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Tuning nanopore surface polarity and rectification properties through enzymatic hydrolysis inside nanoconfined geometries†

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Enzyme-catalyzed reactions inside nanoconfined geometries promote sensitive changes in the surface charge polarity and the concomitant permselective transport properties of the single conical nanopores.

In living organisms, ion channels and pores embedded in the cell membrane regulate and control the passage of ions or particular analyte molecules across the membrane to facilitate the chemical and electrical communication with the extracellular environment, *e.g.* for metabolic and signalling purposes.[1] They work as very sensitive and versatile nanodevices, *e.g.*, (i) diodes for ionic current that restrict ion flow in one direction, (ii) ion pumps, which control ion flow against concentration gradient and (iii) ion sensitive elements, which are permselective, *i.e.*, channels preferentially transport certain ions compared to others.[2] Therefore, they are frequently used in the fabrication of nanodevices for bioanalytical applications such as analysis and sequencing of DNA, biomolecular sensing and separation processes.[2d] These nano sized channels are precisely controlled structures with defined interfacial chemistry, and have therefore proved to be very useful for a variety of interesting applications in nano/biotechnology such as sensing and manipulation of single molecules.[3] However, the fragility and sensitivity of the embedding lipid bilayer restrain their suitability for more practical purposes. Conversely, synthetic nanopores have recently attracted a great deal of interest as their geometry, dimensions and surface chemistry can be tuned on demand and they exhibit excellent chemical and mechanical robustness.[4]

Track-etched single conical nanopores display remarkable transport properties such as permselectivity, voltage-dependent gating mechanism and current rectification, very similar to biological ion channels.[5] The ionic transport through these nanopores is controlled by the nature of the surface chemical groups. Recently, several techniques have been reported for modulation of pore surface chemistry to tune nanopore transport properties.[6,7] These involve the covalent attachment of self-assembled monolayers of functional molecules or polymer brushes that respond to specific external stimuli, *e.g.*, heat,[7b] solution pH,[7c] light irradiation,[6a,c] metal ions,[6d,e] biomolecules[6b,7a] or the modulation of both heat and pH.[7d] However, the integration of enzyme-sensitive nanopores still poses a challenge and addressing it would further broaden the scope and application spectrum of nanoporous systems.

Enzymes are involved in almost all catalytic reactions, chemical interactions and signalling events occurring in living organisms. They have also been considered as promising triggering agents because of their highly selective and efficient action towards specific substrates under physiological conditions.[8] A variety of enzymes are involved in the catalytic breakdown of proteins, peptides and amino acid substrate molecules. Recently, the activity of some of these enzymes has been monitored through the changes in nanopore ionic transport properties.[9] Mayer's group has reported the enzyme-induced modulation of electric charge by the enzymatic cleavage of cationic choline, which led to detectable changes in the pore conductance of channel-forming peptide gramicidin A.[9a]

Herewith, we demonstrate a novel methodology to modulate pore surface polarity and permselectivity through enzyme-catalyzed reactions. For this purpose, single conical nanopores are covalently functionalized with substrates for acetylcholinesterase (AChE) and protease enzymes, and upon enzyme action carboxylic acid groups are generated on the inner pore walls (Fig. 1). Consequently, the success of modification and enzyme-catalyzed reactions can be monitored through the changes in current–voltage (*I–V*) characteristics of these nanopores. To study the AChE enzymatic reaction in a confined environment, a chemical compound *O*-(6-aminohexanoyl)-choline (AHCh), which acted as a possible AChE substrate, was synthesized according to the published procedure (ESI,† Scheme S1).[10]

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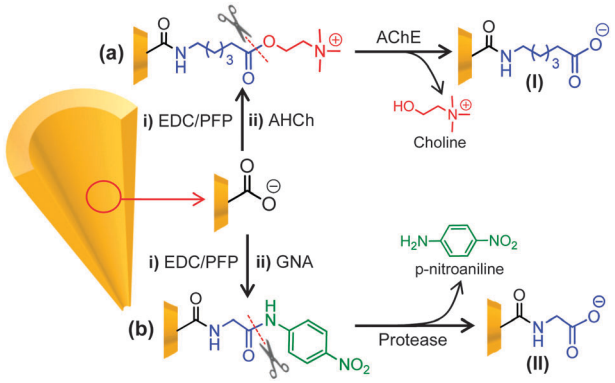

Fig. 1 (a) Functionalization of a single conical nanopore with (a) AChE substrate *O*-(6-aminohexanoyl)-choline (AHCh) and subsequently, the enzymatic cleavage of cationic choline from the inner pore walls; and (b) glycine *p*-nitroanilide (GNA) and subsequently, protease enzyme hydrolyze and cut the uncharged *p*-nitroaniline moieties, leading to the generation of carboxylate groups on the pore surface.

