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Support Information

Precise tetrafunctional streptavidin bioconjugates towards multifaceted drug delivery systems

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Experimental Procedures

Materials: Streptavidin (MW 53361 g/mol) was purchased from Cedarlane Laboratories. Ibio-His-tag (MW 2322 g/mol, iminobiotin-$(His)_{12}$, sequence: iminobiotin-GSGSGSHHHHHHHHHHHH) were synthesized by Pepscan. Folic acid-PEG-biotin (MW 2000 g/mol) was purchased from Nanocs. RPMI-1640 medium without folic acid was purchased from Thermo Fisher Scientific. The Ni^{2+}-NTA column (HisTrap™ HP, column volume 5 mL) was purchased from GE Healthcare Life Sciences. Cell nucleus location sequence (PKKKRKVC, MW 883 g/mol) was purchased from PhtdPeptides Co., Ltd. with 95% purity (Zhengzhou City, China). All organic solvents (acetonitrile (CH_3CN), chloroform ($CHCl_3$), dichlormethane (DCM), dimethylformamide (DMF), dimethyl sulfoxide (DMSO), methanol (MeOH) were obtained from Fisher Scientific and used without further purification (HPLC or analytical grades). All other chemicals were purchased from Sigma-Aldrich. Buffers and aqueous solutions were prepared with Milli-Q grade water.

Preparation of tetrafluorophore streptavidin conjugates: The Cu^{2+}-NTA column was prepared by removing Ni^{2+} ion from a Ni^{2+}-NTA column (HisTrap™ HP, column volume 5 mL) with ethylenediaminetetraacetic acid (EDTA) and reloading it with Cu^{2+} ions. Firstly, 1 µM SA_1 (S: streptavidin, A: atto-565-biotin) prepared as previously described[1] was mixed with 2 µM Ibio-His-tag for 15 min at room temperature to assure the binding of iminobiotin to streptavidin. Then, 2 µM B (atto-425-biotin) was added to the reaction mixture and incubated for another 15 min. 0.5 mL of the reaction mixture was loaded on a Cu^{2+}-NTA column, which was preequilibrated with 50 mL buffer A (50 mM Tris-HCl, 300 mM NaCl, pH=7.4) and then washed with 25 mL buffer A. At last, the different streptavidin conjugates conjugates (S(Ibio-His-tag)$_1A_1B_2$, S(Ibio-His-tag)$_2A_1B_1$, S(Ibio-His-tag)$_3A_1$) were eluted with a linear imidazole gradient from 0 to 120 mM imidazole in buffer A over 60 mL. The elution of different species was monitored by the absorbance at 563 nm (the absorbance of atto-565), 436 nm (the absorbance of atto-425) and different peaks were collected separately. A flow rate of 0.5 ml/min was used throughout the experiment. Each peak was first dialyzed (10 kDa molecule weight cutoff) against 2 L buffer pH=3.5, 50 mM Tris-HCl, 300 mM NaCl solution for 1 h at 4 ºC to remove the Ibio-His-tag and then dialyzed twice against 2 L buffer A for at least 6 h to remove the imidazole. The samples were concentrated using a centrifugal filtration devices (10 kDa molecule weight cutoff) for further studies. Similarly, SA_1B_1 (1 µM) isolated in the previous step was mixed with Ibio-His-tag (1.5 µM) and C (atto-665-biotin, 1.5 µM) to prepare $SA_1B_1C_1$, the elution of different species (S(Ibio-His-tag)$_1A_1B_1C_1$, S(Ibio-His-tag)$_2A_1B_1$) was monitored by the absorbance at 563 nm (the absorbance of atto-565), 436 nm (the absorbance of atto-425) and 663 nm (the absorbance of atto-665). After the same dialysis and concentrating steps, $SA_1B_1C_1$ was isolated. The final fluorophore biotin-5-fluorescein (D) was added stoichiometrically to $SA_1B_1C_1$ as also detailed below to yield $SA_1B_1C_1D_1$.

Determination of open biotin binding pockets: The number of open biotin binding pockets was determined using biotin-5-fluorescein, which's fluorescence is quenched upon binding to streptavidin. Typically, 200 µL of 10 nM of a streptavidin conjugate in buffer A was added to a transparent 96-well plate (Greiner bio-one, F-bottom), different

concentrations (0 to 40 nM) of biotin-5-fluorescein were added to each well and the samples were incubated for 10 min at room temperature. The fluorescence intensity of each well was measured (excitation wavelength 490 nm, emission wavelength 524 nm) using a plate reader (TECAN, infinite M1000).

Förster resonance energy transfer (FRET) measurement: Sample of $SA_1B_1C_1$ (S: streptavidin, A: atto-565-biotin, B: atto-425-biotin and C: atto-665-biotin) was used to measure the FRET fluorescence. Firstly, for determination of the assembled ratio A:B:C, 100 µL 1 µM of the sample were applied into a plate reader (TECAN, infinite M1000), the absorbance spectrum (350 nm-900 nm) of the solution was measured. As control absorbance spectra of same concentration of atto-565-biotin, atto-425-biotin and atto-665-biotin were recorded and subtracted for the calculation. The ratio of A:B:C was determined from the absorption envelopes and a ratio of 0.78:1:0.81 was calculated. Then, 200 µL 1 µM of $SA_1B_1C_1$ in buffer A were added in a transparent 96-well plate (Greiner bio-one, F-bottom), the excitation wavelength was set as 400 nm and the emission spectrum was measured from 420 nm to 800 nm using a plate reader (TECAN, infinite M1000). As a control, 1 µM streptavidin was mixed with 1 µM of each of the fluorophores (atto-425-biotin, atto-565-biotin and atto-665-biotin) for 15 minutes to yield a statistical mixture of streptavidin conjugates.

