Reversible photo-controlled nanopore assembly

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**General information**

All chemicals for synthesis were obtained from commercial sources and used as received unless stated otherwise. DNA was purchased from Integrated DNA Technologies (IDT). Enzymes were acquired from Fermentas and lipids from Avanti Polar Lipids. Technical grade solvents were used for extraction and chromatography. Thin Layer Chromatography (TLC) was performed using commercial Kiesegel 60 F254 silica gel plates with fluorescence-indicator UV254 (Merck, TLC silica gel 60 F254) for normal phase and Nano-Silica gel RP-18W on Alu-foil (Sigma Aldrich) for reversed phase. For detection of components, UV light at \( \lambda = 254 \) nm or \( \lambda = 365 \) nm was used. Alternatively, oxidative staining was performed using a basic solution of potassium permanganate in water or aqueous cerium phosphomolybdic acid solution (Seebach’s stain). Merck silica gel 60 (230–400 mesh ASTM) was used in normal phase flash chromatography. Reveliceris® X2 automatic column was used with Reveliceris® C18 40 µm columns for reversed phase purification. Preparative HPLC purification was performed on a Shimadzu HPLC system with a Phenomenex® Kinetex 5 µm EVO C18 100Å column.

Spectroscopic measurements were made in Uvasol® grade solvents using a quartz cuvette (path length 10.00 mm). UV-Vis measurements were performed on an Agilent 8453 UV-Visible absorption Spectrophotometer. For biological experiments a Synergy H1 plate-reader was used (BioTek). UV-Vis irradiation experiments were carried out using a 365 nm LED (Thorlabs M365F1) and a white light source (Thorlabs Fiber Illuminator OSL1-EC).

NMR spectra were obtained using Agilent Technologies 400-MR (400/54 Premium Shielded) (400 MHz) and Bruker Innova (1H: 600 MHz, 13C: 151 MHz) spectrometers at room temperature (22-24 °C). Chemical shift values (\( \delta \)) are reported in parts per million (ppm) with the solvent resonance as the internal standard (CDCl\(_3\): \( \delta \) 7.26 for \(^1\)H, \( \delta \) 77.16 for \(^13\)C; DMSO: \( \delta \) 2.05 for \(^1\)H, \( \delta \) 39.52 for \(^13\)C). The following abbreviations are used to indicate signal multiplicity: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), brs (broad signal) or dd (doublet of doublets).

Exact mass spectra were recorded on an LTQ Orbitrap XL (ESI+). All reactions requiring an inert atmosphere were carried out under a nitrogen atmosphere using oven dried glassware and standard Schlenk techniques. Dichloromethane and toluene were used from solvent purification system using an MBrAun SPS-800 column. Melting points were determined using Stuart analogue capillary melting point SMP\(_{11}\) apparatus. All errors are given as standard deviations.
Chemical Synthesis

Switch A

\[
\begin{align*}
\text{(E)-2-chloro-N-(4-(phenyldiazenyl)phenyl)acetamide (Switch A)}
\end{align*}
\]

Compound 1 (0.30 mmol, 60 mg) was dissolved in dry dichloromethane (DCM) (8 mL) and the solution cooled to 0 °C. Triethylamine (1.0 eq, 0.30 mmol, 42 µL) was added and the solution was stirred vigorously. Chloroacetyl chloride (1.0 eq, 0.30 mmol, 35 mg) was dissolved in dry DCM (8 mL) and cooled to 0 °C prior to addition and subsequently the reaction mixture was left to stir and warm up to room temperature overnight. The mixture was quenched with water and extracted with DCM (3x 8 mL). The organic layers were washed with 10% aq. HCl and water, dried over MgSO\(_4\) and the solvent was evaporated. The product was purified by flash chromatography on silica gel (hexane : EtOAc = 6:4) to provide 77 mg of product A as a orange solid (yield = 80 %). \textbf{Mp.} 150-153 °C. \textbf{\(^1\)H NMR (600 MHz, DMSO-\textit{d}6))} \delta 10.65 (s, 1H), 7.93 – 7.81 (m, 6H), 7.59 (t, \(J = 7.0\) Hz, 2H), 7.55 (t, \(J = 7.9\) Hz, 1H), 4.32 (s, 2H). \textbf{\(^{13}\)C NMR (600 MHz, DMSO-\textit{d}6))} \delta 165.1, 152.0, 147.9, 141.5, 131.2, 129.4, 123.7, 122.4, 119.6, 43.6. \textbf{HRMS (ESI+)} calc for C\(_{14}\)H\(_{12}\)ClN\(_3\)OH: 274.0742, found 274.0752. The spectroscopic data is in accordance with the literature.\(^1\)
Figure S1. $^1$H NMR (DMSO-$d_6$) spectrum of A:

Figure S2. $^{13}$C NMR (DMSO-$d_6$) spectrum of A:
Switch B

1-nitro-4-nitrosobenzene (3)\textsuperscript{2,3}

\[ \text{p-Nitroaniline (2) (1.5 mmol, 200 mg) was dissolved in DCM (2 mL). Oxone}^{\circledast} (1.95 eq, 5.64 mmol, 1.74 g) was dissolved in water (8 mL) and subsequently added to the aniline solution. The reaction mixture was stirred vigorously overnight at room temperature. The mixture was extracted with DCM, the organic phase was washed with 1M aq. HCl solution, dried over MgSO}_{4} and the solvent was evaporated. The product was purified by flash column chromatography on silica gel (hexane:EtOAc = 5:1) to provide 240 mg of product 3 as a brown solid (yield = 98%). \textbf{Mp.} 126-127 °C. \textbf{1H NMR} (600 MHz, CDCl}_{3} \delta 8.05 (d, \textit{J}=8.79 Hz, 1H), 8.51 (d, \textit{J}=8.75 Hz, 1H). The spectroscopic data is in accordance with the literature.\textsuperscript{2,3} \]

\textbf{(E)-1-(4-methoxyphenyl)-2-(4-nitrophenyl)diazene (4)}\textsuperscript{2,3}

\[ \text{Compound 3 (1.5 mmol, 230 mg) and p-methoxyaniline (1.0 eq, 1.5 mmol, 190 mg) were dissolved in glacial acetic acid (14 mL) and the reaction mixture was stirred overnight at room temperature. The crude mixture was extracted with hexane, the organic phase was washed with aqueous NaHCO}_{3} and dried over MgSO}_{4}. The product was purified by flash column chromatography to yield 380 mg of azobenzene 4 as a brown solid (yield = 70%). \textbf{Mp.} 154 °C. \textbf{1H NMR} (600 MHz, CDCl}_{3} \delta 3.93 (s, 3H), 7.05 (d, \textit{J}=8.96 Hz, 2H), 7.99 (m, 4H), 8.37 (d, \textit{J}=8.97 Hz, 2H). The spectroscopic data is in accordance with the literature.\textsuperscript{2,3} \]

\textbf{(E)-4-((4-methoxyphenyl)diazenyl)aniline (5)}\textsuperscript{4}

\[ \text{Compound 4 (0.4 mmol, 100 mg) was dissolved in methanol (5 ml) and palladium on carbon (5 mol%, 0.02 mmol, 5 mg, 10 wt%) was added. Hydrazine (20 eq, 8 mmol, 0.4 mL) was added dropwise to the solution. An open condenser was attached to a three-neck flask used as reaction} \]
vessel. The heterogeneous orange reaction mixture was left to stir at 40 °C for 3 h and with time became dark brown. The solvent was removed on the rotary evaporator to obtain a green solid. Product 5 was purified by flash column chromatography to provide 60 mg as a orange solid (yield = 60%). \textbf{Mp.}140-142 °C. \textbf{1H NMR} (400 MHz, CDCl$_3$) $\delta$ 7.85 (d, $J=8.9$ Hz, 2H), 7.77 (d, $J=8.6$ Hz, 2H), 6.99 (d, $J=8.9$ Hz, 2H), 6.74 (d, $J=8.6$ Hz, 2H), 4.07 (bs, 2H), 3.88 (s, 3H). The spectroscopic data is in accordance with the literature.\textsuperscript{4}

\textbf{(E)-2-chloro-N-((4-methoxyphenyl)diazenyl)phenyl)acetamide (Switch B)}

Azobenzene 5 (0.13 mmol, 29 mg) and trimethylamine (1 eq, 0.13 mmol, 17 µL) were dissolved in dry DCM (3.3 mL) and the mixture stirred at 0 °C. Chloroacetyl chloride (1 eq, 0.13 mmol, 10 µL) was dissolved in dry DCM (3.3 mL) and added to the reaction mixture. The pale-yellow reaction mixture changed color to dark orange upon addition. The mixture was stirred for 3.5 h at rt. Subsequently water was added to quench the reaction and the product was extracted with DCM, the organic solution washed with 1 M aq. HCl and water and dried over MgSO$_4$. The crude product was purified by flash column chromatography on silica gel to yield 30 mg of product B as a orange solid (yield = 70%). \textbf{Mp.} 166-170 °C. \textbf{1H NMR} (600 MHz, DMSO-$d_6$) $\delta$ 10.61 (s, 1H), 7.89 – 7.85 (m, 4H), 7.80 (d, $J=8.9$ Hz, 2H), 7.14 (d, $J=9.0$ Hz, 2H), 4.31 (s, 2H), 3.87 (s, 3H). \textbf{13C NMR} (600MHz, DMSO-$d_6$) $\delta$ 165.0, 161.7, 148.0, 146.2, 140.8, 124.3, 123.3, 119.6, 114.6, 55.6, 43.6. \textbf{HRMS} (ESI+) calc for C$_{15}$H$_{14}$ClN$_3$O$_2$H: 304.0847, found 304.0862.
Figure S3. $^1$H NMR (DMSO-$d_6$) spectrum of B:

Figure S4. $^{13}$C NMR (DMSO-$d_6$) spectrum of B:
Switch C

\[ \text{(E)-4-((4-(2-chloroacetamido)phenyl)diazenyl)benzenesulfonic acid (Switch C)} \]

A mixture of azobenzene 6 (0.7 mmol, 200 mg) and chloroacetic anhydride (18 eq, 12 mmol, 2.1 g) was heated up to 90 °C and left to stir overnight in a Schlenk flask. The solution was diluted with DCM and the solid was formed and separated by centrifugation. The crude product was purified by reversed phase column chromatography on C18 silica (water : acetonitrile = 9:1) to provide 240 mg of product C as an orange solid (yield = 95%). Mp. > 250 °C. \(^1\)H NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 10.66 (s, 1H), 7.93 (d, \(J = 8.9 \text{ Hz}\), 2H), 7.85 – 7.81 (m, 3H), 7.78 (d, \(J = 8.5 \text{ Hz}\), 2H), 4.32 (s, 2H). \(^{13}\)C NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 165.6, 152.1, 151.2, 148.4, 142.0, 127.2, 124.3, 122.4, 120.1, 44.1. HRMS (ESI-) calc for C\(_{14}\)H\(_{11}\)ClN\(_3\)O\(_4\)S: 352.0153, found 352.0169. The spectroscopic data is in accordance with the literature.\(^5\)
Figure S5 $^1$H NMR (DMSO-$d_6$) spectrum of C:

Figure S6 $^{13}$C NMR (DMSO-$d_6$) spectrum of C:
(E)-N-(4-((4-hydroxyphenyl)diazenyl)phenyl)acetamide (8)\textsuperscript{6}

4-Amino-acetaniline (1.00 g, 6.59 mmol, 1.04 eq) was dissolved in ice-cold 6 M HCl (aq) (7 mL) and subsequently added dropwise to a solution of NaNO\textsubscript{2} (470 mg, 6.81 mmol, 1.02 eq) in water (1.5 mL), to which a few crystals of urea were added. The resulting brown mixture was slowly added to ice-cold solution of phenol (610 mg, 6.48 mmol, 1.0 eq) and NaOH (1.26 g, 31.5 mmol, 4.7 eq) in water (4.5 mL). The mixture was stirred for 2.5 h, left to warm up to room temperature and acidified with 6 M HCl to pH = 6. The precipitate was filtered off with cold water and dried to provide 1.18 g of compound 8 as a brown solid (yield = 71\%). \textbf{Mp.} 195 °C. \textbf{\textsuperscript{1}H NMR (400 MHz, DMSO-\textit{d}_6)} δ 10.20 (s, NH), 7.72-7.76 (m, 6H), 6.90 (d, J = 8.79 Hz, 2H), 2.07 (s, 3H). The spectroscopic data is in accordance with the literature.\textsuperscript{6}

(E)-N-(4-((4-(4-bromobutoxy)phenyl)diazenyl)phenyl)acetamide (9)\textsuperscript{6}

Compound 8 (500 mg, 1.96 mmol), 1,4-dibromobutane (3.92 mL, 32.8 mmol, 17 eq), potassium carbonate (540
mg, 3.91 mmol, 2 eq) and potassium iodide (69.8 mg, 0.420 mmol, 0.1 eq) were dissolved in acetone (20 mL) and the mixture heated under reflux for 3.5 h. After the reaction mixture cooled down, it was diluted with EtOAc (200 mL). The solution was washed with brine (2 x 200 mL), dried over MgSO\(_4\) and the solvent was evaporated. The resulting solid was dissolved in minimum amount of EtOAc to be precipitated by addition of pentane. The solid was filtered and dried to yield 660 mg of compound 9 as an orange solid (yield = 86%). **Mp.** 172-174 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.24 (s, NH), 7.76-7.85 (m, 6H), 7.11 (d, \(J = 8.84\) Hz, 2H), 4.12 (t, \(J = 6.2\) Hz, 2H), 3.63 (t, \(J = 5.89\) Hz, 2H), 2.09 (s, 3H), 2.03-1.96 (m, 2H), 1.93-1.84 (m, 2H). The spectroscopic data is in accordance with the literature.\(^6\)

\(\text{(E)-4-[(4-acetamidophenyl)diazenyl]phenoxy-N,N,N-Trimethylbutan-1-aminium chloride (10)}\)

Azobenzene 9 (250 mg, 0.641 mmol) was dissolved in trimethylamine (4.2 M in EtOH, 15 mL) in a pressure tube and left to stir for 2 d at 50 °C. The vial was cooled down and diethyl ether was added (250 mL). The precipitate was filtered off and washed several times with diethyl ether to provide 230 mg compound 10 as a yellow solid (yield = 97%). **Mp.** >250 °C. \(^1\)H NMR (400 MHz, DMSO-\(d_6\)) \(\delta\) 10.27 (s, NH), 7.87-7.76 (m, 6H), 7.13 (d, \(J = 8.92\) Hz, 2H), 4.14 (t, \(J = 5.94\) Hz, 2H), 3.41-3.36 (m, 2H), 3.07 (s, 9H), 2.10 (s, 3H), 1.93-1.84 (m, 2H), 1.83-1.74 (m, 2H). \(^{13}\)C NMR (600 MHz, DMSO-\(d_6\)) \(\delta\) 168.7, 160.8, 147.4, 146.3, 141.8, 124.2, 123.2, 119.1, 115.0, 67.2, 65.0, 52.2, 25.5, 24.1, 19.2. **HRMS** (ESI+) calc for \(\text{C}_{14}\text{H}_{11}\text{ClN}_3\text{O}_4\): 369.2285, found 369.2288.
Figure S7. $^1$H NMR (DMSO-$d_6$) spectrum of 10:

![H NMR spectrum of 10]

Figure S8. $^{13}$C NMR (DMSO-$d_6$) spectrum of 10:

![C NMR spectrum of 10]
(E)-4-(4-((4-aminophenyl)diazenyl)phenoxy)-N,N,N-trimethylbutan-1-aminium chloride (11)

Compound 10 (200 mg, 0.541 mmol) was dissolved in conc. HCl (15 mL) and left to stir at 40 °C overnight. HCl was evaporated with multiple additions of water and finally the reaction mixture, diluted with water, was freeze-dried. Compound 11 (180 mg) was obtained as a pale yellow solid (quant. yield). **Mp. >250 °C.**

$^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 7.79 (d, $J = 8.86$ Hz, 2H), 7.71 (d, $J = 8.64$ Hz, 2H), 7.10 (d, $J = 8.93$ Hz, 2H), 6.93 (d, $J = 8.47$ Hz, 2H), 4.12 (t, $J = 5.93$, 2H), 3.42-3.37 (m, 2H), 3.08 (s, 9H), 1.91-1.84 (m, 2H), 1.81-1.74 (m, 2H).

$^{13}$C NMR (600 MHz, DMSO-d$_6$) $\delta$ 160.2, 148.7, 146.5, 146.3, 143.2, 126.1, 124.4, 123.8, 123.6, 114.9, 114.7, 67.09, 65.0, 52.2, 25.6, 19.2. HRMS (ESI+) calc for C$_{19}$H$_{27}$N$_4$O: 327.2179, found 327.2181. Compound 11 was used without further purification in the next step.

(11)

(E)-4-(4-((4-(2-chloroacetamido)phenyl)diazenyl)phenoxy)-N,N,N-trimethylbutan-1-aminium chloride (Switch D)

Compound 11 (150 mg, 0.46 mmol) was mixed with chloroacetic anhydride (450 mg, 2.63 mmol, 5.7 eq) in a Schlenck tube and heated to 87 °C under N$_2$ for 4 h. After cooling the obtained sticky brown solid was washed five times with cold ether by using centrifugation to separate the solid from excess chloroacetic anhydride. The obtained product was dissolved in water and freeze-dried to acquire 150 mg of compound D as a pale yellow solid (yield = 81%). **Mp. >250 °C.**

$^1$H NMR (600 MHz, DMSO-d$_6$) $\delta$ 10.73 (s, 1H), 7.78-7.85 (m, 4H), 7.82 (d, $J = 8.93$ Hz, 2H), 7.15 (d, $J = 9$ Hz, 2H), 4.33 (s, 2H), 4.19-4.14 (m, 2H), 3.41-3.38 (m, 2H), 3.08 (s, 9H), 1.92-1.86 (m, 2H), 1.78-1.86 (m, 2H).

$^{13}$C NMR (600 MHz, DMSO-d$_6$) $\delta$ 168.7, 162.1, 152.6, 146.7, 138.8, 124.8, 123.8, 120.1, 115.7, 67.8, 65.4, 52.8, 44.1, 26.0, 19.7. HRMS (ESI+) calc for C$_{21}$H$_{25}$ClN$_4$O$_2$: 403.1895, found 403.1895.
Figure S9. $^1$H NMR (DMSO-$d_6$) spectrum of D:

Figure S10. $^{13}$C NMR (DMSO-$d_6$) spectrum of D:
Switch D was observed as two species present in the NMR (DMSO-d₆), however when the sample was analyzed on the LCMS it clearly showed a single compound was present having a mass fitting switch D (Figure S12). Therefore, we suspected that switch D exists as either: 1) keto-enol equilibrium of the methylene amide moiety, 2) a pair of isomers due to the amide tautomerisation, 3) we observe the rotamers around the amide bond or 4) the second species is observed due to aggregation.

1) **Keto-enol equilibrium of the amide moiety.** The keto-enol tautomerization was excluded based on the ¹H NMR spectroscopy experiment where a small amount of D₂O was added to a solution of switch D in DMSO-d₆ and left overnight. Disappearance of the methylene peak (the carbon connected to the carbonyl group) was expected as substitution of protons with deuterium from water occurs during tautomerization. There was no such phenomenon observed, therefore concluding tautomerism is not observed for switch D.

2) **Amide tautomerization.** Observation of the imidic acid tautomer is possible due to the signals observed in the 2D ROESY NMR spectrum (Figure S15). The methylene peak of one species interacts with its amide proton through space while for the other species, which does
not show an amide proton, there is a cross-peak for the methylene and the closest aromatic protons (Figure S15). Nevertheless, this observation could also indicate rotamers.

3) Rotamers of amide. See above.

4) Aggregation. Since we observe a strong dependence of the species ratio on the concentration (Figure S13), we cannot dismiss the effect of aggregation. However, the DOSY experiment (Figure S16) does not show significant difference in rate of diffusion for the two observed species.

A significant solvent dependence (Figure S20) additionally supports the presence of tautomerism, isomerism to imidic acid as well as rotamers and aggregation.

Hoping the two species would reach coalescence (both for the tautomer, isomer and rotamer), the sample was studied at different temperatures. Upon heating to 50 and 80 °C, only a slight change in ratio of the two species was observed. Degradation of the sample was also observed which could have caused the change of relative ratio of the two species. This indicates that the exchange, if it is present, is too slow to be observed by NMR spectroscopy even at elevated temperatures. Furthermore, in the attempt to observe exchange by magnetization transfer in NMR spectroscopy, the Hoffman-Forsen experiment (Saturation Transfer difference spectroscopy) was performed. In a 1D ROESY NMR experiment, a selective pulse is applied to a selected peak and then the magnetization of peaks that are in exchange with the pulsed peak will absorb the magnetization and change the orientation. However, no exchange was observed for switch D at room temperature, as well as after heating to 80 °C. Therefore, we can only conclude that the exchange, if present, is extremely slow and not observable with magnetization transfer studies with NMR spectroscopy.
Figure S12. A) LCMS traces (ESI+, UV 360 nm) of switch D with water (0.1% formic acid (FA)): acetonitrile (0.1 % FA) as eluent. B) MS traces of the major peak with retention time 12.47 min. C) MS traces of the minor peak with retention time 12.83 min.
Figure S13. Concentration study of D behavior by $^1$H NMR spectroscopy in DMSO-$d_6$ at rt.

Figure S14. Temperature studies of D behavior by $^1$H NMR spectroscopy in DMSO-$d_6$ (74.4 mM).
Figure S15. ROESY NMR of D in DMSO-$d_6$, 74.4 mM at rt.

Figure S16. DOSY NMR of D in DMSO-$d_6$, 74.4 mM at rt.
Figure S17. COSY NMR of D in DMSO-$d_6$, 74.4 mM at rt.

Figure S18. HMBC NMR of D in DMSO-$d_6$, 74.4 mM at rt.
Figure S19. TOSY NMR of D in DMSO-\textit{d}_6, 74.4 mM at rt.

Figure S20. Solvent dependence study of D behavior by \textsuperscript{1}H NMR spectroscopy in A) DMSO-\textit{d}_6, B) D\textsubscript{2}O, C) MeOD, D) acetone-\textit{d}_6 at low concentrations and rt.
**Cloning of cysteine mutants of FraC**

S-FraC-Y138C was prepared by using 100 ng pT7-SC1 plasmid containing the WtFraC gene with the additional mutation W112S (S-FraC)\(^8\) as template for the PCR reaction. The gene was amplified by using REDTaq ReadyMix, 2 µM of Y138Cr and T7 promoter primers (Table S1) in 200 µL reaction volume. The PCR reaction cycling protocol was as following: a pre-incubation step at 98 °C for 90 sec was followed by 30 cycles of denaturation at 98 °C for 15 sec, annealing at 55 °C for 15 sec and extension at 72°C for 2 min. The PCR product was purified using QIAQuick PCR purification kit (Qiagen) and cloned into pT7-SC1 by the MEGAWHOP procedure\(^9\) using 300 ng pT7-SC1 plasmid containing S-FraC gene as template and 500 ng of the purified PCR product as primers. The amplification was carried out with Phire Hot Start II DNA polymerase (Finnzymes) in 50 µL final volume (pre-incubation at 98 °C for 30 sec, then 30 cycles of: denaturation at 98°C for 5 sec, extension at 72 °C for 1.5 min). The circular template was eliminated by incubation with Dpn I (1 FDU) for 2 h at 37 °C. Of the resulting mixture 0.6 µL was transformed into E. cloni® 10G cells (Lucigen) by electroporation. The transformed bacteria were grown overnight at 37 °C on ampicillin (100 µg/ml) containing lysogeny broth (LB) agar plates. The identity of the clones were confirmed by sequencing.

The other cysteine mutants of FraC were prepared as described above using the corresponding reverse primers (G13Cr, K77Cr, S112Cr, Q130Cr, E134Cr, G145Cr, N147Cr and S166Cr, see Table S1) for the first PCR reaction.

**Protein expression and purification**

*E. cloni® EXPRESS BL21 (DE3)* cells were transformed with the pT7-SC1 plasmid containing the appropriate FraC cysteine mutant gene. Transformants were selected after overnight growth at 37 °C on LB agar plates supplemented with 100 mg/L ampicillin. The resulting colonies were inoculated in 2x YT medium containing 100 mg/L of ampicillin. The culture was grown at 37 °C, with shaking at 200 rpm, until it reached an OD\(_{600}\) of ~0.6. The expression of protein was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside and the growth was continued at 20 °C. The next day the bacteria were harvested by centrifugation at 6000 xg for 30 min and pellets were stored at -80 °C.

The pellets containing overexpressed proteins were thawed and resuspended in 20 mL of 15 mM Tris.HCl pH 7.5, 150 mM NaCl, 4 M urea, 20 mM imidazole, 1 mM MgCl\(_2\) and 0.05 M Tris.HCl pH 7.5. The other cysteine mutants of FraC were prepared as described above using the corresponding reverse primers (G13Cr, K77Cr, S112Cr, Q130Cr, E134Cr, G145Cr, N147Cr and S166Cr, see Table S1) for the first PCR reaction.
units/mL of DNase I (Fermentas). Additionally, to avoid oxidation of the cysteine residues, 5 mM Tris(2-carboxyethyl)phosphin (TCEP) was added to the solution. The bacteria suspension was then supplemented with 0.2 mg/mL lysozyme to initiate cell disruption, followed by vigorous shaking at ambient temperature for 1 h. The remaining bacteria were disrupted by probe sonication. The crude lysates were clarified by centrifugation at 6000 xg for 30 min and supernatant mixed with 200 µL (bead volume) of Ni-NTA resin (Qiagen) that was pre-equilibrated with wash buffer (20 mM imidazole, 150 mM NaCl, 15 mM Tris.HCl pH 7.5). After 45 min of gentle mixing at ambient temperature, the resin was loaded onto a column (Micro Bio Spin, Bio-Rad) and washed first with ~10 mL of wash buffer supplemented with 5 mM TCEP and then washed with ~10 mL of wash buffer without reducing agent. Immediately after washing, FraC monomers bound to the Ni-NTA resin were mixed with ~100-fold molar excess of azobenzene derivative, dissolved, 15 mM Tris.HCl pH 7.5, 150 mM NaCl in dimethyl sulfoxide (DMSO, final 20%) and the reaction was allowed to proceed at room temperature overnight. The following day the resin was washed again with ~10 mL of wash buffer supplemented with 5 mM TCEP to reduce unmodified cysteines and then washed with ~10 mL of wash buffer to remove the reducing agent and free azobenzene molecules. Then modified FraC was eluted with approximately 150 µl of 200 mM ethylenediaminetetraacetic acid (EDTA), 15 mM Tris.HCl pH 7.5 and 150 mM NaCl. To assess the extent of modification an aliquot of FraC-azobenzene and the unmodified FraC monomers were treated with 10x PEG-5000-maleimide. The reaction mixture was incubated for 3 h at room temperature and then analyzed by SDS-PAGE electrophoresis.

FraC monomers conjugated to the azobenzene derivative were separated from unmodified monomers by cation exchange chromatography. Modified monomer solutions were first dialyzed for 3 h against buffer A (50 mM Tris.HCl pH 9.5, 2 M urea and 5 mM β-mercaptoethanol, total volume 1500 mL, buffer replaced two times) to reduce the salt concentration and bring the pH to 9.5. Then the sample was applied to a HiTrap SP HP column (GE Healthcare Life Science) connected to Åkta pure chromatography system (GE Healthcare Life Science) equilibrated with buffer A. The protein was eluted with a gradient of buffer B (1 M NaCl and 50 mM Tris.HCl pH 9.5), starting with a step to 5 % and continuing with raising B from 5 to 40 % in 80 min, and then a final step to 100 %. Protein elution was monitored by measuring absorbance at 280 nm and 365 nm. While the first peak at 19.5 % B corresponds to FraC-Y138C-C (280 nm and 365 nm absorbance), the second peak correspond to unmodified FraC (only 280 nm absorbance). Collected fractions of the first peak were combined and
concentrated using Amicon Ultra Centrifugal Filters (10 kDa cut-off). Protein concentration was determined by Bradford assay. All monomers were stored at 4 °C until further use.

**Hemolytic activity assay**

Defibrinated sheep blood (ThermoFisher Scientific) was washed with 150 mM NaCl, 15 mM Tris.HCl pH 7.5 until the supernatant was clear. The erythrocytes were then resuspended with the same buffer to ~1% concentration (OD$_{650}$ 0.6 – 1.0). The suspension (120 μL) was then mixed with the solutions containing 0.3 nM – 11 µM toxin in cis or trans state. Photoisomerization of the azobenzene to the cis-state was achieved by illumination for 5 min with a UV lamp (Thorlabs model M365F1 LED, 365 nm, 4.1 mW). Isomerization back to trans-state was achieved by irradiation for 2 minutes with white light (Thorlabs OSL1-EC Fiber Illuminator, >450nm, 150 W halogen lamp, output 40000 foot-candles).

Hemolytic activity was measured by monitoring the decrease in OD$_{650}$ using a MultiskanTM GO Microplate spectrophotometer (ThermoFisher Scientific) or Synergy H1 Hybrid-Multimode reader (BioTek). Percentage of hemolysis was calculated as followed: % hemolysis = 100*(Abs$_{C~Frac}$-Abs$_{buffer}$)/( Abs$_{triton}$-Abs$_{buffer}$). Experiments were performed in the dark.

**Cell viability assay**

The cell viability assays were performed with the Human squamous carcinoma cell line A431 (Sigma-Aldrich). Detached cell number was determined by cell counting using an improved Neubauer chamber. The viability of cells was measured using (2-2-methoxy-4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) based cell viability assay included in the Cell Counting Kit-8 (CCK-8, Sigma-Aldrich) according to the manufactures instructions.$^{10,11}$

The human squamous carcinoma cell line A431 (Sigma-Aldrich) was routinely maintained at 37 °C, in a humidified incubator under 5% CO$_2$. A431 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM glutamine, 100 μg mL$^{-1}$ gentamycin.

A431 cells were first detached with 5 mL of 0.25 % Trypsin/EDTA solution, centrifuged at 400 xg for 5 minutes, and suspended in 10 mL growth medium, counted as described above and seeded 20.000 cells per well in 96-well tissue culture plates in 200 μL growth medium and
incubated overnight at 37 °C in 5 % CO$_2$ atmosphere. In various concentrations 10 µL toxin was distributed to the cells and incubated 2 hours at 37 °C in 5 % CO$_2$ atmosphere. Cells with only medium were included as control. Next, 5 µL CCK-8 solution was added to each well and the plates were incubated 2 hours before measuring absorbance at 450 nm using the Synergy H1 Hybrid-Multimode reader (BioTek). Experiments were performed in the dark. Results are shown as percentage of control cells and represented as average ± standard deviation of 3 independent experiments with triplicates.

**Electrical recordings in planar lipid bilayers**

The chamber used for electrophysiology experiments contained two compartments, which were separated by a 25 µm thick polytetrafluoroethylene film (Goodfellow Cambridge Limited) containing an orifice of ~100 µm in diameter. Each compartment contained 500 µL of buffer. The aperture was pretreated with ~5 µl of 10% hexadecane in pentane and a sphingomyelin free bilayer was formed by the addition of ~10 µL of 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC) in pentane (10 mg/mL) to both electrophysiology chambers. A potential, which was applied using silver/silver chloride electrodes, refers to the potential of the trans compartment / electrode. FraC nanopores were inserted into lipid bilayers from the cis compartment, which was connected to the ground electrode. The addition of monomeric FraC in a final concentration of 4 µM to the cis compartment was necessary to obtain channels. Electrical recordings were carried out in 1 M NaCl, 15 mM Tris.HCl pH 7.5. For irradiation a UV lamp (Thorlabs model M365F1 LED, 365 nm, 4.1 mW) and a white light (Thorlabs OSL1-EC Fiber Illuminator) were set up inside the Faraday cage.$^{12,13}$

**Data recording and analysis**

Electrical signals from planar bilayer recordings were amplified using an Axopatch 200B patch clamp amplifier (Axon Instruments) and digitized with a Digidata 1440 A/D converter (Axon Instruments). Data were recorded by using Clampex 10.6 software (Molecular Devices) and the subsequent analysis was carried out with Clampfit software (Molecular Devices). Electrical recordings were acquired by applying a 2 kHz low-pass Bessel filter and a 10 kHz sampling rate. Graphs were made with Origin (OriginLab Corporation) or Clampfit software (Molecular Devices).
Table S1. Primer table

<table>
<thead>
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<th>Name of primer</th>
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>S-FraC (protein sequence)

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NSYSNWWNVRYKQKRAIRQMMEEYHHYHRSPFRGDNGWHSRGGLGYGLKSRGM
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>S-FraC (DNA sequence)

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CGCATCATCACCACCACATCAGTA
Additional Figures

Figure S21. Photochemistry of switch A in DMSO. A) $^1$H NMR spectra before (top, trans-A) and after irradiation with UV light at $\lambda=365$ nm (bottom) (1.0 mM). B) UV-Vis absorption spectra before (purple, trans-A) and after irradiation with UV light at $\lambda=365$ nm (red) (20 µM). C) Reversible switching of A by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). D) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).
**Figure S22.** Photochemistry of switch B in DMSO. A) \(^1\)H NMR spectra before (top, trans-B) and after irradiation with UV light at \(\lambda=365\) nm (bottom) (2.0 mM). B) UV-Vis absorption spectra before (purple, trans-B) and after irradiation with UV light at \(\lambda=365\) nm (red) (20 \(\mu\)M). C) Reversible switching of B by cycles of irradiation first with UV light (\(\lambda=365\) nm, orange squares) and then with white light (lavender squares). D) Thermal cis-trans isomerization at 25 \(^\circ\)C (in red exponential fitting).

**Figure S23.** Photochemistry of switch C in DMSO. A) \(^1\)H NMR spectra before (top, trans-C) and after irradiation with UV light at \(\lambda=365\) nm (bottom) (6.0 mM). B) UV-Vis absorption spectra before (purple, trans-C) and after irradiation with UV light at \(\lambda=365\) nm (red) (20 \(\mu\)M). C) Reversible switching of C by cycles of irradiation first with UV light (\(\lambda=365\) nm, orange squares) and then with white light (lavender square). D) Thermal cis-trans isomerization at 25 \(^\circ\)C (in red exponential fitting).
Figure S24. Photochemistry of switch C in buffer (150 mM NaCl, 15 mM Tris.HCl, pH 7.5, 25 °C) A) UV-Vis absorption spectra before (dark blue, trans-C), during and after irradiation with UV light at $\lambda=365$ nm (red). B) Reversible switching of C by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). C) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).

Figure S25. Photochemistry of FraC-Y138C-C. FraC(Y138C)-switch C in buffer (150 mM NaCl, 15 mM Tris.HCl, pH 7.5, 25 °C). A) UV-Vis absorption spectra before (black, FraC-Y138C-trans-C), after irradiation with UV light at $\lambda=365$ nm (red) and after irradiation with white light ($\lambda>450$ nm). B) Reversible switching of FraC-Y138C-C by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). D) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).
Figure S26. Photochemistry of switch D in DMSO. A) $^1$H NMR spectra before (top, trans-D) and after irradiation with UV light at $\lambda=365$ nm (bottom) (4.14 mM). B) UV-Vis absorption spectra before (purple, trans-D) and after irradiation with UV light at $\lambda=365$ nm (red) (20 µM). C) Reversible switching of D by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). D) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).

Figure S27. Photochemistry of switch D in MeOH. A) $^1$H NMR spectra before (top, trans-D) and after irradiation with UV light at $\lambda=365$ nm (bottom) (4.12 mM). B) UV-Vis absorption spectra before (black, trans-D) and after irradiation with UV light at $\lambda=365$ nm (green) (20 µM). C) Reversible switching of D by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). D) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).
Figure S28. Photochemistry of switch D in buffer (150 mM NaCl, 15 mM Tris.HCl, pH 7.5, 25 °C) A) UV-Vis absorption spectra before (dark blue, trans-D), during and after irradiation with UV light at $\lambda=365$ nm (red). B) Reversible switching of D by cycles of irradiation first with UV light ($\lambda=365$ nm, orange squares) and then with white light (lavender square). C) Thermal cis-trans isomerization at 25 °C (in red exponential fitting).
Figure S29. Hemolytic activity of FraC-C constructs. A) Schematic representation of FraC (purple, PDB: 4TSY)\textsuperscript{14} bound to lipids (yellow). Amino acid residues G13, K77, Q130, E134, Y138 G145, N147 and S166 (highlighted as blue spheres) were mutated to cysteine in order to attach an azobenzene at different positions at the membrane interface. B to I) Hemolytic activity of FraC constructs with a substitute cysteine residue at the indicated position (black squares), the same construct modified with switch C (purple spheres), and after irradiation at 365 nm (red triangles). B and C) The introduction of a cysteine residue at position N147 and G145 reduced dramatically the hemolytic activity and attachment of the azobenzene resulted in an inactive toxin. Irradiation with UV light could not restore hemolytic activity. Both residues are at the interface between protomers, most likely important for oligomerization of FraC. D) Substitution with a cysteine residue at position 130 did not change hemolytic activity, all variants lysed blood rapidly, suggesting that this residue has no no direct interaction with the membrane. E) Modification with switch C at position G13 decreased hemolytic activity drastically this effect is most likely due to steric hindrance between the protomers. Isomerization of the azobenzene by UV light (365 nm) did not increase the hemolytic activity. F and G) Modification with switch C at position K77 and S166 decreased hemolytic activity. The \textit{trans} configuration was more active than the \textit{cis} configuration. H) Position E134 is the only mutant with increased hemolytic activity after modification with C. A possible explanation is that E134 interacts electrostatic interaction with the lipid bilayer. Substitution with cysteine reduced the affinity of the monomer for the lipid bilayer. Introduction of switch C then restores the affinity of the monomers with the membrane. Isomerization of the azobenzene from \textit{trans} to \textit{cis} by UV light (365 nm) resulted in a small increase of hemolytic activity. I-J) Modification with switch C at position W112 and Y138 decreased hemolytic activity. Isomerization of the azobenzene from \textit{trans} to \textit{cis} by UV light (365 nm) partially restored the hemolytic activity, as intended. According to the crystal structure (PDB: 4TSY\textsuperscript{14}) both side chains point towards the membrane. All proteins were purified by Ni-NTA affinity chromatography and not further separated from unmodified C-FraC.
Figure S30. Purification of FraC-Y138C-C. A) Chromatogram obtained from eluting FraC-Y138C-C from a cation exchange column (HiTrap SP HP column). Peak 1 corresponds to modified FraC and peak 2 to unmodified FraC monomers. B) Modification of Y138C FraC with switch C examined by 12% SDS-PAGE electrophoresis. Lane 1: Y138C FraC. Lane 2: Y138C FraC modified with PEG-5000-maleimide. Lane 3: protein ladder. Lane 4: FraC-Y138C-C after His-Tag purification. Lane 5: FraC-Y138C-C after His-Tag purification and incubated with PEG-5000-maleimide. Lane 6: concentrated peak 1 from cation exchange chromatography containing FraC-Y138C-C. Lane 7: protein ladder.
Figure S31. Hemolytic activity of purified C-FraC variants. A) Comparison of the hemolysis percentage of Y138C FraC mutants. Introducing a cysteine into FraC at position 138 reduced the hemolytic drastically and attachment of switch C reduced the hemolytic activity even further. Irradiation with UV light (365 nm) regained some hemolytic activity while white light reversed the effect again resulting in comparable hemolytic activities than the nonirradiated toxin. B) Comparison of the 50% hemolysis values of S-FraC, FraC-Y138C, FraC-Y138C-C
and FraC-Y138C-C after irradiation with UV light (365nm). C) Comparison of the hemolysis percentage of W112C FraC mutants. Introducing a cysteine into FraC at position 112 reduced the hemolytic only slightly. Attachment of switch C reduced the hemolytic activity even further. Irradiation with UV light (365nm) regained some hemolytic activity. D) Comparison of the 50% hemolysis values of S-FraC, FraC-W112C, FraC-W112C-C and FraC-W112C-C after irradiation with UV light (365nm). E) Comparison of the hemolysis percentage of E134C FraC mutants. Introducing a cysteine into FraC at position 134 reduced the hemolytic drastically. Attachment of switch C regained the hemolytic activity but irradiation with UV light (365nm) changes hemolytic activity slightly. F) Comparison of the 50% hemolysis values of S-FraC, FraC-E134C, FraC-E134C-C and FraC-E134C-C after irradiation with UV light (365nm).

Figure S32. Hemolytic activity and purification of FraC-Y138C-D. A to C) Hemolytic activity of FraC-Y138C modified with switch D (purple squares), and after irradiation at 365 nm (red spheres) at different concentrations. Faster hemolytic activity of FraC-Y138C-D compared to FraC-Y138C-C was observed, because switch D modification was less efficient and more unmodified FraC was present. D) Chromatogram obtained from eluting FraC-Y138C-D from a cation exchange column (HiTrap SP HP column). Peak 1 corresponds to free azobenzene, peak 2 to unmodified FraC and peak 3 to modified FraC monomers. E) Modification of Y138C FraC with D examined by 12% SDS-PAGE electrophoresis. Lane 1: protein ladder. Lane 2:Y138C FraC. Lane 3: Y138C FraC modified with PEG-5000-maleimide. Lane 4: FraC-Y138C-D after
His-Tag purification. Lane 5: FraC-Y138C-D after His-Tag purification and incubated with PEG-5000-maleimide. Lane 6: protein ladder. Lane 7: concentrated peak 3 from cation exchange chromatography containing FraC-Y138C-D. Lane 8: the flow through from cation exchange chromatography containing no FraC. Lane 9: peak 1 from cation exchange chromatography containing little FraC. Lane 10: peak 2 from cation exchange chromatography containing unmodified FraC. F) Hemolytic activity of cation exchange chromatography purified FraC-Y138C-D in the trans configuration (no irradiation, purple spheres), in the cis configuration (upon irradiation at 365 nm, red triangles).

**Figure S33.** Cell toxicity of FraC mutants. A) Toxicity of FraC mutants towards A431 cells. Cysteine-free FraC lysis A431 cells the fastest. Incorporation of a cysteine at position 138 decreased cell toxicity slightly. Further incorporation of the azobenzene (FraC-Y138C-C) results in no observed cell killing of A431 cells. Irradiation with UV light (365 nm, cis) activates the toxin and recovers toxicity. E) Comparison of the IC\textsubscript{50} values of S-FraC, FraC-Y138C, of FraC-Y138C-C and FraC-Y138C-C irradiated with UV light (365 nm).
Figure S34. Electrophysiology measurements of FraC-S166C-C. A and B) Relationship between current and voltage for single FraC-S166C-C nanopores. The addition of 2 µM of unpurified FraC-S166C-C (trans conformation) to the cis side of an electrophysiology chamber (500 µL) did induce the formation of nanopores. FraC-S166C-C nanopores showed an unitary conductance of 1.4 nS (+50 mV), which most likely corresponded to type II FraC nanopores. The solution used for the electrical recording contained 1 M NaCl, 15 mM TrisHCl, pH 7.5. Current traces were collected applying +50 mV, a Bessel low/pass filter with 2 kHz cutoff and sampled at 10 kHz at room temperature (25 °C).

Figure S35. Electrophysiology measurements of FraC-Y138C-C. A) Histogram of the average number of pores inserted into the membrane after 30 min when 1 µM of FraC-Y138C-C was added to the cis chamber. Recordings were carried out at 25° C in 15 mM Tris.HCl pH 7.5 containing 1 M NaCl.
References


(10) Ishiyama, M.; Miyazono, Y.; Sasamoto, K.; Ohkura, Y.; Ueno, K. A Highly Water-


