition to probing the variations of molecular interaction with the nanopore surfaces.[18,23–25] These systems are dependent on the precise tailoring of nanopore size (diameter and length), in addition to the material it is constructed from. The diameter of the nanopore must be large enough to allow the molecular analyte to fit through, however a very large pore would result in an insignificant signal, making analysis increasingly difficult.[26] The material of the nanopore device also hugely influences the nature of interaction with the molecule as the translocation occurs, as an attraction or repulsion force may be present. This has been shown in many studies which examine nanopore surface functionalisation.[27] The general principle of this approach is to cause an interaction to occur as the molecule translocates through the pore. This can be in an attractive force (including hydrogen bonds and Van Der Waals interactions) used to slow down the translocation speed, or a repulsive force (including large columbic forces), which may act to shrink the appreciable pore diameter.

The precise configuration of successful nanopore devices varies with respect to the specific analyte under investigation, in addition to the environmental conditions of the experiment (i.e. solution concentrations, applied potentials, etc…).[28,29] A result of this is an aspiration for devices to be tuneable, so that a range of molecules can be used without the need for designing, fabricating and testing a new device architecture. Electron beams, plasma etching, material deposition and surface functionalisation have been used to control the shapes and size of nanopores.[30–33] Materials including graphene have been utilised due to their high mechanical

strength, this enables free standing membranes to be formed to support a nanopore, in addition to widely reported low electrical noise and selectivity.[34-40] The graphene membranes used in the construction of these devices is exclusively single/multi-layer graphene sheets suspended over voids. These sheets are then targeted for nanopore milling, primarily using electron and ion beams.

Previous literature reports the ability to select the size of nanopores supported on graphene membranes, however the size of the pore must be determined before any bio-sensing experiment.[8,26,41–43] Electrochemical opening alongside DNA translocation experiments have been reported on molybdenum sulphide and silicon nitride free-standing membranes.[44-46] Recent reports have covered both the electrochemistry and the electrochemical etching of graphene membranes however, the techniques involve a substantial amount of fabrication.[47,48] Relying upon cleanroom fabrication of nanopore devices and single layered graphene.

The work presented in this article aims to use multi-layered graphene films to completely cover our nanopipette (see supplementary information for full experimental details). The aim of the experiments was to completely coat the pore at the end of the nanopipettes using water dispersed graphene nanoflakes (GNFs). The GNFs used in the experiment are small portions of single layered graphene (ø ~ 30 nm) that are able to be dispersed in a solvent (Figure 1C).[49] Graphitic films are formed by annealing the GNF coated nanopipettes in a vacuum oven. The nanopore coating is analysed using ionic conductivity measurements. Alternating current (AC) is used to etch away the membrane material, with the frequency, applied potential and overall treatment length tuned for steady pore opening. The AC opening technique provides the opportunity for graphene membrane etching, electrical testing and DNA translocations to be performed without interruption. The translocation of DNA is carried out as the pores are opened,

any observed difference in DNA behaviour is related to the effect of nanopore size. The reported technique aims to demonstrate precise, in-situ nanopore size control, which would be a vital tool in generating effective and broadly functioning nanopore devices.

The preparation of GNFs were produced by breaking down single-wall carbon.[49] The aqueous GNF dispersions were prepared by dispersing 1, 1.5, and 3 mg of GNFs in 1.00 g of distilled water and gentle stirring. Quartz nanopipettes were engineered from micro-capillaries (inner diameter 0.5mm and outer diameter 1mm). The pipettes were fabricated with a laser based puller.[5,12,50] The pulling diameter generated pipettes with an average nanopore sizes of 25 nm (±2 nm) across 20 pipettes, which was estimated from pore conductance measurements (full details are available in the supplementary information). These pipettes were then dipped into the GNF dispersions and withdrawn at a rate of 10 mm s^{-1}. The pipettes were air dried for 10 min with the pipette tips pointing vertically downwards. The pipettes were then placed in ceramic boats and placed inside a quartz tube attached to a high vacuum system and heated to 900°C over a period of 90 minutes (Figure 1A). Once cooled, the nanopipettes were sealed in air-tight containers and only removed to be analysed.

