USE OF SOLID-STATE NANOPORES TO DETECT DIFFERENT CONFORMATIONAL STATES OF TRANSFERRIN
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ABSTRACT
This paper reports detection of changes in the folding states of transferrin protein at single molecule level using solid-state nanopores. Transferrin protein was detected at transmembrane voltages ranging from 200-1000 mV and also at pHs 5-8. Event characteristics such as current drop, fractional current drop and translocation time were used to infer about the folding state of individual protein molecules. It was observed that transferrin undergoes voltage induced molecular stretching at high transmembrane voltages and switches between open and closed conformations based on pH of the solution.

KEYWORDS: Resistive Pulse Sensor, Nanopore, Protein unfolding, Transferrin

INTRODUCTION
Solid-state nanopores have been used DNA detection, protein detection and studying protein unfolding and unbinding phenomena [1-4]. They are a unique single-molecule platform that allows label-free detection of proteins and sampling of their different conformational states in a dynamic set-up. In this study we use solid-state nanopore for detection and analysis of different conformational states of transferrin protein. Transferrin is an 80 kilo-Daltons serum protein that binds to ferric iron with high affinity ($10^{23}$ M$^{-1}$) at neutral pH and facilitates transferrin-receptor mediated iron uptake by cells. The iron binding ability of transferrin decreases progressively with decreasing pH and both ferric ions get released at endosomal pH of 5.5. In solution, transferrin exists in closed state (while bound to iron) or open state (usually accompanied by iron release) [5]. The closed conformations are compact with relatively small solvent exposed surface area as compared to the open conformations. The distribution of transferrin molecules between the open and the closed conformations depends on the solution pH, presence of iron and chelating agents in the solution. Single molecule detection of transferrin using nanopores help to reveal subpopulations of the protein existing at a certain pH and the shift in the probability of finding the protein in open or closed state based on the pH of the solution.

THEORY
When an insulating membrane with a small nanopore is used to separate two electrolyte chambers and a transmembrane electrical potential is applied, there is flow of ions through the pore. The conductance of such nanopores is given by $G=\sigma\left(\frac{4L}{\pi d^2}\right)+1/d$, where $d$ and $L$ are the diameter and the length of the nanopore, $\sigma$ is the bulk conductivity of the electrolyte. If the open pore current through the unobstructed pore is represented by $I_0$ and the change in current when a protein molecules is present in the pore is represented by $\Delta I$, then $\Delta I/I_0$ or the fractional current drop is proportional to the hydrodynamic volume of the protein present inside the pore.

EXPERIMENTAL
Nanopore chips were fabricated as described before [6] and consisted of 50 nm thick free standing silicon nitride membrane. A solitary 10 nm pore was drilled in each chip using converged beam of JEOL 2100F transmission electron microscope. After the chip was assembled in a flow cell, it was filled with 2 M potassium chloride solution and a transmembrane voltage was applied using Molecular Devices Axopatch 200B. When 100 nM transferrin (holo- transferrin bovine, T1408 Sigma-Aldrich, St. Louis, MO) was added to the flow cell, resistive pulses corresponding to protein translocations were observed (Figure 1 (a) and (c)). The current-time traces were recorded and event magnitude and time were extracted using thresholding method in Clampfit ver. 10.3.1.4 (Molecular Devices).
RESULTS and DISCUSSION

Once the current-time traces were recorded at varying voltages, $\Delta I$ and $Io$ values were extracted and fractional current drop ($\Delta I/Io$) values were calculated. The histograms for $\Delta I/Io$ at different voltages were plotted and were fitted with log normal distribution curves to obtain representative values. It was observed that fractional current drop values decreased with increasing voltages (Figure 2(a)), suggesting that relative current drop caused by the particle was decreasing at higher voltages. This behavior can be explained by fact that proteins are heterogeneously charged and when they experience the high electric field density inside a nanopore, they may get stretched, resulting in voltage induced partial unfolding of the protein. Such voltage dependent unfolding phenomenon can be used to probe stability of single protein molecules at different solution conditions.

Figure 2. (a) Voltage induced protein stretching inside nanopore. The fractional current drop decreases with increasing voltage. Because fractional current drop is a measure of percentage of pore volume occupied by the protein, a decrease in this fraction suggests protein stretches and occupies less volume inside the pore with increasing voltages. (b) pH induced changes in transferrin conformations. Scatter plots for current drop vs. translocation time at pH 5-8. 1 ms was chosen as threshold to classify events as short or long and long events are proposed to be associated with open conformation of the protein. With decreasing pH, Boltzmann weight for open state increases and at pH 5 half of the protein population was observed to be in open state.
As the iron binding and release mechanism as well as the protein folding and unfolding behavior of transferrin is pH dependent, we also performed protein translocation experiments at different pHs. We extracted the $\Delta I$ (current drop) and $\Delta t$ (translocation duration) from current-time traces recorded at pHs 5-8. We hypothesized that closed conformations have relatively small solvent exposed surface area and interact less with the nanopore, resulting in shorter event durations compared to the open or partially unfolded conformations. At pH 8, majority of protein molecules are expected to be in folded conformation and they tend to partially or fully unfold with the decreasing pH. It was observed that 99% of events at pH 8 were shorter than 1 ms and based on this observation 1 ms was used as a threshold to classify events into short (corresponding to folded and compact form) and long (corresponding to partially unfolded form) events. Based on the proposed thresholding criteria percentages of events shorter or longer than 1 ms were calculated at all the pHs (Figure 2(b)). As seen in Figure 2 (b), the distribution of proteins from fully closed state at pH 8 changes to about half open half closed states at pH 5. This is an interesting observation which suggests that only 50% of transferrin molecules release the bound iron and take an open conformation at endosomal pH whereas rest 50% still remain in closed state.

CONCLUSION
We have demonstrated the use of solid-state nanopores to study voltage induced unfolding of proteins and detect different conformational states of transferrin protein in label free solution state, which is difficult to accomplish using conventional protein analysis platforms.

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REFERENCES

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