Single Molecule Detection of Glycosaminoglycan Hyaluronic Acid Oligosaccharides and Depolymerization Enzyme Activity Using a Protein Nanopore

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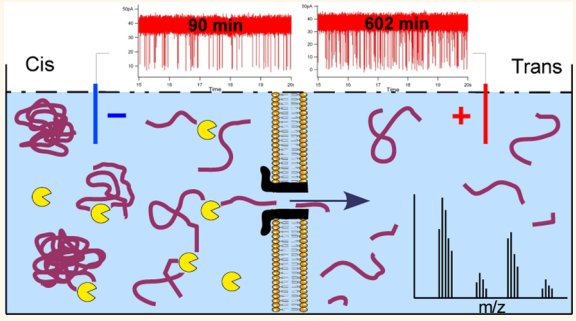
ABSTRACT Glycosaminoglycans are biologically active anionic carbohydrates that are among the most challenging biopolymers with regards to their structural analysis and functional assessment. The potential of newly introduced biosensors using protein nanopores that have been mainly described for nucleic acids and protein analysis to date, has been here applied to this polysaccharide-based third class of bioactive biopolymer. This nanopore approach has been harnessed in this study to analyze the hyaluronic acid glycosamiglycan and its depolymerization-derived oligosaccharides. The translocation of a glycosaminoglycan is reported using aerolysin protein nanopore. Nanopore translocation of hyaluronic acid oligosaccharides was evidenced by the direct detection of translocated molecules accumulated into the arrival compartment using high-resolution mass spectrometry. Anionic oligosaccharides of various polymerization degrees were discriminated through measurement of the dwelling time and translocation frequency. This molecular sizing capability of the protein nanopore device allowed the real-time recording of the enzymatic cleavage of hyaluronic acid polysaccharide. The time-resolved detection of enzymatically produced oligosaccharides was carried out to monitor the depolymerization enzyme reaction at the single-molecule level.



KEYWORDS: nanopore · aerolysin · oligosaccharide · hyaluronic acid · enzyme · hyaluronidase · mass spectrometry

Nanometer-scale protein channels have been recently introduced as ultrasensitive biosensors allowing the detection and the characterization of various analytes at the single molecule level.[1−3] Mainly used for the analysis of proteins,[4−6] nucleic acids[7,8] and nonelectrolyte polymers,[9−13] the nanopore detection is a promising method for deciphering information encoded in linear polymers.[14,15] However this method has been rarely applied to carbohydrate,[16] especially bioactive polysaccharides. We recently reported the detection of nonionic oligosaccharides at the single-molecule level using a protein nanopore.[17] This approach allowed us to discriminate oligosaccharides according to their polymerization degree and glycosidic linkages. This method appears thus promising for the determination of structural features of oligosaccharides, particularly those derived from bioactive polysaccharides such as glycosaminoglycans (GAGs). GAGs are highly anionic linear polysaccharides expressed at the cell surface and in the extra-cellular matrix, which mediate cell−cell and cell−matrix interactions involved in a variety of physiological and pathological functions such as in embryonic development, cell growth and differentiation, homeostasis, inflammatory response, tumor growth, and microbial infection.[18,19] In the study herein, we report on the nanopore analysis of anionic oligosaccharides derived from

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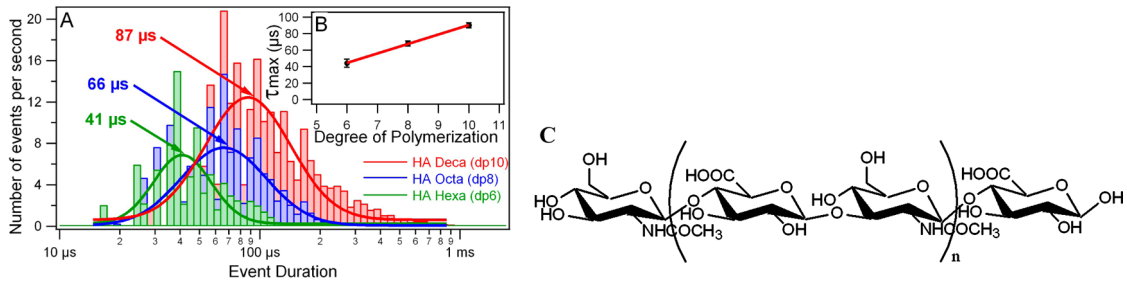