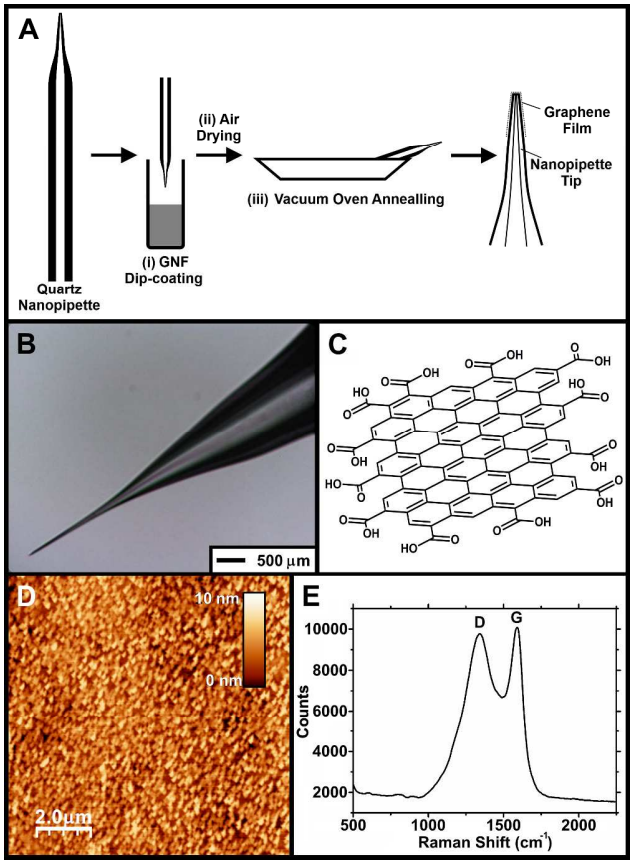


Figure 1. (**A**) Nanopipette coating schematic. Steps include; (i) dip-coating into GNF solutions of various concentrations, (ii) pipettes were left pointing with tips pointing downwards for 10 minutes of air drying and (iii) vacuum annealing was carried out at 900°C and a pressure of ~1.5 x 10^{-5} mbar. (**B**) Shows an optical image of the nanopipette (scale bar inset). (**C**) A cartoon of an individual small GNF. The size of each GNF is ~30 nm, and the edges of the GNF are functionalised with carboxylic acid groups (**D**) AFM image of a spin-coated GNFs annealed on a quartz substrate. The spin coating was carried out using a 1.5 mg mL^{-1} GNF solution, at a spin speed of 5000 rpm for 30 seconds. The individual features (ø - *ca*. 30 nm) are the annealed GNFs, the measured surface roughness indicates a multi-layered arrangement (scale bar inset). (**E**) Raman spectra of the annealed GNF film on a quartz substrates. The characteristic D and G bands present in graphene are indicated on the spectrum.

The characterisation of GNF deposition has been previously reported which showed single sheet flakes of approx. 30 nm distributed homogenously along substrate surfaces.[49] An illustration of an individual GNF is shown in figure 1C. Deposition and the annealing of GNFs on quartz substrates were carried out to validate the resulting graphene materials. Uniform coatings of GNFs were achieved by spin-coating aqueous suspensions of various concentrations,

which were subsequently annealed (Figure 1). The surface roughness of the deposited films was analysed using AFM (Figure 1D). This showed the annealed GNFs (ø ~ 30 nm), and surface features that were no taller than 10 nm. The surface roughness is of the underlying substrate was also analysed using AFM (Supplementary information – Figure S-1). The maximum surface feature was measure as 5 nm, the additional surface roughness (+ 5 nm) caused by the graphene coating suggests a film consisting of multiple graphene layers. The resultant graphitic material was also analysed using Raman spectroscopy (Figure 1E), which showed the D and G-bands expected for a graphene film. The presence of an intense D-band indicates a film containing defects, i.e. not perfectly graphenic carbon. This imperfection is brought about by the 'patchwork' nature of the resulting film, composed of annealed GNFs. XPS analysis was carried out on the coatings (Supplementary information – Figure S-2). In summary, the films were confirmed to be made up of defect-containing graphene, stacked into multiple layers (estimated at ~ 5 nm).

