Isoelectric Point Determination of Proteins Using Track-Etched Single-Nanopore Membrane

İz-Aşındırılmış Tekli Nano Membran Kullanarak Proteinlerin İzoelektrik Noktalarının Belirlenmesi

Research Article

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ABSTRACT

In this study, we have investigated a new method to determine the pI values of proteins compared to the conventional methods. We developed a new sensing platform and paradigm to succeed it. The track-etched poly(ethylene terephthalate) (PET) membranes were used to fabricate both single and multipore membranes. Two-step chemical etching method was prefered to fabricate single nanopore in a reproducible manner. EDC-coupling was used to modify and attach the proteins inside the nanopore surface. By the aid of ion current rectification, the surface charge characteristics of nanopores were identified with and without protein. A close approximation was successfully made to the literature values for the pI values of proteins using nanopore membranes respect.

Key Words
Track-etched polymer membrane, ion-current rectification, nanopore sensor, electrochemical sensor.

ÖZET

Bu çalışmada geleneksel yöntemlere kıyasla proteinlerin pI değerlerinin belirlenmesi için yeni bir yöntem geliştirilmesini araştırdık. Tekli ve çoklu nanogözeneklerin üretilmesi için iz-aşındırılmış polietilen tereftalat membranlar kullanılmıştır. Tekli nanogözeneklerin tekrarlanabilir şekilde üretilmesi için iki basamaklı kimyasal aşındırma yöntemi tercih edilmiştir. Nanogözeneklerin iç yüzeyine proteinlerin tutturulması için EDC bağlanması kullanılmıştır. İyon-akım rektifikasyonun yardımıyla protein varlığında ve yokluğunda yüzey yük karakteristiği belirlenmiştir. Nanogözenek membranlar kullanarak elde edilen veriler ile literatürde ki pI değerlerine başarı ile yaklaşılmıştır.

Anahtar Kelimeler
İz-aşındırılmış polimer membran, iyon-akım rektifikasyonu, nanogözenek sensörü, elektrokimyasal sensör.

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INTRODUCTION

Artificial nanopores have been mimicked the biological ion channels. As the artificial nanopore size becomes similar to the Debye length and/or ionic species, due to the opposite charges of nanopore surface and ions, a particular ion selectivity occurred[1]. Since the ionic transport is dominated by the surface-charge inside the nanopore, a unique phenomenon emerged to selectively control the ion transport through nanopores for various applications. Recent advances in nanopore fabrication also enabled the applications of the nanopore-based sensors. Ion-current rectification (ICR) in nanopores refers to a preferential conduction phenomenon of the ionic current, indicating an asymmetric diode-like current–voltage (I–V) curve[1]. The ICR originates due to the asymmetric geometry of nanopores (i.e., conical) that the charge distribution plays a critical role. The ICR is highly dependent on the surface charge of the nanopore and this property can be used to identify the physical properties of molecules [1-4].

In track-etch method, polymer membranes (or films) are irradiated with accelerated heavy ions, which create latent tracks inside the membranes. These irradiated membranes are then exposed to appropriate etching solution and the latent tracks turn into nanopores. The chemical etching process is controlled by neutralization of the etching solution. Single-pore membranes which are optimal for studying the detection and identification of individual molecules are formed via the chemical etching of a single-ion irradiated film (i.e., 1 ion/membrane). Some of the widely used membranes are poly(ethylene terephthalate) (PET), polycarbonate (PC) and polyimide (PI) [5-8]. These membranes can also be modified through several surface modification techniques to promote the translocation of analytes [9-14]. Preparation of reproducible nanopores of certain sizes and the ability to manipulate their dimensions are the key elements for sensing purposes of desired molecules. The control of nanopore sizes with a good reproducibility in track-etched membranes was successfully shown for conically shaped nanopores using a two-step etching method.

