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Controlling Secretion in Artificial Cells with a Membrane AND Gate

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Abstract

The assembly of channel proteins into vesicle membranes is a useful strategy to control activities of vesicle-based systems. Here, we developed a membrane AND gate that responds to both a fatty acid and a pore-forming channel protein to induce the release of encapsulated cargo. We explored how membrane composition affects the functional assembly of α -hemolysin into phospholipid vesicles as a function of oleic acid content and α -hemolysin concentration. We then showed that we could induce α -hemolysin assembly when we added oleic acid micelles to a specific composition of phospholipid vesicles. Finally, we demonstrated that our membrane AND gate could be coupled to a gene expression system. Our study provides a new method to control the temporal dynamics of vesicle permeability by controlling when the functional assembly of a channel protein into synthetic vesicles occurs. Furthermore, a membrane AND gate that utilizes membrane-associating biomolecules introduces a new way to implement Boolean logic that should complement genetic logic circuits and ultimately enhance the capabilities of artificial cellular systems.

graphical abstract

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Author Contributions

C.E.H., M.L.J., K.R.L., and J.A.P. performed experiments. C.E.H., M.L.J., K.R.L., and N.P.K. analyzed data and wrote the paper.

ASSOCIATED CONTENT

Supporting Information

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Supporting Methods and Figures S1–S11 (PDF)

The authors declare no competing financial interest.

Keywords

artificial cell; protein—membrane interactions; cell-free expression; membrane logic gate

A major focus in synthetic biology has been the use of biological parts to implement logical operations.^{1,2} These operations have been largely implemented using genetic circuitry based on gene transcription and translation events. In contrast, the design of membrane or material-based logical operations have been more limited.^{3,4} As the development of artificial cells and cell-free systems continues, understanding how to design membrane-based logic circuits will become important to improve the specificity and complexity of designed operations of the artificial cellular system as a whole. A membrane function that would be highly useful to execute with a logic gate is membrane permeability: by controlling when and the extent to which membrane permeability is enhanced, the uptake and secretion of molecules from a vesicle can be gated. Such gating would enable higher control over complex functions, such as the release of synthesized or encapsulated molecules in response to environmental signals,^{4–6} or the initiation of encapsulated reactions upon entry of reactive substrates.⁷

A promising approach to control permeability in vesicle membranes has been the use of pore-forming proteins like α -hemolysin (α HL). α HL, a pore forming toxin secreted by *Staphylococcus aureus*, consists of water-soluble peptide monomers that spontaneously assemble in compatible vesicle membranes to form functional, heptameric channels.^{8,9} The functional channel contains a nanometer size, nonspecific pore that allows for the passage of larger cargoes that are otherwise membrane-impermeable, such as ions and DNA.^{10,11} Understanding how to modulate interactions between α HL and synthetic membranes should allow us to dictate not only when channel proteins are incorporated into membranes but also the number that are incorporated, thereby controlling broader features of membrane permeability. These findings could potentially be extended to a range of pore-forming channels to advance the design of artificial cells.

One way to affect the permeability of vesicles using channel proteins is to change the number of channel proteins that functionally insert into vesicle membranes. This number can be controlled by changing the concentration of protein¹² or by changing the affinity of protein—membrane interactions.^{13–17} Toward the former route, Adamala *et al.* recently showed that by varying the concentration of α HL DNA in synthetic vesicles, the expression

of α HL could be adjusted, leading to vesicles with differential permeability.⁶ Toward the latter route, other groups have shown that membrane composition affects protein integration. For example, α HL insertion requires the presence of phosphocholine (PC) head groups at the membrane interface^{12,14} and the presence of cholesterol in the membrane.^{12,18} Most of these latter studies have been directed toward uncovering biological mechanisms of protein activity and, to date, using membrane composition as a strategy to control protein activity in artificial cells and in synthetic biology applications has not been widely explored.

