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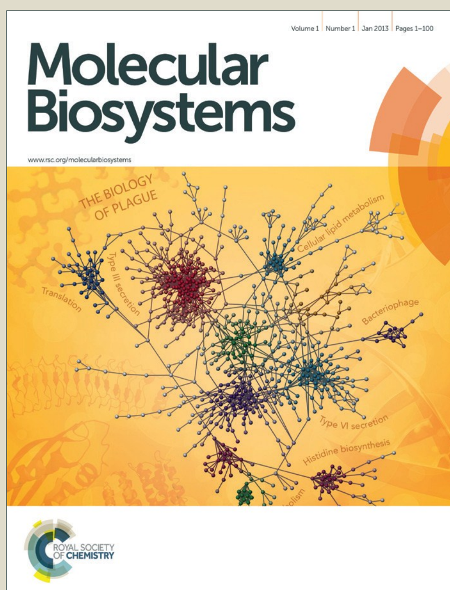

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ARTICLE

Highly Efficient Integration of the Viral Portal Proteins from Different Types of Phages into Planar Bilayers for the Black Lipid Membrane Analysis

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The planar lipid bilayer technology is a technique that yields incredibly useful structural function information about a single channel protein. It is also currently actively utilized as a powerful platform using biological protein nanopores for the development of single-molecule nanopore sensing technology, as well as ultrafast DNA sequencing technology. The portal protein, GP10, from the bacteriophage Φ29 was the first phage portal protein shown to be successfully inserted into planar bilayer membranes, thereby it may inspire more researchers to apply the techniques to portal proteins from the other bacteriophages. However, the technology is far from perfect since the insertion of the channel proteins into planar bilayer membranes is not only technically difficult but also time-consuming. For the fusion of phage portal proteins, vesicles are typically needed to be reconstituted with the portal proteins to form proteoliposomes. However, most of the phage portal proteins have low solubility, and may self-aggregate during the preparation of the proteoliposomes. Furthermore, the fusion of the formed proteoliposomes is sporadic, unpredictable and varied from person to person. Due to the lack of experimental consistency between labs, the results from different methodologies reported for generating fusible proteoliposomes are highly variable. In this research, we propose a new method for the preparation of the fusible proteoliposomes containing portal proteins from bacteriophages, to circumvent the problems aforementioned. Compared to the conventional methods, this method was able to avoid the protein aggregation issues during the vesicle preparation by eliminating the need for detergents and the subsequent time-consuming step for detergent removal. The proteoliposomes prepared by the method were shown to be more efficiently and rapidly inserted into planar bilayer membranes bathed in different conducting buffer solutions including those with nonelectrolytes such as glycerol and PEG. In addition, the method of forming proteoliposoomes has significantly extended the shelf life of the proteoliposomes. To further explore its potentials, we have successfully applied the method to the insertion of a mutant portal protein, GP20, from T4 bacteriophage, a hydrophobic portal protein that has not been explored using the planar lipid bilayer membrane technique. The results suggest that this method could be used to prepare proteoliposomes formed by hydrophobic portal proteins from other bacteriophages.

Introduction

The lipid bilayer is a natural barrier of biological cells and cellular compartments, such as mitochondria, the Golgi-complex, and lysosomes. Consisting of a hydrophilic head and hydrophobic tail, it plays a major role in maintaining homeostasis within the cell. Membrane proteins, usually ion-channel proteins, are integrated in the biological membranes and enable vital cell functions such as signal transduction and transport of ions or small molecules. In order to determine the activity of a protein of interest at defined conditions, the membrane proteins have to be integrated into artificial lipid bilayers, planar bilayers and/or proteoliposomes. The ion flux mediated by the protein channels can be measured using electrical methods.

The planar bilayer technology utilizes a synthetic planar bilayer membrane formed over a small aperture in a partition separating two compartments to measure the ion current mediated by protein molecules embedded in the membrane [1-3]. The technique is very useful for the functional analysis of channel proteins that are naturally expressed in cells at very low levels[4] and offers the possibility of conducting structural investigations of a single protein channel in an *in vitro* environment devoid of any influence from other endogenous protein constituents[5]. Furthermore, the technique is able to visualize conformational changes in individual protein channels in real-time to demonstrate the channel protein's mechanisms of action and its physiological roles when the channel proteins interact with other proteins[6]. Recently, the technique has

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become an increasingly influential single-molecule sensing platform for the development of nanopore biosensors [7-20] and ultrafast DNA sequencing technology [21-27].

Portal protein, also called connector protein, is found in many different types of tailed bacteriophages and herpesvirus. It is one of the critical components in the powerful DNA packaging machines inherent in the tailed bacteriophages that may assist in packaging double-stranded DNA genomes into a capsid [28-30]. Although it is well established that the ATPase energetically fuels DNA translocation in the DNA packaging machines, the portal functions, in particular its role in DNA packaging, are poorly understood[31-33]. For example, there has been considerable debate on whether the portal is mechanistically involved in translocation or merely serves as a channel to transport DNA into and out of the capsid. Moreover, there is little sequence identity among the portal proteins despite the fact that they are structurally conserved, e.g., the portals from phages Φ29[34], T3[35], T4[36], T7[37], P2[38], P22[39-40], SPP1[41-42] are shown as a dodecameric ring by cryo-electron microscopy. Because most portal proteins usually have very low solubility in an aqueous media, crystal structures of the portal proteins so far available are limited to phages, Φ29[43-44], P22[45] and SPP1[46]. Obviously, there remains a tremendous challenge in the investigation of structural function relationships of the portal proteins from the other phages.

