

Ion Conducting Nanopores for (Bio)molecular Sensing: the *iNAPO* Project

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Abstract – Single conical nanopores in polymer foils are fabricated by means of the ion track etching technique. They are chemically modified so that they selectively react with certain molecules to be analyzed. This specific reaction is electrochemically monitored by measuring the electrolyte current flowing through the nanopores in an electrochemical cell. This current is dependent on the presence and concentration of the molecule to be analysed. Thus, a both sensitive and selective molecular sensor is available. The paper describes the fabrication of the nanopores and their use as sensors. As an example, the sensing of hydrogen peroxide is shown. The polymer nanopores are the base of the project *iNAPO*. Its goal is the combination of the polymer-based nanopores with biological protein-based nanopores and their implementation into a micro-device in order to create a new type of sensing tool for analytical or medical applications, e.g. such as environmental water analysis and diagnostics of diseases.

Keywords: radiation damage, ion track etching, polymer membrane, nanopores, biomimetic sensor, nanosensor, current-potential measurements

1. Introduction

Ion conducting nanopores are implemented into the membranes of living cells and regulate mass transport into and out of the cell and also cell communication [1]. They alter their ionic conductance in the presence of certain molecules. Hence, the presence of these molecules can be monitored when the ion conductance of the nanopores is measured [2]. In this way, highly sensitive biomolecular sensors can be realized. A drawback of such natural nanopores for technical applications is that they are embedded in an unstable lipid bilayer. This fragile carrier can be, to a certain extent, replaced by polymer foils. The nanopores in these foils are fabricated by the so-called ion track etching technique. For this purpose, an ion of a heavy element is accelerated to such a high kinetic energy in a particle accelerator that it penetrates the polymer foil in a straight line. It creates a damage track which then is chemically etched open into a nanopore. By an appropriate coupling chemistry, a biomolecular recognition unit is fixed to the nanopore's wall. This is a molecule which specifically reacts with a counter molecule, in a key-lock system. As a consequence of the bioconjugation reaction, the nanopore is changed with respect to features like its diameter or its surface charge. When the nanopore-carrying polymer foil is used as a separation membrane in an electrochemical cell, the transmembrane electrolyte current, i.e. the current based on dissolved ions, such as K^+ , is directly influenced by the presence of the molecule to be analysed [3,4]. Thus, a sensitive and selective nanosensing system is available.

In the following, the fabrication of polymeric nanopores by the track etching technique, the chemical modification of the nanopores, and their use as core part of a molecular sensing device are described.

2. Preparation of nanopores in polymer foils

2.1. Tracks from heavy ion beams in polymers

The nanopores in polymeric foils, used for the present study, stem from interesting historical roots. The technique for the fabrication of these nanopores has been developed for a completely different purpose, namely dosimetry. In space, radiation damage is an important issue, particularly for human beings. Astronauts are hit by galactic cosmic rays (GCR)

and suffer from cell damage. The majority of GCR is light particles, mainly protons and α -particles, but there is also a small percentage of heavier particles, the so-called HZE-ions, meaning "High Z for atomic charge and Energy" ions [5]. The source of these extremely highly energetic projectiles is e.g. supernova explosions. HZE ions are mainly those of the first row of transition metals, particularly iron and nickel. In the United States Apollo program, in missions Apollo 8 and Apollo 12, the so-called "Apollo helmet dosimetry experiment" was carried out [6]. The astronauts' helmets were used as dosimeters. The damage tracks of heavy cosmic-ray nuclei were visualized. The helmets were fabricated of the polymer Lexan, a polycarbonate. When they were hit by HZE ions, these penetrated deeply into the polymer and created damage zones along their trajectories. After return, the Lexan helmets were etched by a solution of ethanol and sodium hydroxide, forming conical etch tracks, i.e. holes. The etch tracks were replicated with silicone rubber and the replicas were coated with gold, creating a very well visible contrast in the stereomicroscope. Comstock et al. presented their results in [6]. They state that most probably the tracks come from ions of iron or the iron group. Fig. 1 shows track etch replicas from the bubble helmet.

