

Nanopore Signatures of High-Molecular-Weight Polysaccharides with Recurring Structural Motifs

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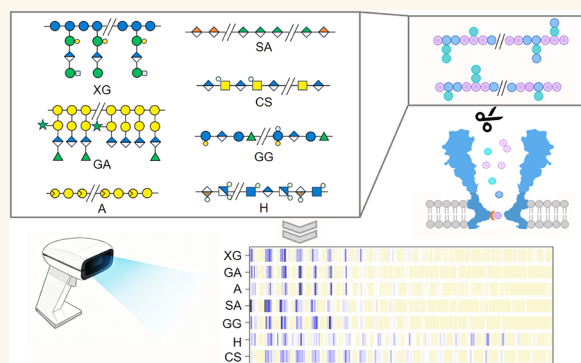
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ABSTRACT: Polysaccharides in nature possess profound biological significance, and their structural complexity renders them of great research value. However, the structural heterogeneity of complex polysaccharides has long posed a great challenge for analysis. Here, a nanopore profiling strategy was developed for polysaccharide characterization. Experimentally, polysaccharides are hydrolyzed into informative fragments, and the distribution of their resulting nanopore events forms a unique profile that serves as a reliable molecular signature. Seven distinct polysaccharides, including xanthan gum, gum arabic, agarose, sodium alginate, gellan gum, heparin, and chondroitin sulfate A, were successfully identified and discriminated. A profile-based machine learning classifier was also developed, which utilizes the overall distribution profile of fragment events as training input, yielding 100% validation accuracy. Further application of the fully constrained least-squares (FCLS) algorithm enabled accurate quantification of polysaccharide mixture composition, allowing for unambiguous analysis of mixtures of polysaccharides in the same assay. Collectively, this work establishes a universal, label-free, and high-resolution framework for decoding the structural complexity of polysaccharides.

KEYWORDS: nanopore, polysaccharides, nanopore glycan profiling, nanopore glycan sequencing, *Mycobacterium smegmatis* porin A



INTRODUCTION

Polysaccharides represent a class of long-chain polymers composed of multiple distinct monosaccharide residues, which are ubiquitously distributed in nature.¹ Owing to their unique physicochemical characteristics, including favorable rheological behavior, thermal stability, and gel-forming properties, alongside diverse bioactive functionalities, these polysaccharides have garnered considerable research attention in fields such as biomedicine,^{2,3} materials science,^{4,5} and food technology.^{6,7} However, the structural characterization and purity evaluation of polysaccharides continue to present substantial analytical challenges, thereby impeding progress in both fundamental scientific investigations and industrial quality control processes.⁸ These difficulties primarily stem from their inherently complex structural features: diverse monosaccharide compositions, intricate linkage patterns, varied functional group substitutions, extensive variations in molecular architectures (from linear to highly branched),^{9,10} and distinct charge properties.^{11,12} Furthermore, polysaccharides derived from plants, animals, and microorganisms typically exhibit high molecular weights, often exceeding several million daltons.¹² The combined effects of such structural heterogeneity, ultrahigh molecular weight, and

broad polydispersity collectively render the comprehensive analysis of polysaccharides significantly challenging.¹³

At present, mass spectrometry (MS) is the primary technique for polysaccharide analysis, yet its application still faces fundamental constraints. Native polysaccharide typically require depolymerization into mono- or oligosaccharides prior to MS characterization,^{14–16} due to their inherent polydispersity and high molecular weight.¹⁷ The pervasive isomeric heterogeneity of these subunits further complicated MS analysis.^{18,19} While advanced MS strategies have been developed,^{20–23} they often come with elevated analytical costs, increased operational complexity, and more intricate data interpretation.^{24–26} Although nuclear magnetic resonance (NMR) spectroscopy could provide atomic-level structural elucidation,^{27–29} its

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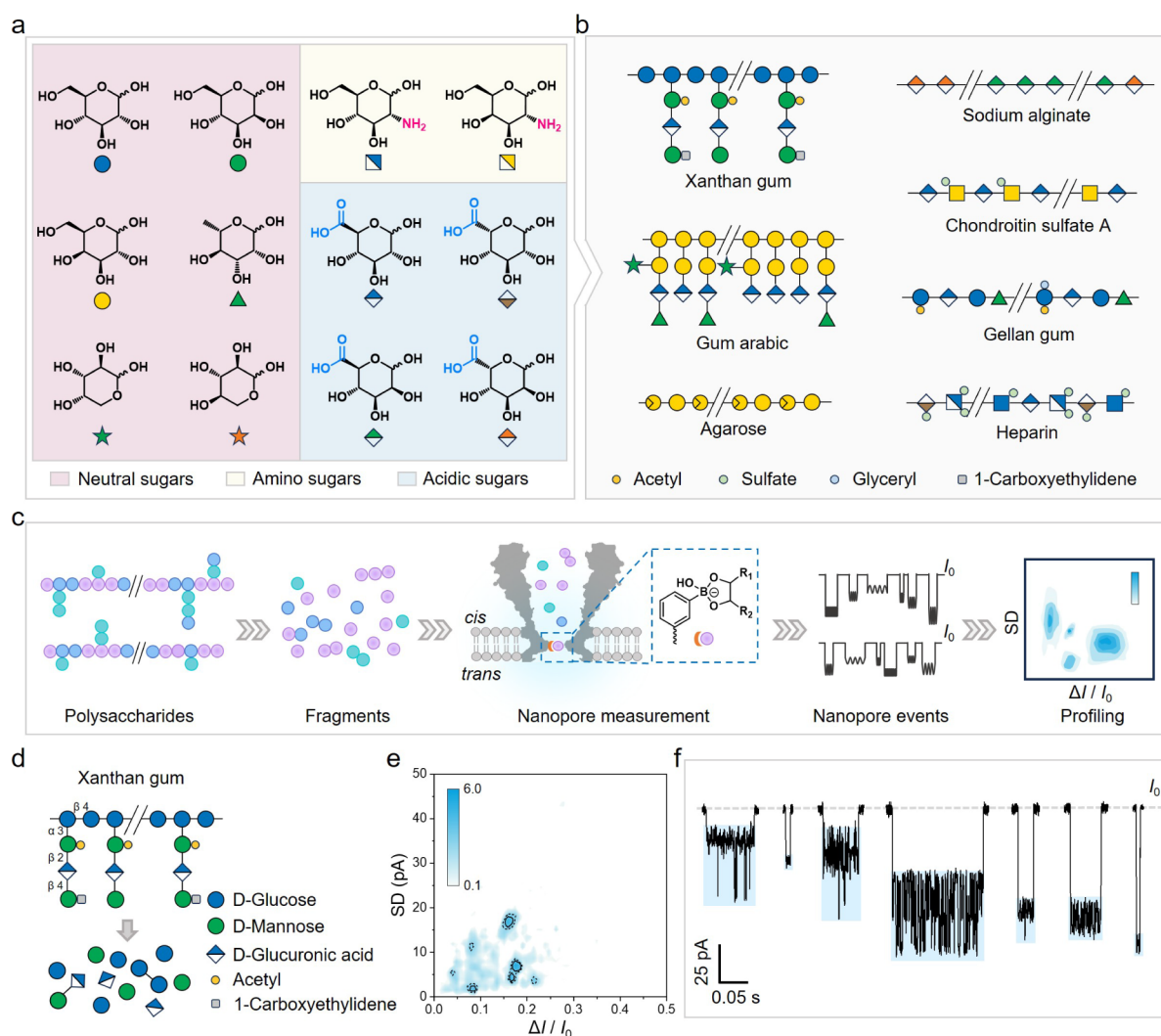