In 2009, using the vesicles reconstituted with the portal protein, an engineered portal protein from the phage Φ29, the GP10 connector, was found to be inserted into a planar bilayer membrane to form a channel, through which DNA can pass under electric voltages[47]. Inspired by the discovery, a handful of studies have been made to explore the roles that the GP10 connector plays in the Φ29 DNA packaging motor [48-53]using the planar lipid bilayer membrane technology. For example, DNA was found to be translocated through the channel of the portal protein unidirectionally[54]. The portal protein experienced three reversible conformation changes after the connector was bound with antibody at its C-terminus[52]. On the other hand, since the portal channel was found to be stable under different buffer conditions[55], Guo and his co-workers explored the possibility of using several mutated portal proteins, GP10 from the phage Φ29, for the single-molecule nanopore sensing technology. The target molecules that have been examined so far included some small chemical molecules[55-56], single stranded/double stranded DNA[57], and a colon cancer antibody[58]. They also made an attempt to apply the portal protein to a lab-on-a-chip (LOC) platform[59]. Although the portal protein seemed to be an excellent candidate for the biological nanopore sensing technology, due to the difficulty in the insertion of the phage portal proteins into planar lipid bilayer membrane, the method reported previously[47] was only used to insert several hydrophilic mutant portal proteins from bacteriophage Φ29 into the planar bilayer membranes.

Because all the phage portal proteins are structurally conserved[33], it is very possible to explore the possibility of using the planar lipid bilayer technique to study the other phage portal proteins. Compared to the traditional research tools, e.g., cyro-electron microscopy and protein crystallography, the technique would provide a unique, and powerful real-time measurement tool for *in-vitro* studies on the structures and the roles of the portal proteins from a variety of the DNA packaging motors inherent in the tailed bacteriophages and herpesvirus. For the development of single-molecule nanopore sensor technology, the portal proteins from different bacteriophages would provide more flexibility in choosing a more appropriate protein channel for the use as a sensing motif. Therefore the phage portal proteins represent a large family of biological nanopore proteins that have not been extensively explored using the planar lipid bilayer membrane technique. Therefore, it would be an emerging research area that may find new applications in the research areas including microbiology, biophysics, nanotechnology and etc.

In spite of its elegance, a critical step for the planar lipid bilayer membrane technology, i.e., the reconstitution of membrane ion channel proteins as well as the portal proteins into planar lipid bilayer membranes, is technically difficult and time-consuming. Normally, once a stable bilayer membrane is formed, the investigator faces the difficulty of long and unpredictable time intervals required for observation of the protein insertions. This capricious parameter is often the rate-limiting step in a successful planar lipid bilayer experiment. Furthermore, the preparation of proteoliposomes is also time-consuming because typical protocols for preparing the vesicles require a step of detergent removal to avoid any possible self-aggregation issues during the proteoliposome preparation. However, the issue was deliberately neglected in the previous attempts to prepare the proteoliposomes reconstituted with a mutant portal protein, GP10 connector, because the mutant connector has a good solubility in aqueous media[47]. Therefore the method would become questionable when utilized to prepare vesicles reconstituted with more hydrophobic phage portal proteins. All of the above issues would make up a tremendous obstacle when one attempts to apply the planar lipid bilayer technique to portal proteins from other bacteriophages for the structural functional studies as well as for their applications in the single-molecule nanopore sensing technology.

In this research, we aim at developing a simple and practical method to prepare vesicles reconstituted with portal proteins from bacteriophages. Using the prepared vesicles, we were able to fuse the portal proteins into planar bilayer membrane more rapidly and more efficiently as compared to the vesicles prepared by the previously reported method[47]. In addition, we found that the shelf life of the prepared vesicles was significantly extended, indicating the possibility of long-time storage of the vesicles for potential commercial use. Finally, using this method, we successfully inserted a very hydrophobic portal protein from T4 bacteriophage into planar bilayer membranes for ionic current measurements.

Experimental

Materials:

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The lipid, 1,2-diphytanoyl-sn-glycero-3 phosphocholine (DPhPC) was purchased from Avanti Polar Lipids (Alabaster,AL). n-Decane and sodium chloride was from Fisher Scientific (Hampton, NH). All other reagents, if not otherwise specified, were from VWR International (Radner, PA). All of the reagents were of reagent grade. The purified mutant portal protein, GP20 (20amN50(Q325am)) [60], from Bacteriophage T4, , was received as a gift from Prof. Venigalla, B. Rao (Department of Biology, Catholic University of America) and used without further purification.

Expression and Purification of C-His GP10 Connectors

The gene of C-His GP10 was from a constructed plasmid pET-21a(+) C-GP 10 (a gift from Prof. N. J. Stonehouse)[61-62]. After the plasmid was transformed into BL21 (DE3) cells (EMD Millipore, Billerica, MA), the protein, C-His GP10 was expressed by the transformed cells cultured in an LB medium with 50 μg/mL Ampicillin (Sigma Pharmaceutial, Rowville, Australia). Isopropyl-β-D-thio-galactoside (IPTG) (Sigma Pharmaceutial, Rowville, Australia) was used to induce the protein expression. The bacterial cells were harvested and resuspended in 50 mM NaH_2PO_4 500 mM NaCl, 10 mM imidazole (pH 7.4) with 15% glycerol and subsequently disrupted by passage of the mixture through a French press. The supernatant was collected by centrifugation at a speed of 16000 rpm for 20 min. Purification of C-His GP10 was performed using a column filled with an HisPur Ni-NTA Resin (Cat# 88221, Thermo Scientific, Waltham, MA). The column was washed with 50 mM NaH_2PO_4 500 mM NaCl, 50 mM imidazole (pH 7.4) with 15% glycerol. Elution was carried out using the buffer, 50 mM NaH_2PO_4 500 mM NaCl, 0.5 M imidazole (pH 7.4) with 15% glycerol. Concentrations of the protein were determined using a Coomassie (Bradford) Protein Assay Kit (Cat# 23220 Thermo Scientific, Waltham, MA).

