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Carbon nanopores for DNA sequencing: a review on nanopore materials

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In the past decades nanometer-scale pores have been employed as a powerful tool for sensing biological molecules. In pursuit of such a technology, a variety of nanotechnology-based approaches have been explored and established, especially nanopore sequencing. In compare to those existing pores from other materials such as Si₃N₄, carbon nanopores have the ability of rapid sensing of various biological molecules at a single molecular resolution and with reduced cost. Different from the most reviews about nanopore sequencing, this article closely on the employed nanopore materials for sequencing applications. After providing an overview on the general issues of nanopore sequencing, this review article concentrated on recent progress and achievements of nanopore sequencing, especially using various carbon nanomaterials such as graphene and carbon nanotubes. The future research directions using carbon nanomaterials for nanopore sequencing are further discussed and outlined.

1 Introduction

Biological molecule sequencing is one of the important approaches to explore the life blueprint on earth.¹ In 1953, Francis Crick and James D. Watson firstly found the double helix structure of DNA molecules, which consists of a deoxyribose sugar and a phosphate backbone with sequences of four nucleic acid bases, namely adenine (A), guanine (G), cytosine (C), and thymine (T).²⁻⁴ The number and specific order of these four nucleobases in DNA strands determine biological information and functionalities. To get genomic information, sequencing of these nucleobases are required since it provides an opportunity to prevent and diagnose various human diseases and further to develop specific and personal medicines.^{5, 6} The process of biological molecule sequencing is thus to precisely determine the amount and distribution of four nucleobases in DNA molecules.⁷⁻⁹ Note that biological genomes have large variations and complexity, due to different biological functions. Taking human genome as an example, it consists of approximately three billion nucleobases.^{10, 11} Developing inexpensive, fast and simple DNA sequencing methods are essential to be capable of detecting entire genomes. This could breakneck pace of genome technology development and revolutionize the world of medicine and technology.^{12, 13} Consequently, the National Human Genome Research Institute of the National Institutes of Health has launched a program widely known as the Advanced Sequencing Technology Program, emerging the development of new DNA sequencing methods. The goal of this program is to reduce the cost of sequencing to \$1,000 and simultaneously increase the accuracy (< 1 error/10,000 bases), long read length (> 10 kb or longer) and high throughput (in the matter of hours or even minutes).

Trigger by such a program, various techniques have been proposed and developed to visualize DNA sequences. In general, they can be classified into four generations: chain-termination based Sanger sequencing as the first generation, amplification-based cyclic-array sequencing as the second generation, single-molecule sequencing as the third generation, and nanopore sequencing as the fourth generation.¹⁵⁻²¹ In the mid 70s, Sanger and Coulson used fluorescently labeled di-deoxynucleotides as chain terminators.²² The variation of Sanger sequencing, such as Maxam and Gilbert sequencing, can shorten the sequencing time by simplifying template preparation.²³ This method was later known as the first generation of sequencing techniques. Its main limitation is a low throughput (80-100 kb per hour). Due to its capillary nature, such a technique is hardly scalable. However, large projects such as Human Genome Project that emerged in 1990 required tremendous workload and extremely high cost.^{24, 25} The second generation of DNA sequencing technologies relies on the sequencing of a dense array of DNA molecules. It was featured by iterative cycles of enzymatic manipulation and imaging-based data collection.²⁶ Such an array-based DNA sequencing enables a much higher degree of parallelism sequencing. In other word, millions of sequencing reads can be obtained in parallel by rastered imaging on effective size. Since it broke through the bottleneck of an electrophoresis process - limited efficiencies of the first generation sequencing technologies²⁷ - this second generation sequencing technology provided the chance to sequence an entire genome at an unprecedented speed with a reasonable cost. In 2005, the pyrosequencing method, developed by 454 Life Sciences (acquired by Roche now), was released on market. It uses the cyclic flowing of nucleotide reagents (repeatedly flowing T, A, C, G) over a platform.^{28, 29} This was the first commercial setup of the second generation sequencing technology. The platform contained approximately one million wells, which have been loaded with sequencing enzymes and primer. The platform was then exposed to a flow of one unlabeled nucleotide, allowing the synthesis of the complementary DNA strand. When a nucleotide is incorporated, pyrophosphate is released. The resultant light emission is monitored in real time. The 454

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72 Sequencer generated about 200 000 reads (20 Mb) of 110 base
73 pairs (bp).^{9, 30} However, the second generation sequencing
74 technologies suffer from low read-length and accuracy, which
75 compare to the first generation Sanger sequencing.^{26, 31}
76 The third generation sequencing technology is based on single
77 molecule sequencing.^{1, 18, 32} Several of them are currently on
78 market, from the companies such as HeliScope Bioscience
79 TIRM and Pacific Biosciences SMART. Compared to the previous
80 generations, single-molecular sequencing does not require
81 cloning, amplification and fluorescent labelling, leading to
82 further reduced costs and increased sequencing speeds.³³⁻³⁵
83 exonuclease enzyme is used to cleave individual nucleotides
84 molecules from the DNA strands. These nucleotides can be
85 identified in the correct order, when they are coupled to an
86 appropriate detection system.³⁶ Such a real-time DNA
87 sequencing technology provides read lengths that are typically
88 exceeded over 5 kb, facilitating high confidence mapping across
89 a greater percentage of the genome. Unfortunately, the
90 individual read accuracy of single-molecular reading length is
91 relatively low (~85%)³⁷, due to the low signal intensity and high
92 background noise. Therefore, single-molecular sequencing
93 technology requires multiple repetitions to calibrate the DNA
94 sequencing results.^{38, 39}
95 The fourth generation sequencing technology is the most widely
96 known as nanopore sequencing.^{21, 40} For such a technology,
97 nanopores, also called nanochannels, nanoribbons or
98 nanopipettes in many cases as well as their arrays are the
99 essential. They actually provide the fundamentals and
100 theoretical concepts of nano-fluidics for future technologies
101 such as single molecule analytics, lab-on-a-chip applications.⁴¹
102 ⁴² Note that these non-nanopore sequencing technologies
103 require complex sample preparation and further complicated
104 algorithms for data processing.^{43, 44} Therefore, the costs of
105 these technologies are high, but their throughput is low and
106 related read lengths are short. Differently, the nanopore
107 sequencing is derived from Coulter counter and ion channels,
108 namely based on the molecular translocation events passing
109 through a tiny nanopore. Nanopore analysis is an emerging
110 technique that allow biological molecules move through a
111 nanopore, and monitoring the change of ionic current.^{45, 46}
112 Under such situations, ionic current signal is reduced or even
113 blocked when a DNA molecule is driving through a nanopore.
114 Determined by the amplitudes of reduced ionic currents, both
115 long length polymers (e.g., single-stranded genomic DNA or
116 RNA) and small-sized molecules (e.g., nucleosides) can be
117 identified and characterized even without amplification and
118 labeling.^{47, 48} Such a unique technology makes inexpensive and
119 rapid DNA sequencing be possible. In past years, lots of progress
120 and achievements on DNA nanopore sequencing have been
121 achieved. In 2014, several companies, including Oxford
122 Nanopore Technologies (ONT) have also commercially
123 marketed nanopore sequencing devices (e.g., MinION), which
124 touched a read length of up to 2Mb.⁴⁹
125 In this review article, we summarize recent advances on DNA
126 sequencing by use of carbon nanopores (Fig.1). In the first part
127 of this paper, an introduction to the technology progress of
128 nanopore sequencing is presented, covering nanopore natures,

employed materials, and existing challenges. In the following
session, recent progress and achievements of the use of carbon
nanomaterials such as graphene and carbon nanotubes (CNTs)
for nanopore sequencing are highlighted. As future
perspectives of nanopore sequencing, the fabrication of novel
members and their nanopores (e.g., ultrathin diamond
membranes and nanopores) as well as their applications for
nanopore sequencing are also discussed and outlined. It is
worth mentioning that this paper focuses on more from
material side with respect to the selection of carbon
nanomaterials as well as the technologies for the nanopore
formation. It will be fully differently from most of published
review articles, of which centers are the performance of
nanopore sequencing events (e.g., sensitivity, devices).

