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pH-Dependent ionic-current-rectification in nanopipettes modified with glutaraldehyde cross-linked protein membranes†

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In this study, we investigated for the first time the influence of an artificial membrane on the ionic current rectification of nanopipettes at various pH levels. The nanopipettes were fabricated and then modified with bovine serum albumin–glutaraldehyde (BSA–GA) artificial membranes. We determined the degree of ionic current rectification of these nanopipettes and compared them with those of bare nanopipettes. In contrast to the bare nanopipettes, the BSA–GA-modified nanopipettes demonstrated pH-dependent ionic current rectification. We also examined the tunability of the degree of rectification using streptavidin (STV) whose isoelectric point differs from that of BSA. The results showed that the ionic current rectification of nanopipettes can be tuned as the addition of STV into the BSA–GA artificial membrane increases the degree of rectification. Using the proposed approach, nanoscale spearhead pH sensors could be fabricated for highly localized extracellular or intracellular pH measurement. Moreover, it is possible to realize the applications of nano-sized channels in relatively larger channels using the present method.

Introduction

Molecular transport through nanopores in cell membranes is vital to many biological processes. The use of these nanopores opens a route to a variety of biotechnological applications such as DNA sequencing.[1] Inspired by biological nanopores, analysis through solid-state nanopores has emerged as a powerful technique, where the change in ionic current through a voltage-biased nanoscale pore is monitored using two electrodes placed on opposite sides of the nanopore. Change in ionic current is attributed to either molecules passing through[2–6] or the interaction of these molecules with recognition sites on the walls of the nanopores, which is likely the case for affinity-based biosensing applications.[3,7–9] Bare solid-state nanopores are usually neither selective nor responsive against biological stimuli such as pH, antigens, or inhibitors. Therefore, prior to being used in biosensing, nanopores must be modified with various biological elements depending on the biosensing application.[7,10] Sensing through nanoscale pores is an attractive technique as there is no requirement for signal amplification or labelling. They can be formed either using track-etching methods or by pulling glass capillaries with a micropuller.[2,7,10–12] Although the track-etching method is preferred as it enables researchers to precisely control the geometry of the nanopore, this method is labor intensive. The fabrication of nanopores from glass capillaries using a micropuller in the form of a nanopipette takes less than a minute and the dimensions of these nanoscale pores can be easily manipulated with high spatial resolution by simply changing the pulling parameters.[13–15]

Ionic current rectification (ICR) is a phenomenon observed with nanopores as asymmetric *I–V* curves, where the ionic currents recorded differ at the same magnitude of applied electrical potentials biased with opposite polarities.[16] Nanopores displaying pH-tunable ICR characteristics can be constructed by functionalizing the surface with pH responsive chemical moieties whose net charge depends on the pH of the surrounding microenvironment. The net charge of chemical moieties on the nanopore controls the transport through the nanopore, resulting in pH dependent *I–V* curves. Up to now, different molecules with pH responsive chemical moieties such as lysine–histidine, poly(amido amine) dendrimer, amphipols and streptavidin have been used for constructing of nanopores with pH dependent ICR characteristics.[7,17–19] The ICR behavior of such nanopores has also been numerically investigated; for instance, Lin and his coworkers theoretically investigated the influence of different parameters such as pH,

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types of ionic species, salt gradient and applied potential bias on ICR behavior in a conical nanopore modified with pH-tunable polyelectrolyte (PE) brushes.[20] According to the results of this study, in addition to the charged conditions of the PE layer, the level of pH, the geometry of nanopore, and the thickness of double layer, the ICR behavior of pH responsive nanopores is significantly influenced by the distribution of ionic species and the local electric field near the nanopore openings. In another study, Ali and his coworkers used a continuous model based on the Poisson and Nernst–Planck (PNP) equations[21] to theoretically investigate the ICR behavior of histidine–lysine modified conical nanopore with pH-tunable property.[17] They found a good agreement between experimental and theoretical results. Theoretical studies can provide a general guideline for designing devices with good ICR characteristics and they are commonly used for interpretation of experimental data.

