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Nanopore-based glycan sequencing: state of the art and future prospects

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Sequencing of biomacromolecules is a crucial cornerstone in life sciences. Glycans, one of the fundamental biomolecules, derive their physiological and pathological functions from their structures. Glycan sequencing faces challenges due to its structural complexity and current detection technology limitations. As a highly sensitive sensor, nanopores can directly convert nucleic acid sequence information into electrical signals, spearheading the revolution of third-generation nucleic acid sequencing technologies. However, their potential for deciphering complex glycans remains untapped. Initial attempts demonstrated the significant sensitivity of nanopores in glycan sensing, which provided the theoretical basis and insights for the realization of nanopore-based glycan sequencing. Here, we present three potential technical routes to employ nanopore technology in glycan sequencing for the first time. The three novel technical routes include: strand sequencing, capturing glycan chains as they translocate through nanopores; sequential hydrolysis sequencing, capturing released monosaccharides one by one; splicing sequencing, mapping signals from hydrolyzed glycan fragments to an oligosaccharide database/library. Designing suitable nanopores, enzymes, and motors, and extracting characteristic signals pose major challenges, potentially aided by artificial intelligence. It would be highly desirable to design an all-in-one high-throughput glycan sequencer instrument by integrating a sample processing unit, nanopore array, and signal acquisition system into a microfluidic device. The nanopore sequencer invention calls for intensive multidisciplinary cooperation including electrochemistry, glycochemistry, engineering, materials, enzymology, *etc.* Advancing glycan sequencing will promote the development of basic research and facilitate the discovery of glycan-based drugs and disease markers, fostering progress in glycoscience and even life sciences.

1. Introduction

Glycans represent the most abundant constituent in all life and are composed of multiple monosaccharides linked through glycosidic bonds.[1,2] Glycans are present either in the form of free glycans or glycan-attached glycoconjugates such as glycoproteins,[3] glycolipids,[4] and glycol-RNA,[5] which play essential roles in biological processes involving energy storage, shape regulation, molecular recognition, *etc.*[6] The complex structure of glycans underpins their diverse functions.[7] Therefore, elucidating the structure of glycans is crucial for understanding their physiological and pathological roles, which can accelerate the discovery of glycan-based disease biomarkers[8,9] and new drugs.[10,11] However, glycan sequencing is still challenging due to the intricate heterogeneity of the glycan structure and the limitations of current detection technologies.[12–14]

The primary structure of glycan, comprising the monosaccharide sequence, chain length, glycosidic linkages, anomeric configurations, substituents, and branch,[14] is much more complex compared to that of linear nucleic acids and peptides. Nowadays, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and related hyphenated methods are still the mainstream tools for glycan structure analysis.[15,16] NMR can be used to conclude α/β anomers, linkage position, and even the monosaccharide order, but the analysis will become more challenging for longer glycans.[16–18] MS and MS-based technologies can also elucidate glycan sequences *via* glycosidic dissociation and cross-ring dissociation, but the resolution requires further improvement, especially for glycans consisting of repeating units.[19–22] These limitations urgently require us to develop innovative technologies for efficient glycan sequencing.[12]

Some emerging technologies just like nanopore technology,[23] recognition tunnelling (RT),[24] and glycan

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microarrays[25] have been employed for glycan structure characterization.[13] Among them, the nanopore technology is the most time-saving, portable, and low-cost method with high sensitivity and high spatial and temporal resolution, providing full composition and structural information of analytes at the single-molecule level.[23,26] During the translocation of different analytes through the same nanopore lumen, the different analyte molecules in nanopore confined space induce characteristic ionic current modulation due to volume exclusion and interaction (Fig. 1).[27,28] The specific features of current signals including the blockage amplitude, dwell time, and stand deviation (Std) are extracted to elucidate the analytes' structural information of composition, charge distribution, sequence, *etc.*[29,30] The nanopore technology offers comprehensive single-molecule structural insights, which makes it particularly advantageous for the sequencing of biomolecules (*e.g.*, glycans and proteins) which cannot be amplified *in vitro* like nucleic acids. Over the past few decades, nanopore technology emerged as a successful platform for long-read-length nucleic acid sequencing[31–33] and a promising approach for protein sequencing.[34,35] Given the compatibility of the size and chemical properties of glycans with nanopores, the nanopore technology holds considerable promise for glycan sequencing as well. The increasingly more attempts at nanopore-based glycan sensing confirmed this speculation to a certain extent,[36–45] which provided the theoretical basis and insights for the realization of nanopore-based glycan sequencing.[38,39] However, the applicability of nanopores towards glycan sequencing has not been formally proposed yet.

Here, we give a comprehensive overview of the application of nanopore technology in glycan sensing from the 1990s to the present and present the perspective of nanopore-based glycan sequencing for the first time. Three potential technical routes of nanopore-based glycan sequencing are proposed including strand sequencing, sequential hydrolysis sequencing, and splicing sequencing. Additionally, we outline the anticipated process of nanopore glycan sequencing. Although there will be some challenges on the way to glycan sequencing, we believe that with interdisciplinary collaboration, nanopore-based glycan sequencing will become a reality someday. The advancements in glycan sequencing technology will promote the development of glycoscience and its applications in medicine and beyond.[46]

2. Nanopore-based glycan sensing: from detection to sequencing

The reports about nanopore-based glycan sensors were summarized and classified into three stages. The first stage is

