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ABSTRACT

Biopolymer sequencing with graphene edge-based tunnel junctions has the potential to overcome current limitations with the third generation of sequencing based on biological nanopores. Detection of nucleotides via (recognition) tunneling with noble metal break junctions shows promising results; however, the bulky nature and a range of physical and chemical instabilities of the electrodes prevent advancing toward long-read sequencing with single base-calling accuracy. Graphene edges as tunnel electrodes may overcome these limitations, with the possibility to reach true single-molecule readout, thanks to their 2D nature. Currently, the development of graphene tunnel sequencers faces challenges in terms of targeted chemical functionalization of the graphene edge to enable recognition tunneling and the eventual integration in a nanopore configuration to realize long-read sequencing of biopolymers. Herein, we discuss the current developments that encourage active research toward graphene edge junctions for single-molecule detection, recognition, and sequencing applications with nucleotides and deoxyribonucleic acid as example.

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INTRODUCTION

Tremendous effort has been spent to enable sequencing of biopolymers [e.g., deoxyribonucleic acid (DNA) and proteins] with single-molecule resolution. With respect to the current state of DNA sequencing, first- and second-generation technology has reached its limit in terms of reducing cost per kilobase sequenced, readout accuracy, and speed.¹ These limitations prompted further developments that resulted in commercially available sequencers, based on the readout of ionic current variations due to blockage of the constriction area of biological nanopores, that have established themselves as a valid alternative to second-generation sequencing. Such devices permit obtaining structural information on DNA polymers, including long-read sequencing information. However, due to the physical dimensions of the nanopores, extensive deconvolution of raw data is required, owing to the lack of single-nucleotide sensitivity, that limits base-calling accuracy.^{2,3} Additionally, the biological device constituents are prone to degradation. This affects the device lifetime, thereby increasing long-term costs.^{4,5} Sequencing devices based on solid-state materials could become an alternative that does not face the same problems.⁶ Especially, monitoring the electrical readout of

conductance variations caused by translocation of a biopolymer through liquid embedded tunnel junctions is a promising approach. Such devices, based on noble metal electrodes, have been able to detect, recognize, and sequence single nucleotides, amino acids, and short microribonucleic acid (miRNA) strands.^{7–9} Complementary to this, an approach named recognition tunneling has been demonstrated to enhance device sensitivity via chemical modification of the electrode surfaces with linker molecules.^{10,11} However, achieving full genome or even long-read sequencing remains out of reach due to inherent limitations in device geometries and the chemical and structural stability of noble metal electrodes.^{10–13} As such, we aim to use recognition tunneling in solid-state nanogap devices with graphene electrodes to overcome the aforementioned limitations.^{14–16}

Graphene conducts electricity and features higher chemical and mechanical stability than noble metal electrodes thanks to its strong carbon-carbon network. These properties make graphene an ideal candidate to replace noble metals for sequencing applications. So far, graphene has been integrated into various tunneling configurations using fabrication methods that are compatible with existing industrial processes.^{17–21} Moreover, there are functionalization strategies that

enable recognition tunneling.²² Unsurprisingly, the development of reliable tunnel devices based on graphene is still a growing field with its own fabrication and engineering challenges. First graphene devices, based on forming a nanopore in a graphene nanoribbon, have been able to detect DNA translocation events via the ionic current across the nanopore as well as by monitoring the in-plane current signals through the nanoribbon. For the latter, transverse tunnel effects via the pore edges have shown to play a role in the in-plane signal response that could theoretically be exploited to distinguish between the different nucleotides. However, the high baseline current around the pore complicates resolving the much smaller nucleotide-specific modulations in transmission current caused by tunnel effects; especially at the level of analyte translocation speed observed.^{23,24} We, on the other hand, demonstrated electron tunneling with completely separated graphene electrodes in a distance adjustable configuration.²⁵ Here, the baseline current is lower, because it only entails the tunnel effects. Such a graphene edge tunnel junction immersed in a liquid environment may not only detect nucleotides but also other biomolecules, to eventually achieve long-read sequencing.