In this study, we investigated ionic current rectification through a BSA–GA artificial membrane in glass nanopipettes using solutions with various pH levels. First, we fabricated the glass nanopipettes using a micro-puller, then modified the tip of the glass nanopipettes with a BSA–GA artificial membrane by immersing the nanopipettes in a freshly prepared BSA–GA solution in order to place the solution into the pore for artificial membrane formation. BSA–GA artificial membranes are used in various applications such as controlled drug delivery[22] and the immobilization of enzymes to build the bio-recognition units of biosensors.[23,24] Schiff bases are formed between the very reactive GA crosslinking agent, the free amine groups of the BSA amino acids and the enzyme of interest, which leads to the formation of an artificial membrane in a matter of minutes. Next, we tested the obtained glass nanopipettes with artificial membranes for their pH responsiveness in solutions with various pH levels (Fig. 1A). In addition, we tuned the pH responsiveness of the glass nanopipettes by adding streptavidin (STV) into the BSA–GA artificial membrane.

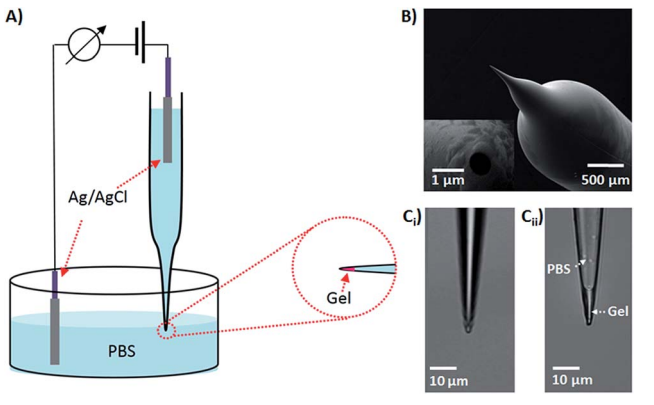

Fig. 1 Schematic illustration of the experimental set-up for ionic current measurement through a glass nanopipette (A). An SEM image of a nanopipette showing the diameter of the tip opening (B). Bare nanopipettes (C_i) were modified with BSA–GA artificial membrane for pH dependent ion current rectification (C_{ii}).

Experimental

Nanopipette fabrication

In order to make glass nanopipettes that have a thin and parallel run to the very end of the tip, we pulled patch-clamp glass capillaries (PG10165-4, World Precision Instrument, USA) with a micropuller (PC-10, Narishige, Japan) using the two-stage pull option. The pulling parameters that yielded fabricated glass nanopipettes with the desired characteristics were as follows: no. 1 heater: 60 °C, no. 2 heater: 39 °C. We took scanning electron microscopy (SEM) images of the nanopipette tips to measure the size of the tip opening. According to the SEM images, the tip opening had a radius of *ca.* 350 nm (Fig. 1B and S1†). Throughout this study, we used the same pulling parameters in order to fabricate uniform glass nanopipettes with similar size tip opening.

Ionic current rectification characteristics of un-modified nanopipettes

Following nanopipette fabrication, we investigated ionic current modulation through the bare tip opening. Using a micro-injector, we filled the nanopipette with 50 mM PBS (0.01 M KH_2PO_4, 0.04 M K_2HPO_4, 0.02 M NaCl, pH: 7) from the back of the nanopipette and then immersed it in PBS solutions with pHs of 3, 7, and 10. To measure the ionic current flowing though the tip opening, we used two Ag/AgCl wires, one of which was placed inside the PBS-filled nanopipette and the other outside. We recorded the ionic current flowing through the tip opening while the potential between the two Ag/AgCl wires was swept linearly from 0.5 to −0.5 V (Autolab PGSTAT101, Metrohm, Switzerland).

Ionic current rectification characteristics of modified nanopipettes

First, we prepared the artificial membrane by mixing 150 μg mL^{-1} of BSA (Amresco, USA) solution and 1% GA in PBS, to realize a final volume ratio of 4 : 1 (BSA : GA).[25,26] After mixing the BSA–GA mixture thoroughly, we immersed the glass nanopipettes into the mixture for 1 s and then the mixture was left to gel in the nanopipette for 40 min. To avoid any loss of BSA–GA mixture at the tip of the nanopipettes, we used a micro-injector to fill the nanopipettes with PBS, beginning a little bit away from the gel, in order to stabilize the gel solution at the tip of the nanopipettes by stopping its free flow (Fig. S2†). After 40 min of cross-linking, we introduced PBS solution into the gel by gently titrating the nanopipette. We then examined the ionic current behavior in the same way as before, using PBS solutions with various pHs (pH: 3, 4, 5, 6, 7, and 10). Next, we investigated the tunability of the degree of ionic current rectification by adding STV into the artificial membrane mixture. Basically, we prepared the artificial membrane by mixing 150 μg mL^{-1} of BSA solution, 2 μg mL^{-1} of STV (Thermo Fisher Scientific, USA), and 1% GA to obtain a final volume ratio of 2 : 2 : 1, following which we immersed the glass nanopipettes into the mixture for 1 s to load the mixture into the glass nanopipettes, as before. The mixture was then left in the glass nanopipettes for 40 min to

allow the GA to cross-link with the BSA. All necessary actions were taken to prevent any loss of mixture inside the glass nanopipettes during gelation, as described above. In order to clarify the impact of STV on the degree of ionic current rectification, the PBS solutions with various pHs (3, 4, 5, 6, 7, and 10) used in the BSA–GA modified glass nanopipettes were also used here to investigate the ionic current modulation.

