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Nanopore Decoding of Oligonucleotides in DNA Computing

Ryuji Kawano

In conventional DNA-computation methods involving logic gate operations, the output molecules are detected and decoded mainly by gel electrophoresis or fluorescence measurements. To employ rapid and label-free decoding, nanopore technology, an emerging methodology for single-molecule detection or DNA sequencing, is proposed as a candidate for electrical and simple decoding of DNA computations. This review describes recent approaches to decoding DNA computation using label-free and electrical nanopore measurements. Several attempts have been successful in DNA decoding with the nanopore either through enzymatic reactions or in water-in-oil droplets. Additionally, DNA computing combined with nanopore decoding has clinical applications, including microRNA detection for early diagnosis of cancers. Because this decoding methodology is still in development and not yet widely accepted, this review aims to inform the scientific community regarding usefulness.

1. Introduction

Polynucleotides contain information encoding amino acid sequence, with this information capable of being transferred and/or copied using chemical and enzymatic reactions. This has garnered the attention of computer scientists desiring to expand their field into wet-lab environments in order to study DNA, RNA, and enzymes. In 1994, a computer scientist, Adleman[1] proposed "DNA-based computing" based on a directed Hamiltonian path problem (**Figure 1**A).[1] The problem solution involves finding a path among several cities on a map, such that each city is visited only once. This was initially transferred to DNA-related problems by preparing short DNAs (20-mers) and assigning each to different cities and paths. Five steps were experimentally performed in order to solve this problem. Step 1: Generate random paths through the graph; with all DNAs mixed, hybridized, and fixed by ligation. Step 2: Remove all paths that do not begin with a start node, "0", and end with an end node, "6", using polymerase chain reaction (PCR). Step 3: The correct length of the DNAs are separated and retrieved by gel electrophoresis. Step 4: Remove any paths that repeat nodes using magnetic bead purification. Step 5: Supply an answer of "Yes" or "No" depending on whether any path remains. This type of problem displays NP complexity, requiring enormous computational resources relative to the number of cities. NP problems are considered beyond the scope of von Neumann-type computers. After proposing this groundbreaking idea, computer scientists collaborated with wet-lab scientists to study 1st generation DNA-based computing,[2–8] which required human intervention to implement each step (i.e., adding solutions, changing reaction temperatures, and observing results via gel electrophoresis).

The 2nd generation of DNA computation allowed autonomous calculations. To create autonomous operations, a method involving "strand displacement" was developed[9,10] that utilized differences in free energy (ΔG) associated with DNA hybridization. When the hybridization energy of DNA strand A-B is larger than that of A-C, the A-C strand is displaced by A-B autonomously. Using this reaction, the operational procedure could be encoded in the DNA sequence by designing the reaction order in terms of the differences in ΔG. Benenson et al.[11,12] proposed a finite automaton system using DNAs and restriction enzymes and that operated on a state transition autonomously. This operation was implemented in a 120-μL volume without additional procedures and at room temperature, with this calculation recorded in the Guinness World Records as representing the "smallest biological computing device."

Logic gate[13,14] implementation is another approach used to construct autonomous DNA-based calculations, given that they are constructed according to a simple binary combination of OR, NOT, and AND gates. This method allows higher-level calculations by combining a number of logic gates, with any logic gate capable of construction through combining multiple NAND (negative-AND) gates. Several researchers have studied complex binary operations using DNAs and enzymes.[15–18] A popular application logic gates involves a game of "tic-tac-toe" using nine wells in a 3×3 matrix. Stojanovic et al.[19,20] constructed a DNA-computational version of "tic-tac-toe" named "MAYA" (Molecular Array of YES and AND-AND-NOT gates) using DNAzyme. This algorithm involved a simplified symmetry pruned game of tic-tac-toe encompassing 19 permissible game plays and using an array of 23 logic gates distributed over eight wells (Figure 1A).

