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Direct Quantitation of Peptide-Mediated Protein Transport across a Droplet−Interface Bilayer

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Supporting Information

ABSTRACT: We introduce a new method for monitoring and quantitating the transport of materials across a model cell membrane. As a proof-of-concept, the cell-penetrating peptide, Pep-1, was used to carry horseradish peroxidase (HRP) across droplet−interface bilayers (DIBs). Two submicroliter, lipid-encased aqueous droplets form a membrane at the contacting interface, through which enzyme−peptide complexes pass during transport. Following transport, the droplets are separated and the captured enzymes are assayed by a fluorogenic reaction. The DIB method recapitulates the findings of earlier studies involving Pep-1, including the dependence of protein transport on voltage and membrane charge, while also contributing new insights. Specifically, we found that leaflet charge symmetry may play a role in Pep-1-mediated protein translocation. We anticipate that the DIB method may be useful for a variety of transport-based studies.

Peptides that enable the transport of large molecular cargo across cell membranes are of immense interest in both fundamental and applied membrane research. In particular, the delivery of functional, therapeutic agents to specific regions within a cell is one of the field's primary driving forces. Since the discovery of Tat in 1988,[1,2] many groups have engaged in vigorous research efforts to describe the mechanism by which cell-penetrating peptides, protein transduction domains, Trojan peptides, poly arginine and other similar peptides permit the passage of large cargoes across cell membranes.[3] In the typical format, several peptides are covalently attached to the cargo (protein, DNA, liposome, nanoparticle) to create a population of complexes. Generally, these complexes are taken into the cell by some form of endocytosis.[3,4]

However, a small group of peptides possess the ability to transport cargo without the requirement of covalent attachment.[5−13] Instead, nonspecific electrostatic and/or hydrophobic interactions drive the assembly of the peptide and cargo to form a complex. Upon crossing the membrane, the peptides and cargo dissociate, leaving the delivered molecule in the cell's cytoplasm.[11] Direct entry into the cytoplasm bypasses the steps required to release cargo from endocytotic vesicles and is therefore a preferable means for delivery. In addition, the cargo is unhindered by covalently attached groups or other unfavorable interactions, thus preserving its functional integrity, such as enzymatic activity or binding specificity.

While molecular transport into cells is the central focus of peptide-based delivery, elucidating the mechanism of action is often challenging using living systems. In particular, noncovalently attached peptides such as Pep-1 and MPG appear to avoid endocytosis entirely, thus prompting the search for a new translocation mechanism.[11] Proposed mechanisms have been difficult to validate conclusively, in part, due to the complexity of experiments using a heterogeneous population of living cells. Model membranes, such as vesicles or planar bilayers, serve as alternatives to live cells and offer the ability to freely manipulate the chemical composition of the membrane environment. In particular, vesicles were used by several groups to study the mechanism of Pep-1.[14−18]

Noncellular studies often investigate the transport of fluorescently labeled peptide into or out of a population of vesicles.[19] However, the presence of a fluorophore inevitably alters a peptide's properties, possibly hindering or promoting transport.[20,21] In general, the transport of the cargo is of key interest, while the ultimate fate of the peptide is mostly secondary. Studies