Results and discussion

The results of un-modified nanopipettes indicated that the ionic current was not rectified in any of the solutions used (Fig. 2A). In other words, solutions with varying pHs did not influence the current flowing through the tip opening. The ionic current rectification phenomenon is usually observed with a radii smaller than 100 nm for unmodified nanopipettes, in which case the phenomenon is dominantly influenced by the inner geometry that can be visualized by TEM and models developed as demonstrated by several groups.[27] However, in this study because relatively large nanopipettes with several hundred nm radii were used, no ionic current rectification was observed.[12,28] As stated above, such glass nanopipettes have the limitation of not being selective, unless they are properly trimmed with bio-recognition elements. In addition, we compared the *I–V* curves of 15 different nanopipettes using PBS at pH 7 to check the uniformity of the nanopores (Fig. S3†). A slight difference was observed between the nanopipettes, but we believe the difference is within the acceptable range. Next, we modified the tip opening with a BSA–GA artificial membrane to investigate the influence of the artificial membrane on ionic current rectification (Fig. 1C_i and C_{ii}). The molecular mass transport in the BSA–GA artificial membrane may differ significantly from that in bulk solution, depending primarily on the concentration of the BSA or GA used to form the membrane.[29] For this reason, the ionic currents acquired with the artificial-membrane-modified nanopipettes were slightly lower than those of the bare nanopipettes (Fig. 2A and B). These ionic current results clearly demonstrate that the ionic current that flowed through the artificial BSA–GA membrane was rectified in a pH-dependent manner. The artificial membrane almost blocked the ionic current flow in the nanopipette opening at lower pHs (pH: 3 and 4) when the potential was swept in the negative direction. The isoelectric point (pI) of BSA is 4.7, thus the net charge of the BSA is positive at pH 4, whereas it is negative at pH 7. In other words, the net charge of the protein BSA is pH-dependent.[30] For this reason, when the nanopipette was immersed in PBS solutions of low pH (pH: 3 or 4), BSA became positively charged and thus blocked the positively charged ion flow through the nanopipette tip opening in negative voltage regions, causing the ionic current to drop. It is worth mentioning that BSA is a major oligonucleotide binding protein and therefore modifying the tip of a nanopipette might present an opportunity to detect oligonucleotides that are negatively charged at low pHs (pH: 3–4).[31] When the nanopipette was immersed in solutions with higher pHs (pH: 5, 6, 7, and 10), the net charge of the BSA changed as expected and so did the ionic current rectification behavior (Fig. 2B). To clarify the rectification behavior in different solutions, we calculated the degree of rectification for each case using the eqn (1) and plotted the data against their corresponding pH values (Fig. 3C_i and C_{ii}).

$$Q = \frac{I(V)}{I(-V)} \quad (1)$$

The degree of ionic rectification of BSA–GA-modified glass nanopipettes was linear for pH levels ranging from 3 to 7, which can be explained by the pH-dependent net charge of BSA. However, the rectification degree of the ionic current at a pH of 10 was not linear between pH levels 3 and 7, and differed from that of pH 7. In contrast to ionic current rectification at lower pHs, the ionic current that flowed through the nanopipette tip opening was blocked at higher pHs (pH: 7 and 10) when the potential was swept in the positive direction. As stated above, BSA becomes negatively charged at higher pHs, which in turn blocks the flow of anions through the tip opening and causes the ionic current to decrease in the positive voltage region. In order to see the stability of the signal, the potential was swept from +0.5 to −0.5 V back and forth 30 times in PBS at pHs of 3 and 7, respectively. The *I–V* curves showed that the behavior of the modified nanopipettes in PBS with different pHs was relatively stable (Fig. S4†). When we tried to evaluate the ionic current rectification degree of different nanopipettes, we observed a slight difference between them which is most likely