The deposition of GNFs onto nanopipettes could not be achieved through a simple modification of a previously used GNF deposition technique (spin-coating or drop-casting). Dip-coating of the pipette tips (Figure 1) offered an adaptable coating method, which could be readily achieved. The spin coating experiments, carried out on flat substrates, were used to estimate the concentration required for a conformal coating. Various concentrations of GNF solutions were used in order to explore a variety coating conditions. Subsequent to dip-coating, the pipettes were left to air dry for 10 minutes, with their tips pointing downward, which demonstrated the most consistent nanopore coverage. Further orientations for pipettes drying were carried out (including; pointing vertically upward and horizontally). However, this did not provide consistent nanopore coverage on the electrical measurement i.e. the I-V curve. When examined

optically, there was no observed presence of coatings on the pipettes when using GNF solutions of 1.5 mg mL^{-1} or less, however there was a slight darkening of pipettes coated using 3 mg mL^{-1}. The annealed nanopipettes showed no change in overall appearance and shape (i.e. taper length, colour and angle of tip). The coatings on the pipettes were imaged using both SEM and TEM (Figure 2). SEM images of nanopipettes before and after the coating process show successful closing of the nanopore. TEM images of the pipette shaft show film thicknesses (~ 3-4 nm) for the deposited material. A full experimental description is given in the supplementary information.

