

# Graphene Nanochannels for Label-Free Protein Detection and Protein-Protein Interaction Analysis

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## Abstract

Resistive pulse sensing, utilizing the electrophoretic movement of proteins through nanopores or nanochannels, has emerged as a promising method for label-free protein detection and characterization. However, traditional solid-state materials, such as Si, SiO<sub>2</sub>, SiN<sub>x</sub>, and polydimethylsiloxane, suffer from significant limitations, including non-specific protein interactions with solid surfaces that cause channel blockage, preventing the long-term reliability of resistive pulse sensing. In contrast, two-dimensional materials have attracted much attention due to their potential in biomolecular detection because of their ultra-thin thickness, ultra-high surface flatness and extremely high mechanical strength. Among them, the extremely high surface flatness helps to reduce the transport resistance of biomolecules moving on its surface. Here, we demonstrate that graphene nanochannels, fabricated via layer assembly, provide exceptional properties for protein analysis, including low noise, high surface smoothness, and minimal non-specific protein adsorption. These attributes make graphene nanochannels an ideal platform for long-term, stable protein characterization. Our findings show that these nanochannels can effectively differentiate between five distinct proteins based on resistive pulse signals. Additionally, we utilized the nanochannels to monitor the binding dynamics of immunoglobulin G (IgG) and the aggregation process of  $\beta$ -lactoglobulin, revealing the capability of

graphene nanochannels in detecting protein-protein interactions and molecular conformational changes. This work highlights the potential of graphene nanochannels as powerful tools for label-free, highly sensitive protein identification and interaction studies, marking a significant advancement in biosensing technology in biomolecular research and diagnosis.

**Keywords:** nanochannel, protein detection, protein-protein interaction, label-free, graphene

Label-free protein identification technologies have attracted more and more attention in the fields of biology, pharmacy and biochemistry, such as nano-optical tweezers, interference scattering microscopic imaging technology, dark-field microscopic imaging technology, resistance pulse sensing, etc., directly image and observe the movement process of biomolecules or fluids, avoiding the interference introduced by markers to biomolecules.

The resistive pulse sensing is an important method for label-free detection and characterization of proteins.<sup>1,2</sup> When a protein passes through the channel driven by the electric field or pressure, it either reduces the number of ions within the channel or brings more ions due to its charge, both of which will introduce fluctuations in the ion current signal that carries proteins size and charge information.<sup>3,4</sup> Different proteins have unique spatial structures, molecular weights and charge characteristics, allowing promoting these pulse signals for protein screening.

Currently, most resistive pulse sensing methods for protein detection are realized using nanopores with thickness comparable to the size of the proteins, so as to maximize the signal-to-noise ratio.<sup>5-9</sup> However, this approach presents several challenges. First, the temporal resolution of the protein signal is affected by the short transmission time (i.e., several microseconds).<sup>10,11</sup> Additionally, the complex surface charge distribution and non-specific adsorption between proteins and nanopore surface make it difficult for proteins to pass through the confined spaces smoothly, which leads to channel blockage and prevents the transport of additional molecules.<sup>12,13</sup>

Prolonging the transmission time can be solved by extending its transmission path. However, a longer transmission path also increases the likelihood of contact between the protein and the solid surface, raising the risk of non-specific adsorption, hence potential channel blockage. Traditional solid material channels struggle to meet these two requirements at the same time, especially when the height of the target channel is equivalent to protein size. A common approach to reducing protein adsorption

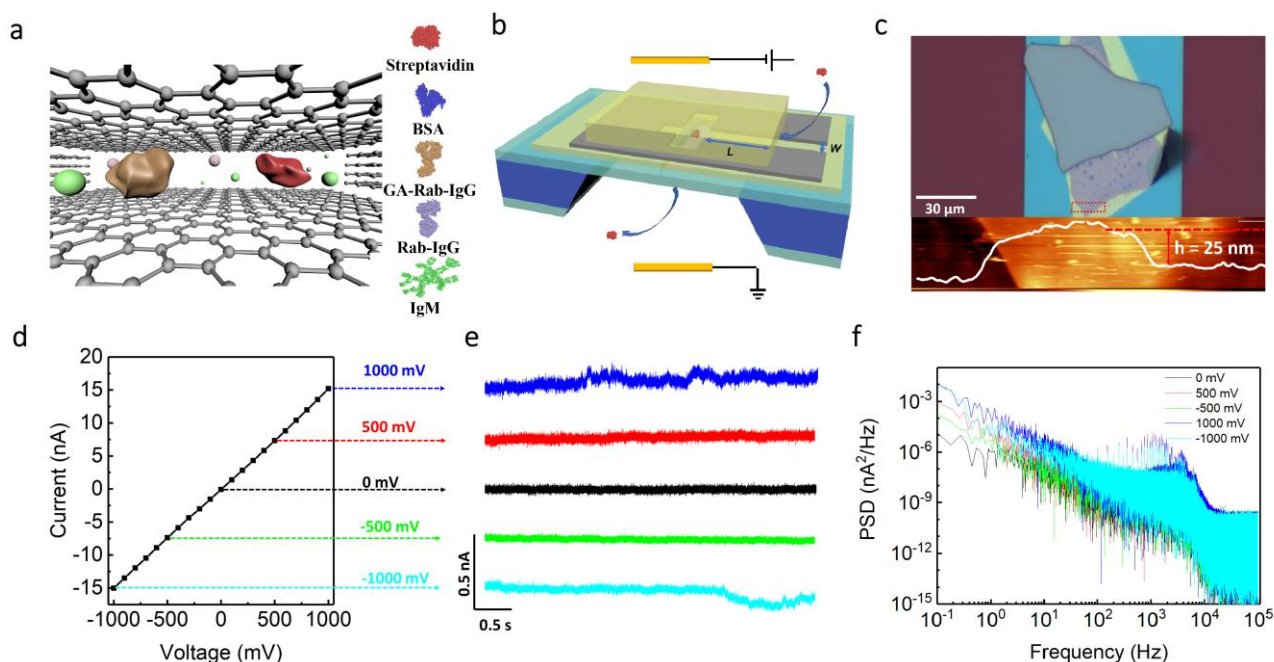
is to coat the surface of the pores with polymers.<sup>14, 15</sup> Although polymer coating can be effective, it is still particularly challenging to achieve complete coverage of the inner surface by infiltrating polymer solution into long restricted channels. In addition, surface coating introduces extra steps, such as sample pretreatment and subsequent cleaning, which increases the risk of sample blockage or damage.<sup>16</sup> Graphene nanochannel provides a promising solution to these problems. The ultra-smooth surface, precise nanoscale size control, and ease of processing make the graphene nanochannel highly advantageous for protein detection.<sup>17, 18</sup> However, defects and contamination on the graphene surface can cause to non-specific adsorption, necessitating exceptionally high standards of surface cleanliness.<sup>19, 20</sup>

