Analysis with biological nanopore: on-pore, off-pore strategies and application in biological fluids

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Abstract

Inspired from ion channels in biology, nanopores have been developed as promising analytical tools. In principle, nanopores provide crucial information from the observation and analysis of ionic current modulations caused by the interaction between target analytes and fluidic pores. In this respect, the biological, chemical and physical parameters of the nanopore regime need to be well-understood and regulated for intermolecular interaction. Because of well-defined molecular structures, biological nanopores consequently are of a focal point, allowing precise interaction analysis at single-molecule level. In this overview, two analytical strategies are summarized and discussed accordingly, upon the challenges arising in case-dependent analysis using biological nanopores. One kind of strategies relies on modification, functionalization and engineering on nanopore confined interface to improve molecular recognition sites (on-pore strategies); The other kind of highlighted strategies concerns to measurement of various chemistry/biochemistry based interactions triggered by employed molecular agents or probes (off-pore strategies). In particularly, a few recent paradigms using these strategies for practical application of accurate analysis of biomarkers in biological fluids are illustrated. To end, the challenging and future outlook of using analytical tools by means of biological nanopores are depicted.

Keywords： Nanopore; On-pore strategies; Off-pore strategies; Molecular interactions; Single-molecule analysis; Biomarkers

1. Introduction

Due to the label-free and real-time analytical capabilities, nanopore technology offers several competitive advantages for the analysis of single molecules. In principle, nanopores provide crucial information from the observation and analysis of ionic current modulations caused by the connection between target analytes and fluidic channels. The changes in the ionic current (Scheme 1), including the mean residence time (τ_{off}), amplitude (I) and frequency of occurrence ($1/\tau_{on}$), are altered with the interactions between the analytes and nanopores [1-5]. It is well documented that both biological nanopores and artificial pores (built on solid-state materials) have been used as novel sensing tools [6-8]. Together with desirable properties and functions, nanopores hold great promise for ultrasensitive, qualitative and quantitative analyses at single molecule level for chemicals and biomolecules [9-11] , etc.

To be a suitable element in stochastic sensing, nanopores need to have clear three-dimensional structures, tolerance to structural modification, moderate single-channel conductance, and clean open channel without transient current spikes. Biological nanopores, most of which are derived from bacterial cytotoxins, have very well-defined structures and can be engineered with sub-nanometer precision using approaches of site-directed mutagenesis. Hence, biological nanopores consequently are of a focal point, allowing precise interaction analysis at single molecule level. They have been ingeniously applied in the field of homeland security [12-13], biomedicine [14-16], environmental monitoring [17-18], etc.

However, accompanied with their unique potential advantages, biological nanopores have limitations and challenges as well. The intrinsic properties of the pores (e.g., 3D structures) will strongly affect the transport behaviors of the analytes and subsequently deteriorate the performance (e.g., resolution,

selectivity and sensitivity) of stochastic sensing [19-21]. Therefore, various approaches have been taken to overcome these hindrances. Thus far, two major strategies have been utilized to improve the performance of nanopore sensors. As illustrated in scheme 1, one relies on modifications or engineering within the nanopore-confined space by accommodating molecular recognition sites (on-pore strategies), which can be achieved by introducing various chemical components and groups, such as adapters [22], aromatic [23] and charged residues [24]. The other kind of strategies takes advantage of external biomolecular agents (off-pore strategies) to detect analytes via various chemical and biochemical interactions, including host-guest [25], protein-ligand [26], nucleic acids hybridization [27], enzymatic proteolysis [28], protein denaturation [29] and chelation reactions [30]. Despite excellent works accomplished by kinds of biological nanopores [31-34], in this review, we aim at introducing the novel analytical methods and conceptualization via some paradigms in particular with α-hemolysin (α-HL), the first proposed and most developed candidate among the biological nanopores. First, some cases were introduced to elucidate the on-pore and off-pore strategies used to improve the performance of nanopore analysis. Second, the optimization of nanopore sensing were depicted through recent paradigms in achieving accurate measurements of biomarkers in biological fluids. The highlighted jobs may inspire people to discover new ways to in-depth research in analytical and bioanalytical chemistry fields.

2. On-pore analytical strategies

The highly well-known and controllable structures of the proteins provide more advantages in construction of biological nanopores, and make them more flexible to propose nanopore-based analytical strategies. In this aspect, modification and engineering of molecular recognition sites in nanopore lumen is a usual strategy to reinforce the interactions between pores and analytes, thus to yield an ultra-high sensing resolution. Herein, the commonly used modification and their designs were presented, including accommodation of molecular adapters, replacement with desired residues mutants (e.g. aromatic, charged and polarized amino acid), and truncated pores via approaches of gene engineering.

2.1. Analysis with nanopores carring molecular adapters

To improve the selectivity and sensitivity of nanopore stochastic sensors, a significant class of biological nanopores has been constructed with molecular adapters that possess internal binding sites for single molecule analytes recognition. In this strategy, an ingenious breakthrough is the employment of molecular adapter β-cyclodextrin (β-CD). β-CD consists of 7 glucose subunits, creating a cone shape structure (diameter 0.60~0.65nm) with rich molecular recognition functions. Due to the structural arrangement, its molecular interior is considerably less hydrophilic than the aqueous environment and consequently able to host hydrophobic molecules. While the hydrophilic exterior offers improved water solubility. Such properties make it feasible to regulate on the uptake or release of trapped molecular species within their cavities [35-36]. Furthermore, β-CD once anchors in the constriction area (~1.4 nm) of the channel, it plays imperative roles in reducing the pore size and subsequently leads to the enhancement of sensing resolution. In this case, it is capable of improving the transport behavior of organic molecules, such as DNA individual bases, which have difficulties to form a connection to the nanopore [22]. For example, Bayley et al. examined the covalent and/or noncovalent interactions between attached β-CD and different α-HL pores. Results demonstrated the mutated α-HL pores showed

much stronger (~104 folds) binding affinity to β-CD than that of the wild-types [37-38]. As shown in Fig. 1A, molecular models show the molecular interaction between engineered α-HL pores and β-CD adapters. Seeing from the model, recognition regions of β-CD responsible for each interaction were identified, including hydrogen bonds, hydrophobic interactions and Π-CH group bindings. In addition to theoretical hypothesis, α-HL pore with β-CD adapter subsequently demonstrated its advantages in the selective identification of DNA bases, which made DNA sequencing possible in the coming future [39-40]. In addition, due to the considerable significance of RNA molecules in exo-sequencing, β-CD assisted nanopores were also implemented in identification of individual RNA nucleotides (Fig. 1B). Different from DNA identification, a processive exoribonuclease (polynucleotide phosphorylase) were employed to sequentially cleave and load ribonucleotide diphosphates to a β-CD carrying α-HL nanopore [41]. On the basis of RNA nucleotides identification, RNAs profiling in cells were afterward implemented, indicating the advantage of nanopores as invaluable tools for biomolecular analysis [42-43].