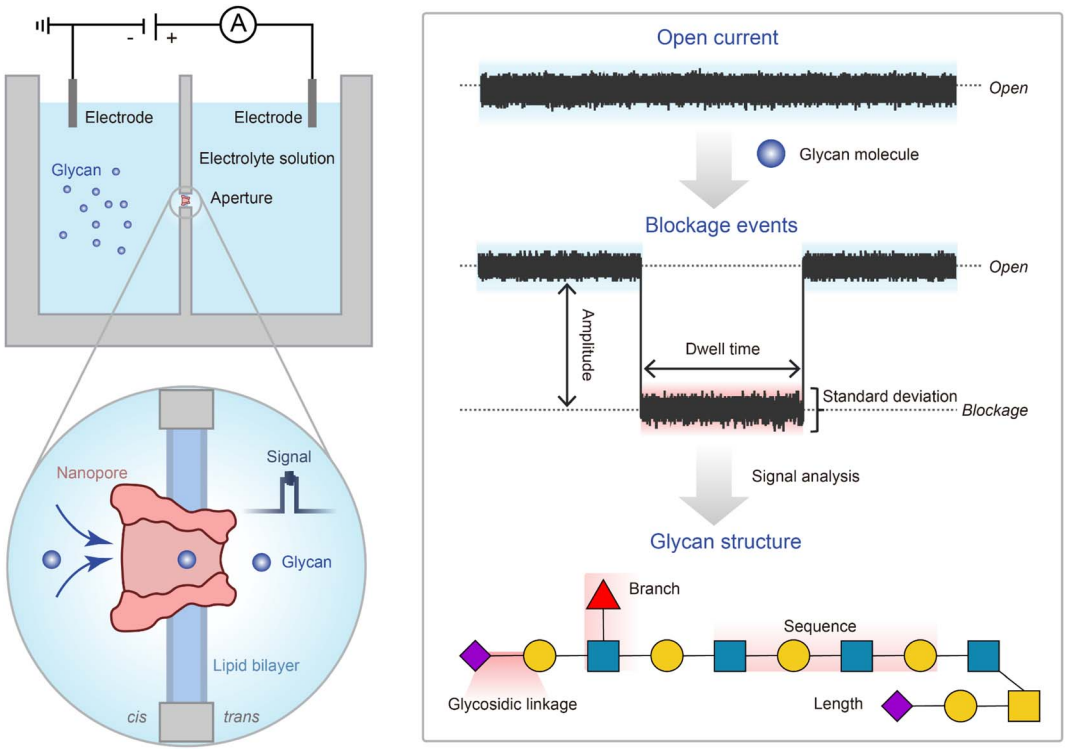

Fig. 1 The principle of nanopore sensing of glycans. (Right) Schematic diagram of commonly used nanopore detection devices and enlarged view of the aperture part. The chamber is divided into two compartments containing electrolyte solution. The two compartments were connected through an aperture on which the lipid bilayer is formed. A constant voltage was applied across the two compartments. The analytes translocate through the single nanopore, inducing characteristic ionic current signals. (Left) The interaction of glycan molecules with the nanopore sensing region induces blockage events based on the nanopore open current. The parameters including the blockage amplitude, dwell time, standard deviation (Std), *etc.* are extracted from the glycan's blockage events to elucidate the full structural information of glycans such as the sequence, glycosidic linkage, branch information, and length.

nanopore discovery since the 1990s when the nanopore was presented as the sensor. In the second stage, from 2010 to day, glycans started to receive attention from the nanopore or channel researchers. Especially starting in 2020, more and more researchers delved into employing nanopores to discriminate glycans with minor structural differences. Lastly, we classify several recent studies proposing the concept of nanopore glycan sequencing and the future development process into the third stage. At the end of the third stage, the goal of nanopore-based glycan sequencing will come true in the future (Fig. 2).

2.1 Nanopore discovery

In general, the natural sources of biological nanopores involve bacterial porins and pore-forming toxin proteins (PFTs).[47–49] The porins refer to the transmembrane proteins located at the outer membrane of Gram-negative bacteria.[48,50] By structurally forming a hollow pore, the porins allow passive diffusion of small biomolecules including oligosaccharides, amino acids, nucleosides, *etc.*[51] According to the specificity, the porins can be classified into general (substrate-nonspecific) porins such as outer membrane protein F (OmpF) and PhoE[52,53] and substrate-specific porins just like the sucrose-specific porin (ScrY).[54] The general porins (<10 Å) non-specifically allow diffusion of solutes with a size lower than 600 Da, while the specific porins have stronger substrate selectivity and the exclusion limit is 200 Da or lower.[48,51] Early in the 1990s, some single-channel studies explored the transport mechanism of glycans *via* porins such as LamB[55] and chitooligosaccharide-uptake porins ChiP