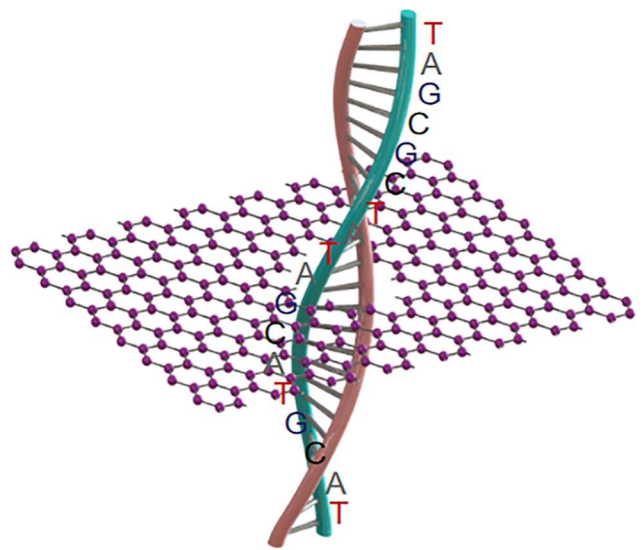


Fig. 1 Schematic DNA sequencing through a carbon nanopore.

1. Nanopore sequencing

1.1 DNA sequencing

DNA is a biological heteropolymer, consisting of four nucleotide monomers: adenine (A), cytosine (C), guanine (G), thymine (T). The DNA sequencing is the process of determining the exact order of these nucleotides in a DNA molecule.

The significance of DNA sequencing lies in its ability to unlock the secrets of the genetic code. This information can be used to understand the genetic basis of various traits, diseases, and conditions. It also helps in the identification of genetic mutations and variations, which can have significant implications for medical diagnosis, treatment, and drug development.

The ultimate goal of DNA sequencing is to achieve cheap, fast and accurate sequencing. One of the most exciting areas of research is the field of personalized medicine, which aims to tailor treatments based on individual genetic information. Nanopores sequencing technologies enabled a better insight of the basis of genetic diseases. For example, DNA sequencing has been used in clinical applications to identify mutations that cause inherited diseases, tumor development pathways⁵⁰ It has also been used to track and diagnose

164 the spread of infectious diseases like novel coronavirus disease 2019
165 (COVID-19).⁵¹

166

167 **1.2 Nanopore natures**

168 Nanometer-scale pores have been widely used for various
169 applications such as energy conversion,⁵² energy storage,⁵³ drug
170 delivery,⁵⁴ enzymology,⁵⁵ polymer data storage,⁵⁶ biosensor,⁵⁷
171 biomarker detection,⁵⁸ nanoparticle fabrication,⁵⁹ and nanoscale
172 chemical reactors.⁶⁰ It has been confirmed that sequencing DNA with
173 nanopores offers exciting potential advantages over other
174 sequencing technologies.^{61, 62} A nanopore sequencing device consists
175 of a nanometer-sized hole in an impermeable membrane, which
176 separates two chambers of an electrolyte solution (e.g., KCl).⁶³ When
177 a voltage is applied across the membrane, ions flow through the
178 pore, resulting in a steady-state ionic current.^{64, 65} The presence of
179 single molecule in the nanopore leads to a transient change in the
180 ionic current, of which change can be detected with an electronic
181 equipment. A distinguishing feature of nanopore sequencing is that
182 it can be used to analyze not only small molecules but also long
183 biopolymers (e.g., DNA, RNA and proteins),⁶⁶⁻⁶⁸ where good
184 understanding of the interactions between the molecules and the
185 nanopore is required. The geometry of a nanopore is thus extremely
186 critical: its depth and diameter. The former depends on the thickness
187 of a membrane, which is determined during the fabrication steps.
188 This characteristic length scale interacts the current and selectivity of
189 the ions in confined area outside of a nanopore.⁶⁹ The latter
190 determines the largest molecule that can move through such a
191 nanopore or the type of molecules that can be analyzed. The local
192 electric field of a nanopore, influenced by the nanopore surface
193 chemistry, is another factor to vary the performance of nanopore
194 sequencing.⁷⁰ For example, the introduction of surface charges
195 (positive, negative or neutral) and/or the variation of wettability
196 (hydrophobic or hydrophilic) of a nanopore by the addition of
197 functional groups (e.g., carboxylic, hydroxide silane, S-H and S-S
198 groups) on the surface of a membrane changed the performance of
199 nanopore sequencing.⁷¹

200 Based on the applied materials, the used nanopores for DNA
201 sequencing can be classified into biological nanopores and solid state
202 ones. A biological nanopore is usually composed of a pore contained
203 protein that is self-assembled or inserted into a transmembrane
204 Such biological nanopores have been widely used in single-molecule
205 detection, disease diagnosis, and DNA sequencing.^{72, 73} As for
206 synthetic solid state nanopores, dielectric materials (e.g., silicon
207 nitride, aluminum oxide) and nanocarbons (e.g., graphene and
208 carbon nanotube) have been frequently selected.

209

210 **1.3 Biological nanopores**

211 A cylindrical nanopore or channel can be naturally formed in a
212 protein membrane.⁷⁴ The repertoire of such biological pores is
213 vast in nature, for example toxins (e.g., α -hemolysin⁷⁵), viral
214 pores (e.g., phi29⁷⁶), mycobacterial porins (e.g., MspA⁷⁷) and
215 nuclear pore complexes (e.g., nucleoporins⁷⁸). Once such a
216 typically biological nanopore is embedded into a soft substrate
217 (e.g., liposome or lipid membrane), *cis* and *trans* events can be
218 separated in a reservoir filled with an electrolyte solution.

Consequently, various biological nanopores have been utilized
for nanopore sequencing.

There are many advantages in using biological nanopores for
DNA sequencing. For example, biological nanopores show well
defined and highly reproducible sizes and structures. Taking α -
hemolysin nanopore as an example, it consists of a 3.6-nm cap
and a 2.6-nm transmembrane β -barrel in diameter. It can be
thus facile inserted into membrane bilayers or other artificial
supporters. Such a narrow and short channel is close to the
diameter (~ 1.3 nm) of a single-stranded DNA (ssDNA) molecule,
allowing the analysis of single nucleotides by use of
reduced/blocked ionic currents inside the nanopore.

