Electronic Supplementary Material

Harnessing Concerted Functions in Confined Environments: Cascading Enzymatic Reactions in Nanofluidic Biosensors for Sensitive Detection of Arginine

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Materials

Poly(ethyleneterephthalate) (PET) foils (Hostaphan RN 12, Hoechst) of 12 μm thickness were irradiated with 2.2 GeV Au ions at the UNILAC accelerator of the GSI Helmholtzzentrum für Schwerionenforschung GmbH (Darmstadt, Germany). Poly(allylamine) hydrochloride (PAH) (Mw 20,000, 20 wt. % in H2O) was purchased from Sigma-Aldrich, potassium chloride and HEPES were purchased from J.T. Baker, L-arginase (Ar) from bovine liver (100 units/mg, Calzyme Laboratories Inc., San Luis Obispo, CA, USA), urease (Ur) from Jackbean (Canavalia ensiformis) (100 units/mg, Calzyme), and surfactant Dowfax 2A1 from Dow Chemicals. L-arginine monohydrochloride, D-arginine monohydrochloride, L-valine, L-alanine, L-phenylalanine, L-proline, and urea (all from Sigma-Aldrich, USA). Glicine from Bio-Rad and glucose from Merck.

Methods

Nanochannel fabrication

For obtaining nanochannels with desired electric properties, first, Poly(ethyleneterephthalate) (PET) polymer foils were exposed to swift heavy ions, leading to the formation of so-called ion tracks, cylindrical zones formed along the path of each projectile. Then, an asymmetrical chemical etching is performed to convert the tracks into open channels by selectively removing the damaged material. One compartment of a two-compartment cell that separates both faces of the polymer film, contains 6 M NaOH, while the other compartment contains 6 M NaOH and 0.05 % v/v Dowfax 2a1. After 6 minutes incubation at 60 °C the nanochannels become cylindrical with a bullet-shape end-segment with the vertex pointing towards the solution containing the surfactant. These nanochannels display nonlinear current–voltage characteristics due to their asymmetric shape and the negative charges stemming from the carboxylate groups generated on the surface after the hydrolysis.

Scanning Electron Microscopy characterization of nanochannels

A JEOL JSM-7401F scanning electron microscope (SEM) was used to take Top-view SEM images from both membrane sides (tip and base side). For the tip-side images, the membrane was covered with a thin gold layer. For cross-section images, the multi-channel PET membrane was firstly sensitized by UV exposure for 72 hours and then, broken down in liquid nitrogen, as explained elsewhere (Laucirica et al., 2023). Finally, the SEM images were obtained by placing a piece of the membrane at 90°. Multi-channel PET membranes were obtained by increasing the fluence during the irradiation (108 ions.cm-2) and then, chemically etched in the same conditions as the single-channel one. Figure S1 shows representative SEM images of nanochannels. Based on these images we can determine that the tip has a final average diameter of 55 ± 5 nm, meanwhile the base diameter is 500 ± 100 nm.

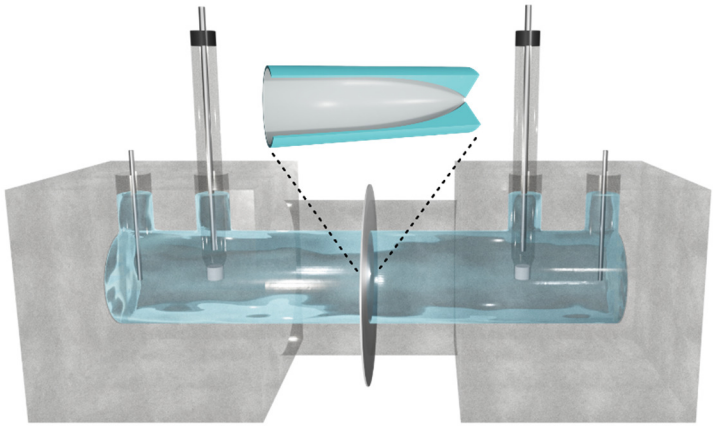
Modifications with PAH and enzymes.