In this study, we proposed an alternative technique to determine the pIs of proteins using nanopores. In order to do that conically shaped nanopores were fabricated with controlled diameters in PET membranes using a two-step etching method. We selected PET membrane because of its stability, mechanical strength and flexibility. The EDC chemistry was used to immobilize the protein on the nanopore surface. The ion-current rectification characteristics were used to determine the charge of protein modified nanopore and related to the pI values. Insulin and bovine hemoglobin were chosen as model molecules because of their importance in living organisms. The effect of immobilization on nanopore surface was investigated and a sensing paradigm to determine the isoelectric point determination was shown.

MATERIALS and METHODS

PET membranes (3 cm diameter, 12 μm thickness) were provided by Gesellshaft für Schwerionenforschung (GSI, Darmstadt-Germany). The membranes had been irradiated with heavy ions (i.e., Au ion, 11.4 MeV) at various ion densities even down to 1 ion/membrane. This was succeeded by defocusing the ion beam and using a metal mask with a 0.1 mm diameter aperture with a shutter system which shuts down the ion beam as the single ion passage was detected. All the membranes were exposed to UV irradiation overnight (λ = 320 nm) to saturate the damages in tracks. All solutions were prepared from deionized water (Millipore Direct-Q 5, Millipore Co.) Formic acid (HCOOH), sodium hydroxide (NaOH), potassium chloride (KCl), Insulin (MW ~5.8 kDa, pI ~5.32), Bovine hemoglobin (MW ~64.5 kDa, pI ~6.8) were purchased from Sigma Aldrich. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (Sulfo-NHS), and 2-(N-morpholino) ethanesulfonic acid buffered saline (MES) were obtained from Pierce. All chemicals were used as received without further purification.

Chemical Etching and Pore Size Calculations

Prior to chemical etching, membranes were

first treated with long-wave UV irradiation overnight, in order to increase the track etching rate and make the pores more homogeneous in size by sensitizing the tracks. Asymmetric etching conditions were previously discussed [9] and base diameter determination was made using multipore PET membrane; in contrast, the current-voltage experiments were performed with single pore PET membranes. Shortly, the membrane was placed in a conductivity cell with one side of the cell facing the UV-treated side of the membrane, filled with etching solution and the other with the stopping solution. Etching solution was chosen as 9 M NaOH and the stopping solution comprised of 1 M HCOOH and 1 M KCl. Platinium (Pt) electrodes were immersed into each cell and 1 V transmembrane potential was applied to monitor the breakthrough moment. The etching process was continued for an hour and then the etching solution was replaced with stopping solution for neutralization. Afterwards both cells were rinsed with di-water to remove residues from the membrane surface. After this etching process, a conically shaped nanopore was obtained with two different sized openings called base (large opening) and tip (small opening). Since the obtained tip sizes ranged between 2-8 nm and the desired tip size was 20 nm, a second step etching was applied which was developed by Wharton et. al [15]. Briefly, after the first step of chemical etching, a more dilute etching solution (1 M NaOH) was introduced in both half cells and a transmembrane potential (1 V) was applied. This second etching step was stopped when the pore reached the desired size by monitoring the current values and once again the etching solution was replaced with the stopping solution of the same HCOOH and KCl content to stop the further widening of the pore. Thereafter, the half cells were rinsed with water and the membrane was then used for sensing experiments.

The large opening of the nanopore (d_{base}) was determined by the SEM images of multipore membranes (10^8 nanopores/cm^2) (Figure 2). Single pore membranes were etched under the same conditions as multipore membranes. The small opening (d_{tip}) was determined through electrochemical measurements [9]. Briefly, each side of the conductivity cell was filled with electrolyte solution (i.e., 1 M KCl, 10 mM PBS buffer at pH = 7). Ag/AgCl electrodes (BAS, West Lafayette, IN) were immersed into both sides of the cell and potential was stepped (50 mV) between -1 V and +1 V (Keithley 6487 picoammeter/voltage source, Cleveland, OH, USA). The resistance of the nanopore (R) is proportional to the conductivity of solution (λ), the length of nanopore (l, thickness of the membrane), d_{base} and d_{tip}. In order to calculate the tip diameter (d_{tip}) equation I was used; all the values were determined and the R value was the reciprocal of the slope of I-V curve (Figure 3) [9].

$$R = \frac{4\rho l}{\pi d_{tip} d_{base}} \quad \text{.....(I)}$$

The average of three sequential measurements was used for the calculations. The conductivity of the solution was measured using a conductivity meter (Mettler-Toledo FE 30, Colombus, OH, USA).