The commercially available *N*-*t*-butoxycarbonyl-6-aminohexanoic acid (**1**) was reacted with 2-chloro-*N*,*N*-dimethylethanamine (**2**) in the presence of potassium carbonate using anhydrous ethyl acetate to yield *N*-dimethyl-*O*-(*N*-*t*-butoxycarbonyl-6-aminohexanoyl)-cholamine (**3**). The crude **3** was refluxed with CH_3I in acetone to get *O*-(*N*-*t*-butoxycarbonyl-6-aminohexanoyl)-choline iodide (**4**). Following the removal of the amine protecting Boc group with dilute acetic HBr solution *O*-(6-aminohexanoyl)-choline bromide hydrobromide (**5**) was obtained. Protease substrate glycine *p*-nitroanilide (GNA) is commercially available and was used without additional purification.

Single conical nanopores were prepared in polyethylene terephthalate (PET) membranes of thickness 12 μm irradiated with swift heavy ions through the well-established asymmetric track-etching technique.[5a] The resulting conical nanopores have small (tip; *d*) and large (base; *D*) openings on the side of the membrane facing neutralizing and etching solutions, respectively. The chemical etching of the ion tracks resulted in the generation of native carboxylic acid (–COOH) groups on the surface and inner pore walls.[11] These groups serve as a starting point to change the chemical characteristics of the nanopore surface by attaching amine-terminated molecules through carbodiimide coupling chemistry.[6,7]

The covalent attachment of amine-terminated enzyme substrate molecules onto the pore surface was carried out in a two-step reaction procedure (Fig. 1). In the first step, –COOH groups were converted into amine-reactive PFP reactive-ester molecules through *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide (EDC)/pentafluorophenol (PFP) coupling chemistry. Subsequently, the PFP-reactive intermediate was covalently coupled with the terminal-amine group of respective enzyme substrate molecules as shown in Fig. 1.

The successful anchoring of the substrate molecules was confirmed by the changes in the *I*–*V* characteristics of the nanopore before and after chemical modification. To perform the *I*–*V* recordings, the single-pore membrane was assembled between the two chambers of the conductivity cell. The electrolyte (100 mM aqueous KCl solution) was filled on both sides of the conical nanopore and the electrodes on each side of the nanopore were arranged in such a way that high currents at positive voltages and low currents at negative voltages were obtained. Fig. 1a shows the *I*–*V* curves of single conical nanopores prior to (black) and after (red) the immobilization of AHCh substrate molecules. As-prepared single conical nanopores exhibit cation-selectivity and rectify the ion current (*i.e.*, cations preferentially flow from the tip towards the base opening) passing through the nanopore due to the presence of ionized $-COO^-$ groups on the pore walls at physiological pH.[5b] The ion current rectification phenomenon in these pores is a consequence of the combined effects of intrinsic geometry and electrostatic asymmetries, which results in different dependences of anion and cation concentrations inside the nanopore when a potential is applied across the membrane.[5c]

In conical nanopores, the direction of ion current rectification is dictated by the electrically charged chemical groups on the inner pore walls.[6,7] Moreover, the degree of rectification (f_{rec}) of the nanopore is directly related to the magnitude of the surface charges. The f_{rec} value is calculated by dividing ionic currents at positive voltages to that of negative voltages and *vice versa*. As expected, the immobilized AHCh molecules onto the pore surface resulted in the flipping of the *I*–*V* curve, *i.e.*, the inversion of current rectification occurred (Fig. 2a). As it can be seen from the *I*–*V* curves, the positive current at +2 V decreased from 1190 pA to 137 pA and conversely, at −2 V applied bias the negative current increased from 220 pA to 811 pA. This clearly indicates the switching of pore surface polarity from negative to positive due to the presence of cationic trimethylammonium head groups, leading to the reversal of permselectivity and consequently, the modified-pore becomes anion-selective – there is preferential transport of anions from the tip to the base opening. The rectification degree calculated from the *I*–*V* curves (Fig. 1a) for as-prepared ($|I$ (+2 V)$|/|I$ (−2 V)$|$) and the modified nanopore ($|I$ (−2 V)$|/|I$ (+2 V)$|$) was ∼5.4 and ∼5.7, respectively. The observed similarity in the f_{rec} values suggested that modification reaction results only in the switching of pore surface charge polarity from negative to positive, while the magnitude of pore surface charges remained almost the same.