After the etching procedure, the nanochannel-containing foils were modified by dip-coating with PAH, urease and arginase. For the PET/PAH/Ur:Ar 1:1 configuration, PAH was incorporated by dip-coating the membrane in a 10 mM solution of PAH (in monomer units) at pH 6 for 1 h, followed by a dip-coating step in a Ur:Ar 1:1 solution (0.5 mg/mL of each protein, *i.e.* 1 mg of total protein/mL, in 10 mM HEPES buffer +10 mM KCl, pH 6.5).

The PET/PAH/Ur/PAH/Ur:Ar 1:1 configuration was made similarly. The first step is a dip-coating with the 10 mM PAH solution at pH 6 for 1h. Then, urease was integrated into the PAH-modified nanochannel by dip-coating in a urease solution for 1h (1 mg/mL in 10 mM HEPES buffer +10 mM KCl, pH 6.5). Thereafter, another dip-coating with the 10 mM PAH solution at pH 6. And finally, the last dip-coating in a urease:arginase 1:1 solution.

Current – Voltage curve measurements

Current–voltage curves were measured using a commercially available potentiostat (Gamry_Reference 600) in a four-electrode setup (working, working sense, reference, and counterelectrode), measuring conductance variations arising from changes in the nanochannel. Both the working and counter electrodes were platinum wires, while the reference and working-sense electrodes were commercial silver/silver chloride (Ag/AgCl/3 M KCl) electrodes. As shown in the following scheme, a conductivity cell comprising two compartments, separated by a single nanochannel membrane, was designed to avoid leakage currents while measuring. In all the experiments, the working electrode was placed at the base of the nanochannel while the counter-electrode was placed at the tip. A 0.1 M KCl solution was used as the electrolyte. All experiments were performed at room temperature, and the I-V curves were measured between +1V and –1 V at 100 mV/s (three cycles).



Rectification Efficiency (f_{rec} and $f_{rec}norm$)

In all experiments, the rectification efficiency (f_{rec}) is defined as follows:

$$f_{rec} = \pm\left|\frac{(1Vor-1V}{I(-1Vor1V)I}\right|) \qquad \text{(S1)}$$

Where the current I in the numerator corresponds to the largest current value in the high conductance state, and the current in the denominator is the lowest current value corresponding to the low conductance state. Additionally, if the higher current corresponds to a negative voltage, the rectification factor is multiplied by -1. This definition allows assigning a negative f_{rec} for a negatively charged nanochannel and a positive f_{rec} for a positively charged one. This notation simplifies the discussion of experimental results in terms of surface charge.

To compare results from different nanochannels, a normalized rectification efficiency ($f_{rec}norm$) is defined by dividing each f_{rec} value from a specific nanochannel by its highest f_{rec} value ($f_{rec,0}$):

$$f_{rec}norm = \frac{f_{rec}}{|f_{rec,0}|} \qquad \text{(S2)}$$

This normalization accounts for slight signal differences due to small differences in the foil, etching procedure, and thus resulting pore size.

Determination of detection and quantification limits.

Based on the linear regression obtained (Fig. 2b) to the PET/PAH/Ur/PAH/Ur:Ar 1:1 configuration (y= 1.26(0.04) - 0.27(0.02) * X) it is possible to define the limit of detection as LoD= 3*σ/m, and the limit of quantification as LoQ=10* σ/m, where m is the slope of the linear calibration (m = 0.27) and σ is the standard deviation of the intercept (σ = 0.04). X represents the log(Concentration). Thus, the LoD = 3μM and LoQ = 30μM.

Measurements in urine

Urine sample was obtained from two male and one female volunteers with no history of renal disease, after obtaining their informed consent. The entire procedure was conducted in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). The urine was filtered and refrigerated at 4°C for use.