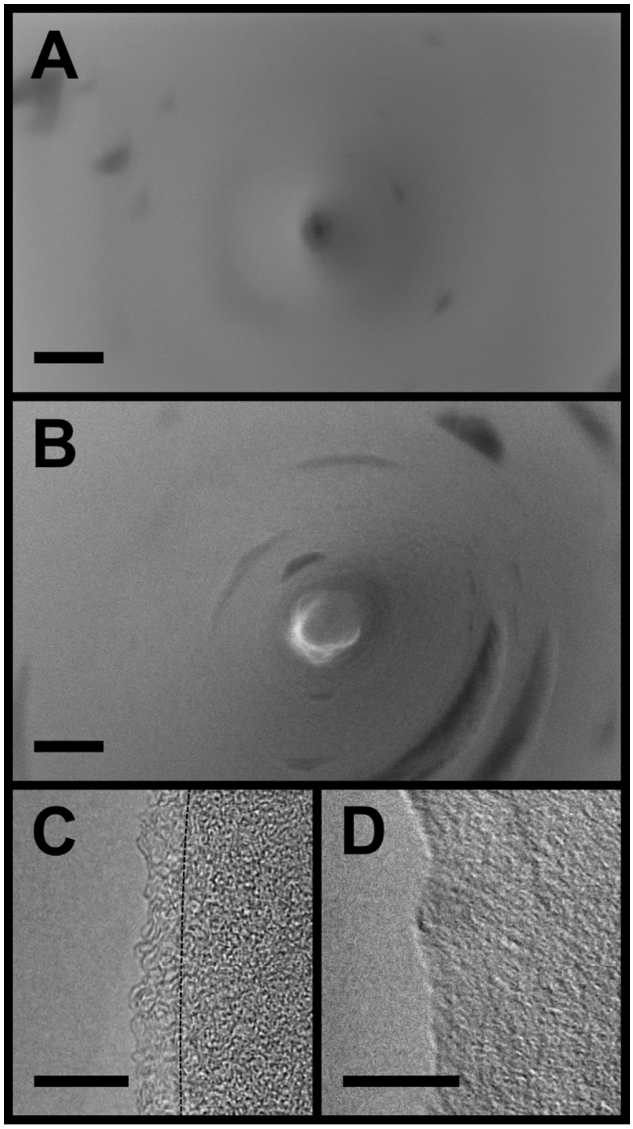


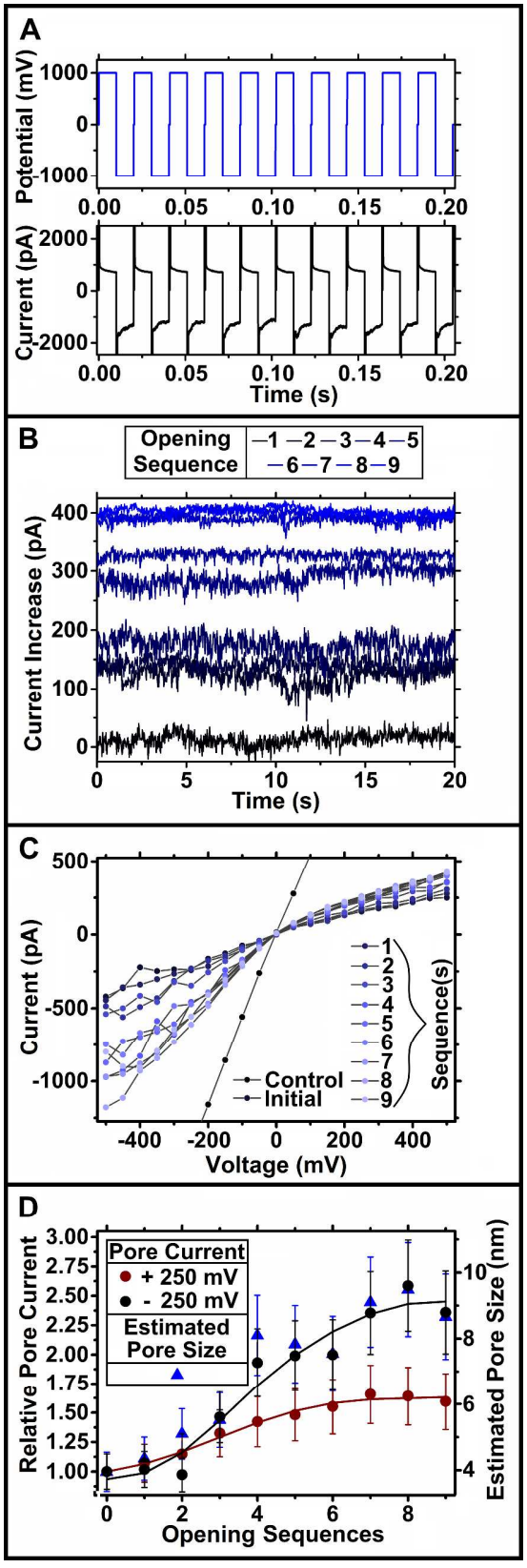
Figure 2. SEM images of (**A**) untreated and (**B**) GNF-coated nanopipettes. The untreated nanopipettes possess an average pore diameter of 25 nm. Scale bars of **A**/**B** show 100 nm. (**C**/**D**) TEM images of (**C**) GNF coated and (**D**) untreated nanopipettes edges. The GNF-coated pipettes have a 3-4 nm coating of material on the surface. The dashed line on image **C** indicates the line of the underlying quartz of the pipette. Scale bars of **C**/**D** show 10 nm. The coated pipettes in the images are treated with 1.5 mg mL^{-1} of GNF solutions before being annealed.

The nanopore size was also estimated through conductivity measurements. The uncoated pipettes showed a conductance of 4.3 nS (± 0.3 nS) at 0.1M KCl. This is estimated to a pore size of 25 nm (± 2 nm) according to the model described by Steinbock et al.[51] The estimated pore

diameters were also comparable to literature recently reported.[5] This value did not change upon undergoing the annealing process. Graphene deposition provided pore blockages for the majority of the treated pipettes at all concentrations, with the relative amounts of blockages increasing with concentration; 50% for 1 mg mL^{-1}, 84 % for 1.5 mg mL^{-1}, 93.5% for 3 mg mL^{-1}. The graphene-coated pipettes showed average conductance value (7 ± 0.8 pS) when electrically tested, indicating that the nanopore is closed. The pore opening process was aimed at steadily opening the membrane covering the nanopore. The protocol designed for opening uses a rapidly alternating current (± 1.0 V at a frequency of 100 Hz), the pore opening was monitored by measuring current flow and subsequent *I-V* measurements (Figure 3). This opening technique was selected as graphene materials has been shown to exhibit delamination and redox chemistry under an applied potential, while rapid reversal of applied potentials ensures a steady opening process.[52,53]

Each opening sequence was carried out over a 200 second period. Typical current vs. time traces are provided in the supplementary information (Figure S-3). The opening of multi-layered graphene films was consistent for each concentration of GNF solutions used for dip coating. The higher concentration solutions however gave films that required a greater number of opening sequences to increase the pore size. The pipettes coated with 1.5 mg mL^{-1} were found to be optimal for opening experiments, providing a large amount of blocked pores, while also providing steady opening for detecting DNA molecules. The average ratio of pore opening (final pore diameter/untreated pore diameter) for these pipettes was ~0.7, which provided a large range of pore sizes as the electroetching progressed. The lower proportion of blocked pipettes at lower concentration is due to incomplete coverage achieved in the dip-coating process, with this principle extended to more substantial GNF coating achieved at higher solution concentrations.