After successful functionalization, we proceeded to study the enzymatic cleavage of choline moieties from the immobilized

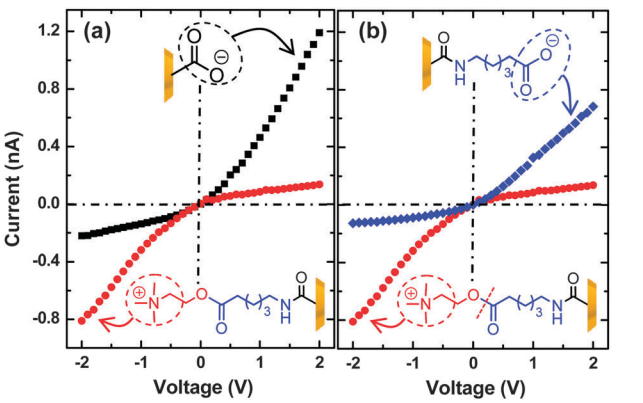

Fig. 2 The *I*–*V* curves of a single conical nanopore ($d = \sim 8 \pm 2$ nm) in 100 mM KCl solution (pH = 7.2) measured (a) before and after AHCh-substrate immobilization, and (b) before and after the AChE-enzymatic cleavage of cationic choline moieties from the pore walls.

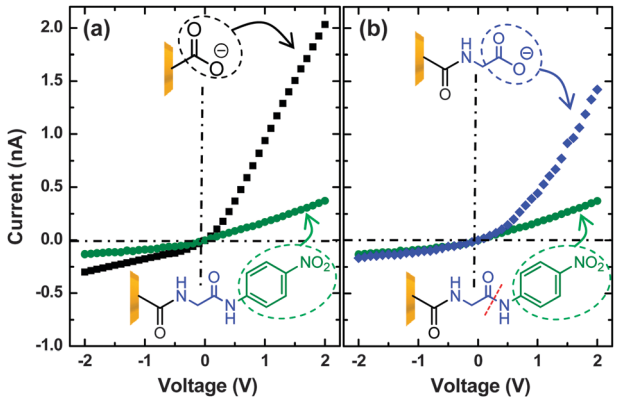

Fig. 3 The *I–V* curves of a single conical nanopore ($d = \sim 10 \pm 3$ nm) in 100 mM KCl solution (pH = 7.2) (a) before and after GNA-substrate immobilization, and (b) before and after treatment with protease enzyme.

substrate in the confined environment. To achieve this, the AHCh-modified nanopore was exposed to 50 U mL^{-1} solution of AChE enzyme, which is known to specifically hydrolyze the ester linkage in choline ester molecules such as acetylcholine.[12] After treating the modified pore with AChE, the cationic choline moieties were hydrolyzed and removed from the pore surface, leading to the generation of carboxyl groups on the inner pore walls (Fig. 1a). *I–V* curves shown in Fig. 2b reveal the recovery of cation-selective and rectification behaviour of the nanopore after AChE enzymatic hydrolysis reaction. The observed current ratio ($|I\ (+2\ V)|/|I\ (-2\ V)|$) in the case of the AChE-treated pore ($f_{rec} = \sim 5.2$) was close to that of the as-prepared nanopore ($f_{rec} = \sim 5.4$), indicating almost complete cleavage and removal of cationic moieties from the inner pore surface.

To further confirm the enzyme-triggered changes in nanopore surface polarity and permselectivity, we have also studied the activity of protease enzyme inside confined geometries by nanopore functionalization with a neutral substrate *i.e.*, glycine *p*-nitroanilide (GNA) as shown in Fig. 1b.

Fig. 3a shows the *I–V* curves of single conical nanopores before (black) and after (olive) the functionalization with GNA molecules. As expected, the nanopore rectification characteristic was almost lost due to terminal uncharged *p*-nitrophenyl moieties. The modified nanopore behaved like an ohmic resistor, indicating the almost zero charge on the pore surface. Upon treatment with protease enzyme, uncharged *p*-nitroaniline moieties were removed from the inner pore walls due to the enzymatic cleavage of the amide bond from the immobilized GNA-substrate molecules.[13] The enzymatic-cleavage reaction resulted in the generation of carboxylic acid groups, which were ionized to $-COO^-$ under physiological conditions (Fig. 1b). The *I–V* curves clearly indicated that the protease catalyzed reaction switched the inner nanopore environment from a hydrophobic (nonconducting) to a hydrophilic (conducting) state and eventually, restored the cation-selective and rectification characteristics of the nanopore (Fig. 3b).

In summary, we have demonstrated the functionalization of single conical nanopores with enzyme substrate molecules. The terminal moieties were removed from the immobilized substrate by exposing the modified pore to respective enzyme. This process led to the generation of functional chemical groups responsible for the permselective ionic transport and rectification properties of the nanopore, which can easily be followed by *I–V* analysis. We believe that such enzyme-responsive nanoporous systems have huge potential for biosensing, drug delivery and design of controlled release platforms, especially when the modulation of nanopore transport properties under biological conditions is desired. Our further efforts will be focused on exploring some of these applications.

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