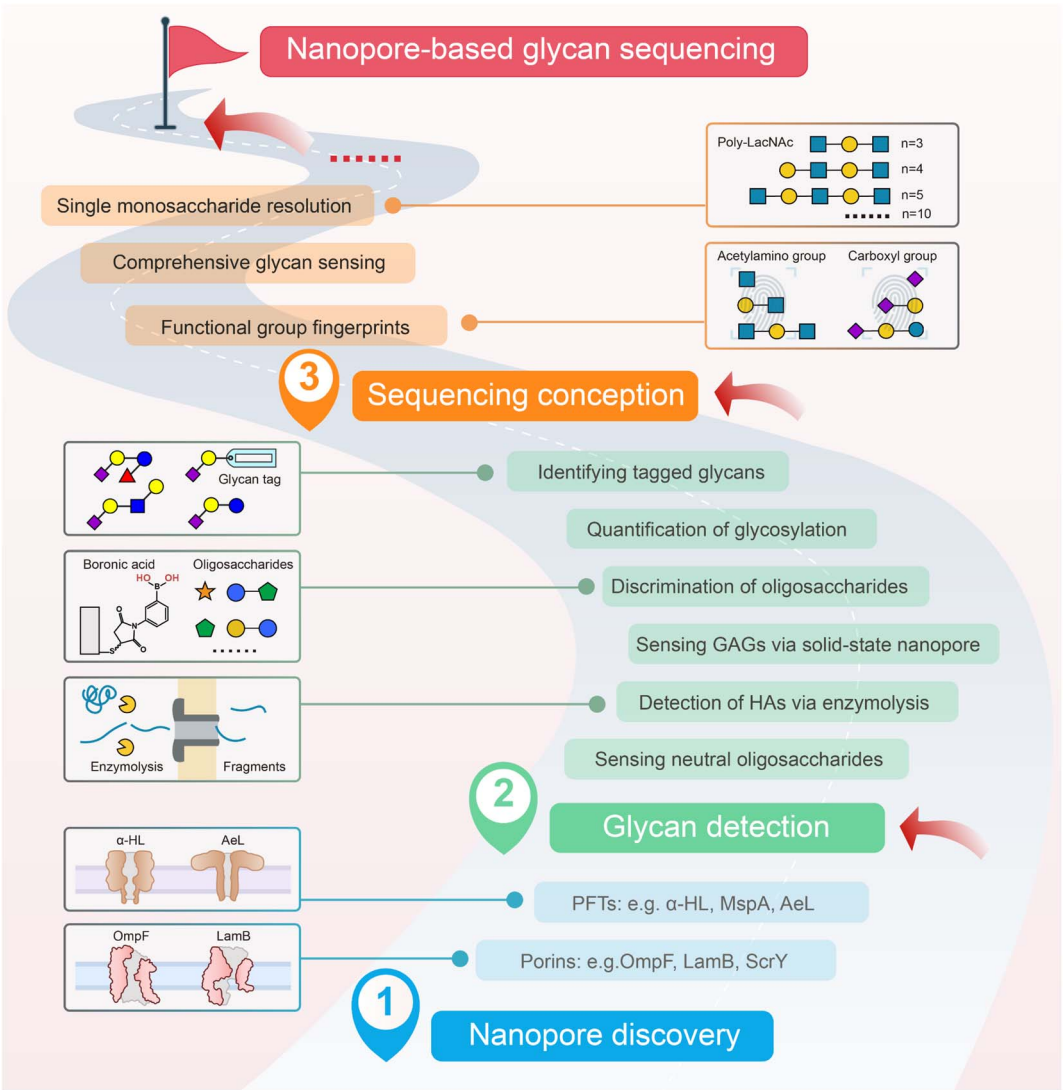

Fig. 2 Development stages of the research on nanopore-based glycan sequencing. This development of nanopore application in glycan analysis was classified into three stages: nanopore discovery, glycan detection, and sequencing conception. The final goal is nanopore-based glycan sequencing. Some representative research studies at each stage were briefly described. The representative reports in seven boxes from the bottom to the top were from ref. 51, ref. 63, ref. 89, ref. 40 and 41, ref. 37, ref. 38, and ref. 39. PFTs, pore-forming toxin proteins. α-HL, α-hemolysin. MspA, *Mycobacterium smegmatis* porin A. AeL, aerolysin. OmpF, outer membrane protein F. LamB, maltoporin. ScrY, sucrose-specific porin. HAs, hyaluronic acids. GAGs, glycosaminoglycans. LacNAc, *N*-acetyl-D-lactosamine.

(chitoporin).[56] Although porins were proposed to be used as the earliest glycan sensors,[57] their low conductance, unavoidable gating signals, strong translocation specificity, and high blockage rate hindered their further application as glycan sensors.[57] By contrast, the general porins OmpF and OmpG with higher signal stability were developed as biosensors[58,59] and possibly applicable for glycan sensing. Nevertheless, the natural transport mechanism of glycan-specific porins seems very enlightening for nanopore design.[56,60] For example, the asymmetric charge distribution within most glycan porins could enhance their sensing resolution.[60] The aromatic residues lining the pore work to assist the glycan translocation with their hydrophobic core.[61] With the help of genetic and protein engineering, the glycan porins may be transformed into satisfactory novel nanopore sensors for glycans.[62]

Compared with the bacterial porins, the nanoscale bacterial pore-forming toxin proteins (PFTs) were developed as widely used nanopores owing to their better perforation activity, stable open state, and strong tolerance of various conditions. Each PFT nanopore consists of several identical subunits that can self-assemble into β-barrel-shaped pores on the phospholipid bilayer,[49,63] with the narrowest region (usually called the constriction site) as the primary sensing region. The adequate diameter with lower substrate selectivity enabled them to be developed as the universal sequencing tools for different biopolymers.[64–69] Since the first heptameric nanopore α-hemolysin (a-HL) from *Staphylococcus aureus* was developed as a single-molecule sensor,[64] more PFTs including *Mycobacterium smegmatis* porin A (MspA),[65,70] aerolysin (AeL),[71,72] fragaceatoxin C (FraC),[73,74] *Escherichia coli* curli transport channel (CsgG),[75,76] *etc.* have been confirmed to allow the detection or/and sequencing of various analytes including peptides, nucleic acids, *etc.*[30,77] For instance, MspA and CsgG have been employed in DNA sequencing because of their narrower sensing region (less than 1 nm) and pore radius (1 nm approximately).[75,78] Although the wild-type nanopores appeared less sensitive to glycans than glycan-specific porins,[48,79] their structure and properties have been elucidated more sufficiently, which will guide us to optimize their performance in glycan sensing.[80]

Besides the above easily accessible and widely used PFT nanopores, it is also possible to obtain novel glycan-matched nanopores through *de novo* design,[81] porin screening or engineering,[82] and shape-tunable nanopores.[83] Beyond biological nanopores, artificial nanopores including solid-state nanopores,[2] nanopipettes,[84] and chemosynthetic membrane channels,[85] are also optional glycan sensing tools. However, site-directed modification on solid-state nanopores is not as convenient as that on biological nanopores. The surface coatings could improve their sensitivity and control the pore diameter.[86] Compared with biological nanopores, most artificial nanopores are quite stable for long-term sequencing, but the fabrication of artificial nanopores with high-precision size is still challenging.[2] A hybrid nanopore, obtained by inserting a single protein nanopore into a solid-state nanopore, shows better robustness while retaining high sensitivity.[87,88] The hybrid nanopore array could be created for high-throughput sequencing.[28]