In conventional DNA computation, recognition of output molecules is mainly performed by four different methods

Prof. R. Kawano
Department of Biotechnology and Life Science
Tokyo University of Agriculture and Technology
Harumicho, Fuchu, Tokyo 183-8538, Japan
E-mail: rjkawano@cc.tuat.ac.jp

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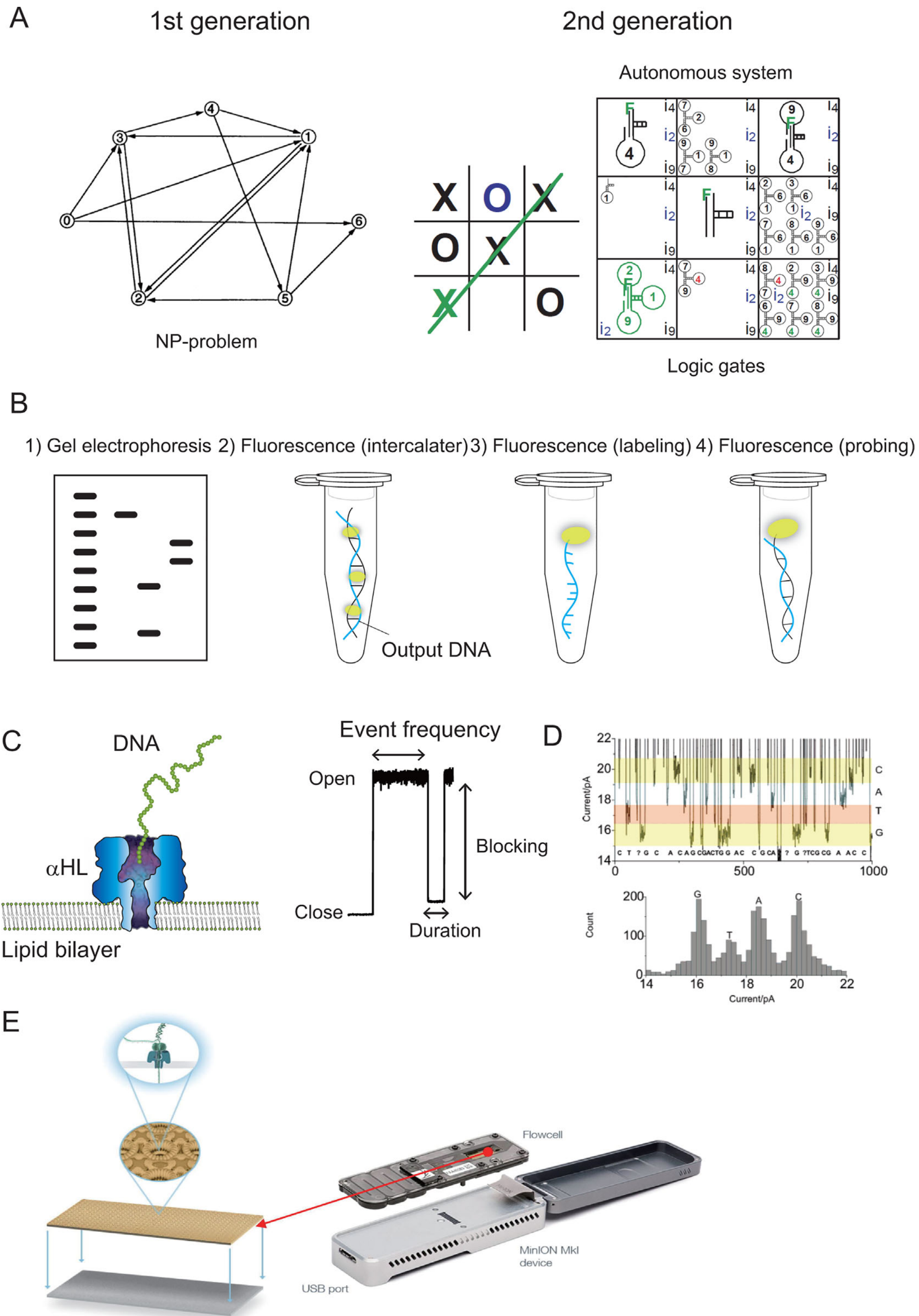

Figure 1. Applications of nanopore technology. A) Adleman[1] described molecular computations using a Hamiltonian path presented as a problem with NP complexity (left). Reproduced with permission.[1] Copyright 1994, the American Association for the Advancement of Science. Tic-tac-toe board representation (right). Reproduced with permission.[20] Copyright 2003, Nature Publishing Group. B) Conventional decoding for DNA computing. C) Schematic illustration of nanopore detection of DNA and current-time trace. D) DNA detection using an αHL nanopore. Four different mononucleotides show individual blocking-current levels. Reproduced with permission.[43] Copyright 2006, the American Chemical Society. E) A commercialized nanopore sequencer with small flow cells (Oxford Nanopore Technologies).