Within this Perspective, we elaborate on the advantages of utilizing graphene edges instead of noble metals as electrodes for recognition tunneling. We then outline the challenges that we foresee and draw a roadmap to tackle these challenges, which consists of (a) construction of stable and well-defined graphene edge junctions in a reproducible and scalable manner; (b) targeted and reliable chemical modification and characterization of the graphene edge; (c) integration of a tunnel junction within a nanopore; and (d) control of the translocation speed of the analyte. We will discuss first the physical principles of electron tunneling in a liquid environment and provide an overview of the current devices used in tunneling detection.

TUNNELING PRINCIPLES

The basic building elements of a tunnel junction are two conductive electrodes, A and B, separated by a (sub)nanometric gap that constitutes a potential barrier preventing classical conduction. Under an applied bias voltage V_b , a tunnel current I emerges between the electrodes:

$$I(V_b, d) = -2e \frac{2\pi}{h} \int_{-\infty}^{\infty} |M|^2 [f(\epsilon) - f(\epsilon - eV_b)] g_A(\epsilon) g_B(\epsilon - eV_b) d\epsilon,$$

with $f(\epsilon)$ being the Fermi–Dirac distribution and $g_{A/B}$ the material specific density of states of A and B. The transition matrix element $M = \langle \varphi_B | H' | \varphi_A \rangle$ is derived from a special application of Fermi's golden rule. It describes the scattering probability of electrons contained in states of A (φ_A), into states of B (φ_B). Here, the scattering is mediated by the transfer Hamiltonian H' , which describes the perturbation of systems A and B by their proximity to each other and by the contents of the tunnel gap. In essence, M encodes the entirety of the geometries of both A and B, their relative positions, and the coupling of their electronic states.²⁶

The trapping of a target molecule, the analyte (e.g., a nucleotide or an amino acid), within the junction proceeds via van der Waals and/or electrostatic interactions with the biased electrodes. Upon trapping, the atomic nuclei of the analyte introduce additional scattering potentials in the potential landscape of the tunnel junction. Consequently, tunnel pathways that bridge the electrodes form

through continuous overlap of molecular orbitals and electrode states. Thus, transmission is modified by coupling between the electronic states of electrodes and analyte, which in itself is deeply interlinked with the type, physical or chemical, and location of the electrode–analyte bond.^{27,28} While an exponential trend between transmission and electrode spacing persists, the conventional 1D approximation for the transmission probability (T) no longer holds as bond locations depend on electrode geometry²⁹ and relative positions, orientations, conformations, and size and shape of the analyte within the tunnel junction.^{30,31}

In biomolecule detection, recognition, and sequencing applications, the tunnel electrodes are typically immersed in a liquid environment consisting of solvent and analyte molecules. Within this environment, the analyte exhibits freedom of motion that enables it to translocate through the tunnel gap via Brownian motion or electrophoresis and its detection via the electrical readout upon subsequent trapping events. During a trapping event, intermolecular interactions induce structural motion of the analyte, such as rotation, wiggling, and tumbling. This results in rapid making and breaking of electrode–analyte bonds, that is, transient bonding, and thus alternating high and low transmission configurations. Consequently, T and, therefore, the tunnel conductance are time dependent and obey a distribution determined by the analyte dynamics. Crucially, if the analyte dynamics are confined to motion around specific orientations with respect to the tunnel electrodes, the distribution may feature peaks. Here, the peak width and shape reflect the degree of confinement of the analyte motion with narrower peaks occurring for more constrained analyte–solvent–electrode systems. Three major sources of possible analyte confinement exist: (1) steric hindrance, i.e., physical constriction of analyte motion by the junction; (2) electrostatic interactions between analyte and the bias induced electric field³² between the tunnel electrodes; and (3) intermolecular interaction (such as hydrogen bonding or van der Waals interaction) between the analyte and electrodes that depends on their respective chemical composition and shape.^{33,34} Therefore, the physical dimensions and the electronic and chemical properties of the analyte determine their possible degrees of confinement. The result is a conductance distribution that is characteristic of the analyte species for a given junction geometry.³⁵