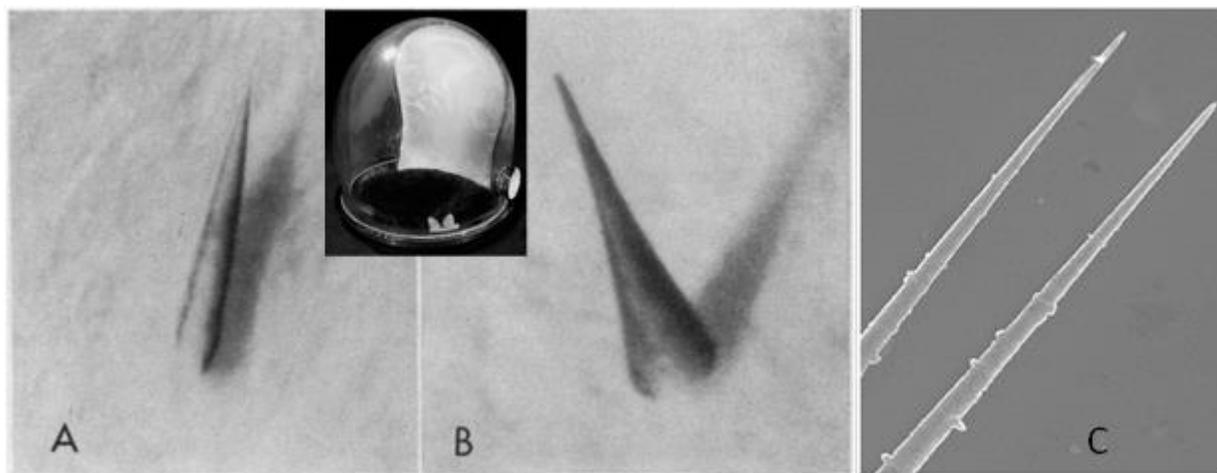


Fig. 1: A, B: Examples of conical replicas of tracks of heavy cosmic rays on the inside of an Apollo space helmet (see insert). The lengths are approximately 500 μm and 700 μm . From Comstock et al., *Science*, vol. 172, p.154, 1971. Reprinted with permission from AAAS. C: Metal replicas of tracks of a heavy ion irradiated polymer foil from the author's lab. The length is 12 μm .

In the author's institution, energetic heavy ions are formed in a linear radiofrequency accelerator, combined with a synchrotron which accelerates all kinds of ions, up to uranium. These can directly be used to study effects and reactions of HZE-like ions. Fig. 1 c shows in comparison the gold metal replica of heavy ion tracks in polycarbonate foil from the author's lab. The foil was irradiated with heavy ions of an energy of GeV. The tracks were etched with sodium hydroxide from one side, forming a conical hole that was galvanically filled with metal. After dissolution of the polymer with a solvent, the replica was freed and inspected under the scanning electron microscope.

When the highly energetic ion of a heavy element passes through a polymer, either in space or in the particle accelerator lab, it releases a part of its kinetic energy into the electronic system of the polymer, leading to dense ionization. The created δ -electrons easily break the chemical bonds between the atoms of the polymer around the ion trajectory, cutting out small molecular fragments [7,8]. Table 1 shows a list of fragments of a polymer, irradiated with high energy heavy ions (e.g. Gold ions of 2 GeV energy, Xe ions of 1.5 GeV energy). The fragments were identified with mass spectrometry. A large number of volatile molecules including hydrogen, hydrocarbons, and carbon dioxide, could be identified. They leave the polymer into vacuum. When a large number of ions is used, the weight loss of the polymer can be quantified. In Fig. 2, the residual mass after irradiation is shown as a function of the number of ions. The polymers suffer from substantial loss by ion induced fragmentation and ion beam sputtering.

Each ion releases a large number of molecules. Since the ion travels through the polymer in a straight line and the damage is close to the core of this trajectory, the formed ion track is a linear cylindrical zone of reduced density and molecular structure. The changed structure has been proven by IR spectroscopy [7]. When the ion beam modified material is subjected to chemical etching, the damage zone is preferentially etched, leading to the observed holes, shown as replicas in Fig. 1.