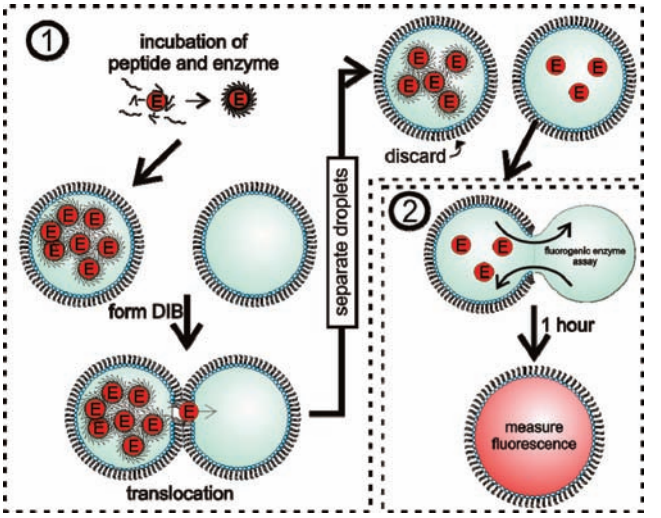

Figure 1. Two-stage process for quantitating peptide-mediated enzyme transport. In stage 1, complexes are formed between Pep-1 and horseradish peroxidase (HRP). Following incubation, the complex is mixed with vesicles submerged under an oil to form a droplet. This is joined to a droplet containing only vesicles to create DIB and enzyme translocation starts. Following translocation, the droplets are separated. In stage 2, the droplet containing captured enzyme is fused with a droplet of fluorogenic substrate. The fluorescence intensity is measured after 1 h and corresponds to the amount of captured enzyme.

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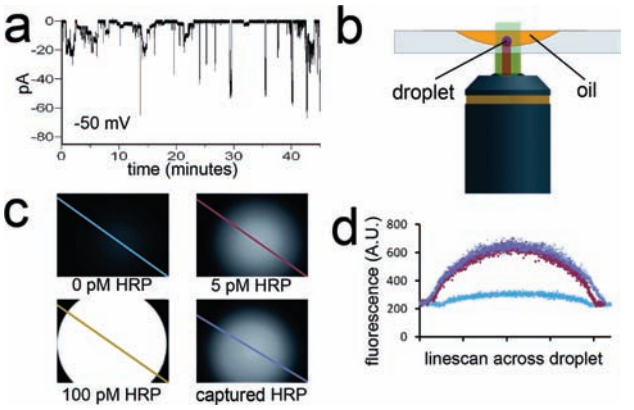

Figure 2. Quantitation of Pep-1-mediated enzyme transport. (a) During translocation, ionic current flowing across the DIB is monitored; at −50 mV, discrete bursts of current are observed. (b) Following droplet separation, the captured enzyme is mixed with a droplet of fluorogenic substrate and situated in a depression well above a microscope objective. (c) After 1 h, the droplet is imaged and the fluorescence intensity is compared to three control substrate droplets with known HRP concentrations. (d) Fluorescence linescans from (c) are plotted and the background is subtracted to obtain relative fluorescence intensities.

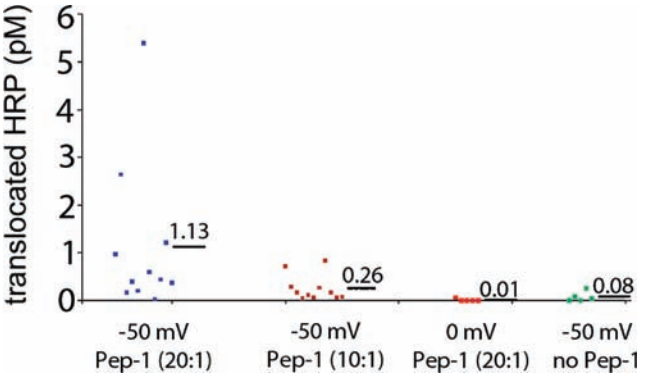

Figure 3. Translocation as a function of voltage and peptide. Data from each experiment is shown as a dot, and the average of each trial is shown as a bar under the numerical value. When a voltage was applied, more transport was observed when using a 20:1 peptide-to-cargo ratio than a 10:1 ratio. If either voltage or Pep-1 was omitted, transport was minimal.

that combine model membranes with Pep-1-mediated cargo detection are rare. In a seminal study, Henriques and co-workers used Pep-1 to carry an enzyme, β-galactosidase, into large unilamellar vesicles (LUVs).[15] Following transport, the captured enzyme was released by disrupting the vesicles with detergent and then detected by addition of a fluorogenic substrate. In agreement with prior research, the authors found that a voltage gradient was required for transport.[15] The voltage gradient in the LUVs was established by incubation with the K^+-specific peptide, valinomycin. Both Pep-1 and valinomycin embed in membranes, and it is not clear whether they interact during enzyme transport. Also, the magnitude and polarity of the voltage gradient created by valinomycin could not be manipulated during translocation. It would be highly desirable to develop a model membrane system where cargo could be transported and quantitated without the need for ionophores and salt gradients.

