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Citation: *Appl. Phys. Lett.* **91**, 053901 (2007); doi: 10.1063/1.2767206

View online: <http://dx.doi.org/10.1063/1.2767206>

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## Electrical characterization of protein molecules by a solid-state nanopore

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(Received 3 April 2007; accepted 9 July 2007; published online 31 July 2007)

The authors measured ionic current blockages caused by protein translocation through voltage-biased silicon nitride nanopores in ionic solution. By calculating the mean amplitude, time duration, and the integral of current blockages, they estimated the relative charge and size of protein molecules at a single molecule level. The authors measured the change in protein charge of bovine serum albumin (BSA) protein induced by pH variation. They also confirmed that BSA molecules indeed traverse nanopores using an improved chemiluminescent analysis. They demonstrated that a larger protein fibrinogen could be distinguished from BSA by a solid-state nanopore measurement. © 2007 American Institute of Physics. [DOI: 10.1063/1.2767206]

Measuring the charge and structural properties of individual protein molecules as well as the distribution of these properties in their native environment is currently a great challenge. Ionic current blockages measured in voltage biased nanofabricated pores have been used to sense nanoparticles and molecules of protein and DNA,<sup>1-7</sup> inspired by the pioneer work on measuring single polymers in protein channels.<sup>8,9</sup> There has been remarkable progress in the study of polymer translocation in protein pores and a recent study shows that polymer size can be resolved at high resolution.<sup>10</sup> However, the ionic current signature and the dynamics of a charged protein molecule moving in a solid-state nanopore need to be studied. In this letter, we report our observations of well-defined current blockage signals due to single protein molecules traversing silicon nitride nanopores. We measured the changes of bovine serum albumin (BSA) as a function of pH, and studied how the protein size and structure affect the blockage signal by comparing a larger fibrinogen protein with BSA. These studies are important milestones in the development of solid-state nanopore devices for fast protein characterization.

The main component of a nanopore sensing system [Fig. 1(a)] is a nanopore in a silicon nitride membrane that separates two chambers connected electrically by ionic solution inside the nanopore. When a voltage is applied across the membrane, a stable open pore current  $I_0$  is observed. After the addition of negatively (or positively) charged protein molecules to the *cis* chamber, the molecules in the vicinity of the nanopore will be captured by the electric field, and forced to traverse the nanopore to the positively (or negatively) biased *trans* chamber. The interaction of protein molecules with the nanopore, either by reversible partitioning into or by translocation through, will result in transient current blockages, as shown in Fig. 1(b). Three parameters are calculated from a current blockage: the mean blockage current  $\Delta I_b$ , the translocation duration  $t_d$ , and the integrated area of a blockage  $A_{\text{ecd}}$ .<sup>6</sup>  $A_{\text{ecd}}$  is the integral of  $\Delta I_b(t)$  with respect to time in units of kiloelectron charge. The current blockages were recorded using an Axopatch 200B system (Molecular Devices) in voltage clamp mode at  $V=120$  mV

with its low pass filter set at 100 kHz for all measurements in this work. For each set of data, about 10 000 blockage events were recorded. The nanopores used in this work were about 10 nm in thickness and slightly larger in diameter than the protein molecules being studied. The details of silicon nitride nanopore fabrication and the single molecule detection apparatus were described in our previous work.<sup>4,6</sup> The concentration of protein molecules placed in the *cis* chamber was approximately 10 nM.