APPLICATION OF TUNNELING SPECTROSCOPY IN A DEVICE

Tunnel devices aimed at recognition or sequencing applications probe the conductance distribution of a translocating analyte via tunnel conductance measurements. For current geometries based on noble metal junctions, the conductance distribution peaks for the building blocks of a biopolymer typically overlap. Therefore, multiple current measurements, often performed over multiple trapping events, are required to reach the desired readout confidence.³⁶ In general, device efficiency is judged in terms of readout accuracy and speed, both of which can be improved by reducing overlap between conductance distribution peaks.

For example, the tendency of nucleotides to form hydrogen bonds has been exploited for recognition tunneling. It was demonstrated that chemically modified gold electrodes with linker molecules that serve as binding sites for a translocating analyte prolong the lifetime of the transient bonds and thereby average trapping time.^{10,11,27,28} This yielded a significant narrowing of the conductance distribution that translated to improved sensitivity and selectivity.³⁷ Despite the implementation of noble metals for recognition tunneling of

nucleotides, the reliability of devices is hampered by the low mechanical stability and sensitivity toward chemical modification and oxidation.¹² The commonly used noble metals can suffer from instabilities due to drift of the atomic arrangements (especially for Au) affecting the reliability of the measurements.^{10,13} Other problematics arise from the fabrication of noble metal electrodes. The junction size needed for nucleotide recognition with reasonable current levels is about 0.5–2 nanometer. Various methods exist to obtain such a tunnel junction. STM-based gaps have been used for DNA analysis³⁷ and for single-molecule detection.^{38,39} Mechanically controlled break junctions (MCBJs) were used to investigate the principles governing electron transport in single-atom and single-molecule junctions.⁴⁰ Finally, tunnel junctions with a fixed gap size can be fabricated by versatile methods, such as nanolithography,¹¹ electron-beam-induced deposition,⁴¹ or feedback controlled electrodeposition.⁴² In all of these approaches, the lateral size of the electrodes is much larger than the gap size. Thus, the actual tunneling takes place at ill-defined contacts and multiple tunnel pathways may exist [Figs. 1(a) and 1(b)], especially in liquids. Furthermore, the bulkiness provides ample reaction sites for the linker molecules, resulting in undefined localization of the molecules employed for functionalization, which subsequently complicates the analysis.⁴³ In order to reach the eventual goal of long-read sequencing of for example DNA strands, the biopolymer bases must be fully guided through the sensing region, that is, be prevented from partially folding or drifting past the tunnel gap. Therefore, integration of the tunnel electrodes within a nanopore is required.⁶ However, fabricating a sufficiently restrictive pore, with a diameter of the size of the tunnel gap, and aligning it with the noble metal tunnel electrodes have so far not been achievable. Pore and electrode fabrication methods are either incompatible or lack the necessary precision or resolution.^{9,11,44} Again, the bulky nature of the fabricated tunnel electrodes is a major complicator. We do not expect any technological advancement in the short term that will resolve these issues.

GRAPHENE-BASED TUNNELING—TRENDS, CHALLENGES AND ROADMAP

Graphene has the potential to overcome the fabrication limitations of noble metal electrodes owing to its superior chemical and mechanical stability. Additionally, when immersing the system in liquid, the amount of parallel tunnel pathways is expected to be more confined to the graphene edge due to the poor charge transfer characteristics of the graphene basal plane, thereby reducing the dimensionality of the system to the one-dimensional graphene edges [Fig. 1(c)].⁴⁵ Moreover, linker molecules may be grafted onto the edge of graphene, confining them laterally, to enable recognition tunneling. So far, electron tunneling between graphene electrodes has been demonstrated in adjustable and fixed configurations.^{25,46} Simulations performed on graphene edges for tunneling applications⁴⁷ show that edge composition,⁴⁸ graphene–substrate interactions,⁴⁹ electric field effects,²⁷ and liquid environment effects³¹ are important factors influencing the tunneling signal. Based on these simulations, it should be possible to distinguish between the different nucleotides and also amino acids.^{8,50,51}

