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Reversibly-triggered protein-ligand assemblies in giant vesicles

Ruud J.R.W. Peters,[a] Marlies Nijemeisland[a] and Jan C.M. van Hest*[a]

Abstract: External small molecule triggers were used to reversibly control dynamic protein-ligand interactions in giant vesicles. An alcohol dehydrogenase was employed to increase or decrease the interior pH upon conversion of two different small molecule substrates, thereby modulating the pH-sensitive interaction between a Ni-NTA ligand on the vesicle membrane and an oligohistidine-tagged protein in the lumen. Upon alternating the small molecule substrates, the interaction could be reversed.

Compartmentalization is a key organizational feature in nature. It allows cells to spatially arrange enzymatic complexes in order for their processes to function more efficiently.[1] In eukaryotic cells, organelles are applied to spatially separate biosynthetic pathways.[2] Nearer to these permanent spatial assemblies of biomolecules, the cell also utilizes a host of dynamic interactions that temporarily interface proteins and ligands to control a cellular process. These dynamic assemblies often form and dissociate in response to changes in their interaction domains.[3] The cues can originate either from elsewhere inside the cell, or from the outer environment.

Cellular responses to external signals often involve signaling pathways facilitated via such a biomolecular assembly process, for example triggered by the binding of the signaling compound to a membrane-bound receptor or other protein and subsequently activating it. Many of these events involve protein-protein, or protein-ligand binding, which causes a change in protein or ligand localization and allows the cell to direct these molecules to the site of action.

Controlling and understanding these complex processes is of vital importance to unraveling the functioning of the cell. Research into protocells and artificial cell-mimics has, among others, already focused on transcription and translation processes,[4] cytoskeletal structures,[5] the use of organelle processes,[6] cytoskeletal structures,[5] the use of organelle compartments to study dynamic protein-ligand interactions.[7] Compartmentalization is a key organizational feature in nature. It allows cells to spatially arrange enzymatic complexes in order for their processes to function more efficiently.[1][13] In eukaryotic cells, organelles are applied to spatially separate biosynthetic pathways.[2] Nearer to these permanent spatial assemblies of biomolecules, the cell also utilizes a host of dynamic interactions that temporarily interface proteins and ligands to control a cellular process. These dynamic assemblies often form and dissociate in response to changes in their interaction domains.[3] The cues can originate either from elsewhere inside the cell, or from the outer environment.

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Gaining spatiotemporal control over these processes can therefore aid in understanding them in more detail,[14][15] but also in designing more complex cell-like systems that can respond to external stimuli.

To devise an artificial cell-mimic of how cells can respond reversibly to external stimuli, i.e. the addition of food sources, or signaling molecules, three elements should be combined: a cell-mimetic compartment, an externally triggered environmental change, and a reversible protein-ligand interaction. We use giant unilamellar vesicles (GUVs) as a cell-mimicking platform where the semipermeable nature of the lipid membrane allows small molecules to passively diffuse into the vesicle lumen and trigger a response inside. Through enzymatic conversion of the small molecule, a pH change can be induced[16] that allows for the selectively triggered assembly and disassembly of a protein-ligand complex. The pH change inside the GUVs can be controlled by using an Alcohol Dehydrogenase (ADH) that requires the natural cofactor NAD(H). During cofactor conversion pros are either consumed or produced, depending on the substrate used, as ADH is capable of both reducing ketones like acetone, and oxidizing alcohols like isopropanol (iPrOH) (Figure 1).

The maximum pH change is therefore also limited by the amount of cofactor present inside the system, and the directionality of the reaction can be reversed upon addition of the opposite trigger. The change in internal pH in the system can subsequently be used to promote a structural change by disturbing or forming a pH-sensitive protein-ligand interaction (Figure 1). A bright and acid stable fluorescent protein called tdTomato[14] containing the commonly employed oligohistidine tag (His-tag) is used to bind a Nickel(II) nitrilotriacetic acid ligand (Ni-NTA).[10] The GUV membrane can be influenced by addition of a Ni-NTA-presenting lipid inside GUVs can be influenced by addition of an external substrate trigger. Upon addition of the trigger, ADH can be used to reversibly change the local pH inside the GUV to modulate the pH-sensitive interaction between Ni-NTA and the His-tagged protein. At acidic pH (A), the interaction is prevented, while upon increasing the pH, the interaction is induced (B) and vice versa.

Supporting information for this article is given via a link at the end of the document.

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The evolution of NADH fluorescence was observed (Figure S2B). These results indicate that the GUVs are permeable to the small molecule substrates, and they retain the larger enzymes and cofactors.

Next, the ability of the system to change and regulate the pH of the inner solution was explored. Bulk studies using a ratiometric pH-sensor were performed to follow pH changes caused by the enzymatic reaction. Either a pH increase (A, C, with acetone) or decrease (B, D, with iPrOH) was induced. Changes in pH are visualized as a change in Fluorescein/TAMRA emission ratio, where a higher ratio represents a higher pH value. Scale bar is 10 μm.

