CHEMISTRY
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Supporting Information

# Dynamic Core–Shell Bioconjugates for Targeted Protein Delivery and Release

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# 1. Overview synthesized molecules and self-assembled bioconjugates

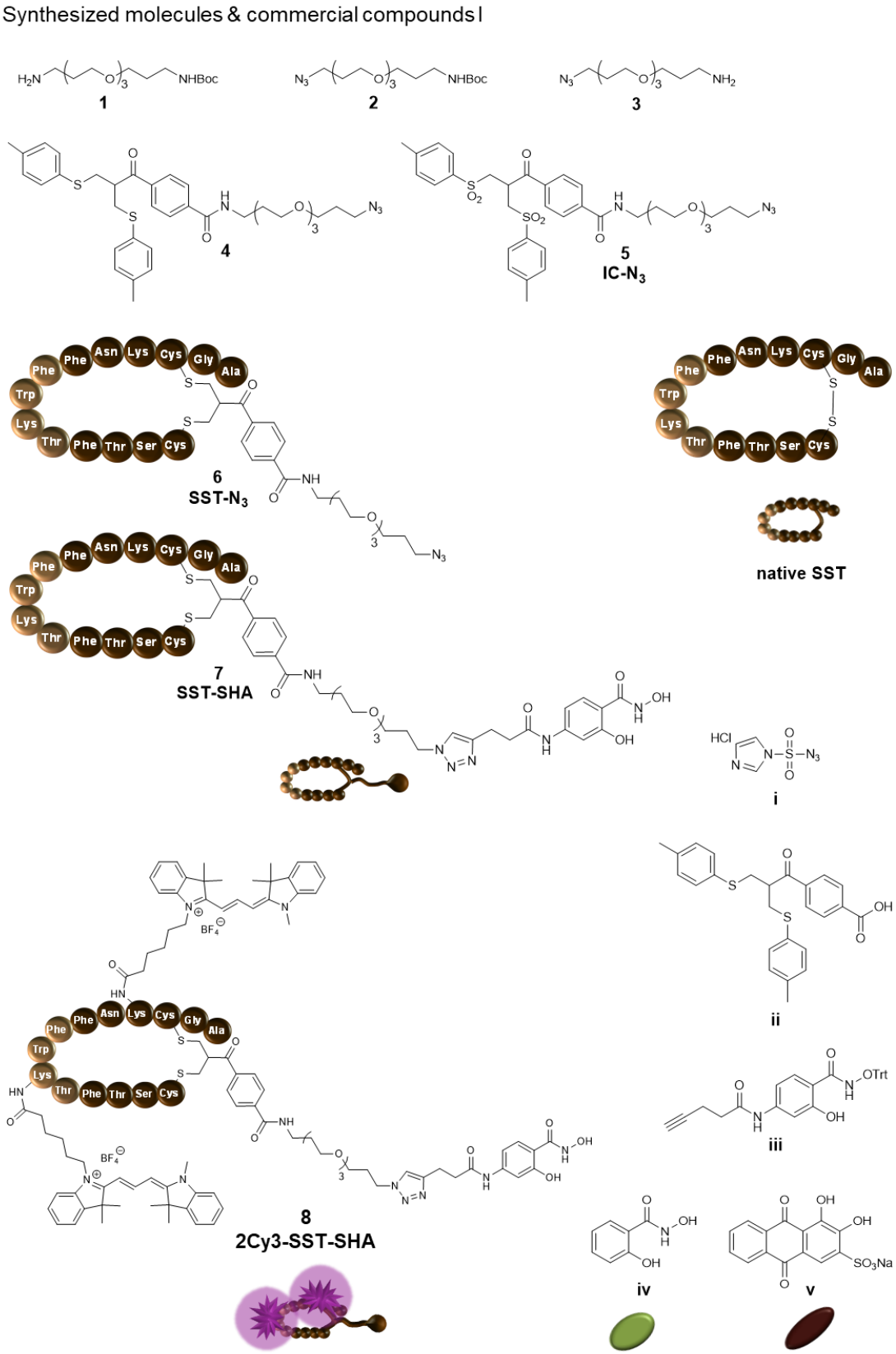


**Figure S1.** Overview about synthesized molecules and commercial compounds part I, which were used for the work herein presented.

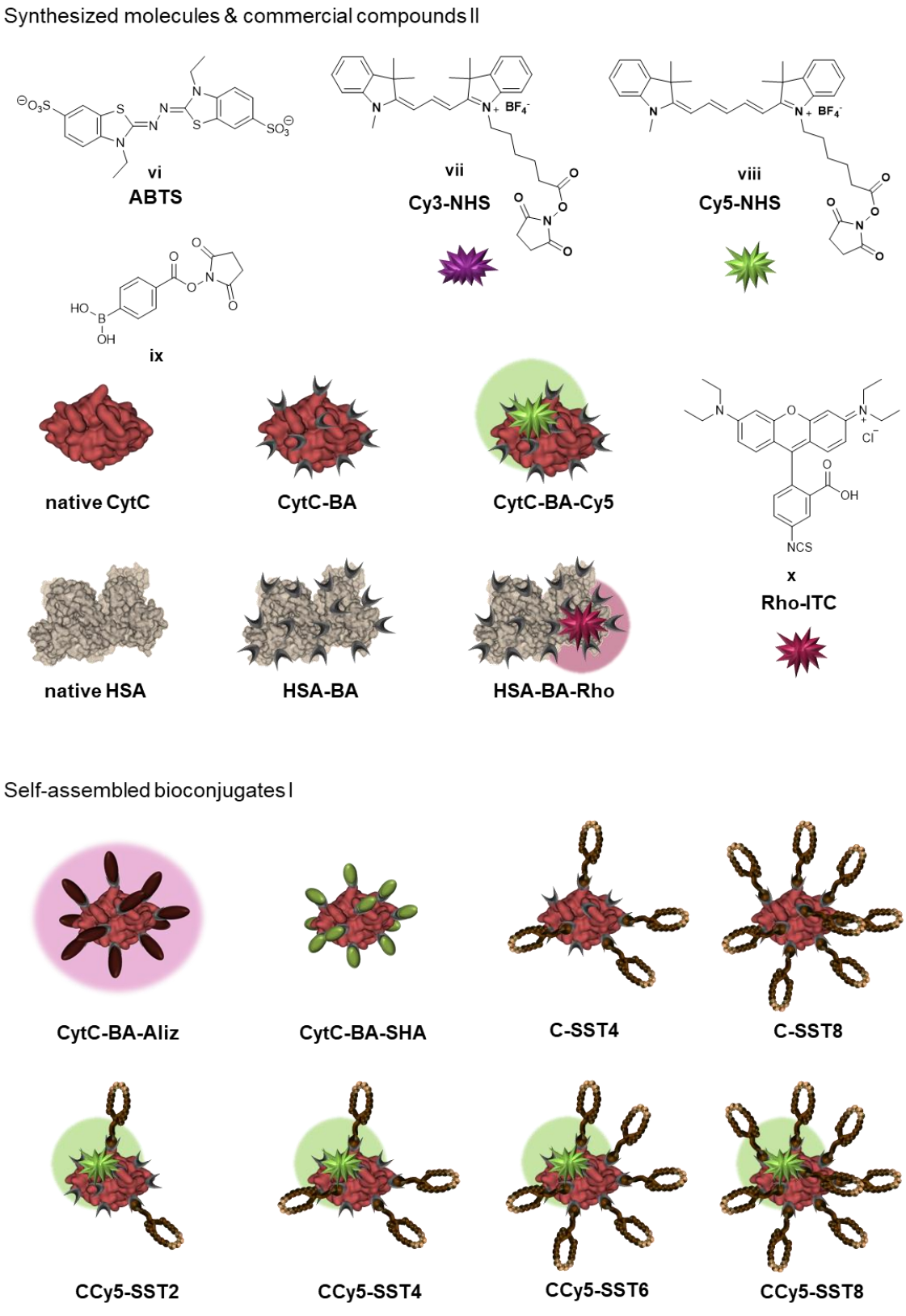


**Figure S2.** Overview about synthesized molecules and commercial compounds part II as well as self-assembled bioconjugates part I, which were used for the work herein presented.

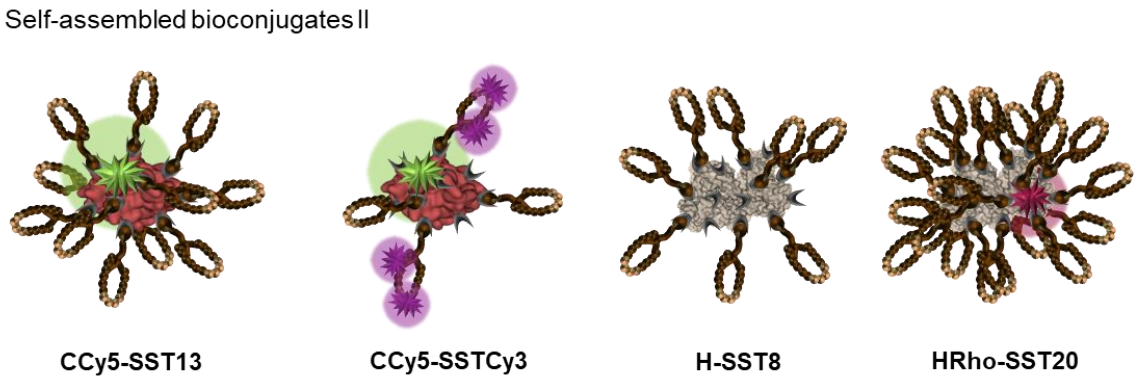


**Figure S3.** Overview about self-assembled bioconjugates part II, which were used for the work herein presented.

## 2. Experimental Section: Chemical Syntheses

### 2.1. Materials and Methods

All solvents and reagents are bought from commercial sources (Merck, Sigma Aldrich, VWR, Fisher Scientific, etc.) and are used directly without further purification unless declared differently. Water for reactions was obtained from a Millipore purification and filtration system. Reaction progress was monitored by thin layer chromatography (TLC) using Merck 60 $F_{254}$ pre-coated silica gel plates and visualized under ultraviolet lamp (254 nm) or using potassium permanganate staining solution. Flash column chromatography was carried out using Merck silica gel 60 mesh. High performance liquid chromatography (HPLC) was carried out using Shimadzu Analytical HPLC system. NMR spectra were recorded using Bruker Avance 300 NMR spectrometer and measured within deuterated solvents as mentioned for each compound individually. Chemical shifts are reported as parts per million referenced with respect to the residual solvent peak. MALDI-TOF-MS spectra were acquired on a Bruker Time-of-flight MS rapifleX. HPLC-ESI-MS analysis was performed on a Shimadzu LC-MS 2020 equipped with an electrospray ionization source, a SPD-20A UV-Vis detector and a Kinetex EVO C18 column (2.1 x 50 mm, 2.6 µm).

### 2.2. Synthesis of azide-functionalized intercalator (IC-$N_3$) 5

The IC-$N_3$ **5** was synthesized like previously described by *Wang et al.*[1] from our group. All synthesis steps yielding in compound **5** were performed as published before[1]. Figure S4 gives the chemical structure and the acquired analyses for characterization and compound purity.

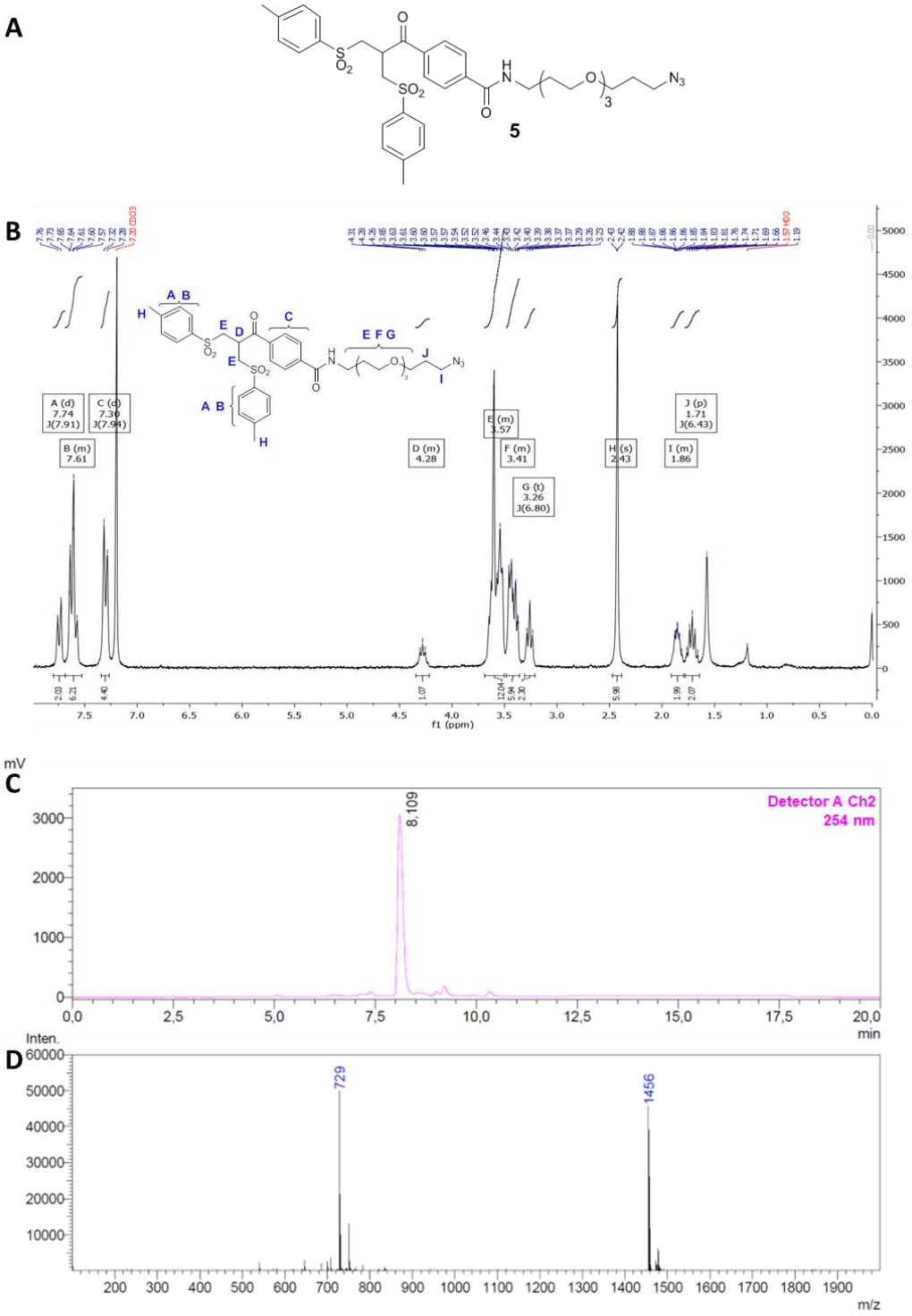


**Figure S4.** Azide-functionalized intercalator **5 A)** Chemical structure, **B)** Proton-NMR spectrum measured in $CDCl_3$, **C)** UV trace during LC-MS analysis – detection at 254 nm, **D)** MS analysis (ESI, LC-MS system) of **5** given by retention time of 8.109 min (formula: $C_{35}H_{44}N_4O_9S_2$, calculated exact mass: 729.25 $[M+H]^+$, 1457.50 $[2M+H]^+$).

