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Recent Application of Nanopores in the Detection of Small Organic Molecules

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Abstract. Nanopore sensors have become a useful tool for detecting small organic molecules, which provides more and more accurate options than traditional analytical techniques. This review outlines and compare the application of various emerging nanopore sensors, which includes solid-state nanopore (silicon nitride and MsO2) and non-solid-state nanopore (α-hemolysin nanopore, aerolysin nanopore, MspA and so on), to identify various organic molecules, including amino acids, nucleotides monosaccharides and others. By taking advantage of the unique current signatures that molecules penerate through nanopores, the researchers have been able to distinguish different types of small molecule with high precision. The differentiation of nucleotides and amino acids is also made use of by innovative methods such as viscosity gradient systems and identification tunnels. Challenges such as nanopore clogging still remains. But it also highlights the further technological need in this field. In summary, nanopore sensors, show great prospect in the field of chemical analysis and have great potential for a variety of applications in the future.

Keywords: nanopore, sensor, small organic molecules.

1. Introduction

Conventionally, analysis of small organic molecules requires sample separation which cosumes a lot of time and advanced analytical instruments such as spectroscopy and chromatography. When a biological nanopore is correctly modified with a reactive adaptor, it is transformed into a nanoreactor capable of responding to chemically suitable tiny molecules. Its analytical principle is based on single molecule detection, which identifies passing molecules by small changes in the current in the nanopore [1]. The nanopores have been applied as a flexible single-molecule sensor for nucleic acids [1], proteins [2], metal ions [3] and others. However, for the detection of mixtures of small organic molecules like the active gradients extracted from herbal medicines, complicated sample separation or purification steps are not necessary. The emergence nanopore has completely change the landscape of chemical analysis, promoting the need for further investigation. While there are many review articles on this topic, most of them are focused on one part of the broad field, such as DNA sequencing, saccharides identification and solid-state nanopore identification. The aim of this account is to overview recent advancement of nanopore detection for a wide variety of small organic molecules, which includes amino acids, saccharides, nucleotides and small organic molecules from herbal medicine.

2. Amino acid

Table 1. The amino acids that are peptidase-digested are sequentially read by a nanopore.

Identification	Type of testing tool	Helper molecule	Advantages and Disadvantages	Reference
five pairs of enantiomers of amino acids	α-hemolysin nanopore	Cu^{II}-phenanthroline	insufficient resolution, cannot distinguish all 20 amino acids	[7]
individual amino acids	tunnel junction	nanopore integrated with	hard to manufacture tunneling junction devices and link them to the nanopore	[8]
oligopeptide	aerolysin nanopore	built-in single-molecule trap	13 out of 20 short peptides can be identified, rather than single amino acids	[9]
fragmented peptides or intact polypeptides	β-barrel nanopore: transmembrane region of bacterial protective antigen combining with mammalian proteasome activator	archaeal 20S proteasome	all proteinogenic amino acids and their PTMs is required	[10]
single amino acid	MspA-NTA-Ni nanopore: hetero-octameric Mycobacterium smegmatis porin A (MspA) nanopore	Ni^{2+}	20 amino acids	[11]

Through recognition tunnelling, single-molecule amino acid identification is possible. Due to splicing and modification, the human proteome has millions of protein variations, many of which are minutely concentrated and associated with diseases. Proteins cannot be amplified, hence single-molecule protein sequencing is essential for finding biomarkers. The recognition tunnelling, a technique that traps amino acids between electrodes coated in recognition molecules, can measure the electronic fingerprints. Enantiomers methylated amino acids, isobaric isomers, and short peptides are all classified by machine learning techniques. This implies that protein sequencing by direct electronic means is possible (**Table 1.**). In 2012, the Bayley group reported that a α-hemolysin (α-HL) nanopore modified with Cu (II)-phenanthroline exhibited direct detection of five pairs of enantiomers of amino acids. If single molecule of amino acids binds to the Cu (II) complex, enantiomers can be distinguished, because the Cu (II) complex provides a chiral environment. During the enzymatic catalytic resolution of racemic mixtures, there will be an increase in enantiomeric excess in real time. However, due to the insufficient resolution of α-HL, it failed to simultaneously distinguish all 20 amino acids [7]. However, the reported incident discrimination is still not good enough. Additional technical obstacles relate to the manufacture consistency at tunnel joints device and its connection to the nanopore sensor.