Nowadays, extensive studies on proteins analysis have also been demonstrated using α-HL nanopore with β-CD adapters. One example is that the kinetics of trypsin was analyzed by real-time monitoring of the current modulations with nanopore sensors (Fig. 1C) [44]. To effectively avoid the disturbance from background signals and specifically recognize hydrolysate, an α-HL nanopore equipped with a polyamine decorated β-cyclodextrin (am_7β-CD) was employed as the sensing platform. The real-time monitoring exhibited the process of trypsin enzymatic cleavage of a substrate N-α-benzoyl-L-arginine ethyl ester (BAEE) at the single molecule level. This β-CD assisted nanopore enables a new scheme to analyze enzyme activity for cleaving small molecules beyond biomolecular substrates.

In addition to biomolecules analysis, Guan et al. developed a simultaneous detection method for small organic agents (Fig. 1D). Cyclohexyl methylphosphonic acid (CMPA) and pinacolyl methylphosphonate (PMPA) are organophosphorus hydrolytes derived from nerve agents Soman and Cyclosarin, respectively [45-46]. As results showed, the distinctive current signals were further produced due to the capture of CMPA and PMPA by β-CD anchored channel. More attractively, the detection limits of CMPA (0.01 mg/mL) and PMPA (0.02 mg/mL) are significantly lower than the discharge limits required by the US Army (0.1% in w/v, i.e., 1000 mg/L) [47]. Further, results indicated this on-pore modification with β-CD adapter should make the direct detection feasible for hard-to-obtain small organic compounds, which is of significant importance for sensing applications in homeland security, environmental surveillance and beyond.

Similarly, other molecular adapter was recently employed for nanopore sensing (Fig. 1E). Kang et al. synthesized a novel artificial receptor, heptakis-[6-deoxy-6-(2-hydroxy-3-trimethylammonion-propyl) amino]-beta-cyclomaltoheptaose, with similar functions of β-CD, which could be harbored in α-HL nanopore [48]. This bio-mimic adapter was designed to carry ATP, ADP and AMP molecules (APs), which are relevant to biological energy storage and signal transduction. Based on the high affinity between APs and molecular adapter, this strategy enabled simultaneous recognition and detection of APs by real-time at single-molecule level, particularly with significant elevation on both sensing selectivity and signal-to-noise ratio.

2.2. Analysis with engineered nanopores carring aromatic residues

Most of nanopore analysis require precision engineering in the pores. According to rational designs, certain amino acids are modified or replaced [49]. In such a way, α-HL nanopore has been modified using site-directed mutagenesis. For example, analysis for peptide mixtures appears to require atomic resolution due to tiny differences in their compositions and lengths, including those differs by only one amino acid among peptide polymers. Therefore, aromatic residues have been engineered along the lumen in native biological pores, which resulted in a strong interactive affinity between peptide molecules and nanopore interiors. This strategy leads to an improvement of sensing resolution due to distinct increase in residence time, amplitude and event frequency of current signals [23]. Fig. 2A shows an engineered α-HL pore with seven aromatic Tyrosine side chains $(M113Y)_7$ that provides peptides with ultra-sensitive connection spots. The respective affinities between the peptides and three different nanopores, $(WT)_7$, $(M113F)_7$ and $(2FN)_7$, were also examined. Our results revealed that the translocation behaviors of the peptides were significantly influenced by the replacement of aromatic residues [50-51]. Conclusion, the enhanced interactive affinity in engineered nanopores allows simultaneous discrimination of peptide mixtures, and makes the analysis available in peptidome.

2.3. Analysis with engineered nanopores carring charged residues

It is worth mentioning that the sensing resolution can be improved by loading extra internal charged residues along the pore lumen, which gives rise to intense interactions between analytes and nanopores. Previous works have already been done on α-HL mutation nanopores, where additional charged residues were engineered in α-HL interface [24, 52]. Also, earlier reports demonstrated the kinetics of polypeptide altered during the translocation through mutant nanopores with some negatively charged rings (named traps). The association rate constant k_{on} indicated that the molecular interactions increased obviously with the help of these traps, especially for hydrophilic polypeptides. Therefore, this engineered nanopore should enable the quantitative analysis of the molecular kinetics to be possible [53].

Another notable example is from Muthukumar et al. (Fig. 2B) who calculated the DNA trajectory in nanopore channel using Langevin dynamics simulations. With positive charged decorations in the nanopore, the step-by-step movements of DNA were calculated [54]. Simulation results agreed well with experimental values for the average translocation time afforded by a single nucleotide [52]. An additional important aspect was that, the DNA velocity was revealed to be altered depending on the location and distance of mutations in the channel. These findings brought new insights into DNA dynamics in nanopores and provided rational engineering strategy for DNA sequencing in the future. Since the impact of engineered nanopores have been felt in diverse precise analysis, other interesting works aiming at constructing and discovering novel pores keep increasing, including Aerolysin, [55] Clytolysin A, [56] MspA, [57] and CsgG nanopores [49].

2.4. Other engineering strategies on nanopores

Previous studies have demonstrated the broader nanopore vestibular area (cavity) allows to capture single molecule in electric field, imparting nanopore analytical power to investigate various macromolecular conformations (G-quadruplexes and fishhook hairpin DNAs), and monitor unzipping kinetics at different locations in the channel [58-59]. The ability to differentiate complicated

nanostructures depends on the overall shapes, sizes, stem lengths and sequence context of the molecules, with no need of molecular engineering in the nanopore cavity. However, recent findings have also suggested that to improve the ability of individual base discrimination, shorter pores should be better [60]. Hence, much of engineering strategies have focused on the transmembrane β-barrel district of α-HL nanopores. In this respect, Bayley et al. explored mutants of the α-HL in which the transmembrane β-barrels were strictly truncated [61]. Truncated barrel mutants (TBM) were made from the α-HL NN mutant (Fig. 2C), residues from β-strands were pairwise detached to yield barrels shortened by 2, 4, 6, 8, and 10 amino acids, respectively. This study demonstrated the truncated proteins could float on the surface of lipid bilayers and form totally different toroidal lipid pores. The truncated pores prompted well-defined transmembrane ionic currents by pores establishment in the underlying lipids. Because short pore with a single constriction is optimal for base discrimination, this design may allow useful improvement in sequencing technology. Another notable engineering example was inspired by the structure of gap junctions in biology. Bayley engineered a dimeric α-HL pore, in which two α-HL heptamers were covalently linked by disulphide bonds. The dimeric pores formed spontaneously due to cysteine residues in cap, allowing cap-to-cap coupling conduit structure [62]. In this structure, one β-barrel inserted into a small lipid vesicle, while the other spanned a planar lipid bilayer. More importantly, this dimetric nanopore provides the capability to serve as a competent candidate for single molecule analysis of smaller molecules and even ions like γ-CD and phosphate anions.