BSA (66 430 Da, Sigma) has an isoelectric point (PI) ranging from pH 5.1 to 5.5;<sup>11</sup> thus the protein has an overall negative charge ( $-18e$ ) at pH 7. Applying 120 mV voltage to an  $\sim 16$  nm diameter pore in a solution of 0.4M KCl at pH 7.0,  $I_0 \sim 7.4$  nA was measured. After addition of BSA to the negatively biased *cis* chamber, downward blockage events occurred [Fig. 1(b)] indicating that the BSA molecules were negatively charged. When the *cis* chamber was positively biased, no blockages were observed at the beginning of the experiment. As the magnitude of the bias potential was decreased, smaller  $\Delta I_b$  and longer  $t_d$  were observed. The cumulative results are presented in an event distribution plot [Fig. 1(d)]. Every dot in Fig. 1(d) represents one blockage event and every blockage is characterized by its  $\Delta I_b$  and  $t_d$ . Figure 1(d) shows that there are two clusters of BSA blockage events: cluster 1 has most probable values of  $\Delta I_b \sim 50$  pA and  $t_d \sim 110$   $\mu$ s, while cluster 2 has smaller  $\Delta I_b \sim 20$  pA and shorter  $t_d \sim 50$   $\mu$ s. We attribute cluster 2 to the events that protein molecules partially entered the pore but failed to pass through it. Cluster 2 events will not be considered further in this study except to note that they can be easily distinguished from cluster 1 events, which were identified as free translocation of protein molecules through nanopores.

When the pH of the chamber solution was lowered to acidic conditions ( $\text{pH} < 5$ ), current blockages disappeared if the *trans* chamber remained positively biased. However, when the *trans* chamber was switched to negatively biased, current blockages appeared again, as shown in Fig. 1(c), indicating that the net charge of BSA protein had changed to positive at  $\text{pH} < 5$ . This measurement is consistent with the fact that BSA is positively charged when the pH is lower than its PI ( $\text{pH} < 5$ ).<sup>12</sup> We studied the translocation of BSA through the same nanopore at three different acidic pH values (4.5, 4.1, and 2.4). The BSA molecules proved to be

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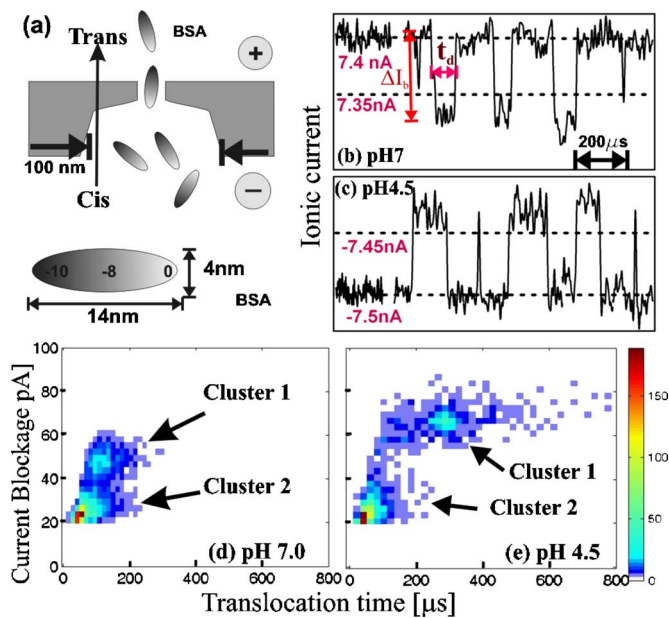


FIG. 1. (Color online) Schematic diagram (not to scale) for nanopore protein analysis experiment (a), base line and typical current blockade events for BSA at  $pH$  7.0 (b) and 4.5 (c). Event distribution plots of  $\Delta I_b$  vs  $t_d$  for BSA at  $pH$  7.0 (d) and  $pH$  4.5 (e).

positively charged at all these  $pH$  values. When the  $pH$  was 4.5, near the PI of BSA, the most probable values are  $\Delta I_b \sim 71$  pA and  $t_d \sim 269$   $\mu s$ , as shown in Fig. 1(e). The same measurement was performed at a higher  $pH$  value of 10.4. The most probable values of  $\Delta I_b$  and  $A_{eccd}$  increased as solution  $pH$  decreased [Fig. 2(a)]. The open pore current  $I_0$  was approximately constant at all the  $pH$  values measured.

