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Protein Modifications

Functionalized Nanocompartments (Synthosomes) with a Reduction-Triggered Release System**

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Biologically derived compartments are constrained in design by their biological functions to ensure life at ambient temperature. Polymer vesicles can be designed to match application demands, such as mechanical stability, organic solvent, substrate and product tolerance, and permeation resistance, that are out of reach for biologically derived vesicles.[1] Synthosomes use, in contrast to polymersomes, a transmembrane channel for controlling the in and out compound fluxes. The block copolymers in synthosomes prevent compound penetration through the polymer shell, whereas polymersomes depend on the diffusion of substrate and product molecules through the polymer shell.

The main advantage of synthosomes over polymersomes is that, through protein engineering, it is possible to design functionalized protein channels. A protein channel that can function as an on/off switch offers opportunities for the design of functional nanocompartments with potential applications in synthetic biology (pathway engineering), medicine (drug release), and industrial biotechnology (chiral nanoreactors, multistep syntheses, bioconversions in nonaqueous environments, and selective product recovery).

The channel proteins FhuA,[2] OmpF,[3–5] and Tsx[6] have been incorporated, in functional active form, into block-copolymer membranes. FhuA, ferric hydroxamate uptake protein component, is a large monomeric transmembrane protein of 714 amino acids folded into 22 antiparallel β strands and made up of two domains. Crystal structures of FhuA have been resolved,[7,8] and a large passive diffusion channel (FhuA Δ1–160) was designed by removing a capping globular domain (deletion of amino acids 5–160).[9,10] FhuA and Tsx were crystallized as monomers and OmpF as a trimer. FhuA and its engineered variants have a significantly wider channel than OmpF (OmpF ≈ 27–38 Å, FhuA ≈ 39–46 Å)[11] and this allows even the translocation of single-stranded DNA.[12]

The aim and novelty of our work is the introduction of a triggering system, by means of a reduction-triggered "release switch" based on an engineered FhuA channel variant. To the best of our knowledge, in none of the reported triggered systems, was a channel protein employed as a switch.

In fact, for polymersomes, a pH trigger,[13] a temperature-assisted pH trigger,[14] and a combined pH/salt trigger[15,16] have been developed. Furthermore, hydrogen peroxide generation was used for polymer-vesicle degradation by glucose oxidase catalyzing glucose oxidation,[17] and a pH-triggered release system for a polypeptide vesicle has been reported.[18] For synthosomes, the activation of an encapsulated phosphatase after a change in the pH value has been reported.[19]

To build up a reduction-triggered release system in synthosomes, the amino-group-labeling agents 3-(2-pyridyldithio)propionic acid *N*-hydroxysuccinimide ester (pyridyl label) and (2-[biotinamido]ethylamido)-3,3′-dithiodipropionic acid *N*-hydroxysuccinimide ester (biotinyl label) were selected, due to size considerations and the presence of a cleavable disulfide bond within the labeling reagents. Reagents for the specific labeling of amino, hydroxy, carboxyl, and sulfhydryl groups have been well studied and are routinely used for protein modifications.[20–22]

The synthosome calcein release system proposed herein is a triggered release system in which the entrapped compound (calcein) is liberated through an engineered transmembrane channel (FhuA Δ1–160) upon addition of a reducing agent. Interestingly, label size played an important role in calcein release. A detection protocol for calcein release from liposomes through wild-type FhuA and FhuA Δ1–160 has been reported.[2] The liposomes were loaded with calcein at a self-quenching concentration (50 mM) and calcein release was achieved by addition of wild-type FhuA and FhuA Δ1–160. The fluorescence generation upon calcein release was used to record the release kinetics.

In order to build a reduction-triggered release system, the amino groups of lysine residues in FhuA Δ1–160 were modified with either a pyridyl or a biotinyl label (see above). Figure 1 illustrates the reactions for FhuA Δ1–160 with eight lysine residues (L167, L226, L344, L364, L455, L537, L556, and L586) chemically modified with pyridyl (left) or biotinyl labels (right).

Upon disulfide-bond reduction with DTT, a 3-thiopropionic amide group remains on the lysine residues of the FhuA Δ1–160 with both labels (Figure 1, upper part). Details