2.2 Sensing strategies for glycan detection

In the 2010s, some researchers began to explore the capability of nanopores to detect glycans. The feasibility was preliminarily confirmed in 2011 by the attempt to discriminate maltose and dextran oligosaccharides with different glycosidic linkages and polymerization degrees according to the differentiated dwell time.[43] In the subsequent years, more and more researchers further explored the feasibility of glycan detection using nanopores. Some ingenious nanopore-based glycan sensing strategies have been devised such as site-directed mutation of nanopores,[38] combination of enzymatic hydrolysis and nanopore sensing,[89,90] capture of glycan molecules by boronic acid covalently bound to nanopores,[40–42] assistance of glycan binding proteins (GBPs),[91,92] surface modification of solid-state nanopores,[45,93–95] and chemical tags of glycans.[37] These approaches aimed to enhance sensitivity and resolution in glycan detection and discrimination. Various types of glycan structural differences were successfully identified using engineered or unmodified nanopores, including diverse building blocks,[38,41,42] distinct glycosidic linkages,[37,39,40] varying lengths,[37–39] and branches.[37] It should be noticed that different strategies could be challenging when applied to different model glycans. For example, boronic acid-bound nanopores expressed high sensitivity in discriminating both monosaccharides and disaccharides.[40,41,96] But they may face challenges in identifying large-sized glycans due to blurred event clusters. Comparatively, wild-type (WT) aerolysin nanopores were utilized to characterize glycosaminoglycan oligosaccharides with various sulfate patterns, glycosidic bonds, and epimers of uronic acid residues, which marked the success of nanopores in comprehensive elucidation of longer charged glycans,[36] although the sensitivity seems not as high as some engineered nanopores. Besides, the analysis ability of solid-state nanopores is also examined on plant polysaccharides[45] and glycosaminoglycans (GAGs),[94,95] but the resolution needs further improvement. Anyway, these meaningful efforts advanced the application of nanopores in glycan detection and provided vitally important insights for future research on nanopore glycan sensing. Beyond these reports related to nanopore glycan sensing, since nanopores are currently most widely used in nucleic acids and peptides, many sensing strategies and experiences such as analyte carriers[97] and host–guest interaction[69] can be learned. Meanwhile, developing glycan-specific sensing strategies remains a crucial task.

2.3 Step towards glycan sequencing

Following glycan detection, our ultimate goal is to achieve nanopore-based glycan sequencing. Glycan sequencing required comprehensive and accurate elucidation of the monosaccharide sequence, anomeric carbons' configuration, glycosidic linkages, branches (position, sequence, and modification pattern), and substituents, demanding the further development of a powerful nanopore platform. Numerous challenges remain to be addressed before achieving glycan sequencing: recognition of the functional group substituent, achieving single monosaccharide resolution on the glycan chain, discrimination of successive addition of single

monosaccharides, *etc.* Previous research demonstrated that the fingerprints of glycan functional groups can be established by nanopores.[38] The glycan fingerprints were utilized for the identification of glycans with different functional groups and varying lengths. In this work, we presented the conception of nanopore-based glycan sequencing for the first time.[38] Subsequently, we screened out a more versatile engineered nanopore for comprehensive identification of the glycan sequence, isomers, and length with a more direct and rapid procedure. The highly sensitive nanopore achieved single monosaccharide resolution on the glycan chain. The resolution of the single building block was considered a necessity for nanopore-based biopolymer sequencing.[98–101] The nanopore also allowed the accurate discrimination of the chain length from pentasaccharides to decasaccharides, which has never been reported and implied the ability to sense the glycan chain. We also discriminated glycan isomers at the tetrasaccharide level.[39] These published studies not only confirmed the comprehensive sensing ability of nanopores for larger-sized glycans but also marked the first step on the way toward future nanopore-based glycan sequencing.

All of the above significant efforts provided the theoretical basis and insights for the realization of nanopore-based glycan sequencing and boosted the progress toward the promising final goal, although it must be admitted that there is still a long way to go. The next task is to design highly general and applicable nanopore sequencing routes that can achieve the determination of unknown glycan sequences, laying the foundation for the ultimate nanopore glycan sequencing machine (glycan sequencer).

3. Technical routes of nanopore-based glycan sequencing

To achieve nanopore-based glycan sequencing, three technique routes were proposed: strand sequencing, sequential hydrolysis sequencing, and splicing sequencing (Fig. 3). We have presented the basic sequencing principles, the key points, and the application scope of each sequencing scheme, respectively.

3.1 Strand sequencing

Nowadays, based on the strand sequencing strategy, Oxford Nanopore Technology (ONT) has achieved long-read DNA sequencing.[28,102,103] Moreover, a few researchers have confirmed the possibility of peptide strand sequencing using nanopores by conjugating the peptide with an oligonucleotide recently.[34,104,105] Similarly, nanopore technology may be also applicable in glycan strand sequencing. The basic principle of glycan strand sequencing is that when the glycan chain translocates through the nanopore, the monosaccharides on the glycan interact with the sensing region in order, which will be reflected in the 'step-by-step' ionic current alteration, each alteration corresponding to an individual monosaccharide if the resolution is high enough, so the sequence information of the glycan can be elucidated manually or automatically with the assistance of machine learning. Theoretically, this strategy can be utilized for sequencing any chained glycan especially when the glycan chain itself is highly charged to make itself fully stretched, such as glycosaminoglycans (GAGs) which are the major linear glycans and participate in various biochemical processes.[106] For neutral glycans with flexible structures, it could be helpful to introduce charged groups to stretch them out. The branched glycans may not fit strand sequencing route as their larger size makes the entrance into the nanopore and translocation difficult.

Several issues in glycan strand sequencing need to be taken into consideration to improve the resolution, many of which are based on the experience in DNA/peptide strand sequencing. First, the interactions between many natural glycans and the nanopore lumen are not strong enough to induce significant signals. The solutions include site-directed mutation based on the amino acid properties inside the nanopore, and chemical modification of the nanopore or the analytes (covalent or non-covalent, including aptamers, chemical tags, *etc.*).[37,99,107–109] Additionally, the dwell time of the analytes in the pore needs to be controlled to make sure that the analytes can interact with the nanopore sufficiently so that the resolution can be improved. Narrowing the translocation path by mutation/modification of the nanopore or the glycan might also work in this issue. The radius of solid-state nanopores can be controlled to the sub-nanometer level, which may be comparable to the size of glycan molecules, but enhancing the interaction needs to be considered carefully.[2] Besides, using a motor protein to control the movement of the glycan molecule is another idea.[110] The development of gene and protein engineering may provide insights into the design of a glycan motor protein. Finally, unlike nucleic acids, many glycans are neutral, without high charge density to induce electrostatic repulsion so they tend to form secondary structures while translocating through the nanopore, which would bring great challenges to nanopore-based glycan sequencing. Modifying the glycans with charged groups might be necessary to keep the glycan chain as straight as possible. In theory, the strand sequencing strategy could be applicable to any chained glycan if the problems above are solved.