If the dimensions of a protein molecule are close to that of a nanopore,  $d_m \sim D_p$  and  $l_m \sim H_{eff}$ , where  $d_m$  and  $l_m$  are the diameter and length of the protein molecule and  $D_p$  and  $H_{eff}$  are the diameter and the effective membrane thickness of the pore; theoretical description for the resistance change of the pore caused by the protein molecule translocation is complicated. However, we neglect the interaction between a protein molecule and the nanopore (free translocation); based on Ohm's law and the work by DeBlois and Bean,<sup>13</sup> the parameters of  $\Delta I_b$ ,  $t_d$ , and  $A_{eccd}$  can be approximately described below,

$$\Delta I_b = \sigma V d_m^2 l_m [1 + f(d_m, D_p, L_m, H_{eff})] / H_{eff}^2 \quad \text{when } l_m < H_{eff}, \quad (1)$$

$$t_d \sim M / QV, \quad (2)$$

$$A_{eccd} = \int_{\text{event}} \Delta I(t) \sim M \Delta I_b / Q. \quad (3)$$

Here  $M$  is the molecular weight,  $Q$  is the effective charge of a protein molecule,  $f(d_m, D_p, L_m, H_{eff})$  is a correction factor that depends on the relative values associated with the dimensions of the molecule and the nanopore, and  $\sigma$  is the conductivity of the solution. Assuming the shape of BSA molecules is globular, as shown in Fig. 1(a), then  $l_m \sim 14$  nm is smaller than  $H_{eff} \sim 20$  nm (estimated from  $I_0$ ). According to Eq. (1), an increase of  $\Delta I_b$  suggests that the product  $d_m^2 l_m$  of BSA molecules increased. The increase in  $d_m^2 l_m$  at lower  $pH$  values implies a change in conformation or

dimensions of BSA molecules. This is consistent with earlier reports that BSA molecules form dimers at low  $pH$  values<sup>14</sup> or that the volume of BSA molecules was expanded at acidic  $pH$  values.<sup>11</sup> From Eq. (2), a change in the charge of BSA ( $Q$ ) can be measured by the change in  $t_d$ . Since  $A_{eccd}$  can be measured more accurately than  $t_d$  for a current pulse, the relative charges  $Q/Q_{pH7}$  were estimated from Eq. (3). The estimated values of  $Q/Q_{pH7}$  are shown in Fig. 2(b).

If we assume that a BSA molecule in a nanopore is a rigid particle diffusing in one dimension under the influence of an electric force  $F = QV/H_{eff}$  with an average drift velocity  $v_{BSA} = H_{eff}/t_d$  using Einstein relation, the effective diffusion constant was estimated to be  $D_{eff} = (k_B T) v_{BSA} / F \sim 10^{-10}$   $cm^2/s$ . This value is three orders of magnitude smaller than it is in bulk solution ( $D \sim 10^{-7}$   $cm^2/s$ ).<sup>15</sup> The estimated characteristic time for a BSA molecule to diffuse a length of  $H_{eff}$  would be  $\tau_{eff} = (H_{eff})^2 / D_{eff} \sim 10$  ms in a nanopore. Comparing to  $t_d \sim 10^2$   $\mu s$  for BSA measured at  $pH$  7, this analysis suggests that there is a strong confinement for a BSA molecule in a nanopore.