Although bacterial toxins are inherently stable, the main
weakness of the biological nanopores comes from their
supporting membranes – lipid bilayers. This is because a bilayer
is very sensitive to temperature, voltage, induced stress and pH.
In other words, it has a short lifetime. Another challenge of
biological nanopores is their limited pore size. For example, a
MspA nanopore has a size of ~ 1.2 nm, while a Phi 29 nanopore
has a pore size in the range of $\sim 3.6 - 6$ nm. Therefore, a reliable
technique needs to control the sizes of biological nanopores.
Since most biological nanopores are formed by repeated
arrangement of the monomers, various nanopore sizes/shape
can be obtained by engineering the protein oligomeric
composition. For example, it was observed that self-assembled
nanopores on Fragaceatoxin C (FraC) can own varied shapes and
size distributions, simply through engineering the protein
oligomeric compositions and the modification of related lipid
interfaces (**Fig. 2a**).⁷⁹ The size of nanopores has been controlled
by mixing three types of FraC nanopores with different
proportions and sizes. Type I FraC exhibits the widest nanopore
with a diameter of 1.6 nm. The nanopore in Type II and Type III
of FraC has a diameter of 1.1 and of 0.84 nm, respectively (**Fig.
2b**). The types of these FraC nanopores were adjusted by using
different preparation conditions. During the oligomerization,
lower concentrations of the monomers increased lower
molecular mass oligomers, leading to smaller nanopores (Type
II and Type III). The oligomerization of monomers under alkaline
conditions (e.g., pH 7.5) pruned to enlarge the pores sizes when
compared to those obtained under acid conditions (e.g., pH
4.5). More importantly, these three nanopores can be
separated by chromatography using an imidazole gradient. The
obtained FraC nanopores allowed direct analysis of a wide range
of peptide lengths with high sequencing speeds.

The use of nanoscopic pores to investigate macromolecules in
solution has been widely researched. The ionic solution (e.g.,
KCl) filled chambers are separated by a voltage-biased
membrane. The negative ions and positive ions are contained
on either side of membrane, which refer to *cis* and *trans*
chambers, respectively. Under applied electric field drives K⁺
ions from the *trans* chamber to the *cis* chamber and Cl⁻ ions
from *cis* to *trans* through the nanopores. Generally, the applied
voltage is positive on the *trans* side. During analysis, the DNA is
electrophoretically driven through biological nanopores from
cis and *trans* chamber to produce an electrical signal containing
sequence information (**Fig. 2c**). Translocation of the
polynucleotide through the nanopore is controlled by a motor

276 enzyme and consequently resulting transient blockade of ion
 277 current (Fig. 2d).⁸⁰
 278

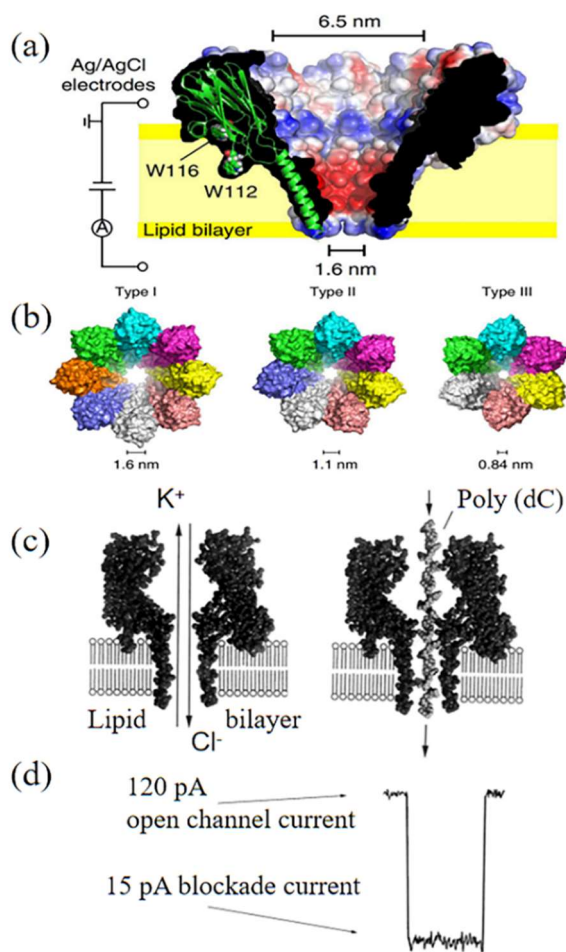


Fig. 2 (a) Cut through of a surface representation of wild type FraC. (b) Molecular models of the three type FraC nanopores constructed from the FraC crystals structure.[79] (c) DNA strand driven through the pore under ionic current of KCl solution, (d) appearance of blockade of ionic current due to the translocation.[80] Figures adapted with permission from National Academy of Sciences, U.S.A., Copyright (1996) and American Chemical Society., Copyright (2022)

279

1.4 Solid state nanopores

280 The solid-state nanopores have attracted more attention than
 281 biological nanopores for the fourth generation DNA sequencing
 282 originating from their high stability in a wide range of analyte
 283 solutions and environments,^{81, 82} their advantages of robustness
 284 and processability over biological nanopores.⁸³ More
 285 importantly, the size and shape of solid-state nanopores can be
 286 flexibly controlled. Solid-state nanopores are usually fabricated
 287 in very thin (< 50 nm) synthetic membranes. Several dielectric
 288 membranes (e.g., Al₂O₃, HfO₂, TiO₂, SiN_x) have been utilized
 289 the supporting membranes for as-fabricated solid-state
 290 nanopores.^{83, 84} Later, SiO₂, polymers MoS₂, hBN, WS₂ and
 291 MXenes have also been applied for nanopore applications.⁸⁵⁻⁸⁹
 292 Several methods have been utilized to fabricate nanopores on
 293 these relatively hard materials.^{90, 91} Coupled with advanced
 294 semiconductor fabrication techniques such as laser etching,⁹²

296 focus ion beam (FIB) milling⁹³, transmission electron microscopy
 297 (TEM) drilling,⁹⁴ the nanopore dimension has been varied to
 298 meet environmental and analyte conditions in a wide range.
 299 Nanopores with a dimension of a few nanometers were firstly
 300 fabricated on a Si₃N₄ membrane by use of reactive ion etching.
 301 Such a nanopore has the bowl-shaped and thus requires further
 302 milling through Ar⁺ ions. Currently, it is more common to drill
 303 nanopores in a solid-state membrane using a TEM (typically
 304 with an accelerated voltage of about 200 - 300 kV). The shape,
 305 dimension, and location of nanopores can be monitored and
 306 controlled in real time. In this regard, electron beam drill
 307 technology conceptually provides the opportunity for the
 308 scalable production of nanopores and their nanopore arrays
 309 with high accuracy (in an order of sub-nanometers) and desired
 310 shapes.⁹⁵ However, electron/ion beams techniques require
 311 expensive precision devices. Due to the physical characteristics
 312 of dielectric materials, fabrication of ultrathin, defect-free and
 313 stress-free membranes is practically difficult.⁸² In addition,
 314 drilling nanopores with the diameters of less than 10 nm is still
 315 challenging. Up to now, DNA sequencing with a single-base
 316 resolution with these materials is still unsuccessful.⁹⁶ The
 317 thickness of these nanopores is usually much thicker than the
 318 length of nucleotide bases, which makes them hard to read
 319 single nucleotide information from a long chain of DNA strands.
 320 The sensitivity of nanopore sequencing technology needs to be
 321 further improved. Therefore, the formation of solid-state
 322 nanopores from other new membrane materials such as carbon
 nanopores is still of high significance.