In this work, we prepared graphene nanochannels with low noise, high stability and high surface flatness, and measured the transport of proteins within them. The experimental results prove that proteins can be smoothly transmitted within graphene channels, and different proteins can be identified based on the current pulses generated during their transmission within the channels. We further applied these channels to the detection of protein-protein binding in protein mixtures and the fibrosis of lactoglobulin. These results highlight the wide application of graphene nanochannels in the detection and characterization of label-free proteins.

## Results and discussion

The structure of the graphene nanochannels is illustrated in Figure 1a and 1b. The top and bottom surfaces consist of mechanically exfoliated graphene, while the middle layer comprises graphene spacers etched by a femtosecond laser. These spacers are three layers of graphene assembled on a free-standing silicon nitride ( $\text{SiN}_x$ ) membrane by fixed-point transfer method.<sup>18</sup> The electric field drives the protein across the membrane through the graphene channel, the only connection between the two sides of the membrane. The ion current of this channel is monitored by a patch clamp amplifier. It is crucial to select the channel size to accommodate the proteins of interest, as low channel prevents larger proteins difficult to pass, while a high channel reduces the signal-to-noise ratio for small proteins. We have tested several samples with different channel dimensions. When the channel height was reduced to 13 nm, even small proteins like BSA (66.5 kDa) struggle to pass smoothly, leading to prolonged channel blockage and makes it difficult to capture transmission signals from other proteins (Figure S1, supporting information). After thorough tests on channels with various dimensions, we concluded that

the optimized channel height of 25 nm (Figure 1c) can effectively detect translocations of proteins with diameters ranging from 5 nm (i.e., streptavidin, 55 kDa) to 30 nm (i.e., IgM, > 900 kDa).

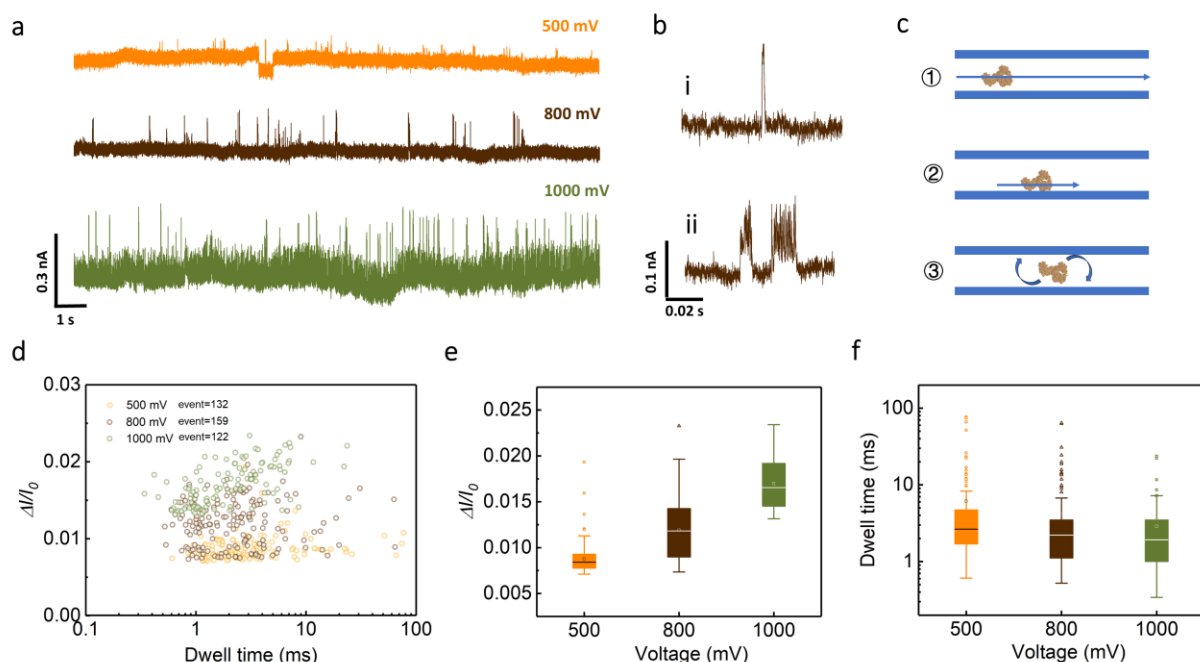


**Figure 1** a) Schematic diagram of proteins transport within a graphene nanochannel. Crystal structures of proteins used in this work, top to bottom: streptavidin, bovine serum albumin (BSA), goat anti-rabbit IgG (GA-Rab-IgG), rabbit IgG (Rab-IgG), immunoglobulin m (IgM). b) Schematic diagram illustrating the structure and current measurement setup of the graphene nanochannel. c) Microscopic image (top) and AFM height scanning (bottom) of the graphene nanochannel ( $h = 25$  nm,  $w = 500$  nm,  $L = 10$   $\mu$ m). d) Current-voltage characteristics of the graphene nanochannel. e) Ionic current traces of the graphene nanochannel at different voltages. f) Power spectral density (PSD) of ion current signals at different voltages.