Figure 1. Dependence of the dwelling time on hyaluronic acid oligosaccharide size. (A) Distributions of the dwelling time of purified HA hexa-, octa-, and decasaccharide. Each distribution was fitted by a log-normal function, allowing the determination of the average dwelling time for each oligosaccharide. (B) Evolution of the average dwelling duration τ according to the degree of polymerization. HA oligosaccharides were 1 mM in the cis compartment in 5 mM HEPES, 1 M KCl, pH 7.5. Trans-membrane potential = 70 mV. (C) Structure of hyaluronic acid oligosaccharide used in this study (n = 2, 3, and 4 for HA hexa-, octa-, and decasaccharide, respectively).

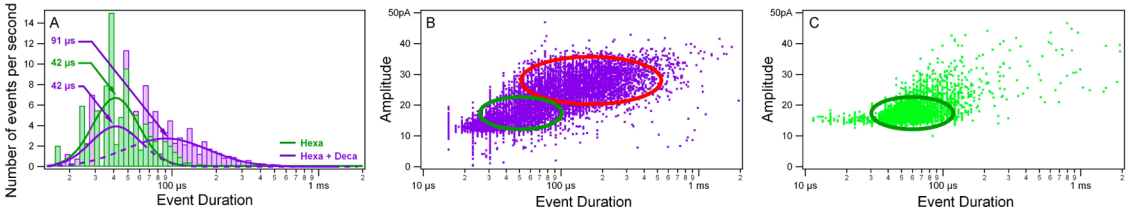

Figure 2. Distributions of the dwelling time of HA hexa- and decasaccharide in mixture. (A) Event duration distributions of HA hexa- and decasaccharide mixture (purple) and purified HA hexasaccharide (green). (B and C) Scatter plot representations of dwelling times for HA hexa- and decasaccharide mixture (B) and purified HA hexasaccharide (C). HA oligosaccharides were 0.5 mM each in the cis compartment in 5 mM HEPES, 1 M KCl, pH 7.5. Trans-membrane potential = 70 mV.

hyaluronic acid (HA), and the real-time nanopore recording of the enzymatic depolymerization of this glycosaminoglycan polysaccharide. HA is a linear polymer composed of disaccharide repeat units of D-glucuronic acid and *N*-acetyl glucosamine (Figure 1). As major GAGs in the extracellular matrix, HA plays an important role in normal tissue homeostasis, and in tissue remodeling during development and disease as well as in tumor progression.[20]

RESULTS AND DISCUSSION

In a typical single nanopore experiment, two compartments filled with 5 mM HEPES, 1 M KCl pH 7.5, were separated by a lipid membrane, in which a single protein nanopore was inserted. This unitary nanopore was built from aerolysin, a toxin from *Aeromonas hydrophila* forming trans-membrane heptameric channels.[21,22] A 70 mV membrane potential was applied (cathode at the cis compartment), allowing the measurement of an ionic current of 32 ± 2 pA in the absence of oligosaccharides, a current value corresponding thus to a single open pore. The addition of HA oligosaccharides in the cis compartment resulted in transient current decreases due to their interaction with the nanopore. These current blockades were characterized by their duration, frequency, and amplitude, which could be related to the structural features of a molecule dwelling within the nanopore.[17] We recorded here the distribution of event durations for purified HA oligosaccharides of different polymerization degrees, that is, hexa- (1136.3 g·mol^{-1}), octa- (1515.4 g·mol^{-1}) and decasaccharide (1894.5 g·mol^{-1}). The data showed an increase of the average dwelling time τ of oligosaccharides according to their polymerization degree (Figure 1), in agreement with previous studies on maltose and dextran oligosaccharides,[17] and poly(ethylene glycol) molecules.[12,13]