2. Carbon nanopores

325 Carbon, the sixth element in the periodic table, forms a variety
 326 of bulk materials (e.g., graphite, diamond) and nanomaterials
 327 (e.g., fullerene, carbon nanotubes, graphene, graphyne).
 328 Among them, carbon nanomaterials are extremely appealing,
 329 stemming from their low mass densities, excellent thermal
 330 conductivities, and high biocompatibility.⁹⁷⁻⁹⁹ Carbon-based
 331 materials provide abundant resources for the design of various
 332 micro and nanostructures like nanopores and nanochannels.
 333 For example, graphene nanopores can be initially generated
 334 through TEM milling of single-layered graphene layers. When
 335 the size of such a graphene nanopore is small enough or
 336 comparable with the sizes of DNA molecules, passing a DNA
 337 molecule through such a pore thus leads to the blockage of the
 338 related ionic currents. To record such blocked ionic current, the
 339 graphene sheet with such a nanopore needs to be inserted into
 340 an electrolyte and a voltage needs to be further applied on the
 341 two sides of this graphene sheet. Due to different properties
 342 (e.g., size and density of electrons), four DNA bases block the
 343 ionic current differently. From the amplitudes and frequencies
 344 of such blocked ionic currents, the type and the order of four
 345 DNA bases inside a DNA molecule can be identified. Such
 346 nanopore sequencing technique has been shown many
 347 potential applications in biomolecular sensing, DNA nanopore
 348 sequencing, and early disease diagnosis. The structures and
 349 properties of different carbon materials are dependent on the
 350 arrangement of carbon atoms, namely their hybrid states.¹⁰⁰⁻¹⁰²

351 These unique properties of carbon nanomaterials have led to
 352 their high potential for sensing and sequencing applications
 353 (Fig. 3).
 354
 355 **2.1 Graphene**
 356 Graphene is a subset of carbon nanomaterials. It contains
 357 hybridized carbon atoms that are positioned in a honeycomb
 358 lattice in two dimensions.¹⁰³ In 2004, British scientist Andre
 359 Geim and Konstantin Novoselov successfully separated
 360 graphene from graphite using a micro-computer peeling
 361 method.¹⁰⁴ The structure of graphene is composed of a layer of
 362 independent sp^2 hybrid carbon atoms, which are arranged in a
 363 hexagonal honeycomb crystal structure.¹⁰⁵ Every carbon atom

graphene¹¹¹ on transition metal substrates such as copper (Cu),
 nickel (Ni) and Cobalt (Co)¹¹²⁻¹¹⁴ has become the most promising
 approach for graphene synthesis. During these CVD processes,
 gas precursors (e.g., a mixture of H_2 and CH_4) are fed into a
 heated CVD reactor, where hydrocarbon precursors are
 decomposed into carbon radicals. Once they are diffused and
 adsorbed on the metal substrate surface, the growth of single-
 layer and few-layers graphene occurs.^{115, 116} During the CVD
 processes, the kinetic of CVD growth of graphene is dependent
 on the used metal substrates (e.g., material type, roughness,
 lattice, purity) and growth parameters (e.g., precursors, gas
 pressure, gas flow rate, growth time, and temperatures).<sup>109, 117-
 119</sup> Since different transition metals own varied catalytic activity
 and solubility, they actually determine the deposition

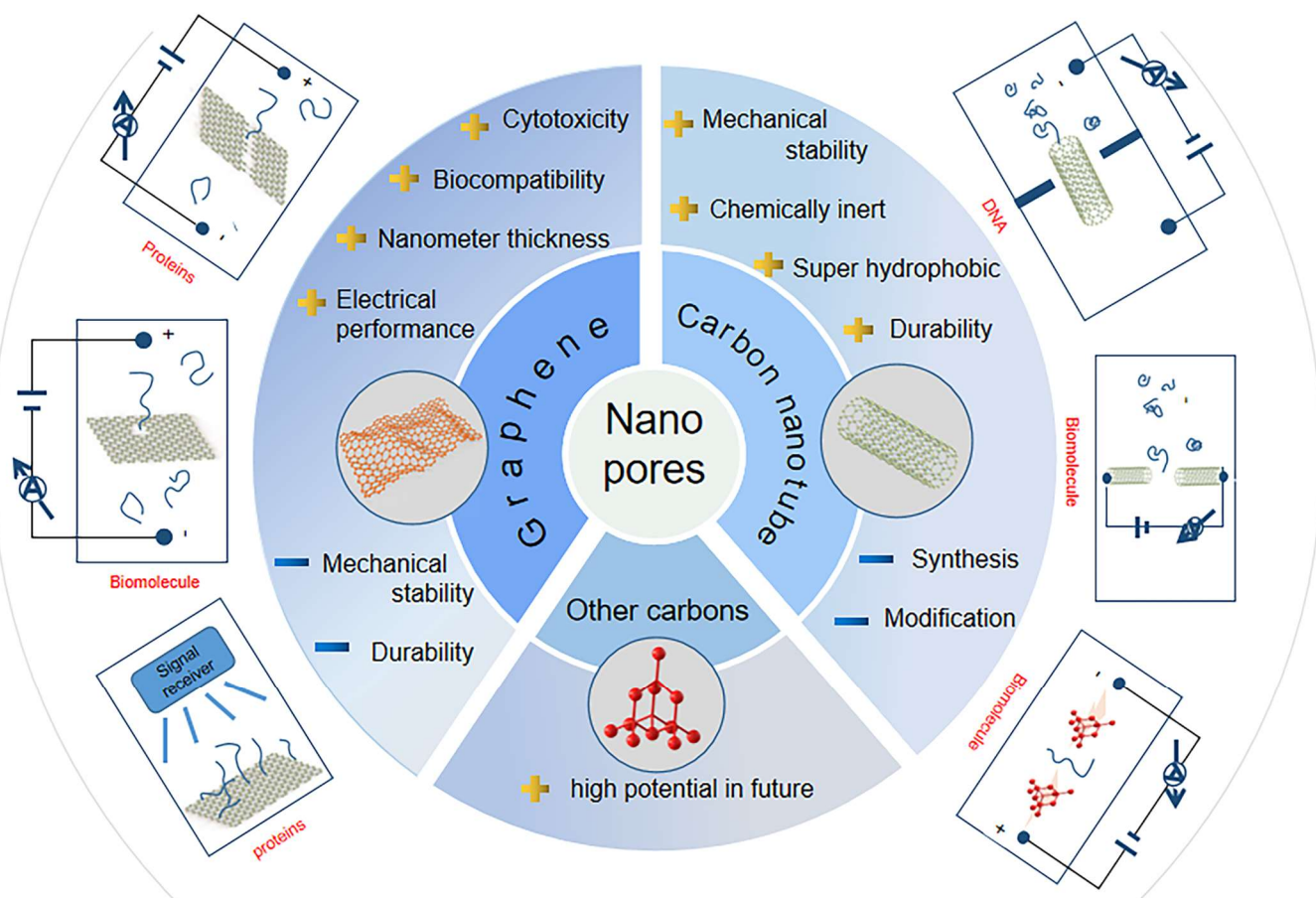


Fig. 3 An overview of carbon nanopores for DNA sequencing technologies.