Preparation of Proteoliposomes Reconstituted with Portal Proteins

We used two methods to prepare C-His GP10 reconstituted proteoliposomes: The control method was reported previously[47]. In brief, a lipid stock solution of DPhPC in chloroform was added to a round-bottom flask. Chloroform was evaporated to form a lipid film under vacuum. Then, a solution of purified protein, C-His GP10, was added to the conducting buffers with 200 mM sucrose and used to rehydrate the lipid film to form a suspension solution of multi-laminar liposomes for a further membrane extrusion.

In the experimental method, the lipid stock solution of DPhPC in chloroform was premixed with purified portal proteins containing 15% (V/V) glycerol (the volume ratio of chloroform vs the aqueous solution: 4:1). The resultant mixture solution was subsequently added to a round-bottom flask. In the flask, two phases, oil phase and water phase, could be visualized clearly and aggregation was not found in either phase. By applying a vacuum for 10 min, the solvent chloroform was completely evaporated which resulted in the formation of a viscous gel. The gel was further rehydrated by a conducting buffer solution containing 15-30% glycerol (V/V) to prepare multi-laminar liposomes. For ease of comparison of the two methods, a final molar ratio of the lipid vs C-His GP10 connector was kept 600:1 in both methods.

To form unilaminar liposomes, an extrusion method was used for the resulting suspension of multilamellar vesicles from the two methods. After the vesicle suspensions were passed through a 100 nm polycarbonate membrane twenty times using the mini extruder (Avanti Polar Lipids, Alabaster,AL), unilaminar preoliposomes were formed and stored as aliquots at -80 °C for long-term use.

Insertion of the Portal Proteins into a Planar Bilayer Membrane

A horizontal bilayer chamber, abbreviated as BLM chamber, (BCH-1A, Eastern Scientific, Rockville,MD) with an LED light below to optically monitor the position of the aperture on a Teflon partition was used for planar bilayer experiments. The BLM chamber was separated into two compartments, cis- vs trans-, by the Teflon partition (200 μm TP-02, Eastern Scientific, Rockville,MD). Both compartments were filled with a conducting buffer, a 5 mM Tris buffer (pH 8.0) with 1 M NaCl if otherwise not specified. The liposomes reconstituted with proteins were added to the *cis*-compartment. As a comparison, a vertical version of BLM chamber was also used for the vesicle insertion experiments. Planar lipid bilayers were formed across a 200 μm aperture in the wall of a Delrin cup (BCH-13A, Warner Instruments; Hamden, CT) with a working volume of 1 mL in the chamber.

To form the bilayer membrane, the aperture of the Teflon partition was pre-painted and air-dried with 0.5 μL 3% (w/v) DPhPC in an n-decane solution three times before the buffer was added. The planar bilayer was formed by depositing a 10 mg/mL of DPhPC in an n-decane solution over the aperture in the buffer. The process for the formation of bilayer membrane on the aperture was monitored by measuring the current response under triangular voltage stimulation since the amplitude of the resulting square wave current response is proportional to the value of bilayer capacitance. The membrane capacitance threshold range was set to be from 80 to 100 pF. Liposomes were introduced to the chamber only after the capacitance value was measured to be within the threshold range. The amount of the proteoliposomes introduced was set to be 1 μL of the stock liposome solution.

Channel Conductance Analysis

The channel conductance was measured through a pair of Ag/AgCl electrodes connected to a BLM setup. The BLM setup included a patch-clamp amplifier with a bilayer headstage (BC-535, Warner Instruments; Hamden, CT), an in-house made Farady cage placed on a vibration-dampening table, and an A/D converter (The Digidata 1440A digitizer, Molecular Device, Sunnyvale, CA). Acquired data were further filtered by an 8 pole low pass Bessel filter (LPF-8, Warner Instruments; Hamden, CT) at 1K Hz under a sampling frequency of 5KHz. The softwares, pClamp 10.2 (Molecular Device, Sunnyvale, CA) and Origin Pro. 9.0 (OriginLab, Corp. Northampton, MA), were used to collect and process the data.

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Results and Discussion

The planar lipid bilayer technique is extremely time-consuming, which stems from two aspects of the experimental technique: the formation of a bilayer on a small aperture and the fusion of protein-reconstituted liposomes into the planar bilayer membranes. These time-consuming problems seem to be mitigated when the techniques are applied for some membrane ion channel proteins capable spontaneously insert into a planar bilayer because the proteins are not needed to form proteoliposomes to facilitate the protein fusion. Therefore, to speed up the protein insertion, a few research groups mainly studied how to rapidly form planar bilayer membranes on the small aperture [63-66]. However, for most membrane ion channel proteins, the planar bilayer membrane experiments would be implemented only after the proteins can be successfully reconstituted into liposomes. Similarly, it has also been proved that direct incubation of the portal protein, GP10, with a well-formed planar bilayer membrane would not result in any protein insertions into the bilayer membrane[47]. Therefore, effectively controlling of the fusion of protein reconstituted vesicles into a well-formed planar bilayer membrane is a key to speeding up the insertion of the portal proteins into the membrane, and thus depends upon the methods of preparing fusible proteoliposomes reconstituted with the portal proteins.