3. Off-pore analytical strategies

The ability of sensing individual molecules is highly desirable in modern biology, chemistry, and beyond. However, some molecules cannot be easily detected by biological nanopores due to their inappropriate dimensions (either too large or too small) or nonspecific interactions with the pores. This disadvantages hinder the usage of nanopore sensing. As a result, there have been various approaches in literature to transmit binding events arising outside the pores in addition to the interior spaces so that the ionic current could be modulated accordingly. One optimized solution towards versatile sensing is to design biomolecular agents (such as aptamers [63-64] or molecular probes [65-66]) for specific coupling to target analytes, therefore analytes can be captured and detected effectively by the pores with enhanced sensing resolution.

3.1 Analysis via aptamer based host-gest interactions

Wu et al. reported a universal nanopore sensing strategy by employing a combination of aptamers and host-guest interactions [25]. In their designation (Fig. 3A), as the host part, an aptamer was first hybridized with a DNA sequence which was modified ahead of time with a ferrocene⊂cucurbituril complex. When a guest analyte was applied to the sensing system, the hybridized duplex of aptamer with a ferrocene⊂cucurbituril-modified sequence will unwind due to the higher affinity between the analyte and aptamer. The competitive binding behavior produced specific current signatures with consecutive multiple substates when the modified DNA molecules translocated through the channel. According to the results of the two types of signature events, the highly sensitive detection of variant molecules was finally implemented via this aptamer-assisted sensing approach. Because aptamers have shown robust

binding affinities with a wide variety of target molecules, this host-guest strategy enables quantitative and selective analysis of different types of analytes within nanopore sensors.

Etiology and pathogenisis of many diseases are often closely associated with the changes in expression of multiple biomarkers. In generally, it requires multiplex detection of series molecules at the same time for precise disease diagnosis and prognosis. Taking lung cancer for example, a most recent work has been presented with designed protein-aptamer binding nanopore strategy on simultaneous detection of three protein biomarkers [67]. As shown in Fig. 3B, the output DNA hybrids (in different length) released for nanopore sensing upon aptamer-target binding and this binding was proved to be a key component of the probe. With more detailed analysis, the distinctive current signals generated in the nanopore provided visual and quantitative discrimination among several proteins (VEGF165: Vascular endothelial growth factor, TB: Thrombin, and PDGF-BB: Platelet-derived growth factor B-chain) even in complex biological samples, and without the need of additional labeling. More attractively, this simple approach allows universal, convenient, and low-cost sensing for different analyte types only need the modulation of the probe composition and length.

3.2. Analysis via nucleotides hybridization

Single-nucleotide polymorphisms (SNPs) are specific nucleotide site mutations where they may have two different nucleotides (including single nucleotide deletion, insertion, substitution or other mutation). When SNPs occur within a gene or in a regulatory region near a gene, they may cause a disease via gene dysfunction. Hence, SNPs are usually used to predict the risk of certain diseases [68-69]. To date, several methods have been developed for the fully and semi-automated discovery of SNPs, including sequencing strategies [70], multiplex reverse dot blots [71], DNA chips [72], and the TaqMan approach [73]. As an attractive choice for obtaining DNA sequence information, nanopore has also emerged as a rapid, direct determination method for detecting SNPs. However, the determining remains a challenge due to extremely short translocation time, low capture rates and signal-to-noise ratio in nanopores. Therefore, molecular probes are introduced to couple with target DNAs to form complex structures that strongly improved the sensing quality [74-75].

Guan et al. developed a novel enzymatic reaction-based method for nanopore sensing of DNA mutations (substitution, deletion and insertion) [76]. The surveyor nuclease was employed since it activates each accurate mismatch site in dsDNA [77-78]. As shown in Fig. 3C, full-matched dsDNA (none mutations) permanently blocked the nanopore regardless of the presence of the nuclease. In contrast, nuclease working on the DNA duplex with mismatches resulted in disappearance of permanent blockage but accompanied with short current events. The phenomena verified surveyor nuclease cleavage in the nanopore. In addition, the dsDNA chains with nucleotides substitution, deletion or insertion were also effectively determined, respectively. It is worth mentioning that this method was also identified to be capable of detecting terminal base-base substitution mismatch, which remains as a challenging task using other determination approaches.

In addition to DNA analysis, tumor-related mRNAs analysis was also completed (e.g. single nucleotide deletion). For example, Baxα, known as a key tumor suppressor gene, is often expressed incorrectly as its isoform, BaxΔ2, which has the same sequence, except for a single base deletion from

eight continuous guanines (G8 to G7) in exon 3 of Bax. It is known that traditional methods, including western blot and reverse transcription polymerase chain reaction, used to detect Baxα and BaxΔ2 are sensitive; however, these techniques also have drawbacks. One significant drawback is the high GC pair content, which often creates artificial mistakes, resulting in truncated or no PCR products. In this case, our nanopore sensor seems to be an optimized analyst candidate without this concern. Based on the complementary base pairs, two DNA probes were designed to selectively distinguish Baxα and BaxΔ2 at the same time [79]. The statistical results of distinguishable events indicated the successful detection of single base deletion between Baxα and BaxΔ2. It further means the occurrence of a potential, rapid and sensitive analytical strategy on other single-base mutations detection in genetic diseases. This probe-assisted nucleic acid sensing strategy was also documented in other literatures [80-82].

3.3. Analysis of enzyme activity via proteolysis

Enzymes are involved in a wide variety of physiological activities in biological environments. Hence, studies on enzymes can help reveal biochemical processes, including enzyme kinetics and proteolysis mechanisms within living cells. These can consequentially provide information for disease state, such as early diagnosis of HIV [83] and ADAMs [84]. Taking pandemic HIV as an example, Fig. 3D shows analytical strategy for the sensitive detection of HIV-1 protease. In our work, peptide-enzyme proteolytic reaction was examined in real time to obtain the kinetic studies of HIV-1 protease, via monitoring the current signals caused by proteolysis. To further evaluate the potential clinical value for disease diagnosis and prognosis, the enzyme in simulated clinical samples with human serum were examined. As expected, this designed nanopore sensor was not affected by human serum and gave advanced high sensitivity. The designed nanopore sensors should provide potential to earlier diagnosis in powerful addition to the current diagnosis via measurement of antibodies in patients. Soon afterward, quantitative measurement of trypsin activity and inhibition with calcium ions was also achieved using the same nanopore strategy [85]. Besides, Long et al. reported their recent effort on a one-step method for determination of protein kinase activity using kinase and phosphopeptides with aerolysin nanopores [86]. In addition, Wu et al. presented their most recent work on monitoring protease activities using a DNA probe that bore a short peptide probe containing phenylalanine [87]. This category of DNA probe permits dual-response to both enzymatic activity and environmental pH. In view of the crucial role of local pH value and protease activity in cancer initiation and metastasis, this nanopore method may be soon explored for specific screening of complex tumor cells.

