Molecular understanding of sterically controlled compound release through an engineered channel protein (FhuA)

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Abstract

Background: Recently we reported a nanocontainer based reduction triggered release system through an engineered transmembrane channel (FhuA Δ1-160; Onaca et al., 2008). Compound fluxes within the FhuA Δ1-160 channel protein are controlled sterically through labeled lysine residues (label: 3-(2-pyridyldithio)propionic-acid-N-hydroxysuccinimide-ester). Quantifying the sterical contribution of each labeled lysine would open up an opportunity for designing compound specific drug release systems.

Results: In total, 12 FhuA Δ1-160 variants were generated to gain insights on sterically controlled compound fluxes: Subset A) six FhuA Δ1-160 variants in which one of the six lysines in the interior of FhuA Δ1-160 was substituted to alanine and Subset B) six FhuA Δ1-160 variants in which only one lysine inside the barrel was not changed to alanine. Translocation efficiencies were quantified with the colorimetric TMB (3,3',5,5'-tetramethylbenzidine) detection system employing horseradish peroxidase (HRP). Investigation of the six subset A variants identified position K556A as sterically important. The K556A substitution increases TMB diffusion from 15 to 97 [nM]/s and reaches nearly the TMB diffusion value of the unlabeled FhuA Δ1-160 (102 [nM]/s). The prominent role of position K556 is confirmed by the corresponding subset B variant which contains only the K556 lysine in the interior of the barrel. Pyridyl labeling of K556 reduces TMB translocation to 16 [nM]/s reaching nearly background levels in liposomes (13 [nM]/s). A first B-factor analysis based on MD simulations confirmed that position K556 is the least fluctuating lysine among the six in the channel interior of FhuA Δ1-160 and therefore well suited for controlling compound fluxes through steric hindrance.

Conclusions: A FhuA Δ1-160 based reduction triggered release system has been shown to control the compound flux by the presence of only one inner channel sterical hindrance based on 3-(2-pyridyldithio)propionic-acid labeling (amino acid position K556). As a consequence, the release kinetic can be modulated by introducing an opportune number of hindrances. The FhuA Δ1-160 channel embedded in liposomes can be advanced to a universal and compound independent release system which allows a size selective compound release through rationally re-engineered channels.
been embedded in a tri-block copolymer membrane PMOXA-PDMS-PMOXA; where PMOXA = poly(2-methyl-2-oxazoline) and PDMS = poly(dimethyl siloxane); and could be opened up through a reduction trig-
gerated system [12]. The reported calcein release kinetics were strongly modulated by the size of employed lysine-labeling reagents [12]. Twenty nine lysines are present in the FhuA Δ1-160; 19 lysines located on the protein surface, 6 are inside the channel, and 4 are at the barrel rim [12]. The 19 lysines on the FhuA surface point into the outer membrane and are after purification covered by oPOE rendering pyridyl-labeling unlikely.

An average of four lysine residues per FhuA Δ1-160 was determined to be pyridyl labeled [12]. Based on the hypothesis that the 6 lysine inside the channel might mainly be responsible for restricting compound fluxes, two subsets of FhuA Δ1-160 variants were generated. In the six subset (A) variants only one of the six lysines in channel interior was substituted by alanine and in the six subset (B) variants only one lysine remained in the channel interior whereas all other five were substituted to ala-
nine. For the in total 12 investigated FhuA Δ1-160 variants a HRP based colorimetric TMB (3,3′,5,5′-tetram-
ethylbenzidine) detection system [13,14] was employed for quantifying the sterical hindrance of pyridyl-labeled lysines on the TMB substrate. The colorimetric HRP/ TMB detection was preferred over the previously reported calcein detection system due to a higher repro-
ducibility [1,12]. Furthermore liposomes instead of a polymeric nanocarrier system were selected for char-
acterizing the 12 FhuA Δ1-160 variants due to more sim-
ple and rapid assay procedures [15], despite drawbacks like leakiness, stability over time [16] and undesired biomolecule adsorption on the surface [17].

However, the better kinetic results reproducibility using liposomes compared to polymersomes, where the FhuA Δ1-160 insertion can be affected by block co-pol-

ermer poly-disperity and traces of residual chemicals, sug-

gested us to use liposomes correcting the kinetic results by the small leakage contribution (see Table 1). To our best knowledge we report a first detailed mutational study on a transmembrane channel protein to gain, on the molecular level, first insights on the sterically con-
trolled diffusion of TMB through the FhuA channel inte-
rior modulated by labeled lysines. Interestingly only one single lysine position is the main responsible of the TMB diffusion.