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(Figure 1B and **Table 1**): 1) gel-electrophoretic detection following PCR amplification; 2) fluorescence detection with isothermal amplification; 3) fluorescence detection by direct labeling without amplification; and 4) fluorescence probing without amplification. The pioneering works of Adleman[1] and Benenson[11] in DNA computation used these methods. Despite the recent development of microscale rapid gel electrophoresis,[21,22] traditional gel electrophoresis is time consuming. As a substitute, several fluorescence techniques have been developed involving specific amplification of output DNA by isothermal reactions and observation by fluorescence labeling.[20,23] Methods 1 and 2 require an amplification step involving enzymes, which requires long reaction times and temperature control, even under constant conditions at 37 °C. Therefore, non-amplification methods (i.e., methods 3 and 4) can be used, where the output DNA is labeled and detected[24] and a specific fluorescence probe is used.[25] Although neither of these methods require an amplification step (considering their implementation at relatively high concentrations), the use of direct labeling or specific probe molecules is required.

Nanopore technology allows the rapid and electrical detection of oligonucleotides in the absence of labeling. Several studies reported methods related to nanopore decoding[26–28] and their applications in diagnosis or clinical settings based on DNA computing. Therefore, nanopore methods represent potential candidate methods for decoding DNA computations.

2. Application of Nanopore Technology for Rapid and Label-Free Decoding

Nanopore technology involves electrical measurement of ion current through a nanopore.[29–34] Biological (proteins) or solid-state nanopores ranging in size from 1 nm to ≥10 nm show open-pore current conductance (Figure 1C). When a molecule passes through or blocks a nanopore, the open-pore current reduces, thereby demonstrating current-signal blockage. The blocking amplitude, duration time, and event frequency provide information regarding the size, mobility, and concentration of target molecules at the single-molecule level. α-Hemolysin (αHL), a channel toxin from *Staphylococcus aureus*,[35] is conventionally used as a biological nanopore for detecting oligonucleotides based on its having a pore size comparable to that of single-stranded DNA (ssDNA) or ssRNA. Extensive studies reported using this nanopore as a label-free, rapid, and electrical method for single-oligonucleotide determination, with one application targeting nanopore sequencing (Figure 1D).[36–40] As the first report in 1996 by Kasianowicz et al.,[36] enormous efforts have been undertaken to apply this method.[37,39,41–54] In 2015, a company named Oxford Nanopore Technologies was launched and provided the first commercially available nanopore sequencer for general use (Figure 1E). Currently, nanopore technology can be utilized not only for single DNA/RNA detection but also for large-scale DNA sequencing.[55]

Nanopore methods allow recognition of oligonucleotides rapidly, electrically, and without the necessity for labeling. Since 2016, several studies reported possible application of this technology for the detection of DNA-computing output. The first study involved detection of the output of a NAND logic operation in a micro-droplet system.[26] This method involved construction of a four-droplet system with a biological nanopore at the droplet-interface bilayer and electrodes in each droplet.[56–59] Input DNAs are injected into two input droplets, calculations are performed in the operation droplet, and these droplets are subsequently passed through the output droplet, with the output monitored electrically by the nanopore (**Figure 2**A). The important feature of this work was that output "1" or "0" was defined according to whether an ssDNA translocated through a nanopore. This method harnessed the unique property of the αHL nanopore, which allowed only ssDNAs to pass through. This method involves conversion of molecular information into electrical signals in a binary system, and while output times associated with fluorescence-based logic gates range from minutes to hours, this nanopore system requires only ≈10 min without any labeling.

Following this preliminary study, Ohara et al.[27,60] proposed a complicated logic operation using enzyme reactions in the micro-droplet of a nanopore system in order to accommodate the necessity for enzymatic reactions in most DNA computations. An enzyme-free system requires rigid operational conditions, because the temperature of the reaction and/or enzyme concentrations are strictly controlled in such reactions. However, enzyme-free operations can be implemented in one-to-one reaction, with one input molecule generating one output molecule using a chain-displacement reaction. By contrast, operations involving enzymes can be implemented in versatile reactions, such as DNA polymerization, amplification, and transcription. Therefore, verification of nanopore decoding is important, especially in cases of DNA calculations involving enzymatic reactions. Ohara et al.[27] constructed an AND gate allowing the input molecules to amplify and transcribe DNA via T7 RNA polymerase when two DNAs are input simultaneously (Figure 2B). Their study showed that four different operations

Table 1. Comparison of conventional fluorescence and nanopore-decoding methods of DNA computation.