Although the average dwelling times of purified hexa- (44 ± 5 μs), octa- (68 ± 3 μs), and decasaccharide (90 ± 3 μs) were distinct for oligosaccharides differing by only one disaccharide repeat unit (Figure 1), differentiation of the oligosaccharides in a mixture was more complex and could be more confidently achieved for oligosaccharides differing by two disaccharide repeats as exemplified in Figure 2. The scatter plot of the duration distribution according to the amplitude of the current blockade for a mixture of hexa- and decasaccharide (0.5 mM each) showed two distinct populations (Figure 2B), one centered at 42 μs (circled in green) and the second one of longer times centered at 91 μs (circled in red). Both the same populations were also distinguished in the event distribution (purple distribution in Figure 2A), showing average duration times very close to purified hexa- and decasaccharide (overlay of the dwelling time distribution of purified hexasacharide in green curve, Figure 2A). This result indicated that both populations at 42 and 90 μs could be respectively attributed to hexasaccharide

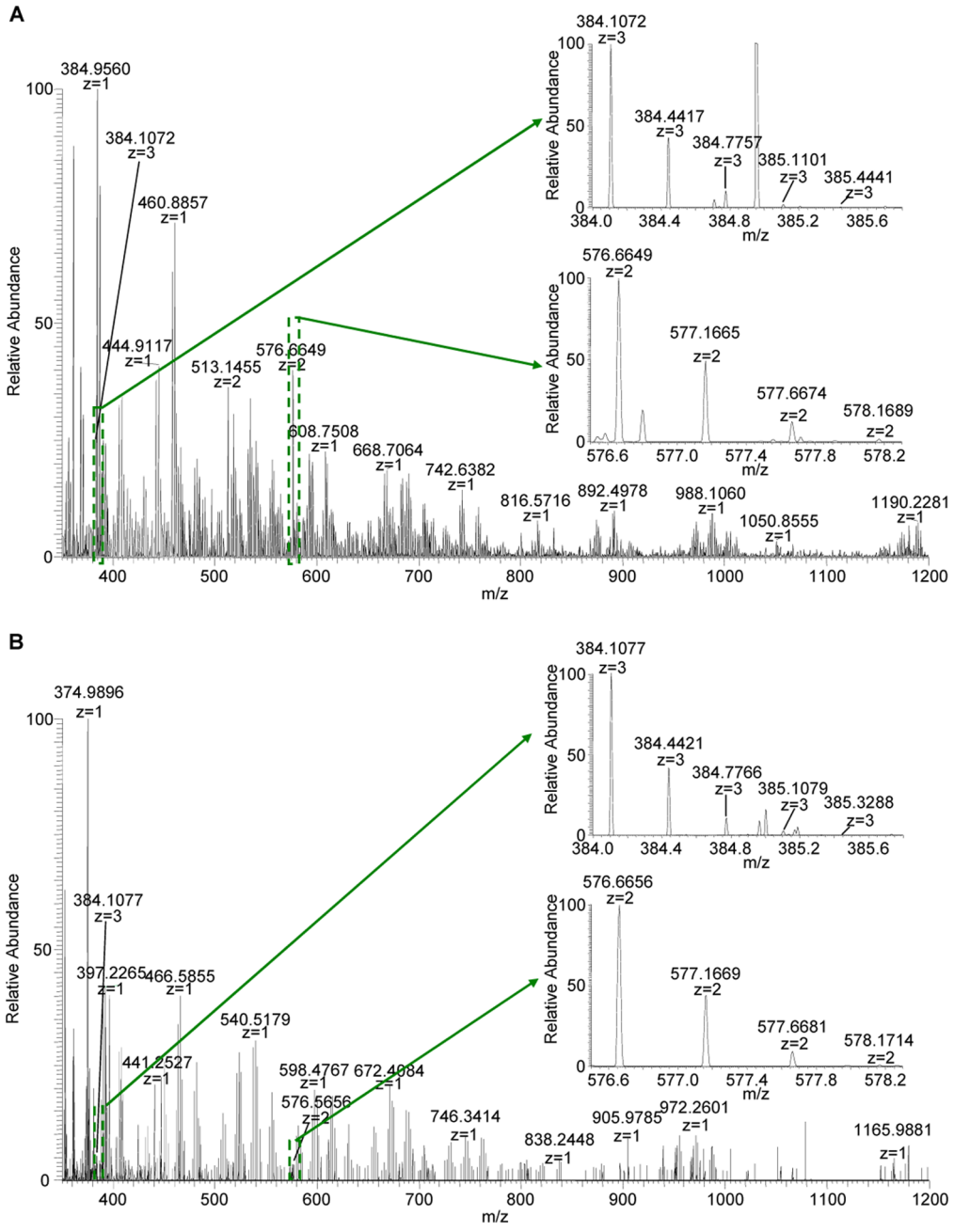

Figure 3. Mass spectrometry detection and analysis of translocated HA hexasaccharide. Full negative ESI mass spectra of the trans compartment content upon hexasaccharide translocation experiment (A), and purified hexasaccharide (1 nM) (B). Inset: enlargement of spectra in mass range containing the two characteristic ions of HA hexasaccharide at *m/z* 384.107 (*z* = 3) and *m/z* 576.665 (*z* = 2). Direct infusion of 5 μL of sample in water/methanol (1/1) solution.

and decasaccharide and they could be detected in the oligosaccharide mixture.

Evidence of the effective translocation of molecules through protein nanopores, leading to their accumulation into the trans compartment, is a recurrent issue in nanopore studies.[14] To address this issue, we have used electrospray mass spectrometry (ESI−MS) to achieve the sensitive detection of translocated HA hexasaccharide molecules in the trans compartment. For that purpose, a high-resolution linear ion trap−orbitrap hybrid ESI-LTQ−Orbitrap mass spectrometer was used, allowing sensitive detection and accurate identification by tandem mass spectrometry (MS/MS) experiments. Trans compartment content corresponding to the pool of seven repeated translocation experiments was dialyzed and freeze-dried, and then analyzed by ESI-MS in negative ionization mode. The negative ion mass spectrum of the trans compartment clearly showed characteristic ions at *m/z* 384.107 and 576.665 (Figure 3A) corresponding to tri- and di-charged species of HA hexasaccharide,[23] in full agreement with the spectrum of purified HA hexasaccharide