3.2 Sequential hydrolysis sequencing

Nanopore-based sequential hydrolysis sequencing was initially proposed for nucleic acid sequencing and used to achieve continuous identification of nucleotides cleaved from ssDNA using an exonuclease.[109] Recently, Zhang *et al.* presented a proof-of-concept demonstration of peptide hydrolysis sequencing using a nanopore with the help of carboxypeptidases.[69] This breakthrough confirmed the feasibility of sequential hydrolysis sequencing in biopolymers. There were also various exoglycosidases (EXGases) functioning to cleave external glycosidic bonds and remove terminal monosaccharides from the glycan non-reducing end, which made glycan sequential hydrolysis sequencing also feasible theoretically. To date, some attempts have been made to identify glycans using EXGases combined with classic detection methods such as MS.[111,112] Presently, we proposed the technical

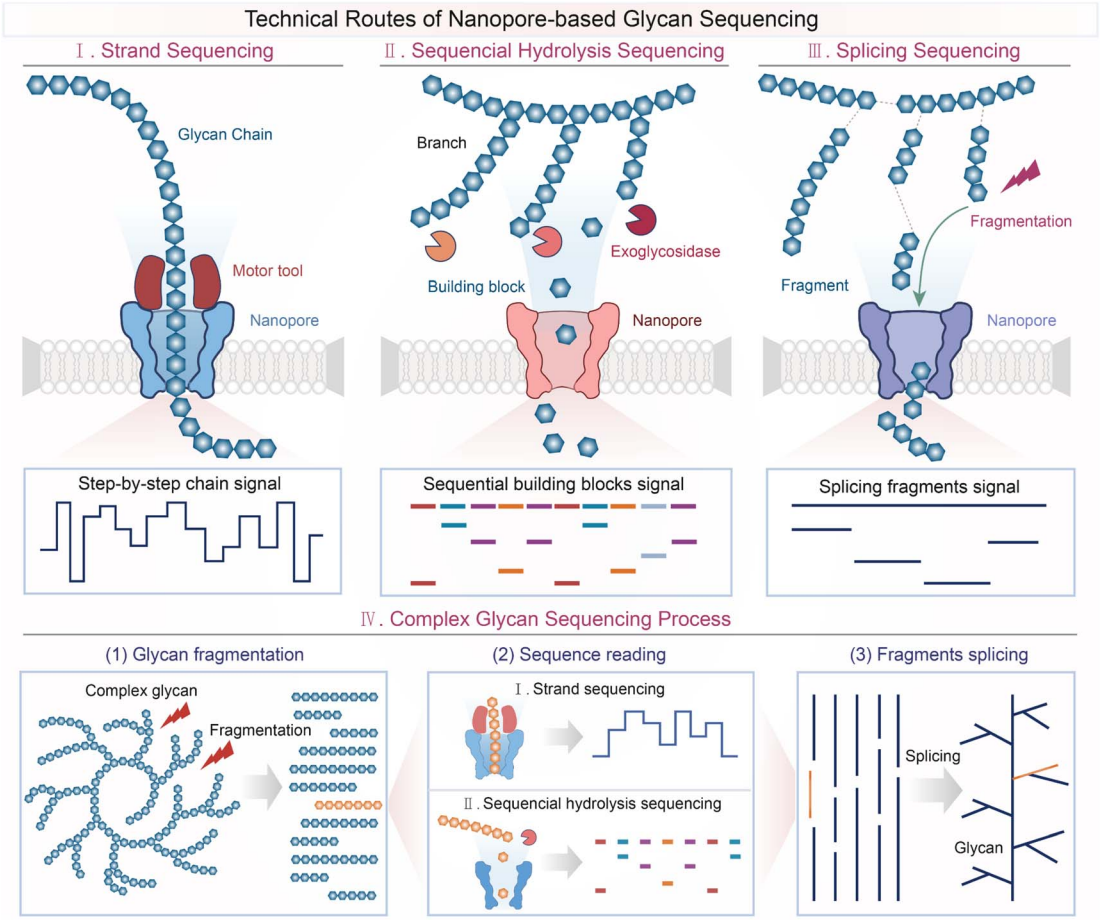

Fig. 3 Technical route of nanopore-based glycan sequencing. (I) Strand sequencing. When the glycan chain translocates through the nanopore with the help of a motor, the glycan building blocks interact with the sensing region in order and induce 'step-by-step' ionic current signals. (II) Sequential hydrolysis sequencing. Sequential hydrolysis of glycans using exoglycosidases causes glycan building blocks to be released and captured by the nanopore and induced monosaccharide signals sequentially. (III) Splicing sequencing. The glycan molecules are broken into shorter fragments which are directly detected by the nanopore and identified by mapping the characteristic signals to the pre-created library of glycan signals. The complete glycan sequence could be obtained by splicing these glycan fragments. (IV) Complex glycan sequencing process. After the glycan molecules are broken incompletely, the fragments are sequenced by the strand sequencing or sequential sequencing method. The full glycan sequence was obtained by splicing the fragment sequence.

route of nanopore-based glycan sequential hydrolysis sequencing. Sequential hydrolysis of glycans using EXGases causes monosaccharides on glycans to be released and captured by the nanopore and induces real-time translocation signals sequentially. The order of distinct translocation signals corresponds to the sequence of monosaccharides while the EXGases specificity corresponds to the stereochemistry information (*e.g.*, conformation and configuration of the glycosidic bond). Compared with strand sequencing, the advantages of sequential hydrolysis sequencing are mainly reflected in smaller-sized and branched oligosaccharides containing limited types of glycosidic linkages. For example, N-glycan and O-glycan which play essential roles in numerous biological processes and participate in immune regulation, signal transduction, pathogenic infections, *etc.*,[113,114] could be sequenced through sequential hydrolysis sequencing.

