Functionalized Solid-State Nanopore Integrated in a Reusable Microfluidic Device for a Better Stability and Nanoparticle Detection

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Supporting Information

ABSTRACT: Electrical detection based on single nanopores is an efficient tool to detect biomolecules, particles and study their morphology. Nevertheless the surface of the solid-state membrane supporting the nanopore should be better controlled. Moreover, nanopore should be integrated within microfluidic architecture to facilitate control fluid exchanges. We built a reusable microfluidic system integrating a decorated membrane, rendering the drain and refill of analytes and buffers easier. This process enhances strongly ionic conductance of the nanopore and its lifetime. We highlight the reliability of this device by detecting gold nanorods and spherical proteins.

KEYWORDS: nanopore functionalization, solid-state nanopore, polymer brush, ionic conductance, nanopore transport, microfluidics, protein

Protein channels, with an electrical detection, have been extensively used to study the dynamics of polymers, polyelectrolytes and biomolecules in nanopores.1–3 This technology is nowadays used for several applications such as fast DNA sequencing,4 mass spectrometry for polymers5,6 and oligonucleotides.7 Insertion of these channels into lipid bilayer lends itself to fluidic miniaturization. The decrease of the bilayer size down to micrometric scale increases strongly its stability, which allows its integration in a microfluidic architecture.8 Efficiency of such a device has been proven with protein channels.9

Meanwhile, biomimetic solid-state nanopores were designed10 and drilled in a silicon-based membrane using a focused ion beam11–13 and more recently by electron beam.14,15 Other materials have been used for these membranes to slow down DNA transport such as aluminum oxide16 or hafnium oxide.17 Design of a viable industrial device requires a better wettability and a longer lifetime. It is then crucial to control the state of these high energy surfaces to avoid nonspecific interaction13,18 and nanopore clogging just after a few hours, even after piranha17 or plasma2,16 cleaning.

Membrane surface treatment could allow overcoming this challenge by avoiding nonspecific interactions of polynucleotides20–22 or proteins.23,24 Another strategy uses lipid coating to enhance protein detection.25,26 An additional challenge, for upcoming applications with solid-state nanopores, is their integration into a microfluidic device that is easy to manufacture. Up to now, few groups used microfluidic devices dedicated to arrays of nanopores or protein detection.27 Grafted nanopores with microfluidic integration makes possible the design of medical devices allowing the detection of specific cancer biomarkers such as circulating microRNAs,28 aberrant DNA methylation15 during cancer development, or prostate specific antigen.29

In this work, we propose to design and manufacture a PDMS (poly(dimethylsiloxane)) microfluidic circuit for solid-state nanopore from a low-cost mold made with a 3D-printer, which is much less expensive and time-consuming than the traditional way. This hermetic setup permits to avoid evaporation and allows experiments to run for a long time (several days). The chamber volume is decreased down to 40 μL to facilitate drain and refill with buffer solution. The membrane surface is controlled by decoration with polymer chains. We control the apparent radius and ionic conductance of the nanopore according to coating density and length of the grafted polymer chains. The control of interactions between nanoparticles (NPs) and the nanopore allows the detection of (1) long gold nanorods, (2) spherical thyroglobulin proteins according to the coated chain length.

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The solid-state nanopore functionalized by PEG (poly(ethylene glycol)) chains and its integration into microfluidic system is shown in Figure 1. This low-cost device with integrated chips is easy to manufacture and highly reproducible (see the Supporting Information). It can last at least two months of intensive use. The role of microfluidics in our setup is to ease the fluid exchanges, decrease the volume of the sample, enhance the reproducibility of the assay, and finally ease the integration of the nanopore chips within the experimental setup. The microfluidic structure is made of three stacked layers of PDMS, which are reversibly assembled: (1) the inlet circuit to the lower side of the chip (in blue Figure 1b), (2) an intermediate thin PDMS plate for the sealing of the solid state nanopore chip, (3) the outlet circuit on the upper side of the chip (in red Figure 1b). These three layers are stacked and assembled using an external shell designed by 3D printer. Changing the buffer solution or cleaning the chip with water is easily and quickly done without unmounting the device thus avoiding any contact with air. Also, the dead volume is easily and quickly done without unmounting the device. This device is dedicated to thus avoiding any contact with air. Also, the dead volume is easily and quickly done without unmounting the device.