(Figure 3B). These diagnostic ions were not detected in the control experiment carried out without oligosaccharide in the cis-compartment (Supporting Information Figure S1). This identification was unambiguously ascertained by multistage fragmentation mass spectrometry MS^n (n = 2 to 4) of these hexasaccharide ions, which led to characteristic fragments (Supporting Information Figures S2 and S3). The control experiment carried out in the absence of an aerolysin channel (oligosaccharides in the cis compartment) led to a mass spectrum devoid of any characteristic HA ions (data not shown), excluding any artifactual translocation of oligosaccharide in the trans compartment. These results showed the direct detection of translocated molecules by mass spectrometry and definitely evidenced translocation of molecules through a protein nanopore. In an attempt to estimate the amount of translocated hexasaccharide, the MS signal of the diagnostic ions was integrated over 1 min from hexasaccharide solutions at 1, 10, and 100 nM. A total of about 200 fmoles of translocated hexasaccharide was estimated in the pooled sample, in agreement with the overall number of recorded translocations events.

The ability of a nanopore to discriminate oligosaccharides at the single-molecule level opens promising analytical applications, notably when various oligosaccharides in mixture have to be distinguished. We took advantage of this capability to monitor the enzyme depolymerization of hyaluronic acid by hyaluronidase (EC 3.2.1.35), which catalyzes the hydrolysis of the $\beta(1\rightarrow4)$ glycosidic bonds between D-glucuronic and *N*-acetyl-D-glucosamine residues. For that purpose, enzyme depolymerization was first performed outside the nanopore. Samples were withdrawn from the reaction mixture during the enzyme reaction, and then added to the cis compartment. The nanopore recording for each sample enabled the monitoring of the variation of duration and frequency of translocation events throughout the depolymerization reaction (Figure 4). It showed a decrease of the event duration upon the enzyme reaction time (Figure 4B), indicating a decrease of the polymerization degree of the translocated oligosaccharides as expected from the enzyme reaction progress. The average dwelling time was 254 μs after 30 min reaction, suggesting the cleavage of the hyaluronic acid polymer into fragments larger than decasaccharide on average at the earlier stage of the enzyme reaction. The dwelling time reached a plateau at an average value of 76 μs indicating the completion of the depolymerization and the accumulation of short oligosaccharides smaller than decasaccharide.