For iontronic measurements, the urine sample (2 mL) was incorporated undiluted and at pH 6 in the compartment facing the tip of the nanochannel, while 0.1M KCl pH 6 was filled in the compartment facing the nanopore base. First, I-V characteristics between +1V and −1 V at 100 mV/s (three cycles) were recorded. Then, 2 µl of different concentrations of arginine were spiked in the 2mL urine sample, followed by I-V curve measurements. For each concentration of arginine, three replicates were measured using a single use nanochannels system. Each of these replicates were done with individual urine samples.

In the case of urine samples, f_{rec}norm is calculated by dividing each f_{rec} from a specific nanochannel by the f_{rec} value of that nanochannel in the presence of just urine (f_{rec},0). Thus, the steady state I-V curves and the rectification factor obtained based on this curve contain precise information that is essential for quantification and background subtraction to remove potential interference from other species in the sample.

Supplementary Figures

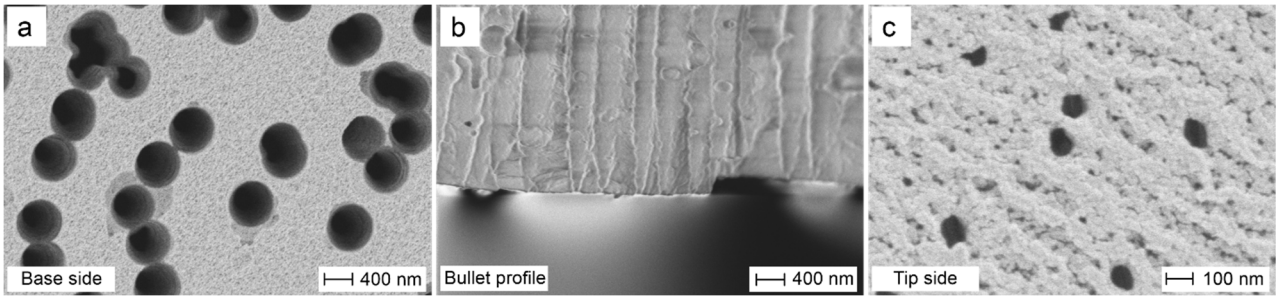


Figure S1. SEM image of multi nanochannels membranes, prepared with the same chemically etched conditions as the single-channel one. **a.** Image of the base side of the membrane. **b.** Image of the tip side of the membrane. **c.** Cross-section images showing a bullet shape of the nanochannels.