Figure 1. Nanopore profiling of polysaccharides. (a) Representative monosaccharide components of polysaccharides, including neutral, amino, and acidic sugars. (b) Schematic representation of polysaccharides investigated in this work. Each polysaccharide is composed of various monosaccharides linked through diverse glycosidic bonds and often modified by functional groups. (c) Workflow for nanopore polysaccharide profiling. The process begins with acidic hydrolysis of the polysaccharide. Then, the fragment mixture is measured by nanopore, yielding a substantial quantity of nanopore events. Computational feature extraction of these events leads to the final polysaccharide profile. (d) Schematic diagram of xanthan gum (XG) hydrolysis. (e) The kernel density plot of $\Delta I/I_0$ versus SD of events acquired with XG hydrolysate. Three independent measurements were performed to generate the plot (Supplementary Figure 3). Contour lines are plotted at 20% (solid line) and 10% (dashed line) of the maximum density value. (f) Representative nanopore events of XG hydrolysates. The measurements were performed with MspA-90PBA in a 1.5 M KCl buffer (1.5 M KCl, 100 mM MOPS, pH 7.0). Xanthan gum was hydrolyzed with 6 M HCl at 95 °C for 4 h (Methods). Twenty μ L hydrolysis product was added to the *cis* chamber to initiate the measurement. A voltage of +160 mV was continually applied.

widespread adoption is constrained by stringent sample demands, spectral overlap that impedes clear interpretation, and considerable instrument expenses.²⁷ Therefore, a universal, low-cost, and simple analytical technique remains a critical requirement for polysaccharide characterization.

Nanopores have emerged as promising single-molecule sensing platforms, with established applications in nucleic acid sequencing,^{30,31} protein identification,^{32–34} and small molecule sensing.^{35,36} However, their extension to polysaccharide analysis remains challenging due to the extraordinary structural complexity of these polymers. Although solid-state nanopores have been used to detect polysaccharides such as hyaluronan,^{37,38} heparin,³⁹ xylan,⁴⁰ and starch,⁴¹ the detection scope is generally limited to negatively charged, soluble glycosaminoglycans, and the spatial and temporal resolution is insufficient for

accurate polysaccharide identification. In contrast, biological nanopores have atomic-scale precision and hold the potential to resolve finer structural features. For example, engineered α -hemolysin (α -HL) can recognize glycans containing specific functional groups,⁴² yet its applicability remains narrow. Aerolysin (AeL) has demonstrated the ability to detect oligo- and polysaccharides,^{43,44} but it suffers from limited detection scope and insufficient resolution to detect small saccharide analytes. Together, these limitations highlight the need for nanopore systems that combine broad detection capability with high resolution for comprehensive polysaccharide analysis.

Unlike proteins and nucleic acids which have well-defined linear sequences, polysaccharides are generally assembled from recurring structural motifs that form ordered yet redundant sequence patterns. This inherent structural feature implies that