The event frequency (event/sec) is linked to the interaction of oligosaccharides with the pore entrance and depends on the concentration of the probed molecules in the cis compartment.[9] No event was recorded before the addition of the enzyme, indicating that none of the substrate molecules was able to

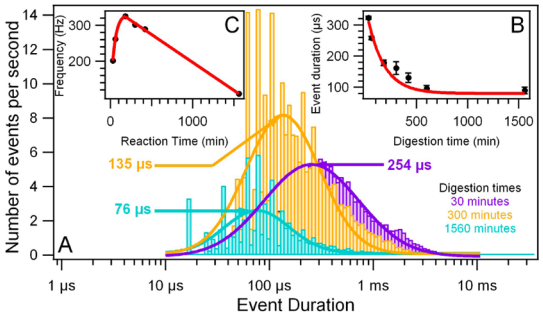

Figure 4. Nanopore recording of hyaluronidase activity. (A) Distribution of dwelling times upon progress of the depolymerization enzyme reaction. Event distributions at 0.5, 5, and 26 h of the enzyme reaction were fitted by a log-normal function allowing the determination of the corresponding average dwelling time. (B and C) Evolution of the average dwelling time and of the event frequency in the course of the enzyme reaction. Depolymerization enzyme reaction of hyaluronic acid (3 mg/mL) catalyzed by hyaluronidase (0.15 mg/mL) in 0.1 M NaAc, 0.15 M NaCl, pH 5.2, 37 °C.

translocate through the nanopore as expected from the high molecular weight of hyaluronic acid polymer (1.63×10^6 g·mol^{-1}). The frequency rose to 200 ± 12 Hz after 30 min of reaction (Figure 4C), indicating a concentration increase in the cis compartment of molecules that can be translocated across the nanopore. The subsequent frequency increases to 260 ± 10 Hz at 60 min, and then to 320 ± 17 Hz after 3 h reflected the reaction progress. In the meantime the concomitant decrease of dwelling time indicated the formation of shorter oligosaccharides. Beyond 5 h of reaction, event frequency decreased, meanwhile translocation duration still diminished. It is likely that the di- and tetrasaccharide limit products were mainly formed at this time of reaction. However, these short oligosaccharides could not be discerned from peak noise given their low polymerization degree, as previously observed with nanopore translocation of purified di- and tetrasaccharides.[17] Therefore, the measurement of event frequency is likely affected by loss of signal at this later time of reaction.

In an attempt to perform a real-time reaction monitoring, the enzyme depolymerization was directly carried out in the cis compartment containing the polysaccharide substrate. For that purpose, enzyme reaction conditions were adapted to allow nanopore translocation and detection, that is using a lower temperature (21 °C in the nanopore device instead of 37 °C) and a higher ionic strength (1 M KCl instead of 0.15 M NaCl). No translocation event was detected before the addition of hyaluronidase to the cis compartment given the high molecular weight of hyaluronic acid. Shortly after hyaluronidase addition, few events of long time duration (millisecond range) were recorded, suggesting the translocation of HA long fragments. Afterward, event frequency greatly rose in parallel with a decrease of the dwelling time along the reaction progress (Supporting Information, Figure S4),

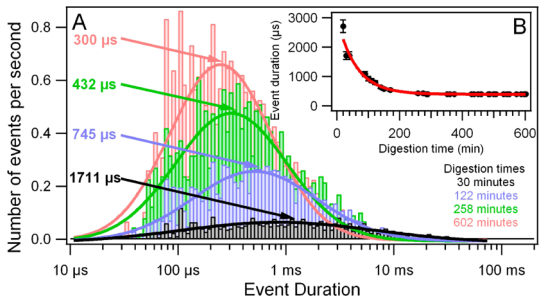

Figure 5. *In situ* real-time nanopore recording of hyaluronidase enzyme activity. (A) Distribution of dwelling times upon progress of the depolymerization enzyme reaction that was directly conducted in cis compartment. Average dwelling times were determined at 30, 122, 258, and 602 min of the reaction course. (B) Evolution of the average dwelling time in the course of enzyme reaction. Depolymerization enzyme reaction of hyaluronic acid (0.75 mg/mL) catalyzed by hyaluronidase (0.075 mg/mL) in 800 μL of 0.1 M sodium acetate buffer, 0.15 M NaCl, pH 5.2 added to 200 μL of 4.5 M KCl, 5 mM HEPES, pH 7.5, 21 °C.

