

# Supporting Information

## Development of polymeric imaging systems for the evaluation of conjugate uptake and cleavage

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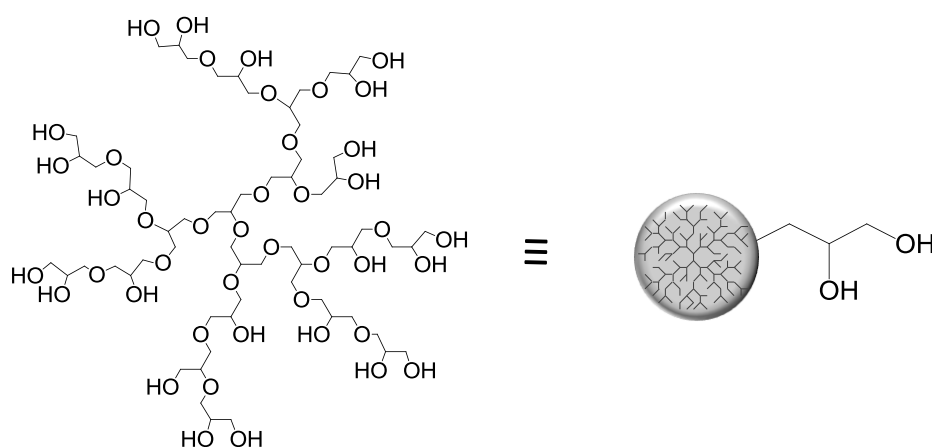
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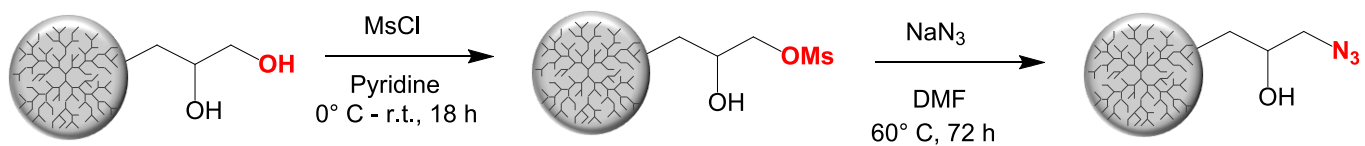
## Equation S1

$$\text{Quenching efficiency} = 100 \times \left[ 1 - \frac{(\text{fluorescence intensity of the probe})}{(\text{fluorescence intensity of the probe after complete cleavage})} \right]$$

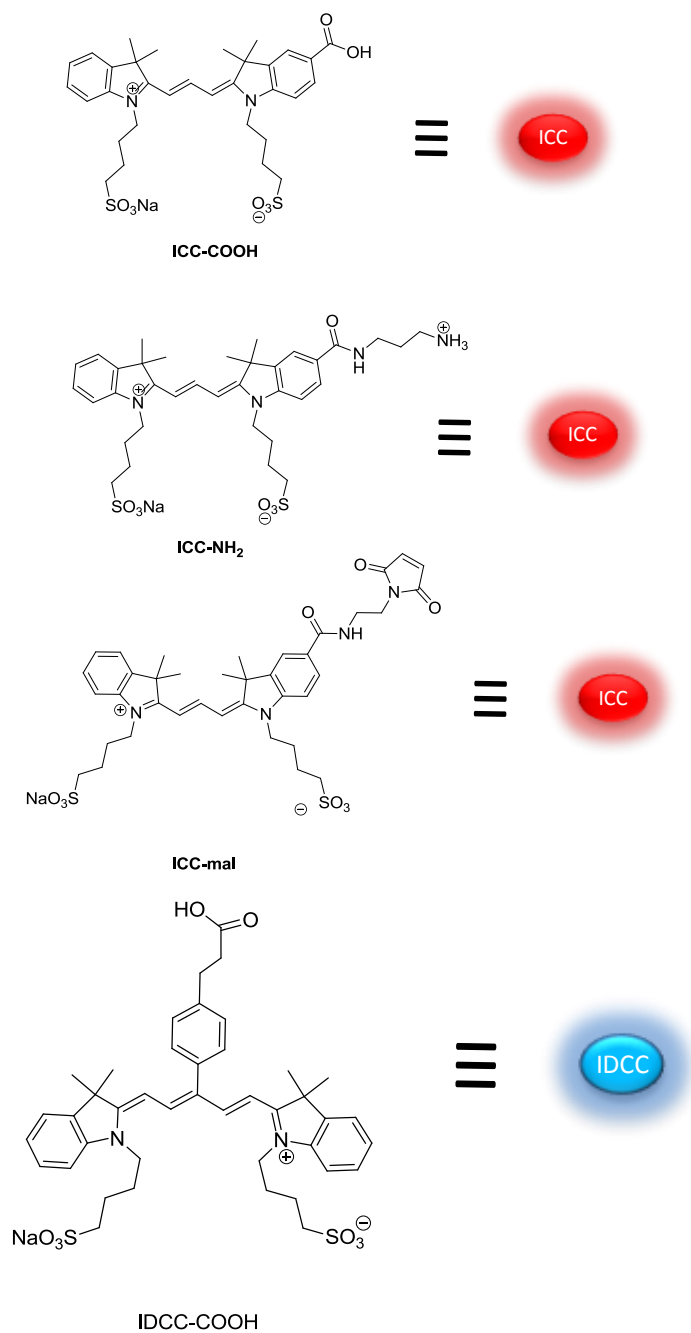
## Chemical structures of the building blocks