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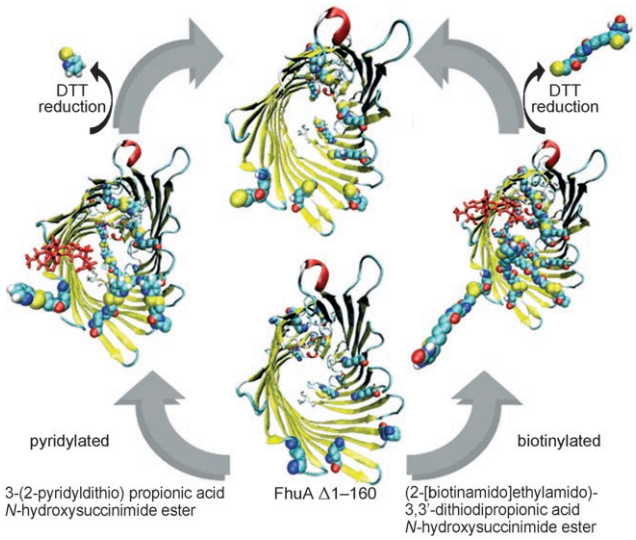

Figure 1. Reduction-triggered release system based on transmembrane channel FhuA Δ1–160. FhuA Δ1–160 is chemically modified with pyridyl or biotinyl labels at lysine residues in the barrel interior and at the rim. The translocation of the calcein molecule (red) through the pyridylated or biotinylated FhuA Δ1–160 is sterically hindered. Upon reduction with 1,4-dithio-D,L-threitol (DTT), the disulfide bond in the linker of the pyridyl or biotinyl label is broken and this results in calcein release. The FhuA Δ1–160 model was prepared from the crystal structure (Protein Data Bank entry: 1BY3) and all lysine residues have been labeled in the model. Further details can be found in the Supporting Information.

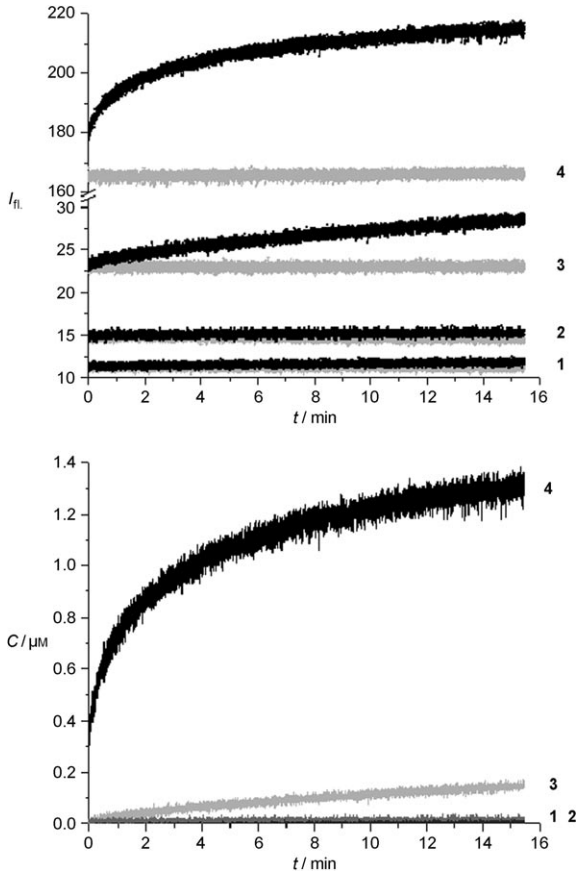

Figure 2. Upper panel: For the recording of the calcein-release kinetics, quadruple sets of data were measured and averaged in all four experiments. For each experiment, a data set with and without DTT addition was recorded for nanocompartments loaded with 50 mM calcein. Each experiment is specified by a number (1–4): 1) Nanocompartments without FhuA Δ1–160 before (——) and after (——) DTT addition; 2) synthosomes harboring FhuA Δ1–160 before (——) and after (——) DTT addition; 3) biotinylated FhuA Δ1–160 synthosomes before (——) and after (——) DTT addition; 4) pyridylated FhuA Δ1–160 synthosomes before (——) and after (——) DTT addition. Lower panel: calcein release in micromolar concentrations upon DTT reduction as calculated from the results in the upper panel: 1) Nanocompartments without (——) and 2) with (——) FhuA Δ1–160, 3) biotinyl-labeled FhuA Δ1–160 (——), and 4) pyridyl-labeled FhuA Δ1–160 (——).

of the chemistry of the pyridyl and biotinyl labeling of FhuA Δ1–160, the protein stability, vesicle dimensions, and FhuA Δ1–160 model generation can be found in the Supporting Information. The top view of the FhuA Δ1–160 channel in Figure 1 provides an impression of how the pyridyl and biotinyl labels restrict translocation after the lysine modification, especially the sterically more demanding biotinyl label. Figure 1 clearly shows how the transmembrane channel might open up after DTT-induced release of the pyridyl and biotinyl labels. However, the FhuA Δ1–160 models do not take into account the possible channel dynamics that have been recorded by conductance measurements.[10]

