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REVIEW

Controlling molecular transport through nanopores

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Nanopores are emerging as powerful tools for the detection and identification of macromolecules in aqueous solution. In this review, we discuss the recent development of active and passive controls over molecular transport through nanopores with emphasis on biosensing applications. We give an overview of the solutions developed to enhance the sensitivity and specificity of the resistive-pulse technique based on biological and solid-state nanopores.

Keywords: nanopore; optical tweezers; translocation; single molecules; sensors

1. INTRODUCTION

The transport of molecules through biological membranes is a tightly regulated process, which is absolutely vital for any living organism. Cells can exert active control over what is imported or exported by expressing specialized membrane proteins or regulating their lipid membrane composition. The basic principle is the same; a molecule due to be transported through the lipid membrane uses a small hole in the membrane for translocation. If these are 'open' proteins, with no or very low selectivity, they often are called nanopores. In the following, a nanopore is a single small hole in a membrane with thickness comparable to the diameter of the nanopore.

The realization that nature produces nanopores, with diameters around 1–2 nm, that remain open for prolonged periods of time inspired the development of molecular sensing via the resistive-pulse technique [1–4]. The sensing process relies only on the change of the ionic current through the nanopore that is altered by the presence of a molecule. This idea was first employed in Coulter counters for the detection of bacteria and is now a standard laboratory technique for counting and sizing cells in solution [5,6]. Although this technique is very powerful, it has some limitations since, owing to its simplicity, it is not specifically tailored for maximum sensitivity or control over the translocation process. In this review, we will address and discuss the solutions developed so far for controlled translocation through nanopores, which are inspired by existing solutions found in living systems.

The emphasis will be on investigating the strategies that have been shown to work in aqueous solution and two main material systems; biological nanopores and solid-state nanopores.

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In the first part of this review, the resistive-pulse technique will be briefly introduced since there exist a number of excellent reviews covering this biosensing technique [2,7–12]. We will also only touch on the availability of nanopores owing to the recent reviews in the literature [8–10,12,13]. The main part of the review first investigates experimental techniques developed for the control of transport through biological nanopores, then the strategies employed in solid-state nanopores are reviewed. In the last part, we will discuss the control of nanopore transport by mechanical manipulation of the molecule using optical or magnetic tweezers and atomic force microscopy. The paper ends with an outlook on future directions of active and passive control techniques over molecules in nanopores.

2. RESISTIVE-PULSE TECHNIQUE

The basis for all the experiments presented here is the resistive-pulse technique. This brilliant and simple idea for the label-free detection of single molecules in solution is a result of improvement of the Coulter counter [5] down to molecular scale. This was demonstrated first by sensing single polymers in solution by Bezrukov *et al.* [3]. This first success lead to increased interest and the first detection of RNA and DNA molecules by Kasianowicz *et al.* [14] a few years later. This latter work, in particular, sparked the imagination of a large number of researchers since it offered a completely new approach to ultrafast sequencing technology with the possibility of long read lengths [11]. The advancement of the nanopore field was further fuelled by the development of solid-state nanopores [15] making use of the powerful arsenal of techniques developed for nanotechnology. In 2010 several groups, almost at the same time, demonstrated graphene nanopores for biosensing both experimentally [16–18] and theoretically for DNA sequencing [19].

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3. NANOPORES

For label-free sensing of single molecules, the availability of suitable nanopores is crucial. Although a number of methods exist to fabricate porous structures and filter membranes, obtaining a single hole in a thin membrane is a significant challenge.

There are two main sources for nanopores—biological protein pores, extracted, for example, from bacteria like *Escherichia coli* (e.g. outer membrane protein F [20,21]) or *Staphylococcus aureus* (e.g. α-haemolysin [2]), and fabricated ones derived from silicon nanotechnology [12]. Despite the abundance of biological nanopores, it was a challenge to find a nanopore that is stable for hours and has large enough inner diameter to enable macromolecules like DNA to pass through them. To date, the most important sensing pore is without doubt α-haemolysin [2], which owing to its commercial availability is used for sensing in an ever-growing number of laboratories around the world.