Table 1: Molecular fragments of polyvinyl acetate released upon irradiation with heavy ions; (r/i) means radical or ion, from [8].

mass	compounds	mass	compounds
2	H ₂ - Hydrogen molecule	26	C ₂ H ₂ - Ethyne
12	C - Carbon (r/i)	28	CO - Carbon monoxide
13-15	CH ₁₋₄ - Hydrocarbon; (r/i): methyl, carbene	29	CHO - Formaldehyde (r/i)
16	O - Oxygen (r/i)	30	CH ₂ O - Formaldehyde
18	H ₂ O - Water	43	C ₂ H ₃ O - Acetaldehyde (r/i)
24-30	C ₂ H ₀₋₆ - Hydro(di)carbon (r/i)	44	CO ₂ - Carbon dioxide

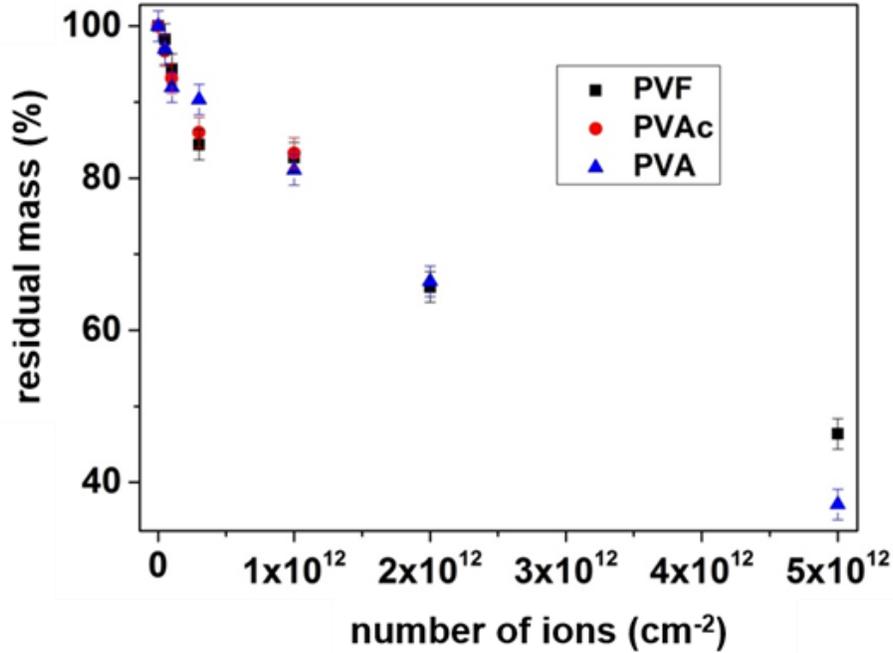


Fig. 2: Mass loss of aliphatic polymers (polyvinyl formal PVF, polyvinyl acetate PVAc, polyvinyl alcohol PVA) under irradiation with high energy heavy ions. The residual mass is shown as a function of the number of ions.

2.2. Fabrication of chemically modified nanopores in polymers

The above mentioned track etching technique can be used for the fabrication of conical nanopores [9]. Polyethyleneterephthalate (PET) foils of 12 μm thickness were through-irradiated at the GSI Helmholtz Centre for Heavy Ion Research with a single Gold ion of a kinetic energy of 2.1 GeV. The latent damage track was sensitized with UV light. PET can chemically be etched with NaOH. However, due to the lower density and the altered chemistry inside the ion damage track, the etch rate V_{track} along the ion damage track is larger than the rate V_{bulk} of the undamaged material. Fig. 3 shows schematically the geometry of etch process. Due to the difference in the etch rates, a conical aperture is formed.

The asymmetric etching was performed with an alkaline etching solution (9 M NaOH) on one side of the foil and an acidic etch stop solution (1 M KCl + 1 M HCOOH) on the other side. This procedure results in a conical pore with a diameter of a few 100 nm of the large aperture and a few nanometers of the small one. A metal replica of such nanopores has been shown in Fig. 1 c.