Here, we demonstrate that the recently developed droplet−interface bilayer (DIB) is well-suited for tackling mechanistic questions regarding peptide-mediated protein transport. For the following proof-of-concept studies, Pep-1 and horseradish peroxidase (HRP) are used as peptide and cargo, respectively, and we anticipate that our approach will be useful for other peptide/cargo combinations. Briefly, a cell membrane mimic is formed by joining two lipid-monolayer-encased aqueous droplets together under an oily hydrocarbon.[22] Lipid vesicles in the aqueous droplets fuse at the oil/water interface to form monolayers.[23] Electrodes within the droplets permit the application of a transmembrane voltage and monitoring of ionic current during translocation. The formation of the membrane is fully reversible; the monolayers may be separated by mechanically pulling the droplets apart. This ability is a key advantage of the DIB membrane system and enables the assay used here (Figure 1). Here, 20 μM Pep-1 was incubated with 1 μM horseradish peroxidase (20:1 ratio)[3,24] in water for 30 min, during which time an enzyme−peptide complex formed. The complex was diluted 10-fold in a buffered vesicle solution, and a 200 nL droplet of this mixture was hung from an Ag/AgCl electrode submerged in hexadecane. On the opposing electrode, a 200 nL droplet of vesicle solution was deposited and both droplets were allowed time for monolayer formation, generally about one minute. The droplets were then joined to create a DIB, initiating the process of translocation. The droplet containing the enzyme−peptide complex is termed the "source" droplet, which, if possible, delivers enzyme to the opposing "capture" droplet through the DIB. The peptide−protein complexes interacted with the DIB over a 45 min period, after which time the droplets were pulled apart and translocation stopped. Critically, the condition of the membrane was monitored by electrophysiology during the entire experiment, ensuring that the membrane did not rupture during DIB formation, translocation or DIB separation (Figure 2a). Following separation, the source droplet was discarded.

To determine the amount of enzyme translocated (if any) by Pep-1, the capture droplet was fused with a freshly prepared 200 nL droplet containing an excess of fluorogenic substrate (Amplex Red) and hydrogen peroxide in buffer. Translocated enzymes reacted with Amplex Red to create the highly fluorescent product, resorufin. After one hour, the droplet was imaged using a fluorescence microscope (Figure 2b). For every experiment, a 200 nL droplet of substrate was fused to each of three control droplets: (i) a droplet containing no enzyme (background), (ii) a droplet containing 5 pM HRP, and (iii) a droplet containing 100 pM HRP (Figure 2c). Each control also incubated for one hour, thus four droplets were imaged for each data point. By comparing the intensity of the capture droplet with the 5 pM HRP standard, the quantity of translocated HRP was determined.

We measured Pep-1-assisted transport of HRP across a pure 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine (DPhPC) bilayer at a potential of −50 mV. Using a 20:1 Pep-1 to HRP ratio, the average concentration of captured HRP was 1.13 pM, or approximately 140,000 molecules (Figure 3). Reducing the peptide−cargo ratio to 10:1 significantly reduced the amount of HRP transported.[24,25] Under either condition, only a tiny fraction of HRP was transported into the adjacent droplet, therefore, the concentration of enzyme in the source droplet remained roughly constant. Removal of either the voltage or the Pep-1 resulted in very little transport of HRP (Figure 3). Therefore, both Pep-1 and voltage are vital for transport across a neutrally charged bilayer. This agrees with previously published results.[11,15,17,26] In separate experiments, we examined DIBs with 2 μM Pep-1 in the