	Gel electrophoresis[1,11]	Fluorescence (intercalator)[23]	Fluorescence (labeling)[24]	Fluorescence (probing)[25]	Nanopore[26,27]
Detection method	Optical	Optical	Optical	Optical	Electrical
Measurement time	Long	Short	Short	Long	Short
Sensitivity	Low	Low	High	High	High
Throughput	Low	Low	High	High	Potentially high
Pre-treatment	Multi-step	Few steps	Multi-step	Multi-step	Few steps
Generality of method	High	High	Medium	Medium	Low

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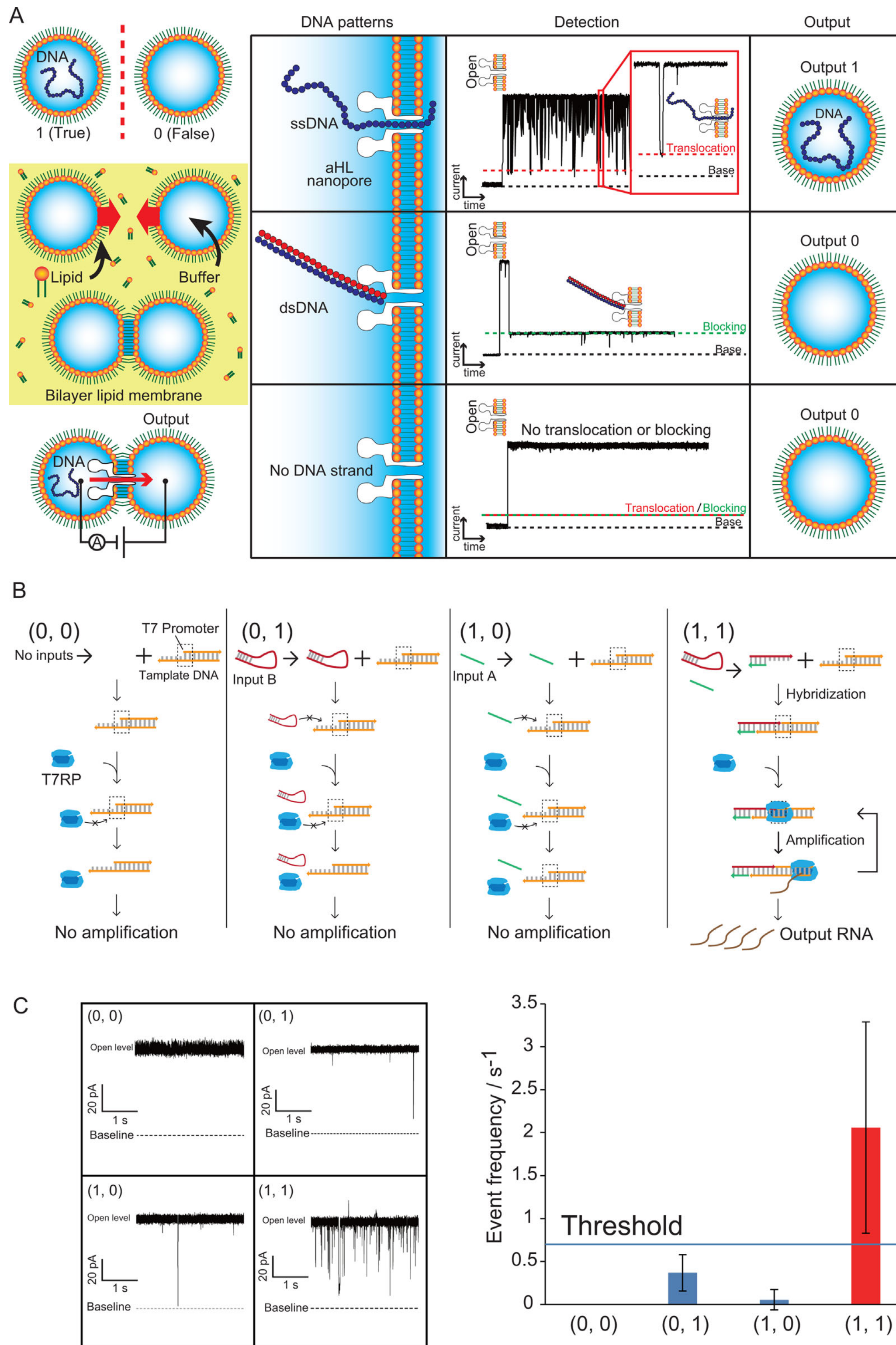