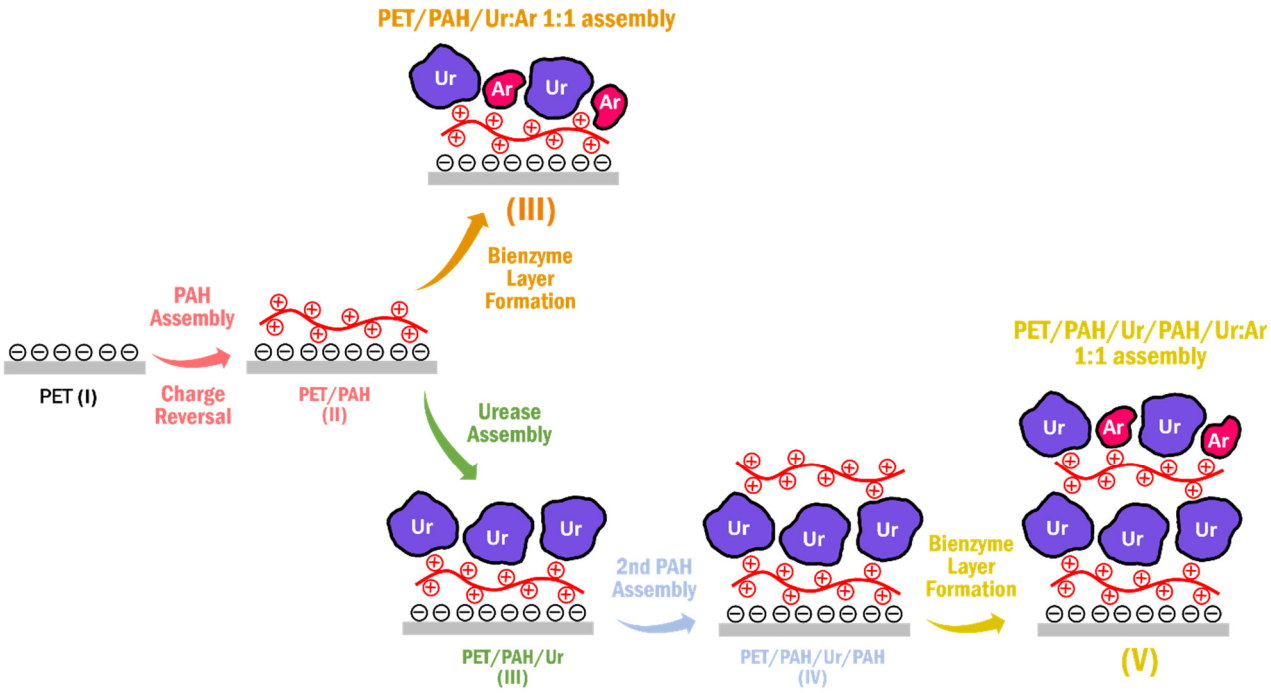


Figure S2. Scheme of the two assemblies inside the nanochannel. In both cases, the first step involves modifying the channel with PAH polyelectrolyte. For PET/PAH/Ur (1:1), a second layer of the enzyme mixture is immobilized. In PET/PAH/Ur/PAH/Ur(1:1), urease is immobilized second, followed by PAH incubation, and finally a layer of the enzyme mixture (Ur: Ar 1:1).

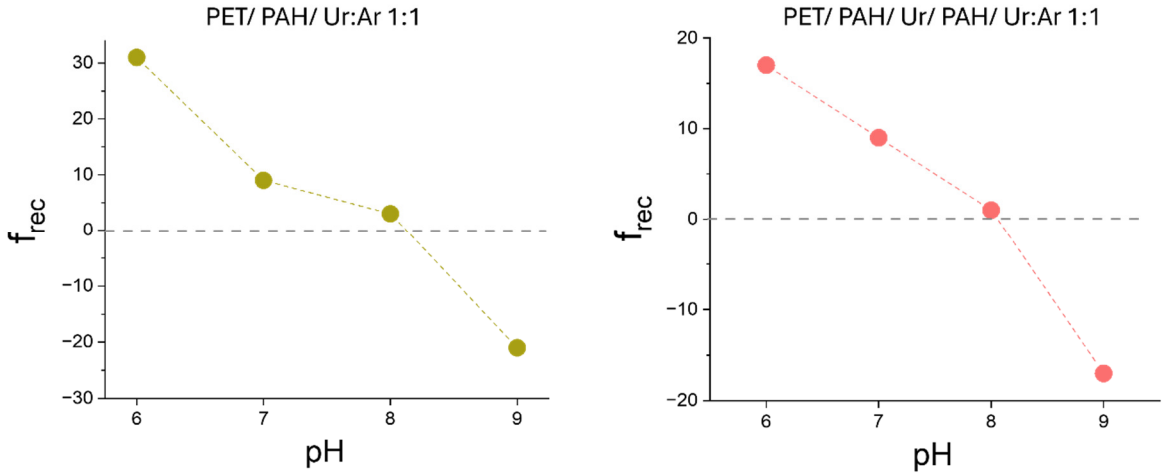


Figure S3. Rectification efficiency versus pH for modified nanochannels: PET/PAH/Ur:Ar 1:1 (left) and PET/PAH/Ur/PAH/Ur:Ar 1:1 (right). Curves were measured in 0.1 M KCl with dropwise addition of NaOH or HCl to reach each pH value.