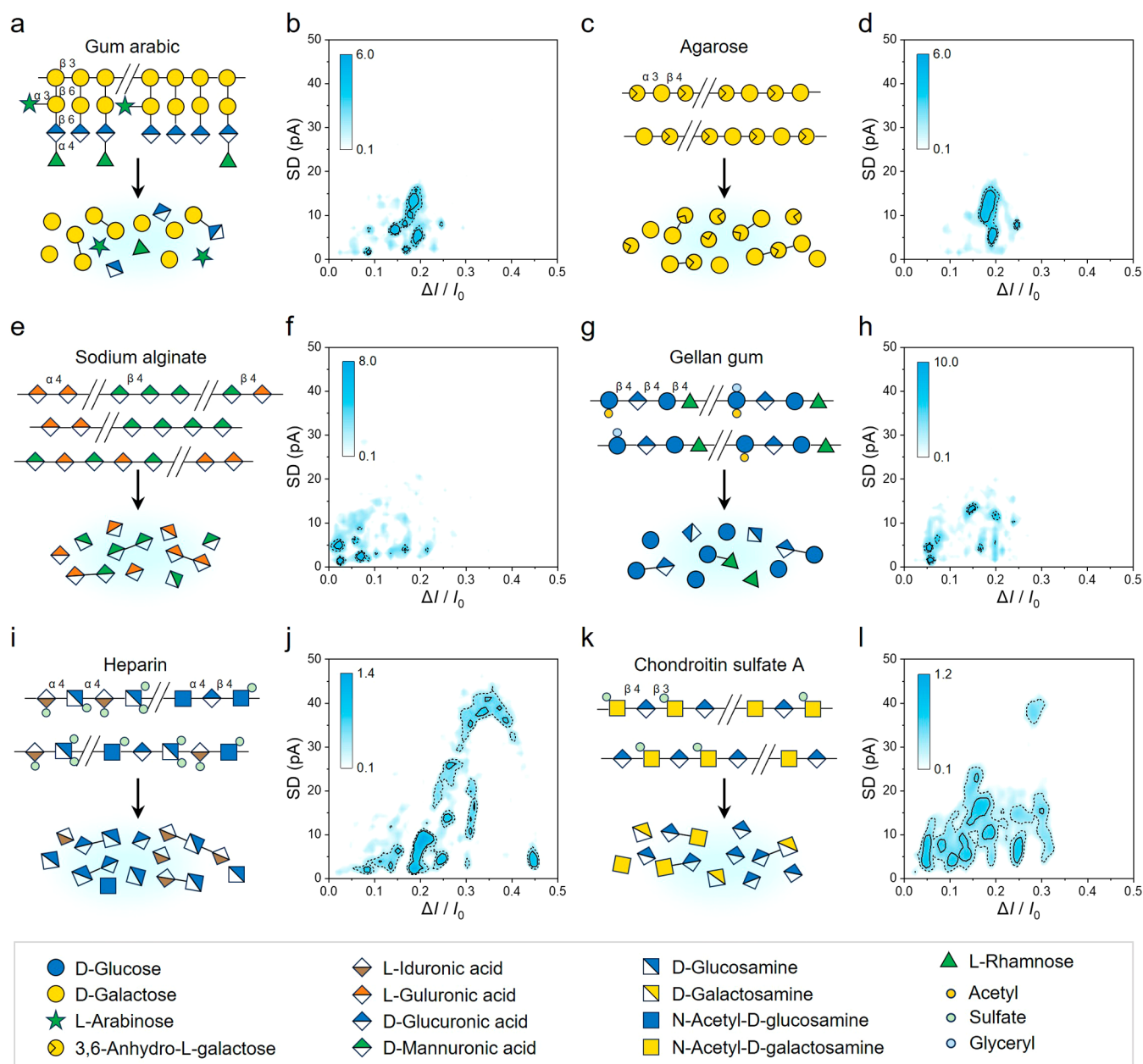


Figure 2. Nanopore identification of structurally diverse polysaccharides. (a, c, e, g, i, k) Schematic diagrams of gum arabic (a), agarose (c), sodium alginate (e), gellan gum (g), heparin (i), and chondroitin sulfate A (k) hydrolysis. The symbol legends for building blocks are placed in the bottom box. (b, d, f, h, j, l) The kernel density plots of events acquired with hydrolysate of gum arabic (b), agarose (d), sodium alginate (f), gellan gum (h), heparin (j), and chondroitin sulfate A (l). Each plot was generated by combining data from three independent measurements (Supplementary Figures 7–12). Contour lines are plotted at 20% (solid line) and 10% (dashed line) of the maximum density value for each panel. Representative events of each polysaccharide profile were demonstrated in Supplementary Figures 13–18. The measurements were performed with MspA-90PBA in a 1.5 M KCl buffer (1.5 M KCl, 100 mM MOPS, pH 7.0). Each polysaccharide was separately treated with 6 M HCl at 95 °C for 4 h (Methods). Twenty μ L hydrolysis product was added to the *cis* chamber for each sample. A voltage of +160 mV was continually applied. Each polysaccharide reports a unique profile in the corresponding kernel density plot results.

full-length *de novo* sequencing of polysaccharides is often unnecessary. For example, in fields such as food authentication, pharmaceutical quality control and functional polysaccharide screening, the primary demand is not the acquisition of complete sequence information, but rather rapid and reliable identification of polysaccharides based on their structural signatures. Therefore, we propose a hydrolysis-based polysaccharide profiling strategy with a functionalized nanopore platform. Briefly, polysaccharides were hydrolyzed under harsh acidic conditions at elevated temperature, yielding low-

molecular-weight saccharide fragments. These fragments can readily enter the nanopore and interact with the chemical adapter located at the pore constriction, generating highly characteristic and reproducible structural profiles that enable robust polysaccharide identification (Figure 1a–c).

RESULTS AND DISCUSSION

Nanopore Profiling of Xanthan Gum

To validate the feasibility of nanopore polysaccharide profiling via fragmentation, xanthan gum (XG) was first selected as the

model analyte. XG consists of a β -1,4-linked D-glucose backbone with a characteristic side chain attached via an α -1,3 linkage. The side chain contains a D-glucuronic acid flanked by two D-mannose residues and is further decorated with acetyl and pyruvate groups.⁴⁵ To fragment XG (Figure 1d), it was treated with 6 M HCl at 95 °C for 4 h. This hydrolysis condition degrades the polysaccharides into saccharide fragments suitable for nanopore analysis, thereby yielding highly characteristic and reproducible profiles. The solution was then neutralized, and the resulting hydrolysate was subjected to ultrafiltration using a 3 kDa molecular weight cutoff membrane. The filtrate was collected for further analysis (Methods).

Nanopore measurements were performed in a buffer containing 1.5 M KCl and 100 mM 3-morpholinopropanesulfonic acid (MOPS) at pH 7.0, and a voltage of +160 mV was continually applied (Methods). By convention, the electrically grounded measurement chamber is denoted as *cis*, while the opposite chamber is referred to as *trans*. A hetero-octameric *Mycobacterium smegmatis* porin A (MspA) equipped with a single phenylboronic acid (PBA) adapter at its pore constriction was used for detection of saccharide fragments. PBA is a well-recognized receptor that can form reversible boronic ester bonds with saccharides (Supplementary Figure 1). This PBA modified MspA nanopore (also referred to as MspA-90PBA) has previously demonstrated precise identification of monosaccharides,⁴⁶ glycosidic linkages,⁴⁷ and oligosaccharides.^{48,49} Upon addition of XG hydrolysate in the *cis* chamber, a substantial number of distinct nanopore events were immediately observed (Supplementary Figure 2). To characterize these events quantitatively, core event parameters were extracted from the events, including the open pore current (I_0), blockage current (I_b), blockage ratio ($\Delta I/I_0 = (I_0 - I_b)/I_0$), standard deviation (SD), and dwell time (t_{off}) (Supplementary Figure 2). A two-dimensional (2D) kernel density plot based on $\Delta I/I_0$ and SD, revealed a unique profile specific to XG (Figure 1e), with strong consistency across three independent measurements (Supplementary Figure 3 and Supplementary Table 1). For in-depth analysis, characteristic regions were identified as distinct, well-defined event clusters with central density greater than 10% of the maximum density (Figure 1e and Supplementary Figure 4). Representative events extracted from these regions serve as the primary structural signatures for XG identification (Figure 1f). These characteristic distributions and representative events collectively establish a critical foundation for the structural profiling of polysaccharides.