The etching process leaves the PET surface with terminal carboxyle groups. These can be used to couple specific molecules to the nanopore's walls, by means of Ethyl-Dimethylaminopropyl Carbodiimide / Pentafluorophenol (EDC/PFP) coupling chemistry. In one of two cases shown here, the PET foil was immersed into a solution of 0.1 M EDC and 0.2 M PFP in ethanol, washed with ethanol and then subjected to reaction with a 50 mM ethanolic solution of Amine-terminated Boronic Ester Carbamate (BEC-NH₂) [11]. This reaction is schematically shown in Fig. 4 a,b. In the other case, the EDC (10 mM) was combined with Hydroxysulfosuccinimide (sulfo-NHS, 20 mM) leading to a reactive intermediate which was used to couple the enzyme Horseradish Peroxidase (HRP, 1 mg/mL) via its lysine residues onto the nanopore walls [12].

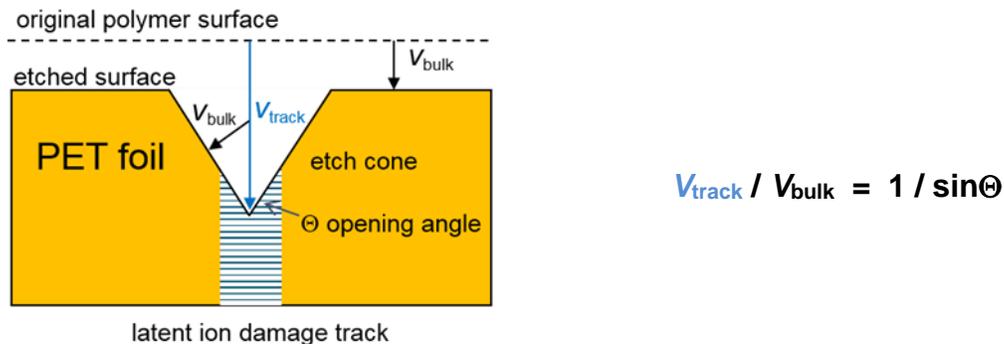


Fig. 3: Schematic presentation of the nanopore etching process. The etch rate along the ion damage track is larger than the one of undamaged bulk material, leading to a conical aperture. When the track/foil is etched through, a conical nanopore is generated. The cone angle Θ is given by the ratio of the etch rates.