Here, we describe an approach to temporally control pore formation in phospholipid vesicles by changing the composition of vesicle membranes in real time. Toward this goal, we were inspired to use fatty acids, which can affect membrane protein behavior and play vital roles in biological systems as signaling molecules.¹⁹ We demonstrate that addition of oleic acid (OA) micelles to phospholipid vesicles results in fatty acid membrane uptake and associated membrane composition changes that promote α HL assembly and pore formation. Finally, we demonstrate our ability to connect the designed membrane response to a genetic system by synthesizing α HL using cell-free protein expression. Our study demonstrates that the remote control of membrane composition imparts a new level of regulation over membrane permeability by affecting when an expressed protein assembles into a functional pore in vesicle membranes.

■ RESULTS AND DISCUSSION

To assess the functional integration of α HL channels into vesicle membranes, we analyzed the release of an encapsulated dye from phospholipid vesicles. Vesicles composed of 18:1 (9) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were prepared containing calcein, a self-quenching dye. Vesicles were extruded to ~100 nm to form small unilamellar vesicles (SUVs) and purified using size exclusion chromatography to remove unencapsulated calcein. We validated our assay by incubating vesicles that encapsulated calcein and that had a membrane composition that promotes α HL integration (1/1 DOPC/cholesterol) in increasing amounts of α HL. We observed increasing calcein release as a function of protein concentration, consistent with previous results (Figure S1).²⁰

We next set out to determine a base lipid composition and compatible, membrane-associating inducer molecule that would enable inducer-specific integration of α HL channel proteins. Our strategy was to design membranes that shift from a state that opposes α HL integration (“inactive”) to one that enables channel integration (“active”) (Figure 1). We hypothesized that membranes with insufficient cholesterol content would generate an inactive membrane that was unable to incorporate α HL. Studies have shown that α HL integration into vesicles is optimal at a 1/1 molar ratio of DOPC/cholesterol.²⁰ Using a calcein dye release assay, we determined the effect of cholesterol content in DOPC vesicle membranes on α HL assembly into functional channels. We observed minimal dye release when DOPC vesicles in Bicine buffer (pH 8.5) contained 20 mol % cholesterol or less and were incubated with α HL, indicating the protein did not spontaneously assemble into functional pores. Importantly, this result was consistent across a wide range of α HL concentrations tested (Figure S2). We therefore used DOPC vesicles with 20 mol % cholesterol as our inactive membrane composition and DOPC vesicles with 50 mol %

cholesterol as our active membrane composition for subsequent studies assessing inducer-specific α HL integration.

Next, we assessed the potential of OA to act as an inducer of α HL assembly by monitoring how OA content in vesicle membranes affected α HL-associated dye release. Cholesterol is a bidirectional regulator of membrane fluidity and as previously mentioned, it enhances α HL assembly into vesicle membranes when used in certain quantities.²¹ Similar to these qualities of cholesterol, OA has been shown to increase membrane fluidity.²² In addition, OA micelles are advantageous as membrane fluidizers in that they can be added at relatively high molar ratios to preformed vesicles to change their membrane composition without destabilizing the vesicles.^{23,24} Due to these qualities, we decided to assess if OA could replace the role of membrane cholesterol and serve as an inducer for α HL assembly. We prepared DOPC vesicles with 20 mol % cholesterol and increasing amounts of OA in order to assess the effect of OA content on α HL integration. When OA was included in DOPC/cholesterol vesicle membranes at various molar ratios, we observed a monotonic increase in dye release from vesicles that depended on both OA content and α HL concentration (Figure 2). Notably, dye release remained low (<10%) across the range of α HL concentrations tested when OA was absent, indicating a lack of functional pore formation. These results therefore support the feasibility of OA addition as a route to induce α HL integration.