Likewise, there are a few key points in this technical route. To begin with, the temperature and substrate concentration should be adequate so that the hydrolysis rate can match the nanopore capture rate to ensure that the order of monosaccharide signals corresponds to the glycan sequence exactly. By manipulating the active site and activity of the exonuclease through enzyme engineering and fusion expression or chemical attachment of the exonuclease to the entrance of the nanopore, it is expected that the released monosaccharides will enter the pore at a suitable trajectory and speed. If the capture rate is lower than the hydrolysis rate, it is better to perform the enzymatic hydrolysis first and then enrich the monosaccharide products for nanopore detection. In this way, the abundance of monosaccharide-specific translocation signals could be analyzed to reflect the glycan sequence information. In addition, as mentioned above, the stereochemistry information of the glycan is concluded based on the specificity of the EXGases, so EXGases with high specificity are needed to ensure accurate sequencing. Nevertheless, the species and specificity of EXGases are quite limited, which could be solved by the collaboration of enzyme engineering and computer-aided

design. Lastly, sequential hydrolysis of one glycan molecule should synchronize with the others to make sure that the hydrolysis product is relatively homogeneous so that the translocation signal can fit the monosaccharide specificity well. Adjusting the hydrolysis time according to signal quality might be helpful.[101] Considering the difficulty of discrimination of all the monosaccharides,[14] the sequential hydrolysis sequencing routes could be initially applied to a series of glycans such as a mammalian glycan which consists of only nine monosaccharides.[115]

3.3 Splicing sequencing

Splicing sequencing is a classic sequencing strategy in DNA sequencing, which has been used as early as the 1990s when Human Genome Project (HGP) was ongoing and was defined as short-gun sequencing.[116,117] At present, this strategy is utilized in almost all whole genome sequencing.[118,119] The basic principle of splicing sequencing is to obtain the complete sequence of biopolymers by splicing their fragment sequence. The molecular masses of some polysaccharide polymers can reach up to 1000 kDa.[115] For glycans with multiple branches or/and extremely large size, it is challenging to sequence them directly with strand sequencing or sequential hydrolysis sequencing due to some limitations (*e.g.*, limited read length in strand sequencing and low specificity of glycosidases in hydrolysis sequencing). Splicing sequencing methods offer a solution to sequencing these large-sized glycan molecules.

In the direct glycan splicing sequencing we defined here, first, the glycan molecules were broken into shorter fragments. The glycan fragmentation methods involve acid hydrolysis, enzymatic hydrolysis (endoglycosidases (ENGases)), ultrasonic treatment, and microwave radiation, and their mechanism and characteristics were reviewed by Chen *et al.*[120] Unlike EXGases in sequential hydrolysis sequencing, the fragmentation induced by these methods occurs inside the glycan molecules rather than at the ends. Next, the fragments will be directly sensed by the nanopore and identified *via* mapping the characteristic signals to the pre-created library of glycan signals. Finally, we could obtain the complete glycan sequence by splicing these glycan fragments. This splicing sequencing method will be relatively easier to implement when the fragments are small enough because the library of smaller oligosaccharides is relatively more accessible. However, building a database of larger fragments or fragments consisting of diverse building blocks would be a tricky business. Therefore, the direct splicing sequencing method is unpractical for glycans which cannot be completely fragmented using existing hydrolysis methods or containing diverse monosaccharides. Considering the unimaginable workload of building the database due to the diverse glycan building blocks, the splicing sequencing methods can be firstly applied to some specific important glycans through signal profiling.[121,122]

Inspired by DNA and protein sequencing,[123,124] the future nanopore-based glycan sequence process would combine several sequencing methods or strategies to cope with the complexity and diversity of glycan structures. The strand sequencing method or sequential sequencing method can be combined with the splicing strategy to achieve sequencing of complex glycans which cannot be elucidated by the direct splicing sequencing method as mentioned above. Specifically speaking, after the glycan molecules are broken incompletely, the fragments could be sequenced by the strand sequencing or sequential sequencing method. Notably, as described above, strand sequencing and sequential sequencing methods are suitable for sequencing distinct types of glycan. At an early stage, combining other technologies such as mass spectrometry (MS) could be helpful for the evaluation and control of glycan fragment size, to determine the applicability of strand sequencing and sequential hydrolysis sequencing. In the future, it is necessary to develop hydrolysis methods and protocols for sample preparation, which could generate glycan fragments suitable for different nanopore sequencing routes. In this way, the full glycan sequence can be obtained by splicing the fragment sequence assisted by bioinformatics tools. This sequencing process will reduce the difficulty of sequencing complex glycans by avoiding the difficulty in chain sequencing caused by branches, the trouble in hydrolysis sequencing caused by long chains, and the adversity in fragment library construction caused by high complexity. Up to this point, we presented three possible methods and proposed a possible sequencing process of glycan sequencing.

Each method has its most suitable type of glycan structure, which depends on their respective characteristics. But they share some common challenges as well. First, the resolution and sensitivity of nanopore sensing to glycans especially neutral glycans need further improvement. It will be solved by nanopore engineering methods, which include site-directed mutation,[38] genetic code expansion (GCE),[125] glycan recognition module grafting,[126,127] integrated adaptors,[128] glycan derivatization including tags with different sizes or properties,[37] reversible host–guest interactions,[129] *etc.*[130] The future novel sensors derived from the *de novo* protein design[81] and glycan porins/transporters will be more glycan-matched.[62] Modification with glycan binding proteins (GBPs) such as lectins and antibodies would enable solid-state nanopores to detect glycans with higher sensitivity.[91,92,131,132] Hybrid nanopores can be designed and fabricated as nanopore arrays for rapid, high-throughput glycan sequencing.[87] Besides, optimization of detection conditions including temperature, applied potentials, and pH values is also helpful to provide additional discrimination capability. Second, the presence of diverse modifications (*e.g.*, sulfation, acetylation, *etc.*), which could have different impacts on the interactions between the analytes and the pore, needs particular attention.[38,133,134] In the future, we will attempt to design a suitable nanopore or even a nanopores platform for different modifications to guarantee a higher accuracy. Third, no matter which sequencing method we use, it is an almost inescapable task to build a huge glycan structure-translocation signal database. The small-sized branched glycan signals are necessary to be concluded in the signal library for attribution of glycan fragment signals to the side or main chain, unless other analysis tools (MS or scanning tunnelling microscopy (STM)) are combined to obtain branch information. In view of the complexity of the sample and the hugeness of the database, processing the sequencing data needs the assistance of artificial