Notably, even at an extremely low voltage of +5 mV, nanopore events remained clearly detectable (Supplementary Figure 5), indicating that saccharide fragments can rapidly and spontaneously reach the PBA adapter at the pore constriction via diffusion to trigger event generation. Similarly, this diffusion-dominated mechanism enables nanopore measurements to be performed at both positive and negative voltages, and well-defined binding events can be clearly resolved under either polarity (Supplementary Figure 6).

Discrimination of Structurally Diverse Polysaccharides

This profiling strategy was further extended to a diverse panel of polysaccharides, spanning branched and linear structures as well as neutral and negatively charged polymers, including gum arabic, agarose, sodium alginate, gellan gum, heparin, and chondroitin sulfate A. Gum arabic (GA) is a branched polysaccharide primarily composed of D-galactose units linked via β -1,3-glycosidic bonds, with side chains containing D-

galactose, D-glucuronic acid, L-arabinose, and L-rhamnose (Figure 2a).^{50,51} Agarose (A) is a linear polysaccharide consisting of alternating D-galactose and 3,6-anhydro-L-galactose units, linked through β -1,4- and α -1,3-glycosidic bonds (Figure 2c).⁵² Sodium alginate (SA),⁵³ also linear, is composed of L-guluronic acid and D-mannuronic acid linked by 1,4-glycosidic bonds (Figure 2e). Gellan gum (GG) is a linear polysaccharide formed by repeating tetrasaccharide units with a molar ratio of D-glucose, D-glucuronic acid, and L-rhamnose of 2:1:1 (Figure 2g).⁵⁴ Some D-glucose residues in the polymer are modified with acetyl and glyceryl groups. Heparin (H) and chondroitin sulfate A (CS) share a highly similar linear glycosaminoglycan (GAG) backbone composed of repeating disaccharide units containing amino sugars and uronic acids (Figure 2i and k).^{55,56} Specifically, heparin is built from D-glucosamine (179.17 Da), L-iduronic and D-glucuronic acids (194.14 Da), whereas CS is composed of D-galactosamine (179.17 Da) and D-glucuronic acid (194.14 Da), posing a great challenge for discriminating these subtle variations.

Experimentally, all polysaccharides were first hydrolyzed under the same condition as xanthan gum was treated (Methods). Subsequently, each hydrolysate was introduced to the *cis* chamber for separate nanopore measurements. Each polysaccharide yields a distinct profile pattern (Figure 2b, d, f, h, j, l), enabling direct identification in a database-independent manner that requires no prior knowledge of the nanopore signatures of hydrolyzed fragments. Each polysaccharide sample was tested in three independent replicates (Supplementary Figures 7–12), and the resulting profiles showed good consistency based on cosine similarity analysis (Methods and Supplementary Table 1). Characteristic regions of these profiles were extracted and assigned with distinct event labels (Supplementary Figures 13–18). Notably, in the case of the structurally similar pair heparin and chondroitin sulfate A, which share isomeric monosaccharide constituents, characteristic events H8, H10, and H11 in H and CS1, CS6, and CS7 in CS served as key discriminators between the two polysaccharides (Supplementary Figures 17–18). Nanopore detection of polysaccharide hydrolysates prepared with different hydrolysis times was also performed, and markedly distinct profiles were obtained (Supplementary Figure 19). These results substantiate that the MspA-90PBA nanopore possesses both superior resolution and extensive utility for the structural elucidation of varied polysaccharide compositions.

Nanopore sensing of untreated polysaccharides was also performed, and no well-defined event clusters were observed (Supplementary Figure 20). This highlights the importance of the hydrolysis operation, which converts high-molecular-weight polysaccharides into smaller, information-rich saccharide fragments. The nanopore events of these fragments collectively form a characteristic fingerprint profile, enabling precise resolution of the structural heterogeneity of polysaccharides.

Deciphering the Polysaccharide Hydrolysis Profiles

Beyond generating unique fingerprints for each polysaccharide, the nanopore profiles also revealed characteristic events that could be directly linked to specific saccharide constituents. To systematically establish this correspondence, a reference collection of nanopore events was assembled from standard monosaccharide measurements. This collection includes L-arabinose (Supplementary Figure 21), D-galactosamine (Supplementary Figure 22), D-glucosamine (Supplementary Figure 23), D-glucuronic acid (Supplementary Figure 24), N-acetyl-D-