First, we tested the stability and conduction performance of the prepared graphene nanochannels. In a 1 M KCl solution, the conductance of this channel can be obtained as approximately  $\sim 15$  nS through the  $I$ - $V$  curve (Figure 1d), which is close to the theoretical conductance value (13.75 nS) when the influence of its surface charge is ignored. The graphene layers stacked by van der Waals forces and thick top layer (more than 100 nm) make the channel more resistant to mechanical vibrations (low frequency noise,  $1/f$  noise) compared to nanopores made from two-dimensional materials. The thick graphene top layer also reduces the total capacitance, thereby, lowering the dielectric noise (high

frequency) of the channel.<sup>21, 22</sup> The current trajectory and power spectral density (Figure 1e and 1f) show that the channel can maintain a low noise level across a wide frequency range even at high voltages. This low-noise characteristic is extremely important for achieving high signal-to-noise ratio, so as to detect signals generated by small proteins or to distinguish various proteins.

To evaluate the capability of graphene nanochannels for protein characterization, we translocated GA-Rab-IgG (2  $\mu\text{g}/\text{ml}$ , 1 M KCl solution, 5 mM Tris, pH=8.1) through a graphene nanochannel ( $h = 25\text{ nm}$ ,  $L = 10\text{ }\mu\text{m}$ ,  $w = 500\text{ nm}$ ). Following the introduction of the protein solution, distinct pulse signals appeared in the current trace (Figure 2a). These pulses, which show short-lasting increases in current, arise from the combination of the protein's charge and space repulsion effects (S1, supporting information).<sup>23, 24</sup> When the spatial repulsion effect dominates, blockage current pulses are generated. Conversely, we observe positive current pulses when the charge effect is significant. In this work, proteins are highly negatively charged in the alkaline electrolyte solution (pH 8.1), which attract a large number of potassium ions into the channel. The influx of the attracted ions into the channel outnumbers the ions repelled by the protein volume, thus generating an ascending current pulse.



**Figure 2** a) Current trace of GA-Rab-IgG at different voltages. b) Two representative pulses collected at 800 mV. c) Illustration of protein transport state in graphene nanochannels. d) Scatter plots of resistive pulses of GA-Rab-IgG translocation at different voltages. e)-f) Statistics of  $\Delta I/I_0$  and dwell time of GA-Rab-IgG translocation events at different voltages. Box plots display the 25th and 75th

percentiles with central mark indicating the median. The whiskers extend to the most extreme data points without considering outliers.

We have observed two typical pulses, labeled as pulses i and ii in Figure 2b. The short and sharp pulse i indicates the smooth passage of a single protein through the channel, with the residence time falling within a few milliseconds (less than 5 ms), corresponding to ① in Figure 2c. Pulse ii demonstrates a typical pulse with a residence time exceeding 10 ms and multiple peaks. Several possibilities can attribute to this multi-peak phenomenon. First, protein adsorption onto the inner surface of the channel causes transmission obstacles, prolonging the transmission time.<sup>3,25</sup> At the same time, positional changes in protein during adsorption lead to multiple peaks (② in Figure 2c). Second, variations in protein orientation within the channel, caused by non-uniform forces or Brownian motion, produce multiple peaks in the current pulse (③ in Figure 2c).<sup>3, 11, 15</sup> Another potential cause of multi-peak signals could be the simultaneous passage of multiple proteins through the channel. However, such occasion would result in changes to the average pulse amplitude as proteins enter or exit the channel, an effect not observed in the two representative multi-peak pulses shown in Figure 2b ii.

Figure 2e and 2f describe the correlations between the  $\Delta I/I_0$  and dwell time with voltage. The current amplification,  $\Delta I/I_0$ , increases with the voltage increasing from 500 mV to 1000 mV (Figure 2e). In resistive-pulse sensing, changes in  $\Delta I/I_0$  under different voltages are often attributed to structural changes of analyte, such as linearization or decomposition.<sup>26-28</sup> However, for proteins with stable structures and large channel size, this increase is less likely to result from proteins conformation changes, as we observed similar voltage-depend increases in  $\Delta I/I_0$  for gold nanoparticles previously.<sup>18, 29</sup> Wen et al. also demonstrated a significant positive correlation between  $\Delta I/I_0$  with voltage in the experiment of trapping particles using ion concentration polarization (ICP).<sup>30</sup> At a higher voltage, strong concentration polarization occurs, resulting in increased ion concentration accumulation on protein's two sides, which leads to a high pulse amplitude during protein transport. At the same time, the dwell time decreases with increasing voltage, suggesting a fast electrophoretic movement of proteins (Figure 2f). The  $\Delta I/I_0$  distribution is relatively concentrated at 500 mV but it becomes dispersed at higher voltages. Low voltage drives protein slowly and creates relative homogenous pulses, which is favored for high-precision protein identification.