Water is easily and quickly done without unmounting the device. The microstructure is made of (ethylene glycol)) chains and its integration into microfluidic device. (c) Current trace measured in the presence of thyroglobulin protein by one nanopore (radius R = 14 nm, length L = 20 nm) grafted by PEG 7 kDa chains. I0 is the mean amplitude of open pore current. ΔI is the mean blockade current, T in is the blockade duration. ΔV = 1 V, 50 mM LiCl, 0.1 M Tris, pH 7.4.

After polymer grafting, we clearly observe a contrast in the IV curve) before and after PEG 1 kDa functionalization shows a linear behavior where the slope corresponds to the conductance between both types of salt. We probe if the functionalized nanopore is able to discriminate the conductance between both types of salt. We show that the slope of these IV curves, i.e., the conductance depends on the salt concentration and on the nature of the salt (Figure 3).

We plot the conductance as a function of salt concentration. The same nanopore is used in the same device with both KCl (29 experiments) and LiCl (22 experiments). Each study is performed with 8–9 different buffer concentrations from 1 μM to 2 M. We observe two regimes at very low and high salt concentrations. In presence of nanoparticles, we observe current blockades characterized by their amplitudes ΔI and durations T in. The current trace of Figure 1c shows several current drops after the addition of thyroglobulin. The shape of these blockades will be discussed in the discussion section. The statistical treatment of these values allows a high resolution for the analysis of spherical biological nanoparticles.

Functionalization allows the control of chemical and physical surface properties.10 The grafting is checked by both microscopic and macroscopic characterizations: HRTEM (high-resolution transmission electron microscopy) and contact angle measurements. This surface modification is also probed by measuring the conductance of the nanopore. We have used well-known methods to functionalize the surface of solid-state nanopores. These coating methods are usually performed in only one step.20 In this work, we follow a two-step pathway to avoid heterogeneities due to steric effects of the grafting polymer chains and to increase the grafting density (Figure 2a).31 This pathway only necessitates silanol groups for Si, SiO2, or SiN membranes or HF–O groups for HfO2 based membranes. These groups are activated by ozonolysis to react with derivates of alkoxysilane (3-chloropropyl-trichlorosilane) and then allow an indirect grafting of the polymer chains. Before the coating, we measure the contact angle (θ = 45.5 ± 0.8°) and the chemical or physical heterogeneities are characterized by receding and advancing angle (θ = 38.3 ± 1.6°, θ = 49.2 ± 1.3°, respectively). The surface is activated by ozonolysis characterized by a low contact angle θ = 6.1 ± 1.2° showing a dense population of silanol groups. After the PEG 600 grafting, the contact angle increases up to θ = 38.4 ± 1.7°, which shows that we increase the hydrophilic nature of the surface. The new receding and advancing angles (θ = 30.0 ± 1.6°, θ = 44.2 ± 0.5°, respectively) lead to the wetting hysteresis calculation θ = 14.2 ± 2°, which is similar to the one before grafting (Figure 2b). This result demonstrates that chemical heterogeneity and roughness of the surface is not affected by the chemical treatment. This observation is confirmed by HRTEM imaging (Figure S1). According to the first step of functionalization, the chemical reaction is successful if there is presence of hydroxyl groups. Then, this process is independent of the substrate nature, according to treatment success with different materials such as SiN (naturally passivated at the surface), SiO2, and HfO2. Moreover, the second step is less dependent on the length of polymer chain.