Surface Modification

EDC chemistry was used to attach the biomolecules on the nanopore surface. The pore etching procedure generates carboxylate groups on the surface nanopore, which helps to form amide bond between carboxylate groups on the surface and the molecule in interest (i.e., protein). This was performed by immersing the membrane into a solution that was 2 mM in EDC and 1.6 mM in sulfo-NHS dissolved in 10 mM MES-buffered saline, pH 6, for 2 hours. The membrane was then rinsed and immersed into a protein solution (2 mg/8 mL) dissolved in 10 mM phosphate buffered saline (PBS), pH 7.6, for 2 hours at 4°C. The EDC reaction for the protein immobilization is shown in Figure 1. The modified nanopore tip diameters were measured using the same electrochemical method described previously. The nanopore tips decrease in diameter after protein modification and the amount of decrease changed depending on the protein type.

Determination of pI Using Ion Current-Rectification (ICR)

The current-voltage curves are able to give information about the surface charge on the pore walls [16]. When the pore wall radius is comparable with electrical double layer, due to the conical shape and negative surface charge, the current-voltage curve would rectify (known

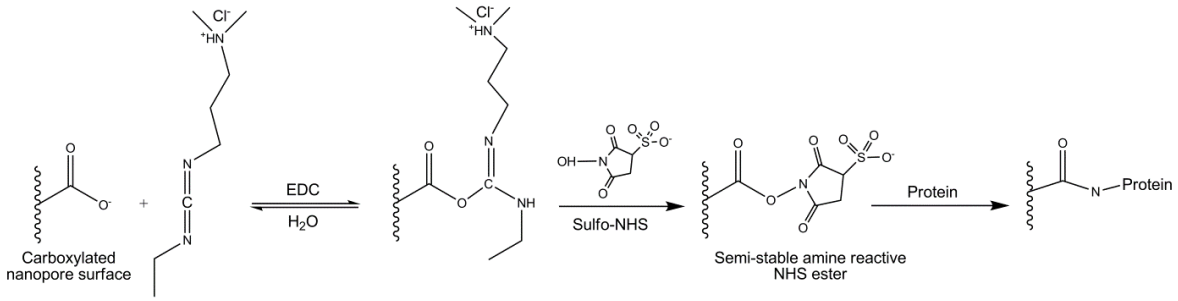

Figure 1. Reaction scheme for the attachment of protein to the carboxylated nanopore surface.

as ion current rectification, ICR). This non-linear behavior can be described as the 'on' and 'off' state of the nanopore. Since this behavior is highly dependent on the surface charge, the ion current rectification becomes linear if the surface charge is removed. PET nanopores carry a negative surface charge at neutral pH due to the carboxylate groups produced during etching. At this pHs, the bare PET nanopores will show a nonlinear current-voltage curve with the "on" state at negative applied potentials and the "off" state at positive potentials. The ion current rectification (ICR) can be quantified by taking the ratio between the current values at -1 V and +1 V (see equation II) [17]. Since the nanopore carries a negative surface charge, the ICR will be higher than 1. In the opposite situation, if the nanopore surface becomes positive, the direction of rectification will be reversed and the "off" state switches to negative potentials and the "on" state switches to positive potentials. So the ICR become less than 1. Proteins also carry charge, and when they are attached to the

$$ICR = {|i_{-1}|}/{|i_{+1}|} \quad \text{.....(II)}$$

nanopore surface, their charge dominates the surface charge characteristic. So, at a certain pI, the charges on the protein become neutral as the surface charge, where a current-voltage curve corresponds to the pI with a linear current-voltage curve. At this pH the ICR value will be equal to one since no rectification is observed. By taking current-voltage curves at various pH values and determining the rectification ratios relative to the pH, the pI of protein films immobilized on the nanopore surface can be determined.