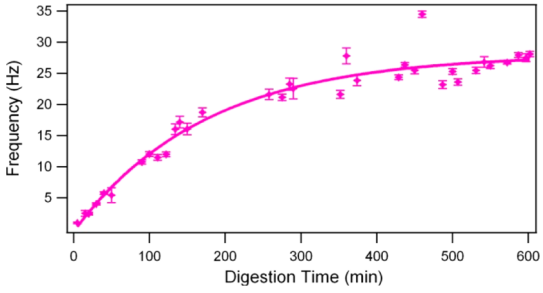

Figure 6. Variation of event frequency during the hyaluronidase-catalyzed HA depolymerization conducted in the cis compartment. Increase of the event frequency all along the enzyme reaction progress showing the increase of the concentration of smaller oligosaccharides in the cis compartment. Depolymerization enzyme reaction of hyaluronic acid (0.75 mg/mL) catalyzed by hyaluronidase (0.075 mg/mL) in 800 μL of 0.1 M NaAc, 0.15 M NaCl, added to 200 μL of 4.5 M KCl, 5 mM HEPES, pH 7.5, 21 °C.

indicating a concentration increase of HA fragments in the cis compartment along with the decrease of their size (Figure 5).

It is worth noting that an average dwelling time of 300 μs was reached after 10 h reaction, while a near value (254 μs) was recorded after only 30 min during the reaction achieved outside the nanopore (Figure 4). This feature pointed to a lower reaction rate of hyaluronidase in the cis compartment, likely due to the lower temperature and the high ionic strength used in the nanopore device, as confirmed by the spectrophotometric assay of the hyaluronidase reaction (Supporting Information Figure S5). Nevertheless, this result evidenced the feasibility of a real-time monitoring of the hyaluronidase-catalyzed HA depolymerization in the conditions required by nanopore translocation and recording. The variation of the event frequency resulting from the increase of reaction products in the cis compartment led to a classic progress curve for an enzyme reaction (Figure 6), allowing the monitoring of depolymerization kinetics at the single-molecule level.

SUMMARY

The translocation of a glycosaminoglycan, hyaluronic acid, through a protein nanopore was observed for the first time. So far, the main evidence for molecule translocation relied mainly on the recording of ionic current variation, since the direct detection of translocated molecules in the trans compartment remains a difficult task due to the inherent low number of translocated molecules. Indirect detection based on an amplification procedure such as PCR has been previously reported for the detection of translocated single-stranded DNA,[14] but such amplification tool is not available for carbohydrates. Here, mass spectrometry analysis was applied for the first time to achieve the direct detection of translocated hyaluronic acid oligosaccharides. The anionic oligosaccharides could be discriminated according to their size through measurement of the dwelling time and event frequency, supporting the potential of nanometer-scale pores for mass spectrometry applications.[12,13] This molecular sieve feature of protein nanopores allowed us to follow the formation of oligosaccharides arising from the enzymatic depolymerization of hyaluronic acid polysaccharide. Single molecule detection of enzyme activity is an emerging application in the field of nanopore biosensors as recently reported for DNA polymerase and peptidase,[14,24,25] yet never described for carbohydrates. Here, the time-resolved recording of ionic current variation arising from the enzyme reaction on a glycosaminoglycan substrate allowed the real-time monitoring of hyaluronidase. GAGs have been recently considered as important biomarkers in metabolic disorders, cancer, and inflammatory and immune diseases.[26,27] These reported results support the nanopore tool as a valuable label-free approach for the detection and characterization of such GAGs biomarkers, which today remain a challenging task in glycosciences.

METHODS

Preparation of Hyaluronic Acid Oligosaccharides. Hyaluronic acid hexa- to decasaccharide (HA-dp6 to HA-dp10, dp = degree of polymerization) was prepared from enzyme digestion of hyaluronic acid sodium salt from *Streptococcus equi* (Sigma) by bovine testes hyaluronidase (E.C. 3.2.1.35, Sigma) as previously described[28] Briefly, 3 mg of hyaluronic acid was solubilized in 1 mL of buffer containing sodium acetate 0.1 M, NaCl 0.15 M, pH 5.2. The solution was shaken for 1 h and let overnight at 4 °C to provide a complete dissolution. This solution was heated at 37 °C for 30 min. Finally, 0.15 mg of hyaluronidase was added and the solution was incubated at 37 °C for 5 h.