364 in graphene is bonded to three adjacent carbon atoms through
 365 a σ bond. The bonding direction is in a lateral plane. Due to
 366 short C-C bond length (~ 0.142 nm), the graphene structure
 367 stable.¹⁰⁶ The thickness of monolayered graphene is 0.34 nm,
 368 which is equivalent to the spatial interval between two adjacent
 369 nucleotides.²⁰ In this context, a graphene nanopore offers
 370 possibility of DNA sequencing at a single-base resolution.
 371
 372 **2.1.1 Graphene synthesis**
 373 There are already numerous methods for graphene synthesis,
 374 including mechanical stripping,¹⁰⁷ liquid-phase exfoliation,¹⁰⁸
 375 chemical vapor deposition (CVD),¹⁰⁹ and epitaxial growth
 376 methods.¹¹⁰ Among these present strategies, CVD growth

mechanisms of graphene on them. In turn, they define the
 morphology (e.g., domain size and boundaries) and thickness of
 as-grown graphene layers. For example, the graphene films
 grown on Ni foils do not belong to uniform monolayers. This is
 because Ni can dissolve carbon atoms, even at their high
 concentrations. The graphene growth thus comes mainly from
 the precipitation during the cooling process. As the result, a
 mixture of graphene monolayers and few-layered graphene are
 obtained in most cases.¹²⁰ On the other hand, a Cu plate is an
 excellent candidate to produce ultrathin graphene films with
 large areas and uniform thicknesses. This is due to the low
 solubility (0.001-0.008 wt% at 1084 °C) of carbon atoms in a Cu
 plate. Since only soft bonds between Cu and carbon can be

404 formed, graphitic carbon formation is then facilitated,⁴⁶¹
 405 ultimately contributing to improved thickness uniformity.⁴⁶²
 406 graphene layers.^{120, 121} To obtain monolayer graphene, ⁴⁶³
 407 crucial to precisely control the number of graphene layers
 408 during the CVD. In most cases, post growth layer transfer ⁴⁶⁴
 409 etching processes (for nanopores opening is very desired) ⁴⁶⁵
 410 required.^{122, 123} It has to highlight here that the CVD method ⁴⁶⁶
 411 inexpensive and thus can be considered as a reliable ⁴⁶⁷
 412 controllable technological process to fabricate large area ⁴⁶⁸
 413 high-quality graphene on transition metals. Up to now, the size
 414 of graphene already reaches as large as 30 inches *via* h the CVD
 415 method.

417 2.1.2 Graphene nanopores

418 Graphene nanopores inherit most the unique properties of
 419 graphene. Due to excellent electrical sensitivity and single-atom
 420 thickness of graphene itself, the transport rates of molecules
 421 through graphene nanopores are expected to be high.¹²⁴ To
 422 achieve high-quality sequencing performance, the technique
 423 for the formation of graphene nanopores needs the following
 424 characteristics. Firstly, the size of as-fabricated graphene
 425 nanopores should be comparable to the diameters of DNA
 426 molecules. Only in such a situation the change in ionic current
 427 can be enhanced when a DNA molecule passes through the
 428 nanopore. Secondly, the method must be effective,
 429 controllable, and economical.¹²⁵ Up to date, the reported
 430 methods to produce graphene nanopores can be categorized
 431 into direct drilling techniques (also called a top-down
 432 approach), chemical etching techniques, and on-surface
 433 synthetic techniques.

434 The direct drilling technique is mainly based on irradiation⁴⁷⁰
 435 graphene with highly energetic electrons or ion beams, such⁴⁷¹
 436 focused ion beam (FIB), focused electron beam (FEB), block⁴⁷²
 437 copolymer lithography (BCL), nano-particle lithography (NPL),⁴⁷³
 438 nano-imprint lithography (NIL) and oxygen plasma etching.⁴⁷⁴
 439 These focused beam irradiation methods produce nanopores⁴⁷⁵
 440 directly on single or multi-layer graphene with only one step.⁴⁷⁶
 441 In 2008, graphene nanopores were firstly fabricated in suspended⁴⁷⁷
 442 multilayer graphene using FEB irradiation in a transmission⁴⁷⁸
 443 electron microscope (TEM).¹²⁶ Utilizing these techniques,⁴⁷⁹
 444 graphene nanopores with various shapes (such as Hall rods,⁴⁸⁰
 445 nanobelts¹²⁸, quantum dots¹²⁹, and nanogap¹³⁰) and sizes have⁴⁸¹
 446 been obtained. The size of graphene nanopores is usually⁴⁸²
 447 determined by the energy of ion/electron irradiation and ⁴⁸³
 448 diameter of beam spot. Therefore, directly “drilling” nanopores⁴⁸⁴
 449 to the desired sizes on graphene layers is theoretically the most⁴⁸⁵
 450 straightforward method to fabricate nanopores. Practically, ⁴⁸⁶
 451 realization of controlled nanometer-scale drilling is ⁴⁸⁷
 452 challenging, especially using FIB. Experimentally, the size ⁴⁸⁸
 453 of graphene nanopores fabricated by traditional FIB is usually ⁴⁸⁹
 454 above 10 nm. To obtain smaller graphene nanopores such ⁴⁹⁰
 455 those with the sizes of sub-5 nm, shrinkage of graphene ⁴⁹¹
 456 nanopores has been realized under a range of temperatures ⁴⁹²
 457 between 400 - 1200 °C by setting irradiation energies.¹³¹ ⁴⁹³
 458 further increase the crystallization of graphene layers, various ⁴⁹⁴
 459 pore-forming temperatures have been even applied in ⁴⁹⁵
 460 apparatus. The utilization of a helium ion beam (HIM) led to ⁴⁹⁶

generation of ultrasmall (~3.7 nm) graphene nanopores since
 the diameter of ion source beam can reach as small as ~0.5 nm
 with an accelerating voltage of 30-35 kV (Fig. 4a).¹³² The size of
 nanopores was easily controlled by various exposure time of the
 HIM. Note that for all these direct drilling techniques methods,
 expensive equipment is required together with experienced
 personnel. Therefore, they cannot be applied for industrial
 production of graphene nanopores in most cases.

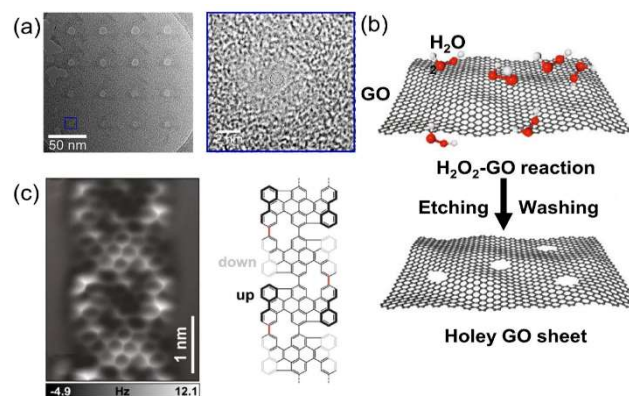


Fig. 4 (a) TEM image of a representative graphene nanopores array and Magnified TEM image of a nanopore with an exposure time of 0.1 s. That uses a helium ion beam microscope to produce single-layer graphene.[132] (b) The carbon atoms in the actively defective zones of GO can be oxidized by H₂O₂, thereby generating nanopores gradually.[144] (c) The AFM image acquired on the graphene nanoribbons segment and its scheme of the chemical structure observe.[145] Figures adapted with permission from Elsevier, Copyright (2021), from Nature Publishing Group, Copyright (2022), John Wiley and Sons, Copyright (2022)