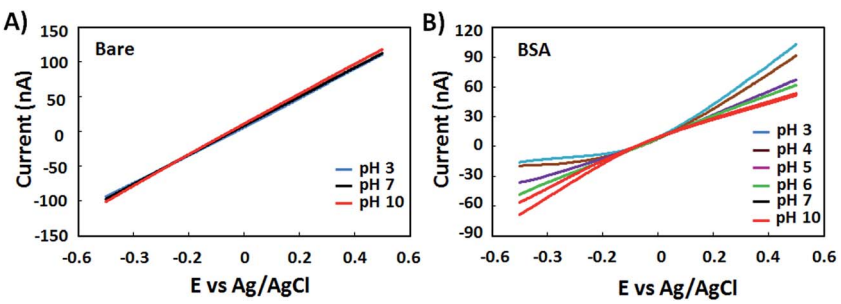

Fig. 2 Ionic current behavior of bare (A) and BSA–GA artificial membrane modified nanopipettes (B) in PBS solutions with various pHs (pH: 3–10). Although, bare nanopipettes did not show any change in ionic current response at different pHs, modified nanopipettes showed a clear pH dependent ionic current rectification.

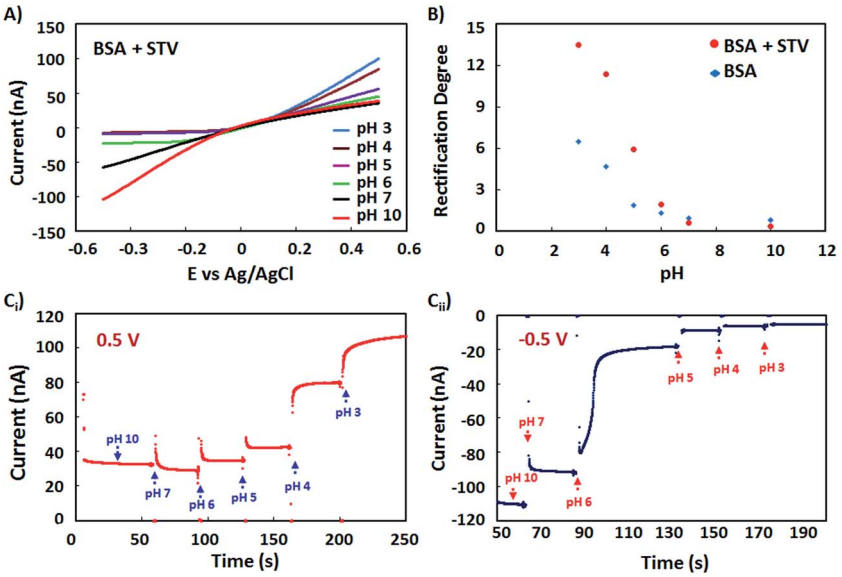

Fig. 3 Ionic current rectification of BSA–STV–GA modified nanopipettes in PBS solutions with various pHs (pH: 3–10) (A). The rectifications degrees of BSA–STV–GA and BSA–GA modified nanopipettes were compared (B). Results clearly showed that the ionic current rectification degree can be tuned using different proteins. Additionally, the ionic current of BSA–STV–GA modified nanopipettes were measured in PBS solutions with various pHs (pH: 3–10) at constant potentials of 0.5 (C_i) and −0.5 (C_{ii}), respectively.

caused by the slight difference between the nanopipette openings and the lack of control over the formation of gel (Fig. S5†). For this reason, there is a need to check the pH responsiveness of each individual modified nanopipettes for more accurate analysis. Subsequently, we investigated the impact of the volume of the artificial membrane in the nanopipette on the ionic current rectification. To increase the volume of the artificial membrane, we immersed and held the glass nanopipettes in a freshly prepared BSA–GA mixture for longer times (3 s and 10 s). The results show that increasing the period in which the mixture is loaded into the glass nanopipettes does increase the volume of the artificial membrane (Fig. S6A†). Next, we kept the mixture inside the glass nanopipettes for 40 min, as described above, and then observed the degree of ionic current rectification in PBS solutions with various pHs (pH: 3 and 7). Although the measured ionic current differed slightly between glass nanopipettes with varying volumes of artificial membrane, the degree of ionic current rectification did not change significantly (Fig. S6A and B†). In other words, small changes in the volume of the artificial membrane had only a slight effect on the degree of ionic current rectification at different pHs (Fig. S6C†). Here, we also checked the impact of the varied KCl concentrations on the ionic current rectification behavior of both bare and BSA–GA modified nanopipettes. Basically, two different conditions were checked; first, the internal and external solutions were kept the same (Fig. S7A$_{i–ii}$†) and then the internal solution was kept the same (PBS) whereas the KCl concentration (PBS, PBS + 0.01 M KCl and PBS + 1 M KCl) of the external solution was varied to form a concentration gradient (Fig. S7B$_{i–ii}$†). Typical *I–V* curves of the two nanopipettes were obtained under these conditions, respectively. According to the results, not only did BSA–GA modified nanopipettes show better ionic current rectification behavior than bare nanopipettes, but also different responses in various KCl concentrations. In addition, typical *I–V* curves of bare (Fig. S7C$_i$†) and BSA–GA modified (Fig. S7C$_{ii}$†) nanopipettes were also obtained in PBS with varying concentrations of NaCl. As expected, no ICR was observed in the case of bare nanopipettes whereas the results of BSA–GA showed similar tendency with those of KCl.

