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**Author contributions**

P.G. conceived and designed the project. A.B, L. Z, and N.B prepared the manuscript, carried out the experiments and analyzed the data. A.B, L.Z., N.B and P.G. cowrote the manuscript, and all authors refined the manuscript.
Voltage Controlled Shutter Regulates Channel Size and Motion Direction of Protein Aperture as Durable Nano-Electric Rectifier

-----A Leading Opinion in Biomimetic Nanoaperture

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ABSTRACT:
In optical devices such as camera or microscope, an aperture is used to regulate light intensity for imaging. Here we report the discovery and construction of a durable bio-aperture at nanometerscale that can regulate current at the pico-ampere scale. The nano-aperture is made of 12 identical protein subunits that form a 3.6-nm channel with a shutter and "one-way traffic" property. This shutter responds to electrical potential differences across the aperture and can be turned off for double stranded DNA translocation. This voltage enables directional control, and three-step regulation for opening and closing. The nano-aperture was constructed in vitro and purified into homogeneity. The aperture was stable at pH2-12, and a temperature of -85C to 60C. When an electrical potential was held, three reproducible discrete steps of current flowing through the channel were recorded. Each step reduced 32% of the channel dimension evident by the reduction of the measured current flowing through the aperture. The current change is due to the change of the resistance of aperture size. The transition between these three distinct steps and the direction of the current was controlled via the polarity of the voltage applied across the aperture. When the C-terminal of the aperture was fused to an antigen, the antibody and antigen interaction resulted in a 32% reduction of the channel size. This phenomenon was used for disease diagnosis since the incubation of the antigen- nano-aperture with a specific cancer antibody resulted in a change of 32% of current. The purified truncated cone-shape aperture automatically self-assembled efficiently into a sheet of the tetragonal array via head-to-tail self-interaction. The nano-
aperture discovery with a controllable shutter, discrete-step current regulation, formation of tetragonal sheet, and one-way current traffic provides a nanoscale electrical circuit rectifier for nanodevices and disease diagnosis.

INTRODUCTION

Life is perpetuated by a set of nanomachines that replicate and adapt with intricate functions\textsuperscript{1}. Protein-based nanomaterials are one of these bioinspired materials that combine the advantage of nanomaterials' size, shape, and surface chemistry, as well as biocompatibility and biodegradability\textsuperscript{2, 3}. There are many exciting developments in bioinspired nanomaterials using proteins for tissue engineering\textsuperscript{4-6}, drug delivery\textsuperscript{7-11}, disease diagnosis\textsuperscript{12, 13}, gene modification, DNA sequencing, single molecule sensing\textsuperscript{7, 8, 14-16} and application in other intelligent device\textsuperscript{17, 18}. This provides the impetus for our foray into applying our understanding of the viral DNA packaging motors, which contain an elegant and elaborate channel complex, onto nanotechnology and nanomedicine.

Protein nanomaterials with a variety of controlled shapes and sizes have been wildly developed\textsuperscript{19, 20}. For example, α-helical motifs can self-assemble into various structures from fibers by star-burst arrangements to function as polynanoreactors\textsuperscript{21}. Proteins can form a variety of sophisticated structures such as ferritin, chaperonins, and viral capsids\textsuperscript{22-24}. A diaphragm model has also been proposed to be related to DNA packaging and control in phage SPP1\textsuperscript{59}. Nanotubulars have attracted tremendous interest due to their unique features, like improving viscosity due to their long length and low diameters, high surface/volume ratio, gelling ability, and biocompatibility\textsuperscript{26-34}. The accumulation of peptides into amyloid fibers in Parkinson's disease inspired the self-assembly of nanotubes, which possess excellent robustness and electrical
conductivity. The one-dimensional protein bundles assembled by metalorganic protein frameworks could mimic collagen assembly in biomineralization processes. Also, genetically engineered bacterial proteins showed great potential for the fabrication of nanotubes.

Here, we reported the finding and application of protein-based nano-aperture towards tumor diagnosis. The nano-aperture is made of 12 identical protein subunits from the Phi29 nano-motor. When an electrical potential was held across the nano-aperture, three reproducible discrete steps of current change were recorded.

RESULTS AND DISCUSSION

Physical property and mechanical structure of the nano-aperture.