**Figure S1** Representative structure of dendritic polyglycerol (dPG)

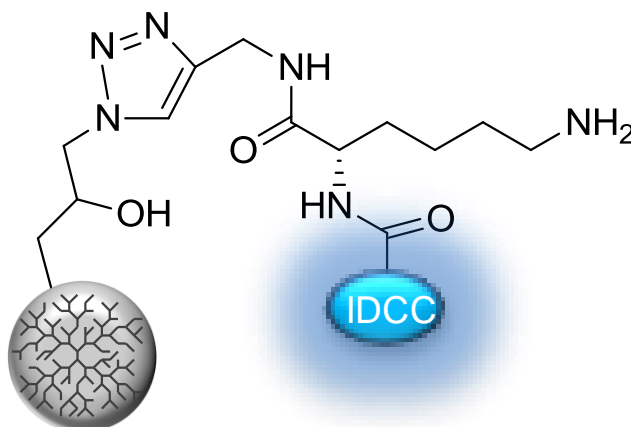


**Scheme S1** Synthesis of azide-bearing polyglycerol



**Figure S2** Chemical structures of the ICC and IDCC derivatives

## Synthesis of IDCC labelled precursor polymer conjugate (4)

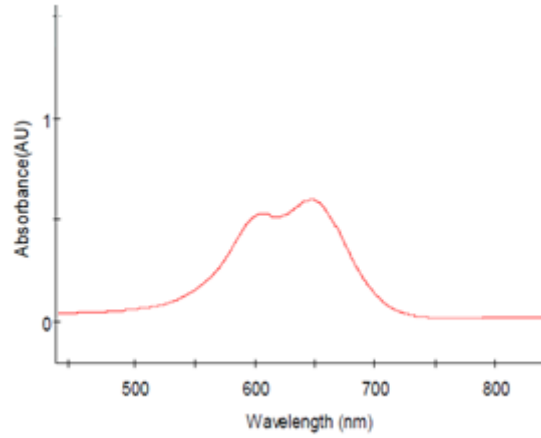


4

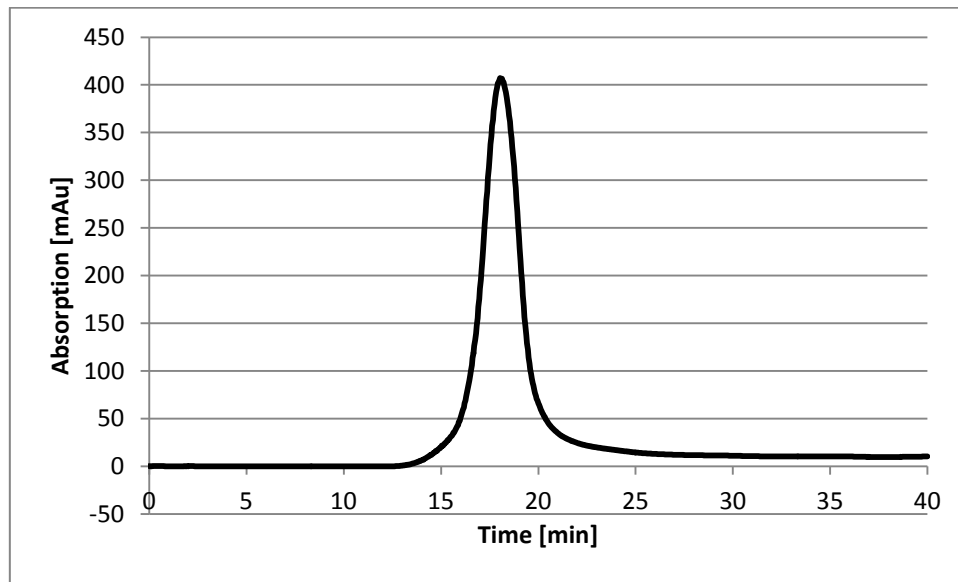
200 kDa PG (0.200 g, 0.001 mmol, 1eq) with 1% of azide functionalization was dissolved in a mixture of 2 mL H<sub>2</sub>O and 2 mL MeOH. To this solution **3** (0.002 g, 0.0019 mmol, 2 eq), sodium ascorbate (0.05 g, 0.0002 mmol, 0.3 eq), and copper sulphate pentahydrate (0.061 g, 0.0003 mmol, 0.45 eq) were added. The pH of the solution was adjusted to pH 8 - 9 by DIPEA, purged with N<sub>2</sub>, and then stirred for one overnight. The crude mixture was purified by ultrafiltration using H<sub>2</sub>O as solvent with one running-circle EDTA solution to remove all copper content. The product was obtained as a blue honey-like solid (0.123 g; 62% yield). The product was characterized by UV-Vis spectroscopy, fluorescence spectroscopy, and FPLC. The conjugate had a loading of 4 µg IDCC per mg conjugate determined by UV/Vis spectroscopy, using the molar extinction coefficient  $\epsilon_{650}=106000 \text{ M}^{-1} \text{ cm}^{-1}$  of IDCC.

Conjugate formation was proven by faster polymer band on SEC compared to the free dye.

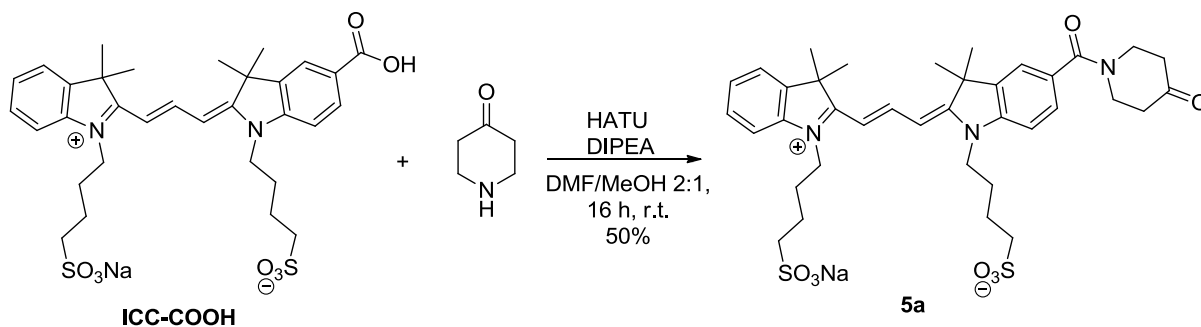
**UV/Vis spectra of IDCC labelled precursor polymer conjugate (4) in H<sub>2</sub>O:**



**FPLC chromatogram of IDCC labelled precursor polymer conjugate (4):**



## Synthesis of ICC-piperidone (5a)



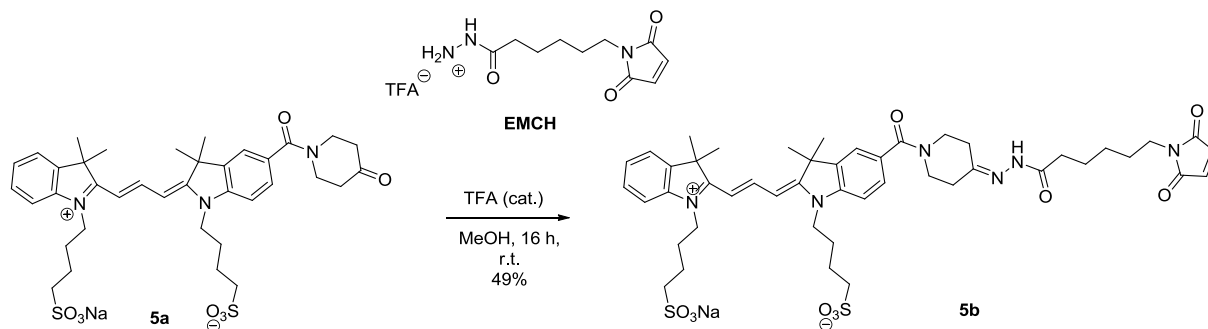
Indocarbocyanine carboxylic acid (10 mg, 0.015 mmol, 1.0 eq) was dissolved in a mixture of 0.5 mL DMF and 0.25 mL methanol. HATU (9.5 mg, 0.022 mmol, 1.5 eq) and two-thirds of the DIPEA (13.9  $\mu\text{L}$ , 0.082 mmol, 5.5 eq) were added and stirred for 30 minutes. Afterwards piperidone and the last third of DIPEA were transferred to the reaction mixture. The solution was stirred for 16 hours at room temperature and then precipitated in diethylether to give the crude product. After centrifugation the precipitate was purified by reversed-phase column in methanol/water (1:2 v/v).