Using the system with 0.5% DGS-NTA-Ni lipid in the vesicle membrane and a starting pH of 4.8 or 7.0, the respective assembly and disassembly studies were performed. To monitor assembly, the ratio of lumen fluorescence intensity over membrane fluorescence intensity was measured. A ratio of 1 would therefore indicate uniform fluorescence in the GUV and complete disassembly, while a ratio close to 0 would indicate a high degree of membrane association for the protein. Both assembly and disassembly were observed upon addition of the respective triggers (Figure 3 and S6), while no change in assembly was observed when ADH was not included in the GUVs, demonstrating the necessity of the pH change to trigger assembly (Figure S7). For membrane assembly, acetone was added, and a clear transition from a lumen/membrane ratio of 1.0 to ~0.3 was observed (Figure 3A-B). This clearly demonstrated the small molecule triggered assembly of tdTomato on the GUV membrane. For GUVs where disassembly was triggered with iPrOH, however, dissociation appeared to be highly dependent on the starting pH in the system. Only the system with a starting pH of 6.1 showed complete disassembly in all vesicles (Figure S6D). At pH 6.5 the response was slower and also not complete (Figure S6A-B), while at pH 7.0, disassembly was even less complete (Figure S6C-D).
pronounced (Figure S6C-D). As the ensemble measurements previously showed that even from pH 7.0, a decrease to pH 5.0 could be achieved (Figure 2B), the GUVs should be capable of reaching the point of disassembly. From our observations in GUVs, however, it appeared that the required pH was not reached when the reactions were started from a pH between 6.5 and 7.0. Therefore it might be possible that the GUVs were unable to build up and subsequently maintain the required pH gradient long enough for disassembly to occur. Generally, liposome-based pH gradients, displaying transmembrane pH differences which can be as high as 2-4 pH units, [18] are maintained by using solutions with a far higher buffering capacity compared to that of the system described here, and their dissipation due to leakage therefore takes a longer time. The durability of the ADH-based gradient not only depends on proton leakage and the weaker buffer, but also might be more limited due to a membrane plasticizing effect of the small molecule substrate, increasing the leakage rate. Also the amount of nicotinamide cofactor that is present, and the rate at which it can be converted in response to the changing pH in the GUV lumen might influence the stability of the gradient over time.

In conclusion, due to the semi-permeable nature of lipid membranes, an enzymatically induced positional assembly process of proteins in GUVs can be started and controlled using small molecules as external stimuli. In this fashion, we showed that ADH was capable of converting its cofactor upon addition of the small molecule substrate, thereby directly influencing the internal vesicle pH. As a result, addition of the enzyme substrates also caused a pH gradient to be established over the GUV membranes that could be applied to reversibly induce or inhibit binding of a His-tagged fluorescent protein, tdTomato, to Ni-NTA ligands positioned on the GUV membrane. Both Ni-NTA ligand density on the membrane, and the regime in which the pH changes were carried out, proved to be critical parameters for the successful reversible assembly of the protein on the membrane. When operated within the right regime, the system can be cycled between an assembled and disassembled state several times, showcasing the reversible and dynamic nature of this system.

Finally, to show the reversibility of the assembly under changing external stimuli, immobilized GUVs containing NADH, ADH and tdTomato in pH 4.8 buffer were exposed to acetone (20 mM), which rapidly triggered tdTomato membrane assembly (Figure 4, blue arrow). After allowing the assembly process to level off and the luminoembrane fluorescence intensity ratio to stabilize, disassembly was induced by the substitution of the acetone solution by an iPrOH solution (20 mM, Figure 4 yellow arrow). The disassembly process was considerably slower compared to assembly, yet this was also expected as a result of the ensemble and GUV-based pH measurements (Figure 2 and S3), where the pH decrease also proceeded considerably slower compared to the pH increase. Furthermore, the multivalent nature of the NTA-SC-functionalized membrane might also further slow down dissociation. After disassembly had been reached, the cycle was started again to show the full reversibility of the system. Similar time frames were observed for assembly and disassembly in all the imaged GUVs and also in the consecutive cycles.

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The GUVs were allowed to settle and bind on the surface for 30 minutes before starting the experiments. Time-lapse imaging was started and the external solution was then substituted with acetone solution at a flow rate of 10 mL/h for 5 minutes using syringe pumps (Screening Devices, Amersfoort, The Netherlands), after which the flow was stopped. After protein assembly was achieved, acetone solution was substituted with iPrOH solution to trigger assembly, again using a 10 mL/h flow rate for 5 minutes. This process was reversed again by changing back to acetone as described before.

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Protein-ligand assemblies in giant vesicles can be reversibly switched on via an enzymatic reaction inside the vesicle, which can be initiated by an externally added small molecule trigger.

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