Figure 2. Nanopore decoder methodology. A) NAND operation in a droplet system for nanopore decoding. The input molecule moves from the input droplet to the output droplet through the nanopore. B) Four individual operations associated with a reverse transcription AND gate involving the T7 RNA polymerase. C) Nanopore measurement enables rapid and label-free detection of the output molecules. The translocation frequency of the output molecules through the nanopore allows discrimination between a (1, 1) system and others. Reproduced with permission.[27] Copyright 2017, the American Chemical Society.

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represented by (0 0), (1 0), (0 1), and (1 1) were implemented in multiple micro-droplet devices, and that the output could be obtained after 90 min, which included a 60-min enzymatic reaction (Figure 2C),[28] suggesting the efficacy of nanopore decoding for operations involving enzymes in micro-droplet systems (summarized in Table 1). However, the reaction efficiency differed between that performed in conventional plastic tubes and droplets with a surrounding lipid bilayer, with reduced efficiency observed in the droplet system.[27] Therefore, these operations need to be improved appropriately with respect to enzymatic reactions performed in a lipid-droplet environment.

3. Application of Nanopore Decoding in Medical Diagnosis

The field of DNA computing was developed largely as a curiosity driven exercise focused on solving mathematics-related problems, including cryptograms and constructing various types of logic gates (AND, OR, NOT, XOR, and NAND). However, this field recently increased in importance due to its potential applications in medical diagnosis.[61–63] Benenson et al.[61] reported autonomous diagnosis and drug-release systems using DNA computing using the following "if-then" logic: "if" certain diagnostic conditions are true, such as low expression levels of certain mRNAs relative to those of others, "then" the antisense drug is released. After this pioneering study, several studies were undertaken focused on application of this technology to diagnosis and therapy.[62] Based on the favorable compatibility of nanopore technology with oligonucleotide detection, strategies utilizing this method for diagnosis using nanopores and DNA have been proposed.

Wang et al.[64] demonstrated nanopore-based detection of microRNAs (miRNAs) from patients with lung cancer (**Figure 3**A).[64] MiRNAs are short noncoding RNAs, the expression levels of which correlate with various diseases and represent potential early diagnostic markers for cancer.[65] Wang et al.[64] used programmable oligonucleotide probes that formed partially hybridized structures with target miRNA (Figure 3B), resulting in partially hybridized DNA/RNA that exhibited different blocking levels against unzipping relative to non-hybridized oligonucleotides. They were specifically able to detect target miRNAs of the let-7 tumor family at picomolar levels, providing a practical demonstration of miRNA detection in cancer patients with cancer and emphasizing that the measurement accuracy of the αHL nanopore was higher than that of quantitative real-time PCR assays.[64] Subsequent studies reported development of specific tags, such as peptide nucleic acid (PNA)[66] or polyethylene glycol (PEG),[28] used to detect miRNA. The PNA-probe method is unique and involves hybridization of a cationic probe to the target miRNA to form a double-stranded structure that can be captured by nanopores exhibiting opposite polarity.[66] Despite the efficacy of this method, simultaneous detection of multiple miRNAs remains challenging. Complementary DNA with a PEG tag was later used to target miRNAs, with PEG tags bound to target miRNAs showing different blocking levels during translocation through the αHL nanopore.[26] Although this method achieved accurate detection of four different miRNAs, it was difficult to measure the amounts of respective miRNAs, because differences in the blocking levels were too close to allow discrimination. Very recently, the analysis of the duration of the blocking instead of the blocking current are proposed for the pattern recognition of miRNA expression using AND logic gate with nanopore technology.[67]

Another advantage of using nanopore technology for miRNA detection is its sensitivity. In conventional analytic methods, such as microscopic or electrochemical methods, sensitivity relies upon signal intensity. In fluorescence measurements, low-intensity results make it difficult to discriminate between noise and signal at low concentrations. Therefore, sensitivity is dependent upon the signal-to-noise ratio (SNR). On the other hand, nanopore measurements at low concentrations result in no changes in the SNR of the current signals, but rather a decrease in the frequency of appearance of the signal. Ideally, even at very low target-molecule concentrations, such as those involving single molecules, the signal will appear during continuous measurement. This implies that the sensitivity of nanopore measurements is dependent upon measurement time, which normally ranges from several minutes to several hours. Consequently, concentration limitations are approximately on the order of one picomolar, even after several attempts at enhancing translocation via the nanopore under asymmetric salt conditions.[68]