One striking feature of biological nanopores is their atomic precision in assembly while providing almost perfect repeatability of nanopore structure. Another main advantage of biological nanopores over man-made structures is the ability to use genetic modification to fine tune the nanopore properties. This enables almost a free choice over their properties down to the single amino acid and even atomic level by mutagenesis. In effect, this was used in one of the earliest demonstrations of control of DNA in a nanopore whereby a single DNA strand in the pore was used to immobilize translocating DNA [22]. A notable disadvantage, however, is most biological nanopores have diameters of less than 2 nm. This is suitable for sensing and sequencing of single-stranded DNA, RNA and unfolded protein chains [23] but impedes the sensing of proteins in their native folded state or even double-stranded DNA. The search for larger diameter biological nanopores that possess tunable diameters is ongoing [24].

Some of the shortcomings of biological nanopores have been addressed by the use of solid-state nanopores, with tunable diameters, first demonstrated by Li *et al.* [15]. Solid-state nanopores can be made in a variety of membrane materials by means of a focused electron or ion beams. The most common as carrier materials for the nanopores are silicon nitride membranes. Diameter, length and shape are only limited by the thickness and robustness of the membrane in salt solutions. For silicon nitride membranes, the thickness was reduced recently to a few nanometres [25], while with graphene nanopores, the nanopore length could be cut down to a single atomic layer [16–18]. Recently, glass nanocapillaries were shown to be a relatively simple alternative approach for DNA sensing [26,27].

4. CONTROL OVER TRANSLOCATION IS NECESSARY

In figure 1*a*, the basic principle of nanopore sensing is summarized. A pore, in this case a biological nanopore embedded in a lipid membrane, connects two reservoirs. The reservoirs contain a solution of, e.g. monovalent salt ions and are electrically insulated from each other by the lipid membrane. All ionic current flows through the small hole in the membrane. The ionic current is driven by electrodes and measured with standard electrophysiology amplifiers commonly employed for the characterization of ion channels in small membranes patches on glass capillaries. In the case of DNA, a voltage difference drives the DNA through the nanopore by electrophoresis. The presence of DNA usually increases the resistance of the nanopore. This leads to a resistive pulse in the ionic current trace depicted on the right in figure 1*a*. It is important to note here that this is a single molecule measurement, and the duration and depth contain information about the charge and length of the molecule (e.g. [28]). Unfortunately, this trace alone does not contain information on which direction this specific molecule passes, e.g. 3′ to 5′ or 5′ to 3′ for DNA, nor the velocity of the molecule as it is going through in the beginning, end or during the process, which is vital for any single-molecule sequencing application. Only analysis of hundreds or thousands of such single molecule traces still yields a deeper insight into the translocation process. Investigating this isolated event, all we can obtain is an average speed and thus duration in addition to the current amplitude. Averaging multiple traces contains valuable information about the identity of molecules and can be used to distinguish DNA and RNA, even with specific sequences [29,30] or translocation direction [31,32]. Nevertheless, the amount of information that can be deduced with just the resistive-pulse technique, beyond these simple characteristics of the molecule, is severely limited. More information can only be obtained when nanopore sensing allows the experimentalist to exert tighter control over the translocation process. This is only achieved by changing either the nanopore, molecule or measurement principle as discussed in the following.

5. CONTROL OVER TRANSLOCATION

5.1. Modification of the translocating molecule

The easiest way to stop and slow down the translocation of a molecule is of course the modification of the molecule itself. Obviously, this takes away some of the beauty and simplicity of the label-free sensing approach when the analyte has to be altered. Nevertheless, this can be very useful for very specific questions about the molecular structure. One of the first experiments by Henrickson *et al.* [33] demonstrated that DNA can be stalled in a protein nanopore in 2000. Avidin was attached to a single stranded piece of DNA which hindered the translocation through the nanopore (figure 1*b*). This allowed determination of the minimum voltage required to keep a DNA–avidin complex trapped in an α-haemolysin nanopore to be around 70 mV [33]. A very similar approach can be used to immobilize a DNA strand and thus identify which of the four bases of DNA is located in the constriction [34,35].