Yield: 50% (5.40 mg)

$^1\text{H-NMR}$  (400MHz; MeOD):  $\delta$  (ppm) = 1.78 (s, 12 H,  $\text{CH}_3$ ), 1.93-2.07 (m, 8 H,  $\text{CH}_2$ ), 2.90 (t, 4 H,  $2 \times \text{N-CH}_2$ ), 3.10 (t, 4 H,  $\text{O=C-CH}_2$  piperidone), 3.49 (m, 4 H,  $\text{N-CH}_2$ -piperidone), 4.19 (t, 2 H,  $\text{SO}_3\text{-CH}_2$ ), 4.24 (t, 2 H,  $\text{SO}_3\text{-CH}_2$ ), 6.53 (d, 1 H, CH), 6.61 (d, 1 H, CH), 7.30-7.36 (m, 1 H, CH), 7.42-7.63 (m, 6 H, arom. H's), 8.55 (t, 1 H, arom. H's).

**MS** (ESI-ToF):  $m/z = 770.2606$  [ $\text{M}+2\text{Na}$ ] $^+$  724.2583 [ $\text{M}$ ] $^-$ , measured), (calcd. for  $\text{C}_{37}\text{H}_{46}\text{N}_3\text{O}_9\text{S}_2^-$ , 724.2726).

## Synthesis of ICC-piperidone-EMCH (5b)



**5a** (5.0 mg, 0.007 mmol, 1 eq) and EMCH were dissolved in 1.0 mL dry methanol in a dried flask. A catalytic amount of TFA was added and the solution was allowed to stir at room temperature overnight. The mixture was purified directly on a reversed-phase column using methanol/water (1:1 v/v) as eluent. After lyophilization a red solid was obtained.

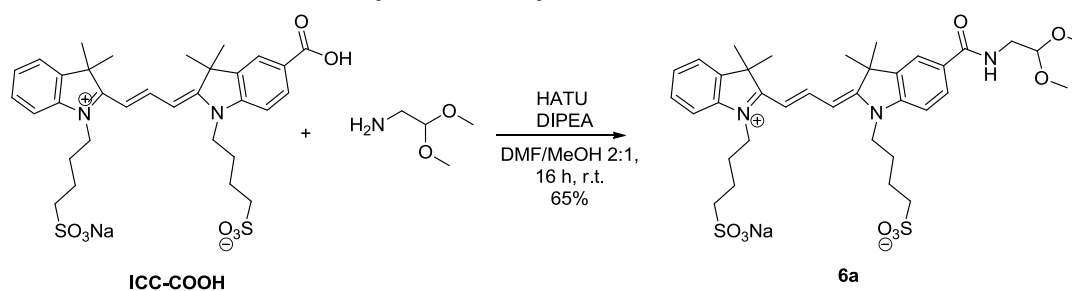
Yield: 49% (3.2 mg)

**MS** (ESI-ToF):  $m/z = 931.3912$   $[M]^-$ ,  $977.3537$   $[M+2Na]^+$ , measured), (calcd. for  $C_{47}H_{59}N_6O_{10}S_2^-$ , 931.3740).

Due to instability of the product in solution no NMR spectra could be recorded.



## Synthesis of ICC-acetaldehyde dimethyl-acetal (6a)



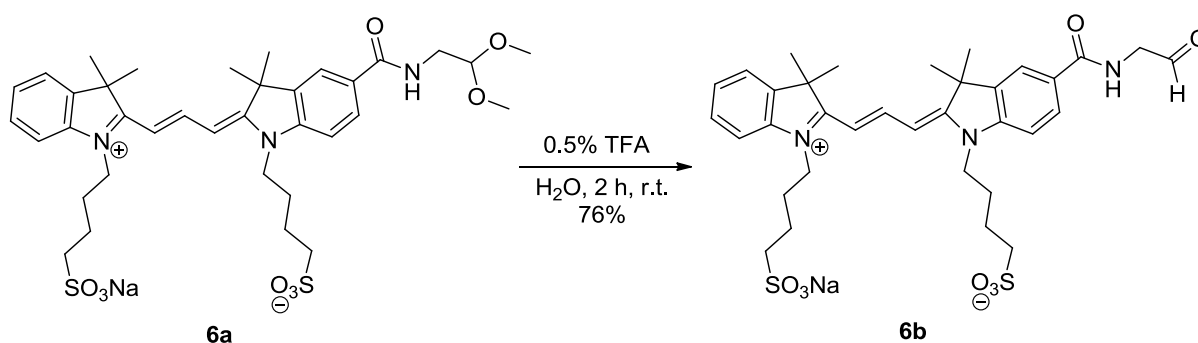
Indocarbocyanine carboxylic acid (10 mg, 0.015 mmol, 1.0 eq) was dissolved in a mixture of 0.5 mL DMF and 0.25 mL methanol. HATU (9.5 mg, 0.022 mmol, 1.5 eq) and two-thirds of the DIPEA (13.9  $\mu$ L, 0.082 mmol, 5.5 eq) were added and stirred for 30 minutes. Afterwards the protected amino acetaldehyde and the last third of DIPEA were transferred to the reaction mixture. The solution was stirred for 16 hours at room temperature and was then precipitated in diethylether to give the crude product. After centrifugation, the precipitate was purified by reversed-phase column using methanol/water (1:2 v/v) as eluent.

Yield: 65% (7.10 mg)

<sup>1</sup>H-NMR (400MHz; D<sub>2</sub>O):  $\delta$  (ppm) = 1.79 (s, 12 H, CH<sub>3</sub>), 1.92-2.08 (m, 8 H, CH<sub>2</sub>), 2.91 (t, 4 H, 2xN-CH<sub>2</sub>), 3.43 (s, 6 H, O-CH<sub>3</sub>), 3.52 (d, 2 H, N-CH<sub>2</sub> acetal), 4.20 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 4.25 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 4.59 (t, 1 H, O-CH), 6.54 (d, 1 H, CH), 6.64 (d, 1 H, CH), 7.32-7.37 (m, 1 H, CH), 7.42-7.50 (m, 3 H, arom. H's), 7.56 (d, 1 H, arom. H's), 7.93 (dd, 1 H, arom. H's), 7.98 (d, 1 H, arom. H's), 8.56 (t, 1 H, arom. H's).

MS (ESI-ToF): m/z = 730.2708 [M]<sup>-</sup>, 776.2746 [M+2Na]<sup>+</sup>, (calcd. for C<sub>36</sub>H<sub>48</sub>N<sub>3</sub>O<sub>9</sub>S<sub>2</sub><sup>-</sup>, 730.2837).