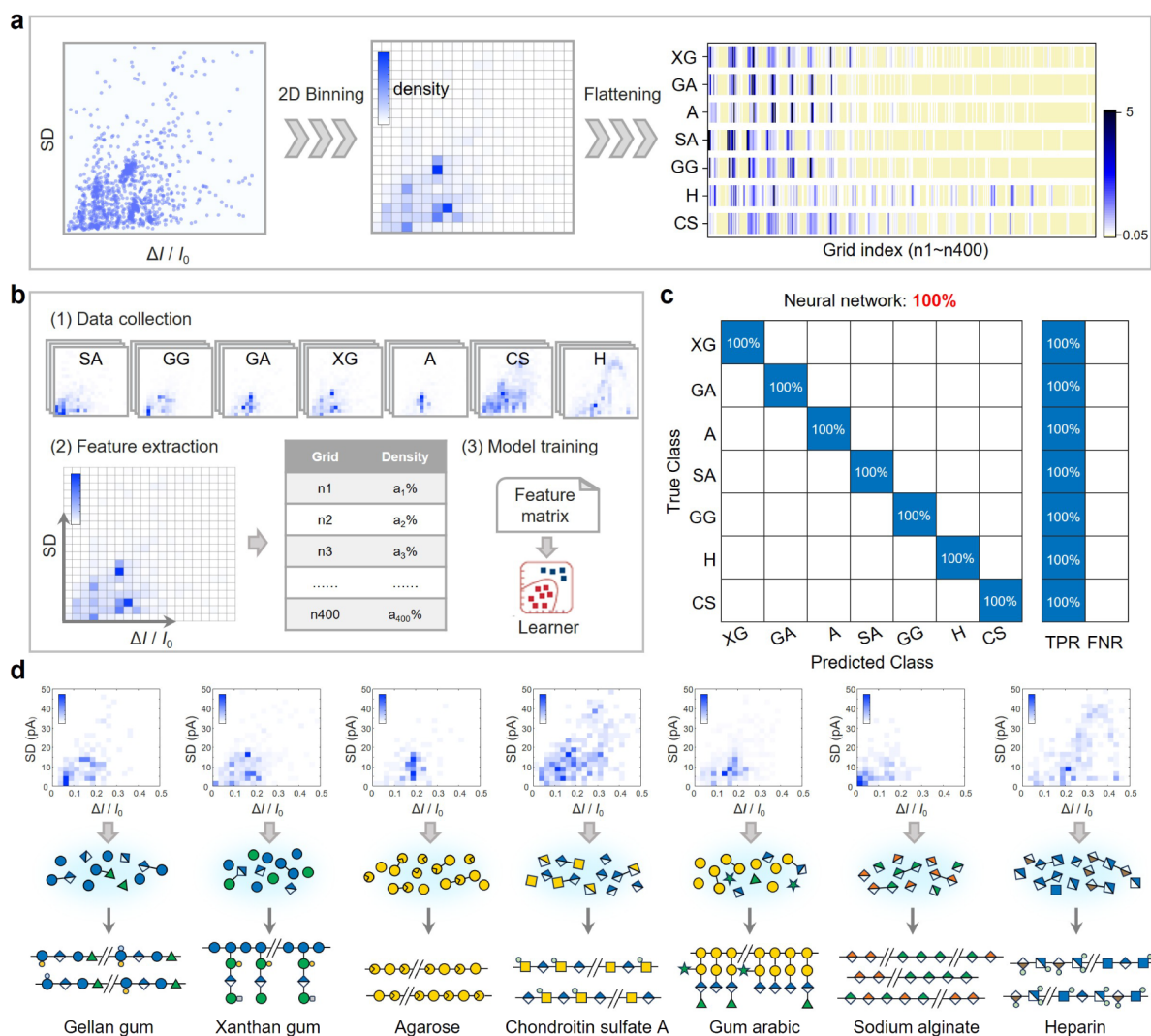


Figure 3. Profile-based machine learning classification of seven polysaccharides. (a) Data preprocessing. Scatter plots of events from each polysaccharide hydrolysate were converted into a 20×20 density-based heatmap. Each heatmap was then flattened into a one-dimensional feature vector by arranging the 400 grid-cell densities in sequence, yielding a unique “barcode” for each polysaccharide. (b) The workflow of machine learning. 100 heatmaps for each type of polysaccharide were collected to form a data set. Each heat map was constructed based on different event counts in the grids. The density value ($a_1\%–a_{400}\%$) in each grid ($n_1–n_{400}$) served as a training feature, together building a 400-dimensional feature matrix. The model was trained and evaluated using the Classification Learner app (MATLAB), with the neural network achieving 100% cross-validation accuracy. (c) Confusion matrix generated by the neural network. TRP and FNR respectively represent the correct or false classification of each true class. (d) Model performance evaluation using the testing set, according to which, all samples were correctly assigned.

glucosamine (Supplementary Figure 25), and a range of previously characterized monosaccharides (D-glucose, D-mannose, D-galactose, N-acetyl-D-galactosamine, D-fructose, L-sorbose, L-rhamnose, D-xylose and D-ribose).⁴⁶ Together, these data cover neutral, acidic, and amino sugars, provide a unified basis for assigning structural origins to features observed in complex polysaccharide hydrolysates.

Through systematic feature matching, specific event clusters in the polysaccharide profiles could be assigned to corresponding monosaccharide constituents (Supplementary Table 2). In the xanthan gum profile, events XG4 and XG6 matched the signatures of D-mannose; XG5 and XG7 corresponded to D-glucose; and XG1 aligned with D-glucuronic acid. Likewise, multiple saccharides were identified across other polysaccharide profiles. D-Galactose was detected in gum arabic and agarose; D-glucuronic acid was found in gum arabic, gellan gum, and chondroitin sulfate A; L-arabinose was identified in gum arabic;

D-glucose and L-rhamnose were observed in gellan gum; D-glucosamine and D-galactosamine were identified in heparin and chondroitin sulfate A, respectively. These results also confirm that the adopted hydrolysis conditions were appropriate and effectively preserved the structural integrity of the saccharide fragments.

Machine Learning-Assisted Identification of Seven Polysaccharides

To enable automated polysaccharide identification, a profile-based machine learning algorithm was developed (Methods). Rather than classifying each individual event of polysaccharide fragments, this approach captures the collective spatial distribution of event ensembles as stable and informative signatures, and the identity of polysaccharide can be encoded in the reproducible statistical pattern of its molecular population.