Results
FhuA Δ1-160 based compound release system
Figure 1 shows a FhuA Δ1-160 based compound release system where FhuA Δ1-160 is embedded in a lipid mem-
brane (left) together with the colorimetric HRP/TMB reporter used for quantifying TMB translocation (right).

HRP based colorimetric TMB detection system
TMB as chromogen has been developed and widely used in enzyme immunoassays (EIA) employing horseradish peroxidase [13,14]. Besides, the colorimetric HRP/TMB detection system proved to be more reproducible than the previously employed calcein assay which generates a fluorescence signal upon release of self-quenching calcein from liposomes into the surrounding solution.

The HRP/TMB detection system is based on a two step consecutive oxidative reactions A→B→C (A = TMB; B and C = first and second TMB oxidation products, see Figure 1) catalyzed by HRP in presence of hydrogen per-

oxide. Each single step is a pseudo-second order rate reaction with a reported second order rate constant (myeloperoxidases) [14] of: \( k_B \rightarrow C = 9.4 \times 10^5 \text{M}^{-1} \text{s}^{-1} \) and \( k_{B \rightarrow C} = 9.4 \times 10^5 \text{M}^{-1} \text{s}^{-1} \). The final TMB oxidation product C is unstable out of very acidic conditions [13] and the intermediate based on the first oxidation product B is used as reaction reporter, explaining the absorbance drop in time (see Additional file 1). The total amount of encap-
sulated HRP was not detectable though using the Soret absorption band. However kinetic data reproducibility was confirmed basing on a three data set for each mea-
surement.

FhuA Δ1-160 lysine positions and diffusion limited TMB translocation
Figure 2 shows the six lysine residues in the FhuA Δ1-160 inner channel which upon labeling might be responsible to modulate sterically the diffusion through the channel protein. In total 12 FhuA Δ1-160 variants were generated to identify the lysine(s) which might limit TMB flux through FhuA Δ1-160 inner channel. Two subsets of six FhuA Δ1-160 variants were generated. Subset A) contains FhuA Δ1-160 variants in which one of the six lysines in the interior of FhuA Δ1-160 was substituted to alanine; subset B) contains six FhuA Δ1-160 variants in which only one lysine was not changed to alanine. Table 1 sum-
marizes for these two subsets the TMB conversions. TMB conversions were determined by diffusion limited trans-
location through the FhuA Δ1-160 [12] inner channel (Additional file 1: Figure S1 and S2) using a previously reported colorimetric HRP/TMB detection system [1,13].

HRP has been entrapped in the liposome harboring FhuA Δ1-160 variants by using film hydration method coupled with extrusion. In this method, the lipid amphiphile is brought in contact with the aqueous medium containing HRP and FhuA Δ1-160 in its dry state and is subsequently hydrated to yield vesicles. After homogenization and purification of the resultant liposomes, the TMB conversion was initiated by supplement-
ing TMB (10 μl) to the aqueous solution. Background conversions of TMB due to liposome instabilities or translocation through the membrane in absence of FhuA
Table 1: Average TMB conversions in liposomes.

| FhuA Δ1-160 variant reconstituted in liposomes | Position(s) of Lys→Ala Substitution(s) | TMB conversion [nM]/s | *True averaged TMB conversion [nM]/s | **TMB conversion ratio |
|-------------------------------------------------|--------------------------------------|-----------------------|--------------------------------------|------------------------|
| Controls                                         |                                      |                       |                                      |                        |
| Lacking FhuA Δ1-160                              |                                      | 13 ± 2                |                                      | 1                      |
| Unlabeled FhuA Δ1-160                            |                                      | 102 ± 5               | 89                                   | 7.9                    |
| Fully labeled FhuA Δ1-160 starting variant        |                                      | 15 ± 4                | 2                                    | 1.2                    |
| Subset A                                         |                                      |                       |                                      |                        |
| K167A                                            | 167                                  | 59 ± 2                | 46                                   | 4.5                    |
| K344A                                            | 344                                  | 52 ± 1                | 39                                   | 4                      |
| K364A                                            | 364                                  | 20 ± 3                | 7                                    | 1.5                    |
| K537A                                            | 537                                  | 76 ± 3                | 63                                   | 5.9                    |
| K556A                                            | 556                                  | 97 ± 4                | 84                                   | 7.5                    |
| K586A                                            | 586                                  | 14 ± 1                | 1                                    | 1.1                    |
| Subset B                                         |                                      |                       |                                      |                        |
| K167                                            | 344, 364, 537, 556, 586               | 22 ± 2                | 9                                    | 1.7                    |
| K344                                            | 167, 364, 537, 556, 586               | 23 ± 1                | 10                                   | 1.8                    |
| K364                                            | 167, 344, 537, 556, 586               | 30 ± 3                | 17                                   | 2.3                    |
| K537                                            | 167, 344, 364, 556, 586               | 21 ± 3                | 8                                    | 1.6                    |
| K556                                            | 167, 344, 364, 537, 586               | 16 ± 1                | 3                                    | 1.2                    |
| K586                                            | 167, 344, 364, 537, 556, 586          | 35 ± 2                | 22                                   | 2.7                    |