### 2.3. Synthesis of azide-functionalized SST (SST-$N_3$) 6

Synthesis and purification of azide-functionalized SST (SST-$N_3$) **6** was published by our group previously[1]. Figure S5 gives the chemical structure and the acquired analyses for characterization and compound purity of **6**.

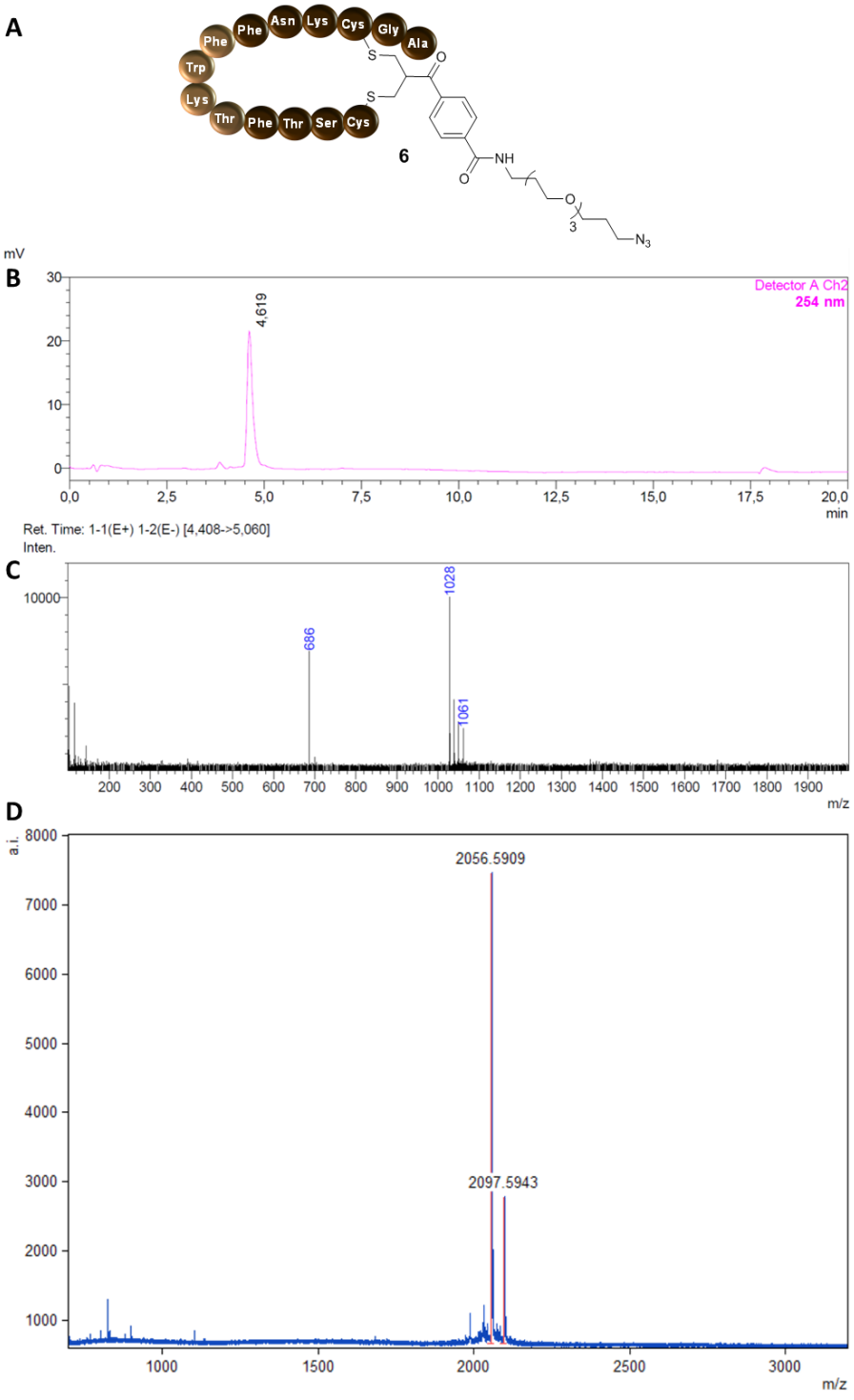


**Figure S5.** Azide-functionalized SST **6 A)** Chemical structure, **B)** UV trace during LC-MS analysis – detection at 254 nm, **C)** MS analysis (ESI, LC-MS system) of **6** given by retention time of 4.619 min (formula: $C_{97}H_{134}N_{22}O_{24}S_2$, calculated exact mass: 2056.37 [M], 1028.47 $[M+2H]^{2+}$, 685.98 $[M+3H]^{3+}$), **D)** HR-Maldi ToF MS analysis of **6**.

### 2.4. Synthesis of SHA-functionalized SST (SST-SHA) 7

Synthesis and purification of salicylhydroxamate-functionalized SST (SST-SHA) **7** was published by our group previously[1]. For this publication, there were slight changes in the procedure to obtain **7**, which is therefore described in detail in the following.

SST-$N_3$ **6** (5.0 mg, 2.4 mmol, 1 eq.) was dissolved in 1 mL of $H_2O$. To this solution, sodium ascorbate (1.4 mg, 7.2 mmol, 3 eq.) and $CuSO_4$ (0.6 mg, 3.6 mmol, 1.5 eq.) were added sequentially. Finally 2-hydroxy-4-pent-4-ynamido-N-(trityloxy)benzamide **iii** (2.4 mg, 4.8 mmol, 2 eq.) dissolved in 1 mL of THF was added. The resulting mixture was gently shaken for 5h at room temperature. The raw product was purified by preparative HPLC using an Atlantis Prep OBD T3 column (19x100 mm, 5 mm) with the mobile phase starting from 60% solvent A (0.1% TFA in water) and 40% solvent B (0.1% TFA in acetonitrile) (0–3 min). Raising to 80% B in 5 min and remaining with 80% B for 5 min then, 100% B was finally reached in further 2.5 min with a flow rate of 10 $mLmin^{-1}$. The absorbance was monitored at 214 and 254 nm. After lyophilization of all product fractions from HPLC purification, 3.5 mg of the trityl-protected SST-SHA was obtained with a retention time of 8.4 min.

The trityl-protected SST-SHA was dissolved in 1 mL of ACN/$H_2O$ (1:1), to which 150 µL TFA and 120 µL of triisopropylsilane were added. After 3h reaction time at room temperature, the reaction solution was concentrated and purified by preparative HPLC using an Atlantis Prep OBD T3 column (19x100 mm, 5 mm) with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) (0–5 min). In further 5 min, phase B was raised to 36% and kept for 8 min long. Finally mobile phase B was raised to 100% within 5 min with a flow rate of 10 $mLmin^{-1}$ in total. The absorbance was monitored at 220 and 280 nm. The deprotected product SST-SHA **7** was obtained from lyophilization in 27% overall yield (1.5 mg) and its retention time was 22.0 min. Detailed analysis data are displayed in Figure S6.

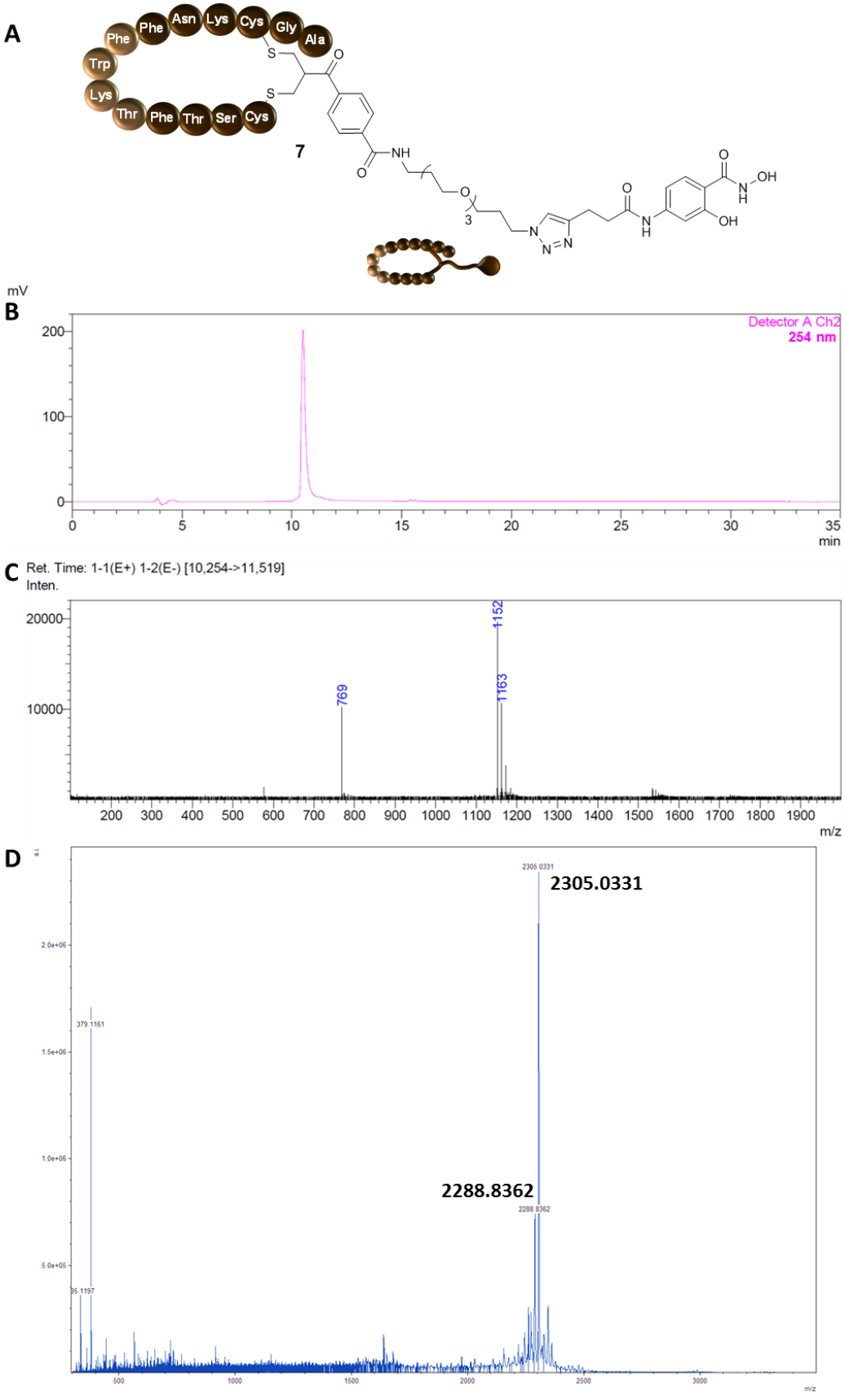


**Figure S6.** SHA-functionalized SST **7** **A)** Chemical structure, **B)** UV trace during LC-MS analysis – detection at 254 nm, **C)** MS analysis (ESI, LC-MS system) of **7** given by retention time of 10.6 min (formula: $C_{109}H_{146}N_{24}O_{28}S_2$, calculated exact mass: 2303.02 [M], 1152.51 $[M+2H]^{2+}$, 768.67 $[M+3H]^{3+}$, 576.76 $[M+4H]^{4+}$), **D)** HR-Maldi ToF MS analysis of **7**.

### 2.5. Synthesis of Cy3 labelled SST-SHA (2Cy3-SST-SHA) 8

SST-SHA **7** (1.00 mg, 0.434 µmol, 1.0 eq) was dissolved in 360 µL of sodium-hydrogencarbonate buffer pH 8.3 (100 mM), to which Cy3-NHS **vii** (0.73 mg, 0.477 µmol, 2.6 eq) pre-dissolved in 60 µL dimethylsulfoxide was added sequentially. After the reaction solution was gently shaken at room temperature overnight, the raw product precipitated out of solution. The precipitates were centrifuged down for 15 min at 10k rpm and the supernatant solution was removed. The crude product was re-dissolved in methanol/acetonitrile (1:1) and purified by semi-preparative HPLC using an Eclipse XDB-C18 Phenomenex column (9.4x250 mm, 5 µm) with the mobile phase starting from 95% solvent A (0.1% TFA in water) and 5% solvent B (0.1% TFA in acetonitrile) (0–1 min). In further 26 min, phase B was raised to 100% and kept for 2 min long. Finally mobile phase B was reduced to 5% within 3 min with a flow rate of 4 $mLmin^{-1}$ in total. The absorbance was monitored at 254 and 550 nm. The product 2Cy3-SST-SHA **8** was obtained from lyophilization in 21% yield (0.29 mg) and its retention time was 19.4 min. Detailed analysis data are displayed in Figure S7.

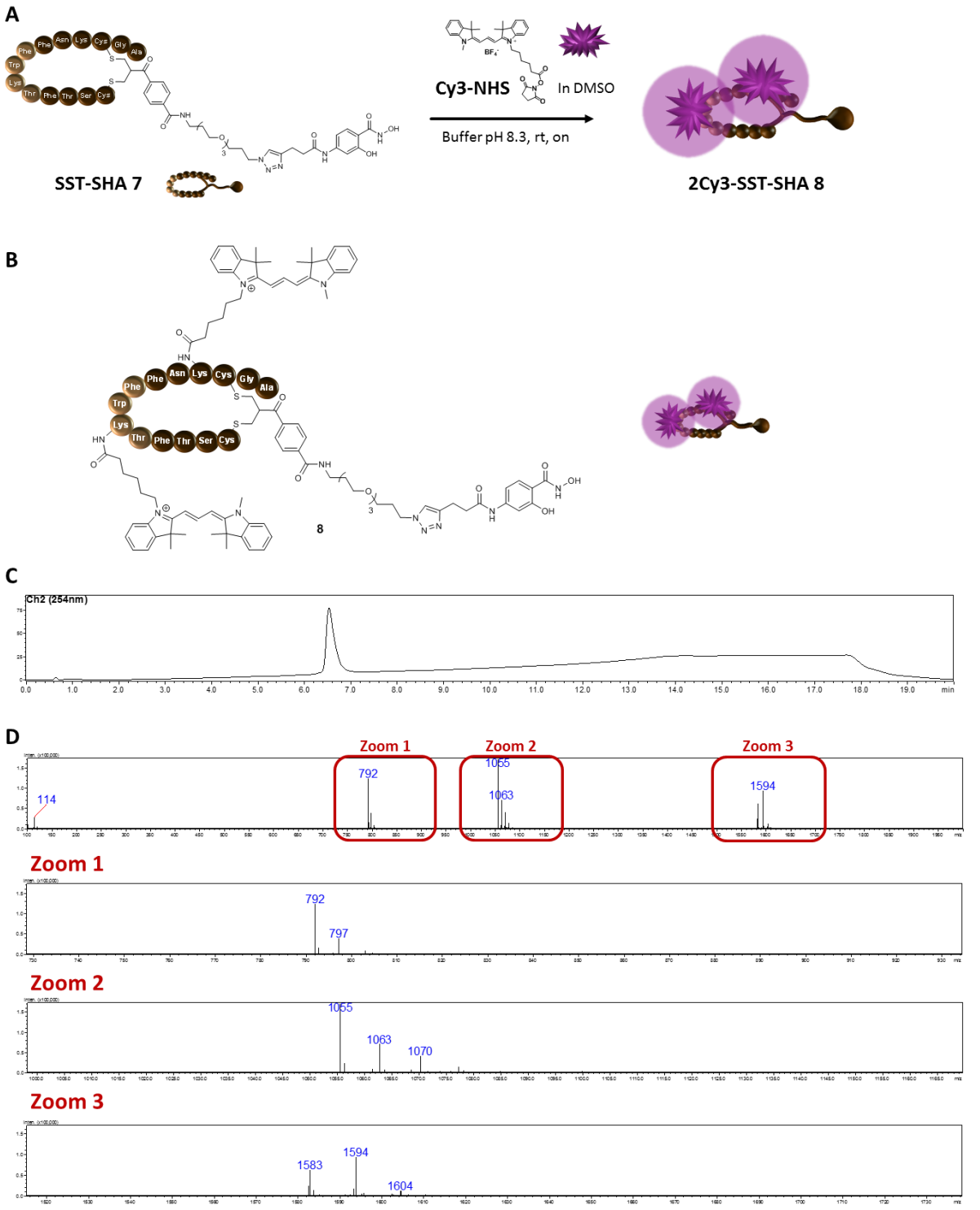


**Figure S7.** Cy3-labelled SST-SHA **8 A)** Reaction scheme, **B)** Chemical structure, **C)** UV trace during LC-MS analysis – detection at 254 nm, **D)** MS analysis (ESI, LC-MS system) of **8** given by retention time of 6.54 min (formula: $C_{169}H_{216}N_{28}O_{30}S_2$, calculated exact mass: 3181.57 [M], 1592.92 $[M+2H]^{2+}$, 1062.29 $[M+3H]^{3+}$, 796.97 $[M+4H]^{4+}$).

## 3. Experimental Section: Protein Modification and Assays

### 3.1. Materials and Methods

Human serum albumin (HSA) and cytochrome c (CytC) from equine heart were purchased from Sigma-Aldrich. All other chemicals/biologicals including alizarin red s, diammonium-2,2′-azino-di-(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS), Cy5-NHS and others are purchased from multiple commercial sources (Merck, Alfa Aesar, Invitrogen, Lumiprobe, etc.) and used directly without further purification. The grade of water ($H_2O$) used is MilliQ ultrapure grade. Ultrafiltration purification is performed using Vivaspin ultrafiltration tubes (GE healthcare) with molecular weight cut-off (MWCO) of 5 kDa (for CytC derivatives) and 30 kDa (for HSA derivatives). Fluorescence and absorbance measurements are taken from Tecan Spark 20M microplate reader. Microscale thermophoresis measurements are performed using a Monolith NT.115 instrument and the data are analysed by NT.Analysis software (both from NanoTemper Technologies GmbH). Dynamic light scattering experiments are carried out using ALV systems equipped with a He-Ne laser (632.8 nm). Mass analyses of proteins were performed using a Bruker Time-of-flight MS rapifleX to obtain MALDI-TOF-MS spectra.

### 3.2. Boronic acid modification of proteins (HSA, CytC)

In Figure S8 the boronic acid modification is displayed exemplary for cytochrome c (CytC), in which the reaction to generate the activated boronic acid **ix** is displayed in part **A**. The compound was prepared as published by our group previously[2] and analytical data were in accordance with the literature.

In Figure S8 **B**, the protein modification is shown, which was conducted to obtain CytC-BA and HSA-BA. The procedure is described exemplary for CytC-BA. The pre-activated boronic acid **ix** (20.18 mg, 0.077 mmol, 19 eq) was dissolved in 100 µL dimethylsulfoxide and added to cytochrome c (50.00 mg, 0.004 mmol, 1 eq), which was already dissolved in 5 mL phosphate buffer (50 mM, pH 8.0). The reaction was shaking overnight at room temperature and purified using Vivaspin ultrafiltration tubes (5 kD MWCO) the next day by washing with MilliQ ultrapure water. This was performed at 3800 rpm and 4 °C to obtain a concentrated, salt free protein solution, which was characterized by MALDI-TOF MS (see Figure S9 for conversion of native CytC to CytC-BA and Figure S10 for conversion of native HSA to HSA-BA). After freeze-drying, the product was obtained in 96 % yield in its powdery form.

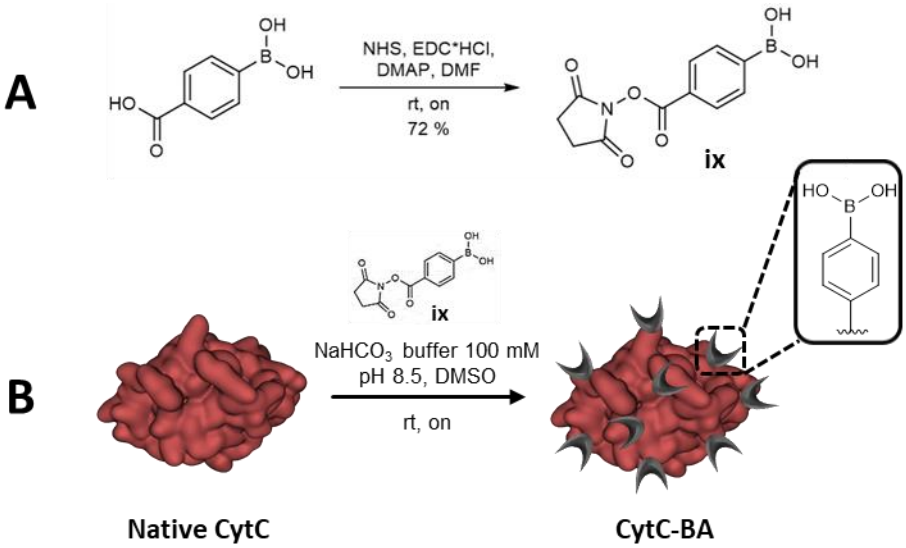


**Figure S8.** Boronic acid functionalization of proteins (HSA, CytC) exemplary shown for CytC; **A)** NHS ester activation of 4-Carboxyphenylboronic acid, **B)** Protein modification in statistical manner via addressing the accessible lysine residues to form an amide bond.

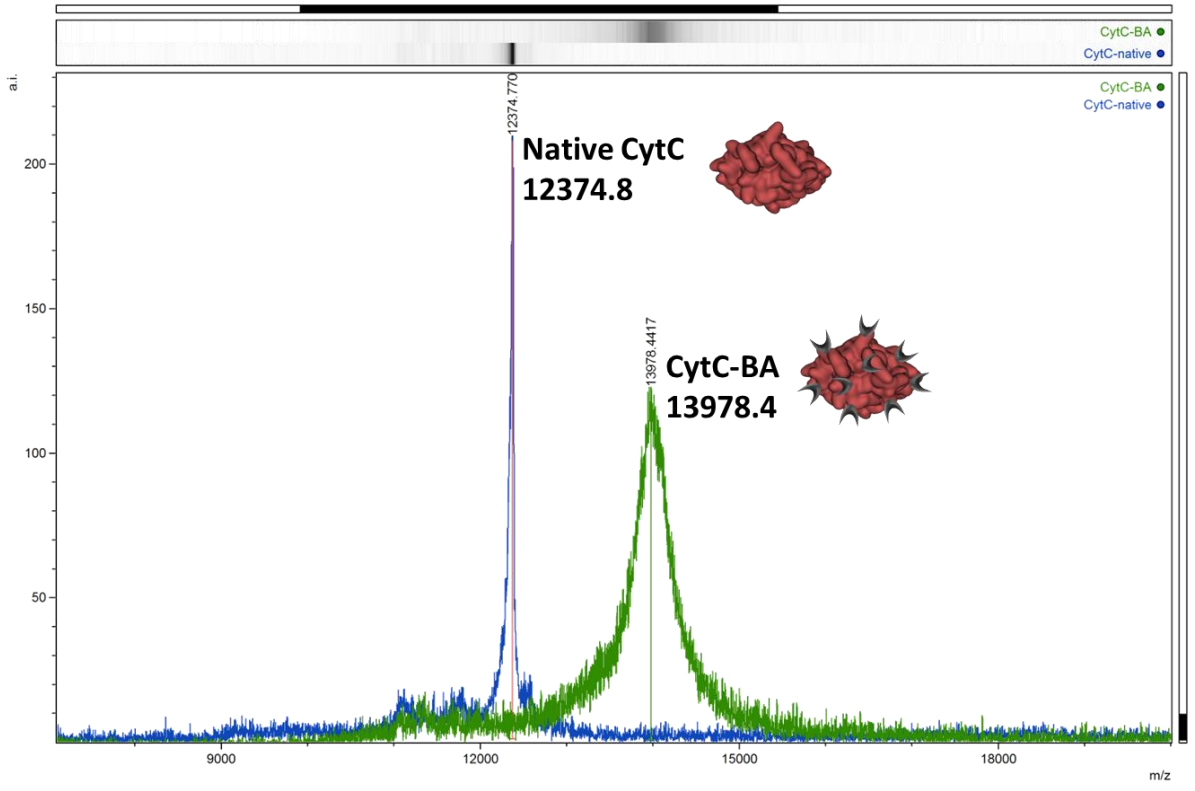


**Figure S9.** Protein characterization via Maldi ToF MS. The native CytC (blue) as well as the CytC-BA (green) were measured and the number of boronic acid groups was calculated via mass difference. CytC-BA was turned out to have 11 BA groups on average.

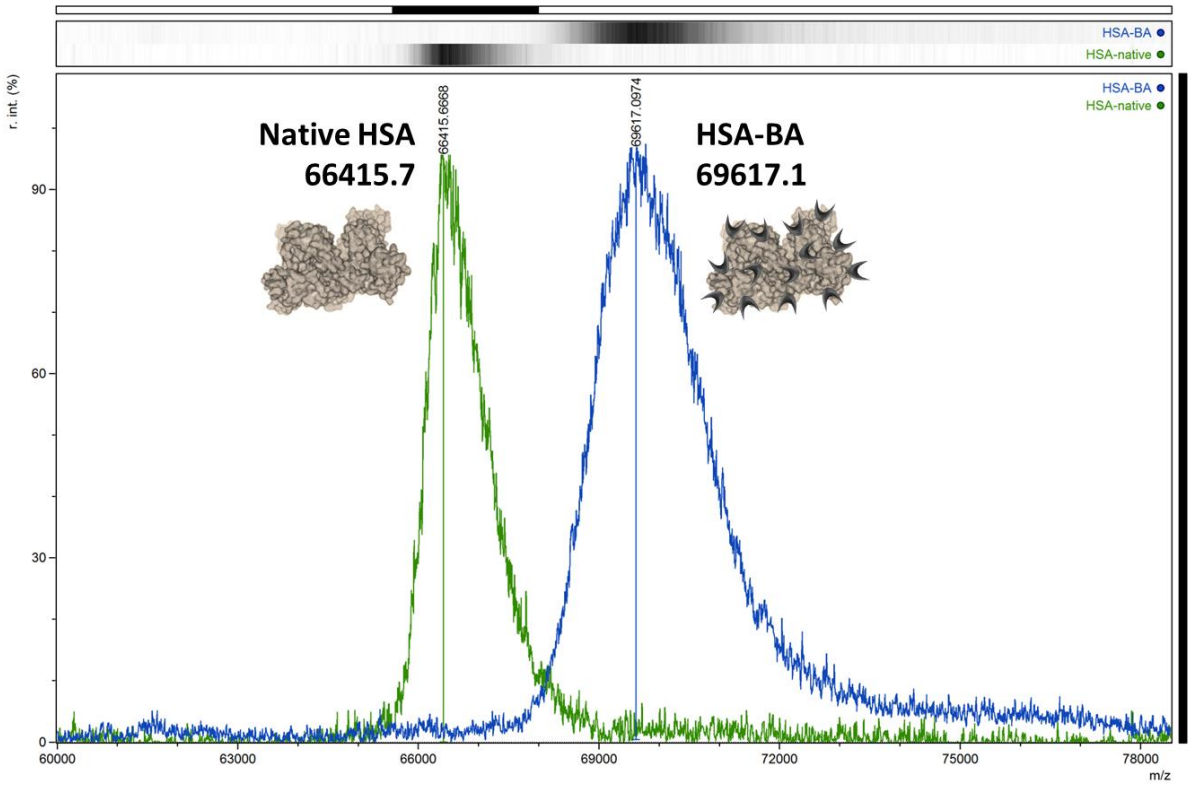


**Figure S10.** Protein characterization via Maldi ToF MS. The native HSA (green) as well as the HSA-BA (blue) were measured and the number of boronic acid groups was calculated via mass difference. HSA-BA was turned out to have 22 BA groups on average.

### 3.3. Dye (rhodamine, cyanine5) labelling of proteins (HSA, CytC)

CytC-BA as well as HSA-BA were labelled with Cy5-NHS **viii** and Rho-ITC **x** as displayed in Figure S11. The procedure to prepare the dye labelling is described exemplary for CytC-BA in the following protocol.

CytC-BA (10.00 mg, 0.72 µmol, 1 eq) was dissolved in 2 mL phosphate buffer (50 mM, pH 8.0) and Cy5-NHS **viii** (0.44 mg, 0.72 µmol, 1 eq., pre-dissolved in dimethylsulfoxide to prepare 10 mg/mL) was added to the protein solution. The reaction mixture was shaken in the dark at 350 rpm overnight at room temperature. Purification was performed using Vivaspin ultrafiltration tubes (5 kD MWCO) by washing with MilliQ ultrapure water until no colour due to free dye was left in the filtrate. The concentrated protein solution was freeze dried to obtain salt free solid in 97% yield.

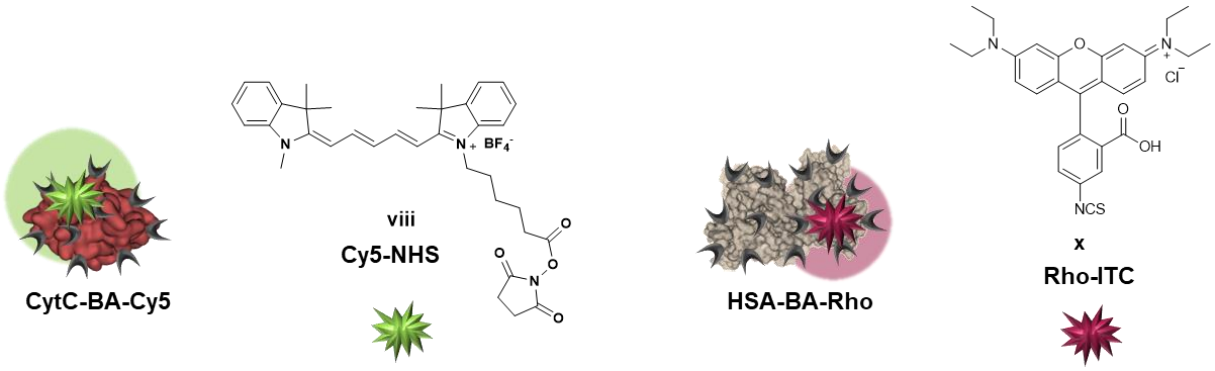


**Figure S11.** The Cy5-NHS **viii** dye was used for labelling of the CytC-BA to obtain CytC-BA-Cy5, whereas HSA-BA was labeled with Rho-ITC **x** to yield in HSA-BA-Rho.

### 3.4. Alizarin red S fluorescence titration (HSA, CytC)

CytC-BA as well as HSA-BA were used for fluorescence titration binding alizarin red s to the boronic acid units firstly and replacing the alizarin red s by salicylhydroxamic acid **iv** in exchange steps secondly. Via drop in fluorescence intensity, this ligand exchange can be seen. The procedure is described exemplary for CytC-BA and was performed adequately with HSA-BA.

CytC-BA (15 µL, 1 mg/mL in 50 mM phosphate buffer pH 7.4) was transferred into a black LoBase clear bottom 384 wellplate and subsequently added alizarin red s (0.5 µL, 10 mg/mL in DMSO ≡ 14 eq). After incubation in an orbital shaker for 10 min, the fluorescence ($\lambda_{ex}$ = 495 nm, $\lambda_{em}$ = 520-720 nm, 2 nm steps) was measured via top reading. Salicylhydroxamic acid (SHA) **iv** (0.32 mg/mL in DMSO) was added in aliquots of 0.5 µL (corresponding to 1 mol. eq per addition), mixing via orbital shaking for 5 min after each addition. Fluorescence scans were taken after each addition & incubation step ($\lambda_{em, max}$ = 600 nm). Addition of **iv** was stopped, when no further emission changes were observed due to the end point of the alizarin red s – SHA **iv** exchange.

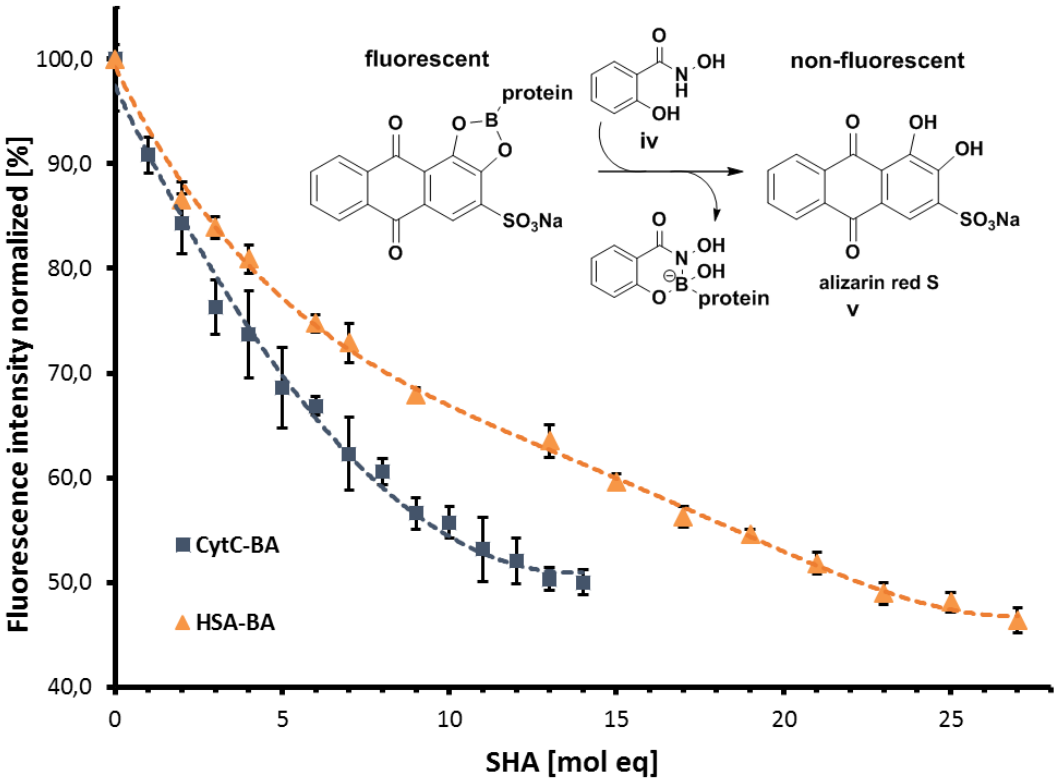


**Figure S12.** Alizarin red s fluorescence titration of CytC-BA (blue, squares) and HSA-BA (orange, triangle) for BA group determination via binding ligand exchange and therefore drop in fluorescence. CytC-BA was shown to have ~ 11 BA groups and HSA-BA to have ~ 22 BA groups.

### 3.5. Microscale thermophoresis for binding affinity determination (CytC)

CytC-BA-Cy5 was used as fluorescent binding partner and generated as described above. Dissolved in microscale thermophoresis (MST) buffer (50 mM TrisHCl pH 7.4, 150 mM NaCl, 10 mM MgCl2), the concentration of CytC-BA-Cy5 was adjusted to 500 nM for the MST measurements. SST-SHA **7** was chosen as ligand and a series of 16 dilutions 1:1 for each sample was prepared using the identical buffer (MST buffer) producing ligand concentrations ranging from 24.4 nM to 800 µM. For thermophoresis, each ligand dilution was mixed with one volume of CytC-BA-Cy5, which leads to a final concentration of fluorescently labelled protein of 250 nM and final ligand concentrations ranging from 12.2 nM to 400 µM. After 2h incubation at room temperature, followed by centrifugation at 13 000 x *g* for 5 min, approximately 8 µL of each solution was filled into Monolith NT Standard Treated Capillaries (NanoTemper Technologies GmbH). Thermophoresis was measured using a Monolith NT.115 instrument (NanoTemper Technologies GmbH) at an ambient temperature of 25 °C with 5 s/30 s/5 s laser off/on/off, respectively. Instrument parameters were adjusted to 30 % LED power and 20 %

MST power. Data of three independently pipetted measurements were analysed (NT.Analysis software, NanoTemper Technologies GmbH) using the fluorescence signal.

Release study (cleavage of SST-SHA **7** from CytC-BA-Cy5 protein core) initiated by drop in pH value due to addition of hydrogenchloride solution was performed using the same solutions, which were measured for binding event determination to obtain the $K_d$. Therefore, 10 µL measurement solution of each dilution step was incubated with 0.2 µL hydrogenchloride solution (1M) for 2h before these solutions were loaded onto Monolith NT Standard Treated Capillaries (NanoTemper Technologies GmbH) and measured using the same parameters & conditions as before.

### 3.6. Colorimetric assay related to enzyme activity (CytC)

The experiment was performed using a 384 transparent, flat bottom microplate (Greiner UV-Star®). C-SST8 (9 µL, 80 µM, 1h pre-incubation in 100 mM phosphate buffer pH 7.4), phosphate buffer pH 7.4 (100 mM, 6 µL) as well as ABTS solution (10 mM, 2 µL) were filled into each well and pre-mixed. As reference to C-SST8, CytC-BA solution (80 µM) was measured adding the same reagents in the same ratio to it. First measurement was performed before addition of hydrogen peroxide solution. The enzyme activity assay was started by addition of hydrogen peroxide solution (10 mM, 2 µL) to each well. Optical images displayed in Figure S13 as well as absorbance scans from 250 nm to 850 nm ($\lambda_{max}$ = 410 nm) were taken before start of the assay and after $ABTS^{\bullet+}$ saturation. To obtain the raising band at 410 nm due to ABTS conversion, kinetic loop measurements were performed. After addition of hydrogen peroxide, orbital shaking for 3s and resting time for 1s was done to homogenize the solutions. Kinetic loop measurements were done afterwards for every 10s by absorbance measurement at 410 nm for 6 min long. Data points were presented in manuscript Figure 3 at 30s, 60s, 90s, 120s, 240s and 360s. All samples were carried out in triplicates.

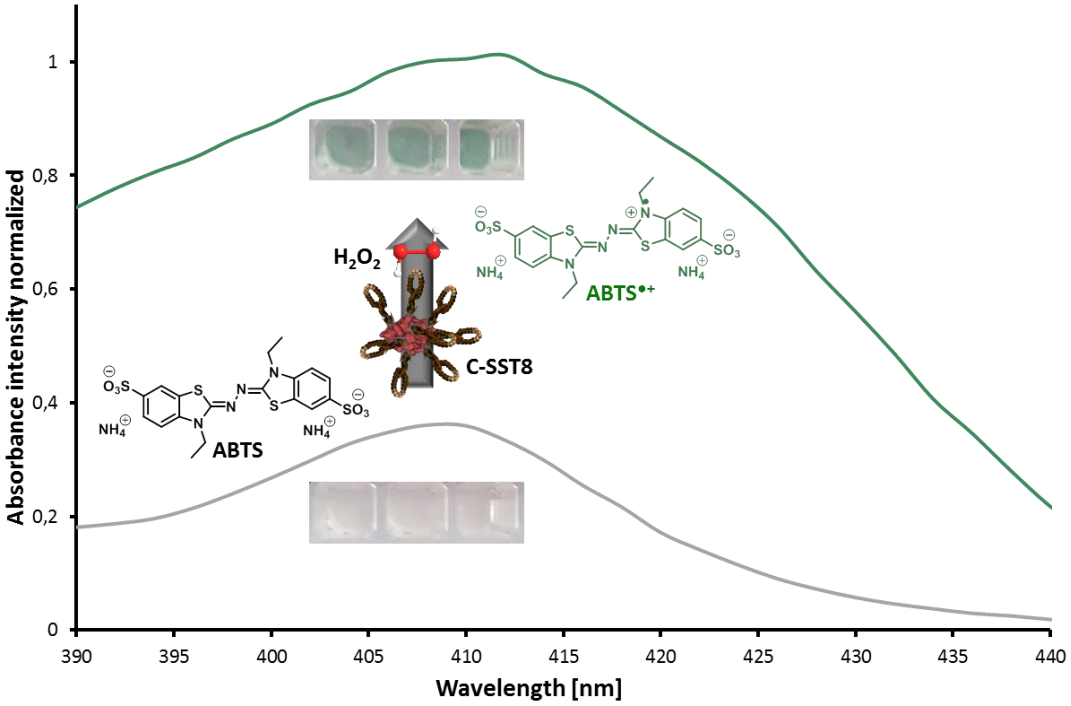


**Figure S13.** Redox-activity of CytC was checked via ABTS assay using C-SST8 as the active species. The absorbance was monitored and increased during conversion of the substrate into its radical cation.

### 3.7. Dynamic light scattering (CytC)

#### 3.7.1. DLS of CytC-nat, CytC-BA and C-SST8 in PB pH 7.4

The hydrodynamic size distribution was characterized exemplary for the protein cytochrome c (native CytC ≡ CytC-nat, CytC-BA and C-SST8) by dynamic light scattering (DLS). Size increase in hydrodynamic radius from CytC-BA to C-SST8 due to assembly of SST-SHA **7** onto the protein surface was investigated. Beforehand, the native CytC (CytC-nat) was analysed. All samples were prepared at 1 mg/mL concentrations in phosphate buffer pH 7.4 (50 mM). They were incubated for 15 min and then filtered through 0.22 µm syringe filters before DLS measurements to avoid dust contamination. For CytC-nat, $R_h$ of 1.9 nm was obtained with a neglegtible amount of protein aggregate, which was monitored due to the high method sensitivity (see Figure S14). Hydrodynamic radii of CytC-BA and C-SST8 turned out to be 3.8 nm and 6.2 nm with neglegtible amount of protein aggregates monitored due to high method sensitivity. The size difference in $R_h$ of 2.4 nm fits to theoretical calculations of SST-SHA **7** (see Figure S15).

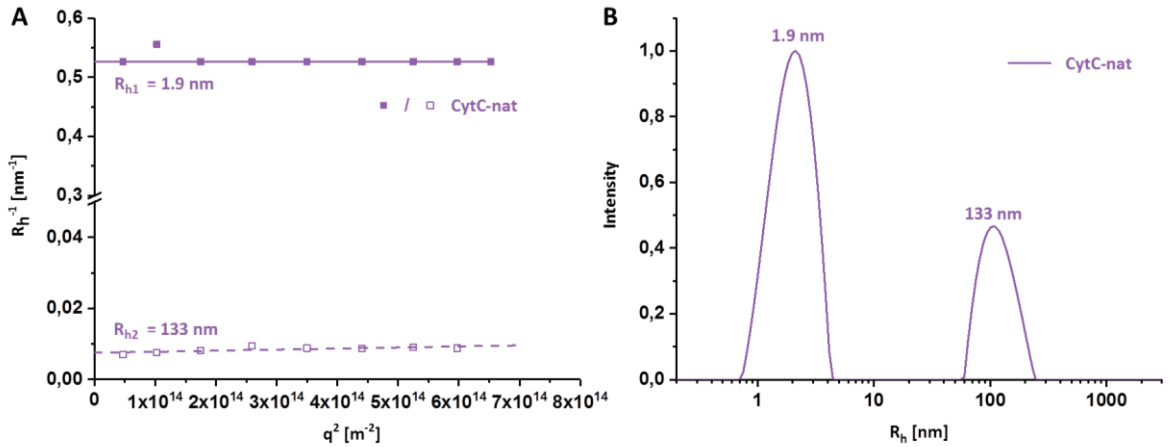


**Figure S14.** DLS data of native CytC (CytC-nat) after incubation in PB pH 7.4 **A)** Hydrodynamic radius of CytC-nat was monitored in 1 mg/mL concentration at room temperature and 632.8 nm to turn out as 1.9 nm (different angles were screened). A neglegtible amount of protein aggregate was formed and monitored due to the high method sensitivity. **B)** Intensity spectrum of CytC-nat is shown for the 90° measurement to highlight the neglegtible amount of the protein aggregate observed.

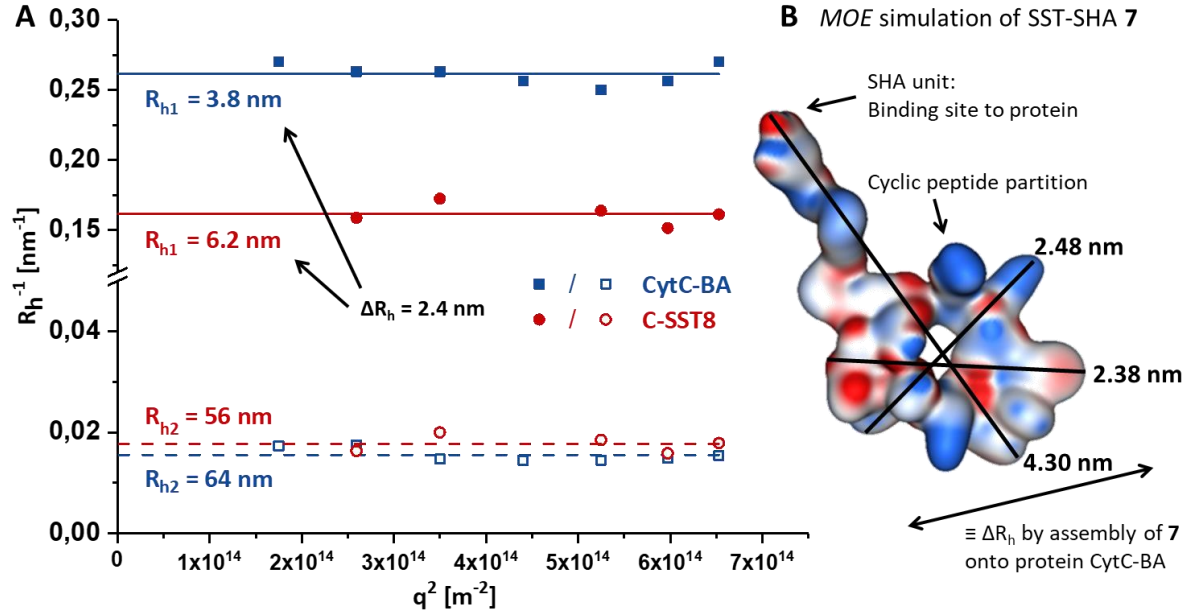


**Figure S15.** DLS data of CytC-BA and C-SST8 after incubation in PB pH 7.4 **A)** Hydrodynamic radius of CytC-BA and C-SST8 was monitored in 1 mg/mL concentration at room temperature and 632.8 nm to turn out as 3.8 nm for CytC-BA and 6.2 nm for C-SST8 (different angles were screened). A neglegtible amount of protein aggregates were formed and monitored due to the high method sensitivity. **B)** The 3D structure of SST-SHA (**7**) with minimized energy was simulated via the software *Molecular Operating Environment (MOE)* and molecule dimensions were measured. The $\Delta R_h$ with 2.4 nm due to SST-assembling on the protein measured by DLS can be proved by the theoretical calculation.

### 3.7.2. DLS of CytC-nat, CytC-BA and C-SST8 in r-SBF

To investigate how such constructs could behave in physiological context, CytC-nat, CytC-BA as well as the self-assembled C-SST8 were studied within *revised simulated body fluid* (r-SBF). As also described in Figure S16, the r-SBF contains the same ion concentrations as blood plasma and was prepared following a literature known preparation protocol.[3]

**Ion concentrations [mM]**

| Ion | Blood Plasma Total | r-SBF |
|---|---|---|
| $Na^+$ | 142,0 | 142,0 |
| $K^+$ | 5,0 | 5,0 |
| $Mg^{2+}$ | 1,5 | 1,5 |
| $Ca^{2+}$ | 2,5 | 2,5 |
| $Cl^-$ | 103,0 | 103,0 |
| $HCO_3^-$ | 27,0 | 27,0 |
| $HPO_4^{2-}$ | 1,0 | 1,0 |
| $SO_4^{2-}$ | 0,5 | 0,5 |

**Figure S16.** Concentration of the listed ions in mM for the revised simulated body fluid (r-SBF) is in accordance with the known ion concentrations within blood plasma.

For all samples (CytC-nat, CytC-BA, C-SST8) the solids were dissolved in r-SBF resulting in 1 mg/mL concentrations and incubated for 1h at room temperature. Before DLS measurements, the samples were filtered through 0.22 µm syringe filters into the DLS cuvettes. All samples were investigated in their hydrodynamic radii after 1.5h, 24h and 48h further incubation. Figure S17 shows the obtained values for CytC-nat. Given as main fraction, the expected 1.6-1.8 nm protein size was observed, whereas protein aggregates increasing in $R_h$ from 163-308 nm over time were also tracked within the sample solution. Such protein clustering upon higher salt concentrations are known, where interaction of salt ions with protein partitions can lead to oligomerization of proteins[4] and therefore lead to higher hydrodynamic radii. Similar effect of protein oligomerization is seen for the CytC-BA and C-SST8 samples as shown in Figure S18, where the $R_h$ increased 4-5 times from the samples incubated at PB pH 7.4 (CytC-BA: 3.8 nm, C-SST8: 6.2 nm) to the samples incubated in r-SBF pH 7.4 (CytC-BA: 18.3-22.0 nm, C-SST8: 24.5-25.2 nm). For the samples CytC-BA and C-SST8 incubated in r-SBF, bigger protein aggregates were not observed, which might be an effect of the boronic acid modification of the proteins preventing bigger aggregates due to interaction of the boronic acids with the ions within r-SBF.

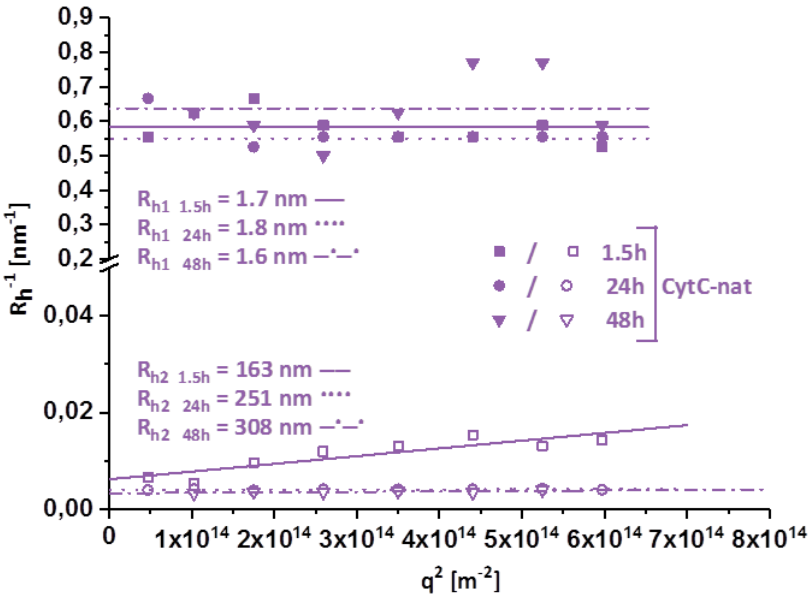


**Figure S17.** DLS data of native CytC (CytC-nat) after incubation in r-SBF. Hydrodynamic radius of CytC-nat was monitored in 1 mg/mL concentration at room temperature and 632.8 nm and was measured after different incubation times (1.5h, 24h, 48h) for different angles. Next to the main fraction of detected structures at around 1.6-1.8 nm in hydrodynamic radius, a small amount of protein aggregates increasing in size over incubation time (163-308 nm) was formed and monitored due to the high method sensitivity.

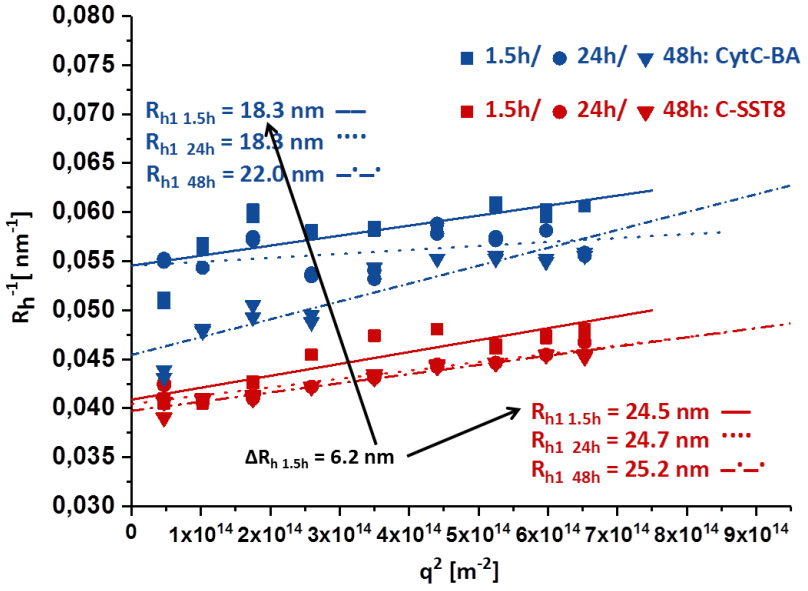


**Figure S18.** DLS data of CytC-BA and C-SST8 after incubation in r-SBF. Hydrodynamic radius of CytC-BA and C-SST8 was monitored in 1 mg/mL concentration at room temperature and 632.8 nm and was measured after different incubation times (1.5h, 24h, 48h) for different angles. The hydrodynamic radius turned out to be 18.3 nm for CytC-BA and 24.5 nm for C-SST8 after 1.5h incubation time, yielding in a difference in $R_h$ of 6.2 nm. Longer incubation times of CytC-BA and C-SST8 within r-SBF led to very slightly increases in hydrodynamic radii as displayed within the diagram.

### 3.8. FRET study on CCy5-SSTCy3 without cells (CytC)

Figure S19**A** displays the three different solutions, which were prepared for the FRET studies: **I**) mixture of **7** / **8**, **II**) CytC-BA-Cy5 and **III**) CCy5-SSTCy3. After the solids were dissolved within PBS pH 7.4 from cell culture (2Cy3-SST-SHA **8** was pre-dissolved in sterile dimethyl sulfoxide to result after dilution with PBS in a solution with less than 2.5 % dimethyl sulfoxide in total), the molecules were incubated for 40 min at room temperature to generate the self-assemblies (**III**) and then transferred into a black LoBase clear bottom 384 wellplate. Concentration therefore was 5 µM of CytC-BA-Cy5, which was diluted 1:1 by the solution of **7** / **8** (2 eq / 2eq) resulting in a 2.5 µM protein solution to generate CCy5-SSTCy3 (**III**). Solution **I** and **II** were diluted 1:1 with buffer to make the concentration dependent signal intensities comparable. The measurements were performed in triplicates and each well was filled with 20 µL of the studied solution. Fluorescence intensity was excited at 535 nm (5 nm bandwidth) and emission was scanned from 550-750 nm (5 nm bandwidth) as shown in Figure S19**B**. Protein release demonstrated by disrupted FRET signal due to peptide shell cleavage (**IV**) was obtained via acidification of solution **III**. Therefore, 1 µL of hydrogenchloric acid (HCl, 0.5 M) was added into each well and orbital shaken for 5 min. Fluorescence intensity scans were taken under conditions as described before at certain time points (0 min, 5 min, 17 min, 30 min) to reveal the full shell cleavage after 30 min, which is sketched in Figure S19**C** and **D**.

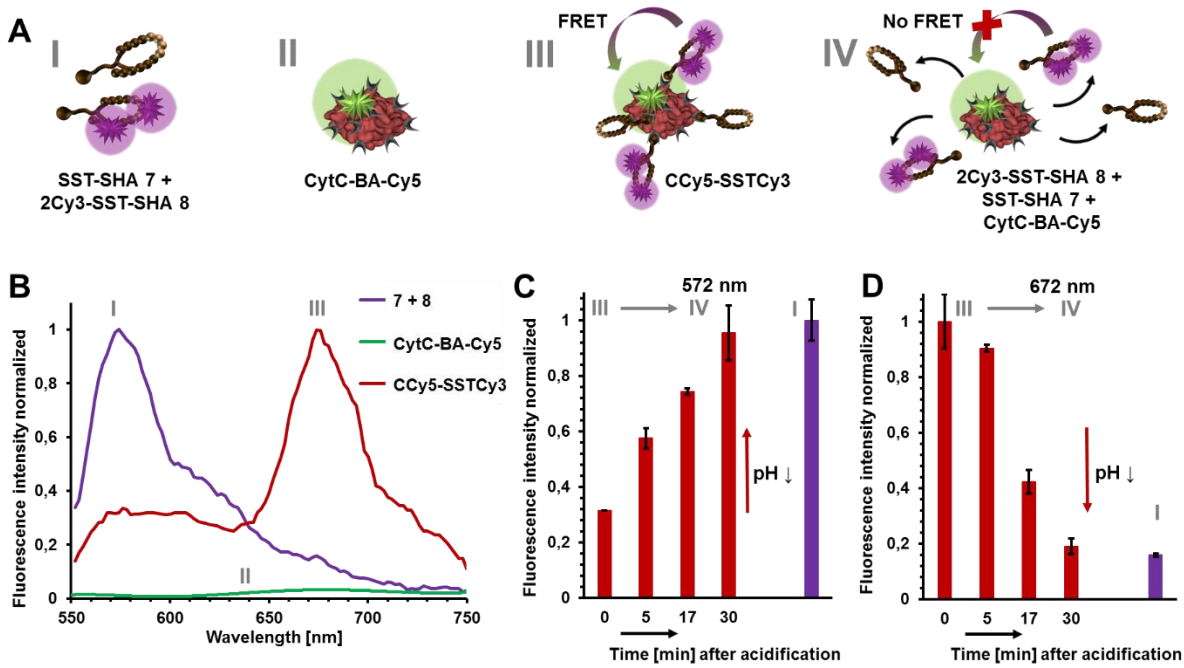


**Figure S19.** FRET study on CCy5-SSTCy3 **A)** Compound solutions, which were prepared to generate CCy5-SSTCy3 (**III**), are a mixture of **7** / **8** (**I**) and the CytC-BA-Cy5 (**II**). After disassembly of **7** / **8** from CytC-BA-Cy5, FRET signal becomes disrupted (**IV**). **B)** Normalized fluorescence intensity spectra at $\lambda_{em}$ = 550-750 nm are displayed for solution **I** (violet line, containing **7** / **8**), **II** (green line, CytC-BA-Cy5) and **III** (red line, CCy5-SSTCy3). Excitation was conducted at $\lambda_{ex}$ = 535 nm (5 nm bandwidth), for which Cy3-labeled molecules (**I**) are possible to detect as well as a FRET-induced fluorescence band of CCy5-SSTCy3 (**III**). **C)** Fluorescence signal increase at $\lambda_{ex}$ = 535 nm/$\lambda_{em}$ = 572 nm for protein release (**III** → **IV**) due to drop in pH; Cy3-related signal intensity of **IV** was comparable to **I** (red bars: CCy5-SSTCy3 ≡ **III** → **IV**; violet bar: **7** + **8** ≡ **I**). **D)** Fluorescence signal decrease at $\lambda_{ex}$ = 535 nm/$\lambda_{em}$ = 672 nm for protein release (**III** → **IV**) due to drop in pH; Cy3-related signal intensity of **IV** was comparable to **I** (red bars: CCy5-SSTCy3 ≡ **III** → **IV**; violet bar: **7** + **8** ≡ **I**).

## 4. Experimental Section: Biological Assays and Cellular Studies

### 4.1. Materials and Methods

All chemicals/biologicals including DMEM cell culture medium, fetal bovine serum and penicillin/streptomycin are purchased from multiple commercial sources (Invitrogen etc.) and used directly without further purification. The grade of water ($H_2O$) used is MilliQ ultrapure grade. Luminescence measurements are taken from Tecan M1000 Infinite microplate reader and the therefore used CellTiter-Glo® solution, which is needed for cell viability assay, was

purchased from Promega and used according to the given protocol. For confocal laser scanning microscopy two different instruments were used: for all experiments except the FRET studies with cells a TCS STED CW super-resolution microscope (equipped with an incubation box and possibility of live cell microscopy) was taken, whereas a TCS SP5 confocal laser scanning microscope (inverse microscope stand) was used for the FRET experiments within A549 cells. Image processing for all confocal laser scanning microscopy experiments was performed with *Image J*. All cell-related experiments were prepared and performed within a flow hood to ensure sterile conditions. Cell culture of A549 and SKUT1 cells was performed in Dulbecco's Modified Eagle's Medium (DMEM, High Glucose) supplemented with 10% FBS, 1% penicillin/streptomycin and 1x MEM non-essential amino acid with incubation conditions set at 37 °C, 5% $CO_2$. The presented studies on binding towards the somatostatin receptor 2 (SSTR2) and hereby induced calcium flux due to receptor stimulation were conducted by the company *Eurofins Cerep*.

### 4.2. Luminescent cell viability assay (CytC)

A549 cancer cells were seeded at 5000 cells/well in a white, flat bottom, half-area 96 wellplate containing 50 µL of DMEM medium each well. Boronic acid modified CytC (CytC-BA, 100 µM stocksolution) was pre-incubated with SST-SHA **7** (400 µM stocksolution, 4 eq) in PBS pH 7.4 from cell culture for 30 min, whereas the samples SST-SHA 7 and doxorubicin (DOX) were only incubated with the described buffer. The solutions of C-SST4, **7** and DOX were diluted with DMEM medium to the final concentrations before addition to the cells. C-SST4 concentrations were given in relation to the CytC concentration (5 µM, 10 µM, 25 µM). Before sample addition to the cells , they were adhered for 24h at 37 °C and 5% $CO_2$. Samples diluted within fresh medium were added after medium of cell incubation with wellplate was removed. Each experiment was conducted in triplicates. The cells were incubated with samples for 4h (except for **7**: 24h incubation) at 37 °C, 5 % $CO_2$ and subjected to CellTiter-Glo® luminescent cell viability kit (Promega) according to manufacturer´s protocol and scanned using a Tecan M1000 Infinite microplate reader.

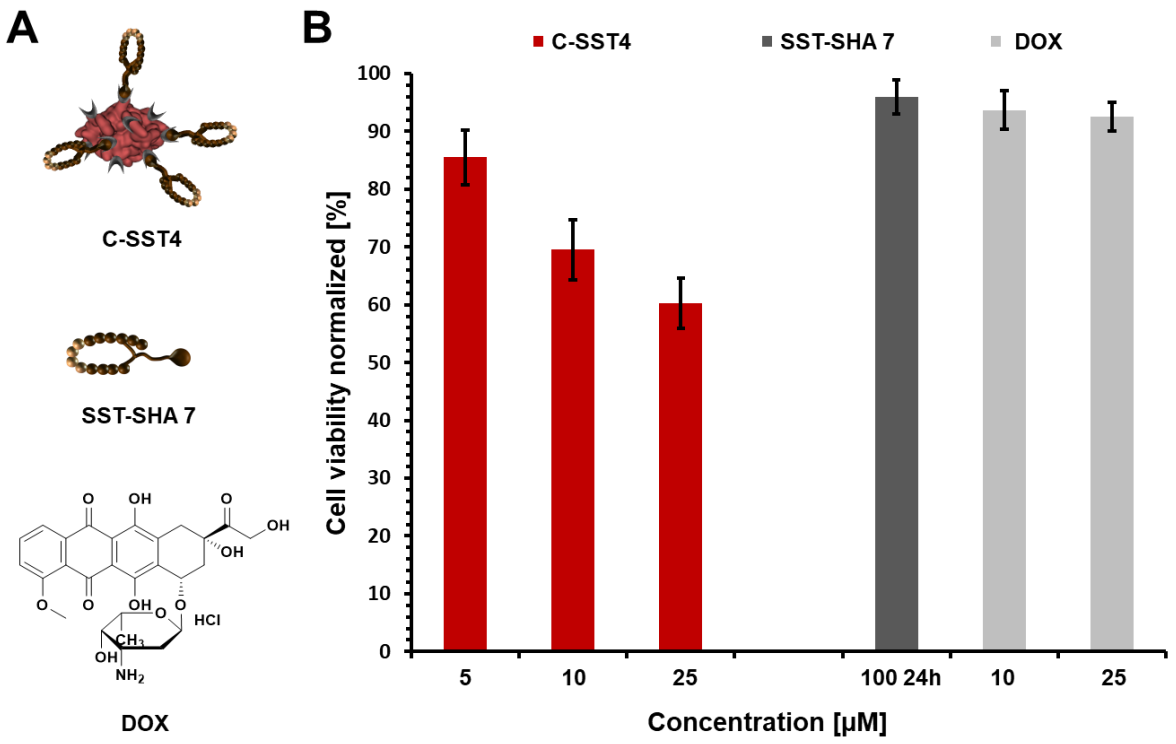


**Figure S20.** Cell viability of A549 cancer cells was investigated after cells were treated with different samples for 4h (SST-SHA **7**: 24h). **A)** Structures of all tested compounds for cell viability assay: C-SST4, SST-SHA **7** and doxorubicin (DOX). **B)** Dose-dependent decrease in cell viability was observed for C-SST4 (red bars), whereas 7 itself results in full cell viability even for 24h incubation (dark grey bar). 4h incubation of A549 cancer cells with the known chemotherapeutic DOX (light grey bars) in comparable concentrations to C-SST4 led to almost full cell viability.

### 4.3. Cellular uptake and localization (HSA, CytC)

A549 (and for one experiment set SKUT1) cells were pre-cultured in high glucose DMEM medium fortified with 10% fetal bovine serum, 1% penicillin/streptomycin and seeded at 10˙000 cells/well in a 10-well confocal microscopy chamber and left to adhere overnight at 37 °C and 5% $CO_2$. The labelled proteins (CytC-based samples: cyanine5-labeling, HSA-based samples: rhodamine-labeling) were pre-incubated with different eq ratios of SST-SHA **7** and/or 2Cy3-SST-SHA **8** (details given at each experiment itself) or only with PBS pH 7.4 from cell culture for the references. Incubation times as well as concentrations were given at the description of the individual experiments. The protein samples were added to the confocal microscopy wellplate after a certain incubation time (see individual experiment protocol), which are containing DMEM medium to afford the final sample concentration. After incubation for 4h at 37 °C and 5 % $CO_2$ (except the FRET studies: 3h incubation with cells), the medium containing the samples was removed and the cells were washed with PBS from cell culture once

and fresh DMEM medium was added before analyzing the wells using a confocal laser scanning microscope.

#### 4.3.1 Cellular uptake of CCy5-SST containing different equivalents of SST-SHA (**7**) into A549 cancer cells

For this experiment set, the cyanine5 labelled protein CytC-BA-Cy5 was used as control and also as core for all bioconjugates. SST-SHA **7** was conjugated in stoichiometrical amounts to CytC-BA-Cy5 using 2, 4, 6, 8 and 13 equivalents of **7** related to CytC-BA-Cy5. In this respect, CCy5-SST2, CCy5-SST4, CCy5-SST6, CCy5-SST8, CCy5-SST13 were generated in 2.5 µM stock concentration by dissolving the compounds under the flow hood in PBS pH 7.4 from cell culture. Afterwards, all samples were incubated for 30 min, before diluted with DMEM medium to the final concentration of 250 nM. After addition of the samples to A549 cancer cells plated on a confocal laser scanning microscopy chamber, they were incubated for 4h at 37 °C and 5% $CO_2$. Before analysis at the confocal laser scanning microscope (CLSM), the samples containing medium was removed and the cells were treated as described in the general protocol given above. After measurement at the CLSM, the images were processed with Image J. The low signal of cellular uptake of CytC-BA-Cy5 was even further reduced for demonstration purposes and all images, which are compared to that within Figure S21, were processed under the same parameters to keep comparability.

For CCy5-SST4, CCy5-SST6 as well as CCy5-SST8, significantly increased cellular uptake into A549 cancer cells was observed due to SSTR2 targeting by **7** conjugated to the Cy5-labelled protein core. For CCy5-SST13, 13 equivalents of **7** were incubated with CytC-BA-Cy5 even though only 11 boronic acid groups are available on protein surface to ensure, that all boronic acids are dynamic covalently connected with **7**. Screening CCy5-SST13 in cellular context, mainly protein aggregates outside of cells were detected. The highest cell internalization of protein conjugates was observed for CCy5-SST4, for which reason CCy5-SST4 and C-SST4 (unlabeled correspondence) were mainly used and investigated for cell-related experiments. Figure S21 shows the cellular uptake efficiency due to SST-SHA **7** equivalents revealing 4 equivalents as the most effectively targeting core-shell hybrid.

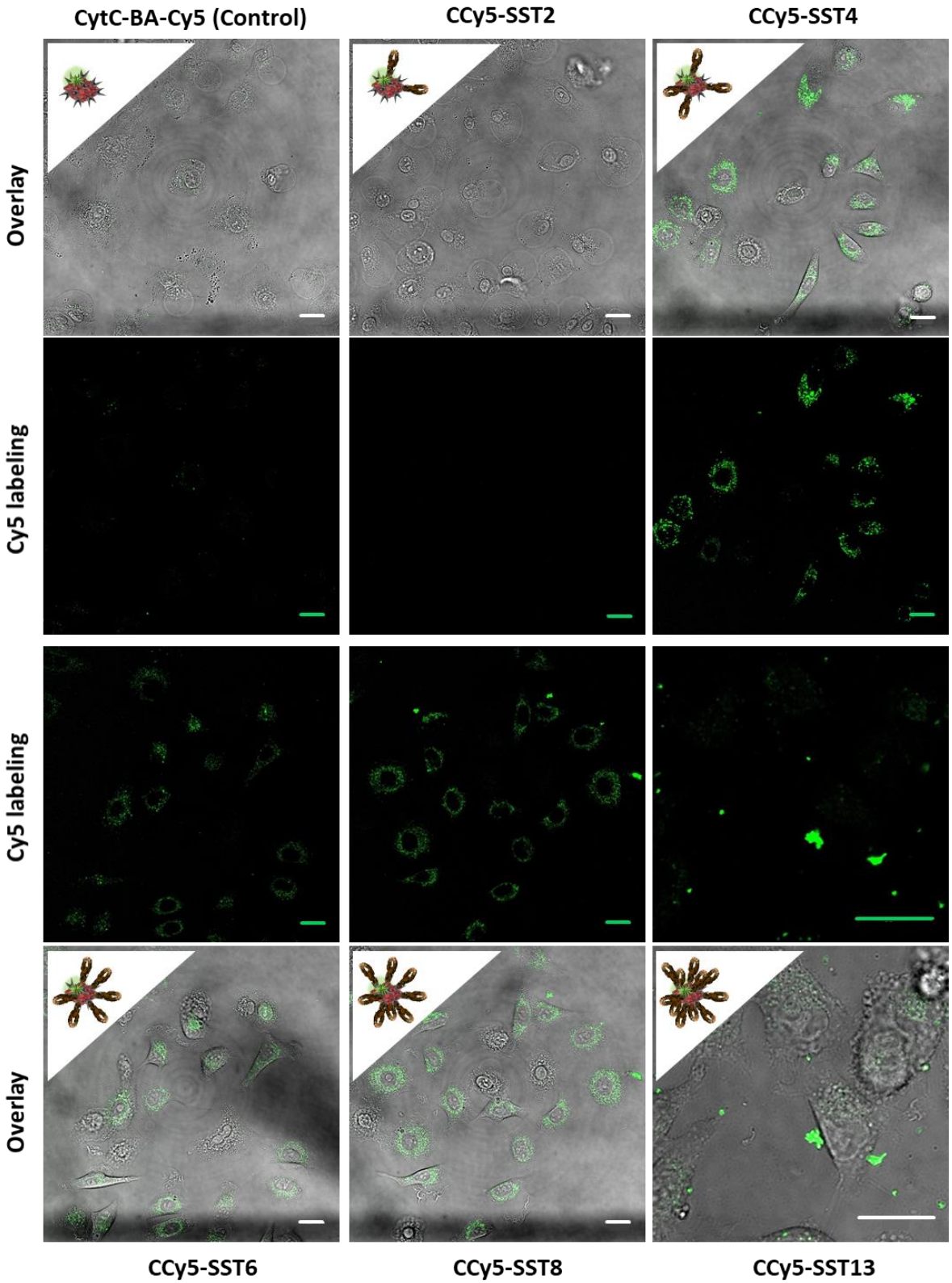


**Figure S21.** CytC-BA-Cy5 as control and different amounts of SST-SHA **7** conjugated to CytC-BA-Cy5 generating CCy5-SST2, CCy5-SST4, CCy5-SST6, CCy5-SST8 and CCy5-SST13 were investigated in their cellular uptake into A549 cancer cells. All samples were incubated for 4h at 250 nM concentration. Fluorescence signal was obtained due to $\lambda_{ex}$ = 646 nm and emission was tracked at $\lambda_{em}$ = 662 nm. The scale bar within each presented image is 20 µm and Cy5 signal is given in green.

### 4.3.2 Cellular uptake of CytC-BA-Cy5 containing different equivalents of native SST within solution into A549 cancer cells

Further investigations were conducted, whether SST-related boost in cellular uptake of core-shell hybrids is based on unspecific adsorption of SST or derivatives (**7**) onto protein surface or whether it is a result of the dynamic covalent attachment of **7** to CytC-BA-Cy5. Therefore

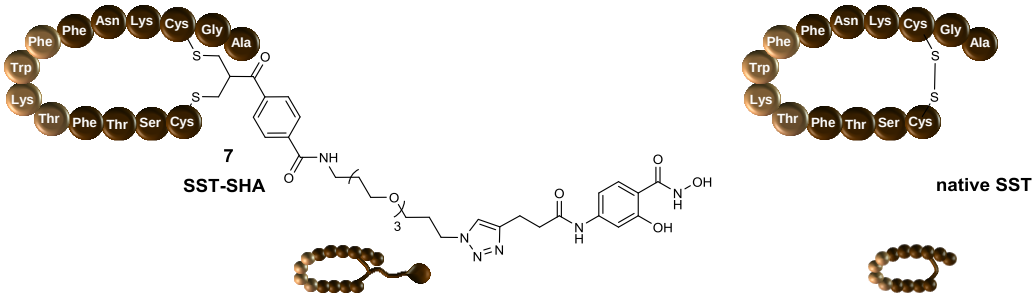


**Figure S22.** Structure of SST-SHA **7** compared to native somatostatin (native SST).

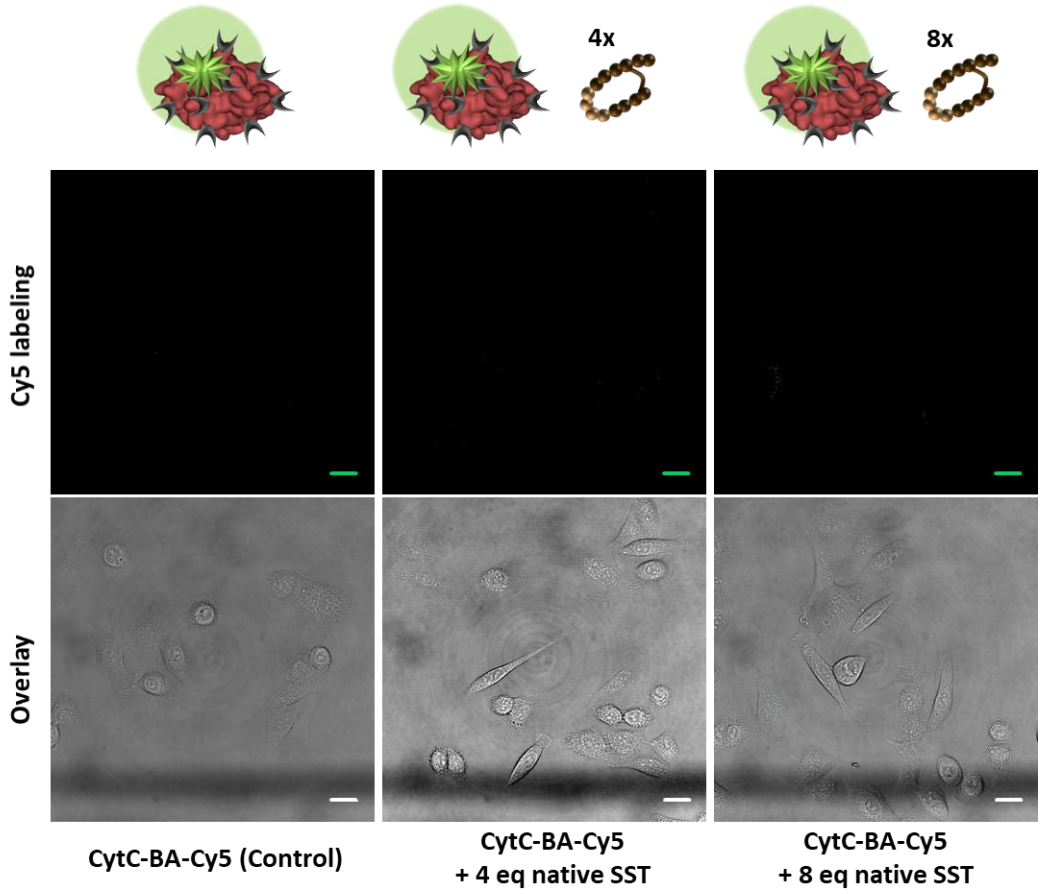


**Figure S23.** CytC-BA-Cy5 as control and CytC-BA-Cy5 pre-incubated with 4 and 8 equivalents of native SST were investigated in their cellular uptake into A549 cancer cells. All samples were incubated with cells for 4h at 250 nM concentration. Fluorescence signal was obtained due to $\lambda_{ex}$ = 646 nm and emission was tracked at $\lambda_{em}$ = 662 nm. The scale bar within each presented image is 20 µm and Cy5 signal would be displayed in green, when detectable.

CytC-BA-Cy5 was pre-incubated with native somatostatin (native SST), which is sketched in Figure S22. Native SST cannot bind to boronic acid groups on CytC-BA-Cy5 due to lack in the needed counterpart – the salicylhydroxamic acid. Figure S23 shows the result of cellular uptake of CytC-BA-Cy5 and CytC-BA-Cy5 pre-incubated with 4 as well as 8 equivalents of native SST. Cell treatment and image processing was done the same way as described in detail beforehand. As shown in Figure S23, the SST-targeting is only effective when the targeting unit is conjugated to the protein.

### 4.3.3 Cellular uptake of HRho-SST20 into A549 cancer cells

Next to SST-modified CytC, which was efficiently uptaken into A549 cells by SSTR2 targeting due to a SST-SHA **7** shell attached, modified human serum albumin (HSA) was incubated with **7** and studied in cellular uptake. Applicability of SST-targeting for selective cell internalization to other proteins was therewith studied by transfer of methodology to HSA. Labeling of HSA was performed with rhodamine isothiocyanate beforehand. HRho-SST20 (structure see Figure S24) was pre-incubated in PBS pH 7.4 from cell culture in 1 µM stock concentration (related to protein concentration) and further diluted with DMEM medium to 250 nM final concentration. After addition of the samples HSA-BA-Rho and HRho-SST20 to A549 cancer cells plated on a confocal laser scanning microscopy wellplate, they were incubated for 4h at 37 °C and 5% $CO_2$. Before analysis at the confocal laser scanning microscope (CLSM), the samples containing medium was removed and the cells were treated as described in the general protocol given above. After measurement at the CLSM, the images were processed with Image J. The low signal of cellular uptake of HSA-BA-Rho was even further reduced for demonstration purposes and the images of HRho-SST20, which are compared to that within Figure S24, were processed under the same parameters to keep comparability.

Due to the SST-SHA **7** shell within the HRho-SST20 bioconjugate, significantly increased cell internalization of protein conjugate into A549 cells was observed and proved the methodology transfer of SST-targeting to another protein class. HSA-based bioconjugates could be of interested as drug carrier systems due to their lipophilic pockets given by the protein architecture.[5] [6]

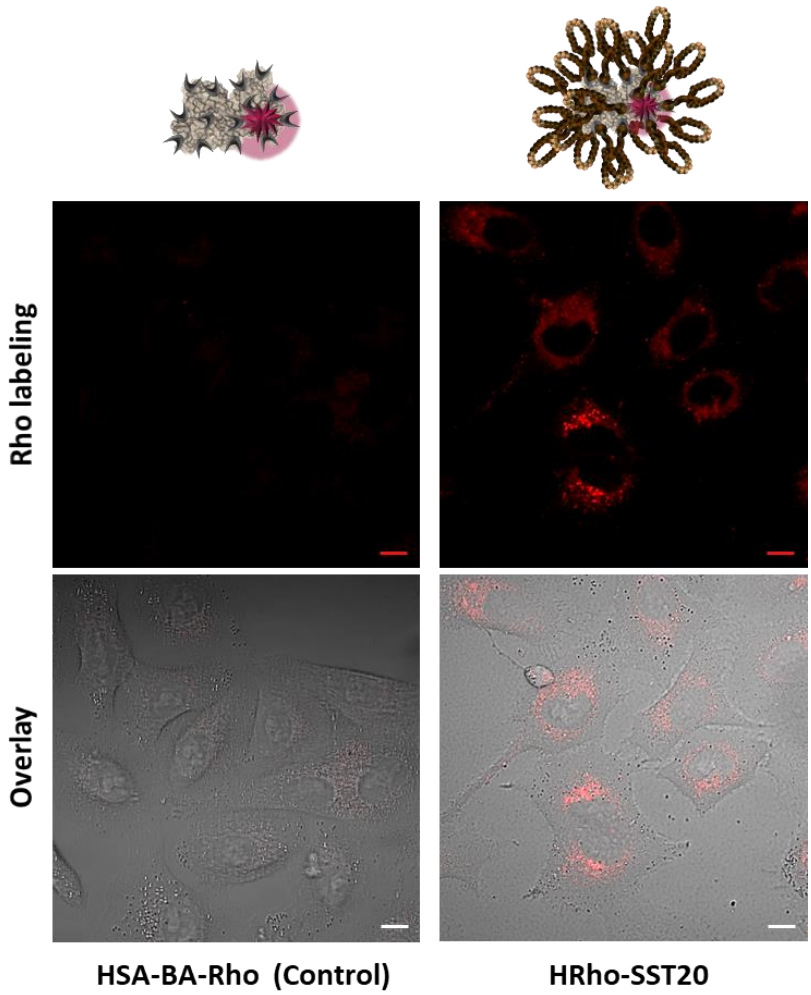


**Figure S24.** HSA-BA-Rho as control and 20 equivalents of SST-SHA **7** conjugated to HSA-BA-Rho generating HRho-SST20 were investigated in their cellular uptake into A549 cancer cells. All samples were incubated for 4h at 250 nM concentration. Fluorescence signal was obtained due to $\lambda_{ex}$ = 540 nm and emission was tracked at $\lambda_{em}$ = 580 nm. The scale bar within each presented image is 10 µm and rhodamine signal is given in red.

### 4.3.4 Cellular uptake of CCy5-SST containing different equivalents of SST-SHA (**7**) into SKUT1 cells

The somatostatin receptor 2 (SSTR2) is the main receptor addressed by somatostatin (SST) and its derivatives (SST-SHA **7**). SKUT1 cells are SSTR2-deficient in contrast to other cancer cell lines like A549 lung cancer, which is a SSTR2-expressing cell line. Therefore, the SKUT1 cells were taken to investigate, whether cellular uptake efficacy of core shell conjugates are SSTR2 dependent as assumed by the already performed experiments with A549 cells and CCy5-SST4 as well as other bioconjugates with SST-SHA peptide shell (see Figure S21 and Figure S24). Therefore, CytC-BA-Cy5, CCy5-SST4 and CCy5-SST8 were incubated with SKUT1 cells. The bioconjugates were pre-incubated in PBS pH 7.4 from cell culture in 2.5 µM for 30 min. Then

all samples were diluted with DMEM medium to have a final concentration of 250 nM. After 4h incubation with the cells at 37 °C and 5% $CO_2$, the samples containing medium was removed and the cells were treated as described in the general protocol given above. After measurement at the CLSM, the images were processed with Image J. The low signal of cellular uptake of CytC-BA-Cy5 was even further reduced for demonstration purposes and all images, which are compared to that within Figure S25, were processed under the same parameters to keep comparability.

No significantly increased cellular uptake of CCy5-SST4 and CCy5-SST8 compared to CytC-BA-Cy5 could be observed for the incubation with SKUT1 cells, which underpins the targeting selectivity of SSTR2 by CCy5-SST4 and other SST-SHA-based self-assembled protein conjugates.

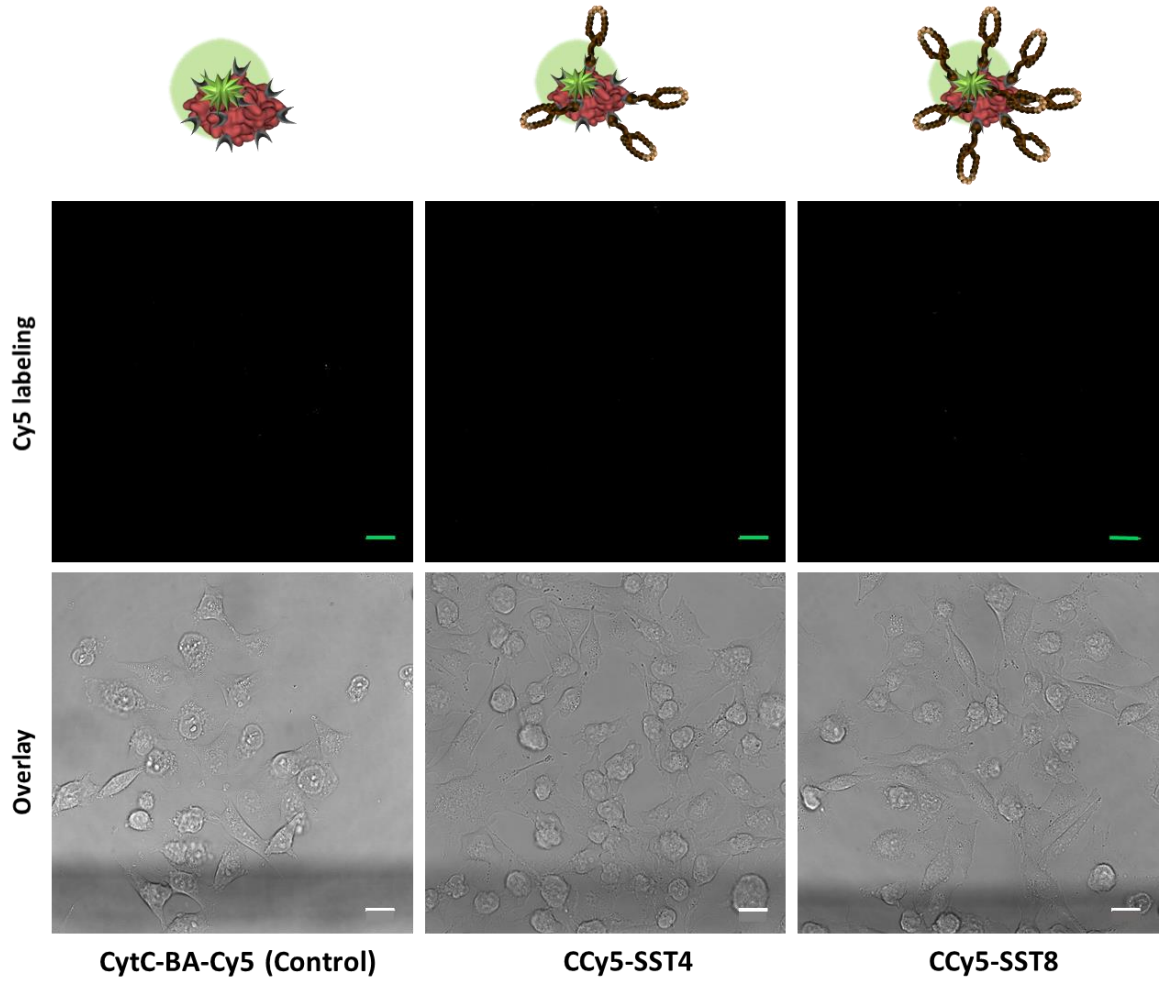


**Figure S25.** CytC-BA-Cy5 as control and 4 as well as 8 equivalents of SST-SHA **7** conjugated to CytC-BA-Cy5 generating CCy5-SST4 and CCy5-SST8 were investigated in their cellular uptake into SKUT1 cells (SSTR2-deficient cell line). All samples were incubated for 4h at 250 nM concentration. Fluorescence signal was obtained due to $\lambda_{ex}$ = 633 nm and emission was tracked at $\lambda_{em}$ = 662 nm. The scale bar within each presented image is 20 µm and Cy5 signal would be displayed in green, when detectable.

4.3.5 Cellular uptake of CCy5-SSTCy3 into A549 cancer cells: FRET study

To demonstrate protein release from core-shell bioconjugates within cancer cells, CCy5-SSTCy3 was built as described in Figure S26**A** in PBS pH 7.4 from cell culture and then incubated with A549 cells at 250 nM concentration for 3h (pre-incubation of CCy5-SSTCy3 at 2.5 µM for 30 min). Peptide shell cleavage induced by drop in pH occurs in acidic compartments in cancer cells due to the nature of boronic acid chemistry, which is sensitive to pH changes. Decreasing from physiological pH to slightly acidic pH range leads to disassembly of CCy5-SSTCy3 into its original components SST-SHA **7,** 2Cy3-SST-SHA **8** and CytC-BA-Cy5. This can be tracked by FRET experiment as described in Figure S26 and in further details in Figure S27 and Figure S28.

After 3h incubation, partial protein release from CCy5-SSTCy3 could be already observed next to intact bioconjugates demonstrated by FRET signal within cellular environment.

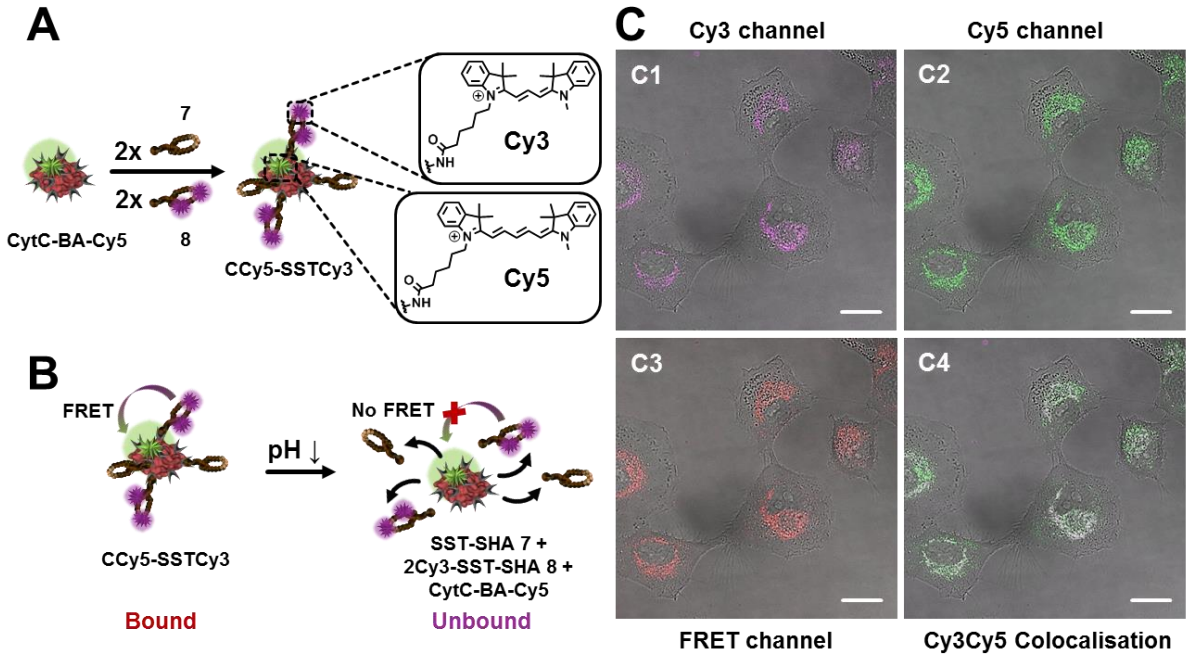


**Figure S26.** FRET study of CCy5-SSTCy3 within A549 cells **A)** Reaction scheme to build up CCy5-SSTCy3 by assembling 2 eq of SST-SHA **7** and 2 eq of 2Cy3-SST-SHA **8** onto CytC-BA-Cy5. **B)** In the bound state of **7/8** to CytC-BA-Cy5, FRET signal is possible to obtain due to proximity of Cy3-dye bound to lysine residues of SST sequence to Cy5-dye attached to protein surface. Disassembly of the CCy5-SSTCy3 due to drop in pH leads to disruption of FRET signal and gives the Cy3 signal for **8** in the unbound state. **C)** Confocal laser scanning microscopy images of CCy5-SSTCy3 incubated at 250 nM with A549 cells for 3h. Fluorescence signal was obtained at $\lambda_{ex}$ = 561 nm with emission tracked at $\lambda_{em}$ = 650-750 nm to detect FRET signal (displayed in red: C3), $\lambda_{ex}$ = 561 nm with emission at $\lambda_{em}$ = 575-630 nm for Cy3 signal (shown in magenta: C1) and $\lambda_{ex}$ = 633 nm tracked at $\lambda_{em}$ = 650-750 nm for Cy5 signal (shown in green: C2). C4 demonstrates colocalisation of Cy3-Cy5 (Cy3 in magenta, C5 in green, overlay in white). The scale bars within the presented images are 20 µm.

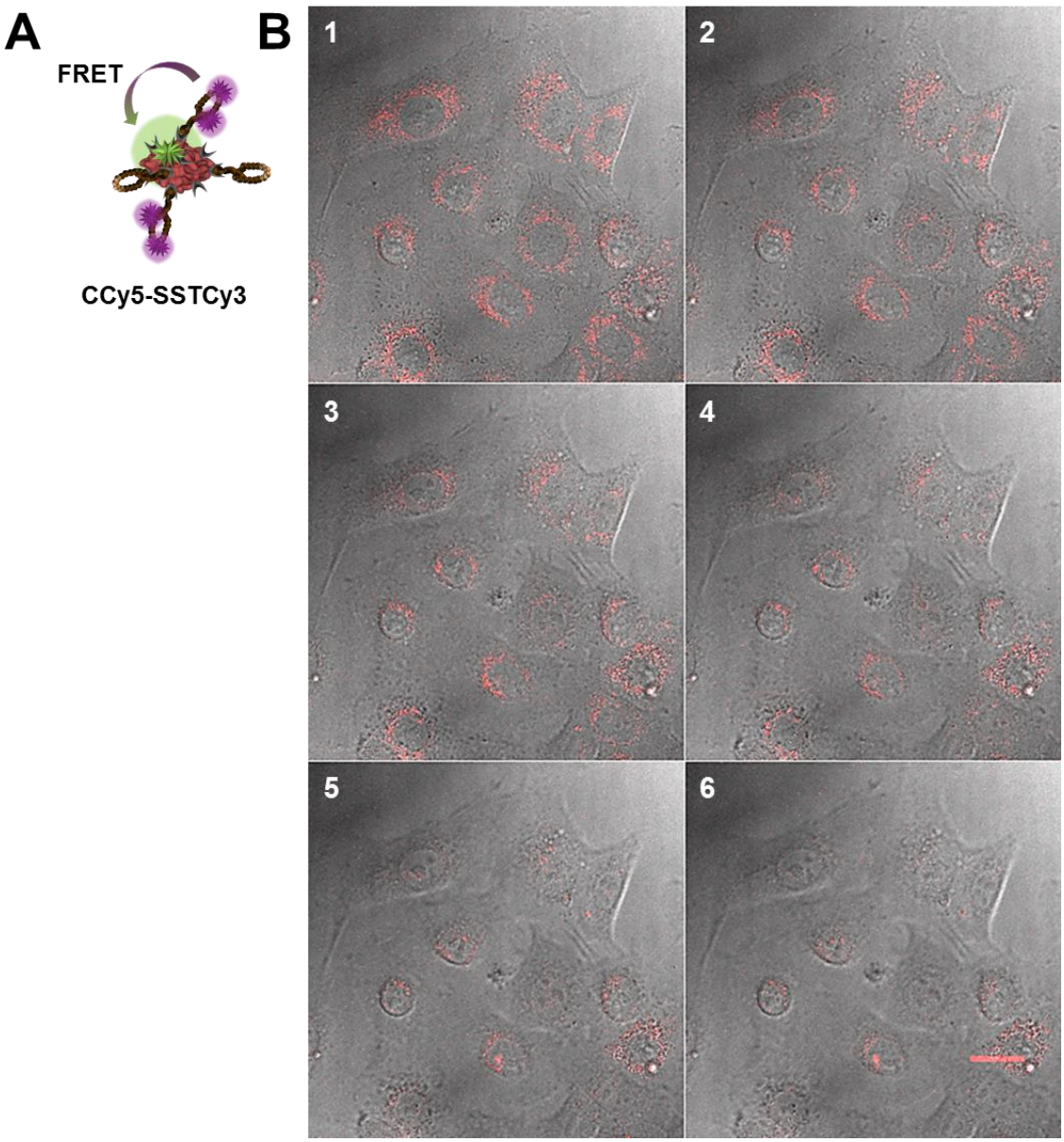


**Figure S27. A)** Structure of CCy5-SSTCy3 sketching the FRET by excitation of the Cy3-dye, which is bond to the lysine residues from the SST peptide sequence of **7**, and energy transfer to Cy5-dye attached to protein surface. **B)** z-Stacks from a confocal laser scanning microscopy spot for proof of cell internalization of CCy5-SSTCy3 at 250 nM into A549 cells after 3h incubation. Fluorescence signal was obtained due to $\lambda_{ex}$ = 561 nm and emission was tracked at $\lambda_{em}$ = 650-750 nm to detect FRET signal. The scale bar within the presented image is 20 µm and the FRET signal is displayed in red.

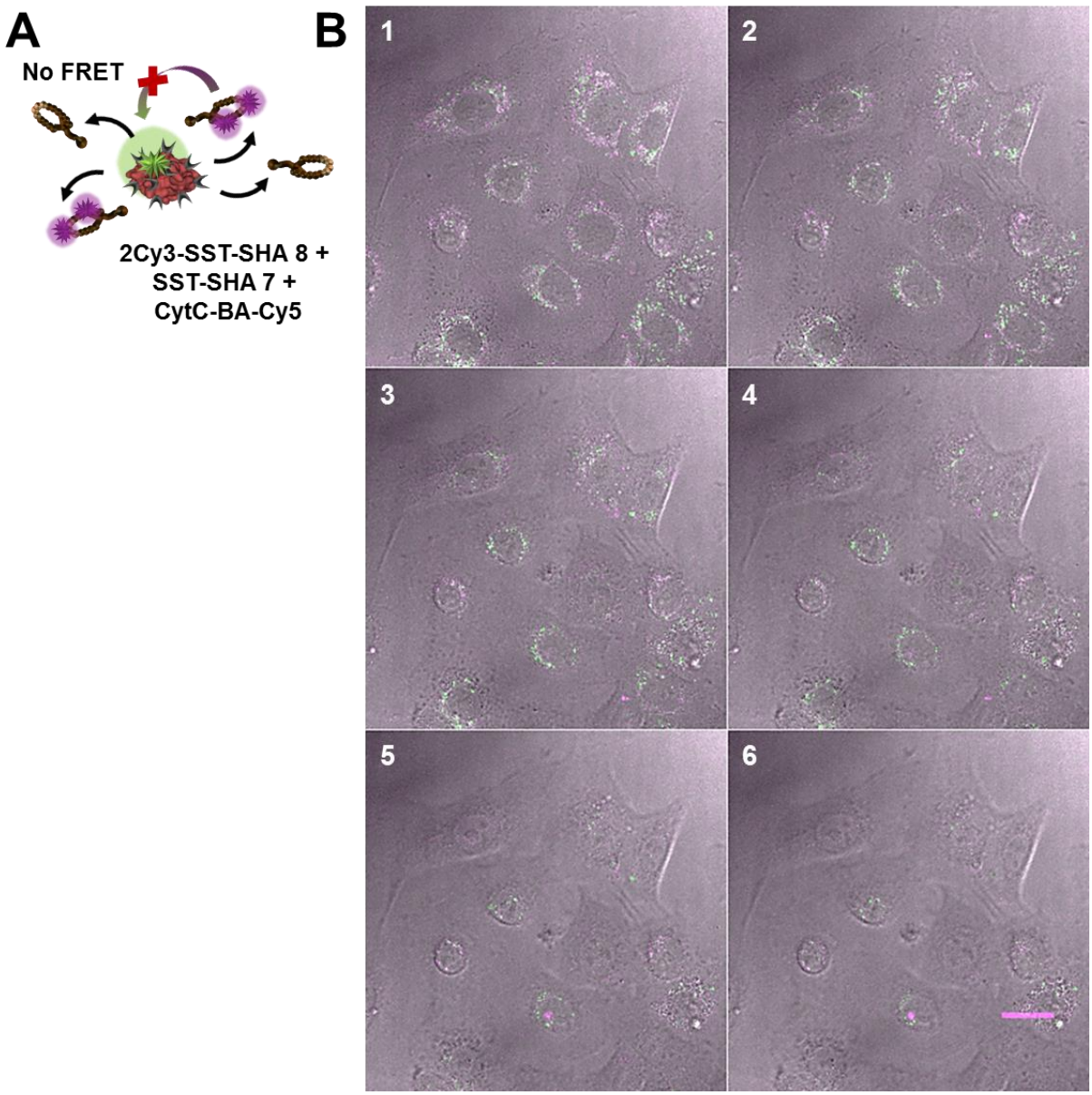


**Figure S28. A)** Disassembly of CCy5-SSTCy3 into its components 2Cy3-SST-SHA **8**, SST-SHA **7** and CytC-BA-Cy5 leading to disruption of FRET by increasing distance between Cy3- and Cy5-dye above the FRET distance limit. **B)** z-Stacks from a confocal laser scanning microscopy spot for proof of cell internalization of CCy5-SSTCy3, which was already partially disassembled leading to protein release due to targeting shell cleavage. The sample was incubated at 250 nM into A549 cells for 3h. Fluorescence signal was obtained due to $\lambda_{ex}$ = 561 nm and emission was tracked at $\lambda_{em}$ = 575-630 nm to detect Cy3 signal and $\lambda_{ex}$ = 633 nm tracked at $\lambda_{em}$ = 650-750 nm to detect Cy5 signal. The scale bar within the presented image is 20 µm and the Cy3 signal is displayed in magenta, Cy5 signal in green and Cy3-Cy5 overlay results in white.

### 4.4. Ca flux assay tracking SST-binding towards SSTR2 (CytC)

Within this assay performed by the company *Eurofins Cerep* in France, CytC-BA as well as C-SST4 (4 eq SST-SHA **7** conjugated to CytC-BA) were tested for calcium (Ca) flux stimulation. They were incubated therefore with two different cell lines: the human recombinant (RBL) cells expressing somatostatin receptors 2 (SSTR2) and RBL null cells (SSTR2-deficient cell line). SSTR2 are the main receptors addressed by somatostatin (SST) or its derivatives (SST-SHA **7**). Therefore, native SST was used as a control compound within the assay and all values of the study are given in relation to the Ca flux upon SSTR2 stimulation induced by native SST. It is referred to as % of control agonist response. Both samples – CytC-BA as well as C-SST4 – were tested for both described cell lines. Only SST-functionalized compounds should be able to show SSTR2-binding after incubation with the SSTR2 expressing cell line (RBL recombinant cells).

The samples were prepared as 30 µM stock solutions within buffer at pH 7.4 and incubated at room temperature, then according to the final concentrations given within the diagram in Figure 4 of the manuscript and Figure S29 diluted down. All samples were prepared in duplicates and six different concentrations were tested.

#### 4.4.1 Ca flux induced by C-SST4 screened in relation to the control agonist response

Results showing a stimulation higher than 50% are considered to represent significant effects of the test compounds. Such effects were observed for C-SST4 incubated with the RBL recombinant cells (SSTR2 expressing cell line) that was targeted by the SST-SHA **7** conjugated to the protein. The % of control agonist response for C-SST4 led to an $EC_{50}$ = 1.4 µM as demonstrated in Figure 4 within the manuscript. As expected, no significant effect was tracked for incubation of C-SST4 with the null cells (SSTR2-deficient cell line).

#### 4.4.2 Ca flux induced by CytC-BA screened in relation to the control agonist response

The % of control agonist response related to CytC-BA incubated with both cell lines (SSTR2 positive and negative cell line) was very low and therefore no significant effect was observed. The expected result of CytC-BA within the Ca flux assay is visualized within Figure S29.

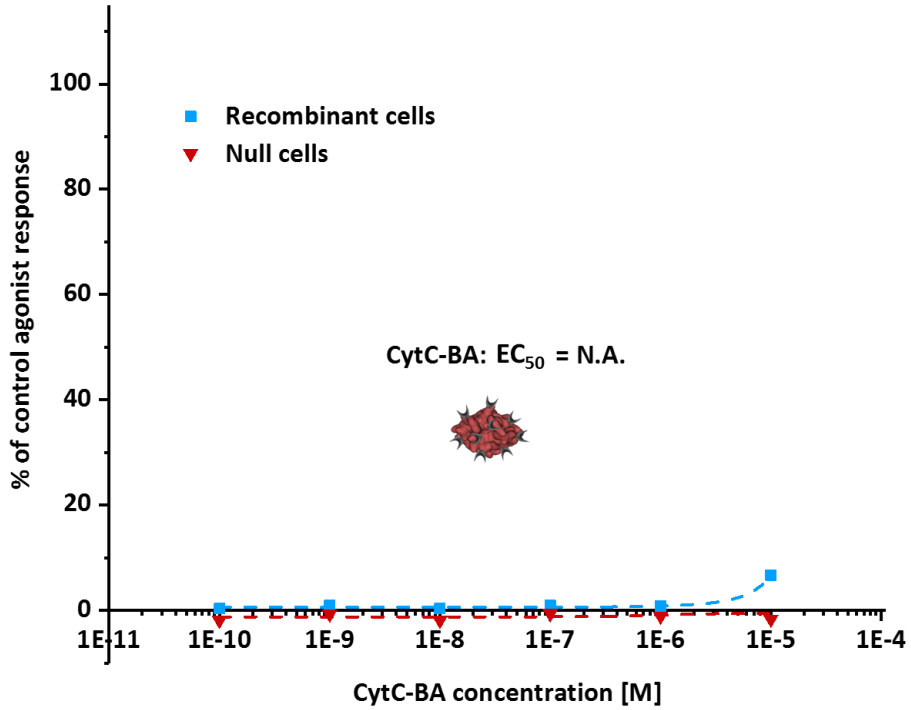


**Figure S29.** Ca flux induced by SSTR2 stimulation given by % of control agonist response was tracked for different concentrations of CytC-BA. The samples were incubated in human recombinant (RBL) cells (SSTR2 expressing cell line, blue squares) as well as in RBL null cells (SSTR2-deficient cell line, red triangles) and resulted for both cases in no measurable SSTR2 binding. The data prove expected values for CytC-BA as no SSTR2 targeting unit was conjugated to the control sample.

## 5. Literature Supporting Information


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