In our experiments, we used two different methods to prepare the proteoliposomes. The mutant GP10 portal protein, C-His GP10, was from the same batch. The fusion of the proteoliposomes was kept under constant conditions throughout. Figure 1A, demonstrates a schematic process for the fusion a single proteoliposome into a planar bilayer membrane. The formation of bilayer membranes can be monitored by a measurement of the membrane capacitance under a triangular voltage input. After the measured capacitance was found to be within the appropriate threshold range (80-100 pF), we subsequently added 1 μL of proteoliposome solution to the *cis*-compartment at the height of 5 mm far from the top of a conducting buffer, 5 mM Tris (pH 8.0) with 1 M NaCl, causing many spikes as shown in Figure 1B. Before protein insertion occurred, the electric current was measured to be 0 pA (Fig. 1B). When a single portal protein was inserted into a planar bilayer membrane by a fusion of a single proteoliposome, a stepwise current jump was recorded (Fig. 1B). We further compared the single-channel conductance of the pores formed by the proteoliposomes prepared by the two methods (Supplementary figure A and B). The statistical results showed that the pores formed by the proteoliposomes from the experimental method had a distribution of the single-channel conductance similar to those formed by the proteoliposomes prepared by the control method under the identical experimental conditions, suggesting that proper protein foldings of the connector were still maintained during the proteolipsome preparation using the experimental method. To identify which of the methods can produce more fusible proteoliposomes, we measured the time elapsed for the 1st insertion into independent lipid bilayer membranes respectively as shown in the example in Fig. 1B. The results from the proteoliposomes prepared by the two methods are shown in Figure 2. They showed that an average of the time elapsed for the 1st insertion using the proteoliposomes prepared by the control method was 8.3±2.6 min (N=48). However when the proteoliposomes prepared by the experimental method were used, the time elapsed for the 1st insertion was significantly decreased, an average of the time elapsed for the 1st insertion was measured to be 0.6±0.5 min (N=56), which indicated that the average time was decreased by 14-fold. Therefore, the proteoliposomes prepared by the experimental method conclusively fused into planar bilayer membranes more rapidly than when prepared by the control method.

Prior research determined that the fusion rate of proteoliposomes into planar bilayer membranes is influenced by two factors, the contact of vesicle with planar bilayer membranes and the diffusion of the vesicles[67]. Several reports have been published on the adjustments of the two factors to increase the proteoliposome fusion rate. These include: creation of osmotic gradient across the planar membrane with vesicle-containing side (*cis*-compartment) hyperosmotic with respect to the opposite (*trans*- compartment) or induction of vesicle swelling by filling into the vesicles with hypertonic solution[68-69], the use of liposomes reconstituted with SNARE protein specializing in mediating intracellular fusion to catalyze the membrane fusion[70], the use of nystatin-induced liposomes[71-74] , the use of cholesterol containing liposomes under low temperature or the use of negatively charged lipid to prepare liposomes or planar bilayer membrane in a Ca2+ ion containing medium[67-68].

As opposed to the previously reported methods[47, 68-69, 71-75], the experimental method in the research is a more feasible and less complicated approach to prepare fusible proteoliposomes. To speed up the vesicle fusion rate, the method only simply used a high concentration of glycerol to replace sucrose, In the experiments shown in Fig. 2, the presence of the high concentration of glycerol (>20% (V/V)) resulted in an huge increase in the osmotic pressure and the density of the prepared liposomes. When the glycerol was used in the experimental method, the molarity of glycerol inside the proteoliposomes was at least 2700 mM, about 13.5 fold greater than the molarity of sucrose (200 mM) in the proteoliposomes using the control method. As a result, the swelling rate for vesicles in contact with planar bilayer membrane increased[69, 76]. In addition, the density of glycerol also played an important role. The 20% (V/V) of glycerol in a water solution at room temperature has a density of more than 1.05 g/mL and 200 mM sucrose has a density of 1.02 g/mL. The density difference would be more favorable for the glycerol-based liposomes in a horizontal bilayer lipid membrane (BLM) chamber to move towards the bilayer membranes under gravitational force in a high-salt conducting buffer, resulting in more vesicles in contact with the membrane within a shorter time. To confirm the explanation, in another independent experiment, we replaced the horizontal chamber with a vertical BLM chamber to perform

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the planar bilayer membrane experiments using the proteoliposomes prepared by the experimental method. Because a 200 um aperture was located on the sidewall of the vertical chamber, after we added the proteolipsomes to the aperture of the chamber, most of the high-density proteoliposomes settled on the bottom of the chamber under the gravitational force. In our experiments, under the mechanical stirring condition, only one out of 36 independent bilayer membranes showed that the protein insertion happened. The results indicated the proteoliposomes prepared by the experimental method would be more appropriate for the planar lipid bilayer experiments when horizontal BLM chambers are used.

It should be noted that the use of more than 200 mM sucrose to prepare the liposomes in the control method would also result in an increase in the density and osmotic pressure of the formed proteoliposomes. However, unlike the experimental method where glycerol was used, such an increase, is limited by the solubility of sucrose in a buffer used. For example, a maximum concentration of 300 mM of sucrose was reported to be used to prepare the proteoliposomes reconstituted with a membrane channel protein, voltage-gated K channel KAT1[77] or the mutant portal protein, GP10[47] .