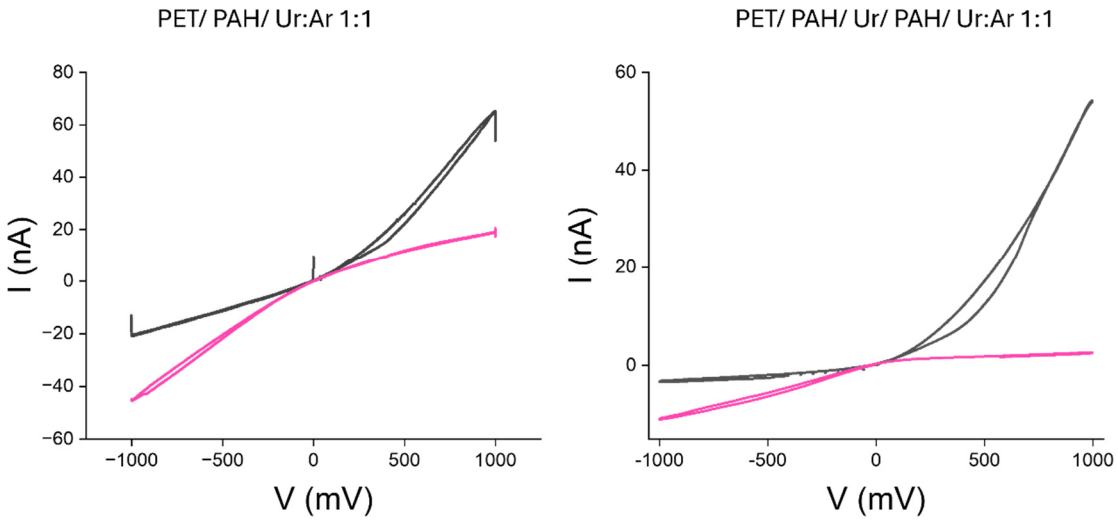


Figure S4. I-V curves before (grey) and after (pink) incubation with 3 mM Urea solution in 0.1 M KCl pH = 6 for both constructions: PET/PAH/Ur:Ar 1:1 (left) and PET/PAH/Ur/PAH/Ur:Ar 1:1 (right).

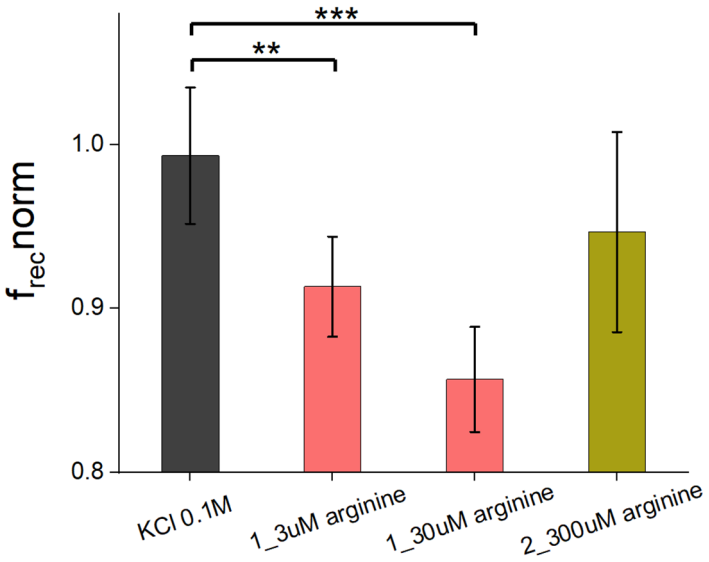


Figure S5. Normalized rectification efficiency in different conditions. Grey represents the mean and SD of f_{rec}norm measure only in the electrolyte solution, while in red are represented the mean and SD of f_{rec}norm when the PET/PAH/Ur/PAH/Ur:Ar 1:1 configuration is used to measure 3 and 30uM arginine; and in yellow, when the PET/PAH/Ur:Ar 1:1 configuration is used to measure 300uM arginine. two-tailed Student's t test; **p<0.01, ***p<0.001, bars represent mean ± SD.