Figure 2 shows the calcein-release kinetics (upper panel) and the absolute calcein concentrations (lower panel) in the synthosomes before and after addition of the reduction trigger (DTT). There is no detectable calcein release before and after DTT addition in absence of the FhuA Δ1-160 (Figure 2, data set 1). In the case of the unlabeled FhuA Δ1–160, one would expect that the calcein would translocate through FhuA Δ1–160 as previously shown,[2] and it is therefore lost during synthosome purification (Figure 2, data set 2). For the biotinyl-labeled FhuA Δ1–160, a linear release kinetic is observed after DTT reduction. A greater than 30-fold faster and exponential initial calcein release is observed upon reduction of the less bulky pyridyl label, which leads to the same 3-thiopropionic amide labeled FhuA Δ1–160 (Figure 1). The strong size dependence of the initial release kinetics indicates that the biotinyl labels stay bound to the FhuA Δ1–160 channel upon DTT reduction. Interestingly, after approximately six minutes, the release kinetics reached a nearly constant increase for both labels, and this increase remained constant for 2 h (data not shown). The release kinetics trend is characteristic of passive diffusion processes and can be modeled by the monoexponential defined in Equation (1),[23] in which C is the calcein concentration versus time (t) and $P1$, $P2$, and $P3$ are fitting constants (Figure 2; Table 1).

$$C = -P1\exp\left(-\frac{t}{P2}\right)+P3 \qquad (1)$$

Constant $P1$ depends on the calcein concentration gradient inside and outside of the nanocompartments in the bulk suspension (Table 1). The significantly higher values for the pyridyl-labeled FhuA Δ1–160 channel (28.53 μ for pyridyl versus 7.24 μ for biotinyl) can be attributed to FhuA Δ1–160

Table 1: *P*1, *P*2, and *P*3 constants from Equation (1), calculated by fitting the recorded release kinetics data (Figure 2).

Nanocompartment system	*P*1 [μM][a]	*P*2 [min][a]	*P*3[a]
nanocompartments without FhuA Δ1–160	–	–	11.58
synthosomes with FhuA Δ1–160	–	–	14.53
synthosomes with FhuA Δ1–160 and biotinyl label	7.24	13.88	23.76
synthosomes with FhuA Δ1–160 and pyridyl label	28.53	3.99	165.72

[a] Further details given in the text.

limited diffusion because the employed samples were analyzed after purification with a Zeta-Sizer and, in quantity, were normalized by elution areas. The *P*2 value represents the time constant of the calcein-release process and describes the efflux from the nanocompartment sample through the FhuA Δ1–160 channel protein (Table 1); it is dependent on the number of FhuA Δ1–160 molecules per nanocompartment, the channel properties (size, charge, dynamics, chemical labeling), and the DTT concentrations. Upon unblocking, pyridyl-labeled FhuA Δ1–160 shows a time constant (3.99 min) that is four times shorter than that of biotinyl-labeled FhuA Δ1–160 (13.88 min). Apart from the labeled amino groups, all other factors were identical and, therefore, differences are directly connected to the nature of the labeling reagents. The *P*3 constant describes the background fluorescence of the nanocompartment systems (Table 1). The significantly higher background values for the pyridyl-labeled FhuA Δ1–160 channel (Figure 2) can be attributed to a slow release of calcein during storage. The pyridyl-labeled FhuA Δ1–160 suspension shows a calcein fluorescence buildup after storage overnight, which is in contrast to the biotinyl-labeled FhuA Δ1–160 suspension. A 4 h incubation period after DTT addition results in a further increase in the absolute fluorescence difference of less than 15% for both pyridyl- and biotinyl-labeled FhuA Δ1–160 synthosomes.

At the moment, it is unknown which lysine residue(s) is (are) decisive for blocking the calcein translocation in pyridyl- and biotinyl-labeled FhuA Δ1–160. The biotinylation degree found per single FhuA Δ1–160 molecule is 3.6 (see the Supporting Information). Over the 29 lysines contained in FhuA Δ1–160, 19 are on the protein surface which might be covered by detergent; and probably not avoidable to biotinylation. Another six are located in the channel interior and only four are on the channel rim. It is reasonable that the biotinylation occurs on the latter group because they are more accessible to the reagent. Future investigations by lysine-site-specific mutagenesis and modeling studies will shed light onto this problem.

In summary, a synthosome reduction-triggered release system with an engineered and chemically labeled FhuA Δ1–160 channel has been developed and validated by calcein release. Two labeling reagents of different sizes have been used. The release kinetics of calcein were strongly modulated by the size of the lysine-labeling reagents. In general, such on/off switches would be of high value for controlling and modulating cellular biosynthetic pathways and might become attractive for applications in the pharmaceutical and/or chemical industries. In ongoing studies, we aim to generate a reversible switch by chemically "remodifying" the free sulfhydryl groups that are formed upon reduction in the FhuA Δ1–160 transmembrane channel protein.

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