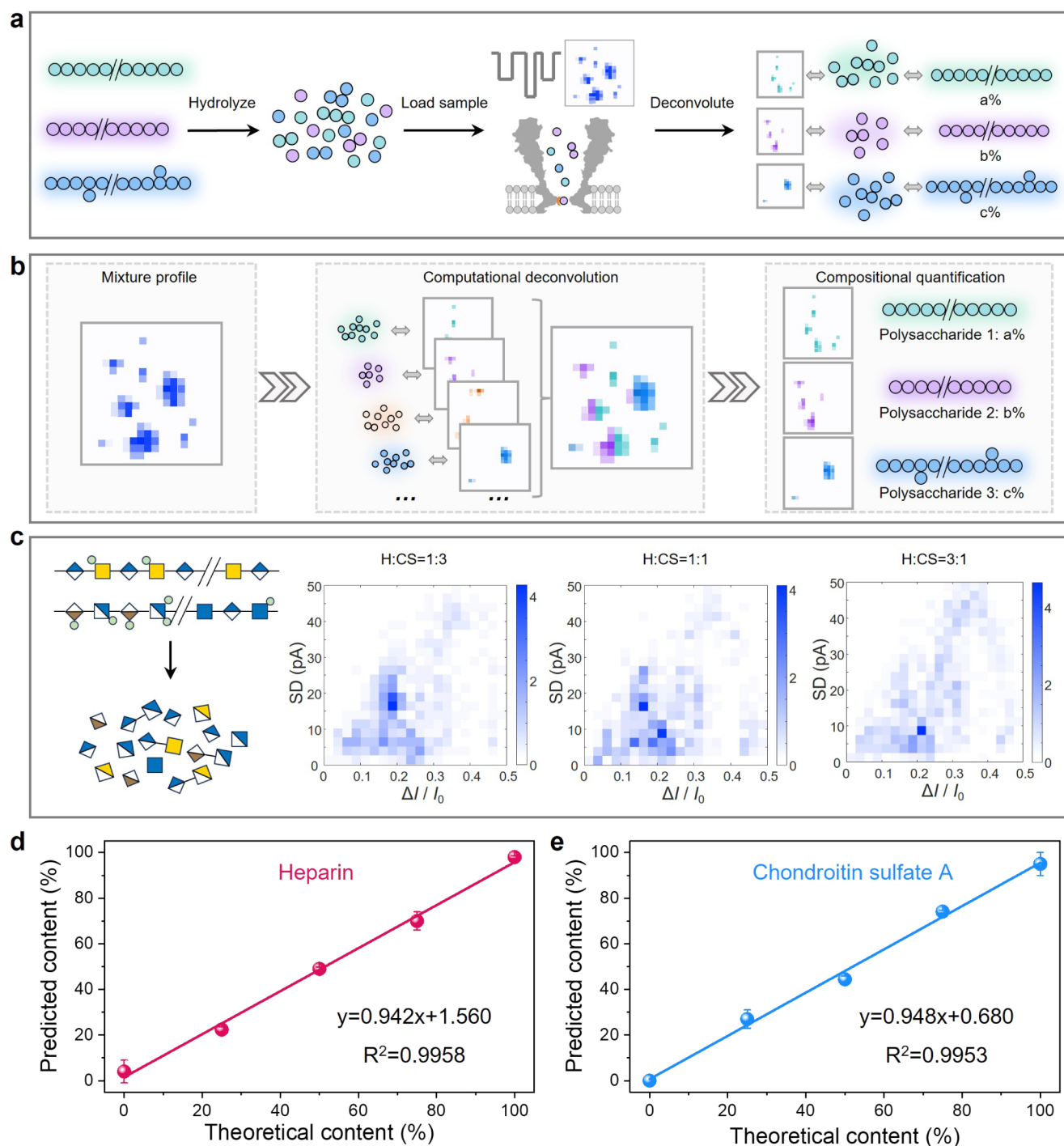


Figure 4. Quantitative analysis of polysaccharide mixtures. (a) Workflow for quantitative analysis of polysaccharide mixtures. Briefly, polysaccharide mixtures were first hydrolyzed, and the resulting fragments were analyzed by nanopore to obtain the profile. The profile was subsequently deconvoluted to resolve the characteristic profile of each individual polysaccharide, enabling quantification of their relative proportions. (b) Workflow for the deconvolution of the mixture profile. The heatmap acquired from polysaccharide mixture fragments was decomposed into a linear combination of reference heatmaps of individual polysaccharides via the fully constrained least-squares algorithm (Methods). The derived contribution coefficients directly indicate the identities and relative proportions of the polysaccharides in the mixture. (c) Representative heatmaps acquired from heparin (H)/chondroitin sulfate A (CS) mixtures at varying ratios. (d) Predicted versus theoretical content for heparin. (e) Predicted versus theoretical content for chondroitin sulfate A. In both plots, the slopes approximate 1, confirming the accuracy of quantitation.

Specifically, for each polysaccharide, 100 random segments of consecutive events were collected, with each segment containing varying event numbers. Each segment was projected onto a fixed 2D coordinate space defined by $\Delta I / I_0$ and SD (Figure 3a), and then converted into a 20×20 density-based heatmap

(Supplementary Figures 26–27). For demonstration, the 2D-heatmap was flattened into a one-dimensional feature vector by sequentially arranging the 400 grid-cell densities (Figure 3a), which serves as a unique “barcode” for each polysaccharide. The pattern of high- and low-density regions within this barcode

constitutes a stable, specific molecular fingerprint. Besides, these barcode profiles exhibit robustness to variations in the number of events used for its construction (Supplementary Figure 28) and high reproducibility across independent experimental replicates (Supplementary Figure 29).

For machine learning, data from seven polysaccharides were consolidated into a 700×400 feature matrix (Figure 3b), where each polysaccharide was represented by 100 heatmaps, and each heatmap was characterized by 400 features (the density value within each grid of the heatmap). This matrix was randomly partitioned into a training set (80%) and a testing set (20%) for model construction and evaluation, respectively. Machine learning was implemented using the Classification Learner app in MATLAB. Six inbuilt classifiers, including ensemble, support vector machine (SVM), decision trees, naïve bayes, neural network, and k-nearest neighbor (KNN), were trained. Model performance was assessed via 10-fold cross-validation (Supplementary Table 3). All classifiers achieved an accuracy exceeding 99%, with the neural network model attaining 100% validation accuracy (Figure 3c). Learning curve analysis revealed that 40 training samples were sufficient to reach 95% validation accuracy (Supplementary Figure 30). The trained model was further applied to the held-out testing data (Figure 3d). Each hydrolysate profile was accurately assigned to its corresponding source polysaccharide, confirming the practical utility of this method.