The greater effort required to open the graphene films formed using higher GNF solution concentration was caused by the resultant carbonised coating being thicker and more consistent around the nanopipette. The pore opening process, could be primarily monitored through the current allowed to pass during the AC opening sequence (Figure 3A). The mean current flow would increase as the opening sequence was carried out (Figure 3B). This current flow is a direct indicator of the pore size, allowing for progressive monitoring as the opening sequence was applied. This was also used to gauge if a pore had reached its maximal size, with no increase observed throughout the sequence. After each opening sequence an *I-V* measurements was taken to more accurately estimate the pore size (Figure 3C). Pipette coatings carried out using 1.5 mg mL^{-1} could be opened with relative ease. Consecutive opening sequences resulted in the opening of the pore from fully closed to an estimated 9 nm in 9 sequences (Figure 3D). The thicker coatings generated by using 3 mg mL^{-1} were harder to open (Supplementary information – Figure S-4). The fully closed pores were commonly not open at all by numerous opening sequences. An optimal opening rate of 1.2 (± 0.17) nm per sequence was achieved using an initial GNF solution of 1.5 mg mL^{-1}, this allowed for the size of the nanopore to be precisely tuned to detect specific analyte.


A
Potential (mV)
1000
0
-1000
0.00
0.05
0.10
0.15
0.20
Current (pA)
2000
0
-2000
Time (s)
B
Opening Sequence
1 2 3 4 5
6 7 8 9
Current Increase (pA)
400
300
200
100
0
0
5
10
15
20
Time (s)
C
Current (pA)
500
0
-500
-1000
-400
-200
0
200
400
Voltage (mV)
Control
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Sequence(s)
1
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5
6
7
8
9
D
Relative Pore Current
3.00
2.75
2.50
2.25
2.00
1.75
1.50
1.25
1.00
Pore Current
+ 250 mV
- 250 mV
Estimated Pore Size
Estimated Pore Size (nm)
10
8
6
4
0
2
4
6
8
Opening Sequences

Figure 3 - Pore opening data. (**A**) Shows a plot of the repeating square wave potential applied to the multi-layered graphene membranes. A corresponding current trace from a nanopore coated using 1.5 mg mL^{-1} of GNF solution is shown. (**B**) Shows the current increase/time trace for the first 20 seconds of each pore opening sequences for the same pipette, generated by using the average positive current flow (shown in 3A, from ~0-10 ms in the square wave cycle). The trend shows a general increase in current after each opening sequence. (**C**) Nanopipette *I-V* plots after subsequent nanopore opening sequences. The pore opens from completely closed to a final estimate size of 8.7 nm after 9 sequences. (**D**) Shows a plot of the pore current at positive and negative potentials, as the pore is opened. The corresponding estimated pore size is also shown.

The mechanism for the membrane opening could follow two main mechanisms, either *via* atom-by-atom removal or by sporadic flaking of the membrane, brought about by the electroetching of the graphene.[52,53] The opening rate of the multi-layered graphene membranes is somewhat consistent between opening sequences (Figure 3D). However, some fluctuation is seen in the current increase during the opening sequences (Figure 3B). Current flow was stable upon completion of the opening sequences, with similar baseline noise levels observed for both GNF-treated and un-treated nanopipettes (Supplementary information – Figure S-5). Opening occurring through an atom-by-atom removal would provide linear increases during the opening sequence. As this is not the case, a mechanism whereby masses of the graphene membrane is removed intermittently is most likely. However, pore opening data does not indicate the precise nature of this.

The translocation behaviour of DNA was then studied, the aim was to monitor variations in translocation behaviour as the graphene membrane was opened. All of the reported DNA translocations were carried out using one type of DNA (10 kbp). With full characterisation of the translocation behaviour of the DNA carried out on untreated pipettes. Both the DNA concentration and ionic strength of the solution were kept constant throughout all reported experiments, however the potential applied to drive the translocations was varied. This change in applied potential provides differences in observed translocations (Figure 4). The current-time

traces show a positive spike in current as the DNA passes through, this is due to the extra charge carried by the DNA molecule. The features of each spike is characteristic of the pore properties, in addition to the conformation of the DNA as it passes through the nanopore (Figure 4A). The most important features of these traces are the dwell time (the total time for a translocation event), peak amplitude (the maximum height of a translocation peak from the baseline) and charge (the integrated area underneath the plotted translocation event). Detailed analysis of multiple translocation events reveals typical values of 0.37 (±0.02) ms, 59.3 (±3.6) pA and 17.1 (±2.17) fA for dwell time, peak amplitude and charge respectively, at an applied potential of 300 mV, using untreated nanopipettes.