RESULTS and DISCUSSION

Fabrication of Track-Etched PET Membranes

Track-etched PET membranes were etched with alkali solution (i.e., 9 M NaOH). After chemical etching, the base diameters of nanopores were determined by SEM images of these membranes (average of minimum 10 pores). The base diameter of the membranes with only 9 M NaOH as etchant was found to be 514±29 nm.

Current-Voltage Behavior of Protein Modified Track-Etched PET Nanopores

Tip size comparisons were made for each nanopore before and after protein modification. Nanopores modified with insulin showed a decrease in tip diameter of ~5±3 nm and with bovine hemoglobin showed a decrease of ~8±2 nm. After protein modification, current-voltage curves were again taken in 10 mM KCl with 10 mM PBS from pHs [4-8]. The change in rectification characteristics can be seen by comparing the current-voltage curves taken before modification (bare PET nanopore). The current-voltage curves obtained before and after modification, at various pHs, are shown in Figure 4 and 5 for two protein-modified nanopores. The current-voltage curves for the insulin modified nanopore showed rectification at positive potential which indicates the change of surface charge to the positive due to the protein attachment. The known pI is ~5.32 for free insulin and under this value it is the nanopore surface become positively charged (see Figure 4A). As the pH increased to 8, both bare nanopore and protein modified form rectified in the same

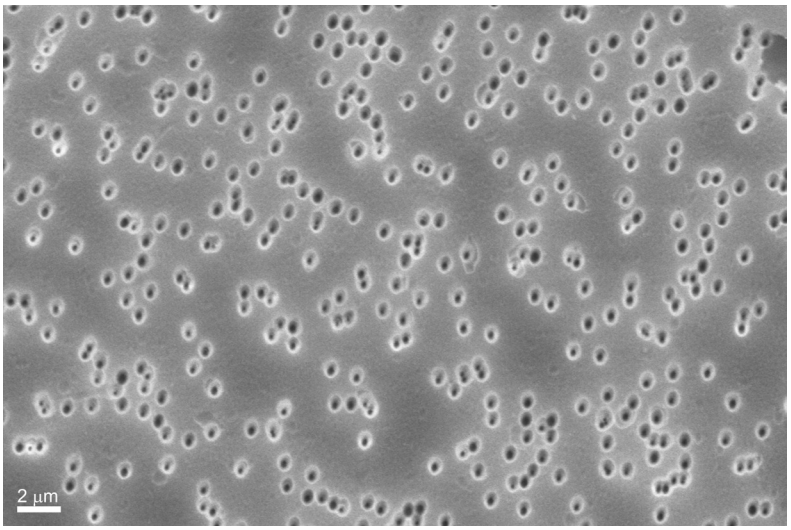

Figure 2. SEM images of chemically etched multipore PET membrane surface.