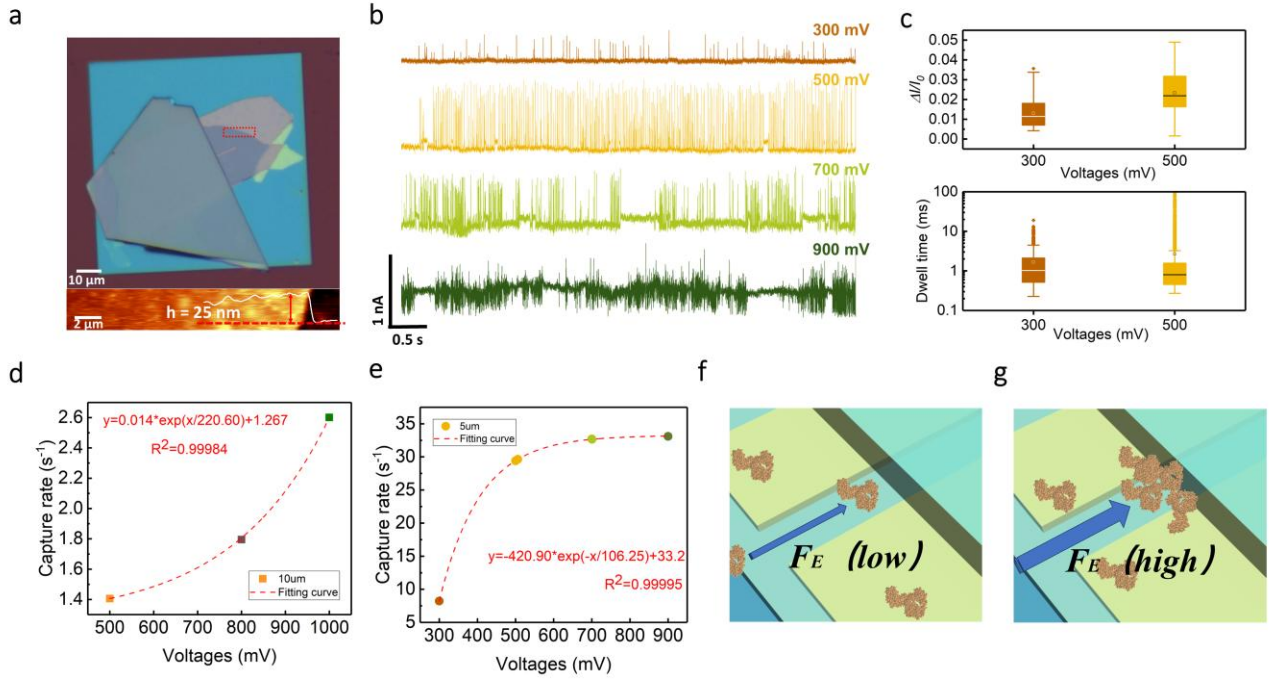


Figure 3. a) Microscopic image and AFM height scanning of the graphene nanochannel ( $h = 25$  nm,  $w = 500$  nm,  $L = 5.5$  μm). b) Current trace of GA-Rab-IgG at different voltages. c) Statistics of  $\Delta I/I_0$  and dwell time of GA-Rab-IgG translocation events at different voltages. Box plots display the 25th and 75th percentiles with central mark indicating the median. The whiskers extend to the most extreme data points without considering outliers. d) Protein capture rate of 10 μm channel at different voltages with fitting to exponential growth. e) Protein capture rate of 5.5 μm channel at different voltages with fitting to exponential decay. f) - g) Schematic diagram of protein transport state at the entrance of channel under different electric force intensities.

For channels with the same height, shortening the channel length to 5.5 μm ( $h = 25$  nm) still allowed the detection of GA-Rab-IgG signals (Figure 3a). The reduced channel length almost doubled the electric field intensity in the channel. At 300 mV, the current baseline remained stable, and the pulse signal indicates a smooth transport of protein (Figure 3b, 300 mV). When the voltage increased to 500 mV, the pulse frequency increased, and also show some baseline fluctuations and some long-term pluses with low amplitude (Figure 3b, 500 mV). We analyzed the pulse signals at 300 mV and 500 mV, and observed a positive correlation of  $\Delta I/I_0$  and voltage (Figure 3c, top), consistent with those observed in the 10-μm channel (Figure 2d). The  $\Delta I/I_0$  values observed in the 5.5-μm channel are

almost twice those of the 10- $\mu\text{m}$  channel, suggesting that the protein occupies a higher volume and charge ratio in the shorter channels (Figure S2, supporting information). As expected, the dwell time in the 5.5- $\mu\text{m}$  channel is approximately half that observed in the 10- $\mu\text{m}$  channel (Figure 3c, bottom).