In 2014, an aerolysin-based nanopore was employed by the Lindsay group to discern octapeptides with a solitary terminal amino acid alteration. The inherent single-molecule retention feature of the aerolysin nanopore confines the interacting amino acid within its sensing domain. Precise volumetric measurements of the amino acid within the nanopore, a process that might be time-consuming, are essential. This method, however, revealed that merely 13 out of 20 brief peptides, not individual amino acids, were acknowledged as analytes. [8]

In 2020 the Oukhaled group reported a β-barrel nanopore composed of a eukaryotic proteasome activator modified transmembrane segment of bacterial protective antigen. The archaeal 20S proteasome facilitates protein unfolding and linear traversal through the nanopore. Target proteins, either fragmented peptides or intact polypeptides, are processed within the proteasomal chamber. Nevertheless, an aspiration yet to be realized is the development of a nanopore capable of unequivocally identifying all natural amino acids and their modifications. This comparison highlights limitations and challenges inherent to diverse amino acid analysis techniques. [9]

In 2021, the Maglia group reported that all 20 natural amino acids and modifications could be distinguished with a unique hetero-octameric Mycobacterium smegmatis porin A (MspA) nanopore, which incorporates one single Ni^{2+} alteration. By interacting with the amino acids and their alterations as they travel through the nanopore, this modification—known as MspA-NTA-Ni—plays a crucial part in the sensing configuration [10]. The production of very distinctive and repeatable amino acid events is facilitated by the NTA- Ni^{2+} complex and the conical lumen shape of MspA. Combining a proprietary machine learning technique with the distinct nanopore architecture of MspA-NTA-Ni, the combination achieves a general accuracy of 98.6% in differentiating between amino acids and their modifications. The durability and stability of the MspA-NTA-Ni nanopore enables reliable and continuous observation for several hours. The MspA-NTA-Ni nanopore has excellent durability and stability, allowing reliable and continuous observation within hours. Applications of this nanopore-based approach include nutritional analysis, c linical diagnosis, and even examining amino acids in peptides or proteins destroyed by proteases. Because the designed MspA-NTA-Ni nanopore can distinguish between all 20 natural amino acids and modifications, it can be used to assess both amino acids digested from peptides and amino acid supplement tablets. Overall, the high accuracy and discrimination of MspA-NTA-Ni nanopore makes it an effective tool for evaluating complex amino acid mixtures, whether found in supplement tablets or as a result of peptide digestion. Proteomic research, nutritional analysis and clinical diagnosis are three areas where the technology shows promises [11].

3. Nucleotides

There is little discrimination between the four types of different deoxyribonucleotides, Adenine, Guanine, Cytosine and thymine, and the four types of ribonucleotides, Adenine, Guanine, Cytosine and urcail, so the identification of these single nucleotides remains a challenge. Solid-state nanopores such as Boron Nitride, Silicon Nitride, Graphene, MoS2 and Silicon oxide [9] have drawn much attention due to the desire for nanopore with better robustness, more straightforward modification and controlled size. Non-solid nanopores will also be introduced in this paper.

Table 1. Nanopore sensors for small organic molecules.

Identification	Type of nanopores		Method	Advantages and Disadvantages	Reference
ribonucleotides	non-solid nanopore	α-hemolysin protein nanopores (containing amino-cyclodextrin adapters)	ionic current measurement	failed to show an actual difference between cytosine diphosphate and uridine diphosphate.	[13]
monophosphates ribonucleosides (5-methylcytidine, N6-methyladenosine, N7-methylguanosine, N1-methyladenosine, inosine, pseudouridine and dihydrouridine)		hetero-octameric Mycobacterium smegmatis porin A nanopore (phenylboronic acid-modified)		higher resolution	[14]
deoxyribonucleotides	solid-state nanopore	nanopore fabricated in the thin MoS_2 membrane	current measurement (during the transient residence)	hydrophobic interactions result in nanopore clogging, the high translocation velocity limits the usability of DNA sequencing.	[15]
		Silicon Nitride	blockade current measurement	challenges in discriminating between certain nucleotides.	[12]

In 2013 the Bayley group reported that when a ribonucleotide monophosphate or diphosphate penetrates through α-hemolysin protein nanopores containing amino-cyclodextrin adapters, they would interact with the adapters leading to a valid ionic current change. Due to the residual currents and dwell times, nanopores can distinguish between ribonucleotides rGDP, rADP, rCDP and rUDP. In the envisioned exon sequencing approach, a polynucleotide phosphorylase is used for sequential cleavage of ribonucleoside diphosphates from molecules. These cleaved nucleotides are then presented to the nanopore for identification, allowing for direct RNA sequencing [13].

In 2022 the Huang group reported that application of cyclodextrin-embedded alpha-hemaglycetin (alpha-HL) detection of nucleotides failed to show an actual difference between uridine diphosphate and cytosine diphosphate, as well as identification of RNA modification. This low resolution whould caused by the cylindrical lumen geometry of α-HL34. In contrast, Mycobacterium branched-chain A (MspA), a cone-shaped pore is more favourable. When MspA nanopore's phenylboronic acid groups interact with nucleotide's cis-diol groups, reversible covalent complexes are formed. This specific interaction occurs only in nucleotides containing ribose moieties. It can change the ionic current that is different between various nucleotides. An algorithm helps to distinguish them, including modified nucleotides. This approach provides a comprehensive understanding of the types and abundance of nucleotide modifications in the RNA molecules [14].