The planar bilayer membrane technique has been proved be an effective measurement method for the determination of physical dimension of pores formed by some ion channel proteins[78-81]. The measurement is particularly useful to the approximation of the channel size of the proteins whose crystal data have not been available yet. In the size approximation measurement, nonelectrolytes including glycerol as well as PEGs with different molecular weights are typically mixed with a conducting buffer containing KCl. Measurements of the protein channel conductance would be dependent upon the size of the nonelectrolytes[82]. Polymers, e.g., PEGs, that are sufficiently small to enter the channel's pore will decrease the channel's conductance. Polymers with radii larger than those of the two pore entrances rarely partition into the pore, and thus will not affect the channel conductance. However, on the other hand, the nonelectrolytes always decrease the bulk conductivity of the conducting buffer solutions. As a result, the dependence of the channel conductance on the polymer molecular mass can be used to deduce the pore's radius using a mathematical relationship between the measured channel conductance and the conductivity of the bulk conducting buffer solutions used. To date, the method has been successfully applied for *Borrelia burgdorferi* P13 Porin and P66 Porin[83-84], *Colicin Ia* channels[81], *Bacillus anthracis* PA63 channels[85], Engineered *FhuA Δc/Δ4L* Protein[86], *epsilon* toxin[87], *staphylococcal* α-toxin as well as to *cholera* toxin channels[81] and etc. However, the size measurements were seldom used for the channel proteins that require forming proteoliposomes to facilitate the protein fusion into planar bilayer membranes. One reason would be attributed to the difficulty in the fusion of the proteoliposomes into the bilayer membranes in the buffer solutions containing nonelectrolytes, such as glycerol or PEG. When the conducting buffers are mixed with a high concentration of nonelectrolytes, the density and the viscosity of the buffers are increased, which would significantly slow down the rate for the fusion of the proteoliposomes into the bilayer membranes. For example, we found that, when the GP10 proteoliposomes prepared by the control method was added to a BLM chamber bathed in a 1M KCl 5 mM HEPES (pH 7.4) containing 20% glycerol, no connector insertion was found to occur (Data not shown).

The development of a method to prepare fusible proteoliposomes in the conducting buffers containing nonelectrolytes would be of particular significance to the structural studies on the phage portal proteins because the crystal data of most phage portal proteins are currently still unavailable. The proposed method in this paper provided us with a solution to the issue. In the experiments shown in Figure 3, considering that the density and viscosity were significantly increased in the conducting buffer containing 20% (V/V) glycerol, we specially used a rehydration buffer, 1 M KCl 5 mM HEPES (pH 7.4) with 25% glycerol (V/V), to prepare the GP10 proteoliposomes for the planar bilayer experiments. It was found that there were still a constant of supply of vesicles in the viscous buffer and steady rates of fusions were also observed (Fig. 3). To test how the channel conductance is affected by the size of nonelectrolytes used, we used the proteoliposomes to measure the single channel conductance of GP10 in the buffer containing PEG (MW:8000) . A summary of the results is shown in Table 1. In the table, the average conductance of single connector channel was measured to be 2.81±0.09 nS (N=41) in the presence of 20% glycerol vs 4.46±0.12 nS (N=52) in the absence of glycerol. The decrease in the single channel conductance was a result of the occupancy by the non-conducting glycerol molecules in the channel of the GP10 connector. However when 20% PEG (MW: 8000) was used, the conductance measured was 4.28±0.15 nS (N=65), close to 4.46±0.12 nS (N=52) in the buffer without the PEG, which indicated that the polymer molecules were excluded by the channel of the GP10 connector channel due to its bulk size. From the above results, it can be concluded that the experimental method was highly effective method to prepare fusible proteoliposomes for the use in the buffers containing nonelectrolytes.

The control method was the first reported technique to prepare fusible vesicles reconstituted with, a mutant phage portal protein, GP10 connector, for the planar bilayer membrane experiments[47]. In fact, it is a highly simplified dehydration-rehydration method. In the conventional dehydration-rehydration methods, an appropriate amount of detergents is typically added to rehydration buffers to help to increase the solubility of hydrophobic channel proteins when they are in contact with the lipid molecules during the rehydration step [88-92]. Proteoliposomes are typically formed by means of a time-consuming step for detergent removal using reagents, such as Biobeads. Clearly, the control method took less time, however it ignored the protein self-aggregation issue during the rehydration step. Consequently, we found that several mutant GP10 portal proteins, for example, N-His

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tagged connector, self-aggregated during the rehydration step when we used the control method (Data not shown).

To explore whether the experimental method could be used to prepare proteoliposomes reconstituted with more hydrophobic portal proteins, we further applied it to the preparation of proteoliposomes using a purified mutant portal protein, GP20 (20amN50(Q325am)) [60], from bacteriophage T4 (A generous gift from Prof. Venigalla, B. Rao from Department of Biology, Catholic University of America). The portal protein is structurally well-conserved and also forms the dodecameric portal channel through which DNA enters during packaging and exists during infection[29, 60, 93]. During assembly of T4 prohead, the protein needs to be bound to cell membrane to initiate scaffolding core assembly. GP20 is very hydrophobic and thus its solubility is very limited[36]. Using the experimental method, we prepared and added the proteoliposomes to a 1 M NaCl 5 mM Tris (pH 8.0) buffer. The results are demonstrated in Figure 4A. A current jump indicating an insertion of a single GP20 protein molecule was recorded. Under the voltages applied, the portal channels were kept open under the experimental conditions. The distribution of the conductance of a single channel of the mutant GP20 is shown in Fig. 4B. The average conductance for the mutant GP20 was 2.25 ±1.03 nS (N=23), lower than the average of the single channel conductance, 3.04±0.56 nS, from C-His GP10 in the same buffer reported previously[54]. Although the crystal data of the mutant GP20 is currently unavailable due to the hydrophobicity and low solubility, the conductance measurement results indicated that the size of the mutant GP20 channel could be 26% smaller than that of C-His GP10, which is consistent with the results in a recently published paper[94] where the diameter at the constriction in the channel of the native GP20 was reported to be 2.8 nm, less than 3.6 nm in the channel of the Φ29 connector[44]. To the authors' knowledge, it is the first time to prove that the portal protein, GP20, from bacteriophage T4 can be inserted into planar bilayer membrane, indicating that the planar lipid bilayer membrane technique would be a new research tool for researchers to perform structural-function study on the roles that GP20 plays in the head assembly, genome packaging, neck/tail attachment and genome ejection in Bacteriophage T4. The results also suggested that although all the phage portal proteins are structurally conserved, due to little sequence identity among the portal proteins, the size and channel properties of the portal proteins would vary among different bacteriophages. Therefore the large family of the underexplored portal proteins from bacteriophages and herpesvirus would provide more flexibility of choosing appropriate biological pores as a sensing motif for the single-molecule nanopore technology.