## Synthesis of ICC-acetaldehyd (6b)



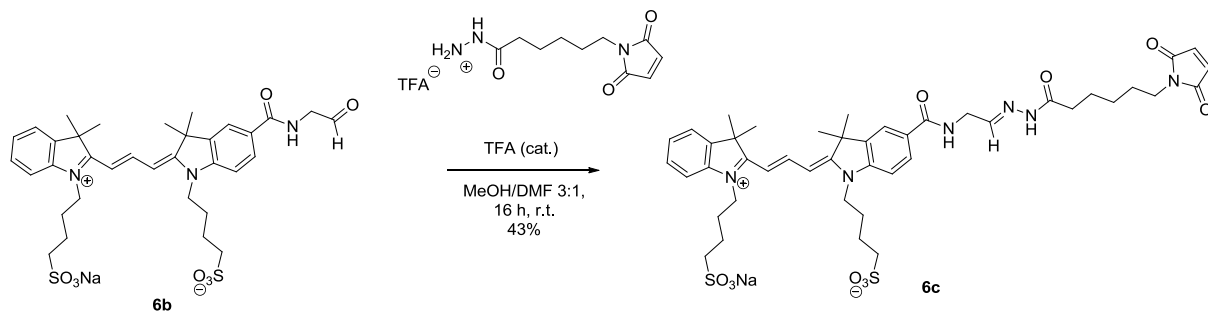
**6a** (7 mg, 0.001 mmol) was dissolved in 5 mL methanol and 0.5% TFA (25  $\mu$ L). After 1 hour stirring at room temperature the solvent and TFA were removed in vacuum. The crude product was purified by reversed-phase chromatography using methanol/water (1:2 v/v) as eluent.

Yield: 76% (5.0 mg)

<sup>1</sup>H-NMR (400MHz; D<sub>2</sub>O):  $\delta$  (ppm) = 1.69 (s, 12 H, CH<sub>3</sub>), 1.85-1.90 (m, 4 H, CH<sub>2</sub>), 1.93-1.99 (m, 4 H, CH<sub>2</sub>), 2.97 (t, 4 H, 2xN-CH<sub>2</sub>), 3.53 (d, 2 H, N-CH<sub>2</sub> acetal), 4.09 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 4.15 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 5.26 (t, 1 H, CHO), 6.31 (d, 1 H, CH), 6.41 (d, 1 H, CH), 7.30-7.31 (m, 1 H, CH), 7.35-7.38 (m, 2 H, arom. H's), 7.46 (t, 1 H, arom. H's), 7.56 (d, 1 H, arom. H's), 7.81 (d, 1 H, arom. H's), 7.87-7.88 (m, 1 H, arom. H's), 8.47 (t, 1 H, arom. H's).

MS (ESI-ToF):  $m/z$  = 684.2428 [M]<sup>-</sup>, measured), (calcd. for C<sub>34</sub>H<sub>42</sub>N<sub>3</sub>O<sub>8</sub>S<sub>2</sub><sup>-</sup>, 684.2419).

## Synthesis of ICC-Acetaldehyd-EMCH (6c)

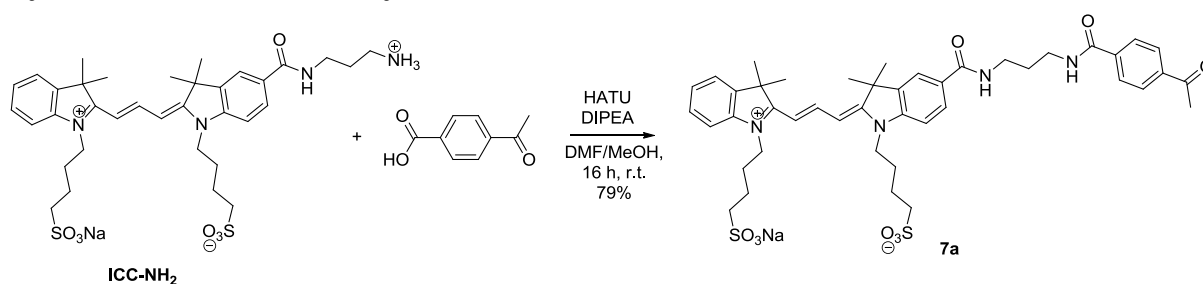


**6b** (5.0 mg, 0.007 mmol, 1 eq) was diluted in methanol/DMF 3:1. Then EMCH (7.4 mg, 0.022 mmol, 3 eq) was added in 1 mL dry methanol. A catalytic amount of TFA was added and the solution was allowed to stir at room temperature overnight. The mixture was given directly on a reversed-phase column and was eluted with methanol/water (3:1 → 1:1 v/v). The methanol was removed in vacuo under mild conditions (30 °C bath temperature) and the remaining water was lyophilized to give a red solid.

Yield: 43% (2.8 mg)

**MS** (ESI-ToF):  $m/z = 891.3752 [M]^+$ , measured), (calcd. for  $C_{44}H_{55}N_6O_{10}S_2^-$ , 891.3427).

## Synthesis of ICC-4-acetylbenzamido (7a)



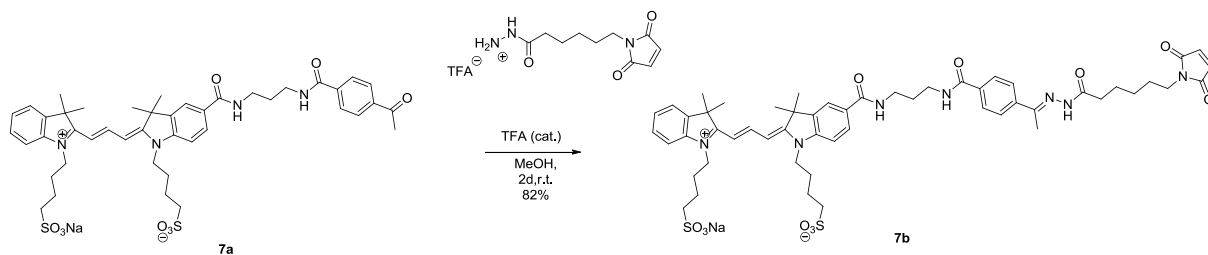
4-acetyl benzoic acid (7.0 mg,  $4.3 \cdot 10^{-2}$  mmol, 1.5 eq) and HATU (18.5 mg,  $6.5 \cdot 10^{-2}$  mmol, 1.5 eq) were dissolved in 3 mL DMF and two-thirds of DIPEA (17.6  $\mu\text{L}$ , 0.1 mmol, 3.6 eq) were. After 30 minutes the indocarbocyanine amine derivative dissolved in 8 mL DMF/methanol 1:1 and 1 mL of water and the last third of DIPEA (8.8  $\mu\text{L}$ , 0.05 mmol, 1.82 eq) were added to the mixture which was allowed to stir for 16 h at room temperature. Afterwards the solution was precipitated in diethylether to give the crude product. After centrifugation the precipitate was purified by reversed-phase column with methanol/water (1:1 v/v).