The spontaneous insertion of fatty acids from micelles presents a route to alter membrane composition in real time.^{23,24} We therefore wondered if the exogenous addition of OA micelles to preformed vesicle membranes could induce α HL integration. We monitored OA insertion using microscopy (Figure S3) and a bulk Förster Resonance Energy Transfer (FRET) assay that measures changes in membrane surface area. The FRET assay uses the distance dependence of nonradiative energy transfer between fluorescent, lipid-conjugated donor and acceptor dyes to report increases in lipid separation during membrane growth (Figure 3a). A standard curve relating mol % dye in the membrane to FRET efficiency enables quantitative measurements of changes in membrane surface area (Figure 3b). We observed that adding increasing amounts of OA micelles to DOPC vesicles leads to increases in membrane surface area, confirming the insertion of OA in DOPC membranes (Figure 3c). We then verified that OA added to the outside of inactive vesicles can flip to the inner leaflet of the membrane using the pH sensitive dye HPTS (pyranine) as an indicator for OA flipping (Figure S4). The flipping of OA to the inner leaflet of the membrane is an important feature of our system because it indicates that our method should be compatible in vesicle systems with encapsulated protein or expression systems. Finally, we incubated inactive membranes with α HL and remotely added OA micelles. α HL assembly into the membrane was measured by monitoring the release of encapsulated calcein (Figure 3d). We observed that both the presence of OA micelles and α HL monomers were required to induce calcein release and that calcein release increased with increasing OA concentration. This effect was reproducible when vesicle diameter was increased to 1 μ m (Figure S5a). These results demonstrate that external OA addition to phospholipid vesicles is a viable strategy to control the functional assembly of α HL into vesicle membranes.

Combining inducible genetic circuits with membrane logic gates would create systems with more specificity and complexity, especially for functions, like membrane secretion, that

utilize vesicle membranes and membrane proteins. An advantage of using channel proteins to induce membrane permeability is that protein activity can be connected to genetic circuits by programming the output of the circuit to be protein synthesis.^{25–30} Specifically, by placing a gene for α HL downstream of a genetic circuit, α HL production has been used to couple genetic circuits to membrane secretion.^{5,27,31,32} In the past, however, it has been difficult to control when α HL integrates into vesicle membranes since the protein spontaneously assembles into pores as it is expressed. We wondered if our membrane AND gate could be used to better control when expressed α HL integrates into vesicle membranes, thereby imparting higher order control over the temporal dynamics of membrane secretion, as well as demonstrating the potential to connect designed membranes to genetic systems.

We investigated the potential of OA addition to temporally regulate the integration of expressed proteins into vesicles present in the reaction mixture. We performed cell-free reactions using the PURExpress system in the presence of inactive and active vesicles. First, we confirmed that OA-induced α HL assembly into vesicle membranes was reproducible in the 50 mM HEPES buffer (pH 7.6) used with PURExpress reactions (Figure S5b). We then expressed a fluorescent fusion protein of α HL to confirm α HL synthesis in the presence of vesicles and to determine the kinetics of α HL expression. We expressed α HL-eGFP in the presence of our inactive vesicles and observed increased eGFP fluorescence over time, plateauing at just over 2 h (Figure S6a). A Western blot was used to confirm α HL-eGFP expression (Figure S6b). We observed that expressing α HL-eGFP in the presence of vesicles premade with OA caused a decrease in expression, presumably because OA interferes with expression machinery.³³ This inhibitory behavior does not interfere with our AND gate, however, if OA is added as an inducer postexpression. Our results confirm that the presence of vesicles with the inactive membrane composition does not inhibit the synthesis of α HL protein.

We then expressed α HL without the eGFP tag in the presence of inactive or active vesicles containing calcein. In active vesicles, we observed calcein release soon after the reaction started. This behavior is consistent with the kinetics of α HL-eGFP expression and indicates that α HL assembles into functional pores in active membranes as it is expressed (Figure 4). This result also highlights a previously mentioned challenge with using genetic circuits that express channel proteins. Mainly, because expressed membrane proteins spontaneously insert into vesicle membranes as they are synthesized, it has been difficult to control when and the extent to which expressed membrane proteins integrate into vesicles that are present during the reaction.³⁴ In contrast, we observed minimal calcein leakage (<15%) from inactive vesicles, consistent with leakage that occurs when adding cell-free reaction components to vesicles in the absence of α HL (Figure S7). After 2.5 h, we added OA micelles to inactive vesicles at a 3:10 molar ratio of micelles: vesicles and observed a rapid increase in calcein release. An equivalent volume of NaOH buffer used to prepare OA micelles was also added to confirm that changes in calcein fluorescence were not due to other components in the micelle buffer. Total vesicle leakage was quantified by lysing vesicles with TritonX-100 to determine 100% release. We observed that OA addition to inactive membranes can cause nonspecific calcein leakage dependent on OA concentration, but that nonspecific leakage could be reduced to low (<5%) levels while still inducing α HL-associated release by adjusting the OA:vesicle ratio (Figure S8). Finally, we observed that by