The successful insertion of the mutant GP20 into the planar bilayer membranes in Fig. 4B would be a consequence of the use of higher concentration of glycerol to prepare the proteoliposomes in the experimental method. It was also used to successfully insert hydrophobic mutant GP10 connectors, e.g., N-His GP10, into bilayer membranes (Data not shown). Glycerol is known to be a good co-solvent for the portal protein, GP 10, and it can prevent the protein from self-aggregation during the protein purification. In the dehydration step, the portal proteins mixed with 15% glycerol were added to the lipid, DPhPc, in a chloroform solution. The resulting mixture showed two phases, organic and aqueous phase. In both of the phases, no aggregation was visualized. The connector protein was found intact and not denaturized due to that the distribution of the single-channel conductance of the protein pores in the bilayer membranes was not affected (Supplementary figure A and B). The phenomenon could be explained as follows: The lipid, DPhPC formed a monolayer membrane at the interface between the organic phase and the aqueous phase which blocked most of the chloroform molecules in the organic phase from entering the aqueous phase to denature the protein. Under a vacuum condition, chloroform and water are removed. The evaporation rate for chloroform is faster than that of water because of the significant difference in their vapor pressure. As a consequence, a lipid film may rapidly form, and a higher concentration of glycerol was subsequently left to be with the portal proteins, resulting in the formation of a gel-like material. In the material, the interactions between the highly concentrated glycerol and the hydrophobic portal proteins could provide protective functions for the protein: A molecular dynamics simulation has confirmed that glycerol preferentially interacts with large patches of contiguous hydrophobicity on a protein surface[95]. The glycerol-protein interaction might shift the protein native conformation towards more compact conformations, in which glycerol orientation are preferred whereby the glycerol carbon atoms are in direct contact with the hydrophobic surface and the glycerol oxygen atoms point toward the solvent, resulting in that relatively few water oxygen atoms make contact with the protein surface. After the rehydration buffer mixed with glycerol was added, glycerol may act as an energetically favorable amphiphilic interface between the hydrophobic surface and polar solvent atoms to enhance the solubility of the proteins and to prevent the protein from aggregation and unfolding. Obviously, the glycerol in the experimental method improved the solubility of the hydrophobic portal proteins during the rehydration step, which is similar to the roles that the detergents play in the rehydration step for the conventional hydration-dehydration methods. However, the experimental method is a faster proteoliposome preparation method because it does not need to remove the glycerol and thus can avoid the time-consuming step for detergent removal used in the conventional hydration-dehydration methods.

Finally, it should be noted that the shelf life of proteoliposomes prepared by the experimental method was found longer, >2 years at -80°C or 3-4 months at 4 °C, than those prepared using the conventional dehydration-rehydration methods, < 6 months at -80°C and 1-2 weeks at 4°C, reported by different research groups[5,91,96-98]. The prolonged shelf life should be attributed to the use of high concentration of glycerol that can strongly interacts with water by hydrogen bonding, resulting in great changes in the colligative properties of solutions. For cryopreservation of the

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proteoliposomes at -80°C, the freezing point of water in the vesicle solution was decreased so that it can reduce the amount of ice formed at any given temperature, which minimize the probability of destroying the liposome membrane under direct mechanical action. In addition, because sucrose was replaced with glycerol that is membrane permeable, the osmotic pressure across the vesicle membrane could be adjusted continuously to keep the vesicles intact even as water gradually evaporates under long time cryopreservation. At 4 °C, the longer shelf life for the glycerol-based proteoliposomes should be a consequence of lower water vapor pressure caused by the high concentration of glycerol, which substantially prevents water from evaporation. The extended shelf life results suggested that the proteoliposomes prepared by the experimental method can be transported and shared more conveniently between research labs, which would also be of greater value to future commercialization of the proteoliposomes for different biomedical applications[99].

Conclusions

In summary, we proposed a new method to prepare liposomes reconstituted with portal proteins from bacteriophage and determined that it was more advantageous in at least four ways than the conventional dehydration-rehydration methods used for decades. The first improvement lay in changing from glycerol to replace sucrose, which imparted the stability of proteins and prevention of proteins from aggregation. Second, the density and osmotic pressure of the formed proteoliposomes could be adjusted by the use of different concentrations of glycerol in the rehydration step, which would control the rate for the contact of the proteoliposmes with planar bilayer membranes and the subsequent proteoliposome fusion into the membrane when different buffers were used. Therefore the insertion of phage portal proteins has become fast, controllable and predictable in horizontal BLM chambers. Our experiments showed that we were able to decrease the average time elapsed for the first portal protein insertion, by 14 fold, to less than 1 min. The third benefit from utilizing our experimental method is an increase in the longevity of the formed liposomes owing to the use of high concentration of glycerol. Storage and transport of liposomes would become more convenient. The feature would be very useful when the proteoliposomes can be commercialized for different biomedical applications, e.g., liposome-based drug delivery system, liposome-based nanopore sensing motifs and etc. Finally, the method was practical but not complicated. It did not need to use a specially made lipid molecules[98] or an engineered portal protein[47], as previously reported, to facilitate the portal protein insertion. Furthermore, in principle, it could be potentially used to prepare proteoliposomes reconstituted with hydrophobic phage portal proteins from bacteriophages as well as hydrophobic channel proteins from a larger family of cell membrane proteins if these proteins have a good solubility in the co-solvent, glycerol. Therefore, the method in the paper may provide a rapid, simple and practical approach not only to explore the possibility of screening more appropriate phage portal proteins for the single-molecule nanopore sensing technique but also to perform in-vitro real-time structural functional studies on the roles that portal proteins play in the head assembly, DNA packaging and DNA ejection among a variety of the tailed bacteriophages and herpesviruses. The technical advance will no doubt have a sufficiently significant impact on the research areas of nanotechnology, biophysics, and microbiology.