A useful technique associated with DNA computation involves amplification, which can enhance detection of targets at low concentrations. Zhang et al.[68] reported the successful detection of low concentrations of miR-20a, which is secreted in small-cell lung cancer (SCLC), by combining isothermal amplification of the oligonucleotide along with nanopore-based methods (Figure 3C).[69] Their method amplified stable ssDNA from miR-20a at concentrations ranging from one femtomolar to 10 picomolar using an isothermal enzymatic reaction, with the output DNA capable of quantification by nanopore measurement according to the translocation frequency. Based on this methodology, any cancer-specific miRNAs can potentially be specifically amplified and detected by changing the nucleotide sequences of the DNA template and primer according to the target miRNA.

Another interesting aspect of DNA computing used in clinical applications is "theranostics," which describes a system of simultaneously combining diagnosis and therapy. Benenson et al.[61] reported an autonomous diagnosis and drug-release system using DNA computing and involving a one-to-one reaction (i.e., a single input molecule generates a single output molecule), which is incompatible with the requirements of most therapies, where the concentration of drug molecule (output) needs to be higher than that of the diagnostic molecule (input). Hiratani et al.[70] demonstrated a theranostic system for SCLC using isothermal amplification from target miRNA to an antisense oligonucleotide, which was treated as a DNA-based drug (Figure 3D). Isothermal amplification is an emerging technique in DNA computation that allows DNA amplification at a constant temperature. A previous study described generation and amplification of a DNA-drug molecule (output) using enzyme-mediated strand-displacement amplification following detection of the target (miR-20a; input) from an SCLC patient, with the generated DNA drug (oblimersen) monitored and quantified by nanopore-based measurement in real time.[70] The results of nanopore quantification showed that oblimersen was

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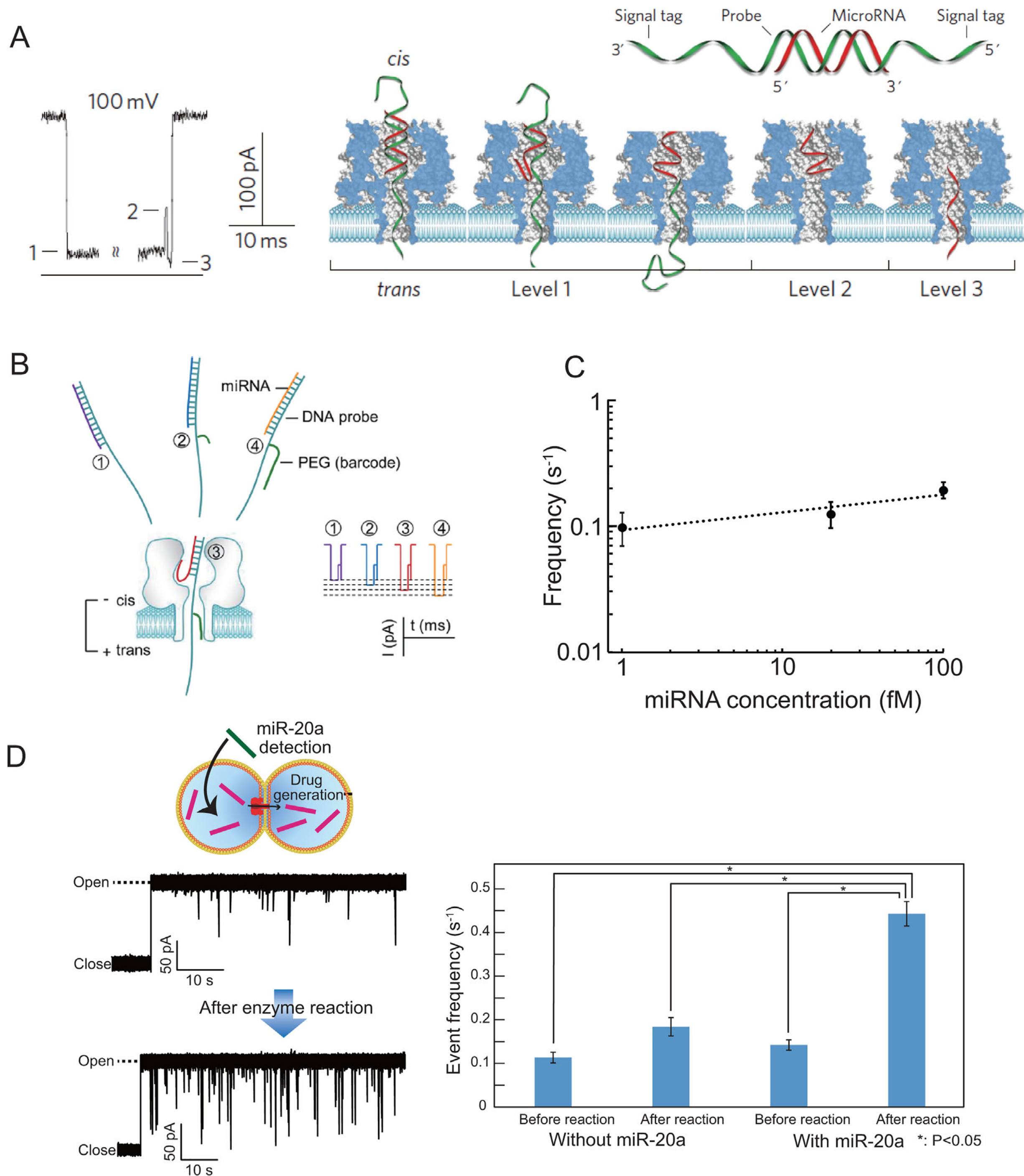