In 2015 the Radenovic group reported molybdenum disulfide nanopores exploiting the unique current signature generated by each deoxyribonucleotide during its brief residence in the nanopores. The transfer kinetics of nucleotides through nanoscale pores in atomically thin molybdenum disulfide membranes were controlled by introducing a viscosity gradient system composed of room temperature ionic liquids (RTILs). This system permits statistical identification of all four types of nucleotides from DNA based on the current signatures recorded during the moment they pass through the nanopore. The high viscosity of RTILs in the nanopore slows down nucleotide translocation and allows nucleotide types to be distinguished due to the distinct current characteristics. Electrostatic interactions and hydrophobic binding within the nanopore, which can be known as the interactions between the nucleotides and the pore walls, can contribute to the unique current patterns exhibited by each nucleotide type. By analysing these features of current, adenine, cytosine, thymine and guanine nucleotides passing through the molybdenum disulphide nanopore can be distinguished under the influence of a viscosity gradient system. This innovative approach opens up new possibilities for high-precision DNA sequencing technologies. But there are still some challenges such as hydrophobic interactions which can result in nanopore clogging and the high translocation velocity that limits the usability of DNA sequencing applications [15].

In the case of a Silicon Nitride nanopore with a diameter of 1.8nm, a distinct blockade current is observed upon the passage of individual nucleotides. Upon applying a bias voltage, these nucleotides traverse the nanopore under the influence of an electrophoretic flow, not the force, enabling the distinction between the four unique nucleotides based on their volumetric differences. Enhanced by the electroosmotic flow, a single nucleotide can remain within the pore for hundreds of microseconds, facilitating single nucleotide identification. The residence time distributions adhere to the first-passage time distribution of the 1D Fokker-Planck equation, facilitating the determination of each nucleotide's diffusion coefficient and speed [16].

Molecular dynamics simulations confirm that larger nucleotide translocates inside the nanopore more quickly than the smaller one. This is likely due to the larger nucleotide's higher surface area, which could result in a larger drag force from the electroosmotic flow. However, there are still some limitation and challenges in discriminating between certain nucleotides, like Thymine and Adenine [13].

4. Monosaccharides

Because of the weak affinity, tiny size and complex and similar glycosomal structures, conventionally it is hard to identify single-molecular saccharides directly. In 2022 the Huang group reported that

MspA nanopore recognizes sugars through a unique mechanism. The phenylboric acid attached to the nanopore forms reversible bonds with sugars, especially with hydroxyl groups in sugar molecules. By detecting current changes caused by binding and deionization events, the sensor can distinguish between different types of sugars based on their specific interactions with phenylboric acid within the nanopore. This innovative approach allows for the direct identification of sugars at one single-molecule level with excellent precision and resolution [17].

At the same year the same group reported that MspA nanopore could also distinguish between 13 different types of sugar alcohols. A complete database of sugar alcohol sensing characteristics was formed, including l-galactitol, arabitol, xylitol, glycerol and others. [18].

In 2024 the Xie group applied carbon nitride nanopore device to recognize monosaccharide (GlcNAcpN) among the trisaccharide molecules (LeApN and SLeCpN), disaccharide molecule (LacNAcpN) and monosaccharide molecule (GlcNAcpN), indicating that this nanopore possess a monosaccharide-level resolution [19].

5. Small organic molecules from herbal medicines

Natural herbs containing pharmacologically active compound have been applied as traditional medicines. However, time-consuming sample separation as well as analytical instruments such as high-performance liquid chromatography, liquid chromatography-mass spectrometry and Fourier transform infrared spectroscopy are required for the analysis of chemical components. In 2024, the Huang group applied MspA nanopore with a phenylboronic acid adapter to identify and quantify a variety of bioactive salvianolic acids, including protocatechuic acid, caffeic acid, protocatechualdehyde, rosmarinic acid, salvianic acid A, salvianolic acid A, salvianolic acid B and lithospermic acid. Salvia miltiorrhiza, rosemary and Prunella vulgaris are a few examples of natural herbs that are also used in this sensing concept. The PBA modification on the nanopore allows reversible capture and release of the analyte during the recording process, which can extend the time of residence. This modification can let the sensor alter to bind to compounds that contain 1, 2-diol groups, which can be found commonly in salvianolic acids. Thes selective binding and decoupling process improve the ability for the sensors to distinguish between different salvianolic acids. In addition, the high resolution of the nanopore which is cooperated with customized mechanism with learning algorithms, can ensure the accurate identification of bioactive compounds without using method of complex sample preparation or purification. [20].

6. Conclusion

A viable way for quick and accurate analysis which doesn’t need to prepare laborious samples is to use Nanopore sensors in the detection of tiny organic compounds. Nanopore technology has demonstrated to have the ability of detecting different chemicals as single molecule based on their interactions within the nanopore, including saccharides, nucleotides, amino acids and even specific herbal ingredients. However, there are still some difficulties and challenges to distinguish between some specific compounds. Recent advances in nanopore design have indicated that improving the specificity and resolution of the identification can be expected soon.

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