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Figure 1

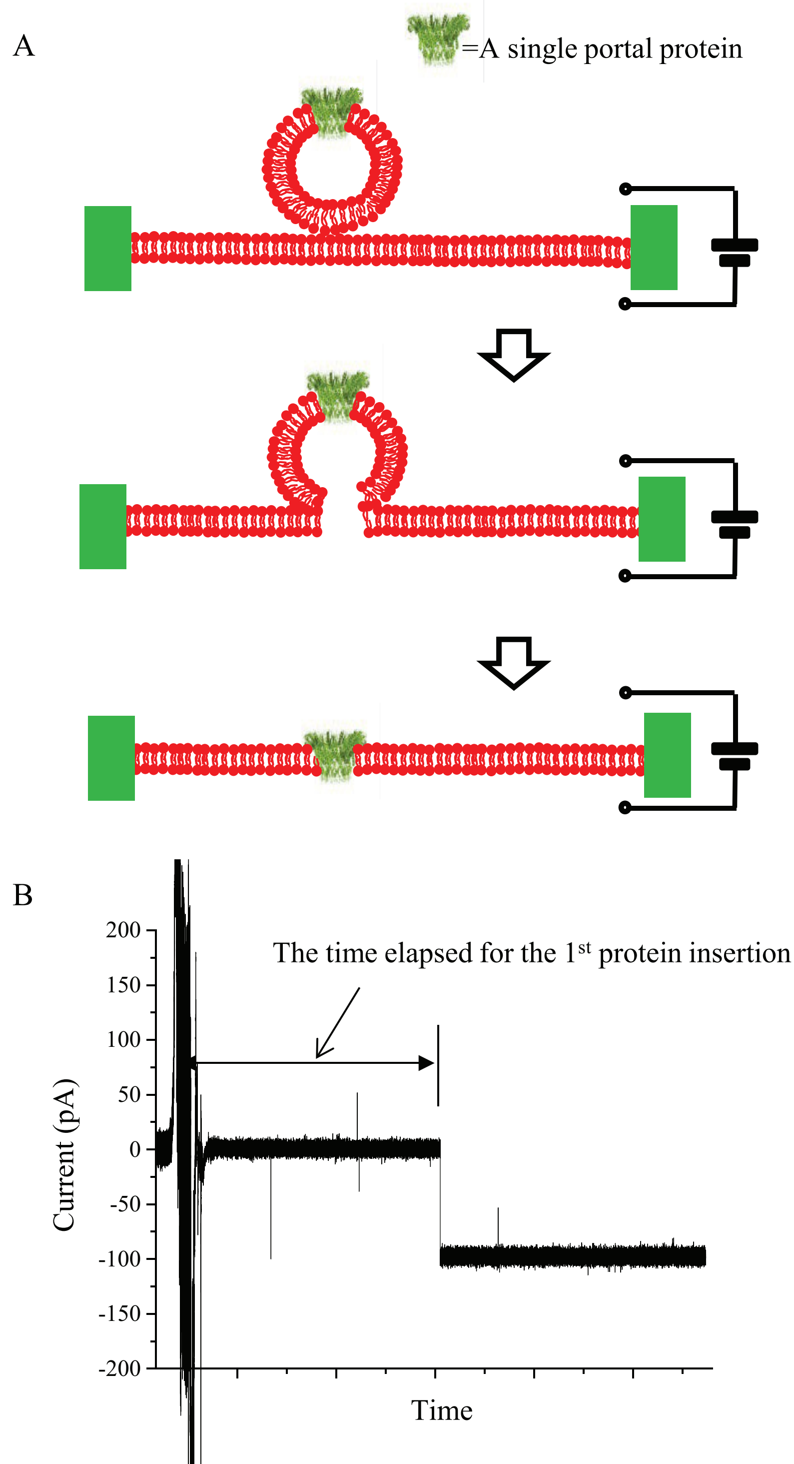

Figure 1 A: A schematic for the process of a single proteoliposome fused into a planar lipid bilayer membrane; B: A typical record of current trace showing one portal channel inserted into a planar bilayer membrane after proteoliposomes were added to the *cis*-chamber.

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Figure 2

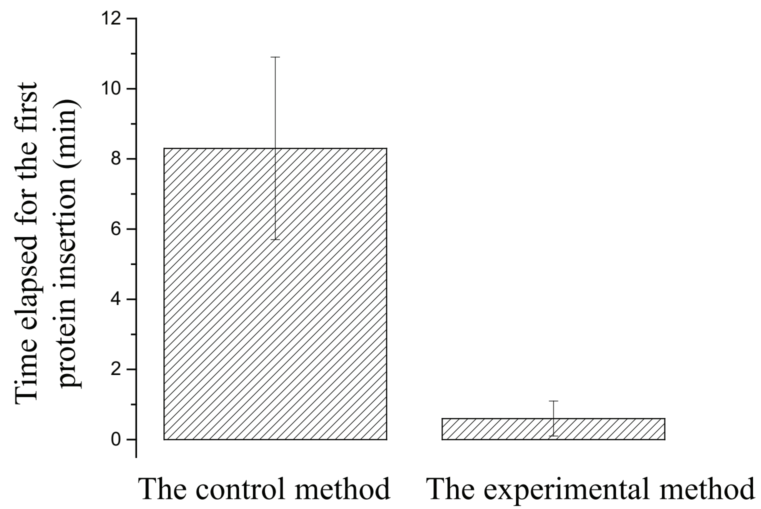

Figure 2: A comparison of the time elapsed for the 1st insertion after the proteoliposomes prepared by the control method and the experimental method were added to the BLM chambers respectively.