Digestion products were then size-separated using a Bio-Gel $P\overline{6}$ column (Bio-Rad; 5 × 100 cm) equilibrated with NH_4HCO_3 0.2 M (pH 8.3) and run at 1 mL/min. Elution performed using the same buffer was followed by refractive index detection (RI-71, Merck). Each isolated oligosaccharide fraction from tetra- to decasaccharide was freeze-dried with two intermediate lyophilization steps, and then suspended in ultrapure water and stored at −80 °C. Ultrapure water (Milli-Q, Millipore, Milford, USA) was used. Other chemicals and reagents were obtained from commercial sources at the highest purity available.

Single Nanopore Formation and Recording of Oligosaccharide Transit. In a typical single nanopore experiment, two compartments filled with buffered electrolyte were separated by a lipid bilayer. The membrane lipid bilayer was formed according to a previously described method, using 1% of diphytanoyl phosphatidylcholine−lecithine (Avanti Polar Lipids) in decane (SDS).[29] The lipid solution was spread over an aperture 90 mm in diameter size perforated in a polysulfone plate (Warner Instruments, USA). The planar bilayer was formed upon thinning of the decane film at room temperature. The polysulfone plate was then inserted in a device divided in two compartments, cis and trans (the cis compartment is connected to the ground). Both compartments were filled with 1 mL of 5 mM Hepes and 1 M KCl pH 7.5. A 70 mV potential was applied, using Ag−AgCl electrodes in contact with the electrolyte within each compartment. The lipid membrane was then thinned until having 65 ± 5 pF capacitance corresponding to 4.85 ± 0.4 nm in thickness. The formation of a single protein nanopore was initiated by the addition of 2 μL of 3 μM aerolysin to the cis-compartment.[6] Aerolysin was produced in *Escherichia coli* as proaerolysin as described previously.[30,31] It was activated by digestion with trypsin 10 min at room temperature prior to introduction in the cis-compartment to eliminate propeptide sequence and to allow polymerization of the aerolysin monomer.[32] A 70 mV potential was applied, and the ionic current through the pore was measured by a Dagan current amplifier, filtered at 10 kHz and sampled at 200 kHz. Under these conditions, an ionic current of $I_0 = 32 \pm 2$ pA was recorded indicating that a single nanopore was inserted in the membrane. This current value corresponded to the open pore in an unoccupied state.

For the nanotransit experiment, 100 μM purified oligosaccharide was added to the cis-compartment containing 5 mM Hepes buffer, 1 M KCl pH 7.5. The addition of oligosaccharides in the cis compartment resulted in transient current decreases due to interaction of the sugars with the nanopore. The current blockades were characterized according to the following parameters, duration, frequency, and amplitude, which were determined for each purified oligosaccharide ranging from hexa- to decasaccharide. Current traces were analyzed with Igor Pro software (WaveMetrics, USA).

Nanopore Recording of Hyaluronic Acid Depolymerization Catalyzed by Hyaluronidase. Aliquots were withdrawn from the reaction mixture during the enzyme depolymerization of hyaluronic acid performed as above-described.[28] The reaction was stopped by heating the aliquots at 100 °C for 1 min. Then, a 800 μL aliquot was mixed with 200 μL of 4.5 M KCl, 5 mM HEPES, pH 7.5, and the mixture was added to the cis compartment at ambient temperature. Alternatively, enzyme depolymerization of hyaluronic acid was directly performed into the cis compartment to record the real-time progress of the enzyme reaction. For that purpose, the cis compartment was filled with 800 μL of 1 $mg \cdot ml^{-1}$ hyaluronic acid in 0.1 M sodium acetate, 0.15 M NaCl, pH 5.2 mixed to 200 μL of 4.5 M KCl, 5 mM HEPES, pH 7.5. The enzyme reaction was initiated by the addition of 7.5 μg hyaluronidase. A 70 mV potential was applied, and the ionic current was measured all along the enzyme reaction.