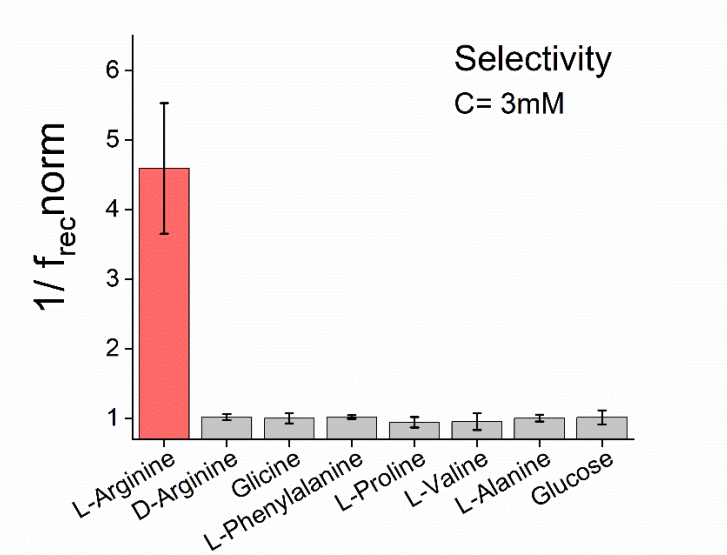


Figure S6. Selectivity assay. Inverse of f_{rec}norm obtained for L-arginine, 5 non-target amino acids, glucose and D-arginine. The concentration of small molecules is 3mM. n = 3 technical replicates (mean ± SD)

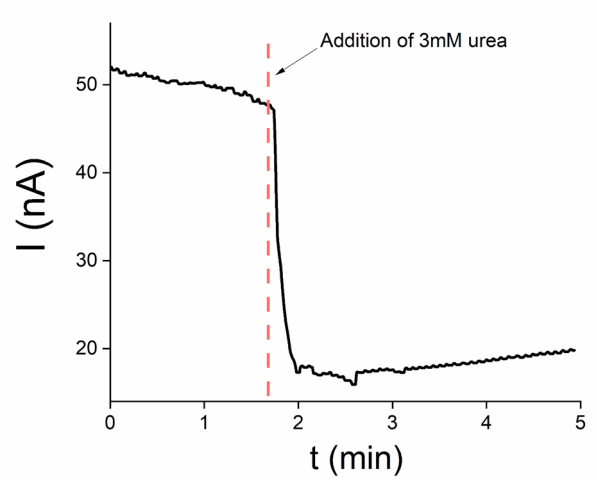


Figure S7. Kinetic response of a PET/PAH/Ur/PAH/Ur:Ar 1:1 modified nanochannel to the addition of 3 mM urea in solution while measuring the transmembrane current at 1 V.

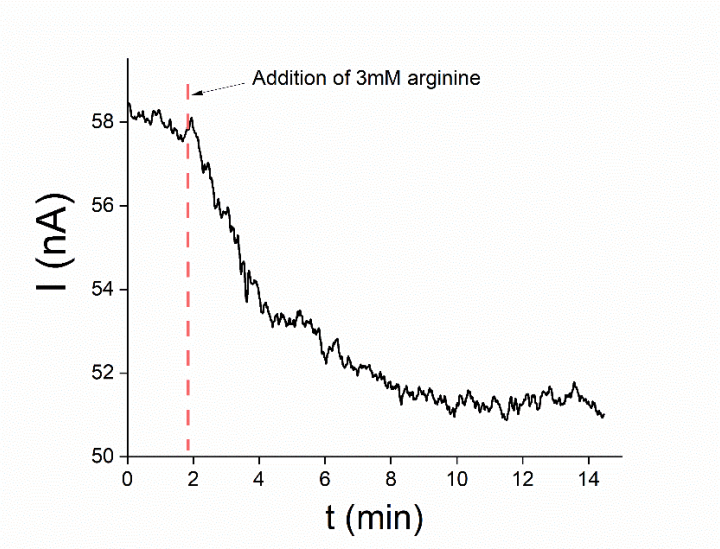


Figure S8. Kinetic response of a PET/PAH/UrPAH/Ur:Ar 1:1 modified nanochannel to the addition of 3 mM arginine in solution while measuring the transmembrane current at 1 V in KCl 0.1M pH 6.