intelligence and multi-team collaboration.[135] Unification of data standards is necessary for convenient data sharing. Last but not least, the low availability of high-purity glycans impedes the sequencing method training, so developing highly efficient synthesis methods such as automatic glycan synthesis[136] and purification methods of natural or synthesized glycans[137,138] is urgently needed. With developments in related methods, we believe there will be some excellent proof-of-concept studies of nanopore glycan sequencing based on these technical routes or possibly other ingenious designs in the near future.

4. Outlook and future prospects

4.1 Glycan sequencer instrument

Compared with classic analysis technology NMR and MS, nanopore sensors are more easily miniaturized and integrated into portable devices.[139,140] The incorporation of complementary metal-oxide-semiconductor (CMOS)-integrated electronics with high-integration microfluidic systems offers an opportunity for parallel and high-throughput nanopore glycan sequencing.[140] It is possible to integrate the sample processing unit and nanopore array (including the necessary molecules, *e.g.*, enzymes) with the current signal detecting and processing unit to assemble a portable glycan sequencing machine. As this field is still in its early stages, massive obstacles are waiting to be overcome. The methods surrounding the nanopore-based glycan sequencer are presented in Fig. 4. The essential nanopore sensors, motor protein or highly specific EXGases, model glycans, sequencing algorithm, and instruments need to be either redesigned or improved. Despite the advantages of nanopore-based glycan sequencing, it must be admitted that MS and NMR have their unique advantages, *e.g.*, MS can be used

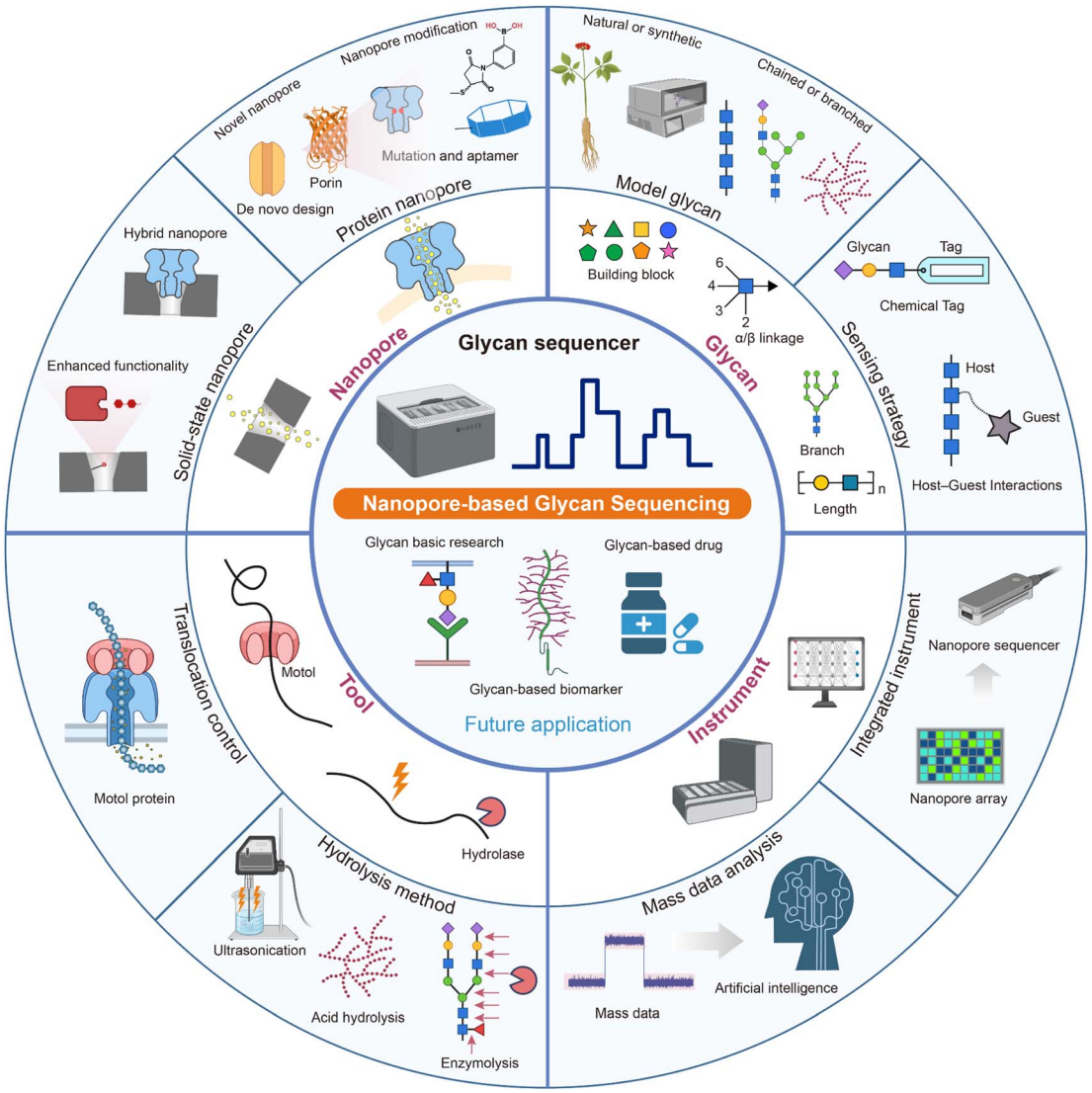

Fig. 4 Methods surrounding biological nanopore technologies for glycan sequencing and possible future applications. Four potential components in the development of nanopore glycan sequencing include nanopores, sequencing tools, glycans, and instruments. The peripheral methods of each of the four components have the potential to contribute to the realization of glycan sequencing. Created with https://www.biorender.com.