Chemical etching technique is the second approach to prepare
 graphene nanopores, which allows massive production of
 graphene nanopores with low costs and less time.¹³³ For
 example, graphene nanopores with the diameter as small as 2
 nm were fabricated in both exfoliated and CVD-grown graphene
 layers.¹³⁴⁻¹³⁸ As one of derivations of graphene, graphene oxide
 (GO) has been utilized to produce or just as graphene
 nanopores. It is comprised of carbon and oxygen atom in plate-
 like structure.¹³⁹⁻¹⁴¹ It is often prepared using Hummers
 method, where a strong oxidant mixture (e.g., a combination of
 potassium permanganate and sulfuric acid) is used to oxidize
 graphite.^{142, 143} These atomically thin sheets or flakes are
 stacked into a laminate structure with atomic-scale point
 defects and pathways, allowing molecular transport (Fig. 4b).¹⁴⁴
 In contrast, an exfoliated graphene layer contains defects,
 enabling the selection of graphene sheets with a range of
 thicknesses. During chemical etching processes, the shape and
 size of graphene nanopores are determined by the
 concentration of the etching solution and the etching time or
 temperature. Clearly, chemical etching is very hard to precisely
 control the size, shape, and density of graphene nanopores.
 Very recently, on-surface synthesis under ultrahigh vacuum
 condition or at the solid-liquid or solid-vapor interface has been
 extensively used as a new approach to fabricate low
 dimensional carbon nanostructures.¹⁴⁵ The most representative
 on-surface reaction is Ullmann coupling (Fig. 4c), which has
 been applied to the fabrication of a variety of graphene-related

497 nanostructures.¹⁴⁶⁻¹⁴⁸ This technology requires a careful design
 498 of the monomer precursors (e.g., diphenyl-10,10'-dibromo
 499 9,9'-bianthracene¹⁴⁶, 2,7,11,16-tetrabromotetrabenzo
 500 These monomers are further for related polymer chain
 501 reactions on selected substrates, usually on the gold surface
 502 The polymerized graphene nanoribbons are then activated
 503 through thermal treatment/reactions. In the last step, ordered
 504 graphene nanopore arrays can be obtained *via* interconnection
 505 of graphene nanoribbons, of which sizes are within a range
 506 nm. Depending on the inner edge structure, these graphene
 507 nanopore can have either a planar or a nonplanar geometry
 508 In this context, the size, density, and structure of the
 509 graphene nanopores are defined with atomic precision once
 510 monomer precursors are carefully designed/selected.

non-hexagon, graphene-like structures. The resultant graphene
 nanopores have irregular geometries and are not stable.¹⁵⁸ In
 other words, graphene nanopores might have poor stability and
 their sizes might change during the sequencing processes.

To overcome the noise of graphene membranes, one effect way
 is to increase the sensitivity of graphene nanopores (e.g., by
 their surface modification).¹⁵⁹⁻¹⁶² For example, carboxyl group
 terminated graphene nanochannels were obtained by
 immersing graphene nanochannels in a mixture of 1 %
 polyethylenimine (PEI) and zirconium acetate solution.¹⁶³ The
 functionalized graphene nanochannel is then positively
 charged, due to the presence of PEI and Zr^{4+} ions on its surface
 (Fig. 5). Under an external electric field, the long-chain
 molecules are easily accumulated on the nanochannel surface
via the electrostatic interaction. The adsorption of negative
 charged of dsDNA molecules altered the charges of the
 nanochannel surface with only a small amount of target miRNA.

In this way, the detection signal can be enhanced. Its detection
 concentration was in the range from 100 aM to 1 pM.¹⁷²
 It must point it out that using traditional experimental analysis
 tools such as atomic force microscopy (AFM), TEM, X-ray
 diffraction (XRD) it is directly difficult to observe the migration
 of molecules through nanopores in solutions. Understanding
 the dynamic behavior of molecules inside nanopores and
 related signal variation during the process of atomic-scale
 transport is thus extremely important since it can provide
 important guidance to optimize nanopore sequencing
 technology.^{150, 153, 160, 164} For example, molecular dynamics (MD)
 can directly track the trajectory of each molecule, ion, or water
 molecule inside a nanopore.^{165, 166} Dynamic transport of DNA
 molecules through the nanopore and the corresponding ionic
 current can be simulated. In addition, the computational
 methods of quantum mechanics, such as density functional
 theory (DFT), can accurately predict the interaction of
 molecules with nanopores.¹⁶⁷ This technique is based on the
 nuclear electron interaction mechanism and the principles of
 quantum mechanics.¹⁶⁸ Combine with the nonequilibrium
 Green function, transverse conductance or current within the
 nanopore can be calculated. The interatomic interactions
 between the analyte and nanopores can be calculated and
 predicted even without real tests. By use of MD, information
 such as the interaction between the DNA and nanopore during
 the translocation process has been revealed.^{159, 169-171} For
 example, the simulation of graphene nanoribbon based
 microfluid distinguished different Peptide bonds.¹⁵⁰ The
 nanopores located at different positions in the graphene
 nanoribbon array were used to detect different parts of the
 peptide chain. The nanopore in middle of the array was
 specifically used to collect signals triggered from other

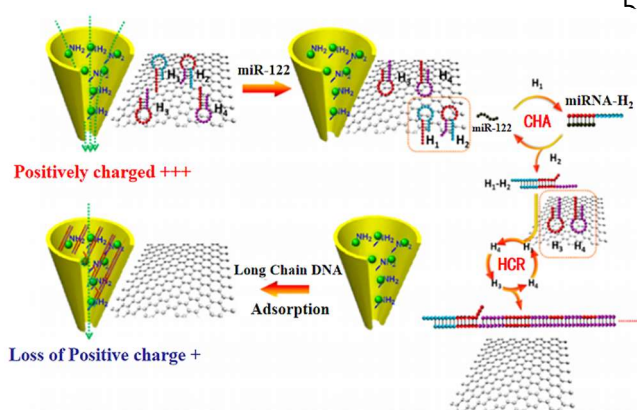


Fig. 5 Schematic of the Sensing Strategy Based on Zr^{4+} -PEI-Coated Nanochannel Biosensor for miR-122.[163] Figure adapted with permission from American Chemical Society, Copyright (2020)

512
 513 **2.1.3 Challenges of graphene nanopore**
 514 Previous studies have clearly shown that graphene nanopores
 515 are extremely promising for DNA sequencing. Table 1
 516 summarizes the graphene nanopores that either experimentally
 517 fabricated or simulated calculation for various DNA sequencing.
 518 Unfortunately, the signal-to noise ratio (SNR) of such an
 519 approach is typically lower than 10.¹⁵⁰⁻¹⁵⁴ This is because
 520 graphene nanopore sits at high ionic current noise levels, which
 521 are several orders of magnitude larger than dielectric materials
 522 (e.g., silicon nitride).¹⁵⁵ In general, the noise spectrum is
 523 composed of both a high frequency regime ($f > 1$ kHz) and a
 524 low frequency regime ($f < 1$ kHz).¹⁵⁶ The former is associated with the membrane
 525 capacitance, whereas the latter with current fluctuation due to
 526 $1/f$ characteristics.¹⁵⁵ For graphene nanopores, the noises mostly
 527 come from both regimes. Moreover, graphene contains various
 528 surface defects.¹⁵⁷ During the irradiation process, graphene
 529 nanopores are shown to heal spontaneously by filling up with

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Table 1. Different graphene nanostructures and pore-forming processes used for DNA sequencing.