To confirm the current blockages observed were due to BSA molecules translocating from the *cis* to the *trans* chamber via a nanopore, the *trans* chamber solution was collected after a BSA translocation experiment and subsequently used for an improved chemiluminescent BSA enzyme linked immunosorbent assay (supplemental materials). An 18 nm diameter pore with 200 mV bias voltage and an increased concentration of BSA protein (100  $\mu M$ ) in the *cis* chamber were used to increase the rate of blockage events. The translocation experiment lasted  $\sim 50$  h and the estimated number of blockages was  $\sim 2 \times 10^7$ . The event trigger level was set so that cluster 2 events were not counted. An immunoenzymetric assay kit (Cygnus Technologies, Catalog No. F030) and a chemiluminescent substrate (Lumigen PS-atto, Lumigen) were used to quantify the amount of BSA in the *trans* sample. The luminescence signal generated from the reaction of the substrate with the horse radish peroxidase (HRP) on the anti-BSA/HRP-labeled antibody was measured. The chemiluminescent intensity analysis (Fig. 3) indicated that approximately  $1.8 \pm 0.3$   $\mu g$  of BSA was in the *trans* chamber. This amount of BSA corresponds to  $\sim 10^7$  molecules, in good agreement with the number of blockages estimated. This assay demonstrated that BSA molecules were detected in the *trans* chamber; hence the current blockages observed were the result of protein traversing the nanopore.

To characterize how the size ( $M$ ) and the structure of protein molecules change current blockages, fibrinogen, a protein with charge ( $-16e$ ) similar to BSA but larger in size (340 000 Da, Sigma) was studied. A schematic model of the

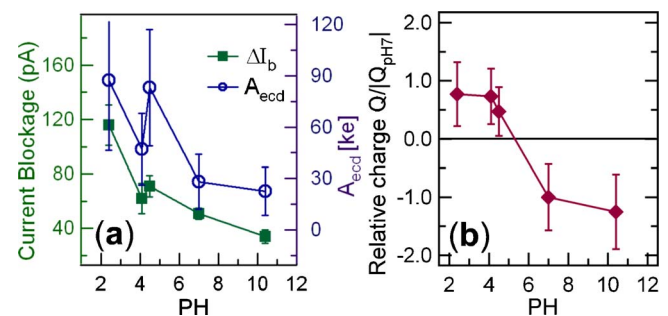


FIG. 2. (Color online) Plot of peak values of  $\Delta I_b$  and  $A_{eccd}$  vs  $pH$  (a) and plot of  $Q/Q_{pH7}$  estimated from Eq. (3) vs  $pH$  (b).

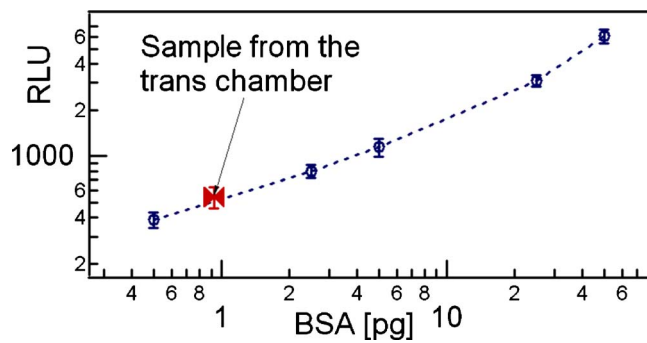


FIG. 3. (Color online) Luminescence intensity calibration curve for BSA standards (○) and for half of the sample collected from the *trans* chamber (as indicated).

fibrinogen structure<sup>16</sup> describes it as an elongated molecule  $\sim 47.5$  nm long, featuring three nodule regions, as illustrated in Fig. 4(a). In this study, an  $\sim 18$  nm diameter nanopore was used. First, BSA molecules were measured in this pore, subsequently the *cis* chamber was washed, and the fibrinogen was added and measured. Several typical current blockages are shown in Fig. 4(a) and the event distribution plot for fibrinogen is shown in Fig. 4(b). Most blockages do not reveal the nodular structure of fibrinogen except for a few of long  $t_d \sim 1$  ms events [inset of Fig. 4(b)]. When cluster 1 events of fibrinogen are compared with those of BSA, fibrinogen has larger  $\Delta I_b$  (Fig. 4(c)), longer  $t_d$  [Fig. 4(d)] and greater  $A_{\text{eccd}}$  [Fig. 4(e)] with much broader distributions. For BSA, the peak values of these parameters are  $\Delta I_b = 52 \pm 13$  pA,  $t_d = 95 \pm 28$   $\mu\text{s}$ , and  $A_{\text{eccd}} = 27 \pm 12$  ke. For fibrinogen, these values are  $\Delta I_b = 95 \pm 29$  pA,  $t_d = 170 \pm 48$   $\mu\text{s}$ , and  $A_{\text{eccd}} = 56 \pm 32$  ke. Thus, using these parameters we could clearly distinguish fibrinogen from BSA by a nanopore experiment.