We identify five primary milestones to enable graphene tunnel junctions as a device for systematic recognition of analytes (initially nucleotides) and biopolymers with single-molecule resolution:

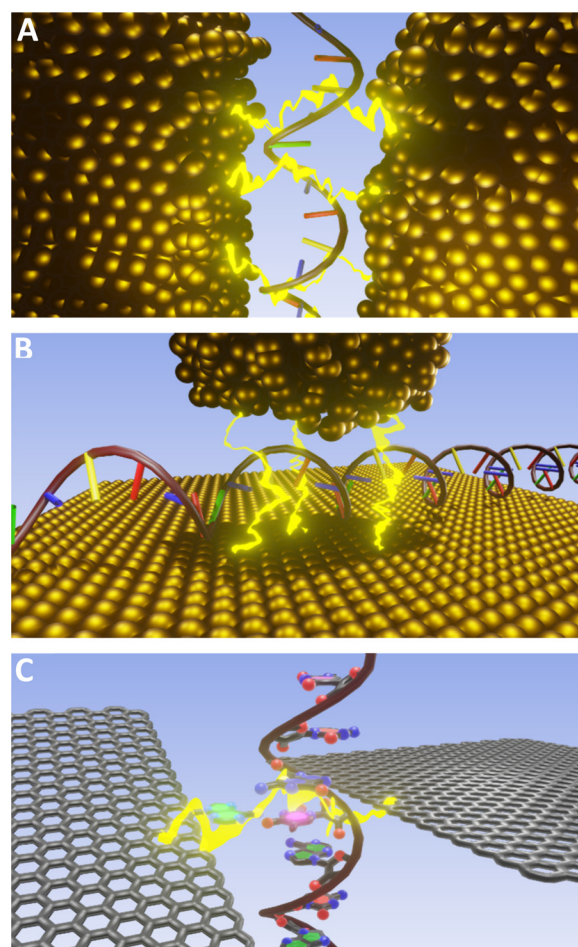


FIG. 1. Different approaches to use electron tunneling for nucleotide detection and identification. (a) Mechanically controlled break junction (MCBJ). (b) Scanning tunneling microscopy (STM) gaps. (c) Graphene tunnel junction that can be edge functionalized with linker molecules.

Fabrication of graphene tunnel junctions that are stable in the liquid environment

In order to be integrated into a tunneling sequencing device, graphene electrodes need to be supported up to the edge on a preferably insulating substrate. While graphene is a remarkably robust material, it is prone to damage during electrode fabrication and/or due to delamination from its substrate.⁵² Therefore, adhesion to the substrate is a crucial parameter.

Delamination is specifically observed in aqueous solutions, for example due to water intercalation. To produce reliable graphene tunnel junctions that are stable in liquids like water, employing strategies to reinforce graphene–substrate interactions is of paramount importance. Chemical modification of the substrate surface with, for example, a molecular layer of pyrene molecules strengthens the adhesion between graphene and substrate by introducing π – π interactions and reducing water intercalation.^{52,53} By implementing a similar approach, we have obtained preliminary results on the immersion of our

controllable twisted graphene edge junction²⁵ in aqueous solution to yield stable devices. Therefore, we are confident that this approach with graphene electrodes can be implemented for nucleotide recognition in aqueous conditions in the near future, thereby catching up with the field of noble metal break junctions.

Controlling the chemistry on the graphene edge

Similar to recognition tunneling with noble metals, chemical modification of the graphene edge can be used to ameliorate the signal and/or reduce overlap of the conductance distribution peaks by reducing the structural motion of an analyte within the junction. For example, nitrogenated edges were predicted to increase transmission through nucleotides as compared to hydrogenated edges, resulting in higher conductance peaks.⁴⁸ Based on the type of analyte, selective linker molecules can be designed to achieve the desired type of interaction (e.g., hydrogen bonding) to reduce the structural motion. Out of the different basal plane functionalization²² strategies employed, electrochemical grafting of modified aryl diazonium salts with proper functionalities to allow recognition tunneling may be an appealing approach due to the ease of preparation and ability to monitor the diazonium activation and depletion via cyclic voltammetry. However, insulation of the basal plane is required to translate the employed chemistry to the edge of graphene exclusively as, for example, diazonium chemistry is a common way to passivate the basal plane as well (especially when performed electrochemically). Furthermore, the device architecture should be developed in such a way that it allows for both functionalization of the graphene edge and incorporation of the graphene into a tunnel junction.