changing the concentration of α HL plasmid in the PURExpress reaction we could further tune the extent of calcein release (Figure S8, S9). Specifically, by increasing or decreasing the concentration of α HL plasmid in the reaction, we were able to achieve commensurate changes in calcein release upon OA addition to inactive vesicles. Plasmid concentration therefore provides yet another handle to modulate secretion from inactive vesicles by controlling the total number of channel proteins that are expressed.

Finally, as the previously described studies were conducted outside vesicles, we set out to confirm that OA-induced release could be achieved when the cell-free reaction was encapsulated inside inactive vesicles, an important feature when designing artificial cells or organelles (Figure S10). Because high concentrations of calcein can interfere with the activity of cell-free reaction systems, we used the release of D-biotin as a reporter for α HL assembly. We encapsulated the cell free reaction system, the α HL plasmid, and D-biotin inside inactive vesicles and confirmed that the addition of OA to these vesicles induced biotin release by monitoring the fluorescence of a biotin-reporter added outside of vesicles. Taken together, these results show that vesicle composition can affect the extent to which expressed channel proteins integrate into vesicles present during a cell-free reaction. In addition, our results indicate that OA micelle addition is a feasible strategy to temporally control the integration of expressed α HL into vesicles. Furthermore, our results serve as a first step toward connecting a membrane gate to genetic circuits by showing that the output of a cell-free expression system can be used as an input into a membrane AND gate.

In the present study, we hypothesize that OA contributes to α HL pore assembly in the membrane by enhancing membrane fluidity and affecting cholesterol distribution. α HL is a member of the cholesterol-dependent cytolysin (CDC) family, a large family of pore-forming toxins.¹⁸ Membrane cholesterol is necessary for CDC incorporation; however, the exact role of cholesterol remains unclear. One theory posits that cholesterol increases membrane fluidity, an important feature for membrane protein assembly.³⁵ Another model states that the distribution of cholesterol in the membrane may also be important for CDC binding.^{14,18,20} A third theory is that cholesterol promotes CDC transition from the prepore state to an assembled pore, without which CDC oligomers are trapped in the prepore state.³⁶ In addition, OA could also contribute to changes in membrane curvature that could influence channel protein dynamics, including the propensity of channels to insert into membranes as well as oligomerize.^{37,38} Finally, there is also the possibility that the insertion of α HL monomers and OA together could lead to encapsulant leakage due to membrane packing defects instead of functional pore assembly. Although we did not explore other cholesterol-dependent cytolysins, we expect that OA has the potential to be used as a trigger for spontaneous insertion of these types of membrane proteins into membranes with depleted cholesterol levels, similar to α HL. Similarly, while we used OA as an inducer molecule to change membrane composition, a range of other fatty acids, like myristoleic acid (Figure S11) and other membrane-associating molecules could potentially serve as inducers of channel assembly.

In summary, we designed vesicles that incorporate a functional channel protein upon external addition of a fatty acid. We showed that an inducible change in vesicle membrane composition can be used to control content release by modulating the assembly of α HL. Our

approach presents the design of a membrane AND gate that incorporates membrane-associating molecules as inputs that work in conjunction to control vesicle secretion. In the design of artificial cells, biosensors, or nanocarriers, understanding how to regulate membrane protein activity will be important for controlling sensing and transport, among other activities.³⁹ Toward this goal, our study highlights one way that membrane composition can be used to temporally control the integration of an expressed channel protein into preformed vesicle membranes. Our study demonstrates how membrane composition has the potential to be used as a new circuit element in artificial cells, which may be coupled to existing genetic circuits.^{27,32,40} This work opens the door to a range of new studies exploring how membrane composition could be used to control membrane protein activity for functions beyond secretion.

■ MATERIALS AND METHODS

Materials.