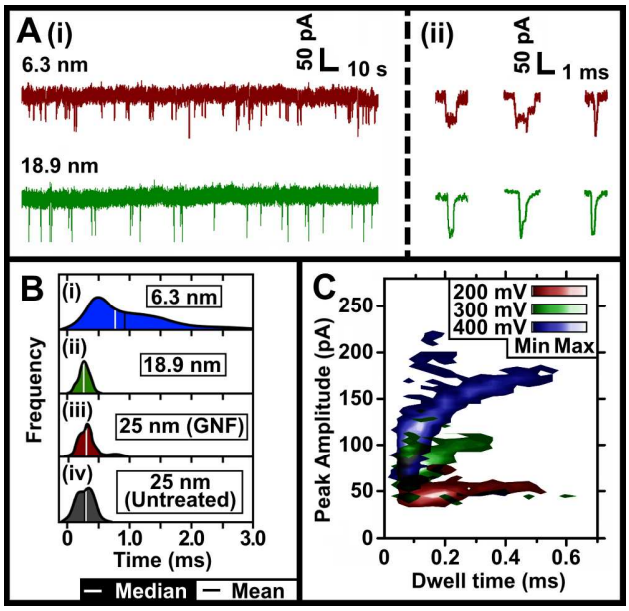

Figure 4. Translocation data for 10 kbp DNA through nanopores treated with multi-layered graphene membrane. (**A**) Current-time traces of DNA translocations through a GNF coated nanopores. Individual translocation events are also shown. (**B**) 'Half-violin' plots showing the average dwell time at different size of pore at various stages of opening, the overall trend shows the dwell time decreases as the pore diameter increases. (**C**) Translocation data from pipette membranes fabricated using an initial 1.5 mg mL^{-1} GNF solution. The data shows the separation of DNA conformations as the applied potential is varied. All of the applied potentials have translocation events that occur at ~ 50 pA, this splits into two populations for both the 300 mV (~ 75 pA) and 400 mV (~125 pA) cases. The estimated pore size for this was ~ 22 nm.

The translocation of DNA was carried out in conjunction with the pore-opening experiments described above, with translocations attempted at each stage of opening process. The DNA solution used in each pipette was introduced using a MicroFil needle, the same DNA solution was used for all steps of opening and DNA sensing and was not exchanged at any point. The tip of the MicroFil needle was thin enough to reach the tip of the pipette, and so no air was trapped inside upon filling. The partially open pores, were then examined for differences in translocation behaviour. The current flow through the pore during translocations was found to be stable for the vast majority of nanopores at the various stages of opening. This made the observation of translocation events straightforward (Figure 4A). The systematic analysis of the coated pipettes found that there were no observed translocations using pipettes with pore sizes less than 6.3 nm, however there were bumping events at 5.52 nm, or alternatively this might due to the GNF flakes interacting with the DNA near the pore entrance. Additionally, fully opened nanopipettes showed moderate differences in translocation behaviour compared to measurements taken using non-coated pipettes (Figure 4B). This indicates that there was some interaction between the graphene coating and translocating DNA once the pipettes were fully opened. The major differences between these two cases include an increased average dwell time (from 0.37 ms to 0.83 ms) at 300 mV, and increased the distribution of dwell times. These values compare well with reported nanopore devices fabricated from graphene membranes.[54,55] As the pore size was opened past 6.3 nm in size, translocations were observed. At these initially small nanopore sizes, the translocation events had longer dwell times (Figure 4B). This longer dwell time is caused by a range of factors, this includes the effect of confining the translocation volume, in addition to chemical interaction between the DNA and the graphene membrane. The reduction in pore size increases the energy barrier for DNA translocation. Another effect of the small pore sizes is that

the DNA must unravel in order to translocate, this conformation intrinsically has a longer translocation time compared to a more constricted conformation. Additionally, the DNA may be interacting significantly with the graphene coating as it passes through. The electrochemical process used to open the pore may cause hydrophilic groups appearing at the surface, these groups would act to attract the DNA molecules toward the membrane material – an effect that would be greatest at smaller pore sizes.[56] The overall dwell time is reduced as the nanopore is fully opened, which supports this concept.