3.4. Analysis of protein conformational change via denaturation

Exploring the interaction mechanism between proteins at the single molecule level remains one of the mostly fundamental problems in biology. For instance, aggregated proteins need to be unfolded to transport through narrow pores in membranes, and then refolded within a recipient cellular compartment, such as toxin proteins into host cells [88]. Previous studies including experiments and theories have explored protein translocation mechanisms through nanopores [89-91], but one of the main difficulties is to experimentally explore the conformational change in transport process. In this context, Pelta et al. proposed nanopore electrical method to study the protein folding/unfolding at the single molecule level, which permits the complete separation of all the conformations in denaturant unfolding [29]. In their

examination (Fig. 3E), they compared the unfolding transition of the wild type and a destabilized variant of maltose binding protein using two different channels, α-HL and aerolysin. The distinguishable current signals were capable to differentiate unfolded states from partially folded ones. They found that the unfolding transition curves of the destabilized variant protein were shifted toward the lower values of the denaturant agent compared to the wild type protein. According to their results, they also proved that the nanopore structure, geometry, and net charge did not influence the folding transition but change the transport dynamics. Soon after, Pelta et al. also explored the protein unfolding in thermal denaturation using same protein and nanopore models [92]. The sigmoid function fits the normalized frequency of occurrence for both nanopores (Fig. 3F), indicating the protein unfolding sped up by thermal motion, and did not depend on the nanopore characters neither. All of the above suggest that such real time elucidate protein folding-unfolding transition using nanopore strategies can obviously lead to exciting developments in research area of protein exploration and designation.

3.5. Analysis of metal ions via chelation

While nanopores have been designed for studying different biomolecules in various ways, they also enable the sensitive and selective detection of analytes as small as metal ions. To achieve this application, one of the simplest paradigms is the employment of an appropriate molecular probe, which can selectively chelate with target metal ions. Concise and convenient cases using this approach have been demonstrated, including specifically designed DNA strands for mercury (Fig. 4A)[66], lead and barium ions (Fig. 4B) [93], or molecular probes for the discrimination of Copper, Zinc, Nickel and Cobalt ions (Fig. 4C) [94-97]. However, metal ion detection using a chelating molecular probe remains challenging, as without a clear understanding of how the molecular probe nature changes after chelation (e.g., conformational changes or net charges). This change is correlated with characteristics of current signals, including residence time and blockage amplitude, further makes the current signals unpredictable and results in difficulty for designing sensors.

For the purpose of optimizing this chelation-based nanopore detection method, as shown in Fig. 4D, we proposed a computation-assisted approach for highly sensitive and selective detection of thorium ions (Th^{4+}), a well-known radioactive and chemically toxic element [30]. The computational prediction indicated the most significant changes in the net-charge of peptide probe (before/after the addition of Th^{4+}) occurred at pH 4.5. In addition, Th^{4+} is prone to form eight-coordinate 1: 2 (Th/D-12) stoichiometric complexes, especially in acidic solutions. Consequently, Th^{4+} was detected at a concentration of ~ 250-fold less than those of other interfering ion species in the optimal pH condition. Furthermore, the detection limit in a 10 min examination using this nanopore strategy was sufficient for the analysis of thorium in environmental samples. Similarly, we also developed a label-free method for the detection of uranyl ions (UO_2^{2+}) by monitoring the peptide-ions chelation [98]. The detection of uranyl ions is significant not only for environmental monitoring, but also for radioactive nonproliferation. In conclusion, driven by the need of an advanced platform for metal ions analysis (especially heavy and radioactive metal ions), this development of nanopore sensors provides a great potential in a wide range of applications.

4. Application in biological fluids

Based on substantial academic effort, nanopore technique has been developed as an outstanding analysis tool not only in fundamental sciences but also going to commercialization [49]. Given a massive effort in sequencing field, there is no doubt that additional areas can be commercialized, including the stochastic sensing in real word. However, despite the advances, to reach the full application in real world using nanopore analysis, several technical challenges still need to be resolved. For example, in terms of disease diagnosis and prognosis, it requires precision selectivity and ultra-high sensitivity for biomarkers tests with complex biological samples. In this case, one issue is that blood components usually block pores and affect the stability of lipid membranes, accordingly hinders the sensing resolutions [99]. Another snag falls into that trace amount analyte detection in biological fluids prolongs the sampling, and also accompanied with nonspecific background signals decrease the analysis efficiency [100]. Hence, the optimization of nanopore sensing strategies is of more importance in achieving the accurate measurement of a range of biomarkers in biological fluids.

At the example of the renin protease, which is a diagnostically relevant hydrolytic enzyme and involved in regulation of blood pressure and homeostasis, Howorka et al. presented a new nanopore-based analysis strategy for matrix containing serum samples [99]. Using their strategy (Fig. 5A), the renin enzymatic activity was electrically detected with the help of single spin-column, within where the enzyme-cleaved substrate was affinity-purified using multifunctional resin to discard the analytically harmful interferent from blood serum. This method overcame limitations arised from blood component-induced membrane instability and poor signal-to-noise ratio. As they demonstrated this strategy using a multifunctional resin spin-column can very likely be extended to other hydrolytic enzymes dissolved in any analyte matrix and be exploited for analytical read-out methods other than nanopore sensing.

Second example concerns for mRNAs detection in biological fluids, which is recognized valuable for predicting cancers [43]. In Fig. 5B, we depicted the selective extraction and accurate detection of mRNAs using displacement-chemistry strategy with the help of nano-mag-beads and two-probes (capture and release probe) [101]. Despite the loss of mRNA molecules in sample preparation, mRNAs recovery could be significantly improved using a simple method of increasing the molar ratio between probes and target mRNAs. Notably, compared to other approaches [43, 99, 102-103], our method is crystal simple and not involved in molecular structure functionalization, complicated molecular probes designation and tedious physical preparation (e.g. centrifugation and elution) [59].

Third example in aspect of biomarker analysis, Wu et. al. proposed a DNA-assisted nanopore strategy on simultaneous quantification of multiple cancer biomarkers in blood samples [104-105]. In their experiments, five barcode DNAs were thoughtfully designed to label different gold nanoparticles that can selectively bind to specific antigens (Fig. 5C). After the completion of the sandwich assay, barcode DNAs were released and subjected to nanopore translocation test. This approach was determined very useful for accurate and multiplexed quantification of cancer-associated antigens at picomolar level in clinical samples, including prostate-specific antigen (PSA), carcinoembryonic antigen (CEA), alpha-fetoprotein (AFP), neuron specific enolase (NSE) and carbohydrate antigen 19-9 (CA19-9).

Noteworthily, nanopore sensing was soon afterwards applied to analysis with real cancer cells [106]. At this example, Xi et.al. reported a sensing strategy to probe the human 8-oxoguanine DNA glycosylase (hOGG1) activity in human lung adenocarcinoma cells, by employing an enzyme-catalytic cleavage

reaction of DNA substrates. As shown in Fig. 5D, the hOGG1 specifically catalyzed the removal of the 8-hydroxyguanine (8-oxoG) and cleaved the DNA substrates immobilized on magnetic beads, following the release of output DNA for quantitative test in α-HL nanopore. This strategy exhibited the excellent performances for discriminating hOGG1 from the interferent. According to statistical results of a linear correlation in the range from 100 to 10000 cells, this approach shows impressive practical capability for quantitative detection of enzyme activity in complex cell systems. Besides, in addition to bio-nanopores sensing, the ultrasensitive detection of antigens in blood samples was recently reported using nanoparticles-assisted SiN_x nanopores [107]. Taken together, it can be concluded that the success of nanopore sensing in complex biological media opens the door towards nanopore development of commercial devices for potential point-of-care diagnostics.