Geometries of nanopores	Pore-forming process	Analyte	Analytic method	Reference
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1.4~2.2 nm nanopore	Helium ion beam	sDNA of poly-dN20, poly-dN5, poly-dN3, and dNTP	Ionic current based sensing, current in bias of 200 mV	132
4.5~48 nm nanopore	FIB drilling and shrinking in SEM	homopolymer DNA	Ionic current based sensing, current in bias of 1000 mV	172
5 nm nanopore, 30 nm nanoribbons	E-beam lithography and oxygen plasma etching	DNA	Ionic current based sensing, current at resistance and capacitance in bias of 300mV	154
10~25 nm nanopore	Electrochemical etching	λ -DNA	Ionic current based sensing, translocation time in 200 mV	173
1.6~2.1 nm nanopore	MD simulation	Poly ssDNAs	Ionic current based sensing, current in bias of 2 V	174
5 nm nanopore	Helium ion beam	poly(dA), poly(dG), poly(dC), and poly(dT)	Ionic current based sensing, current in bias of 500 mV	175
5 nm nanopore	MD simulation	DNA methylation	Ionic current based sensing and Field-effect based sensing, in energy window from -0.2 to 0.1 eV	176
1 nm hybridnanopore	MD simulation	ssDNA	Field-effect based sensing, the corresponding binding energy for each target molecule	177
1.5, 2.1, 3.1, 4.1, and 5.1 nm nanopore	Simulation	dsDNA	Ionic current based sensing, current in bias of 2 V	178
1~2 nm nanopore in nanoribbons	Simulation	DNA	Field-effect based sensing, the corresponding binding energy for each target molecule	179
1.4 nm nanopore	Simulation	ssDNA	Field-effect based sensing, current sensitivity in bias of 1.1 V	180

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nanopores during translocation.¹⁶⁵ The non-equilibrium Green's function method based on DFT was used to simulate the collected signals. The sequence information of peptide chain and the sequencing principle of graphene nanoribbon array was thus obtained through MD and simulations.

2.2 Carbon nanotubes

Carbon nanotubes (CNTs) consist of cylindrical nanostructures, made up of carbon atoms arranged in a unique pattern. Due to their high strength, thermal and electrical conductivity, and unique electronic properties, CNTs have gained attention in a wide range of applications such as DNA sequencing. The CNTs offer potential advantages over other nanopore materials, such as improved signal-to-noise ratios and enhanced translocation speeds. For example, when a DNA molecule passes through a CNT, a large increase of the net ion current can be observed. This is because the large electro-osmotic flow from the CNTs can be turned into a large net current, rather than a current blockage. Meanwhile, the construction of nanopores is relatively simple once CNTs are employed. Since discovered in late 20 century, carbon nanotubes (CNTs) are the mostly studied one-dimensional (1D) nanostructures.¹⁸¹⁻¹⁸⁴ They are comprised of sp^2 carbon atoms, in the form of either single-wall nanotubes (SWNTs) or multi-walled nanotubes (MWNTs).¹⁸⁵ The SWNT consists of a single graphene sheet, involving only hexagonal rings with double and single carbon-carbon

bonding.¹⁸⁶ The CNTs were primarily produced by arc discharge,¹⁸⁷ laser ablation,¹⁸⁸ and catalyzed CVD method.¹⁸⁹ The former two methods only obtained low yields of CNTs. Similar to graphene, the CVD method is a more reliable technique for large-scale production of CNTs.¹⁹⁰ The CVD growth of CNTs involves the following basic steps: the dissociation of hydrocarbon gas molecules, atomic carbon saturation on the surface of catalytic nanoparticles, and carbon atom diffusion. The morphologies, structures, and properties of CNTs are thus determined by both the catalysts preparation and subsequent growth conditions. For example, the catalyst is very critical for the CVD growth of CNTs. Different compositions and sizes of catalysts can lead to the as-grown CNTs with different morphologies. In more detail, the size of the catalyst often determines the diameter of the grown CNTs. A number of transition metals (e.g., Fe, Mo, Co, Ni) have been applied for the catalytic growth of SWNTs, owing to high solubility of carbon atoms as well as high diffusion rates of carbon atoms in these metallic catalysts.¹⁹¹ With respect to carbon sources, the most commonly fed gas are methane (CH_4), ethylene (C_2H_4) and acetylene (C_2H_2). Their flow rates and related growth conditions (e.g., temperature, growth time) affected the length and morphology of the CNTs. For the CVD growth of CNTs, there are three growth modes of CNTs: tip growth, base growth, and symmetrical growth. According to different growth modes, the

encapsulated catalytic nanoparticle is located at top, bottom and middle of a CNT.¹⁹²

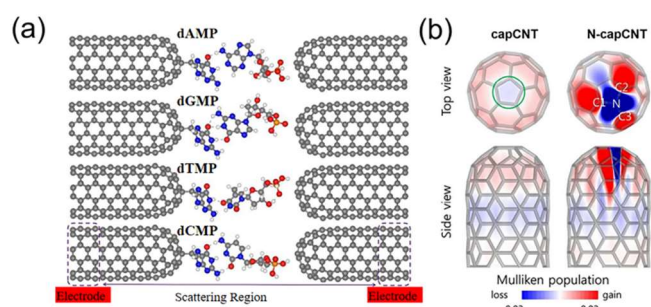


Fig. 6 (a) Atomic structure of the functionalized closed-end cap CNT based nanogap setup for the detection of four different nucleotides (dAMP, dGMP, dTMP, and dCMP). The CNT electrodes (left and right) are semi-infinite and periodic along the transport direction (z-axis).¹⁹⁶ (b) Charge distributions within the pristine and N-doped capped CNTs.¹⁹⁷ Figures adapted with permission from American Chemical Society, Copyright (2018) and Royal Society of Chemistry, Copyright (2020)

It has been reported that long-length CNTs, especially those with big inner diameters (> 50 nm) are not suitable for translocation of biological molecules.^{193, 194} Since the fabrication of ultrashort CNTs is still technically challenging, it is important to develop a precise and effective “cutting” method to have ultrashort CNTs. Meanwhile, such a “cutting” method must avoid the formation of defects on the CNT walls. In this regard, various cutting processes such as sonication-assisted, chemical and plasma etching have been used to shorten ultralong CNTs.^{193, 195} Using a mechanical shear force, long CNTs were cut into short ones.¹⁹⁴ The obtained CNTs were further used to fabricate nanofluidic chips, revealing high potential for sensing single molecules, cations and ssDNA strands.

Another way to read the sequence of DNA molecules using CNTs is to let a DNA molecule pass through a nanogap between two aligned and functionalized CNTs (**Fig. 6a**).^{196, 197} The current recorded on the CNT electrodes is from a tunneling current conducted *via* molecules passing through the membrane. Here, the CNTs act as transverse tunneling tips (**Fig. 6b**).¹⁹⁷ By selecting the potential of between CNT electrodes, the speed of the molecule translocation can easily be controlled. Through such transverse tunneling, the current from CNTs was measured in the range of nano-ampere, which can probably solve the problem for fast translocation speed of a DNA molecule since the generated ionic current is only in range of pico-ampere, especially at a high frequency area.¹⁹⁸ In these cases, the movement of molecules in electrolyte is only dependent on the gravity and drag force. Four DNA bases can be distinguishable by their different electrical resistances.¹⁹⁶⁻¹⁹⁸

However, many challenges exist and hinder the development and practical applications of CNT nanopore sequencers. For example, the fabrication of large scaled CNTs with a particular structure still remains a major challenge. This is because the CNTs are often prepared with flow-through heated reactive gases. In other words, the size and geometrical uniformity of the CNTs, which determines the performance of CNT nanopore

sequencers, is hard to be precisely controlled. The separation of different CNTs, especially in a particular structure is still difficult. Furthermore, the interactions between DNA and CNTs are varied case by case, dramatically affecting the sensitivity of the CNT nanopore sequencers. Note that the properties of CNTs are even strongly dependent on the physical and chemical properties of the applied electrolytes. Once the temperature, content, concentration of the electrolyte are changed during sequencing analysis, the accuracy of the sequencing results using a CNT nanopore sequencer is thus possible to be altered.