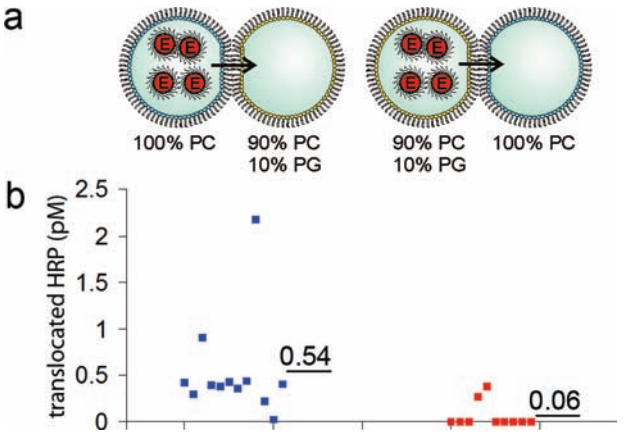

Figure 4. Translocation as a function of bilayer asymmetry. (a) DIBs were created with 10% negatively charged lipids either facing away (left) or toward (right) the source of enzyme−peptide complex. No voltage was applied in either trial. (b) When negative charge was present in the far leaflet, HRP transport was observed. Negative charge on the near leaflet did not promote transport.

absence of HRP (Supporting Information) and found that transient pores formed in the membrane at a potential of −50 mV. The ionic conductance of membranes in the presence of Pep-1 has been reported previously by others.[17]

Next, we examined the role of negatively charged lipids on Pep-1-mediated HRP transport. DIBs were formed where one of the monolayers contained 10% 1,2-diphytanoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DPhPG) and 90% DPhPC and the other monolayer was pure DPhPC. Previous DIB experiments have shown that membrane asymmetry can be maintained for at least several hours.[23] The location of the negatively charged DPhPG monolayer was found to have a surprising effect on transport (Figure 4a). When the negative leaflet was oriented away from the source of the Pep-1/HRP complex, substantial transport of enzyme was observed (Figure 4b). However, when the negative leaflet faced the source of the complex, very little transport was observed. Both experiments were conducted at zero applied voltage. This experiment suggests that lipid composition on the *inner* leaflet of a cell may play a predominant role in Pep-1-assisted molecular transport. The ability to form an asymmetric membrane using the DIB system will allow this finding to be probed in greater detail quickly and reliably.[23]

To the best of our knowledge, this is the first time peptide-mediated cargo translocation has been quantified using individual artificially formed membranes. Previous studies have relied on populations of vesicles.[15] Also, this is the first example of a coupled electrophysiology−quantitation experiment using artificial membranes. By observing the conductance of the membrane during translocation, we hope to find a correlation between the bursts in current (duration and magnitude, Figure 2a) and the amount of HRP transported. Such a relationship might be able to explain the high variability of transport seen under certain conditions (Figure 3). Ongoing experiments in our lab are focusing on this possibility.

The DIB approach enables the efficient capture of cargo in a submicroliter volume, greatly reducing the dilution of translocated materials when compared to planar bilayers. Because the droplets are easily separated, the entire volume of captured enzymes can be isolated without the risk of contamination or the need for complex protein/membrane digestion schemes.[15] Instead, analysis simply requires fusing the isolated droplet with a reagent droplet, followed by fluorescence detection. Importantly, the condition of each membrane is monitored during the entire translocation process, providing a new window for mechanistic insights. Though preliminary, the DIB approach has already provided an interesting clue regarding Pep-1's mechanism, opening the possibility that the inner leaflet may help to pull peptide and cargo molecules into the cell.

In summary, we have shown that the droplet−interface bilayer approach has the potential to contribute new insights in membrane translocation investigations. Our initial experiments with Pep-1 indicate that voltage or negative charge in the far leaflet can act as independent driving forces for protein transport. We anticipate that this method may be applicable to a variety of transport studies, including other cell-penetrating peptides and cargoes, protein-pore-based translocation (such as toxins) or other cases where the quantitation of transported materials is desired.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Detailed description of experimental procedures and controls. This material is available free of charge via the Internet at http://pubs.acs.org.

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