DOPC (18:1 (9) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, 25 mg/mL), cholesterol (ovine wool >98%) and Lissamine-Rhodamine PE (18:1 1,2-dioleoyl-*sn*-glycero-3-phosphoethanoamine-*N*-(lissamine rhodamine B sulfonyl)) were obtained from Avanti Polar Lipids. NBD PE (NBD-phosphoethanolamine, triethylammonium salt), Triton X-100, and calcein were obtained from Thermo Fisher Scientific. Phosphate-buffered Saline (PBS), Bicine (*N,N*-Bis(2-hydroxyethyl)glycine), Sepharose 4B (45–165 μm bead diameter), and α -Hemolysin from *Staphylococcus aureus* (lyophilized powder, Protein ~60% by Lowry, 10 000 units/mg protein) were obtained from Sigma-Aldrich. Oleic acid (OA) was obtained from NU-Chek Prep, Inc. PURExpress In Vitro Protein Synthesis Kit was purchased from New England Biolabs. DNA plasmids pIVEX2.3d- α -hemolysin and pIVEX2.3d- α -hemolysin-eGFP were a generous gift from the Noireaux Lab at the University of Minnesota and are described previously.²⁴

Preparation of Vesicles.

Small unilamellar vesicles (SUVs) were prepared using thin film hydration.⁴¹ Membrane components (DOPC, cholesterol, and OA) were mixed in chloroform, added to glass vials, dried down uniformly under a stream of nitrogen, then dehydrated in a vacuum oven for >5 h to form thin films. Vesicles were rehydrated with 0.2 M Bicine (pH 8.5), or a 20 mM calcein solution in Bicine to a final concentration of 5 mM of lipid and incubated at 60 °C overnight. All vesicles were vortexed briefly then extruded 9 passes through 100 nm polycarbonate filters for size uniformity. Dye-containing vesicles were subjected to freeze—thaw cycles 4 times to increase encapsulation efficiency and purified using a size-exclusion column packed with 5 mLs of Sepharose 4B. An FC 204 Fraction Collector (Gilson) was used to collect vesicle fractions purified away from unencapsulated dye.

Preparation of Micelles.

OA micelles were prepared by mixing neat OA with 100 mM NaOH to a final concentration of 100 mM OA.

Calcein Release Assays with α -Hemolysin.

A stock solution of α HL was prepared in Milli-QH₂O (250 μ g/mL). Calcein release assays with α -Hemolysin were conducted by incubating 100 μ M of purified SUVs containing 20 mM calcein with varied amounts of α HL (5–30 μ g/mL) in 0.2 M Bicine, pH 8.5. After incubation for 30 min, calcein fluorescence was read on a plate reader at 25 °C (ex. 480 nm, em. 520 nm). Calcein release was calculated as %release = $\frac{I_t - I_0}{I_{\text{Triton}} - I_0} \times 100$. For studies that utilized OA addition to membranes, α HL (15 μ g/mL) was added first, followed by OA addition. For all studies that utilized TritonX-100 to report calcein release upon vesicle lysis, 1 μ L of 10% Triton X-100 was added to a 30 μ L sample.

FRET Assays for OA Uptake.

Vesicles labeled with FRET membrane dyes were prepared by mixing Lissamine-Rhodamine PE/NBD PE with DOPC/cholesterol in chloroform as described previously.³² Briefly, a standard curve was produced by adding FRET dyes (at a final concentration of 0.025, 0.05, 0.1, 0.125, 0.175, and 0.2 mol %) to 100 μ M 80/20 DOPC/cholesterol vesicles and mixed prior to removal of solvent. Post-evaporation, the FRET-labeled films were hydrated with 0.2 M Bicine and extruded as described above. Vesicles were excited at 463 nm and emission was measured at 517 nm to measure donor emission and at 590 nm to measure acceptor emission. FRET ratios were calculated by analyzing donor fluorescence over acceptor fluorescence where FRET efficiency = $\frac{f_{\text{donor}}}{f_{\text{acceptor}}}$. This ratio of fluorescence was compared to standard curve samples to translate changes in FRET efficiency to membrane surface area changes where surface area = (mol % dye_{initial}/mol % dye_{final}). Studies assessing OA micelles uptake were conducted with 100 μ M vesicles containing 0.1 mol % FRET dyes.