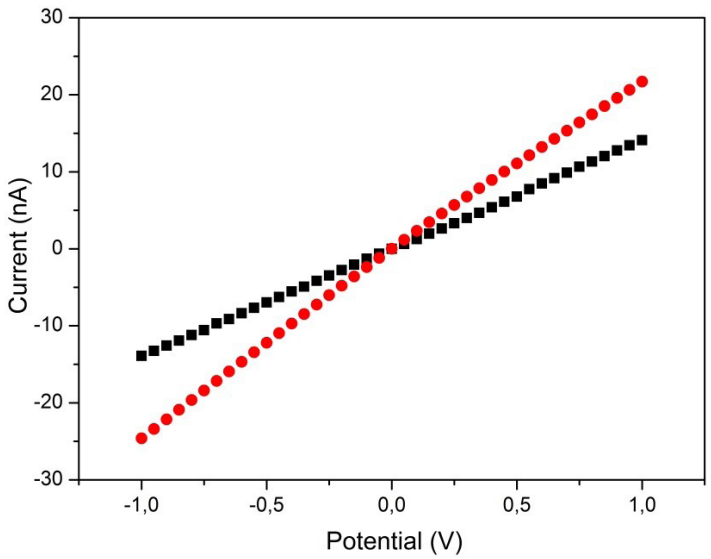

Figure 3. Current-voltage curves taken in 1 M KCl before and after modification to measure the change in tip diameter (black ■: d~22 nm), after modification with insulin (red ●: d~17 nm).

direction, at negative potentials (Figure 4B). The current-voltage behavior of bovine hemoglobin at pH 4, showed similar rectification characteristics observed as the insulin [18], however, it rectified relatively higher than insulin due to its higher pI. At pH ~ 8, its rectification characteristic matched with bare PET nanopore since the pH is over its pI value (i.e., ~ 6.8). The pI values farthest away from the bare PET surface would change the surface charge of the nanopore. In this respect, hemoglobin has higher pI value than insulin and its attachment on the surface would be more decisive.

The plots of ion current rectification vs. pH for each of the protein-modified nanopore membranes are shown in Figure 6. As with the bare PET membranes, the data was plotted on a semi-log plot and fit linearly. From the linear fit, the pH corresponding to the pI was extracted. The experimentally determined pIs of the insulin and bovine hemoglobin modified nanopores were 4.91±0.11 and 6.23±0.23, respectively. The experimentally determined pI values are slightly lower than the literature pI values for the free

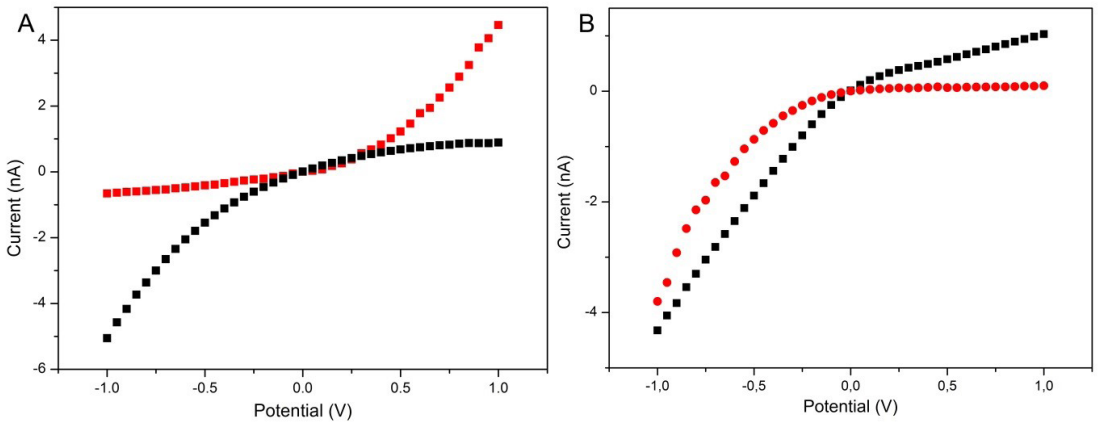

Figure 4. Current-voltage curves taken in 10 mM KCl, 10 mM PBS buffer before and after modification with insulin at pH 4 (A), pH 8 (B). (Before modification = black, After modification = red).