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Figure 3

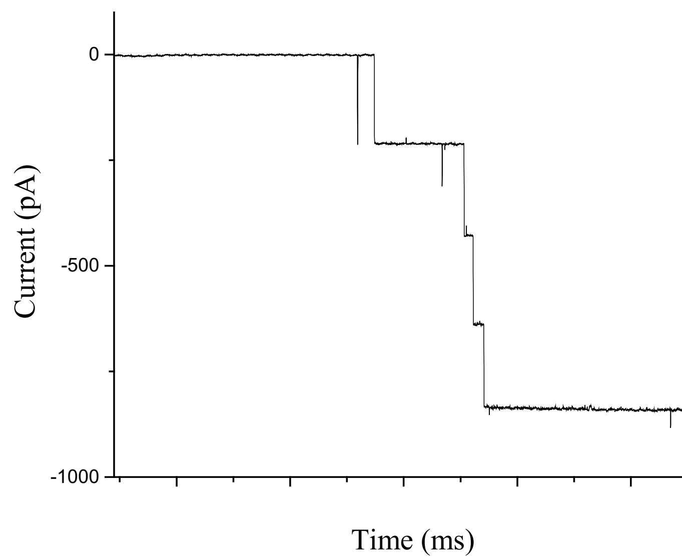

Figure 3: A current record showing continuous C-His GP10 connector insertion into a planar bilayer membrane in a 1 M KCl (pH 7.4 5 mM HEPES buffer) containing 20% glycerol (V/V) solution

Figure 4

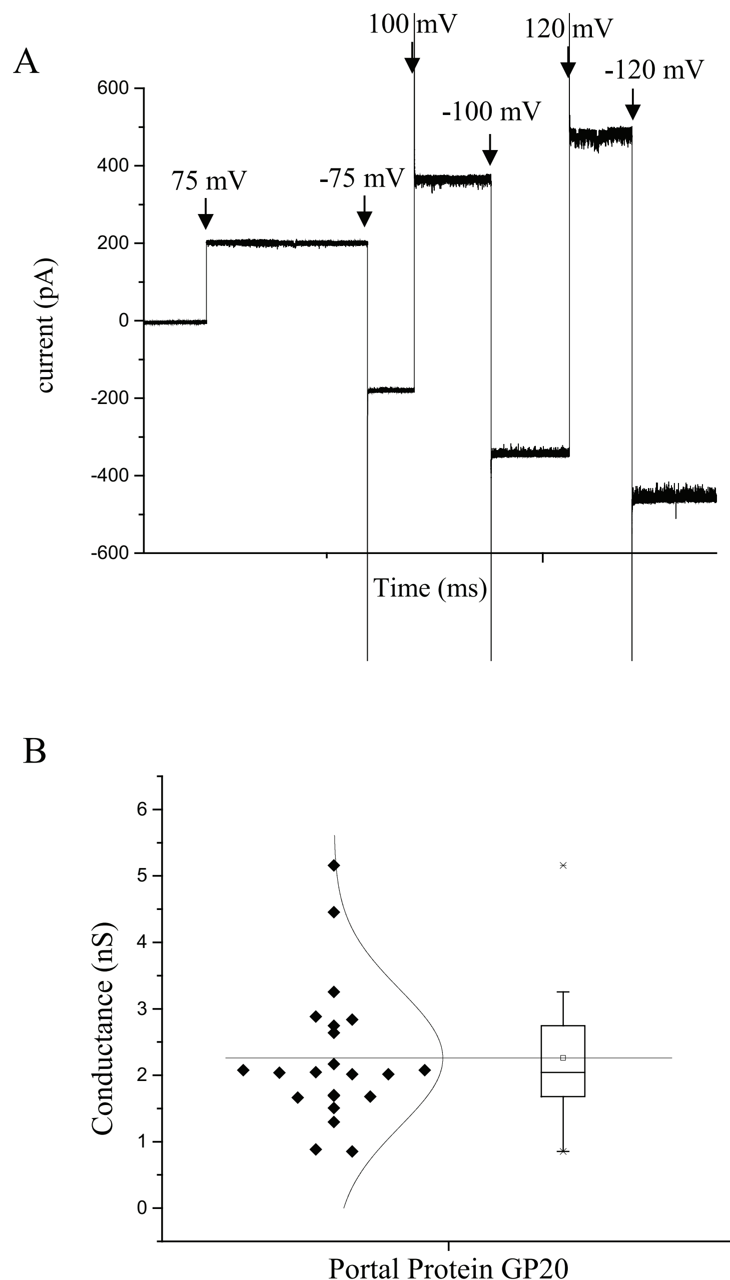

Figure 4 A: A Single Insertion of the Mutant Portal Protein, GP20 (20amN50(Q325am)), from Bacteriophage T4 in a Planar Bilayer Membrane under Different Voltages; B: A Box Plot Showing a Distribution of Measurements for Single Channel Conductance of GP20 (20amN50(Q325am)) in a Buffer, 1 M NaCl (5 mM Tris pH 8.0) under -75 mV.

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Table 1: Measurement of Single Channel Conductance of GP10 Connector in different conducting buffers

Conducting Buffer	Conductance (nS)
1 M KCl (5 mM HEPES pH 7.4)	4.46±0.12 (N=52)
1 M KCl (5 mM HEPES pH 7.4) with 20% PEG (MW: 8000)	4.28±0.15 (N=65)
1 M KCl (5 mM HEPES pH 7.4) with 20% glycerol	2.81±0.09 (N=41)

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