Another successful idea is the introduction of double-stranded DNA molecules into the pore, as indicated by the sketch in figure 1*c*, since the α-haemolysin nanopore only allows the passage of single-stranded DNA. This

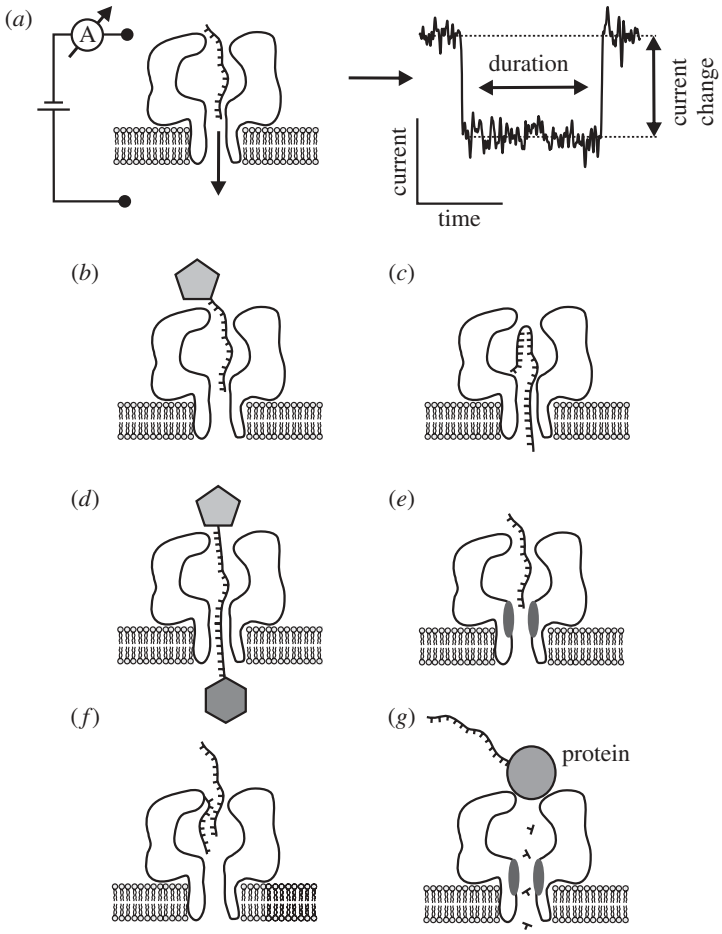

Figure 1. Control of molecular transport though biological nanopores. (*a*) Basic measurement principle, the nanopore connects the reservoirs with ionic salt solution separated by an impermeable lipid membrane. An ionic current is applied and changes if a molecule, here single-stranded DNA is translocating. A typical measurement trace is shown on the right. The main observables are the current change and the duration. (*b*) Translocation of the molecule is controlled by attaching a larger protein at the end. (*c*) A hairpin is used to stop and control the translocation. (*d*) The introduction of a dumbbell structure allows a molecule to be arrested almost indefinitely in the nanopore. (*e*) Genetic modification can be used for tuning of the translocation velocity. (*f*) A complementary strand of DNA can detect single basepair mismatches. (*g*) Combination of an endonuclease and a nanopore might be used to detect the DNA sequence one nucleotide at a time.

technique was employed by Vercoutere *et al.* [36] to study and discriminate between DNA hairpins and detect the duplex stem length, base pair mismatches and loop length. The same group could also distinguish between the number of hydrogen bonds at the end of the hairpins [37]. Several other groups used the α-haemolysin pore for the unzipping of double-stranded DNA molecules, which in essence always stalls the molecules for the unzipping time. This prolongation of the residence time of the molecule by several orders of magnitude makes analysis of the intrinsically noisy data much more accurate [38–42]. It is also possible to study the helix-coil transition with the double-stranded DNA stalling approach [43].

A very interesting technique to completely arrest a molecule in the nanopore is the introduction of blocking groups on both ends of the molecule. This could be like the structure depicted in figure 1*d*, where modification of the molecule on both ends leads to formation of a dumbbell. This can be either formed by adding a specific antibody to the lower reservoir or a complementary strand of DNA. This will hybridize to the DNA sticking out of the nanopore and prevent the DNA from leaving. This nice trick can be used to prolong the presence of molecules in the nanopores and notably allows even the massive parallelization of nanopore sensing as was demonstrated by Marziali *et al.* [44–46]. In a very similar way, the dumbbell approach was used by the Ghadiri group to detect the incorporation of single bases into DNA by a polymerase [47,48]. This effectively transforms the nanopore into a molecular ruler.