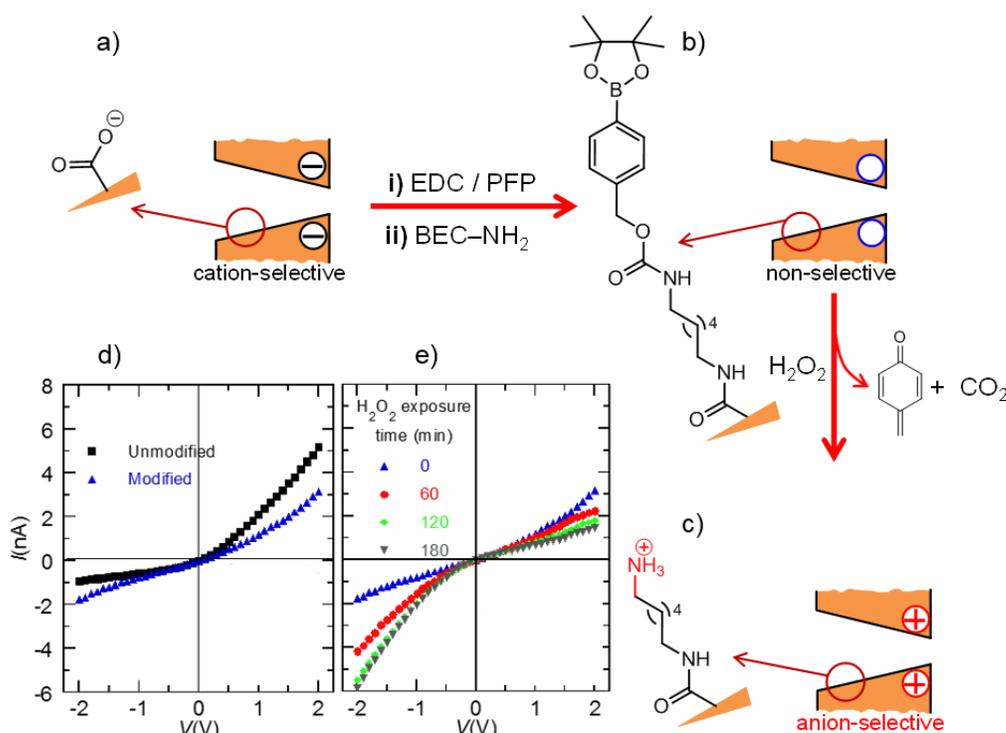


Fig. 4: Schematic presentation of chemical modification of nanopore walls for hydrogen peroxide sensing, a) Coupling of BEC to terminal negatively charged carboxylate groups, leading to a neutral surface b) Splitting of BEC by H₂O₂, leading to c) ammonium-termination with positive surface charge, d) current-voltage (I/V) curves of carboxylate-terminated nanopore (unmodified) and BEC-terminated nanopores (modified); d) I/V -curves in the presence of H₂O₂, with different exposure times; adapted from [11].

3. Chemically modified nanopores as sensors for Hydrogen Peroxide

The polymer foil with the modified nanopore was mounted as separation membrane between the two halves of an electrochemical cell, with 0.1 M aqueous KCl solution as the electrolyte. Each half-cell contained a Ag/AgCl standard reference electrode, connected to a voltage source and a sensitive current meter (picoammeter 6487, Keithley Instruments). A voltage ramp from -2 V to +2 V was applied between the electrodes in steps of 0.1 V, and the resulting current flowing through the nanopore was recorded.

In order to measure the effect of H₂O₂ on the system, a hydrogen peroxide solution (0.8 mM; $pH = 8.2$ in carbonate buffer) was added to the electrochemical cell. After exposure for different time periods, the transmembrane current was

measured with KCl solution. The reaction that was specifically induced by H_2O_2 is schematically shown in Fig. 4 c. The peroxide hydrolyzes the boronic ester, eventually leading to positively charged ammonium groups on the nanopore walls. While this specific reaction has been developed by Lo and Chu for sensing H_2O_2 on the base of cleaved measurable fluorescent groups [13], here it is the change in the surface charge which is used.

The resulting current-potential (I/V) curves are shown in Fig. 4 d and e. Due to a negative surface charge, the carboxylate groups of the unmodified nanopores walls interact with the electrolyte ions (K^+ , Cl^-), leading to an asymmetric current distribution. With a negative surface charge, the nanopore becomes cation-selective; the positive branch of the I/V curve is steeper and shows larger values. With the neutral BEC-molecule, the current is more symmetric (Fig. 4 d). When, however, the BEC-group is selectively removed by hydrogen peroxide, a positively charged ammonium group is left on the surface. This inverts the current flow, leading to significantly reduced positive and increased negative current values (Fig. 4 e). Hence, the curve is a direct result of the presence of H_2O_2 , and the reaction can be used for selective H_2O_2 sensing.

Another approach is the use of a H_2O_2 -specific enzyme, the Horseradish Peroxidase. HRP is a metalloenzyme that reduces peroxides while oxidizing other compounds. The hydrogen peroxide bonds to a vacant position on the HRP iron complex. However, the HRP alone cannot be used for sensing. Another molecule, a substrate, is required. It is oxidized by HRP in the presence of hydrogen peroxide. It is the change of the substrate that is measured, mostly spectrometrically with a coloration of the chromogenic substrate [14]. The substrate used for the present case was Azino-bis-ethylbenzothiazoline-sulphonate (ABTS). It is an electron donor for the reduction of hydrogen peroxide and becomes oxidized at an amino group into a green radical cation which can photometrically be determined. In the present case, however, it was again the charge which was utilized [12]. Fig. 5 shows the reaction schematically. HRP was immobilized on the nanopores walls by means of EDC/NHS coupling chemistry. The sensing solution contained ABTS (1.5 mM). When H_2O_2 was added, ABTS was enzymatically turned into the cation, see Fig. 5 b. Being charged, the molecule is accumulated in the nanopore leading to a reduction of the transmembrane current. Fig. 5 c shows that the current is a direct function of the H_2O_2 concentration. With this set-up, hydrogen peroxide is quantitatively measured. The data show that a concentration of only 10 nM H_2O_2 can easily be distinguished from the blank solution without H_2O_2 (shown in white).