5. Conclusions, Challenges, and Future Outlooks

Nanopores have shown great potential as fast, label-free and ultrasensitive analysis elements for individual molecules at single-molecule level. It is, at least in principle, easy based on the Coulter counting technique using the current-pulse sensing method in the electric field. As mentioned above, this biology-inspired monitoring technique leads to surprising observations when molecules or ions are driven through the nanoscaled channels. However, several challenges remain to be overcome to extend the availability of nanopore sensing assays for various analyses. Accordingly, we herein elucidate the solutions to those snags through recent paradigms, including on-pore strategies (intrinsic engineering with the pore), off-pore strategies (extrinsic interaction factors) and practical analysis in biological fluids. These strategies exhibit great diversity and rapid advancement in fields of analytical and bioanalytical chemistry.

In terms of advances of an ideal analytical tool, nanopore technology can provide flexible and versatile properties for molecular analysis in a range of great interest. However, there is, of course, much more work that remains to be done to build such an ideal tool using nanopore elements. To the best of our knowledge, it is fundamental that two factors play imperative roles in nanopore analysis, the pore itself and the analysis methodology. In terms of the pores, one of the most attractive aspects is that this biology-derived element enables many sensitive and selective analyses in biological systems (from bio to bio). In addition, it is highly reproducible and easy to regenerate through site-directed genetic engineering. In contrast, the biological properties may become the weaknesses as well as the advances. In particular, the suitability of a nanopore for stochastic sensing is mostly affected by 3D structures and other instinct properties. That includes the opening, the vestibule, the latch and the constriction area of the pore. These factors make the channel more specific (ion selectivity similar to that of potassium, sodium ion channels) and subsequently deteriorate the performance (confined space for molecules capturing, e.g. chiral molecules). One strategy should fall in the discovery of other nanopores from microbial origin. The novel exploited nanopores should provide multiple opportunities and be expected to offer great benefits to bioanalyses. As an alternative, the other solution is the construction of nanopores using molecular self-assembling nanotechnology. Among these structures, the most competitive ones are DNA-based programmable nanopores and α-helical peptide-based nanopores. The structures of self-assembling nanopores are very clear at the atom level and allow flexibility in tuning the properties of each

component, including pore size, chirality, hydrophilic and hydrophobic characters, host of functional groups, etc. With such advantages, the sensing resolution and practical feasibility of these artificially built bio-pores can be improved in case-dependent analyses.

The optimization of the analysis methodology is an optional strategy in addition to pore design and construction. There are many very good works regarding interdisciplinary applications in the combination of classic methods with nanopore platforms. For example, excellent jobs have been reported regarding nanopore analysis coupled with back titration chemistry, proteolysis chemistry, chelation chemistry, oxidation-reduction chemistry and displacement chemistry, etching chemistry, etc. These great efforts may inspire people to make fast progress in developing an advanced and functional analytical tools using nanopore devices.

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Declaration of competing interest

The authors declare no competing financial interest.

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Abbreviations

α-HL	α-hemolysin
3D	three dimensional
β-CD	β-cyclodextrin
BAEE	N-α-benzoyl-L-arginine ethyl ester
CMPA	cyclohexyl methylphosphonic acid
PMPA	pinacolyl methylphosphonate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
AMP	adenosine monophosphate

TBM	truncated barrel mutants
WT	wild-type
CsgG	curli specific genes G
MspA	mycobacterium smegmatis porin A
VEGF165	vascular endothelial growth factor
TB	thrombin
PDGF-BB	platelet-derived growth factor B-chain
SNPs	single-nucleotide polymorphisms
PSA	prostate-specific antigen
CEA	carcinoembryonic antigen,
AFP	alpha-fetoprotein
NSE	neuron specific enolase
CA19-9	carbohydrate antigen 19-9
hOGG1	human 8-oxoguanine DNA glycosylase

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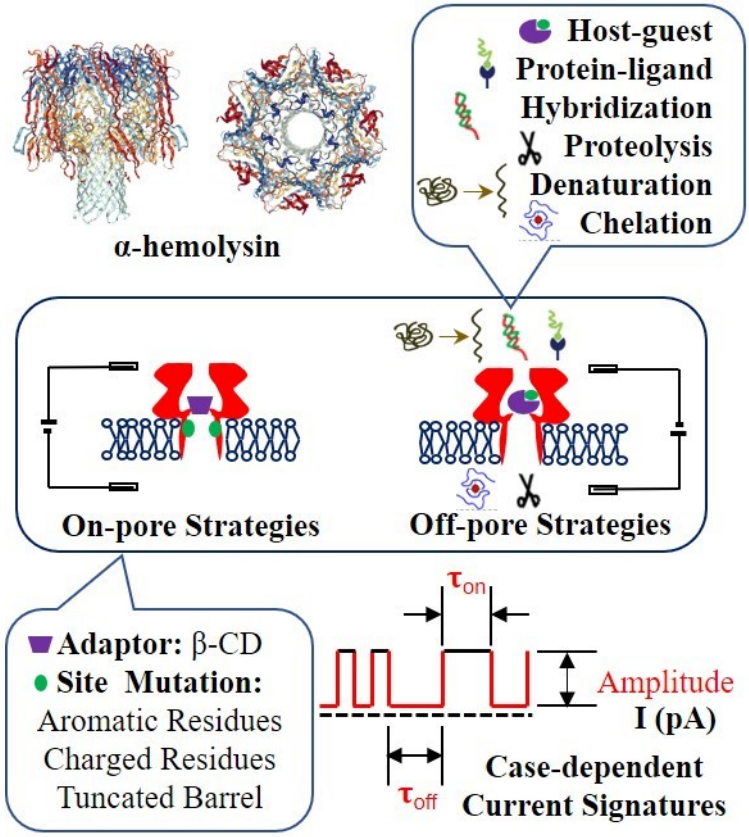

Scheme 1. Schematic representation of nanopore analysis using α-hemolysin (PDB ID: 3ANZ) embedded into a planar lipid bilayer. It principally takes advantage of recording ionic current modulations by the passage of target analytes that are driven through a single fluidic channel. The analysis strategies rely on modifications within the nanopore-confined space by accommodating molecular recognition sites (on-pore strategies), which can be achieved by introducing various chemical components and groups, including adapters, aromatic and charged residues. The other uses are external biomolecular agents (off-pore strategies) that detects analytes based on various chemical and biochemical interactions, including host-guest, protein-ligand, hybridization, enzymatic proteolysis, and chelation reactions.