5.2. Modification of biological nanopores

As mentioned before, modification of the molecule to be analysed by the resistive-pulse technique requires prior knowledge of what we are looking for. Another, potentially more versatile approach is to modify the nanopore itself by mutagenesis or chemical modification. It was shown that the translocation of DNA could be slowed down considerably [49] by introducing positive charges into the constriction of the α-haemolysin channel as indicated in figure 1*e*. The attractive interaction between the positive channel and the negative DNA backbone has a profound influence on the translocation speed [49]. The molecule can even be completely stalled if too

many charges are introduced. However, introduction of additional charged amino acids is not the only possibility. Bayley and co-workers [50] modified nanopores with cyclodextrin; this allowed for a clear distinction between the four mono-phosphates, the basic building blocks of DNA.

Soon after the first demonstration of RNA and DNA sensing using the resistive-pulse technique with the prototype biological nanopore α-haemolysin, it became clear that detection of single bases could be facilitated by genetic modification of the α-haemolysin nanopore. Since the crystal structure and amino acid sequence of the α-haemolysin pore are well known [1], genetic modification allows for the placement of almost any compatible molecule in the pore. Bayley and co-workers [51] introduced the approach to modify the pore by attaching a polymer to the top part of α-haemolysin. After initial experiments with polyethylene glycol chains [51], they were able to add a single-stranded piece of DNA to the nanopore. With this genetically modified pore, the binding kinetics of translocating DNA strands were detected [22] and even sequence-specific detection of DNA strands was demonstrated [52]. In practice, this elegant method allowed them to determine the voltage distribution inside the α-haemolysin nanopore [53]. Finally, it should be possible to add an endonuclease onto the α-haemolysin nanopore, which itself was modified at the constriction region (figure 1*g*). The endonuclease would cut the DNA into single nucleotides which then could be unambiguously analysed by the modified nanopore. This might allow for sequencing of the cut off bases, given they reach the constriction region. Combination of α-haemolysin with processive enzymes was recently demonstrated [54,55], so this approach holds great promise for DNA sequencing. It is even possible to map the extension of DNA templates with 0.5 nm resolution [56–58]. This is another indication of the versatility of the nanopores sensing approach and its great sensitivity for changes on molecular length scales.

5.3. Exterior parameters

Another obvious control mechanism is the applied voltage. However, since the ionic current is the only experimental observable, and thermal motion and entropy would drive the molecule out of the nanopore, this is not very promising. This is still feasible when the molecule is modified or in combination with another single-molecule technique-like optical tweezers, as will be discussed below. However, with calculations *in silico* by molecular dynamics (MD) simulations, this proved to be a very successful approach especially in the solid-state nanopore field (see below).

Control of translocation by regulating the driving voltage was demonstrated for a number of different systems both for biological nanopores [59–63], investigating hairpin unzipping and translocation direction, and for solid-state nanopores for recapturing of DNA [64]. Since the ionic current is proportional to the applied voltage, the main experimental observable to assess the translocation progress is directly dependent on the control parameter. This is not ideal for the quantitative analysis of the shape and charge of molecules. With all-atom molecular dynamics simulations, this still could be used to prove that the directional dependence of DNA motion through α-haemolysin is due to the tilting of the bases during the translocation process [31].

The same limitations apply, in part, to adjustments of the viscosity of the solution, which is directly linked to its ionic conductivity. Increasing the viscosity in order to slow down the molecules could be promising. In an impressive paper from 2001, Meller *et al.* [65] demonstrated that DNA can be slowed down to the extent that even the passage of small DNA oligos consisting of only four nucleotides could be detected by increasing the viscosity of the solution. This was achieved by cooling the nanopore system down to temperatures of 2°C [65]. This was also investigated for the solid-state nanopore system [66]. Addition of molecules to the reservoirs is possible as well, but polymers like polyethylene glycol require extreme salt conditions allowing for detection of polyelectrolytes like DNA by the resistive-pulse technique [3,67,68].