Lambert Beer law was used with an extinction coefficient of 3.9 × 10⁻⁴ M⁻¹ cm⁻¹ for the first TMB oxidation product. Two subsets (A & B) of FhuA Δ1-160 variants were apart from controls analyzed. FhuA Δ1-160 variants of subset A contain a single lysine to alanine substitution while subset B contain five lysine to alanine substitutions. All FhuA Δ1-160 variants are pyridyl-labeled except two controls (liposome lacking FhuA Δ1-160 and the unlabeled FhuA Δ1-160). ***: The true TMB conversion is calculated from the TMB-conversion of FhuA Δ1-160 variant subtracted by the TMB conversion of the background lacking FhuA Δ1-160; ****: TMB conversion ratio represents a ratio between TMB conversions of pyridyl-labeled FhuA Δ1-160 variants and the liposome control lacking FhuA Δ1-160.

Δ1-160 were determined to be 13 [nM]/s (Table 1). Further control experiments were based on liposomes harboring unlabeled FhuA Δ1-160 and the fully pyridyl-labeled FhuA Δ1-160 (starting variant). A TMB conversion of 102 [nM]/s (unlabeled FhuA Δ1-160) and 15 [nM]/s (pyridyl-labeled FhuA Δ1-160; starting variant) were reached, upon optimizing liposome preparation, and the TMB assay. The 7.9-fold higher TMB conversion in the unlabeled FhuA Δ1-160 translates an excellent detection system to monitor differences in TMB translocation through the twelve FhuA Δ1-160 variants.

TMB conversion of the six subset A variants
The aa-position 556 has a major impact on TMB conversion: K556A substitution increases TMB conversion to 97 [nM]/s which is close to the value of the FhuA Δ1-160 unlabeled variant. A further TMB important blocking position is found by the substitution K537A increasing
TMB conversion to 76 [nM]/s. In summary the following order of increased TMB conversion has been observed for subset A variants: 586 < 364 < 344 < 167 < 537 < 556.

**TMB conversion of the six subset B) variants**

Subset B variants of FhuA Δ1-160 have in the inner channel only one labeled pyridyl-lysine. For pyridylated position 556, a reduction of the translocation to 16 [nM]/s was achieved. The latter proves impressively that a single labeled lysine can efficiently and independently from all other labeled lysines block TMB translocation through FhuA Δ1-160. For position 537 a cooperative effect can be observed since the subset B variant shows a significantly less pronounced TMB blocking as expected from the corresponding subset A variant. Similar to the subset A) variants the following increased TMB conversion has been observed for the subset B variants: 586 > 364 > 344 > 167 > 537 > 556.

Differences in the absolute values between the two experimental data sets can likely be attributed to pyridyl labeling efficiencies, *i.e.* inner channel sites have a lower probability to get labeled once lysines on the protein ores are labeled or when multiple sites are labeled (Additional file 1: Figure S3). Further experimental details on the TMB conversion calculations and controls (Additional file: Figure S1 and S2), CD-spectral measurements on secondary structure stability of FhuA Δ1-160 (Additional file 1: Figure S3), size measurements of liposomes (Additional file 1: Figure S4 and S5) and simulation details, can be found in the Additional file 1.