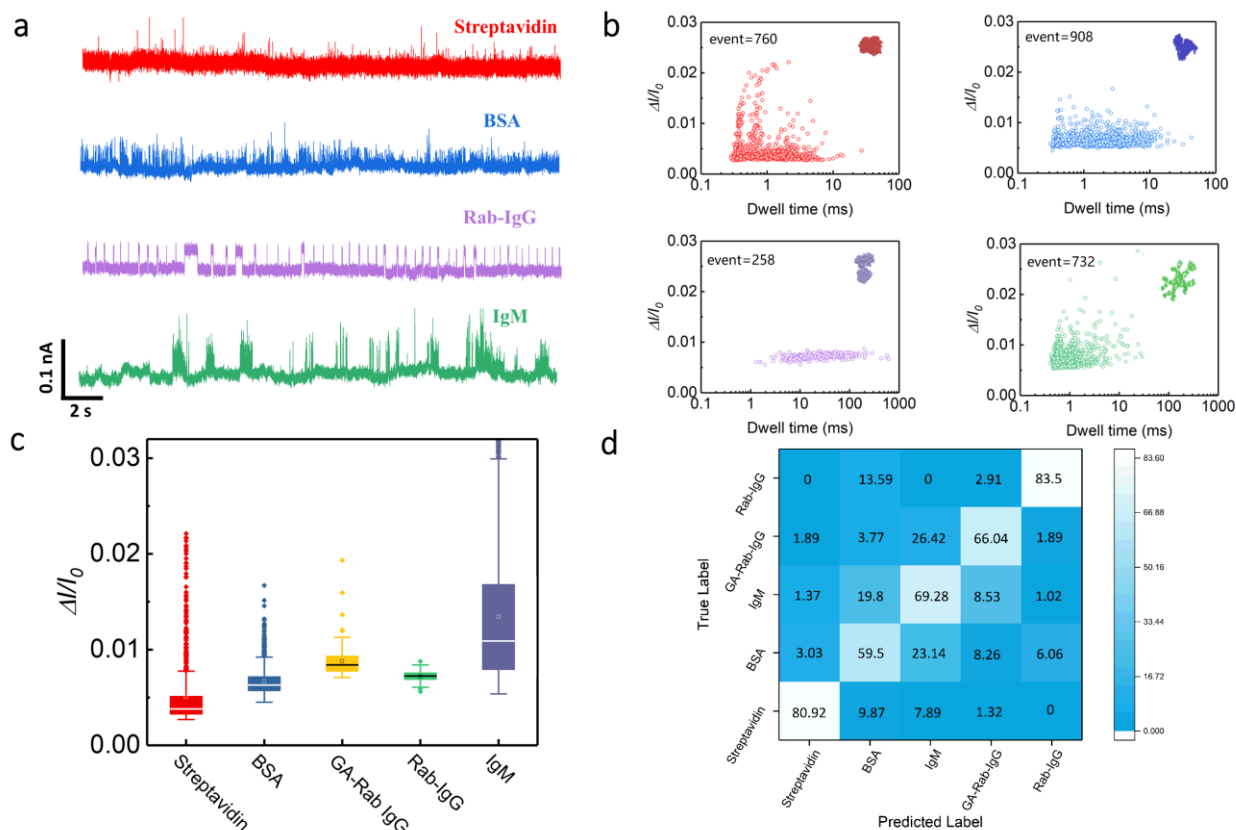
As the voltage further increased to 700 mV, the frequency and duration of these low-amplitude long-lived current pulses also increased (Figure 3b, 700 mV). These observation agrees well with the results of BSA translocating through  $\text{SiN}_x$  nanopores reported by David et al.<sup>25</sup> They attributed similar phenomena to protein adsorption at the nanopore entrance without passing through. In the case of nanochannel here, the proteins absorption within the channel produces long multi-peak pulses when moving into the channel, and the pulse height is consistent, instead of low-amplitude long-lived current blocking.

Theoretical analysis predicts a positive exponential growth of the protein capture frequency with voltage (S2, supporting information), which is consistent with our observation in 10- $\mu\text{m}$  channel (Figure 3d). Kevin et al.<sup>31</sup> and Lindsay et al.<sup>32</sup> respectively obtained similar conclusions in the protein capture experiment using solid state nanopores. For 5.5- $\mu\text{m}$  long channel, however, its capture rates show  $-A \exp(-\alpha V)$  with the voltage, as show in Figure 3e. These signals likely arise from protein aggregation at the channel entrance. At low voltage, the weak driving force on the protein results in a low capture frequency of the channel. Under this condition such as 300 mV and 500 mV, the channel entrance captures protein one at a time, enabling smooth translocation (Figure 3b and 3f). As the voltage increases to 700 mV, the stronger driving force pushes protein accumulating at the channel entrance, which prevents protein from passing through the channel (Figure 3b and 3g). As the voltage increase further to 900 mV, proteins accumulated at the entrance completely blocks the channel, resulting in noise-like fluctuations without clear translocation signals (900 mV in Figure 3b). During the process of testing this sample, whether it is from 900 mV to 300 mV or from 300 mV to 900 mV, we can observe the trend of this signal, and this signal is not a random.

According to equation (S8), there should be a significant positive correlation between protein concentration and signal frequency. However, due to the influence of charge accumulation and protein accumulation at the entrance of the channel, the high concentration of protein is more likely to lead to signal capture difficulties. Although the theoretical capture probability decreases at low concentration, the blocking and channel repulsion effects at the hole are weakened. It might be easier to enter the

transmission channel. Our low concentration experiments found that the frequency of protein signal is actually higher at low concentration (Figure S3, supporting information).

The precise structural design of the nanochannel is essential for capturing the translocation signals of diverse proteins and minimize the impact of protein accumulation at channel entrance and exit, or from adsorption to the surface. Optimizing electric field intensity and protein/ion concentrations further improves the protein translocation efficiency and capture rate.



**Figure 4** a) Current traces of different proteins translocating through a graphene nanochannel at 500 mV. b) Scatter plots of resistive pulse signals for different proteins. c) Statistics of  $\Delta I/I_0$  for different proteins corresponding to b. d) The confusion matrix of the testing set generated by the Ensemble model of machine learning.

We used a 10- $\mu$ m graphene channel to detect the translocation of four other proteins at 500 mV, including 2  $\mu$ g/ mL Streptavidin, 20  $\mu$ g/ mL BSA, 4  $\mu$ g/ mL Rab-IgG, and 8  $\mu$ g/ mL IgM, all in 1 M KCl, 5 mM Tris buffered at pH=8.1. Figure 4a shows variation in baseline noise for different proteins, reflecting the varying levels of difficulty in protein transport. Compared to other proteins, BSA is more

prone to non-specific adsorption on solid surfaces, resulting in greater baseline perturbations.<sup>25</sup> IgM shows certain differences in signaling compared to the other four proteins, with some signals forming clustered fluctuations. We think this is due to the larger molecular size of IgM (30 nm in long axis). The other four kinds of proteins are smaller than the size of the channel in any dimension, so they can be transported anywhere in the channel without being too hindered at the entrance of the channel. However, IgM has a disc-shaped structure, and the diameter corresponding to its long axis is higher than the height of the channel. Therefore, when entering the channel to transmit, its transmitting position is limited by the channel, and it is necessary to enter the channel in a parallel, as shown in Figure S4, supporting information. During the process of posture change, the collision with the entrance will produce continuous fluctuations, forming a cluster of pulse signals. Also, the large size of the protein will also cause severe friction or collision with the channel surface, thus generating signal fluctuations.