5.4. Solid-state nanopores

Until now, we mainly focused on experiments with biological nanopores. In the following, we present the most promising approaches developed for solid-state nanopores. Figure 2 summarizes the three main new concepts that can be employed in these systems. One of the easiest and most obvious control mechanisms is surface modification [69]. The binding of polymers to the nanopore is a particularly successful and thus promising example (figure 2*a*). Interestingly, this trick is borrowed from natural nanopores, like the nuclear pore complex, which regulates the traffic between the nucleus and the cytoplasm in a very similar fashion. As recently demonstrated [70], binding of a purified subunit of the nuclear pore complex allowed for the functionality and thus translocation control to be mimicked. Other groups used this idea to detect the sequence of DNA by binding either DNA [71,72] or peptide nucleic acid (PNA) [73] to the interior of solid-state nanopores. Alternatively, the transport of ions through nanopores can be controlled by adding temperature-dependent polymers to the nanopore walls [74]. These brushes dehydrate and thus collapse at high temperatures, which allows the nanopore diameter and its transport properties to be tuned [74]. Interestingly, it was shown that even the flow of protons could be regulated in these systems [75]. These polymers can be voltage responsive, which leads to a range of interesting effects recently reviewed in Siwy & Howorka [13].

The robustness of solid-state nanopores against the application of harsh chemical conditions and high voltages of several volts improves the sensing capabilities enormously. The exceptional voltage stability alone allows for an extension of the attainable forces by orders of magnitude to hundreds of picoNewton and even nanoNewton compared with their biological counterparts. This enables the stretching and denaturation of double-stranded DNA in small solid-state nanopores as depicted in figure 2*b* [76]. By shrinking the nanopore diameter to 1.8–2 nm, less than the DNA diameter, double-stranded DNA has to denature while pulled through the pore by voltages over 1.5 V [76]. This idea was then employed to specifically slow down the translocation of double-stranded DNA

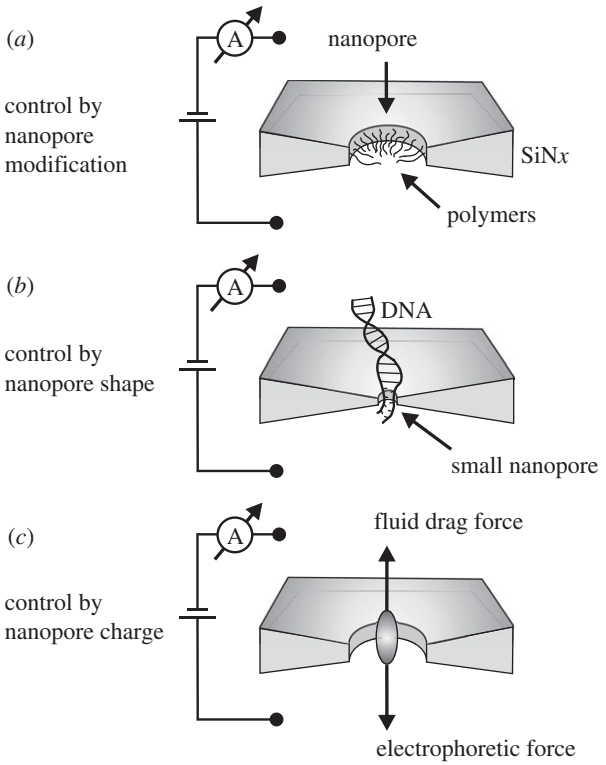

Figure 2. Solid-state nanopore modification. (*a*) Control of the surface properties makes the nanopore specific for molecules or excludes transport completely. (*b*) Tunable solid-state nanopore diameter leads to the denaturing and slow translocation of double-stranded DNA. (*c*) The surface charge leads to a hydrodynamic drag and thus slows down the translocation.