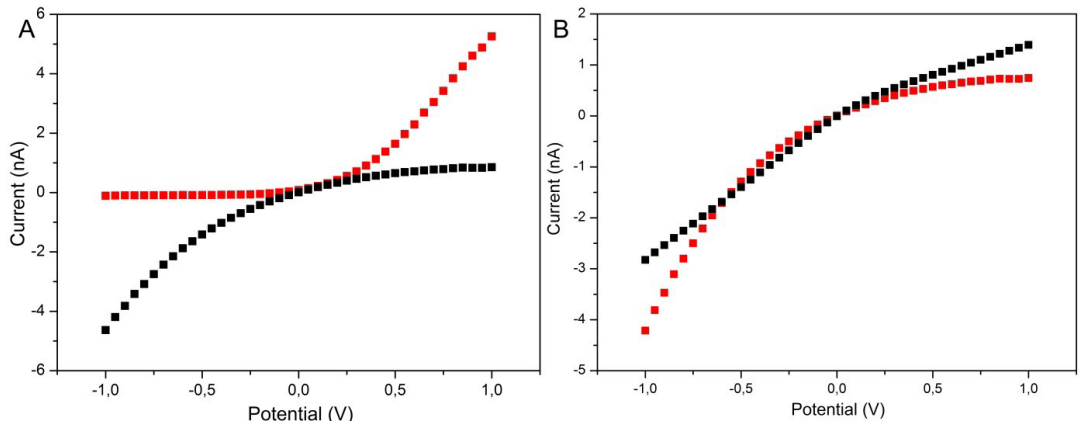

Figure 5. Current-voltage curves taken in 10 mM KCl, 10 mM PBS buffer before and after modification with bovine hemoglobin at pH 4 (A), pH 8 (B). (Before modification = black, After modification = red).

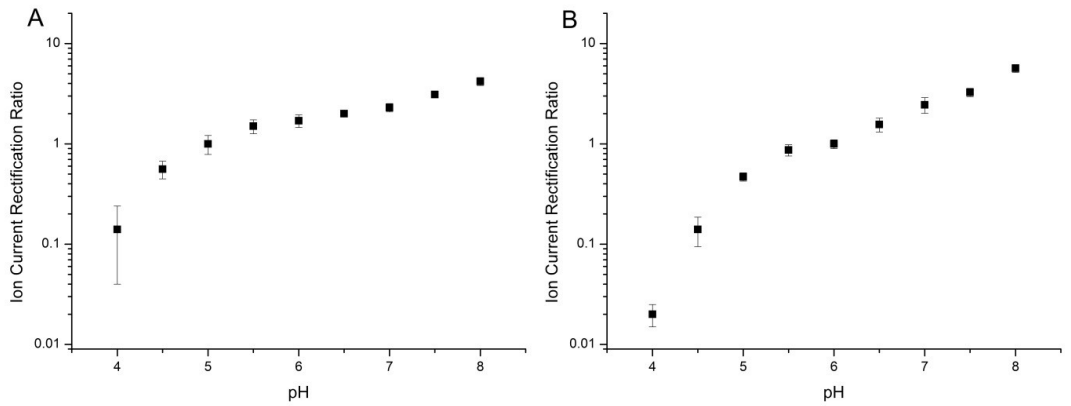

Figure 6. Plot of Ion-current rectification versus pH of Insulin (A), Bovine Hemoglobin (B).

proteins. The possible explanation for the deviation of pI values from the literature values may be the unfunctionalized carboxyl groups on the nanopore surface after protein modification. This would directly alter the net charge of nanopore and interfere with the proteins' charge. The other reason can be the chemistry of EDC and sulfo-NHS, this groups may also effect the amine groups on the proteins and cause changes of charges on proteins.

Conclusion

In this study, we showed that track-etched single nanopore membranes could be used for the determination of pI values of proteins. The proteins were successfully attached on nanopore surface, which confirmed by the change in current-voltage curves. The EDC coupling supported with sulfo-NHS was used for protein immobilization on the nanopore surface. The ion current rectification ratio was used to determine the pI values of insulin and bovine hemoglobin. The reasons we obtain different pI values for the immobilized and free proteins are still being studied. However, the possible reasons may originate from the interference from PET nanopore surface or the immobilization dynamics of the proteins. It is also possible that due to the formation of air-oxide films [19], the pH may differ few units respect to the reported pIs. The results showed that this technique has a potential to use the nanopore sensors for the determination of pI of proteins and surface isoelectric point.

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