The baseline fluctuations come from the noise of the device itself, the adsorption or friction between proteins and the surface, the collision between proteins and the orifice, etc. In addition, when it comes to the signal transmission of different proteins, the baseline fluctuation of different protein is also different due to the differences of its own charges or adhesion. The baseline fluctuations of BSA are relatively strong, but that of streptavidin and the two IgG proteins is relatively weak. However, this is obviously different from the blocking effect caused by strong nonspecific adsorption in solid-state nanopores reported.<sup>3, 25</sup> Proteins still show a continuous state of transportation, and there is no strong obstacle.

Figure 4b shows that the  $\Delta I/I_0$  distributions for all proteins are relatively concentrated, while the dwell time presents a wide range of variability. Although the same kind of proteins processes fixed volume and charge, the probability of it contacting and collision with the channel surface is random, which leads to a wide distribution of dwell time. Figure 4c shows distinguishable  $\Delta I/I_0$  values for the five proteins. Given that the pulse amplitude correlates with protein charge, S3 (supporting information) lists the estimated charge of the five proteins. Streptavidin, with 2.4 charges, exhibits the lowest  $\Delta I/I_0$ , while IgM, carrying 130 charges, has the highest  $\Delta I/I_0$ . Although BSA carries about 8 more charges than the two IgG proteins, the  $\Delta I/I_0$  values for two IgGs are slightly higher than BSA due to their molecular weight being 2.3 times greater. Due to the ICP effect, when carrying a similar charge,

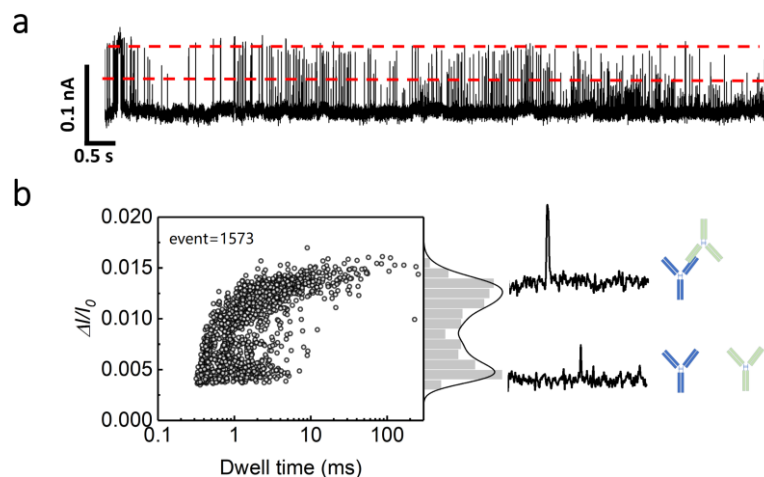
proteins with larger spatial structures attract more ions on both sides, thus enhancing the channel conductance.

Because of the long scale of the channel, there is indeed signal overlapping caused by the simultaneous transmission of multiple proteins. The process of simultaneous transmission of various proteins can be judged by the peak value and shape of the signal. The most obvious difference between the pulses of overlapping events and those of independent events lies in the pulse height, which will cause the pulse height to increase by multiple and distinguish it from most independent events. For example, when multiple proteins are transmitted simultaneously, an increase in pulse height is bound to occur. As shown in the Figure 4b, some data obviously deviate from the whole, showing high blocking efficiency, which may contain overlapping transmission signals. Although there is signal superposition, in the box plot, the obviously deviated signals are excluded. Statistics from the box plot of  $\Delta I/I_0$ , for the channel with a height of 25 nm, the values displayed by 75% is less than twice that displayed by 25%, and the pulse height of the main part is relatively concentrated, which is within the reasonable range of single molecule transmission.

In order to achieve accurate protein recognition, we build a machine learning algorithm based on decision tree method. The model was trained and tested using the blocking efficiency  $\Delta I/I_0$  and dwell time of the protein transmission signal, and the corresponding confusion matrix results were given (Figure 4d). Under the influence of protein adhesion and baseline fluctuation, the recognition accuracy of BSA was the lowest, reaching 59.5%, while that of other proteins is over 60%, and the recognition efficiency of streptavidin and Rab-igg even reached 80%. The results show that graphene nanochannels have a good recognition efficiency for proteins. We also used this channel to test the mixed solutions of proteins with different molecular weights (Figure S5, supporting information). From the current trajectory, multiple pulses of different heights could be identified, and the current test of a single protein showed that the pulse heights were relatively uniform. From the scatter plots, it is manifested as a mixed signal of multiple proteins.

In many clinical or diagnostic settings, target proteins are present in the nanogram per milliliter range or even lower. In order to verify the signal capture ability at lower concentration, we diluted the GA-Rab IgG protein samples by  $10^2$  and  $10^4$  times on the basis of the current concentration, reaching the nano-molar level, and tested it in graphene nanochannel ( $h=36$  nm,  $L=10$   $\mu$ m), the current trace

and statistics as show in Figure S6-S7, supporting information. The protein transport signals were captured at both concentrations, which proved that this channel is suitable for low concentration detection.