2.3 Alternative carbon materials

The development of nanopore sequencing technologies is known to be strongly relied on the used materials for the nanopore formation as well as the supporting membranes. In comparison to those existing and reported materials, diamond membranes are extremely attractive. They are expected to own many advantages for nanopores sequencing, such as their excellent chemical stability, well biocompatibility, and long-term stability under extremely harsh conditions.¹⁹⁹⁻²⁰² Moreover, diamond films or free-standing diamond membranes feature the flexibility upon the reduction of film thickness.²¹ Modification of diamond surface (e.g., hydrophilic or hydrophobic surface) can easily be achieved through varying diamond terminations or adding functional groups onto its surface.⁸ The doping during the CVD growth of diamond can make diamond films own various electronic conductivity and electrochemical potential windows.^{203, 204} For example, boron-doped diamond exhibits high stability for physisorption and chemisorption.²⁰⁵ Note that one of the reoccurring problems of current nanopores is the reproducibility of the measurements. During the translocation under an electric field, a lot of molecules stack and block the nanopores, leading to insufficient spatial/temporal resolution and “biofouling” the sequencing devices. Meanwhile, the reuse and regeneration of conventional microfluid devices require complex processing, which might even damage the core component of fragile bilayers. Furthermore, the reactivation of diamond membranes can be easily realized by applying high currents/potentials under ambient conditions, which generates strong oxidant (OH radicals) in electrolyte solutions and mineralizes (or “cold burn”) organic substances on the diamond surface.^{156, 203} All these advantages make diamond films/membranes extremely attractive for the nanopore formation as well as for DNA sequencing technologies. To realize diamond nanopore sequencing, the growth of ultrathin diamond films/membrane and subsequent formation of well-shaped diamond nanopores are the keys. Unfortunately, both issues have not been well solved up to date.

On the other side, tremendous effort have been devoted to the development of synthesis processes for (ultra-)thin diamond membranes with controlled film thickness.²⁰⁶ Different from thermal CVD growth of graphene, microwave chemical vapor deposition (MWCVD) and hot filament chemical vapor deposition (HFCVD) technique are widely used for the synthesis of ultrathin diamond on non-diamond substrates. During these

CVD processes, gaseous reactants (e.g., methane and hydrogen in most cases) are fed into the CVD reactor. The diffusion and adsorption of activated or initiated species by a hot filament or plasma leads to diamond growth. Such growth is performed by two major processing steps: nucleation and growth of diamond. For example, diamond nanoparticles (e.g., few nanometers in diameter) act as nucleuses. Diamond deposition is controlled and optimized independently by adjusting process parameters, such as gas composition and concentration (or flow rate), chamber pressure, growth temperature and time. For fabricate ultrathin diamond, a slow growth rate is more favorable. Namely, ultrathin diamond films might be grown at low temperature (down to 300 °C) and a long growth time (to hours) during CVD process. Ultra-thin diamond films need feature either insulating or semiconductive properties. The thicknesses of diamond films must be comparable to other 2D materials. For DNA sequencing with high resolutions, diamond films must be as thin as few Angstroms, the same scale as the spacing between DNA bases. To obtain pure diamond membranes, the substrates need to be removed or separated by wet-chemical etching in boiled solutions (e.g., 30 wt% NaOH solution at 80 °C to remove Si substrate) or precise laser cutting technique.²⁰⁷

To generate diamond nanopores, there exist different nanotechnologies such as top-down etching method and bottom-up overgrowth approach.²⁰⁸ As for top-down etching methods, diamond films are etched by plasma (oxygen) or thermos-catalytic (graphitization or burning) reaction through a porous mask, resulting in the generation of porous diamond films.²⁰⁹ The bottom-up growth is either guided by diamond nucleation/deposition at selective areas or is achieved by direct diamond growth on a porous template (e.g., silica spheres,²¹⁰ ²¹¹ SiO₂ nanofibers,²¹² carbon foam,²¹³ titan foam²¹⁴). The quality of obtained diamond pores from the top-down approaches are mainly determined by the etching masks (e.g., nature, size and shape) and etching conditions (e.g., time, temperature, pressure). Since the pore sizes of these porous templates can range from few nanometers to micrometers, generated diamond pores from the bottom-up overgrowth approach are thus expected to have right pore sizes for DNA sequencing. Note that nanopore size is extremely important to accomplish translocated molecule with required selectivity and sensitivity. Unfortunately, the creation of diamond nanopores remains a technological challenge, due to high hardness of diamond and its chemical inertness. Up to date, there is no setup or demonstrator available with respect to diamond nanopore sequencing.

Conclusions

As the fourth-generation sequencing technique, the concept of nanopore sequencing has witnessed unprecedented advances in measuring the structure of nucleotides in DNA molecules. As a label free DNA sequencing technology, nanopore sequencing is expected to achieve long read lengths and meanwhile high sequencing speeds. For such potential sequencing technology, the employed nanopore plays the key role. In addition to

biological nanopores, artificially fabricated solid-state nanopores seem to be more promising. These fabricated on carbon nanoparticles shine light toward a right direction and a bright future of DNA nanopore sequencing. Three mostly used carbon materials, namely graphene, CNT and diamond have been summarized and discussed for their potential nanopore sequencing applications. It is known that they own varied physical, chemical, electrical, and mechanical properties, stemming from their different hybrid states of carbon atoms and geometric features. Among them, graphene is regarded as the best pore and membrane material. This is because graphene layers can represent as both the membrane and the electrode for DNA sequencing. The interactions of DNA molecules with graphene sheets and nanopores are too complicated, depending heavily on existing surface charges, defects, and functional groups. The CNTs are possible to provide nanopores with similar dimensions of DNA molecules. However, they suffer from poor uniformity, leading to uncertain sequencing performance. A free-standing diamond film reveals excellent chemical stability, well biocompatibility, and long-term stability under extremely harsh conditions. It is hard and therefore diamond nanopores can be fabricated as required. For example, with advanced nanotechnology the production of diamond nanopores in the range of sub-nanometers to few nanometers are expected to be possible. However, the formation of ultrathin and large-sized diamond membranes, especially those with similar dimensions of the height of DNA bases is still impossible using currently available chemical vapor deposition methods. We expect that this review article brings readers more thoughts with respect to the selection of carbon materials for nanopore sequencing in their future studies. Many new exciting discoveries of molecular biology are expected at the single-molecule scale when right carbon nanopores are designed, produced and employed.

Author Contributions

Jing Xu: Writing – original draft; Xin Jiang & Nianjun Yang: Writing – review & editing

Conflicts of interest

In accordance with our policy on [Conflicts of interest](#) please ensure that a conflicts of interest statement is included in your manuscript here. Please note that this statement is required for all submitted manuscripts. If no conflicts exist, please state that “There are no conflicts to declare”.

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