The aperture of a camera or microscope alters its diameter to control the amount of light flowing through its opening (Figure 1A-D). The voltage-controlled nano-aperture, which is a component of nano-motor (Phi29 DNA packaging motor), described here displays a structure and appearance similar to the cameral aperture (Figure 1E), albeit at a nanometer scale. The twelve identical subunits of the aperture tilt 30° in a spiral configuration with a left-handed chirality, thus facilitating the directional control for one-way traffic through the aperture. The X-ray crystallography (PDB ID: IFOU) shows this protein nano-aperture appears as a truncated cone (Figure 2D), with a length of 7.5 nm, a diameter of 13.8 nm for the top (C-Terminal), and 7.8 nm for the bottom (N-Terminal) (Figure 2D). When the nano-aperture is completely open, the narrowest center channel is 3.6 nm in diameter. In nature, an anti-parallel arrangement of an aperture with a left-handed configuration facilitates the right-handed dsDNA helix to pass through the aperture via a revolving mechanism. In each step of the revolution that moves the dsDNA to the next subunit, the dsDNA physically moves to a second point on the aperture channel wall,
keeping a 30° angle between the two segments of the DNA strand (Figure 1F). This structural arrangement enables the dsDNA to touch each of the 12 connector subunits (GP10 protein) in 12 discrete steps of 30° transitions for each helical pitch (Figure 1F).

**Fabrication, production, and purification of the durable homogeneous nano-aperture in large scale.**

This protein nano-aperture was produced on a large scale and purified into homogeneity, with $10^{17}$ nano-aperture particles per milliliter by nickel column purification. Industrial-scale fabrication is feasible. The purified nano-aperture can efficiently self-assemble into regular tetragonal arrays via the up-and-down alternating arrangement, as observed by transmission electron microscopy (Figure 2A-C). Deepview/Swiss-PDBViewer program$^{45}$ was used to construct 3D models of regular tetragonal arrays models. In this model, the subunit of each aperture was arranged in an alternating up and down arrangement (Figure 2E and F). The distance between each dodecamer was adjusted to 165 Å. The nano-aperture is stable at -80 to +60°C and resistant to a pH 2-14. The homogeneity of the observed nanopore from TEM indicates high yield and reproducibility across the sample’s constituency.

**Two-way traffic of peptide translocation was found in the channel of the mutant nano-aperture.**

The nano-motor uses a "Revolving Through One-Way Valve" mechanism$^{46}$. The protein nano-aperture with left-handed chirality functions as a one-way valve. This special structure allows DNA, to be transported with a single directional motion through the channel$^{42}$. However, the one-
way traffic property of the aperture can be changed into two-way traffic by the removal of 17 amino acid residues (aa 229-246) from the internal channel wall (Figure 3A), evidenced by the two-way traffic property of a peptide through the mutant nano-aperture channel (Figure 3B). The unfolded peptides is about 2 nm in diameter, similar to the diameter of dsDNA. The current blockage spike looks similar to the dsDNA indicating translocation in a similar manner. However, the rate of peptide translocation from the C-terminus to the N-terminus is lower than the translocation from the N to C, suggesting the 30-degree left chirality of the channel wall still plays a role in affecting the traffic direction. The sensing and translocation of peptides is still hampered by a lack of unique signatures that are one-to-one and onto each of the amino acids. Further investigation into how the aperture can be adjusted using the one- and two-way traffic to better discriminate against amino acids for differentiation will enable significantly improved diagnostics.

Three steps of gate size transition were confirmed by electrical conductance assays.