To investigate the tunability of the degree of ionic current rectification, we added STV into the artificial membrane mixture. STV has a neutral pI, which is higher than that of BSA (pI: 4.7). The net charge of STV is also pH-dependent and its impact on the degree of ionic current rectification has already been demonstrated, whereby STV-modified nanopores exhibited pH-dependent ionic current rectification.[7] The results clearly demonstrate that the ionic current modulation was also pH-dependent (Fig. 3A). In other words, we observed that the ionic current was rectified in a pH-dependent manner. In contrast to the BSA–GA modified glass nanopipettes, the ionic current modulation yielded a better degree of ionic current rectification, which demonstrates its tunability by the use of proteins with different pI values (Fig. 3B). In addition, unlike GA–BSA modified nanopipettes, a drastic decrease in the ionic current of GA–BSA–STV modified nanopipettes was observed when dipped into PBS at pH 6. We believe it is because of the pI of STV, which is around 7. The results showed that at pHs below 7, the probe tip becomes positively charged blocking the flow of ions in negative voltage regions. The ICR behavior of both GA–BSA and GA–BSA–STV modified nanopipettes observed in this study showed a similar tendency with the results of the previously reported both theoretical and experimental studies where the charge condition of the nanopore was determined by the pH of the solution.[17–19] Lastly, we analyzed the pH-dependent ionic

current response behavior of the BSA–STV–GA-artificial-membrane-modified glass nanopipettes over time at constant potentials, for which we acquired the ionic current of nanopipettes for a certain period of time (40–60 s) in PBS solutions with varying pHs (pH: 10, 7, 6, 5, 4, and 3) at 0.5 V and −0.5 V, respectively. Even though we used a hydrogel membrane to modify the glass nanopipettes, the ionic current changed quite rapidly with changing pH and then found a steady state (Fig. $3C_i$ and C_{ii}). In other words, the net charge of the proteins in the artificial membrane changed quickly enough in PBS solutions with different pHs to gain a steady state within a matter of seconds. Based on these obtained results, we believe that pH-responsive glass nanopipettes can be easily produced to measure pH in very small volumes, such as in the intracellular or extracellular spaces of single cells. As demonstrated with STV, using proteins with different pI values could yield a means for fabricating more sensitive pH nanoprobes that could operate over a larger pH range. Since the degree of ionic current rectification can be tuned using different proteins, it is likely that molecules that can interact with BSA protein might also influence the degree of rectification. Therefore, the proposed strategy could be used for the detection of these molecules as well. Moreover, when it comes to the applications of nano-sized channels, the unique ion transport characteristics of such channels' pores is what attracts researchers. It is possible to realize such applications in larger channels using the present method. Larger pipettes or channels are easier to manipulate for laboratories with no or limited resources. And, it is highly unlikely for nanopores to be blocked when modified with the artificial membrane as long as it is not dried.

Conclusion

In conclusion, we investigated the ionic current modulation of glass nanopipettes modified with artificial membranes in PBS solutions with different pHs. Because BSA has a low pI value, the ionic current that flowed through the nanopipette opening in solutions with low pHs were blocked, thus causing ionic current rectification. The addition of STV into the artificial membrane changed the rectification of the ionic current, which resulted in a higher degree of rectification than that of glass nanopipettes modified with BSA–GA. In other words, the addition of STV demonstrated the tunability of the degree of ionic current rectification, which is a property that could be used to modify the response of a nanopipette in certain desired cases. To the best of our knowledge, this is the first study that demonstrates the potential for using artificial membranes in the modulation of ionic current, which could have a high potential for applications in the fields of chemistry and biosensing.

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