for the assessment and control of glycan size and help identify branches' positions with higher efficiency. Therefore, the combination of nanopores with other classic analysis methods (MS and NMR) or even optical tools[141–143] will help to fully resolve the full structure of complex glycans. To sum up, the nanopore sequencer invention calls for interdisciplinary collaborations including chemistry, glycochemistry, engineering, materials, enzymology, *etc.*

4.2 Future applications

4.2.1 Basic research. Glycans are widely distributed on cell surfaces and in extracellular matrices and play a vital role in cellular interactions[14] and mediate various biological processes. The biological functions of glycans were mainly determined by their complex structure.[6,14] A thorough understanding of the glycan structures helps to correlate them to their respective function. The advent of nanopore technology will offer a method that promises fast, accurate, and comprehensive analysis of glycan sequences and stands to revolutionize glycomics profiling.[144] Therefore, nanopore-based glycan sequencing will boost the basic research on the structure–activity relationship of glycans. This will help us understand how the glycans act with their receptors and shape biological processes.

4.2.2 Applied research

4.2.2.1 Disease diagnosis kit. The interactions between glycans and proteins are implicated in the development of diverse diseases, such as pathogen infection,[145] inflammation,[146] and cancer.[9] Therefore, glycans hold considerable promise as a disease marker.[147,148] On the one hand, through nanopore glycan sequencing, various undefined physiological and pathological processes related to glycans will be elucidated more comprehensively, which will promote the discovery of some reliable disease markers. On the other hand, the glycan-related disease biomarkers provide an application scenario for nanopore-based glycan sequencing. Nanopore-based glycan sequencing methods might be developed as a diagnosis tool to analyze specific glycan biomarkers in body fluid samples directly.[149] With the development of methods of sample handling, nanopore sequencing, and signal processing, a fast, disease diagnosis kit might be developed, which will improve the convenience and compliance of the diagnosis for both the doctors and the patients.

4.2.2.2 Glycan-based drugs. Glycans have great promise in drug discovery.[10] The glycan-based drugs and therapeutics represent a great market.[150] The approved drugs include polysaccharides/oligosaccharides, glycomimetics, small molecule glycosides, glycoproteins, glycopeptides, and glycan-based vaccines.[11,151,152] On the one hand, nanopore-based glycan sequencing could provide access to well-defined glycans (endogenous or exogenous), which will make the design of glycan-based drugs more rational and less risky. On the other hand, even subtle differences in glycan molecular weight, monosaccharide compositions, and glycosidic linkages could affect their biological activities.[152] Heparin, one of the most successful anticoagulant drugs, has drawn global attention due to the heparin contamination crisis in 2007, which suggested the importance of precise glycan structure elucidation for the quality and safety of glycan-based drugs.[153] Actually, quality analysis of heparin and other drugs using nanopores has been reported in recent years,[36,154] which indicates its potential in drug quality control. The nanopore-based glycan sequencer will be widely applied to the quality control of glycan-based drugs.

4.2.3 Cost-effectiveness of nanopore-based glycan sequencing. Generally speaking, the cost of nanopore-based glycan sequencing mainly arises from three aspects: device, consumable and data analysis. As the sequencing device may be technologically immature, its price would be high in the initial stage.[28,155] Besides, pre-processing of the sample needs specific reagents, and detection of the sample is completed on a disposable chip, which cannot be avoided as long as we use this method. Moreover, data analysis would require a lot of computing power and quite a long time due to the complexity of glycan sequences. But we believe that the overall cost will be reduced with the improvement of the manufacturing and related techniques. Compared to the cost, the effectiveness involves many more aspects including all the upstream and downstream markets. For instance, the wide use of nanopore-based glycan sequencers will expand the scale of manufacturing of related devices (processing, assembly, and quality control from raw materials to terminal devices). Meanwhile, this method will promote the development of both basic and applied research, including glycomics analysis (related to identification of glycan biological roles and disease biomarkers),[147] disease diagnosis,[149,156] drug discovery,[11,157] microorganism detection,[158,159] environmental pollutant control,[160] *etc.* Though nanopore-based glycan sequencing is faced with many challenges in its early stage, we believe that the development of this area could promote the progress of various disciplines and industries.

5. Conclusion

Glycoscience, a pivotal and burgeoning field, intricately intertwines with all branches of biology.[46] However, challenges in glycan sequencing impede the development progress of glycoscience. Recently, significant progress has been made in nanopore-based glycan sensing, shedding light on the feasibility of nanopore-based glycan sequencing. We presented the conception of nanopore-based glycan sequencing in our recent work for the first time.[38] In this perspective, we summarized the major advances in nanopore-based glycan analysis. These meaningful reports were manually classified into three stages: nanopore discovery, glycan detection, and sequencing conception. These efforts paved the way to the ultimate goal of nanopore-based glycan sequencing, although there is still a long way to go. Based on the characteristics of the glycan structure and the nanopore sequencing technology, we presented three potential glycan sequencing technical routes and a complex glycan sequencing process. To simplify the sequencing process, we proposed the idea of integrating all the necessary units into a glycan sequencing machine (glycan sequencer). Undoubtedly, the invention of a glycan sequencer

will meet different hurdles and require multidisciplinary collaborations. We provided some strategies to improve the sensitivity and resolution of nanopores for glycan sensing, which could provide some insights for overcoming the technical barriers in nanopore-based glycan sequencing. Finally, an outlook was given on the future application of nanopore-based glycan sequencing. We believe that the advancement of glycan sequencing would facilitate the development of glycoscience and even the entire life sciences.

Author contributions

Z. Gao and B. Xia provided the key advice and supervised the preparation of the manuscript. G. Yao and W. Ke conducted literature search and analysis of published results. All the authors wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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