Quantitative Analysis of Polysaccharide Mixtures

The nanopore-based profiling strategy was further applied to quantitative analysis of polysaccharide mixtures. Polysaccharide mixtures were first subjected to hydrolysis, and the resulting fragments were then analyzed by nanopore, generating a composite profile. This profile was then deconvoluted into the characteristic fragment profiles of each polysaccharide, thereby enabling the quantitative determination of the original components in the mixture (Figure 4a–b). For demonstration, heparin (H) and chondroitin sulfate A (CS) were chosen as representative polysaccharides. Both are widely used glycosaminoglycan therapeutics, and their structural resemblance has historically resulted in serious contamination problems,⁵⁷ emphasizing the critical need for reliable analytical techniques for quality control of polysaccharide pharmaceuticals.

Experimentally, H/CS mixtures with varying ratios were prepared and hydrolyzed under identical conditions used for individual polysaccharides (Methods). The resulting hydrolysates were then analyzed by MspA-90PBA, yielding distinct event distributions (Figure 4c and Supplementary Figures 31–33). To deconvolute the mixture profile, each mixture heatmap was modeled as a linear combination of reference heatmaps from individual polysaccharide (Figure 4b). The fully constrained least-squares (FCLS) algorithm was used to estimate the relative abundance of each component by minimizing the least-squares error between the reconstructed and measured mixture profiles subject to non-negativity and sum-to-one constraints (Methods).⁵⁸ The resulting coefficients directly represent the estimated composition. Quantitative analysis of heparin and chondroitin sulfate mixtures using this approach yielded estimates that closely matched the theoretical values (Figure 4d–e and Supplementary Table 4), validating the accuracy of this strategy for determining compositional ratios in mixtures of structurally similar polysaccharides.

CONCLUSION

In summary, this work establishes a universal polysaccharide identification strategy built on the intrinsic statistical distribution profiles of hydrolytic fragment events, featuring broad applicability, enhanced resolving power, and unique polysaccharide-specific signal fingerprints. Experimentally, high-molecular-weight polysaccharides, which yield limited structural information when analyzed in their intact form, were hydrolyzed into smaller and information-rich fragments. The collective nanopore events of these fragments form a unique and reproducible molecular fingerprint, enabling unambiguous identification of different types of polysaccharides. Comparison with monosaccharide standards enables assignment of specific events within the profile, further enhancing the identification. To enable automated and unbiased classification, a machine learning algorithm was developed. Rather than identifying each individual event, the model learns from the overall distribution of each polysaccharide's hydrolysate profile, achieving 100% validation accuracy across seven common polysaccharides, including xanthan gum, gum arabic, agarose, sodium alginate, gellan gum, heparin, and chondroitin sulfate A. Furthermore, this approach was extended to the quantitative analysis of polysaccharide mixtures. By deconvoluting the mixture profile into the standard reference contributions, accurate quantification of heparin/chondroitin sulfate A mixtures was demonstrated, providing a new pathway for quality control of polysaccharide pharmaceuticals.

Acknowledging the chemical specificity conferred by the PBA adapter, the system exhibits strong anti-interference capability and is not affected by common biomolecules such as amino acids, peptides, and nucleic acids (Supplementary Figure 34). For molecules that can potentially interact with the PBA adapter, supervised machine learning algorithms such as One-Class SVM could be employed to effectively filter out interfering events, further enhancing the anti-interference performance of this platform. Although this study only employed acidic hydrolysis, future investigations may explore milder and more universal hydrolysis strategies, such as enzymatic digestion with single or combined glycosidases, or enzyme–acid coupled hydrolysis. In addition, controlled hydrolysis conditions (e.g., varying time, temperature, or acid/enzyme concentration) could be systematically optimized to ensure efficient hydrolysis while preserving richer structural information. Furthermore, more event parameters could be incorporated to construct multidimensional feature profiles, thereby providing higher precision and robustness when dealing with more complex sensing scenarios. Future engineering efforts such as optimization of experimental parameters, rational pore engineering, and the implementation of parallelized nanopore arrays may further improve the sensitivity. Besides, expanding the coverage of reference polysaccharide profiles and integrating orthogonal techniques may further broaden the applicability of this method. As profile libraries grow and artificial intelligence-driven event interpretation advances, these profiles may evolve into rich data sets for inferring oligosaccharide sequences and domain structures, opening new avenues to investigate the structure–function relationships of polysaccharides.

METHODS

Nanopore Preparation

(N90C)₁(M2)₇ is a hetero-octameric MspA protein, composed of one N90C MspA-H6 monomer and seven M2MspA-D16H6 mono-

mers.^{59,60} Both genes were custom synthesized and simultaneously inserted in a pETDuet-1 plasmid for coexpression. The recombinant plasmid was expressed with *E. coli* strain BL21(DE3) pLysS competent cells, and the expression products were purified via nickel affinity chromatography. Different hetero-octameric assemblies were further separated by polyacrylamide gel electrophoresis, and the band corresponding to (N90C)₁(M2)₇ was excised and recovered. The collected (N90C)₁(M2)₇ was either used immediately or stored at −80 °C for long-term storages.

To introduce a phenylboronic acid (PBA) adapter to (N90C)₁(M2)₇, 1 μL 500 mM MPBA, 2.5 μL (N90C)₁(M2)₇ and 21.5 μL KCl buffer were mixed and incubated at room temperature for 10 min⁴⁶. The resulting product, referred to as MspA-90PBA, was directly used in downstream nanopore measurements.

Nanopore Measurements

To minimize environmental noise interference, all nanopore measurements were carried out in a homemade Faraday cage fixed on an optical table (Jiangxi Liansheng Technology). The measurement device consists of two chambers separated by a Teflon film containing a centrally drilled aperture (~100 μm in diameter). Conventionally, the electrically grounded chamber is defined as *cis* while the opposing chamber is defined as *trans*. The aperture was pretreated with 0.5% (v/v) hexadecane in pentane prior to each use. Then, each chamber was filled with 0.5 mL KCl buffer (1.5 M KCl, 100 mM CHES, pH 7.0). A pair of Ag/AgCl electrodes were placed in both chambers and in contact with the buffer to form a closed circuit. A drop of DPhPC (5 mg/mL in pentane) was then added to each chamber to form a lipid bilayer on the aperture. Subsequently, the prepared nanopores were added to the *cis* chamber to initiate pore insertions. Upon a single nanopore insertion, the *cis* chamber was immediately exchanged with fresh KCl buffer to prevent further pore insertions.