Protein Expression and Analysis.

Cell-free protein expression was performed using the PURExpress system prepared as directed by the New England Biolabs protocol. Experiments were performed in the presence of 10 mM DOPC vesicles that were previously hydrated with 20 mM calcein in Milli-Q water, extruded, and purified to remove unencapsulated calcein. Prior to purification, vesicles at 260 mM total amphiphile concentration containing 20 mM calcein were purified resulting in a final concentration of 10 mM vesicles in the reaction sample. pIVEX2.3d- α -hemolysin (0.9 Mg, 200 μ g/ μ L) was expressed in the reaction and calcein release was analyzed using a SpectraMax i3x plate reader at 37 °C (ex. 480 nm, em. 520 nm). The reaction wells were sealed and measurements were recorded every minute for 3 h. After the reaction had plateaued, OA micelles were added from a stock solution of 100 mM OA in 100 mM NaOH to completed PURExpress reactions at a final working concentration of 3 mM OA. Equivalent volumes of water or NaOH were added to control samples. Calcein release was calculated as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic of a membrane AND gate. (a) A membrane logic gate employs changes in membrane composition from oleic acid (OA) and a pore-forming protein (α HL) to control protein activity. (b) An inactive membrane is unable to assemble functional pores, while an active membrane composition assembles functional pores. (c) An inactive membrane can be transformed to an active membrane via addition of oleic acid, thus triggering pore assembly, and release of encapsulated cargo.

Figure 2.

α HL assembly is dependent on oleic acid content and α HL concentration in vesicles.

Increasing molar amounts of OA in 100 μM DOPC/cholesterol membranes leads to greater α HL incorporation into the membrane and subsequent release of calcein from vesicles.

Figure legends reflect the molar ratio of DOPC/cholesterol/oleic acid in preassembled SUV membranes prior to exposure to α HL. Studies were conducted in 0.2 M Bicine buffer at pH 8.5, 25 $^{\circ}\text{C}$. All samples are normalized to calcein release after vesicle lysis with TritonX-100. $n = 3$; error bars represent standard deviation.

Figure 3.

Remote addition of OA leads to vesicle uptake and α HL assembly. (a) FRET measurements allow monitoring of OA incorporation into vesicle membranes, which leads to membrane surface area increases, and a corresponding decrease in FRET signal. (b) FRET standard curve providing a reference for corresponding FRET ratios ($f_{\text{donor}}/f_{\text{acceptor}}$) with a change in m^o % dye. (c) Increasing OA addition to vesicles leads to increased membrane surface area. (d) Triggered calcein release from vesicles in the presence of 15 $\mu\text{g/mL}$ α HL or no α HL as a function of added concentration of OA micelles. Samples are normalized to total calcein

release after TritonX-100 lysis. The end-point values between the two samples are statistically significant, $*p < 0.001$ as determined by an unpaired t test. All studies were conducted with 100 μ M 80/20 DOPC/cholesterol vesicles in 0.2 M Bicine buffer at pH 8.5, 25 °C. $n = 3$; error bars represent standard deviation.

Figure 4.

OA addition induces calcein release from vesicles present in a cell-free expression reaction. (Top) Schematic of membrane composition of vesicles present in the reaction. (Bottom) Calcein release from vesicles present during the cell-free expression of α HL is monitored over time as a function of vesicle composition and reported normalized to calcein release with TritonX-100 vesicle lysis. At 2.5 h, equivalent volumes ($0.9 \mu\text{L}$) of 100 mM OA, 100 mM NaOH, or water were added to vesicles. TritonX-100 addition reports complete calcein release upon vesicle lysis. Vesicles (10 mM) containing 20 mM calcein were incubated with a PURE system. Figure legends reflect the molar ratio of DOPC/cholesterol in preassembled SUV membranes prior to exposure to the PURE system containing α HL plasmid ($200 \text{ ng}/\mu\text{L}$). Studies were conducted in 50 mM HEPES buffer at pH 7.6, 37°C .