Yield: 79% (19.1 mg)

**<sup>1</sup>H-NMR** (400MHz; MeOD):  $\delta$  (ppm) = 1.78 (s, 12 H, CH<sub>3</sub>), 1.91-2.09 (m, 10 H, CH<sub>2</sub>), 2.64 (s, 3 H, O=C-CH<sub>3</sub>), 2.91 (t, 4 H, N-CH<sub>2</sub>), 3.52 (t, 4 H, O=C-NH-CH<sub>2</sub>), 4.19 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 4.26 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 6.54 (d, 1 H, CH), 6.64 (d, 1 H, CH), 7.32-7.37 (m, 1 H, CH), 7.42-7.50 (m, 3 H, arom. H's), 7.56 (d, 1 H, arom. H's), 7.93-8.01 (m, 4 H, arom. H's), 8.05-8.09 (m, 2 H, arom. H's), 8.56 (t, 1 H, arom. H's).

**MS** (ESI-ToF):  $m/z = 845.3511$  [M]<sup>-</sup>, measured), (calcd. for C<sub>44</sub>H<sub>53</sub>N<sub>4</sub>O<sub>9</sub>S<sub>2</sub><sup>-</sup>, 845.3259).

## Synthesis of ICC-4-acetylbenzamido-EMCH (7b)



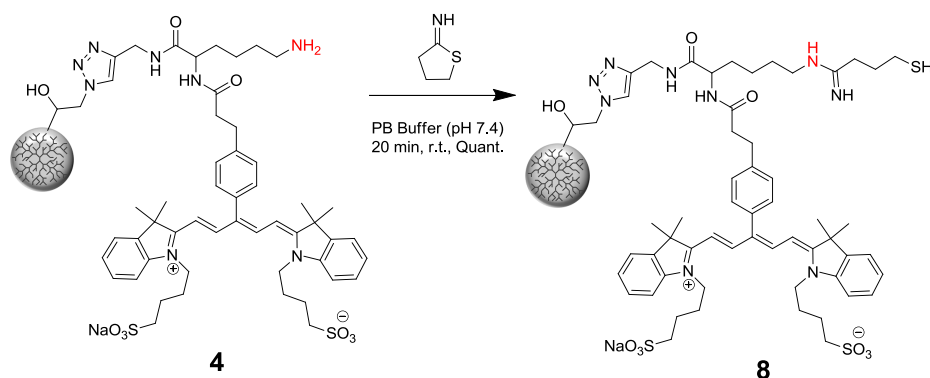
**7a** (21.6 mg, 0.025 mmol, 1 eq) and EMCH (24.2 mg, 0.075 mmol, 3 eq) were dissolved in methanol. A catalytic amount of TFA (1:2000) was added and the solution was allowed to stir for two days at room temperature. The mixture was diluted with water and given directly on a reversed-phase column which was eluted with methanol/water (3:1  $\rightarrow$  1:1 v/v). The methanol was removed in vacuum under mild conditions (30 °C bath temperature) and the remaining water was lyophilized. A red solid was obtained with a yield of 82%.

Yield: 82% (19.2 mg)

**<sup>1</sup>H-NMR** (400MHz; D<sub>2</sub>O/MeOD):  $\delta$  (ppm) = 1.32-1.37 (m, 2 H, CH<sub>2</sub> caproyl), 1.59 (qi, 2 H, CH<sub>2</sub> caproyl), 1.70 (qi, 2 H, CH<sub>2</sub> caproyl), 1.76 (s, 12 H, CH<sub>3</sub>), 1.90-2.05 (m, 10 H, CH<sub>2</sub>), 2.35 (s, 3 H, N=C-CH<sub>3</sub>), 2.43 (t, 2 H, O=C-CH<sub>2</sub> EMCH), 2.92 (t, 4 H, N-CH<sub>2</sub>), 3.49 (m, 2 H, N-CH<sub>2</sub> EMCH), 3.52 (t, 4 H, O=C-NH-CH<sub>2</sub>), 4.17 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 4.25 (t, 2 H, SO<sub>3</sub>-CH<sub>2</sub>), 6.47 (d, 1 H, CH), 6.58 (d, 1 H, CH), 6.78 (s, 1 H, maleimido), 6.82 (s, 1 H, maleimido), 7.34-7.40 (m, 2 H, CH + arom. H's), 7.44-7.50 (m, 2 H, arom. H's), 7.57 (d, 1 H, arom. H's), 7.80-7.88 (m, 3 H, arom. H's), 7.89-7.96 (m, 3 H, arom. H's), 8.54 (t, 1 H, arom. H's).

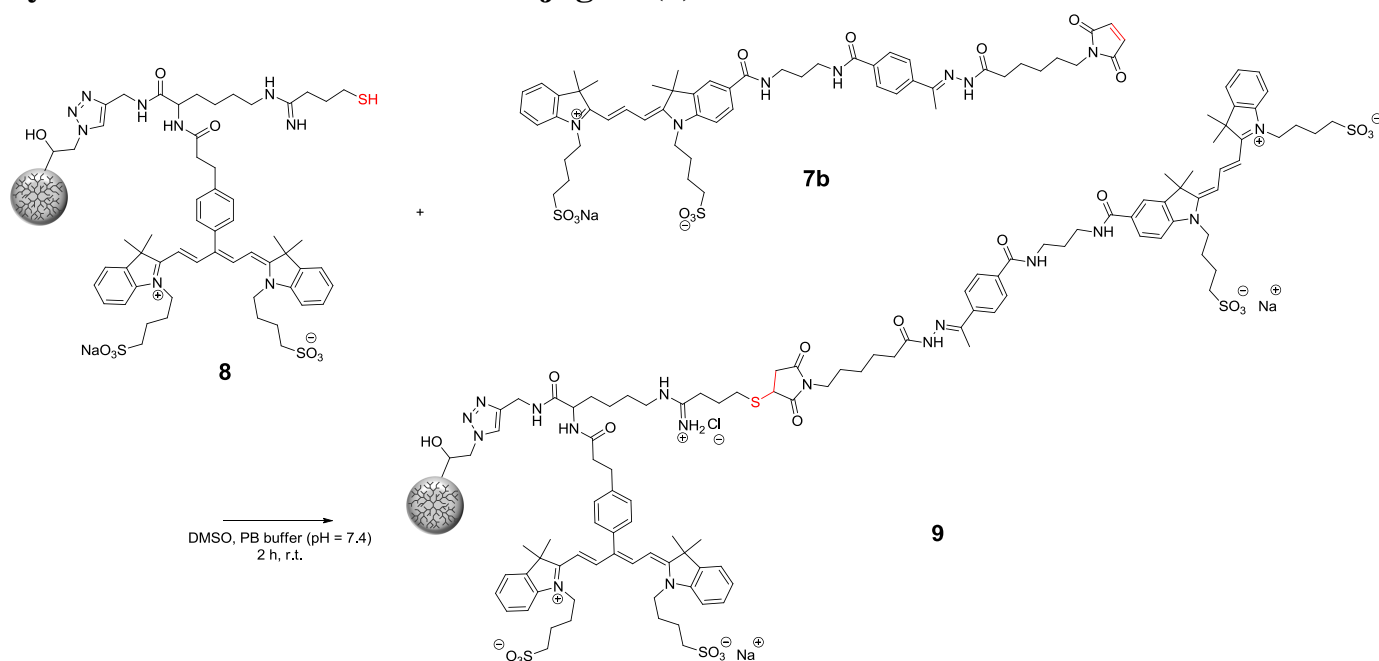
**MS** (ESI-ToF):  $m/z = 1052.4271$  [M]<sup>-</sup>, measured), (calcd. for C<sub>14</sub>H<sub>25</sub>N<sub>3</sub>O<sub>3</sub>, 1052.4267).