**Molecular Dynamics simulations to investigate the key modulating position 556**

A working hypothesis for controlling the compound flux in the inner FhuA Δ1-160 precisely is a defined and rigid conformation of the blocking lysine residue. Lysine fluctuations of all six FhuA Δ1-160 have been directly correlated to the B factors deduced from Molecular Dynamics MD trajectories in a first simulation (see Additional file 1). The B factor analysis indicates the dynamic mobility of an atom or group of atoms. The concept is derived from the X-ray scattering/crystallography theory, alternatively known as "temperature-factor" or "Debye-Waller factor" [18]. Table 1 suggests that the amino acid position 556 is a key residue in modulating the compound flux through the inner channel. Interestingly a general trend between low B factors values of the unlabeled FhuA Δ1-160 (Figure 3) and translocation importance (experimental results; Table 1), has been found. Again the most important position 556 is in the B factor analysis the least mobile one.
In detail, FhuA Δ1-160 is a β-barrel with a cross-section of 39 Å and 46 Å on the "top" part and a reduced cross-section on the "lower" exit of the barrel, 29 Å and 19 Å. K556 is placed in a rigid β-barrel at the "lower" cross-section (Figure 2) which originally interacts with the ferrichrome peptide and TonB protein [19] for further iron translocation. Other lysines are located on the opposite site K167, K537 and K586 (placed near the top cross-section) or underneath (K344; lower cross-section). As indicated by B values the positions K556, K167 and K537 have the higher blocking effects which are in accordance to the experimental results in corresponding subset A and B variants though no simulations on the pyridyl-labeled starting variant FhuA Δ1-160, has been performed (see Additional file 1 for further discussion).

In summary, experimental results and first computational simulations indicate that the rigidity of the labeled positions play an important role in generating FhuA Δ1-160 channels with a defined and "non-fluctuating" pore size. Fluctuations in pore sizes of FhuA Δ1-160 will reduce the discriminating power to control compound fluxes and are therefore an important prerequisite for a universal compound release system that can rapidly be re-engineered for a "time and dose-dependent" compound release.

Conclusions
Molecular understanding of the sterically controlled diffusion in FhuA Δ1-160’s inner channel is an important prerequisite to develop a universal compound release system that can rapidly be re-engineered for a "time and dose-dependent" compound release.

Six lysine residues were systematically analyzed in two subsets of engineered FhuA Δ1-160 channels. Analysis of 12 variants identified position K556 as a key substitution to sterically control compound fluxes through the inner channel of FhuA Δ1-160 embedded in liposome membrane. A first B-factor analysis based on MD simulations identified position K556 as the least fluctuating lysine among the six investigated lysines suggesting a correlation between flexibility and steric control of TMB compound translocation through the inner FhuA Δ1-160 channel. The subset B variant K556 of FhuA Δ1-160 represents therefore an excellent starting point to under-
stand channel dynamics and to sterically control compound flux through engineered FhuA Δ1-160. Based on these results it seems promising that the reduction triggered release system can be advanced to a universal and compound independent release system which allows a size selective compound release through rationally re-engineered FhuA Δ1-160 channels.

Methods

All chemicals used were of analytical reagent grade or higher quality, purchased from Sigma-Aldrich Chemie (Taufkirchen, Germany) and Applichem (Darmstadt, Germany) if not stated otherwise. A thermal cycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) and thin-wall PCR tubes (Multi-ultra tubes; 0,2 ml; Carl Roth, Karlsruhe, Germany) were used in all PCRs.

1. Site-directed mutagenesis

Six lysines located in the FhuA Δ1-160 channel were substituted by alanine using QuikChange (developed by Stratagene; La Jolla, CA, USA) [20] derived SDM protocol generating two subsets (A & B; Table 1) of FhuA Δ1-160 variants. Table 2 lists the primers employed for SDM. The SDM was performed by using a two-stage PCR protocol [21]: first stage: one cycle (95°C, 1 min), three cycles (95°C, 30 s; 55°C, 1 min; 68°C, 2 min) and a second stage: one cycle (95°C, 1 min), 15 cycles (95°C, 30 s; 55°C, 1 min; 68°C, 2 min) and one cycle (68°C, 25 min). In each reaction were employed template FhuA Δ1-160 (25 ng), a primer set (see Table 2; 200 nM each), dNTP mix (200 μM) and Pfu DNA polymerase (1 U) in Pfu reaction buffer (2 × 25 μl total volume, for stage one). In stage one for each primer the extension reaction is performed in a separate PCR tube and subsequently pooled for the stage 2 PCR. In Stage 2 additional Pfu DNA polymerase (0.02 U) is supplemented before starting the second PCR. For digestion of parental DNA, DpnI (10 U; 1 h, 37°C) is supplemented to the PCR mix. All 12 FhuA Δ1-160 variants were fully sequenced to assure lysine to alanine substitutions and lack of additional mutations. Amount of DNA after PCR was quantified using a NanoDrop photometer (NanoDrop Technologies, Waltham, Massachusetts, USA).