All electrophysiological measurements were performed with an Axopatch 200B patch-clamp amplifier paired with a Digidata 1550B digitizer, sampling at 25 kHz with 1 kHz low-pass filtering. This configuration efficiently suppresses high-frequency noise while faithfully capturing valid nanopore events without signal distortion. Unless otherwise stated, all measurements were performed in a KCl buffer (1.5 M KCl, 100 mM MOPS, pH 7.0) and a continually applied voltage of +160 mV at room temperature. All analytes were added to the *cis* chamber to the desired final concentration.

Data Analysis

All nanopore events were detected by the “Single-Channel Search” function in Clampfit 10.7 (Molecular Devices). Events with a dwell time <10 ms were ignored. Event features including $\Delta I/I_0$, *SD*, and *t*_{off} were extracted from all events using a custom MATLAB code. All subsequent data processing was performed with Origin 2025.

To evaluate the reproducibility of the kernel density profiles, cosine similarity analysis was performed on the triplicate measurements for each polysaccharide (Supplementary Figures 3, 7–12). Cosine similarity is a standard metric for quantifying the similarity between high-dimensional vectors and is widely used in chemometric fingerprint analysis.^{61,62} For each polysaccharide, each of the three density matrices was flattened into a vector, and pairwise cosine similarities were calculated between replicates. The average of the three pairwise values was taken as the quantitative measure of profile reproducibility. The results were summarized in Supplementary Table 1.

To generate a standardized data set for machine learning classification, nanopore events from seven polysaccharide hydrolysates were collected. For each polysaccharide hydrolysate, 100 random subsets, each comprising 100 to 800 consecutive events—were extracted from the continuous recordings. Each subset was converted into a two-dimensional heatmap in the coordinate space defined by $\Delta I/I_0$ (range: 0–0.5) versus *SD* (range: 0–50 pA). This space was discretized into a uniform 20 × 20 grid, yielding 400 cells. The feature value for each cell was defined as the relative event density within that cell, calculated as the percentage of events in the cell relative to the total number of events of that subset. Thus, each heatmap corresponds to a 400-dimensional feature vector, labeled by its source polysaccharide. The final compiled data set comprises 700 heatmaps (7 polysaccharide

× 100 heatmaps per analyte), forming a feature matrix of 700 rows × 400 columns. The feature matrix was then randomly split into a training set (80%) and a testing set (20%), respectively used for model training and model testing. Machine learning was conducted in the Classification Learner app of MATLAB. Multiple built-in classifiers, encompassing Decision trees, Support Vector Machine (SVM), Neural Network, K-Nearest Neighbor (KNN), Naïve Bayes, and Ensemble, were evaluated. To prevent overfitting, 10-fold cross-validation was applied, from which validation accuracy and test accuracy were determined. The model exhibiting the superior cross-validation accuracy was chosen for subsequent data prediction tasks.

A computational deconvolution strategy based on the fully constrained least-squares (FCLS) algorithm was employed to resolve the compositional profiles of mixtures.⁵⁸ Pre-established two-dimensional heatmaps for the seven individual polysaccharides (Supplementary Figure 27) served as reference distributions. Prior to deconvolution, each reference map was scaled by a correction factor derived from the relative event frequency of each polysaccharide profile, normalized to that of chondroitin sulfate A. For unmixing, the mixture data was projected into the same two-dimensional descriptor space, and the empirical probability distribution (*f*_{mix}), was computed analogously. This distribution was modeled as a linear combination of the seven preprocessed reference distributions, which together form a matrix *F*. The proportional contribution of each reference to the mixture is described by a weight vector *w* = [*w*₁, *w*₂, ..., *w*₇], obtained by solving a constrained linear least-squares problem that minimizes the sum of squared residuals between *f*_{mix} and the reconstructed *Fw*, subject to non-negativity and sum-to-one constraints (i.e., *w*_{*i*} ≥ 0 and $\sum w_i = 1$). The optimization was performed in MATLAB using the *lsqlin* function. The resultant optimal weight vector, *w*_{opt}, directly provides the estimated fractional composition of the unknown mixture.

Acid Hydrolysis of Polysaccharides

To achieve efficient fragmentation of polysaccharides, acid hydrolysis was performed. 50 mg sodium chondroitin sulfate A, sodium heparin, xanthan gum, gellan gum, sodium alginate, gum arabic, agarose or their mixture was separately dissolved in 1 mL of 6 M HCl, and incubated at 95 °C for 4 h. Following hydrolysis, the pH of the solution was neutralized to 7.0 using KOH. The samples were then completely lyophilized, redissolved in 1 mL of ultrapure water, and subjected to ultrafiltration using a 3 kDa molecular weight cutoff membrane at 9000 rpm for 0.5 h. The obtained filtrate was collected and either used immediately or stored at 4 °C for subsequent analysis. To initiate nanopore measurements, 20 μL of the filtrate was added to the *cis* chamber, corresponding to a theoretical final concentration of approximately 2 mg/mL.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsnano.6c07846>.

Details of Figures and Tables; Materials; statistics of event core parameters; and continuous current traces during single-channel recordings (PDF)

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Author Contributions

[#]W.L. and Y.X. contributed equally. S.H., K.W., and W.L. conceived the project. W.L. prepared the MspA nanopores. W.L. and X.G. performed the measurements. W.L. and Y.X. designed the machine-learning algorithms. P.Z. set up the instruments. S.H., K.W., and W.L. wrote the paper. S.H. supervised the project.

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Notes

The authors declare the following competing financial interest(s): S.H. and K.F.W. have filed patents describing the preparation of heterogeneous MspA and its applications. The remaining authors declare no competing interests.

ABBREVIATIONS

FCLS, fully constrained least-squares; MS, mass spectrometry; NMR, nuclear magnetic resonance; α -HL, α -hemolysin; AeL, aerolysin; XG, xanthan gum; MOPS, 3-morpholinopropanesulfonic acid; MspA, *Mycobacterium smegmatis* porin A; PBA, phenylboronic acid; GA, gum arabic; A, agarose; SA, sodium

alginate; GG, gellan gum; H, heparin; CS, chondroitin sulfate A; GAG, glycosaminoglycan

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