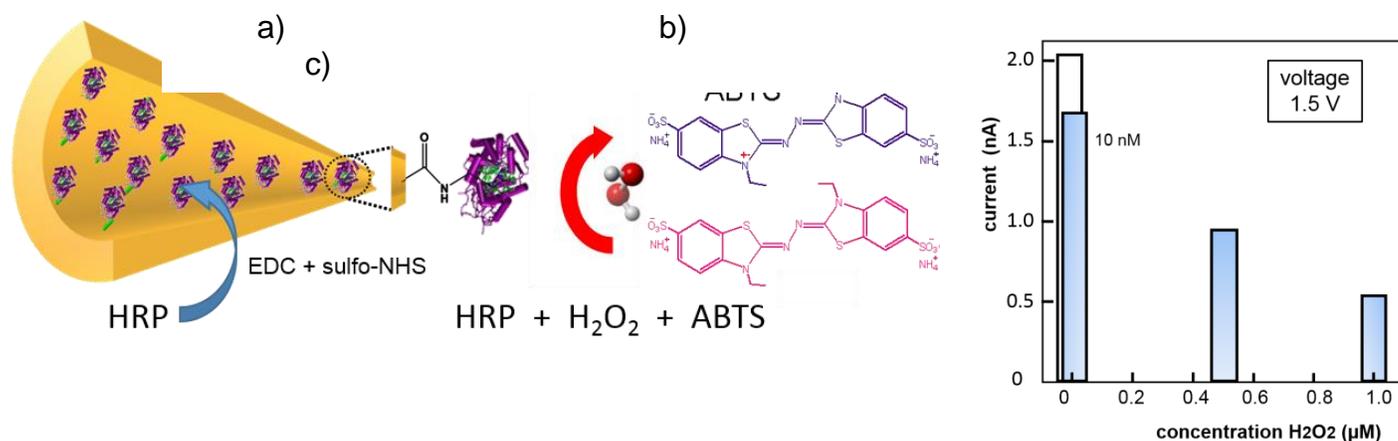


Fig. 5: a) Schematic presentation of HRP coupling on the nanopore wall, b) Reaction of ABTS with HRP in the presence of H_2O_2 to ABTS radical cation, c) Transmembrane current at a voltage of +1.5 V with different concentrations of H_2O_2 ; the current value of the blank sample without H_2O_2 is shown as white bar.

The given examples of nanopores-based analysis dealt with a very small molecule. Those are, in general, difficult to analyse. On the other hand, H_2O_2 shows a very specific redox-behaviour which can analytically be used.

Even more promising than the sensing of small molecules are large biomolecules. In this case, bioconjugation is used. The biomolecule to be analysed reacts specifically with a counter-molecule that selectively fits to it, like a key-lock system.

When the biorecognition molecule is immobilized within the nanopore, the presence of the analyte molecule affects the transmembrane current, giving a selective sensor. This has been demonstrated with carbohydrate and protein sensing,

e.g. in a model reaction with mannose-mediated recognition of the protein Concanavalin A [15] and aptamer-protein conjugation for Lysozyme sensing [16].

4. Conclusion

Chemically modified conical nanopores formed in polymer foils by the asymmetric track etching technique in combination with electrochemical measurement technique constitute a powerful tool for future miniaturized sensor technology. At present, the proof-of-concept has been shown with a variety of examples. With a compact electrochemical cell, the analytical volume is already quite small. Also, the sensitivity and selectivity values obtained are very promising.

A step further is the task of the *iNAPO* project. Here, two approaches are being investigated by a group of materials scientists, biologists, physicists, chemists and electro engineers. One is the combination of natural protein nanopores with the polymer nanopores in order to make use of the unique analytical selectivity and sensitivity of nature's nanopores. The other one is the implementation of the hybrid nanopores into micro devices with very small analytical volumes and power consumption making use of micro-nano-integration technology. The goal is to develop second-generation bio-synthetic nano sensors, with applications in the fields of e.g. environmental analysis and medical diagnostics.

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