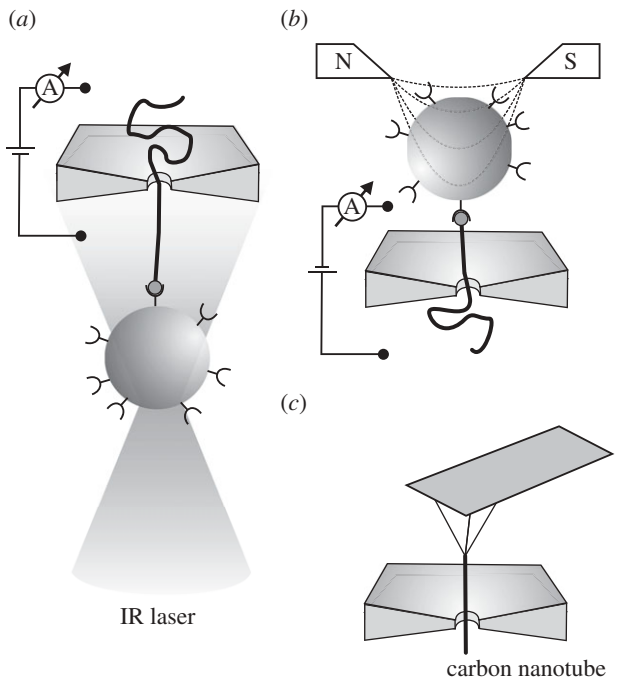

Figure 3. Mechanical control over translocation. (*a*) Optical tweezers stall DNA translocation through a solid-state nanopore. (*b*) Magnetic tweezers can stop and reverse translocation direction by application of a constant force. (*c*) Atomic force microscope tip with a carbon nanotube controls the position of the molecule with sub-nanometre precision.

through nanopores. The molecule has to be denatured during this process and so should yield information about the primary sequence of the molecule [77]. In brief, ionic current measurements in combination with MD simulations show that the translocation time and current depend on the DNA sequence [77]. This is a combination of both primary and secondary structures of DNA. It is interesting to note here that the effect of secondary structure was employed in the first demonstrations that DNA and RNA can be discriminated in biological nanopores [29,30].

However, there is another way to influence and tune the transport through solid-state nanopores that uses the surface charge of the material. As a matter of fact, nanopores with charged surfaces act like fluid pumps with flow speeds that can easily reach several millimetres per second. The surface charge and thus the electro-osmotic flow can be tuned by the salt concentration and the pH of the buffer used. Following experiments on protein translocation through solid-state nanopores [78], Rant and co-workers [79] used this idea to facilitate the translocation of proteins through nanopores and could even reverse the translocation direction by adjusting the buffer parameters. This is a direct consequence of the complex interplay between hydrodynamic and electrokinetic forces in nanopores [80]. Potentially, proteins with varying surface charge and molecular volume could be distinguished by simply investigating their translocation direction and depth of the resistive pulse. The surface charge of a nanopore can give rise to a hydrodynamic flow and thus enhance the number of molecules translocating through a solid-state nanopore by the introduction of differing salt concentrations in both reservoirs [81].

By exploiting the hydrodynamic coupling between the translocating molecule and the nanopore, a simple change in diameter of the nanopore should allow for control of the translocation time [80] as experimentally shown for nanopores with diameters less than 7 nm [82]. Apart from the diameter, control of the nanopore surface could also provide the means to influence the translocation time. As recently demonstrated [83], reduction of the surface charge is possible by using aluminium oxide as nanopore surface.

5.5. Mechanical control

Until now, we discussed methods to control the molecular motion involving either the nanopore, the translocating molecule or other experimental parameters. Although these approaches are promising and very successful, basic questions about the actual forces and position of the molecule in the nanopore can be inferred only indirectly by modelling.

Several groups came up with solutions to achieve mechanical control over molecules in nanopores. The first demonstration of a molecular tug of war between electrophoretic and mechanical forces was published in 2006. Keyser *et al.* [84,85] designed and built optical tweezers to hold the end of a long strand of double-stranded DNA and subsequently insert it into a nanopore. For the first time, it was possible to directly measure the force on a single DNA molecule during translocation

(figure 3*a*). A laser focused into a diffraction-limited spot traps a dielectric colloid coated with DNA. This system acts as a simple Hooke spring and balances the electrophoretic forces pulling the DNA through the nanopore. Thus, the DNA can be stalled and its position relative to the nanopore be controlled with nanometre precision [85].

This, then unique, experiment allowed models for the translocation process to be tested and, in a subsequent paper, van Dorp *et al.* [86] showed that hydrodynamic interactions and thus the nanopore diameter determines the force on the molecule. The somewhat counterintuitive result is that the larger the nanopore the smaller the electrophoretic force becomes. The optical tweezers–nanopore combination was demonstrated by several other laboratories [87–89], extended to DNA–protein complexes [90] and single RNA molecules [91]. Recent results combining nanocapillaries with optical tweezers [26,27] extend the range of possible applications and will facilitate incorporation of other optical techniques, such as single molecule fluorescence detection.