## Synthesis of thiolated IDCC labeled precursor polymer conjugate (8)



To a solution of **4** (10 mg,  $7.5 \cdot 10^{-5}$  mmol relating to IDCC loading, 1 eq) in 0.3 mL PB (pH 7.4) 2-iminothiolane (0.031 mg,  $2.3 \cdot 10^{-4}$  mmol, 3 eq) was added from a stock solution and the reaction mixture was stirred for 20 min. The following step was performed in situ.

## Synthesis of functional FRET conjugate (9)

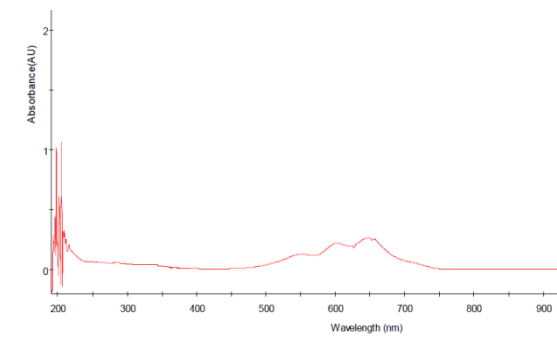


To the stirring mixture of **8** a solution of **7b** (0.24 mg,  $2.3 \cdot 10^{-4}$  mmol, 5 eq) in DMSO was added and allowed to stir for 2 hour. The solution was purified by FPLC using Sephacryl S-100 gel. The product fraction was lyophilized and reconstituted in PB for analysis.

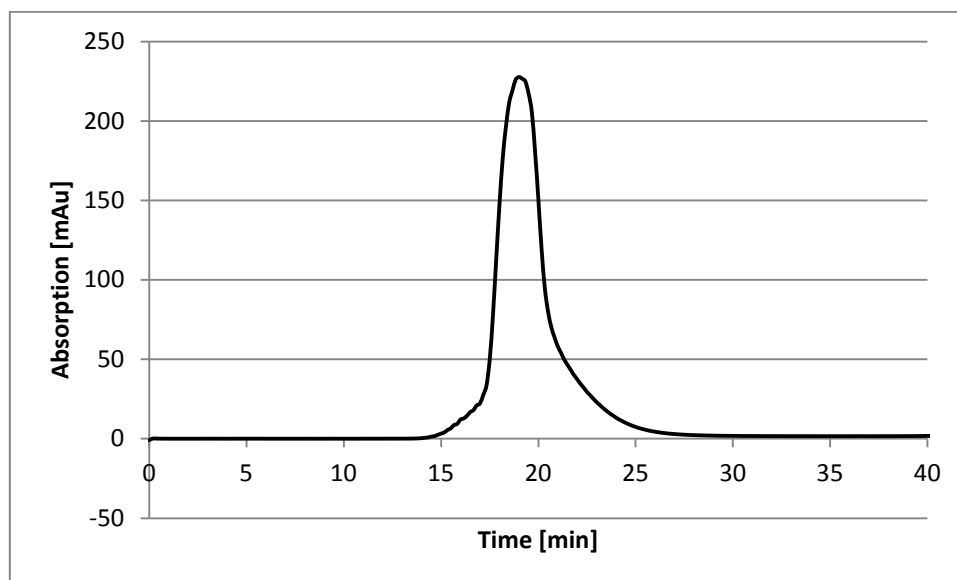
Yield: 69% (5.8 mg)

Conjugate formation was proven by faster polymer bond compared to free dye by FPLC.  
**UV/Vis-spectrum** was recorded and showed the characteristic band of IDCC and ICC. 1 mg conjugate contains 1.75  $\mu\text{g}$  ICC and 4.1  $\mu\text{g}$  IDCC.  
**Fluorescence spectra** were recorded.

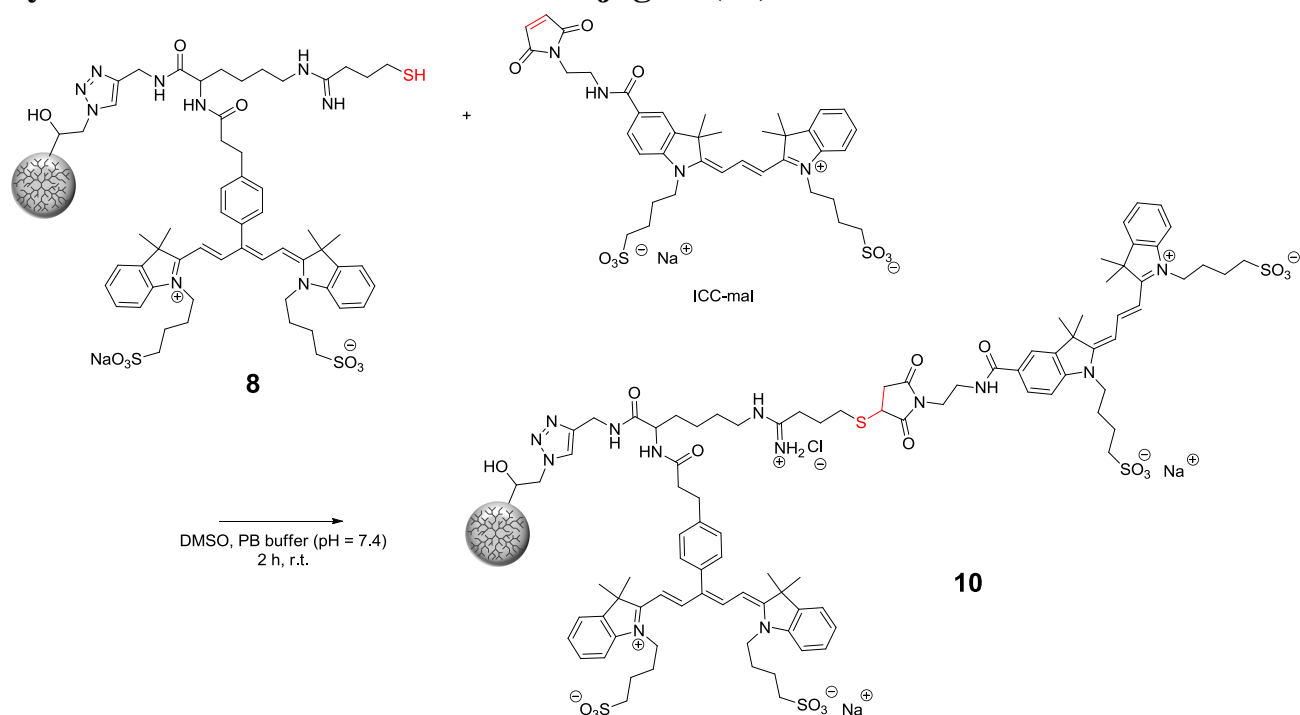
**UV/Vis spectra of functional FRET conjugate (9) in PB:**



**FPLC chromatogram of functional FRET conjugate (9):**



## Synthesis of non-cleavable FRET conjugate (10)



To the stirring mixture of **8** a solution of **ICC-mal** (0.23 mg,  $2.5 \cdot 10^{-4}$  mmol, 5 equiv.) in DMSO was added and allowed to stir for 2 hour. The solution was purified by FPLC using Sephacryl S-100 gel. The product fraction was lyophilized and reconstituted in PB for analysis.