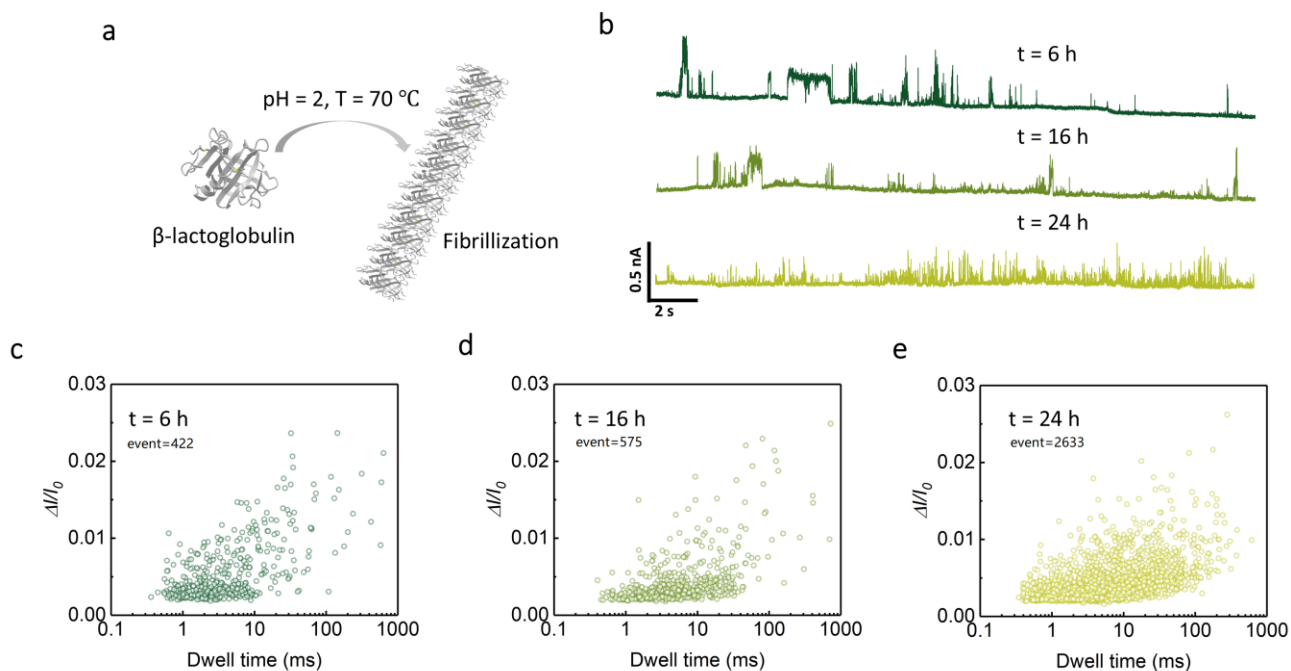


**Figure 5** a) Current trace of GA-Rab-IgG and Rab-IgG mixture translocating through a graphene nanochannel. b) The scatter plots of  $\Delta I/I_0$  and dwell time for GA-Rab-IgG and Rab-IgG mixture, with representative pulses shown on the right.

In the aspect of disease diagnosis, the study of specific protein-protein combination is helpful to find biomarkers closely related to diseases.<sup>9, 28, 33-35</sup> These biomarkers can be the key protein involved in the occurrence and development of diseases, or the protein complex whose binding state can reflect the disease state. By monitoring the combination of these protein, doctors can identify the existence of diseases earlier and more accurately, so as to take timely and effective treatment measures. Rapid identification of pathogenic protein is very important for early diagnosis and improvement of therapeutic effect. Surface plasmon resonance (SPR) technology and grating coupled interference (GCI) technology can also be used to identify the specific binding of proteins. It measures the change of surface refractive index before and after protein binding by optical methods. It has a high sensitivity to surface reactions and depends on the binding ability of proteins on the surface. It is necessary to balance the concentration of the analyte and the affinity. The channel directly detects the transport of proteins, so it is necessary to balance the concentration and detection throughput in the test. In principle, the channel has a higher sensitivity for protein binding recognition and is suitable for the detection of low concentrations. Furthermore, these two methods detect the collective responses brought about by

a large number of immune responses and are difficult to delve into the single-molecule level. The channels can delve into the single-molecule scale to analyze the conformational changes of proteins.

Given previous success of graphene channels in identifying proteins based on their molecular weights, they are excellent candidates for studying specific binding of proteins. In this study, we prepared a mixed solution (4  $\mu\text{g}/\text{ml}$  of Rab-IgG and 2  $\mu\text{g}/\text{ml}$  of GA-Rab-IgG, and the ratio of the mixture was 1:1, 1 M KCl, 5 mM Tris, pH=8.1). Theoretically, this mixture should contain unbound proteins and their specifically bound products. Figure 5 depicts the current trajectories and statistical data of this protein mixture translocating through the graphene nanochannel ( $h=25\text{ nm}$ ,  $L=10\text{ }\mu\text{m}$ ). The current trajectories reveal two different pulse amplitudes indicated by two dashed lines. Both scattering plot and the histogram exhibits two distinct peaks in  $\Delta I/I_0$  value. The lower  $\Delta I/I_0$  value corresponds to the translocation of unbound proteins, which is consistent with that obtained from separate tests of GA-Rab-IgG and Rab-IgG (Figure 4c). The higher  $\Delta I/I_0$  value arises from the translocation of protein complex. The application of graphene channel in the study of protein specific binding will not only deepen our understanding of the interaction mechanism between biomolecules, but also open up a new perspective for disease diagnosis and treatment.



**Figure 6** a) Illustration of  $\beta$ -lactoglobulin fibrillization process at pH 2.0, at a temperature of 70 °C in water bath. b) Current traces at 1000 mV for  $\beta$ -lactoglobulin incubated for different durations. c)-e) Scatter plots of  $\Delta I/I_0$  and dwell time for  $\beta$ -lactoglobulin after incubated for different durations.