The main advantage of the optical tweezers approach lies in the true three-dimensional spatial control of the trapped particle and thus the molecule attached to the colloid. In practice, optical tweezers can be used to measure position with sub-nanometre accuracy [92], which should enable base by base detection. It is important to note here that the main challenge is the intrinsic thermal motion of the DNA molecule. For stiff double-stranded DNA, this is not as severe as for single-stranded DNA with its approximately 50 times smaller persistence length. The fluctuations of the molecule lead to an intrinsic positional noise in the ionic current signal, which hinders straightforward reading of the primary DNA structure. In reality, this is the major problem for all sequencing techniques based on nanopore translocation.

Although optical tweezers allow for the possibility to work in parallel [93], which would be beneficial for technological applications, magnetic tweezers are promising candidates for massively parallel force spectroscopy. The control of a single DNA strand in a nanopore by magnetic tweezers (figure 3*b*) was demonstrated by Peng & Ling [94]. Two magnets with a small gap can be used to create a gradient in the magnetic field, which leads to a constant force on a magnetic colloid [95] (figure 3*b*). The translocation of DNA could be stalled and the DNA be effectively retracted from the nanopore [94]. One of the potential advantages is that hundreds of colloids and thus DNA molecules could be controlled within hundreds of nanopores simultaneously with magnetic tweezers. This could speed up the analysis process by orders of magnitude. One obvious disadvantage of the magnetic tweezers approach is the lack of three-dimensional control of the molecules compared with optical tweezers. In essence, the magnetic tweezers pull the colloid with roughly constant force towards the magnets, which might be interesting for processes where a constant force is required.

As we already discussed, both magnetic and optical tweezers have their specific advantages and disadvantages. One intriguing idea was demonstrated by King & Golovchenko [96] who inserted a single carbon nanotube attached to an AFM tip into a solid-state nanopore (figure 3*c*). They could locate the nanopore by ionic current measurements and suggested that thermal motion of the DNA could be suppressed by wrapping the molecule around the much stiffer carbon nanotube and thus, owing to superior stiffness of the AFM cantilever, position the molecules with sub-ångström accuracy in the nanopore [96]. However, a demonstration of these experiments is still elusive.

6. FUTURE PERSPECTIVES

In this review, we focused on the technological aspects of controlling nanopore transport. The techniques described here can be used to shed light on the uptake of DNA into bacteria [97] or into the cell nucleus [98]. Owing to the vast variety of membrane proteins, there remain a vast number of processes that remain under intense investigation. One prominent example is the translocation of proteins in and out of both the cell nucleus and mitochondria. The same is true for the ejection and packaging of DNA into the capsid of a virus. These processes are essential for the viral function and thus need to be tightly controlled [99]. The variety of other specific membrane nanopores like calcium-, potassium- or proton-channels can now be mimicked and their mechanisms modelled by artificial nanopores [100]. With surface modification, it is possible to build both fluidic diodes [101] and transistors [102].

The combination of biological and solid-state nanopores is another exciting direction recently shown by the Dekker group [103]. Most importantly, these pores offer the best of both worlds, exquisite control of the nanopore surface and excellent robustness of the carrier membrane. Another approach combined tunnelling electrodes with nanopores explored by several groups and holds the promise for DNA sequencing technology [104–108]. The use of alternating electric fields might have some potential for analysis and control of the translocation process [109], but more work is needed to clarify the mechanism and feasibility. There are recent reports of controlling insertion of single-stranded DNA in carbon nanotubes [110]—which might open another avenue for the mass production of nanopores.

The combination of single-molecule sensing techniques is the most promising route to take full advantage of the unique capabilities of nanopore detection. Control is in any case associated with knowledge about the position, orientation and velocity of the molecule in the nanopore. As a consequence, any fluorescence technology that might give direct information about these parameters would be extremely powerful [111,112]. First steps in this direction are already under active development [113]. It is also conceivable that these experiments would help investigations about antibiotics transport into bacteria [114,115].

In conclusion, active and passive controls over nanopore translocation allow for exciting new applications for the complete characterization of macromolecules. These techniques will without doubt shed new light on the fundamental processes and interactions governing the translocation process. Since all techniques described will be compatible with lab-on-a-chip technology and thus nano- and microfluidics, nanopores will become important sensors at the interface between physics, chemistry and biology.

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