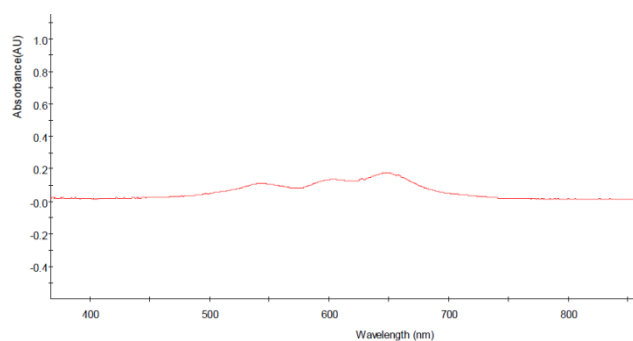
Yield: 72% (6.1 mg)

Conjugate formation was proven by faster polymer bond compared to free dye by FPLC.

**UV/Vis-spectrum** was recorded and showed the characteristic band of IDCC and ICC.

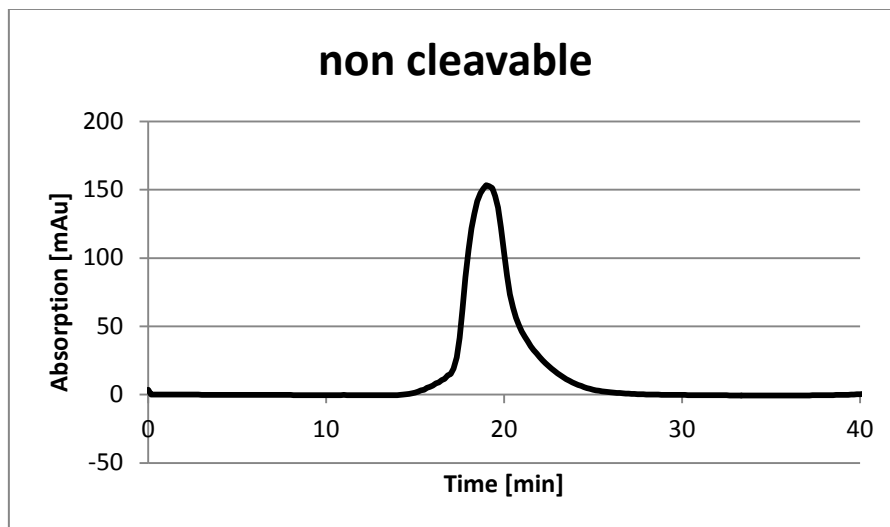
**Fluorescence spectra** were recorded.