When the nano-aperture was inserted into a membrane, the aperture's closure can be controlled by holding the voltage, (the electrical potential difference across the connector) to specific values. This was true in the connectors from the Phi29, SPP1, T3 and T4. Here, a 3-step channel size reduction was also observed when a high potential was applied to the wild type Phi29 (120 mV), and T7 (140 mV), as well as the N-terminal (N-Δ14-N-His Phi29 with the deletion of the first 14 amino acids at the N-terminus) (80 mV) and C-terminal (C-Δ25-C-Strep Phi29 with the deletion of the last 25 amino acids at the C-terminus) (150 mV) loop deleted Phi29 connectors (Figure 4 A, B, D, E). Each step reduces the channel dimension by 32%, as evidenced by the decrease in
applied current. The resistance across the membrane increases in response to the closing channel, like ion channels in neurons. An exact 32%, 64% and 96% current change was detected when a formula of Ia/Io was applied to calculate the current variation in response to translocation and conformational changes, where Io is the original current and Ia is the instantaneous current. Interestingly, a high enough voltage is not all that is needed to trigger gating, a nano-aperture with the C-terminal modified with a urokinase-type plasminogen activator receptor (uPAR) binding peptide, once incubated with the uPAR protein, can undergo gating in its entirety (Figure 4C). It should be mentioned that uPAR has proven to be predictive biomarkers in several types of cancer, including breast cancer, pancreatic cancer, soft-tissue sarcoma, and pulmonary adenocarcinoma. Three-step reopening of a closed nano-aperture has been observed after applying a low voltage, which can be readily described in a similar manner with three steps (Fig. 4F).

It is very interesting to find that in all three discrete steps of channel size reduction, the rate of reduction is all about 32%. The 32% of reduction is a rate almost identical to the rate of current blockage by dsDNA. Although the mechanism that leads to such novelty of the three 32%-reduction steps is a mystery, it is hypothesized that, during the viral evolution, the conformational change in change size reduction is a correspondence to the size of the dsDNA. That is, dsDNA has served as a mold for the adaptation of the conformational change during the mutation and adaptation process. Alternative mechanisms include that the connector subunits may independently and ubiquitously undergo conformational changes in the presence of a potential difference across the connectors, evident of an induced fit model of dsDNA translocation. The three steps would then represent the three conformational states the connector adopts in the process of dsDNA translocation.
Application of the nano-aperture for cancer biomarkers detection at the single molecule level.

We then sought out towards using this method of rapid detection of conformational change of the connector via a 32% reduction towards cancer biomarker detection. A conductance assay using an aperture conjugated with a six-histidine His-tag to the C-terminal and incubated with an anti-his tag antibody or nanogold coated with Ni-NTA, triggered one-step of conformational change; similar to the first step of gating shown in Figure 4, which inspired the application of the nano-aperture for single-molecule detection of antigens or antibodies by antigen-antibody interaction. Utilization of engineered protein nano-apertures with an 18-amino acid Epithelial Cell Adhesion Molecule (EpCAM) detection peptide at its C-terminal (Figure 5A), colon cancer-specific EpCAM antibody-specific binding events could be detected in real-time at the single-molecule level in 0.4 M KCl, 5 mM HEPES, pH 7.4 conductance buffer (Figure 5B). This engineered protein nano-aperture can discriminate the signal from background events with high sensitivity (Figure 5C). The result also supported the conclusion that the shutter is located at the C-terminal (Figure 5). This conclusion was also supported by the fact that the deletion of 25 amino acids at the C-terminal disabled the three-step gating capacity.

The nano-aperture was also re-engineered with uPAR binding peptide to its C-terminal. uPAR is the biomarker of breast cancer, pancreatic cancer, soft-tissue sarcoma, and pulmonary adenocarcinoma. Once incubated with the uPAR protein in 0.15 M KCl, 5 mM HEPES, pH 7.4 conductance buffer, the binding signals of the uPAR protein to the C-terminal peptide were observed, evidenced by three steps gating (Figure 3C), current blockage histogram, and scatter plot result.
These nano-apertures are larger than 3 nm in diameter, making them more amenable as a single-molecule sensing platform with an advantage over other biological membrane pores which are about 1.2 nm. The uniform dimensions and homogeneous pore size make the sensing and other practical applications more reproducible. Generally, the nanopore sensing mechanism is dependent on the resistive pulse technique. Once an electrical potential is applied across the aperture, the sample molecules translocating through the aperture will generate a unique electrical current signature due to the changes in ion flow caused by the blockage. Thus, measuring the current alternation and dwell time (length of the event) of translocation events will serve as the signature of the analytes. When different molecules, such as DNA, RNA, or peptides pass through the nano-aperture, unique, distinctive current signatures and dwell times will be generated, which can serve as the fingerprint for the identification of the corresponding molecules. Nano-apertures have been applied to the sensing of RNA, DNA, chemical. The gating mechanism could be harnessed to prove a unique third variable to the blockage signature of the translocating analytes for high-resolution discrimination. It has also been used for the differentiation of peptides with only one amino acid difference in length or in composition. The nano-aperture is capable of differentiating between diverse conformations of a protein. It has also been used for quantitative analysis of the kinetic process of peptide oligomerization in real-time at the single molecule level.