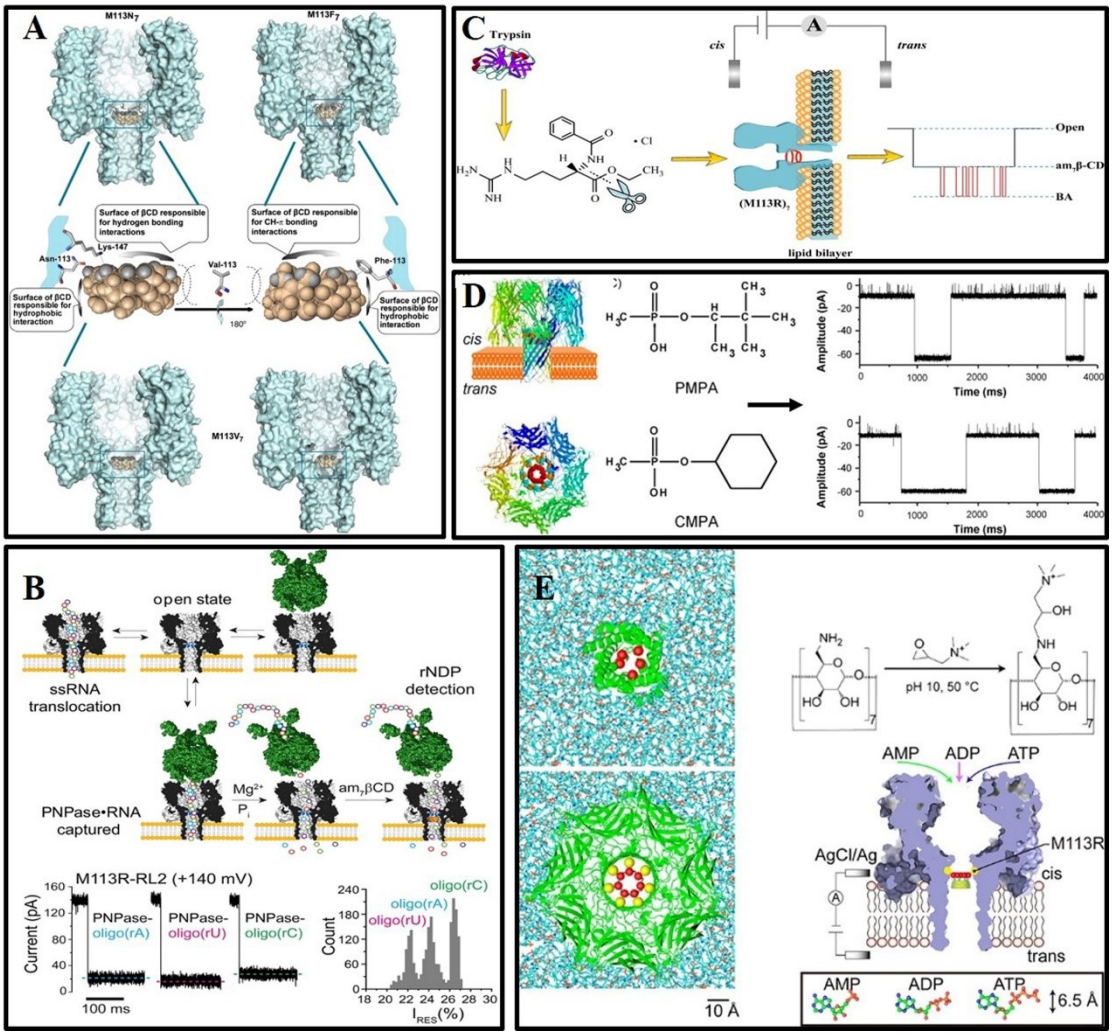

Fig. 1. (A) Molecular model showing interactions involved between α-HL pores and molecular adapters, including hydrogen bonding, hydrophobic interactions and Π-CH group bonding interactions. Figure adapted with permission from Ref. [37] Copyright (2010) National Academy of. Sciences. (B) Direct identification of individual RNA nucleotides with processive exoribonuclease in a β-CD carrying α-HL nanopore. Figure adapted with permission from Ref. [41] Copyright (2013) American Chemical Society. (C) α-hemolysin nanopore equipped with (am_7β-CD) for the kinetics studies of trypsin. Figure adapted with permission from Ref. [44] Copyright (2019) American Chemical Society. (D) Simultaneous detection method for small organic agents. Figure adapted with permission from Ref. [45] Copyright (2009) Elsevier. (E) Simultaneously recognize and detect ATP, ADP and AMP. Figure adapted with permission from Ref. [48] Copyright (2020) American Chemical Society.

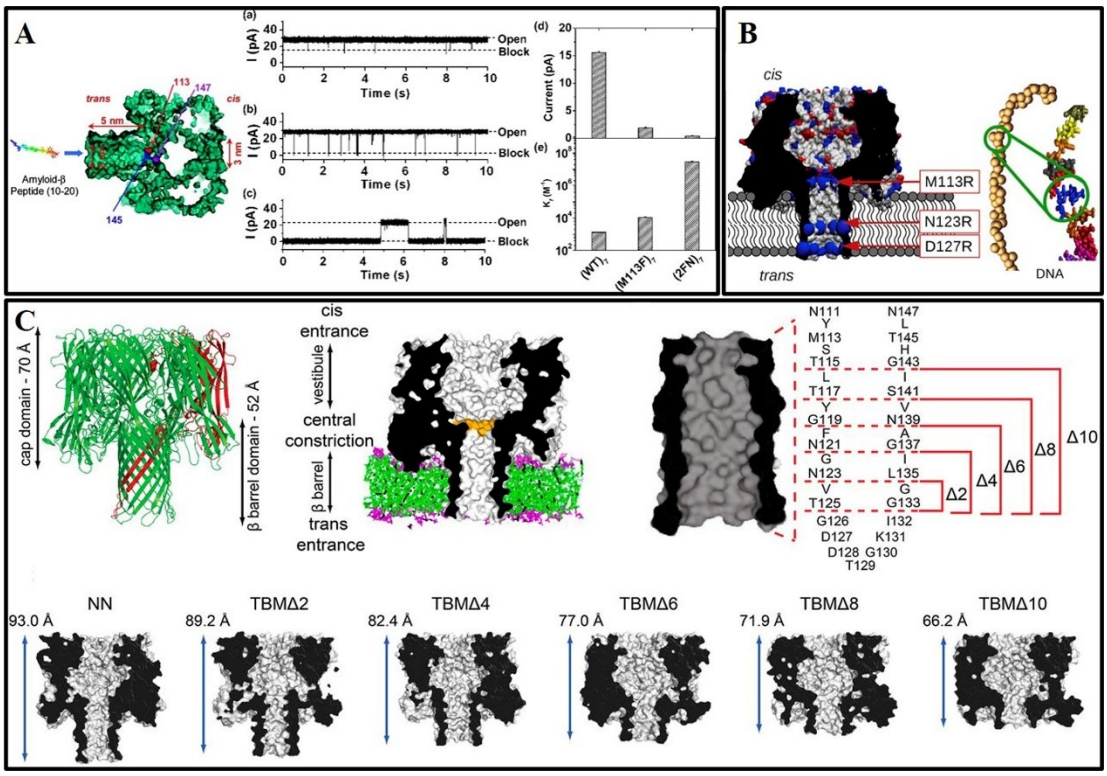

Fig. 2. (A) A model of the wild-type α-HL pore and representative amyloid-β peptide(10−20). The three mutation positions (113, 145, and 147) are highlighted. Current traces showing the structural effect of the pore on the peptide translocation. Figure adapted with permission from Ref. [51] Copyright (2009) American Chemical Society. (B) The α-HL pore mutations with additional positive charged residues in the β-barrel. The blue beads represent positively charged mutations. The red beads are negatively charged, and the gray beads are neutral. The ssDNA is modeled using three beads per nucleotide. Figure adapted with permission from Ref. [54] Copyright (2017) Cell Press. (C) Cartoon and cut-through representations of the WT α-HL pore (PDB ID:7AHL) and truncated barrel mutants shortened by 2, 4, 6, 8, and 10 amino acids. Figure adapted with permission from Ref. [61] Copyright (2014) National Academy of Sciences.