2. Expression, extraction and purification of FhuA variants

FhuA Δ1-160 variants were expressed, extracted and purified as previously described [1] with several modifi-
pPR1-FhuA Δ1-160 plasmid is freshly transformed into the expression host *Escherichia coli* BL strain BL 21 (DE3) omp8 (F’ *hsdS*<sup>B</sup> *m<sup>R</sup>* gal *ompT dcm* (DE3) Δ*λ*ompF::Tn5 ΔompA ΔompC) [22]. An overnight culture (TY media, 25 ml) [12] was prepared and used to inoculate expression media (inoculate 20 ml; TY medium, 250 ml) for FhuA Δ1-160 production (1-L shaking flask; 250 rpm, 37°C, 70°C humidity//Infors HT Multitron, Bottmingen, Switzerland). When the OD<sub>578</sub> reached 0.7, FhuA Δ1-160 protein expression was induced with IPTG (final concentration of 1 mM). Cells were grown (37°C) until the OD<sub>578</sub> reached 2.0-2.5 and harvested (20 min, 3220 rcf, 4°C//Eppendorf 5810R; Hamburg, Germany). Cells were resuspended in lysis buffer (12 ml; pH 8.0, 20 mM Tris, 2.5 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1 mM PMSF), cooled on ice and disrupted by passing through a high-pressure homogenizer (3×, 2000 bar//Emulsiflex-C3, Avestin Inc., Ottawa, Canada). The disrupted cell suspension was mixed with FhuA Δ1-160 extraction buffer (1 ml; pH 8.0, 20 mM phosphate buffer, 2.5 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 20% Triton X-100) and incubated (1 h, 100 rpm, 37°C//Infors HT Multitron, Bottmingen, Switzerland). The outer membrane fractions were isolated by centrifugation (45 min, 39,700 rcf, 4°C//Avanti J-20XP, Beckman Coulter, Fullerton, USA) and resuspended in pre-solubilization buffer (9 ml; pH 8.0, 20 mM phosphate buffer, 1 mM EDTA, 0.1% oPOE, 1 mM PMSF) [23]. The resuspended outer membrane fractions were subjected to a further incubation (1 h, 200 rpm, 37°C//Infors HT Multitron, Bottmingen, Switzerland) and were subsequently isolated by centrifugation (45 min, 109,760 rcf, 4°C//Beckman Optima LE-80K Ultracentrifuge, Fullerton, USA). In the final step the isolated pellet was resuspended in solubilization buffer (9 ml; pH 8.0, 20 mM phosphate, 1 mM EDTA, 3% oPOE, 1 mM PMSF) and membrane fractions were removed by centrifugation (45 min, 109,760 rcf, 4°C//Avanti J-20XP, Beckman Coulter, Fullerton, USA). The supernatant containing FhuA Δ1-160 was concentrated using ultra-filtration (20 min, 3220 rcf, RT//Eppendorf 5810R Centricon YM30; Millipore, Bedford, USA). Purity of extracted fractions was controlled by protein gel electrophoresis and comparable to previously reported values [24]. Protein concentrations were determined using the standard BCA kit (Pierce Chemical Co, Rockford, USA).

| Sequence Name | Sequence 5’ to 3’ |
|---------------|-------------------|
| FhuA Δ1-160 K167A Fwd | CCGCTGAAAGAAGTTCAGTTTGCGGCCGGTACTGACAGCC |
| FhuA Δ1-160 K167A Rev | GGCTGTCAGTACCGGCCGCAAACTGAACTTCTTTCAGCGG |
| FhuA Δ1-160 K344A Fwd | GGCCATTATCTGGGCACGTGCTAGCTGTTGATGATGAGAAG |
| FhuA Δ1-160 K344A Rev | CTTCTCATCATTACGACCAGTCAGCTGCAACTTGGATATG |
| FhuA Δ1-160 K364A Fwd | GATACCCAGTTGCAAGCGCCCGGTTCACCTGCAACTTGGATATG |
| FhuA Δ1-160 K364A Rev | CGATATCGCCAGTTGCAAAACCGGCTCTGGCAACTTGGATATG |
| FhuA Δ1-160 K537A Fwd | GCAGTATGAAGTCGCTGCGTGTGATGATGAGAAG |
| FhuA Δ1-160 K537A Rev | CGATCTTCCCCGATTACGACCGGACTTCAATCTG |
| FhuA Δ1-160 K556A Fwd | GCCGTGTATAATCTCAGTGGCAACCGAACCCTGATGGCCG |
| FhuA Δ1-160 K556A Rev | CGCCATCATGATGTGGATGTGGACAGAATTATACACCGG |
| FhuA Δ1-160 K586A Fwd | CGTAGAAATCGAAGCGGGCGGCAGCAGCTGGTCGCGAG |
| FhuA Δ1-160 K586A Rev | CTCGCGGACAGCGCGCGCGCGCCGCTGGTCGCGAG |
3. FhuA Δ1-160 labeling and nanocompartment formation