A larger  $\Delta I_b$  for fibrinogen can be attributed to a larger  $d_m^2 l_m$  as indicated in Eq. (1), although when  $l_m H_{\text{eff}}$ , Eq. (1) is not accurate. A longer  $t_d$  was expected based on Eq. (2) because the ratio of  $M/Q$  is about five times larger for fibrinogen than BSA. Considering the charge distribution and the structure [Fig. 4(a)], a fibrinogen molecule could enter a nanopore with many possible configurations. This would result in different levels of  $\Delta I_b$  and values of  $t_d$ , thus a broad distribution in the  $\Delta I_b$  vs  $t_d$  plot [Fig. 4(b)] was compared to BSA [Fig. 1(d)].

In summary, by measuring the change in current caused by protein molecules traversing a nanopore, this work demonstrated that the relative charge and size of protein molecules could be estimated based on the values of  $\Delta I_b$ ,  $t_d$ , and  $A_{\text{eccd}}$ . This nanopore technique can measure properties of individual protein molecules sequentially and would allow one to determine the distribution of these properties in real time and under natural conditions. The results presented here suggest that by using a marker protein with a known charge, conformation, and size, a solid-state nanopore can be used to characterize unknown proteins. Furthermore, if the spatial

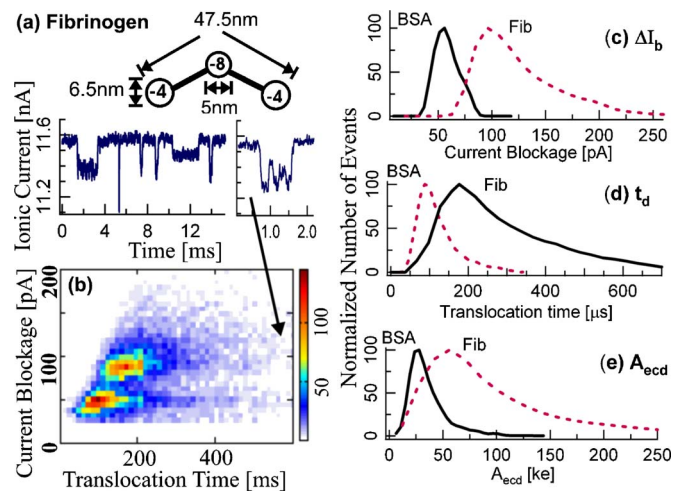


FIG. 4. (Color online) Schematic drawing of a fibrinogen molecule and several typical current blockages for fibrinogen (a), event distribution plot for fibrinogen (b), distribution comparisons of BSA with fibrinogen for  $\Delta I_b$  (c),  $t_d$  (d), and  $A_{\text{eccd}}$  (e). The experiment was performed in a 0.4M KCl and TE buffered solution at  $\text{pH}=7.0$ .

and temporal resolutions of the nanopore sensing system can be improved, structural features of protein molecules could be measured in more detail.

The authors thank J. Golovchenko for the assistance of focused ion beam hole preparation and valuable comments in this letter, and M. Xiao, S. Singh, and Ryan Rollings for helpful comments. This work was supported by NSF/MRSEC under Contract Nos. 0080054, ABI-PT06, and NIH1R21HG003290.

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