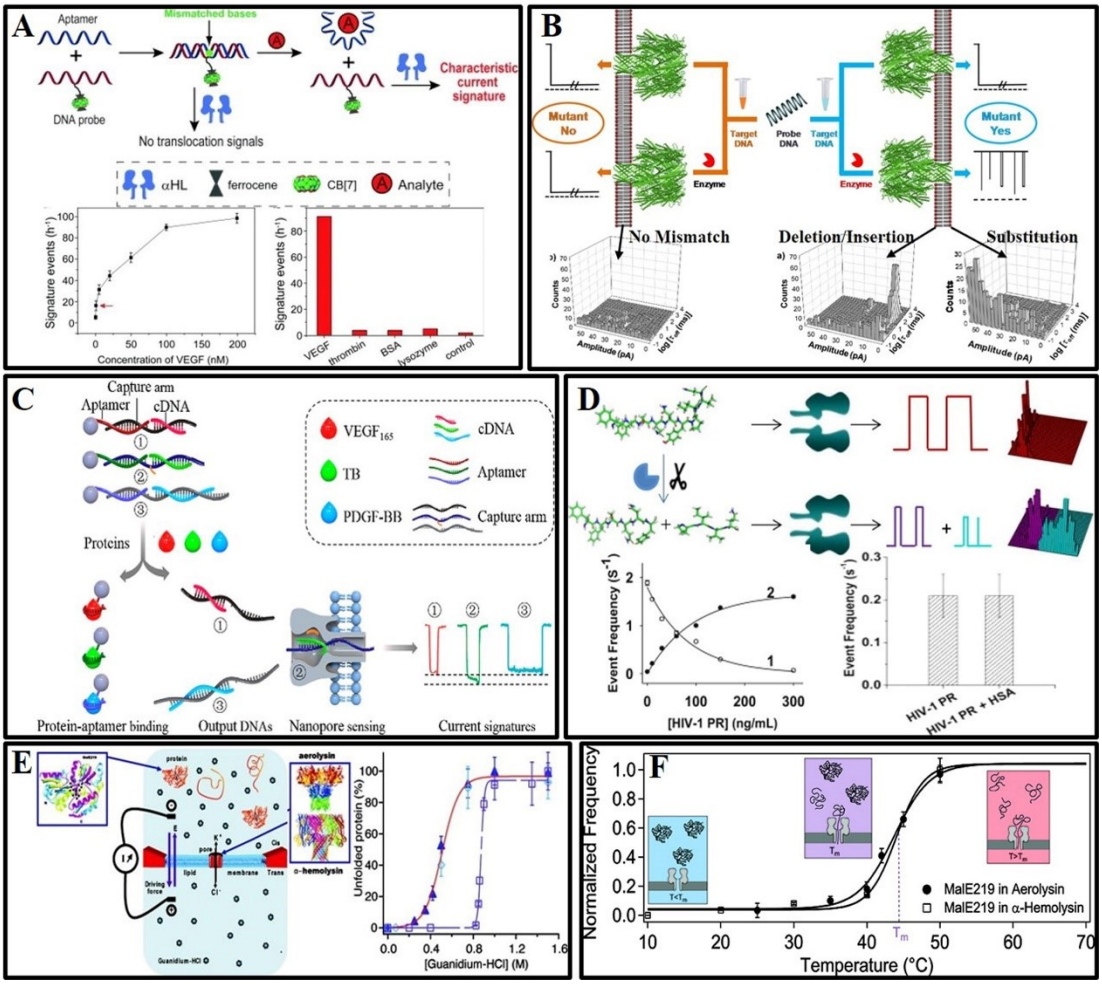

Fig. 3. (A) The sensing strategy for quantitative and selective determination of VEGF121. The icon legends are shown in the dashed rectangle box. Figure adapted with permission from Ref. [25] Copyright (2015) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim (B) Illustration of the nanopore assay used for simultaneous detection of multiple protein biomarkers with a series of dsDNA-based probes. Figure adapted with permission from Ref. [67] Copyright (2020) American Chemical Society. (C) Schematic representation of the nanopore detection of dsDNA mutations (substitution, deletion and insertion) with nuclease. Figure adapted with permission from Ref. [76] Copyright (2018) American Chemical Society. (D) Schematic representation showing the detection of HIV-1 protease in a nanopore sensor through a strategy for enzyme-peptide proteolysis (Top), Plot of event frequency vs. the concentration of HIV-1 PR and interference study with BSA samples (Bottom). Figure adapted with permission from Ref. [83] Copyright (2014) Elsevier B.V. (E) Schematic representation showing the principle of protein conformation detection using the nanopore device and unfolding curves using different nanopores and different proteins. Figure adapted with permission from Ref. [29] Copyright (2012) American Chemical Society. (F) Schematic representation showing thermal unfolding transition curves of MalE219 detected by α-hemolysin and aerolysin nanopores. Figure adapted with permission from Ref. [92] Copyright (2012) American Chemical Society.