At the microscopy level, pore size is controlled by TEM drilling with subnanometer accuracy in several materials such as SiO2 (Figure 2c), SiN (Figure 2d), and HfO2 (Figure 2e). After polymer grafting, we clearly observe a contrast modification inside the nanopore in comparison with its initial state as it is shown in Figure 2c with SiO2 membrane. Examining the effect of the surface modification with electrical measurement, the plot of current as a function of applied voltage (IV curve) before and after PEG 1 kDa functionalization shows a linear behavior where the slope corresponds to the conductance G of the nanopore.30 In the case of the native membrane, the ionic conductance G = 0.47 ± 10^-3 nS is very low compared to the theoretical conductance G = 58 nS according to eq 1 below. In Figure 2f, right, we see that the conductance decreases from G = 0.5 ± 10^-3 nS down to G = 0.08 ± 10^-3 nS after 2 h, whereas the conductance jumps up to G = 29.9 ± 10^-3 nS after the treatment and remains steady (27.7 ± 10^-3 nS) for at least 6 h. This value is more consistent with the one calculated from eq 1. The modification of surface increases the stability of the nanopore conductance.

To determine if the conductance is due to ion transport through the nanopore, we perform IV-curve as a function of salt concentration. Previous publications19 have shown different ionic conductance values as a function of the nature of the salt such as KCl versus LiCl. We probe if the functionalized nanopore is able to discriminate the conductance between both these types of salt. We show that the slope of these IV curves, i.e., the conductance depends on the salt concentration and on the nature of the salt (Figure 3).
concentration. In the first regime, the conductance is constant and independent of salt concentration between 1 to 100 μM. In the second one, between 0.5 and 2 M, conductance increases linearly with salt concentration (dotted lines in Figure 3c). This behavior could be interpreted according to a simplified model considering both the excess counterion conductance on the nanopore surface at very low concentration \( G_{\text{surface}} = \pi (R/L) \mu_+ e/2 \) and the ionic conductance of electrolyte in the nanopore at high concentration \( G_{\text{nanopore}} = \pi \left( R^2/L \right) K_0 \) where \( R = 21 \pm 1 \) nm and \( L = 20 \pm 1 \) nm are nanopore radius and length, respectively. \( K_0 = (\mu_+ + \mu_-) c e^2 \) is the bulk conductivity, \( e \) is the elementary charge, \( c \) the ion concentration, and \( \mu_\pm \) the mobility of positive (\( \mu_{K^+} = 4.76 \times 10^{11} \) m²S/J, \( \mu_{Li^+} = 2.50 \times 10^{11} \) m²S/J) and negative ions (\( \mu_{Cl^-} = 4.94 \times 10^{11} \) m²S/J). The total conductance \( G_0 \) reads

\[
G_0 = G_{\text{nanopore}} + G_{\text{surface}}
\]

This expression is plotted on Figure 3c) and leads to the evaluation of surface charge \( \sigma = 0.022 \pm 0.001 \) C/m² for both KCl and LiCl buffer. This surface charge has the same magnitude that the one we could observe in SiNₓ nanopore (σ

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**Figure 2.** Surface state control of membranes. (a) Chemical pathway to graft PEG chains on the membrane surface. (b) Contact angle of a sessile water droplet on a Si based surface before and after the PEG 600 grafting. Measurements of advancing and receding contact angles. White bar = 1 cm. (c) Left: HRTEM picture of a bare SiO₂ nanopore; right: STEM picture with high-angle annular dark-field imaging (HAADF-STEM) of the same pore after PEG 600 grafting. The red arrow points to a PEG layer visible inside the nanopore. (d) HRTEM view of a SiNx nanopore. (e) HAADF-STEM imaging of a HfO₂/SiNx nanopore. (f) Evolution of IV curves before and after the grafting of PEG 600 on SiO₂ nanopore. Left: IV curves before (warm colors) and after (cool colors) grafting. Right: focus on IV curves before grafting. 0.1 M KCl 0.1 M Tris pH 8, nanopore \( R = 17.5 \) nm \( L = 30 \) nm.
Figure 3d shows that the normalized conductance $G/G_0$ does not fluctuate during 2 days according to the error bars. Error bars and variations are due to the difference between experimental data and the theoretical curve calculated from eq 1. Despite these variations, dotted lines are obtained by fitting data with a constant value equal to $0.99 \pm 0.1$ for KCl and $1.0 \pm 0.1$ for LiCl buffer. This duration could be extended until 3 weeks (Figure S3).