### UV/Vis spectra of non-cleavable FRET conjugate (10) in PB:

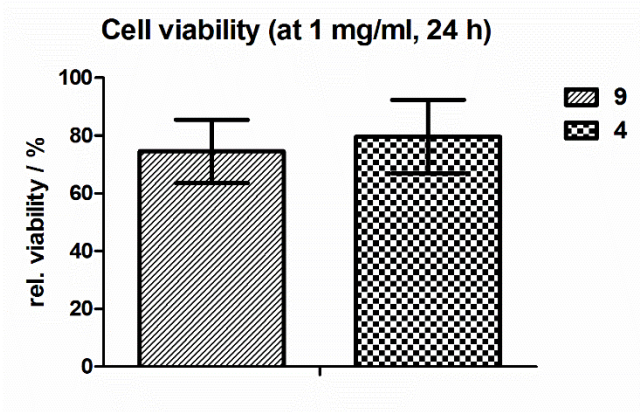




**FPLC chromatogram of non-cleavable FRET conjugate (10):**



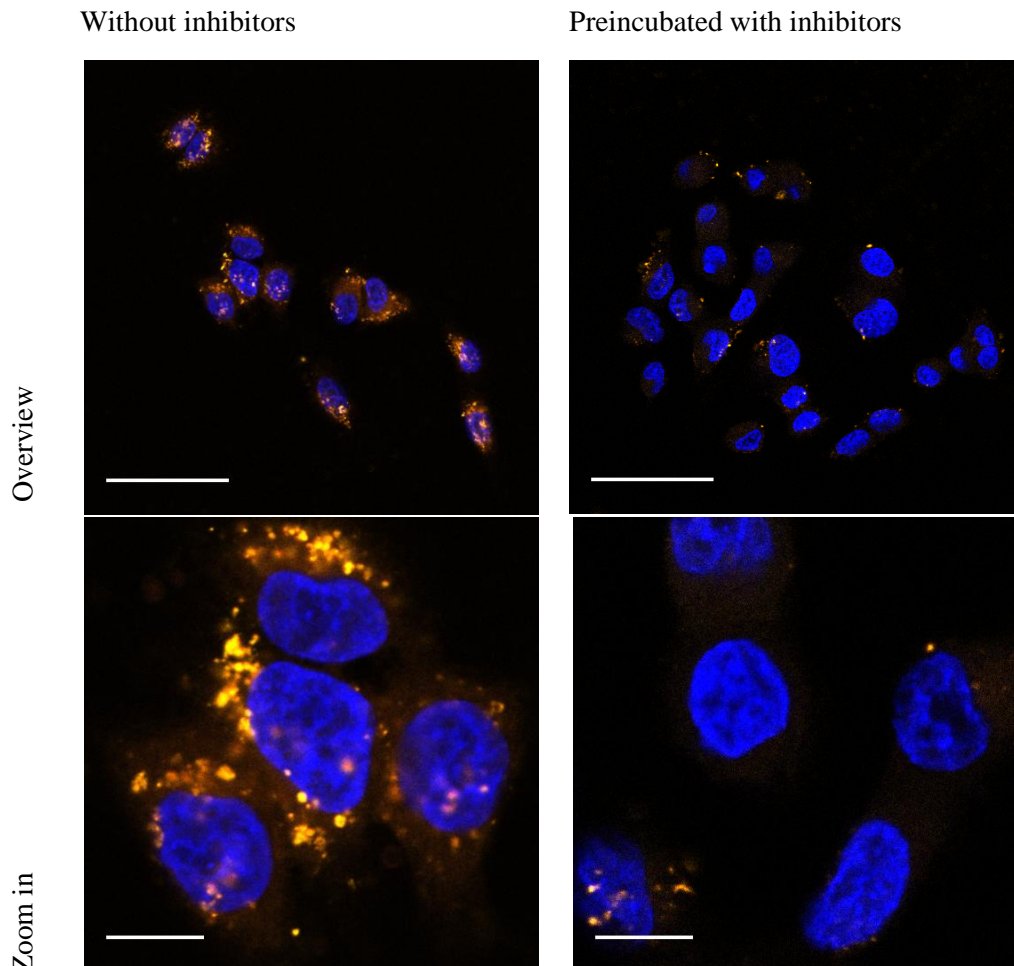
# Cell viability study



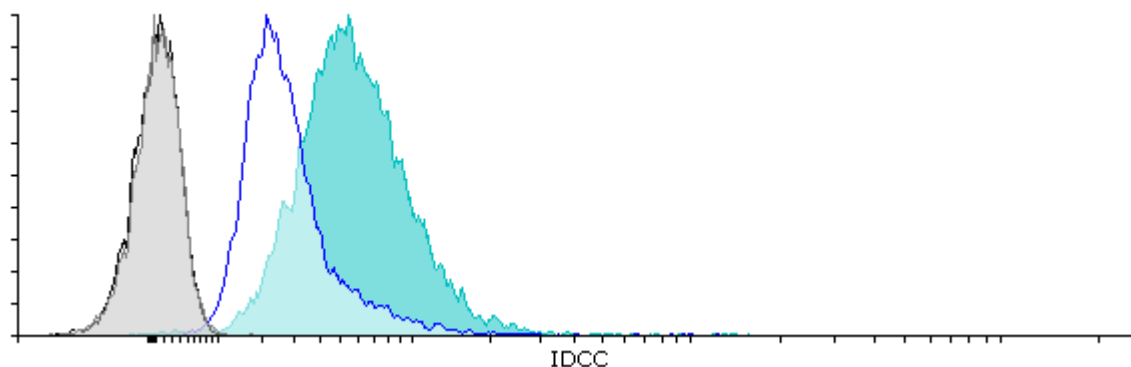
**Figure S3:** Cell viability assay determined by CellTiter-Glo® Luminescent Cell Viability Assay (Promega).

## Cell uptake and inhibition studies

Cell uptake and inhibition was studied by confocal laser scanning microscopy and flow cytometry. A549 cells were seeded onto cover slips in 24-well plates and grown over night in DMEM with 10% fetal calf serum (FCS) and 1% Penicillin / Streptomycin at 37 °C and 5% CO<sub>2</sub>. Parallel samples for the flow cytometry were processed in the same 24 well plate. After incubation over night, cells were washed 3x with PBS and either incubated with the compounds alone in DMEM (without FCS and phenol red) or were pretreated with 20mM methyl- $\beta$ -cyclodextrin (Sigma) in DMEM for 15 minutes at 37 °C. Pretreated cells were then washed 3 times with PBS, and the compounds were incubated for 4 hours at 1 mg ml<sup>-1</sup> in DMEM including 0.4  $\mu$ g ml<sup>-1</sup> Lovastatin (Sigma). Cells on coverslips were then washed 3 times with 1 mL PBS and fixed with 4% PFA for 20 minutes. After washing again with 4% PFA, cells were stained with DAPI (2,5  $\mu$ g ml<sup>-1</sup>, Carl Roth), washed again and mounted on microscope slides with ProTaq<sub>s</sub> Mount Fluor mounting medium. Samples for flow cytometry were washed with PBS, trypsinized and centrifuged 5 minutes at 138xg. The supernatant was aspirated, 2 ml PBS was added and centrifuged again. After removing the buffer, cells were fixed with 4% PFA for 10 minutes. 1 mL PBS was added and centrifuged. Cells were resuspended in PBS without Ca and Mg with 1% FCS and 0.1% sodium azide and stored at 4 °C until measurement. 10000 events were counted for each sample.

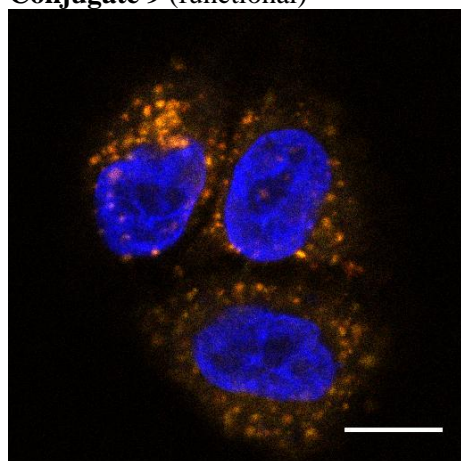


**Figure S4:** Confocal laser scanning microscopy images of A549 cells incubated with conjugate **9** for 4h. Overview on the top row, more detailed view on the bottom row. Blue color indicates DAPI stained cell nuclei. Orange color results from ICC (yellow) and IDCC (red) fluorescence of the conjugate. Punctate patterns in the perinuclear region shows uptake of the conjugate into compartments of the cells. Cells on the right side had been preincubated with the endocytosis inhibitors methyl- $\beta$ -cyclodextrin and Lovastatin. In comparison, much fewer signals of the conjugate is seen and those which are visible are more at the cell periphery. Scale bar upper row: 50 microns, lower row: 10 microns

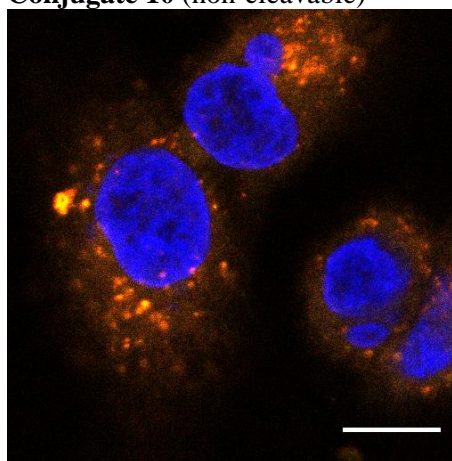


**Figure S5:** Overlay histograms of flow cytometric data of A549 cells incubated with compound **9** or control cells. Relative fluorescence values of untreated cells (grey line, filled) and cells pretreated with inhibitors (black line, empty) were similar. When cells were incubated with compound **9** alone (cyan line, filled) the resulting histogram is shifted to higher fluorescence values, indicating cell uptake. When cells were pretreated with inhibitors (blue line, empty), the population is shifted to a lower fluorescence average, indicating inhibition of the cell uptake.

**Conjugate 9 (functional)**



**Conjugate 10 (non-cleavable)**



**Figure S6:** Confocal laser scanning images of A549 cells incubated with **9** and **10** for 8 hours. Overlay images: blue: cell nuclei, red: IDCC, yellow: ICC, (overlay leads to orange color). Scale bar: 10 microns.