CONCLUSION

A nano-aperture with a voltage-dependent shutter system for size control has been identified and constructed. The adjustable channel size and trafficking direction made it possible to alter the aperture from one-way traffic to two-way traffic. These controllable characteristics in pore size
and motion direction unleash the potential for the nano-aperture to serve as a nano-electric rectifier. The production of electrical signature from such nano-apertures have been applied to the fingerprinting of DNA, RNA, protein, chemical, and antibody. This concept enables investigation towards more precise differentiation of small molecule blockage signatures during translocation for better discrimination between small analytes. The system can be used for diagnosis via the detection of detect current change driven by small molecule binding.

Conflicts of Interest
P.G. is the consultant of Oxford Nanopore Technologies; the cofounder of Shenzhen P&Z Biomedical Co. Ltd., as well as co-founder and board member of ExonanoRNA, LLC and its subsidiary Weina Biomedical LLC.

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Author contributions
P.G. conceived, designed the project and initiated the manuscript. L. Z. and N.B carried out the experiments. A.B, and L. Z, helped analyzed the data and finalizing the manuscript. X, C. enhanced the figure quality and resolution.

Reference
Fig. 1. Structure of the camera-like nano-aperture. (A-D) Structure of a camera aperture with stepwise-closing. (E) Crystal structure of the connector of phi29 DNA packaging motor showing the tilting of the 12 protein-subunit with 30° angle between two adjacent connector subunits. (F) Top: Illustration of one 360°-circle divided into 12 sections, showing the shift of 30° angle between two adjacent sections. Bottom: Illustration of one 360°-helical turn of dsDNA divided into 12 steps, showing the shift of 30° angle between two sequential steps.
Fig. 2. Images of the pacified nano-aperture. (A-C) TEM images of the fabricated purified nano-aperture, self-assembled into a tetragonal array. The scale bar is 25 nm. (D) Side view of the nano-aperture crystal structure. (E-F) Computationally constructed structure displaying the array structure assembled by alternative up and down arrangement, showing dimensions and polarity.
Fig. 3. One-way traffic of a nano-aperture. (A-B) “One-way” DNA translocation of the connector of phi29 DNA packaging motor under a ramping potential from -100mV to +100mV (data from\textsuperscript{42}). The change of the connector orientation leads to the appearance of current blockage spikes when dsDNA was placed at both trans and cis sides of the chamber (data from\textsuperscript{42}). (C) No DNA in either chamber as negative control (data from\textsuperscript{42}). (D) Alteration of voltage polarity to demonstrate that DNA can only be translocated from one side at one kind of polarity, when DNA was placed in both trans and cis chambers(data from\textsuperscript{42}). (E) Illustration showing the flexible inner channel loops (green color) of the phi29 connector. (F) Two-way traffic property of peptide through the internal loop-deleted Phi29 connector. Figures were adapted with permission from\textsuperscript{42} Copyright 2013 American Chemical Society.
Fig. 4. Three-step gating of connector nano-aperture of phage DNA packaging motors. The observed dip in (A) into the 2nd step of the phi29 conformation while still in the 1st step indicates that the gating conformational changes are reversible and there are varying degrees of stability within each of the conformational states.
Fig. 5. Real-time sensing of EpCAM antibody (Ab) interactions with EpCAM antigen (Ag) using engineered phi29 connector nano-aperture, demonstrating the C-terminal is the shutter triggering the step-wise conformational change. (A) Schematic diagram showing the placement of the colon cancer Ag into the C-terminal of the phi29 connector for real-time detection of the Ag with its corresponding Ab. (B) Histogram of current blockage events caused by the addition of diluted EpCAM Ab. (C) Histogram plotting of current transition events of specific (green) and nonspecific (red) binding to the shutter at the C–terminal caused by Ab/Ag interactions.
Declaration of interests

x The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: