SUPPORTING INFORMATION

A Whole Cell *E. coli* Display Platform for Artificial Metalloenzymes: Poly(phenylacetylene) Production with a Rhodium–Nitrobindin Metalloprotein

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SUPPORTING INFORMATION

General Comments

All chemical experiments were performed under a nitrogen or argon atmosphere using standard Schlenk techniques or a MBraun nitrogen-filled glovebox. ^{1}H NMR spectra were recorded on a Bruker Avance III 400 NMR spectrometer. Chemical shifts are reported in ppm relative to the residual solvent resonances[1]. Gel permeation chromatography (GPC) was performed on a TOSOH SC8020 apparatus with a refractive index (RI) detector with a TOSOH TSKGel G5000H HR column. All OD_{600} measurements were performed with a BioPhotometer plus device (eppendorf). All cells used in this work were lyophilized for 24 h in a CHRIST Alpha 1-2 LDplus lyophilizer (CHRIST) after the harvested cells were centrifuged (4000 g, 4 °C, 20 min) and frozen at -20 °C. Prior to further processing, all lyophilized cells were rehydrated in swelling buffer for at least 30 min in a process called swelling.

Experimental Section

Reagents and media. The PBS buffer consisted of 140 mM NaCl, 10 mM KCl, 6.4 mM Na_2HPO_4, 2 mM KH_2PO_4. In cases when BSA-supplemented PBS buffer was employed, 0.2% (w/v) BSA were added. The swelling buffer for the cells consisted of 20 mM Tris, 100 mM NaCl at pH 8. The LB medium employed for genetic engineering and expression was composed of 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl. In the case of LB agar plates, 20 g/l agar were added. The medium was then autoclaved at 121 °C for 20 min. All components were obtained from AppliChem.

Cloning. The primers employed for the cloning are listed in Tables S1-S5. The composition of and conditions for the polymerase chain reaction (PCR) experiments are detailed in Tables S6 and S7 respectively. To insert the gene for NB4 (insert) into a pET22 plasmid bearing only the

CSD system (vector), both the insert and vector were first amplified using the primers in Table S1 via PCR (Tables S6-S7). Restriction digestion was performed with NheI-HF (NEB) and AscI (NEB) (Table S8). The mixture was incubated at 37 °C for 6 h. Then, an agarose gel electrophoresis was run (0.8% agarose, 80 V, 90 min) and a gel extraction was performed (see NucleoSpin Gel and PCR Clean-up, Macherey-Nagel). For the ligation a ratio of 1:4 (w:w) of vector to insert was set and the ligation mixture was assembled with 1 µl T4 DNA ligase (400000 units/ml, NEB), 2 µl 10x T4 DNA ligase reaction buffer (NEB), the insert and vector up to 20 µl with ddH_2O. The ligation mixture was incubated at room temperature for 1 h and then used for transformation. Subsequently, a TEV protease cleavage site was introduced between NB4 and the E-tag antibody binding site via PCR (TEV site encoded in employed primers, Table S3). The EstA esterase used as an autotransporter for cell surface display was inactivated via a substitution in the catalytic triad (serine to alanine; Table S4). Transformation to chemically competent *E. coli* DH5α cells (heat shock at 42 °C, 45 second) and sequencing was performed after every PCR. The final pET22 csdNB4 TEV variant was then transformed to chemically competent *E. coli* BL21 (DE3) gold cells (heat shock at 42 °C, 20 seconds) for expression. As a negative control, the cysteine C96 in csdNB4 TEV was substituted to glutamine via site directed mutagenesis (Table S5).

Protein expression. The LB medium employed for genetic engineering, expression pre- and main cultures was composed of 10 g/l tryptone, 5 g/l yeast extract and 10 g/l NaCl. In the case of LB agar plates, 20 g/l agar was added. The medium was then autoclaved at 121 °C for 20 min. All components were obtained from AppliChem. Shake flask expressions started with the inoculation of 10 ml LB_{Amp} in a test tube with 2 µl from a glycerol stock of *E. coli* BL21 (DE3) gold pET22b csdNB4 (C96Q) TEV as a preculture. This preculture was shaken at 37 °C, 250

rpm overnight. The preculture was then used to inoculate 50 ml LB_{Amp} main culture in a 1 l Erlenmeyer flask to a starting OD_{600} of 0.02. The main culture was shaken at 37 °C, 250 rpm, and when the OD_{600} had reached 1.3, it was induced with 0.2 mM IPTG. The shaking of the main culture was continued at 18 °C, 250 rpm for 20 hours. The cells were then harvested and centrifuged at 4000 g, 4 °C for 30 min. The cell pellets were frozen at -20 °C.

Outer membrane fractionation and quantification. The cells with the outer membrane target protein were resuspended in swelling buffer. They were then sonicated at 40% amplitude 15 second pulses with 15 second breaks, for 5 min – 10 min. The sonicated suspension was then centrifuged at 3220 g, 4 °C, 1 h. The supernatant was subsequently centrifuged at 100,000 g, 4 °C, 30 min. The pellet was resuspended in PBS supplemented with 0.01 mM $MgCl_2$, 2% Triton X-100 and incubated at room temperature for 30 min. The suspension was then again centrifuged at 100,000 g, 4 °C, 30 min. The resulting pellet corresponded to the outer membrane fraction and was resuspended in buffer for further use. This method was based on a protocol by Maurer *et al.*[2]. For the quantification of the isolated OMFs, an Experion Automated Electrophoresis Station (Bio-Rad) was employed, with Experion Pro260 Chips (Bio-Rad). Samples for the Experion device were prepared according to the Experion Pro260 Analysis Kit Quick Guide (Bio-Rad). To calculate the number of csdNB4 TEV proteins per cell, $7*10^8$ *E. coli* cells per ml at an OD_{600} of 1 were assumed according to literature[3].

Immunofluorescence staining and detection. For fluorescence staining purposes, a fluorescein derivative-conjugated antibody specific for the E-tag peptide sequence was used (Epitope-Tag Antibody conjugated with FITC, Novus Biologicals). With the E-tag sequence present in csdNB4 TEV, this allowed for fluorescent staining of the expressing cells. First, 10 mg of the lyophilized cells were resuspended in 5 ml PBS with 0.2% BSA and centrifuged at 3200 g,

4 °C, 10 min two times. The cells were then resuspended in 167 µl of a 1:100 diluted E-Tag antibody (Epitope-Tag Antibody conjugated with FITC, Novus Biologicals) solution. In the case of the unstained negative control, PBS with 0.2% BSA was used for this step. The cells were incubated on ice for 10 min, after which 8.5 ml PBS with 0.2% BSA was added. The suspension was then centrifuged at 3200 g, 4 °C, 10 min. Finally, the cells were resuspended in the volume of PBS with 0.2% BSA necessary to set OD_{600} 75 and used for analysis. For detection with the Tecan Infinite M1000 96-well plate reader (Tecan), the excitation wavelength λ_{ex} was 495 nm, and the emission wavelength λ_{em} 519 nm.

TEV cleavage of csdNB4 TEV. AcTEV Protease (ThermoFisher Scientific) was employed for cleavage of the TEV sequence in the csdNB4 TEV fusion protein based on the following protocol: After outer membrane fractionation, the outer membrane fraction was resuspended in 10 ml swelling buffer and subjected to ultracentrifugation again at 100,000 g, 4 °C, 30 min. The pellet was then resuspended in the digestion mixture composed of 15 µl 20x AcTEV buffer (ThermoFisher Scientific), 3 µl 0.1 M DTT (ThermoFisher Scientific), 2 µl (20 U) AcTEV, 280 µl ddH_2O. The digestion mixture was shaken at 25 °C, 150 rpm, 24 h.

Rh(cp)(cod) coupling. Coupling of Rh(cp)(cod) **1** to csdNB4 TEV whole cells was performed in a glovebox under nitrogen atmosphere. 250 mg lyophilized csdNB4 TEV whole cells were resuspended in 10 ml swelling buffer and stirred at 150 rpm, 30 min. 100 equiv. Rh(cp)(cod) **1** in THF (10% (v/v)) were then added to the suspension. The mixture was stirred at 150 rpm, 30 min at room temperature, then centrifuged at 2300 g, 20 min. Three THF wash steps were then performed by discarding the supernatant, resuspending the pellet in 20 ml 50% THF/swelling buffer and centrifuging at 3200 g, 4 °C, 10 min. In case cellular components were extracted during the THF washing procedure, these components would be discarded with the supernatants.

Finally, two buffer wash steps were performed by discarding the supernatant, resuspending the pellet in 20 ml swelling buffer and centrifuging at 3200 g, 4 °C, 20 min.

ICP-OES quantification of cellular rhodium content. After coupling of Rh(cp)(cod) **1** to csdNB4 TEV whole cells, the washed cells were lyophilized again and dissolved in 10 ml aqua regia (1 volume HNO_3, 3 volumes HCl). The mixture was incubated at 100 °C overnight, then filtered (glass frit) for ICP-OES. The obtained ppm values were then converted to mol values and divided by the number of csdNB4 TEV whole cells used for coupling.

Polymerization of phenylacetylene. After coupling, the ArMt bugs were resuspended in 5 ml swelling buffer, 100 µl phenylacetylene **3** were added and the reaction mixture was stirred at 150 rpm, 12 h at room temperature under air. After reaction time indicated, 100 eq. acetic acid were added to quench the catalyst. The polymer and the monomer were extracted with chloroform, dried over magnesium sulfate and dried under vacuum. The obtained polymer was analyzed by ^{1}H NMR spectroscopy in $CDCl_3$ prior by analysis with GPC in THF.

Results

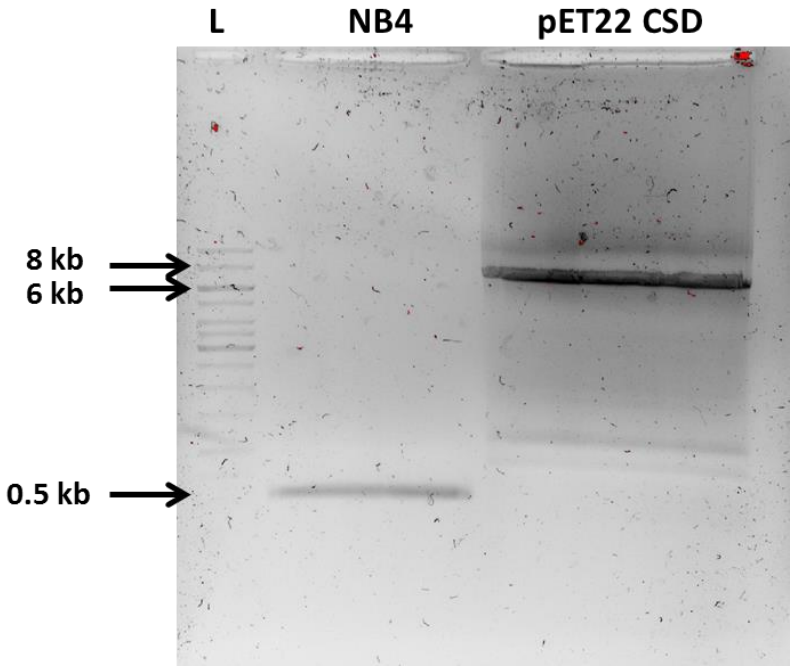


Figure S1. NB4 insert and pET22 CSD after restriction enzyme digestion. L: Ladder (GeneRuler^{TM} 1 kb DNA Ladder (Thermo Scientific)).

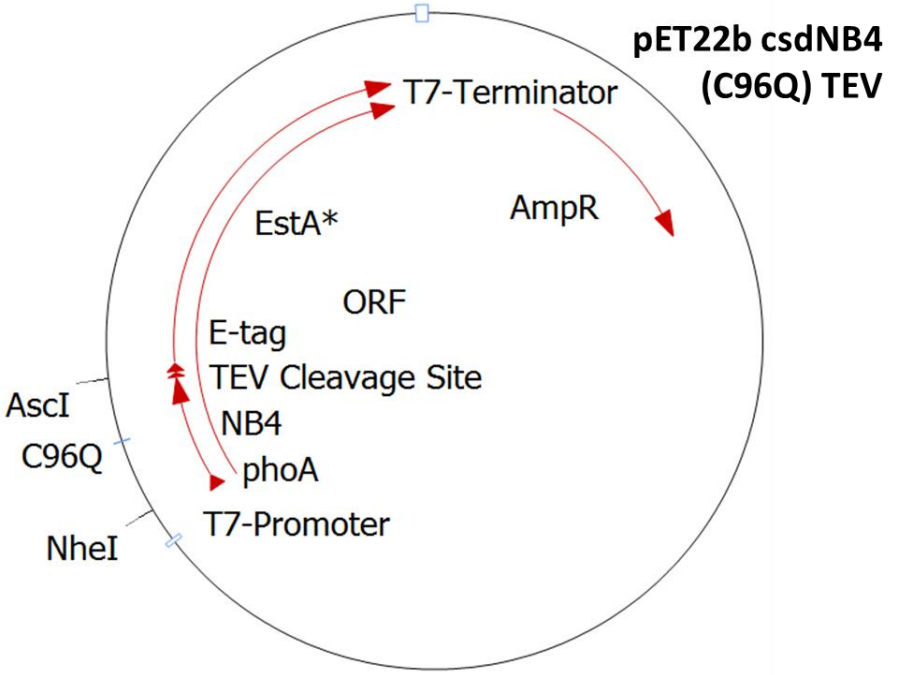


Figure S2. Vector maps for cell surface display of nitrobindin variant NB4. The amino acid sequence of the csdNB4 TEV fusion protein is given below (legend: phoA leader, NB4, TEV site, E-tag, EstA*).

MKQSTIALALLPLLFTPVTKAASNQLQQLQNPGESPPVHPFVAPLSYLLGTWRGQGEGE
YPTIPSFRYGEEIRFSHSGKPVIAYTQKTWKLESGAPLLAESGYFRPRPDGSIEVVIACSTG
LVEVQKGTYNVDEQSIKLKSDLVGNASKVKEISREFELVDGKLSYVVRLSTTTNPLQPLL
KAILDKLGSENLYFQGSGGAPVPYPDPLEPRAASAPSPYSTLVVFGDALSDAGQFPDPAG
PAGSTSRFTNRVGPTYQNGSGEIFGPTAPMLLGNQLGIAPGDLAASTSPVNAQQGIADGN
NWAVGGYRTDQIYDSITAANGSLIERDNTLLRSRDGYLVDRARQGLGADPNALYYITG
GGNDFLQGRILNDVQAQQAAGRLVDSVQALQQAGARYIVVWLLPDLGLTPATFGGPLQ
PFASQLSGTFNAELTAQLSQAGANVIPLNIPLLLKEGMANPASFGLAADQNLIGTCFSGN
GCTMNPTYGINGSTPDPSKLLFNDSVHPTITGQRLIADYTYSLLSAPWELTLLPEMAHGT
LRAYQDELRSQWQADWENWQNVGQWRGFVGGGGQRLDFDSQDSAASGDGNGYNLT
LGGSYRIDEAWRAGVAAGFYRQKLEAGAKDSDYRMNSYMASAFVQYQENRWWADA
ALTGGYLDYDDLKRKFALGGGERSEKGDTNGHLWAFSARLGYDIAQQADSPWHLSPF
VSADYARVEVDGYSEKGASATALDYDDQKRSSKRLGAGLQGKYAFGSDTQLFAEYAH
EREYEDDTQDLTMSLNSLPGNRFTLEGYTPQDHLNRVSLGFSQKLAPELSLRGGYNWR
KGEDDTQQSVSLALSLDF

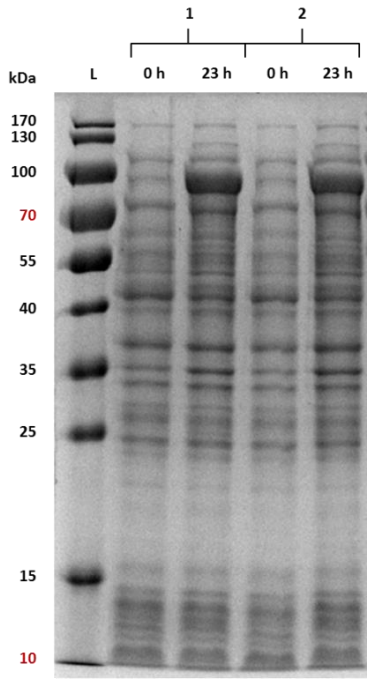


Figure S3. SDS-PAGE of two csdNB4 TEV expressions (1, 2). The molecular weight of csdNB4 TEV was calculated to be 90524.62 g/mol (ExPASy ProtParam tool). L: Ladder (PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific)).

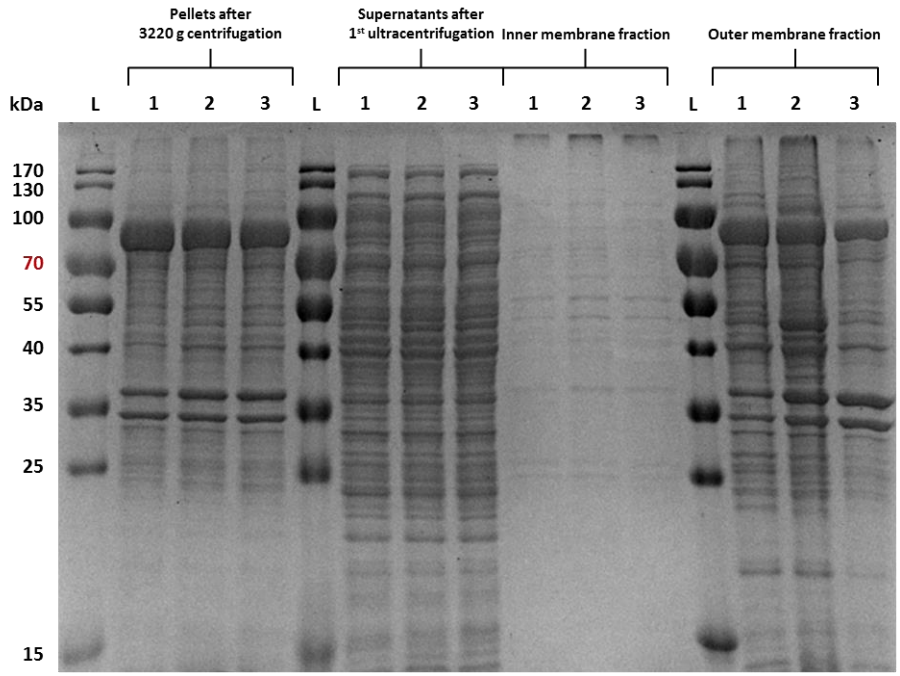


Figure S4. SDS-PAGE of samples taken during outer membrane fractionation of three csdNB4 TEV expression batches (batch 1, batch 2, batch 3). 100 mg lyophilized cells of each batch were used for outer membrane fractionation. L: Ladder (PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific)). Samples from left to right: Pellets after slow 3220 g centrifugation (= cell debris); Supernatants after first 100,000 g ultracentrifugation; Supernatants after second 100,000 g ultracentrifugation (= inner membrane fraction); Pellets after second 100,000 g ultracentrifugation (= outer membrane fraction). Protocol according to Maurer *et al.*[2].

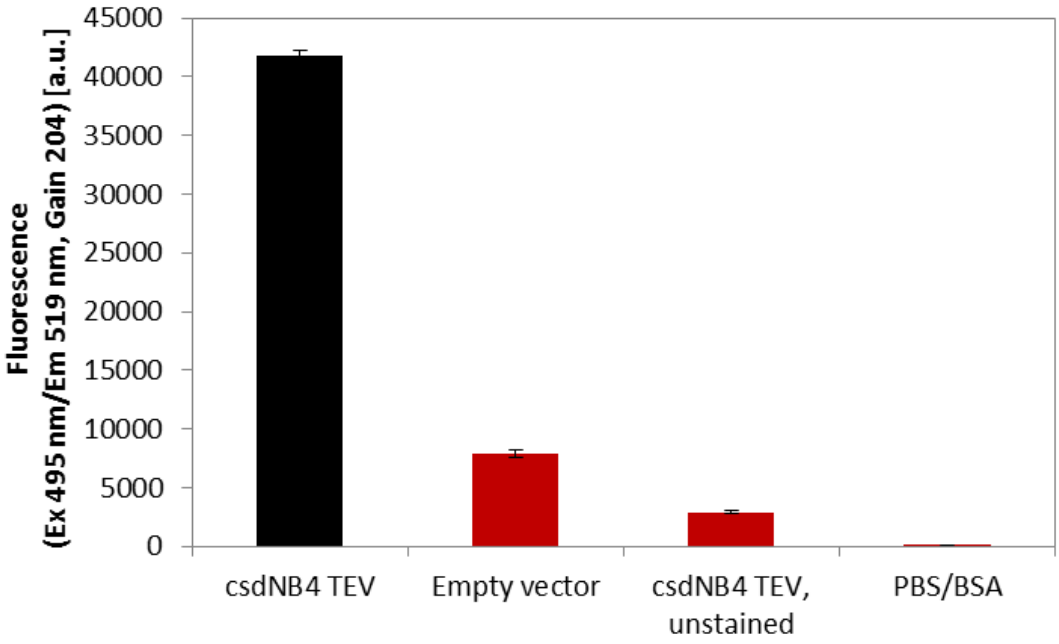


Figure S5. Immunofluorescence staining of csdNB4 TEV whole cells. Red columns represent negative controls. OD_{600} 75 was set for all cell samples. Fluorescence exhibited by unstained csdNB4 TEV cells is expected to be mainly caused by endogenous flavins[4]. To evaluate the extent of periplasmic display of outer membrane csdNB4 TEV, an E-tag antibody staining was also performed with csdNB4 TEV whole cells and subsequently again with the isolated outer membrane fraction. The membrane fraction experiment shows a < 10% increase in fluorescence after washing (1462 a.u. after staining of whole cells, 1590 a.u. after staining of isolated membrane fraction; Tecan Infinite M200 plate reader, λ_{ex} 490 nm, λ_{em} 525 nm, gain 142). The low difference in fluorescence intensity is a strong indication that most of the outer membrane csdNB4 TEV is displayed on the *E. coli* cell surface.

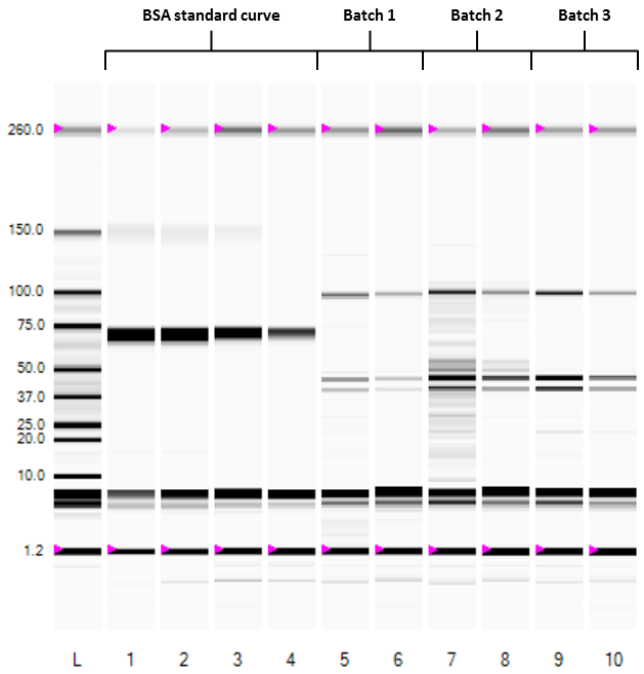


Figure S6. Experion results of outer membrane fractionation of *E. coli* BL21 (DE3) gold pET22 csdNB4 TEV. Quantification procedure of outer membrane fraction described in "outer membrane fractionation and quantification" in the experimental section, also see calculation in caption of Figure S7.

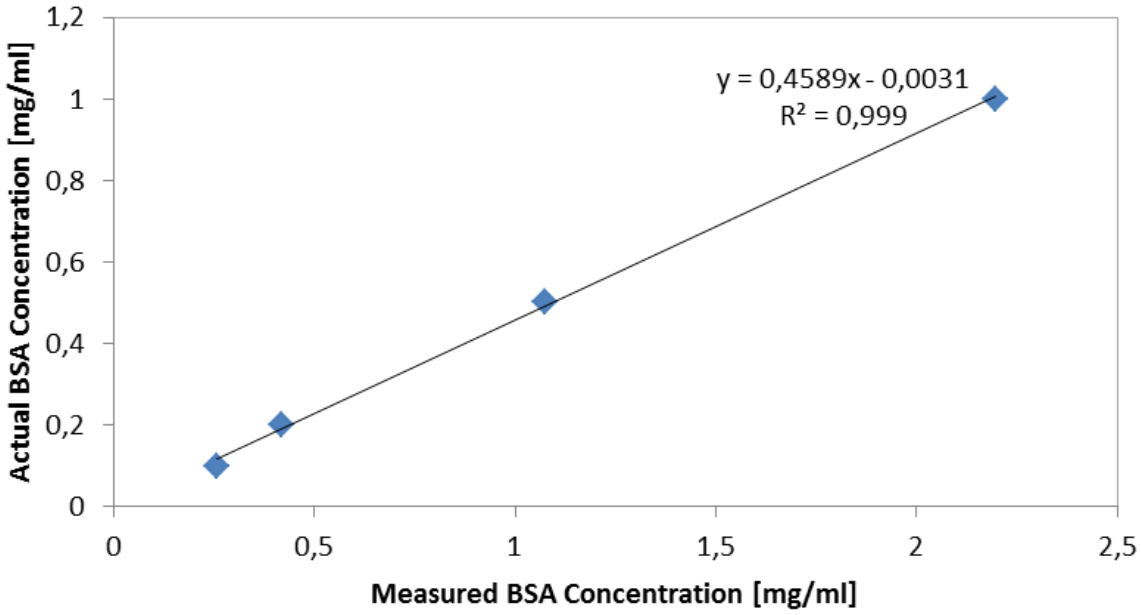


Figure S7. BSA standard curve with Experion. Using the equation of the BSA standard curve (actual BSA concentration [mg/ml] = 0.4589 · measured BSA concentration [mg/ml] – 0.0031), the csdNB4 TEV content in relation to the lyophilized cell mass in three expression batches was determined to be 0.14%, 0.31% and 0.26%. The resulting average of 0.24% ($g_{csdNB4\ TEV}/g_{lyophilized\ cells}$) was then converted to a molar concentration of 26.23 $nmol_{csdNB4\ TEV}/g_{lyophilized\ cells}$. At an *E. coli* cell density of $7 \cdot 10^8$ cells/ml at an OD_{600} of 1, that corresponds to approximately 12867 csdNB4 TEV molecules per *E. coli* cell[3].

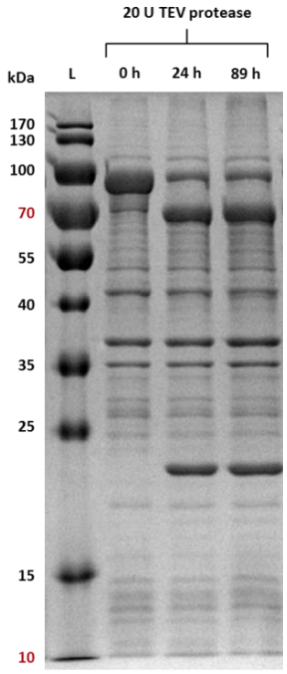


Figure S8. TEV cleavage of OMF csdNB4 TEV. A band corresponding in size to the cleaved NB4 domain appears after 24 h incubation of outer membrane fraction csdNB4 TEV with 20 U TEV protease (calculated: 21581.7 g/mol). L: Ladder (PageRuler^{TM} Prestained Protein Ladder (Thermo Scientific)).

	m/z calculated	m/z found
NB4	21581.7	21582.4
1@NB4	22017.2 (HCl adduct)	22017.7

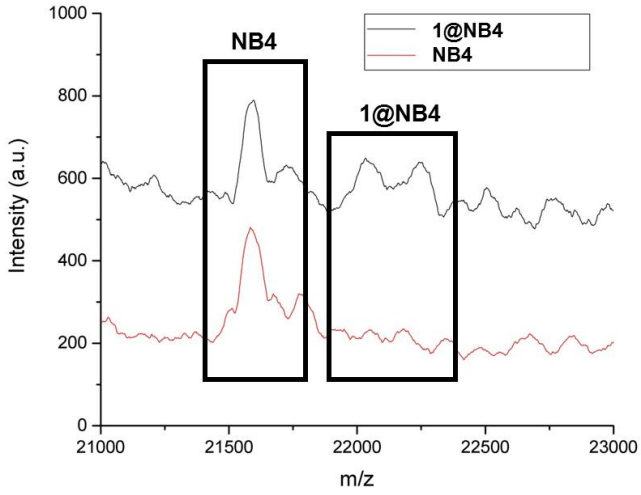


Figure S9. MALDI-ToF-MS spectra of uncoupled and coupled TEV-cleaved NB4 in outer membrane fraction using 100 mg lyophilized cells after TEV protease cleavage. The twin peaks in the coupled **1**@NB4 sample is expected to result from cleavage of the cyclooctadiene (cod) ligand from Rh(cp)(cod) **1** during MS analysis.

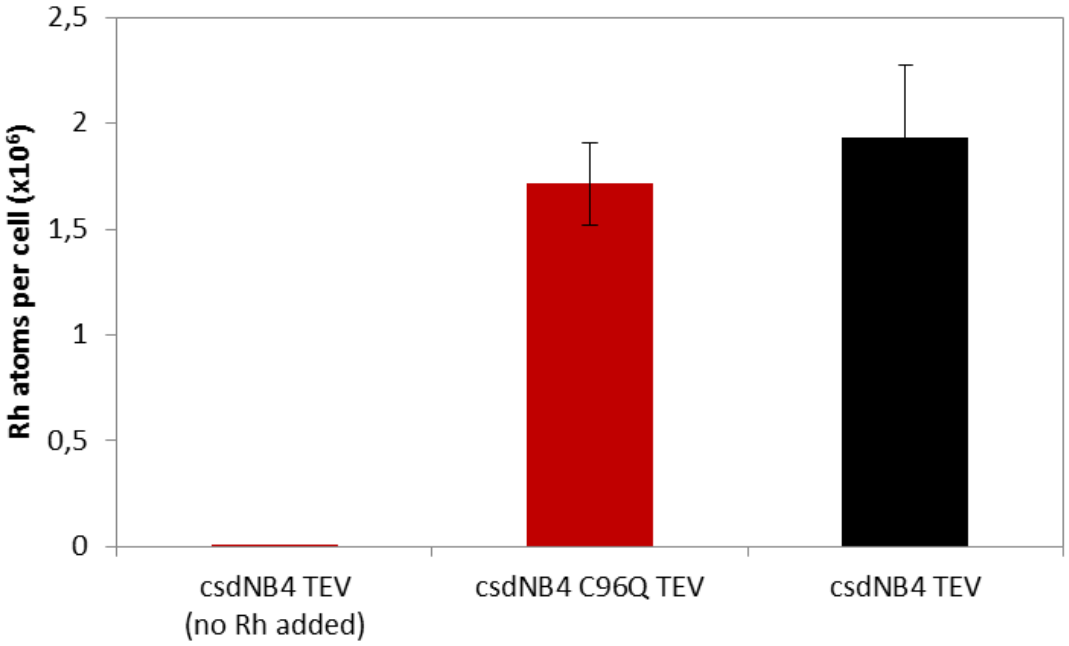


Figure S10. ICP-OES quantification of cellular Rh content. While coupled csdNB4 TEV whole cells (ArMt bugs; black bar) produced 80% *trans* polyphenylacetylene (TON per cell = $39*10^6$ $cell^{-1}$, Table 1, entry 5), no product formation could be detected with coupled csdNB4 C96Q TEV whole cells (Table 1, entry 6; red bar). csdNB4 C96Q TEV differs from csdNB4 TEV in one respect only: It does not contain the cysteine residue at position 96 in NB4 which is necessary for covalent coupling of the maleimide-bearing Rh(cp)(cod) catalyst. By employing *E. coli* cells with displayed csdNB4 C96Q TEV instead of empty vector cells, differences (e.g. unspecific binding in the hydrophobic NB4 cavity) between the negative control (csdNB4 C96Q TEV) and the cells displaying csdNB4 TEV are minimized. As an additional negative control, no Rh(cp)(cod) catalyst 1 was added to the csdNB4 TEV whole cells.

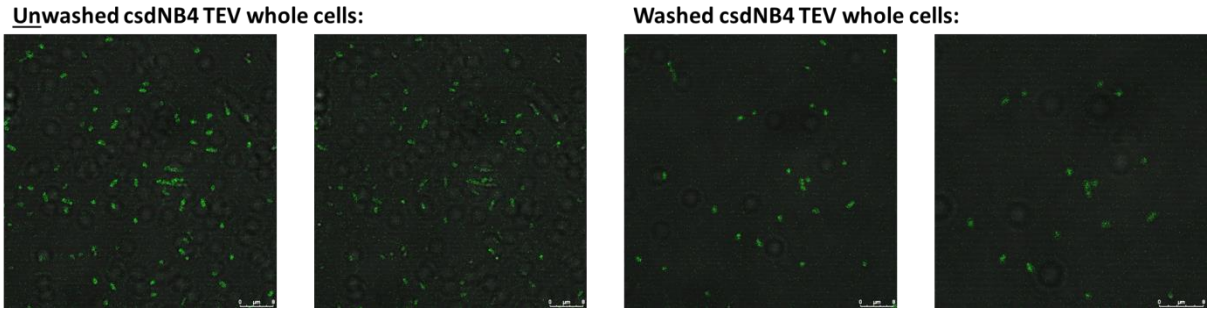


Figure S11. Washed and unwashed (control) *E. coli* csdNB4 TEV whole cells to show what the cells look like after the washing procedure (see "Rh(cp)(cod) coupling" in the experimental section). Lyophilized *E. coli* csdNB4 TEV whole cells were stained with 15 µM ThioGlo®-1 and analyzed with a TCS SP8 confocal microscope (λ_{ex} = 485 nm, argon laser intensity = 4%, λ_{em} = 520 nm, gain = 1250, HyD1 detector; Leica Microsystems CMS GmbH, Mannheim, Germany). Transmission and fluorescence images were generated and overlayed with the Leica Application Suite X software.

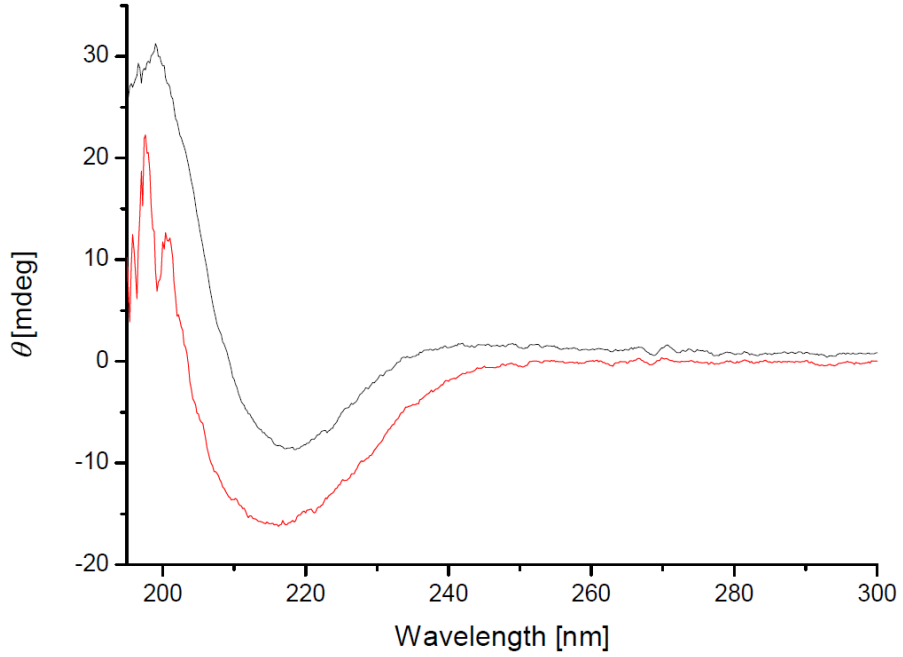


Figure S12. CD spectra of 1 mg/ml NB4 in 20 mM Tris, 50 mM NaCl, pH 8 (black) and 50% THF (red).

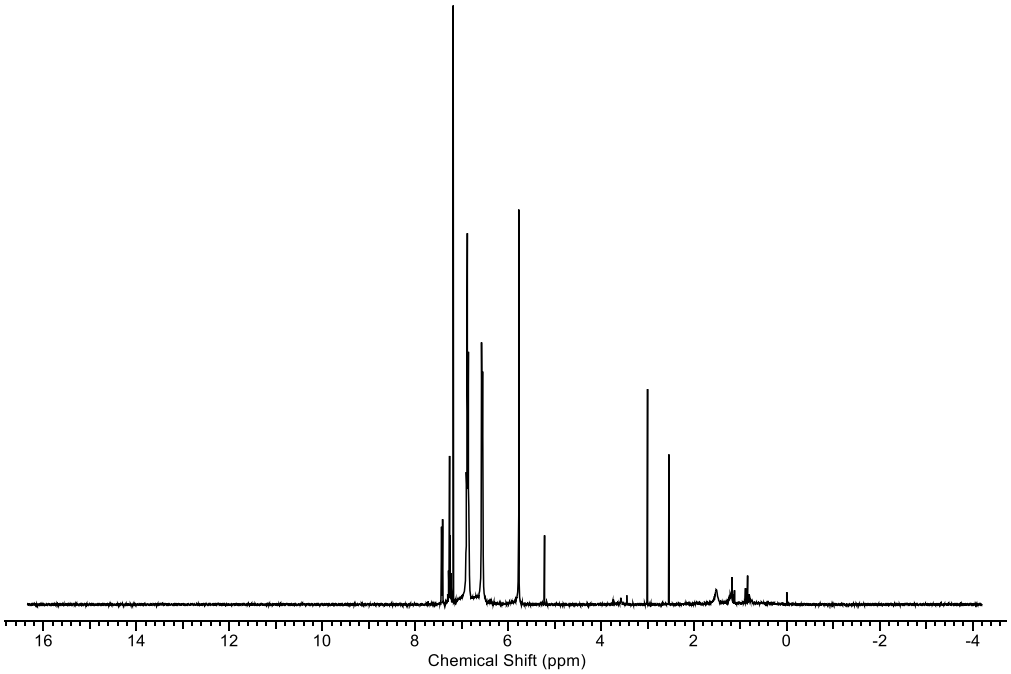


Figure S13. Representative ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 23 °C) of *cis* polyphenylacetylene (Table S9, entry 1).

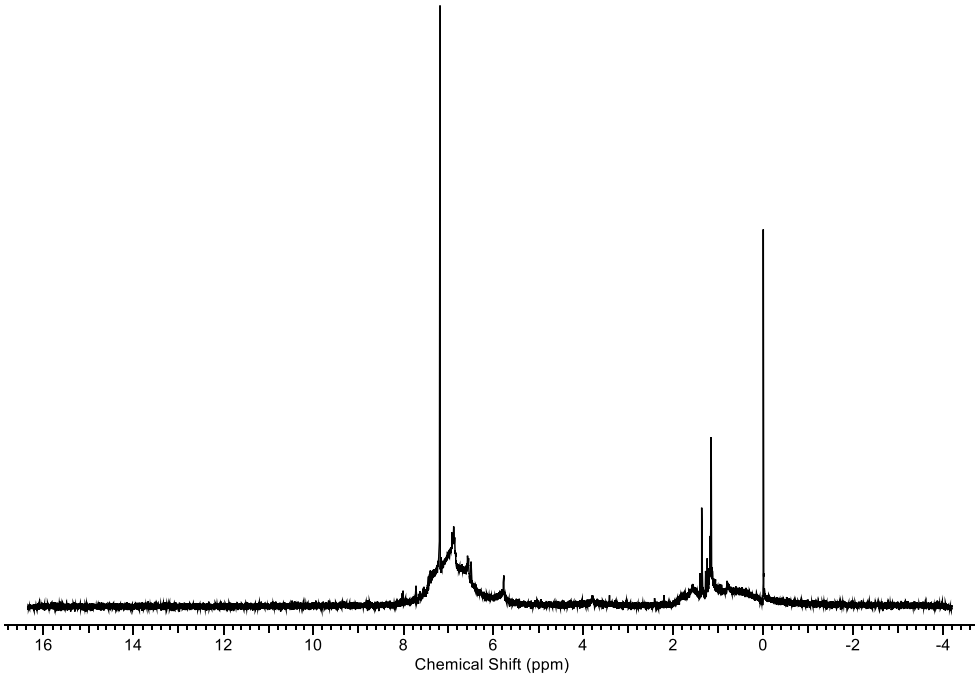


Figure S14. Representative ^{1}H NMR spectrum (400 MHz, $CDCl_3$, 23 °C) of *trans* polyphenylacetylene produced by ArMt bugs (80% *trans*; Table S9, entry 6).

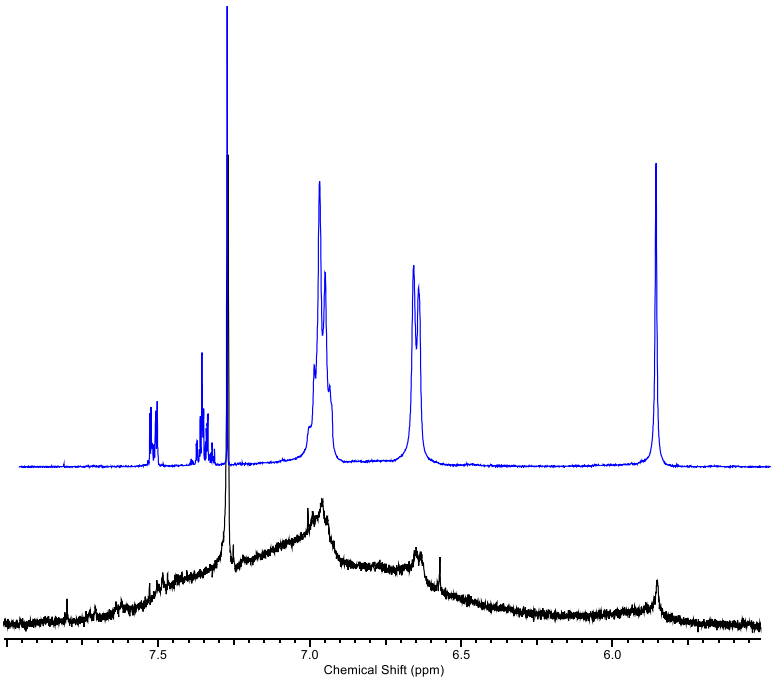


Figure S15. Comparison of ^{1}H NMR spectra (400 MHz, $CDCl_3$, 23 °C) of *cis* (blue) and *trans* (black) polyphenylacetylene.

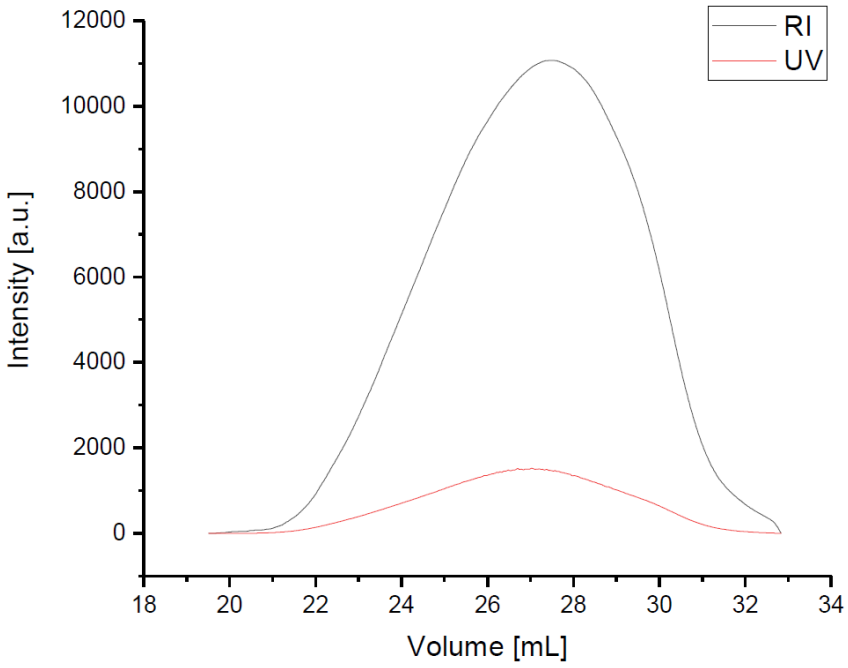


Figure S16. GPC trace of polyphenylacetylene produced by ArMt bugs (Table S9, entry 6).

Table S1. Primers for the amplification of pET22 CSD vector and NB4 insert.

Primer	**Sequence (5' → 3')**	**T_m**
FWD vector	AAT GGC GCG CCG GTG CCG	65.1 °C
REV vector	AAA GCT AGC CGC TTT GGT CAC CGG	66.1 °C
FWD insert	GGG GCT AGC AAT CAA CTG CAA CAA CTG	66.5 °C
REV insert	AAA GGC GCG CCC AGT TTG TCC AGG	67.8 °C

Table S2. Primers for the deletion of excess bases at either end of the NB4 gene in the CSD construct on pET22. The primers were named after the restriction site in the proximity of which the excess bases were observed.

Primer	**Sequence (5' → 3')**	**T_m**
FWD NheI	ACC AAA GCG GCT AGC AAT CAA CTG CAA CAA CTG	69.5 °C
REV NheI	CAG TTG TTG CAG TTG ATT GCT AGC CGC TTT GGT	69.5 °C
FWD AscI	AAA CTG GGC GCG CCG GTG CCG TAT C	71.2 °C
REV AscI	GAT ACG GCA CCG GCG CGC CCA GTT T	71.2 °C

Table S3. Primers for the insertion of TEV cleavage site in pET22 csdNB4.

Primer	**Sequence (5' → 3')**	**T_m**
FWD	C ATC CTG GAC AAA CTG GGC AGC GAA AAC CTG TAC TTC CAG GGG AGC GGC GGC GCG CCG GTG CCG TAT C	> 75 °C
REV	G ATA CGG CAC CGG CGC GCC GCC GCT CCC CTG GAA GTA CAG GTT TTC GCT GCC CAG TTT GTC CAG GAT G	> 75 °C

Table S4. Primers for the inactivation of esterase in pET22 csdNB4 TEV.

Primer	**Sequence (5' → 3')**	**T_m**
FWD	G TTT GGC GAT GCG CTG AGC GAT G	66 °C
REV	C ATC GCT CAG CGC ATC GCC AAA C	66 °C

Table S5. Primers for the removal of the covalent coupling site (C96Q) in NB4, in pET22 csdNB4 TEV.

Primer	**Sequence (5' → 3')**	**T_m**
FWD C96Q	GTT ATC GCA CAG TCG ACC GGT C	64 °C
REV C96Q	G ACC GGT CGA CTG TGC GAT AAC	64 °C

Table S6. General PCR Mixture Composition.

Component	**Stock Concentration**	**Volume [µl]**
PfuS buffer	10x	5
Primer	10 µM	2
dNTPs	10 mM	1
Template	20 ng/µl – 100 ng/µl	1
ddH_2O	-	38.5
PfuS	2 U/µl	0.5
Total volume		50

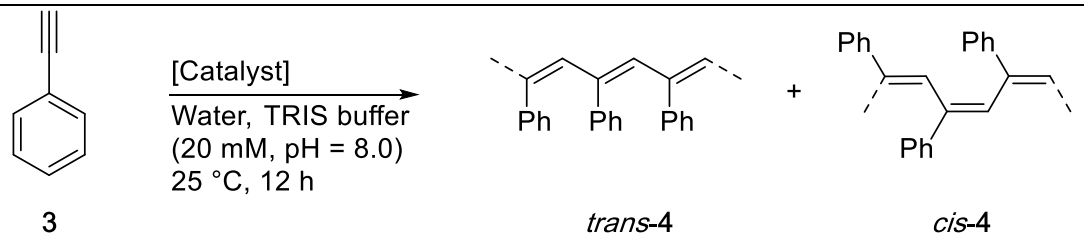
Table S7. General PCR Protocol with PfuS. Highlighted cycle (denaturation, annealing, elongation) was repeated 25 times.

Step	Temperature	Duration
Initial denaturation	98 °C	2 min
Denaturation	98 °C	10 sec
Annealing	Primer T_m – 5 °C	30 sec
Elongation	72 °C	30 sec/kb
Final elongation	72 °C	2*30 sec/kb
Cooling	4 °C	-

Table S8. Restriction mixture for cloning of NB4 into pET22 CSD.

Component	Stock Concentration	Volume [µl]
Amplified DNA (insert or vector)	-	40
NheI-HF	10,000 units/ml	1
AscI	10,000 units/ml	1
10x CutSmart buffer	10x	5
ddH_2O	-	3
Total volume		50

Table S9. Results for polymerization of phenylacetylene.



3 *trans*-4 *cis*-4

Entry	Protein	Rh	Coupling Time (h)	*cis* : *trans*	M_n	PDI	Yield (mg)	Reaction Time (h)
1[a]	-	Yes	-	95 : 5	6400	3.0	50	12
2[b]	-	Yes	-	93 : 7	22900	2.6	-	24
3[c]	Mb(A125C)	Yes	0.5	91 : 9	46500	2.3	0.1040	12
4[c]	NB4	Yes	0.5	18 : 82	38900	2.4	0.0065	12
5	csdNB4	-	-	-	-	-	-	12 or 96
6[d]	csdNB4 TEV	Yes	0.5	20 : 80	7600	2.7	2.1	12
7[e]	csdNB4 TEV	Yes	0.5	-	-	-	-	12
8	csdNB4 C96Q TEV	-	-	-	-	-	-	12
9	csdNB4 C96Q TEV	Yes	1 or 24	-	-	-	-	12
10	Intracellular NB4	Yes		-	-	-	-	-
11	pET22	-	-	-	-	-	-	12
12	pET22	Yes	1 or 24	-	-	-	-	12
13[f]	-	-	-	-	-	-	-	-
14[g]	-	-	-	-	-	-	-	-

Extending of the reaction to 24 h did not lead to a significant increase of the molecular weight M_n. [a]Δyield = 1 mg. [b]Onoda *et al.* (2012)[5]. [c]Fukumoto *et al.* (2014)[6]. [d]ΔM_n = ±34%, ΔPDI = ±29%. Δyield = 0.3 mg. [e]No rehydration (swelling) of lyophilized csdNB4 TEV whole cells before coupling. [f]Water. [g]Tris buffer.

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