Characterization of the (chemically modified) graphene edge

For functionalization, the implemented chemical strategy (i.e., diazonium chemistry) overcomes the intrinsic reactivity difference of the edge configuration (armchair/zigzag),⁵⁴ especially when achieved via electrochemical reduction of the used diazonium salt. However, the chemical state of the edge prior to functionalization (e.g., hydrogenated edges) should be well defined to gain more understanding about the chemical reactivity. At the same time, the attachment of the molecule has to be proven. Therefore, there is a need for characterization methods that can determine the chemical composition and provide the spatial resolution necessary to verify that the linker molecule has reacted with the graphene edge. Promising tools that are expected to achieve such characterization include surface-enhanced Raman spectroscopy with signal enhancing particles exclusively at the graphene edge. Additionally, tip-enhanced Raman spectroscopy could serve as a way to characterize the linker molecules without additional sample modification.

Integration of graphene tunnel junctions into a nanopore^{9,44}

Long-read sequencing of biopolymers (such as DNA) requires guidance and to prevent folding. To gain control over these problems, the degrees of freedom of the analyte have to be restricted. The most promising approach is integration of the tunnel junction into a nanometric pore, a fabrication challenge that limited progress in noble metal tunnel sequencers. Graphene devices are naturally reduced by

one dimension compared to noble metal devices, overcoming the challenge of device bulkiness. However, many of the geometries proposed so far still require sub-nanometer precision in electrode and nanopore alignment,^{50,51} which is beyond the current capacity of nanofabrication. However, our earlier work on graphene tunnel junctions suggests that the mechanical stability and chemical stability of graphene enable expansion of research into macroscopic approaches without the need for nanoscale engineering beyond the current capacity. Here, the emphasis lies in obtaining preliminary results of biopolymer recognition and sequencing with graphene tunnel junctions.

For example, our previous work on a nanopore configuration⁵⁵ could allow for direct integration of graphene-based tunnel electrodes. This type of pore can be created in a straightforward manner by stacking two polymer slabs that have an embedded gold nanofilm; after etching away the gold, the pore is formed [Figs. 2(a) and 2(b)]. Placing graphene between the slabs and etching the graphene through the slits would create a set of four tunnel electrodes directly aligned with the nanopore [Fig. 2(c)]; this platform could also be used for recognition tunneling if the electrodes are functionalized [Fig. 2(d)]. The challenge lies in obtaining the pore dimensions necessary for reading out the tunneling current. Currently, we are working on reducing the thickness of the etch mask as we are limited to a thickness of 10+ nm due to evaporation of the gold film. In this regard, we are exploring the possibility to obtain etchable masks down to two nanometers via low-angle ion beam milling. Alternatively, by atomic layer deposition (ALD), etch masks can be produced from a wide array of materials.⁵⁶ With these methods, we can work toward the required dimensions for graphene-based tunnel junctions.

With the described fabrication method, it is possible to rapidly produce many devices on a lab scale, as polymer slab production is simple. However, once biopolymer sequencing is achieved with the device, research into alternative device architectures that may be scaled into an industrial setting, while reducing (electronic) noise and permitting fast readout,⁵⁵ should be pursued. Producing tunnel devices via macroscopic fabrication methods should simplify the ability to scale-up the devices compared to nano-engineering approaches.