Spectrophotometric Assay of Hyaluronic Acid Depolymerization. The assay solution was composed of 1 mL of 3 mg/mL hyaluronic acid, in 0.1 M sodium acetate buffer, NaCl 0.15 M, pH 5.2. Enzyme depolymerization was initiated by the addition of 15 μL of 0.1 mg/mL hyaluronidase, and the reaction mixture was incubated at 37 °C. For comparison purposes, the enzyme reaction was also carried out in conditions used for the real-time recording in the cis compartment. Assay solution was composed of 800 μL of 1 mg/mL hyaluronic acid, in 0.1 M sodium acetate buffer, NaCl 0.15 M, pH 5.2 HA mixed to 200 μL of 4.5 M KCl, 5 mM HEPES, pH 7.5. Enzyme depolymerization was initiated by the addition of 7.5 μg of hyaluronidase, and the reaction mixture was incubated at 21 °C.

Samples were withdrawn all along the enzyme reaction and heated at 100 °C for enzyme inactivation. 30 μL of 1/200 (reaction at 37 °C) or 1/100 (reaction at 21 °C) diluted samples were mixed with 30 μL of 1.1 mg/mL WST-1 reagent (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, water-soluble tetrazolium salt WST-1, Fisher scientific) in 0.1 M. NaOH. The absorbance at 584 nm was then measured after incubation at 60 °C for 60 min (Supporting Information, Figure S5).[33]

ESI-LTQ-Orbitrap Mass Spectrometry Analysis of Translocated Oligosaccharide Molecules in Trans-Compartment. The trans-compartment content corresponding to 7 consecutive translocation experiments of hyaluronic acid hexasaccharide was pooled and gel filtrated on Sephadex G-25 column equilibrated in water beforehand (PD MidiTrap G-10, GE Healthcare, Uppsala, Sweden). The desalted sample was freeze-dried and suspended in 15 μL of a water/methanol 1/1 mixture prior to ESI-MS analysis. A control experiment was carried out without oligosaccharide in the cis-compartment (Supporting Information, Figure S1).

ESI-MS experiments were carried out using a LTQ-Orbitrap XL from Thermo Scientific (San Jose, CA, USA) and operated in negative ionization mode, with a spray voltage at −3 kV, and a sheath gas flow rate of 11 arbitrary units. A water/methanol 1/1 mixture was continuously infused using a 250 μL-syringe at 5 μL/min flow rate through a 10 μL loop. A 5 μL sample in water/methanol 1/1 was injected, providing a consistent signal for at least 1 min. Applied voltages were −19 and −120 V for the ion transfer capillary and the tube lens, respectively. The ion transfer capillary was held at 275 °C. Resolution was set to 30 000 (at *m/z* 400) for all studies, and the *m/z* ranges were set to 300−1200 in profile mode and in the high mass range during full scan experiments. Spectra were analyzed using the acquisition software XCalibur 2.0.7 (Thermo Scientific, San Jose, CA, USA), without smoothing and background subtracts. During MS/MS scans, collision induce dissociation (CID) fragmentation occurred in the linear ion trap analyzer and detection in Orbitrap with centroid mode. For CID fragmentation, an activation *Q* value (*Q*) of 0.25 and an activation time (*T*) equal to 30 ms were used. Normalized collision energy (NCE) was set at 20% (MS/MS), 15% (MS^3), and 20% for MS^4 for the doubly charged species, and at 15% for all MS^n (with $n = 2$ to 4) steps for the triply charged species.

The automatic gain control (AGC) allowed accumulation up to 1×10^6 ions for FTMS scans, 2×10^5 ions for $FTMS^n$ scans and 1×10^4 ions for $ITMS^n$ scans. Maximum injection time was set to 500 ms for both FTMS and $FTMS^n$ scans and 100 ms for $ITMS^n$ scans. For all scan modes, 1 microscan was acquired. The precursor selection window was 2 Da during MS^2 experiments. Supporting Information, Figures S3 and S4 display negative mass spectra upon sequential fragmentation of translocated hyaluronic hexassaccharide.

Conflict of Interest: The authors declare no competing financial interest.

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Supporting Information Available: Multistage fragmentation mass spectrometry MS^n of hyaluronic acid hexasaccharide and spectrophotometric assay of hyaluronidase. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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