There is a close relationship between amyloid fibrils and many degenerative diseases, among which the most well-known are Alzheimer's disease and Parkinson's disease. These two diseases belong to the category of neurodegenerative diseases, which have a great impact on the quality of life of patients.<sup>36-38</sup> Amyloid fibril is a fibrous structure formed by misfolding and aggregation of specific protein. In the brain of patients with Alzheimer's disease,  $\beta$ -amyloid will abnormally aggregate to form amyloid fibrils. The deposition of these fibrils in the brain will lead to the impairment of neuronal function, which will lead to a series of symptoms such as memory loss and cognitive impairment. Balme *et al* utilized orbital etching to create functionalized conical nanopores for tracking amyloid growth.<sup>37</sup> Due to the strong absorbability of amyloid, surface grafting of PEG is required to prevent non-specific adsorption. Graphene channels, with their high conductivity, low non-specific adsorption and high-sensitive protein recognition, present a promising approach for monitoring the long-term amyloid fibril formation. Here, we utilized Graphene nanochannels ( $h=25$  nm,  $L=10$   $\mu$ m) to follow the fibril formations process of  $\beta$ -lactoglobulin.  $\beta$ -lactoglobulin fibers were cultured at 70 °C at a concentration of 10 mg/L, in deionized water (pH 2.0) (Figure 6a). Samples incubated for different durations were diluted 40 folds in 1 M KCl solution (pH 2.0). Current trajectories (Figure 6b) reveal pulses of different amplitudes for different incubation time, indicating a heterogeneous protein mixture with different molecular weights.

The scatter plots in Figure 6c show that data points are concentrated in lower  $\Delta I/I_0$  values and shorter dwell time after incubation for 6 hours. As the incubation time increases to 16 hours (Figure 6d), both  $\Delta I/I_0$  and dwell times shift to higher values, indicating partially aggregation of lactoglobulin and fibril formation. This trend is further confirmed by Figure 6e, which demonstrated that longer incubation time leads to a larger  $\Delta I/I_0$  values and longer translocation times for lactoglobulin fibrils. These results suggest that this channel can effectively monitor processes like protein aggregation, fibril formation or fibrosis.

## Conclusion

In summary, we utilized a layer assembly method to create highly stable and low-noise graphene nanochannels. The exceptional surface smoothness and minimized non-specific adsorption of nanochannels allow them to transport proteins without clogging and, as demonstrated here, to identify

five different proteins based on their current pulses. We provided experimental evidence of these channels for investigating protein-protein binding and the fibrotic processes of lactoglobulin, highlighting the significant potential of graphene nanochannels for label-free protein characterization. Our findings demonstrate that graphene nanochannels offer a compelling alternative to traditional nanopores for resistive pulse sensing, providing an extended transmission path, prolonged dwell time, and the capability for long-duration translocation experiments. In the future, this method is expected to be combined with microfluidic technology, enabling highly sensitive extraction and recognition of specific pathogenic proteins in the blood to achieve rapid diagnosis of diseases.

## **Materials and methods**

**Materials.** Graphene was purchased from XFNANO. Potassium chloride (>99%) was obtained from Sigma-Aldrich. DI water (PH = 6.8) was obtained from Watsons. The SiN<sub>x</sub> chips are customized by Suzhou Wenhao Co., Ltd. (<https://www.whchip.com/>). Streptavidin was obtained from Coolaber. BSA was obtained from Sigma-Aldrich. GA-Rab-IgG was obtained from Solarbio. Rab-IgG was obtained from Beyotime Biotechnology. IgM was obtained from Gene Teah.  $\beta$ -lactoglobulin was obtained from Sigma-Aldrich. KCl solutions of different pH are titrated with 1% HCl and 0.1 M KOH solution.

**Characterization:** AFM characterization of nanochannel was done with atomic force microscopes (Witec Alpha 300 RAS).

**Preparation of graphene nanochannels.** The graphene nanochannels were prepared following the method described in our previous work.<sup>18</sup> First, graphene was exfoliated onto a silicon wafer, and the substrate with an appropriate thickness was transferred onto a SiN<sub>x</sub> chip to serve as the underlying layer, cover the through hole on the free-standing SiN<sub>x</sub> membrane. A graphene layer of suitable thickness was then selected as the middle layer, and the channel structure was etched using a femtosecond laser (800 nm, 70 fs, 60 mW). The etched channel was transferred onto the underlying graphene. Subsequently, a through-hole junction in the channel and the underlying graphene was created by laser milling. Finally, the channel was sealed by adding an upper graphene layer.

**Nanochannel Translocation Experiments.** The SiN<sub>x</sub> chip containing the graphene nanochannel was encapsulated within a fluidic cell, with the joints sealed with rubber rings to prevent leakage. Either side of fluidic cell held 200  $\mu$ l of electrolyte, connected solely by the graphene nanochannel. Ag/AgCl

electrodes were immersed in the solution on both sides to apply voltage and record current. We used a patch clamp amplifier (Heka EPC-10) for applying voltage and signal acquisition. Current traces were recorded at a sampling rate of 200 kHz and filtered at 2 kHz using a low-pass Bessel filter. Current pulses were extracted and analyzed using the open-source software Transalyzer (<https://github.com/voyn/transalyzer/>, Calin Plesa).<sup>39</sup>

**Calculation of protein charge:** We estimated protein charges at pH 8.1 using PROTEIN CALCULATOR v3.4 (<https://protcalc.sourceforge.net/>).

## ASSOCIATED CONTENT

### Supporting Information

Supporting Information Available: The following files are available free of charge.

The detailed derivation process of the relationship between pulses and volume and charge, as well as the relationship between capture frequency and applied voltage. Experiment on protein transport in channels of different sizes. The simulation results of the transmission states of different structural proteins at the entrance of the channel. And the experimental results supporting the experimental part of the main text.

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Z.L. and Y.C. planned and conducted the experiments, and they wrote the manuscript. Y.C. and L.G fabricated the samples and characterized and tested them. Z.L. and J.T. supervised and directed the research. C.Y. provided theoretical and technical support for the experiment and put forward reasonable suggestions for the experiment. The manuscript was written based on contributions by all authors. All authors have given approval to the final version of the manuscript.

## **Notes**

The authors declare no competing financial interest.

## **ACKNOWLEDGMENTS**

This work is supported by the National Natural Science Foundation of China (Grant 12174207).

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TOC Figure