To probe the sensor capabilities for nanoparticle detection, we compare now two different nanoparticle shapes and materials: gold nanorods and spherical thyroglobulin protein. In the first case, the membrane is grafted with PEG 600 chains. Current traces show some spikes in the presence of nanorods. We plot the blockade current $\Delta I$ of each blockade as a function of the corresponding dwell time. From this cloud, we extract three types of events according to blockade shapes described by Wu et al.\textsuperscript{35} The first one is associated with short blockades with low magnitudes and could be interpreted as bumping (inset b-red, 62% of blockade number). The second one is characterized by longer durations with low amplitudes and is attributed to one nanorod oscillating at the entrance of the nanopore without reaching its exit (inset b-green, 34% of blockade number). Finally, just a few events are defined by large duration and high amplitude corresponding to a conductance decrease due to the transport through the channel (inset b-blue, 4% of blockade number).

In presence of thyroglobulin (Ø = 18 nm), the current trace remains steady when the membrane is decorated with PEG 1 kDa (Figure S4). Considering the protein charge ($-140$ e), we could evaluate the electrophoretic time $T_{\text{elec}} \approx 3$ ns and the diffusion time $T_{\text{diff}} \approx 10$ $\mu$s. As these times are below or similar to the sampling time (5 $\mu$s), we are not able to detect these blockades.\textsuperscript{13} To detect proteins, we must increase translocation time. First, we change the material using a SiNx membrane.
covered by a 5 nm of HfO2 and grafted with PEG 1 kDa chains. In presence of gold nanoparticles (r = 5 nm, 1 mM), we observe current blockades which are mostly attributed to bumping (Figure S5). Then we use a biomimetic hairy channel obtained after the grafting of long PEG 7 kDa chains to increase friction applied to protein inside the nanopore.36 In presence of thyroglobulin, we mainly observe very well-defined current decrease with high blockade ratio around 99% and long durations larger 8.6 ± 0.4 ms (Figure 4 d-blue, Figure S6). This ratio is much larger that the theoretical one (25%) considering the nanopore without polymer grafting. These events constitute the larger part of blockades (67%) and are associated with the dwelling of thyroglobulin. We observe two other populations. The first one with amplitudes around 25 nA and duration about 3.5 ± 0.1 ms (Figure 4 d-green, 21% of all the blockades) is attributed to presence of thyroglobulin at the entrance of the pore. The second one has a small amplitude around 12 nA with short times 220 ± 10 µs (Figure 4d-red, 12% of all the blockades), attributed to bumping of proteins at the pore entrance.

Reclaiming solid-state nanopores requires aggressive piranha treatment17 or oxygen plasma.2,13,19 After this process, surface energy is increased allowing a better wetting, but impurities can also be attracted, decreasing the lifetime and reliability of device based on this family of nanopore (inset Figure 2f). Several approaches have been performed to improve the ionic conductance stability of solid state nanopores to control pore diameter, selectivity and transport properties. The main advantages of a solid-state nanopore decorated with a fluid lipid bilayer26,37 are the control of the thickness and surface chemistry of the lipid coating by the choice of lipids in the liposome preparation, the possibility to incorporate ligands in the membrane to confer specificity and slowdown the dynamics of protein. The main disadvantage is the difficulty to coat lipid bilayer on the solid surface from a liposome. The other approach is to perform a chemical modification, i.e., covalent functionalization, with polymer chain,20 chemical groups,21 protein, or antibody.23,24 The main advantages are also a best control of the pore diameter, dynamics of proteins, DNA transport through the nanopore, and the selectivity. The main disadvantage is due to chemical reaction and the control of the number of chemical groups grafted to the surface membrane and the difficulty to functionalize inside the nanopore.