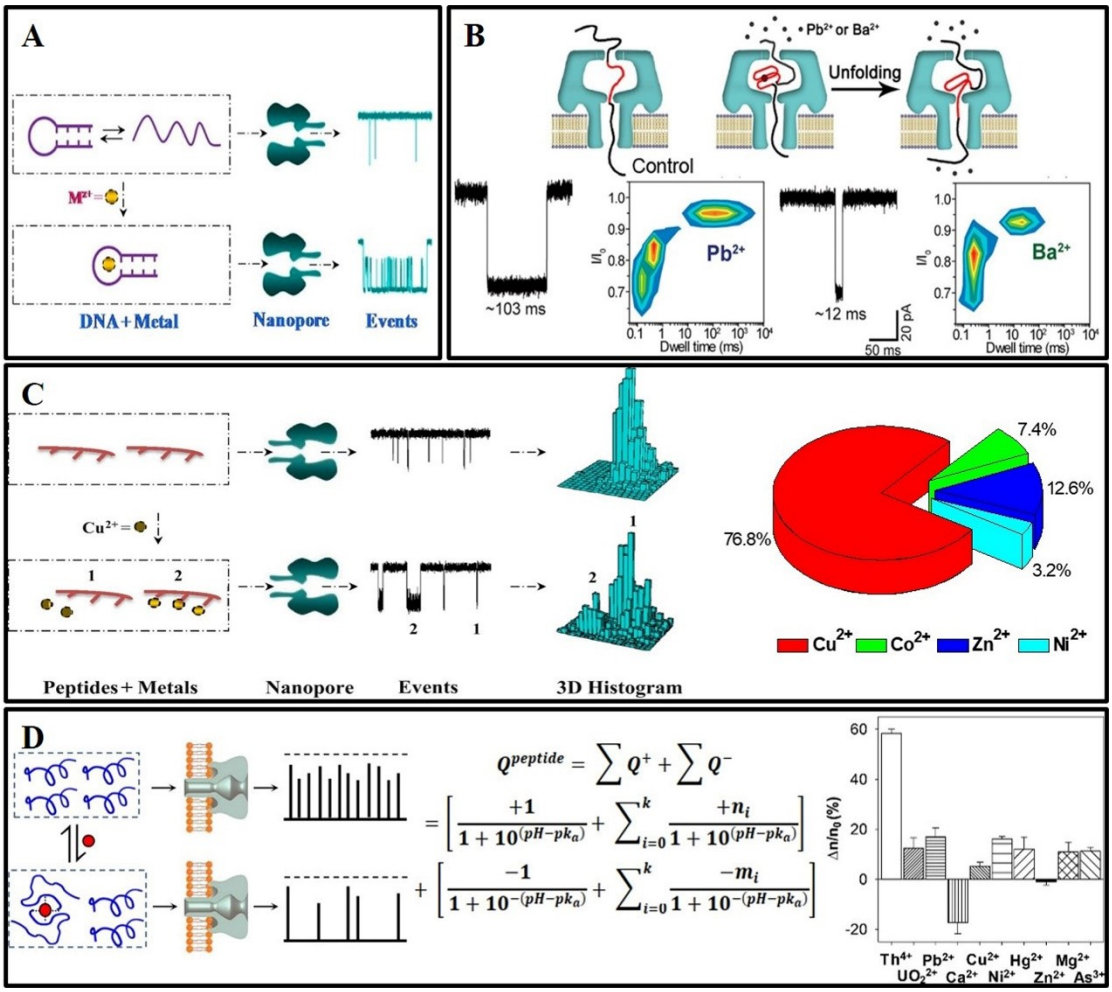

Fig. 4. (A) Specifically designed DNA strands for mercury detection in nanopores. Figure adapted with permission from Ref. [66] Copyright (2013) American Chemical Society. (B) Highly sensitive simultaneous detection of lead (II) and barium (II) with DNA G-quadruplex in α-HL nanopore. Figure adapted with permission from Ref. [93] Copyright (2013) American Chemical Society. (C) Nanopore detection of Cu^{2+}, Zn^{2+}, Ni^{2+} and Co^{2+} ions using a polyhistidine probe. Figure adapted with permission from Ref. [95] Copyright (2014) Elsevier B.V. (D) Computation-assisted nanopore detection of thorium ions with calculation of the charge state of peptide probes and interference study of the Th^{4+} in nanopores. Figure adapted with permission from Ref. [30] Copyright (2018) American Chemical Society.

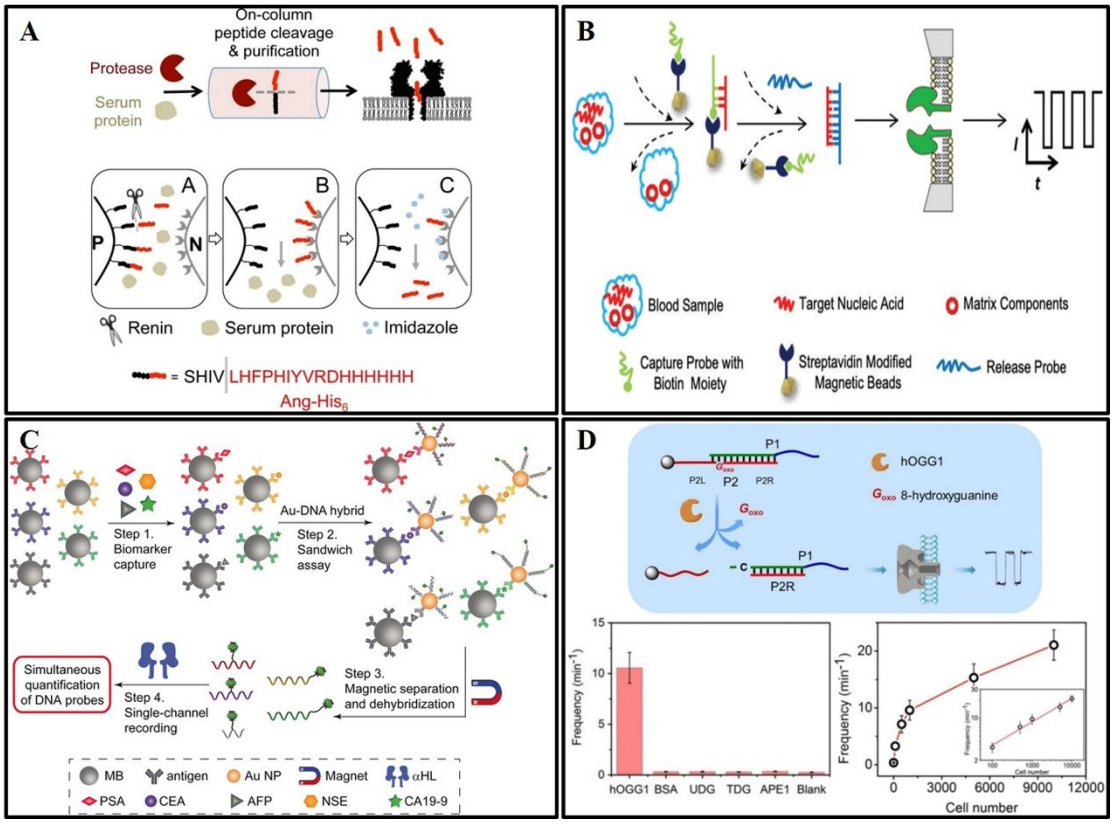

Fig. 5. (A) Scheme illustrates the sensing strategy for the protease renin. Steps A-C are conducted using one spin column containing a mixture of beads. Figure adapted with permission from Ref. [99] Copyright (2015) American Chemical Society. (B) Scheme represents the principle of displacement chemistry-based nanopore analysis of nucleic acids in complicated matrices. Figure adapted with permission from Ref. [101] Copyright (2018) Royal Chemical Society. (C) Schematic sandwich assay for the detection and quantification of multiple cancer biomarkers. Figure adapted with permission from Ref. [105] Copyright (2018) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. (D) Schematic illustration of the nanopore-based assay for the detection of cellular hOGG1 activity. Figure adapted with permission from Ref. [106] Copyright (2018) American Chemical Society.