Translocation speed of the analyte

The integration of a tunnel sensor and a nanopore produces a challenge for eventual sequencing application. In order to access the tunnel gap, biopolymers must be driven into and through the pore. Therefore, control of their translocation speed is crucial to achieve reasonable dwell times within the sensing volume for single base resolution. Aiming for a dwell time per base of ten times the response time (τ) of the transimpedance amplifier circuit that detects the tunnel current can provide a simple estimate of the upper bound for translocation speed ($v_{\max}^T$). With noble metal-based tunnel junctions, a nucleotide-specific average tunnel conductance on the order of 100 pS has been observed experimentally when sequencing short oligonucleotide sequences. Based on these average tunnel conductance values, a bandwidth $BW = 10$ kHz of the amplifier is a reasonable value to maintain a good signal-to-noise ratio.^{7,11} For an idealized tunnel conductance signal in the form of a step function, $\tau \approx 0.35/BW = 35 \mu\text{s}$. Consequently, we estimate $v_{\max}^T = 1 \text{ base}/10\tau \approx 3 \text{ bases/ms}$. At this translocation speed, standard sampling rates ranging from 100 ks/s to 1 Ms/s of the data acquisition electronics at the amplifier output should suffice to capture enough of the base-specific conductance fluctuations

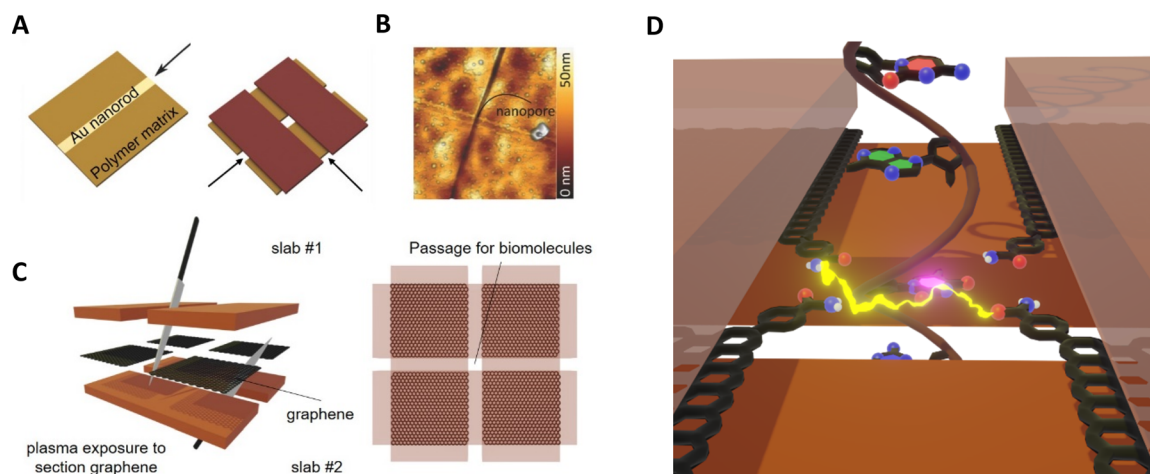


FIG. 2. Integration of graphene tunnel junctions in a nanopore. (a) Schematic representation of the fabrication of an interfacial nanopore via incorporation of a gold nanorod in a polymer slab. Two such slabs are stacked with the nanorod perpendicularly, and the gold is then etched away to create a nanopore at the intersection of the rods. (b) AFM image of a nanopore created via the interfacial pore method.⁵⁵ (c) Fabrication of graphene electrodes for tunneling inside the interfacial nanopore. A graphene sheet is placed in the slab stack and etched to create quadrants with nanosized separation. (d) Artist's impression of recognition tunneling in the interfacial nanopore via opposing graphene quadrants. Here, the quadrants are functionalized with linker molecules to transiently bind with the nucleotides in the DNA molecule. Image (a) adapted from and image (b) reproduced from Arjmandi-Tash *et al.*⁵⁵ with permission.

occurring in real systems to reach competitive base-calling accuracy.³⁰ In order to judge the feasibility of reaching the necessary translocation speed, we can orient ourselves on the adjacent field of solid-state nanopore sequencing.⁵⁷