Figure 3. Nanopore detection of miRNAs. A) MiRNA detection using DNA probes. The αHL nanopore recognizes the complex according to specific current signals. Reproduced with permission.[64] Copyright 2011, Nature Publishing Group. B) PEG-tagged DNA probes provide different blocking currents to allow discrimination between specific miRNAs. Reproduced with permission.[28] Copyright 2014, the American Chemical Society. C) Detection of ultra-low concentrations (on order of 1 fM) of miRNAs using nanopores and DNA computing. Reproduced with permission.[69] Copyright 2017, the Royal Society of Chemistry. D) Theranostic system for SCLC using DNA computing. An antisense DNA drug generated upon miR-20a detection; followed by nanopore-based measurement to quantify drug concentration using a label-free and real time method. Reproduced with permission.[70] Copyright 2017, the American Chemical Society.

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amplified by >20-fold from miR-20a, thereby meeting the dosage requirement for SCLC therapy and suggesting this autonomous amplification strategy as a potential candidate for broad-range theranostics using antisense oligonucleotides .

Nanopore decoding might also contribute to molecular robotics.[71,72] Molecular robots represent next-generation biochemical machines comprised of biomaterials, such as DNA, proteins, and lipids, with the prerequisites of sensors, intelligence, and actuators proposed as requirements for the construction of such robots. To develop sensors necessary to apply a level of "intelligence" to these robots, output decoding, using nanopores will be a valuable tool used to constructing the necessary parts.

4. Conclusions

In this review, we described recent developments in nanopore decoding methods for DNA computation and their applications in clinical fields. Nanopore technology does not require labeling, and the decoding time is relatively rapid compared with conventional fluorescence methods. However, for laboratory scale measurements, reconstitution of biological nanopores in lipid bilayers requires training that might be time consuming. Although the droplet-contact method enables rapid, reproducible, and stable nanopore measurements, it requires experience and training. Powerful strategies based on micro-fabrication have been recently introduced allowing preparation of massive numbers of nanopore chambers in a small device in order to acquire the required data exclusively from the appropriate chambers. This strategy addresses current nanopore-specific issues and can be potentially applied to other nanopore technologies, including nanopore decoding of DNA computation on an industrialized scale. Nanopore technology represents a valuable methodology for enhancing the decoding of DNA computations.

Abbreviations

αHL, α-hemolysin; DNA; microRNA, miRNA; PCR, polymerase chain reaction; PEG, polyethylene glycol; PNA, peptide nucleic acid; SCLC, small-cell lung cancer; SNR, signal-to-noise ratio; ssDNA, single-stranded DNA.

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Conflict of Interest

The authors declare no commercial or financial conflict of interest.

Keywords

DNA logic gates, DNA sequencing, lipid bilayers, nanopores, single molecule detection

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