DMSO containing 3-(2-pyridyldithio) propionic acid N-hydroxysuccinimide ester (250 μl, 38 mM) was added drop-wise into FhuA Δ1-160 (750 μl, 4.3 μM) in phosphate buffer (pH 7.4, 0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄, 3% oPOE) and stirred (1 h, 3000 rpm, RT//RCT basic IKAMAG, IKA-Werke GmbH, Staufen, Germany). Final concentration of DMSO and oPOE in the solution was 25% and 1.5%, respectively. The latter solution was used for formation of nanocompartments loaded with HRP (2.9 U/ml).

4. Liposome preparation methodology

The film hydration method coupled with the mechanical dispersion technique by filter extrusion was used [25] for liposome preparation. Conventional methods of liposome production involves three basic stages: drying of the lipid solution from organic solvents, dispersion of lipids into the aqueous media, homogenization, and purification of the resultant liposomes with subsequent analysis of the final product [26]. E. coli total lipid extract (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA) is a chloroform extract of the respective tissue. A mixture of E. coli total lipid extract (500 μl, 10 mg) and methanol (1:1, v/v) were used to form a thin lipid film on round-bottom flask under reduced pressure by using a rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). The aqueous solution containing phosphate buffer (pH 7.4, 0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄), FhuA Δ1-160 (3.2 μM final concentration) and HRP (2.9 U/ml) for entrapment in the interior of the vesicles was supplemented and the thin lipid film was hydrated overnight in a 30°C water bath. Nanocompartments encapsulating HRP, harboring FhuA Δ1-160 as well as amino group labeled FhuA Δ1-160 were extruded using Avanti Lipid 1 ml syringes (Alabaster, Alabama, USA), an Avanti Lipid extrusion apparatus (Alabaster, Alabama, USA) and a Bibby Heating block (Staffordshire, UK). Three polycarbonate membranes (Millipore Corporation, Bedford, MA, USA) with pore sizes of 1 μm, 0.4 μm and 0.2 μm were used with the extrusion equipment in a sequential manner to form uniform spherically shaped nanocompartments [27]. Nanocompartments were purified by gel filtration using Sepharose 4B (Fluka, Cat. no. 84962) in phosphate buffer (pH 7.4, 0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄). Average diameters of nanocompartments were routinely determined using a Zeta-Sizer (Zeta-Sizer Nano Series; Malvern, Worcestershire, UK//Additional file 1: Figure S5).

5. TMB assay with nanocompartments

TMB (Sigma Cat. N°: T 0440) assay was selected as a conversion reporter system. Pre-prepared TMB/H₂O₂ solution were used in the kinetic measurement of the TMB oxidation by the HRP [13,14]. The oxidation of TMB by the HRP/H₂O₂ system yields a blue and subsequently a yellow colored reaction product. Initial TMB oxidation kinetics are quantified by measuring an absorption maximum at 370 nm [14] using a microtitre plate reader (Omega Series; BMG LABTECH; Offenburg, Germany). TMB solution (10 μl) was supplemented to a 100 μl dispersion consisting of purified nanocompartments (in phosphate buffer, pH 7.4, 0.2 M Na₂HPO₄, 0.2 M NaH₂PO₄). Detailed kinetic results of the TMB assay of FhuA Δ1-160 variants are available in Additional file 1: Figure S1.

Additional material

Additional file 1 Molecular understanding of sterically controlled compound release through an engineered channel protein (FhuA)

Additional file 1 contains a summary of kinetic data for TMB diffusion and experimental details on TMB conversion calculations and controls. Furthermore CD spectral measurements on FhuA Δ1-160 secondary structure stability, simulation details and size measurements of liposomes are presented.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

AG carried out design, study and drafting of the manuscript. MF and BH performed data analysis and drafting the manuscript. US carried out design, study and drafting of the manuscript. All authors read and approved the final manuscript.

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