DNA translocation detection depends heavily on the size of the nanopore used.[28,29] It was found throughout the experiments that specific nanopore sizes lead to atypical observations of DNA behaviour. This is exemplified by an example shown in figure 4C. The particular nanopore was fabricated using an initial 1.5 mg mL^{-1} GNF, the pore was opened to an estimated diameter of 22 nm. At an applied potential of 200 mV a single DNA population was observed indicating an unfolded state of the molecules passes through the pore, however second populations appeared when increased voltage at both 300 and 400 mV. Usually, we would expect this is due to different conformations of the DNA passes the pore, however the excluded charge (integrated current area per translocation event) values were very similar to 200 mV (5.43 ± 2.2 fAs), only increased by 1.5 fA as the voltage increased by 100 mV indicating other factors are affecting. Another supporting information is that the uncoated nanopipette at 25 nm had an excluded charge of 13.95 ± 3.01 fAs at 200 mV which in agreement with other literature reported.[12] We hypotheses this is due to the shape of the opening pore. Under typical circumstances the nanopore would be thought to be circular, however the irregular multi-layer graphene coating may have caused a non-circular opening in this case. As the size estimations are modelled on

circular pores, this behaviour may be caused by a 'letter box' shaped pore, confining the translocation in one dimension, hence affecting the time of DNA passes through the pore.

The electrostatic interaction of the mobile ions with the nanopore surface charge is a factor that plays an important role in translocation behaviour in these devices to determine the device selectivity and rectification.[57] The untreated pipette has a rectification ratio of 1.83 $\pm$ 0.3 at 0.1M KCl at pH 8.0 and this is consistent with other reported literatures.[58,59] The rectification ratio indicates the K^+ ions flow more freely at negative applied potentials than positive potentials. The modified pipette at the same pH has a rectification ratio of 2.67 $\pm$ 0.52 which is slightly higher than untreated nanopipette. This further enhances the selectivity and having a more negatively charged surface on the nanopipette. This may be caused by a number of factors, including; as the graphene coating is electro-etched and opened, there may be a functionality added to the surface such as negative carboxylate groups. This carboxylate groups could have respond to the electric field which then change the effective diameter of the pore and even closing it hence resulting higher rectification ratio.[60] Other characteristics that may contribute to surface charge effects include the electron density of the graphene coating.[26]

Conclusion

The present work demonstrates the *in-situ* controlled opening of nanopores *via* electroetching. The multi-layered graphene membranes are shown to be able to provide nanopores of any size between that of complete closure and fully opened pores. Through this we have achieve *in-situ* nanopore opening, allowing for the size to be varied as translocation experiments are carried out. The nanopipette devices demonstrate differences in DNA translocations, with small pores demonstrating very different in dwell time. These properties are facilitated by a targeted coating

using GNFs (30 nm in diameter), to coat the nanopipette devices with a 25 nm diameter nanopore. The GNF films were annealed to form fully characterised graphene films. The film thickness was optimised to provide consistent coating and ease of opening. This was found to be obtained by using a 1.5 mg mL^{-1} GNF solution for dip-coating, which provided a 3-4 nm thick film. Targeted nanopore opening will avoid the necessity to design and fabricate new nanopore architectures, as it is possible to tune the pore size to the analyte being probed. The technology reported also provides key benefits with respect to other reported techniques, such that the nanopipette fabrication and coating technique are both readily achieved and do not require any cleanroom fabrication. Use of graphene membranes is also compatible with surface functionalisation, and would provide a platform where surface chemistry could be tune to further explore a molecule of interest.

Supporting Information. The accompanying supporting information detail the following; experimental details, in addition to further materials and electrical characterisation. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENTS

This work was supported in part by an ERC starting investigator grant (NanoP - 279818) and BBSRC grant (BB/L017865/1) to J.B.E. C.R.C. acknowledges support from the Ramsay Memorial Trust and C.G.S. the Royal Society (UF100144).

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