We describe here for Si, SiNx, SiOx, or HfO2 nanopores that polymer grafting leads to a better wettability of the membranes, which may be due to a decrease of the surface energy (Figure 2). This pathway could be applied to other oxidized surfaces such as Al2O3.38 Each step of this surface treatment on a Si wafer, which is oxidized on the surface, is followed by contact angle measurement. At the nanometric level, the grafting is illustrated by high-angle annular dark-field imaging (HAADF). It enhances strongly both the nanopore conductance (Figure 2) and its reliability (Figure 3) in various electrolytes (KCl or LiCl). We focus on the ionic conduction at the inner surface of the nanopore to determine if the PEG 1 kDa grafting occurs inside the nanopore or at the membrane surface (Figure 3). At high ionic concentration, the conduction is mostly governed by ionic flow. At low ionic concentration, it is mainly due to the counterions at the inner surface. If grafting had occurred into the nanopore, we could expect a very low surface charge density. As we measure a value corresponding to a SiN0 surface, σ = 0.022 ± 0.001 C/m2 (KCl buffer) after PEG 1 kDa grafting, we think these chains are grafted just at the entrance of the pore, but not into the nanopore. Another argument comes from the complexation of K+ ions by PEG 1 kDa chains. If this complexation took place, we should measure a different surface charge in the presence of K+ and Li+ ions, knowing the latter does not complex with PEG 1 kDa chains.39 As the surface charge is the same in both cases, PEG 1 kDa chains do not graft the inner part of the nanopore. These chains favor the access of ions into the nanopore (Figure 2f), but modify neither their transport through this channel nor their conductance at low ionic concentrations.

Our nanopore chip, thus functionalized and integrated within a microfluidic connecting structure, was tested with the following experiments: first we measured the ionic current through a nanopore grafted by PEG 1 kDa in the presence of long gold nanorods. We mainly observe interactions between the nanorods and the nanopore and only a few transport events. Then, we grafted longer PEG chains to increase the interactions between the grafted chains and the nanoparticles. The pore is partially blocked in its initial state presumably by the PEG chains.40 In the presence of thyroglobulin, we only observe a few bumps between the proteins and the pore and mainly blockades with high amplitude, which have not the same shape than the one observed with nanorods. When thyroglobulin enters the pore, it almost completely blocks the pore. Upon leave, the pore apparent diameter transiently increases. We presume that PEG conformation in the pore is changed by thyroglobulin and then goes back to its initial state. Considering the blockade current and dwell time, the friction between the nanopore and the nanoparticle is strongly increased by PEG grafting. As the protein radius is 9 nm, we could then suppose that the apparent inner size of the nanopore has the same value. This decrease means that the PEG layer is around 5 nm thick. Considering that polymer chain behaves as ideal coil, its end-to-end length is equal to 4.4 nm, thus in good agreement with electrical measurements. Then, PEG conformation on the membrane surface is the one of a coil slightly deformed by the ionic flow. Moreover, as thyroglobulin is strongly charged (−140e), it is surrounded by its counterions, which contributes to the current increase after each blockade (Figure 4 c).39

The integration of solid-state nanopore into a low-cost microfluidic circuit made by a 3D-printer allows to obtain a powerful reusable system with a high signal-to-noise ratio, which is capable of being disassembled and reassembled. This setup increases the reliability of solid-state nanopore based devices by avoiding the eventuality of external contamination. When hermetically closed, it can be used during several days. The capability to drain and fill the circuit is applied to polymer chain grafted nanopores. The sample volume is very low; a few microliters. We show that the grafting of polymer chains takes place on the membrane surface, but not on the inner surface of the channel. This surface treatment protects the nanopore and allows long time experiments. This device allows the detection of gold nanorods and spherical thyroglobulin. It discriminates each nanoparticle type according to the blockade shape. Long polymer chains favor long nanoparticle dwell time. This decorated nanopore constitutes a first step toward biomimeticism of natural channel such as nuclear pore complex.53 We plan to apply this approach for the detection of various nanoparticles as potential biomarkers or environment pollutants.
Principle for TEM piercing and observation; detailed description of the surface grafting, the contact angle measurements, microfluidic device and data acquisition; stability of nanopore conductance; conductance of decorated SiN nanopore; dwelling of gold nanoparticles in decorated HfO2/SiN nanopore; detailed view of current blockade in the presence of thyroglobulin protein into decorated SiN nanopore with PEG 7 kDa (PDF)

**REFERENCES**