Here, the sequencing paradigm relies on the blockage of a nanopore by a translocating DNA strand that causes a detectable drop in ionic current when driven across the pore by electrophoresis. We note that the targeted translocation speed for nanopore sequencing is on the order of $v_{\max}^T$ (our estimate) due to similar conductance levels and pore diameter requirements. Typically, the observed translocation speeds are orders of magnitude higher than $v_{\max}^T$ and attempts at sufficiently slowing down the analyte have been fruitless so far.⁵⁷ The main hurdle here is that the ionic current serves as both the driving mechanism as well as the readout. Therefore, options to slow down the translocation speed are limited as the ionic current must be maintained at a detectable level.

However, for nanopore embedded tunnel junctions, the ionic current levels are not relevant. Although it can be used as a mechanism for verification of capturing of an analyte within the pore, it is the transmission current that probes the analyte. Therefore, drive voltages may be pushed well below the threshold in nanopore sequencing. Additionally, simple approaches to slow down translocation can be reconsidered, among others reduction of temperature,^{58,59} choice and concentration of electrolyte,⁶⁰ application of an asymmetric salt gradient,⁶¹ increase in viscosity,⁶² use of molecular motors,⁶³ and combinations thereof.

Furthermore, the spatial resolution of the sensor is independent of pore depth. Therefore, deeper pores might be employed to harness friction between the analyte and pore walls. Similarly, inducing intermolecular (hydrogen) bonding, by adding a coating around the pore, has shown to increase the residence time of a traversing oligonucleotide in a solid-state nanopore.⁶⁴ As such, we are confident that the introduction of linker molecules onto graphene junctions for

recognition tunneling will increase the residence time of the analyte through intermolecular interactions, similar to how the introduction of linker molecules onto gold junctions has shown to increase the residence time of nucleotides within the junction.⁵⁷ In conclusion, we expect that controlling the translocation speed in tunnel devices is likely to be less problematic than in nanopore sequencing.

We expect that achieving the stated milestones will enable long-read sequencing of DNA strands with base-calling accuracy and also form the stepping stones for single-molecule detection of other biopolymers such as peptides/proteins and carbohydrates.

CONCLUSION

We conclude that graphene-based recognition tunneling shows potential for (single) molecule detection and biopolymer sequencing, owing to its chemical and mechanical stability, the theoretical predictions and the developments in the field. Reducing the dimensionality of the tunnel system to the one-dimensional graphene edge should inherently increase the spatial resolution. The possibility to modify the edge of graphene allows for the introduction of functional groups to detect the analyte by recognition tunneling to further enhance device sensitivity. Furthermore, the robust nature of the material makes it possible to continue exploring fabrication methods for the integration of a nanopore in a nanogap. Finding a combination of fabrication methods to obtain well-aligned six-membered rings (i.e., the graphene edge) while being able to tune the edge termination will be a crucial step in the fabrication of graphene tunnel junctions. Furthermore, there are fundamental challenges to be solved regarding device stability, functionalization and characterization of the graphene edge, and scalability and control of translocation speed. Once these are cleared, a pathway is opened to integrate a nanopore into a nanogap to allow sequencing by recognition tunneling with graphene and develop next-generation solid-state biopolymer sequencing devices.

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AUTHOR DECLARATIONS

Conflict of Interest

The authors have no conflicts to disclose.

Author Contributions

Batuhan S. Can and Norman V. V. Blümel contributed equally to this work.

Batuhan S. Can: Writing – original draft (equal); Writing – review & editing (equal). **Norman V. V. Blümel:** Writing – original draft (equal); Writing – review & editing (equal). **Erik P. van Geest:** Writing – original draft (supporting); Writing – review & editing (supporting). **Max Makurat:** Writing – original draft (supporting); Writing – review & editing (supporting). **Jan M. van Ruitenbeek:** Supervision (lead); Writing – review & editing (supporting). **Grégory F. Schneider:** Supervision (lead); Writing – review & editing (supporting).

DATA AVAILABILITY

The data that support the findings of this study are available within the article.

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