Preparation of doxorubicin-biotin: Doxorubicin-biotin was synthesized according to literature.[2] Typically, to an ice-cold solution of biotin-N-hydroxysuccinimide ester (0.14g, 0.41 mmmol) in DMF (10 ml) was added doxorubicin (0.3 g, 0.41 mmol) under argon atmosphere. After stirring for 30 min, triethylamine (0.5ml, 2 mmol) was added to this reaction mixture and was allowed to stir for another 12 h at room temperature. The reaction was monitored by TLC (Merck Silica 60, HF 254, 20: 80 methanoldichloromethane v/v). After completion of the reaction, excess diethyl ether (100 ml) was added to the reaction mixture. The red solid thus obtained was filtered and washed three times with diethyl ether (50 ml, three times). This red solid was then subjected to column chromatography using methanol-dichloromethane (20:80, v/v) as an eluent to obtain 0.25 g (Yield = 78%). LC-MS: T_R= 5.5 min, ESI m/z: calculated for. $C_{37}H_{43}N_3O_{13}S$: 769; found 792 $[M+Na]^+$, 809 $[M+K]^+$. MALDI-ToF (DHB): m/z: calculated for. $C_{37}H_{43}N_3O_{13}S$: 769; found 792 $[M+Na]^+$, 809 $[M+K]^+$. ^{1}H NMR (MeOD-d4, 300 MHz): 7.91 (d, 2H, aromatic), 7.50 (d, 1H, aromatic), 5.38 (brs, 1H, OH), 5.25 (brs, 1H, OH), 4.97 (s, 2H, -CH2-OH), 4.33 (brs, 2H, OH), 4.07 (m, 2H, CH, CH), 4.16-4.13 (m, 1H, CH), 3.94 (s, 3H, OCH3), 3.41 (m, 1H, CH), 3.09 (brs, 2H, OH), 3.10-3.00 (m, 4H, CH2, CH), 2.88-2.54 (m, 3H, CH2, CH), 2.20-2.00 (m, 1H, CH), 1.41-1.13 (m, 8H, CH2, CH3, CH2).

Preparation of biotin-NH-PKKKRKVC-COOH: The cell nucleus location sequence (PKKKRKVC) is a peptide derived from the simian virus 40 large tumor antigen (SV40 large T antigen), to enhance nuclear entry. For biotinylation, 5 mg (5.08 µmol, 1 mol. equiv.) of peptide was dissolved in 50 mM phosphate buffer pH 6.5 and 5 eq (13.4 mg, 25.4 µmol) Biotin-TEG-MI was added and shaken for 4h at RT. Product was subjected to reversed phase HPLC using a XDB-C18 column with the mobile phase starting from 100% solvent A (0.1% TFA in water) and 0% solvent B (0.1% TFA in acetonitrile) with a flow rate of 4 mL per minute, raising to 5% solvent B in five minutes, 15% solvent B in 10 minutes, and then reaching 100% solvent B after 29 minutes. It remained in this state for one minute. Solvent B concentration was then finally lowered to 5 % in five minutes. Absorbance was monitored at 280 nm and 254 nm. The retention time for biotin-NH–PKKKRKVC–COOH was 10.5 minutes, and 2.10 mg (1,39 µmol, 30%) of the product was obtained after lyophilization. LC-MS:T_R: 3.91, m/z = $[M+ H]^+$ 1511. MALDI-ToF-MS (CHCA): m/z **=** calculated. for $C_{67}H_{121}N_{19}O_{16}S_2$: 1511, $[M+H]^+$ 1511, $[M+Na]^+$ 1534.

Separation of tetrafunctional streptavidin conjugate ($SA_1F_1D_1C_1$) with atto-565 (A), folic-acid (F), doxorubicin (D) and nucleus penetrating peptide (C): 10 µM SA_1 (S: streptavidin, A: atto-565-biotin) prepared as previously described[1] was mixed with 20 µM Ibio-His-tag for 15 min at room temperature to assure the binding of iminobiotin to streptavidin. Then, 20 µM F (folic acid-PEG-biotin) was added to the reaction mixture and incubated for another 15 min.

0.5 mL of the reaction mixture was loaded on a Cu^{2+}-NTA column, which was pre-equilibrated with 50 mL buffer A and then washed with 25 mL buffer A. At last, the different streptavidin conjugates ($S(Ibio\text{-}His\text{-}tag)_1A_1F_2$, $S(Ibio\text{-}His\text{-}tag)_2A_1F_1$, $S(Ibio\text{-}His\text{-}tag)_3A_1$) were eluted with a linear imidazole gradient from 0 to 120 mM imidazole in buffer A over 60 mL. The elution of different species was monitored by the absorbance at 280 nm and different peaks were collected separately. Each peak was first dialyzed against (10 kDa molecule weight cutoff) 2 L buffer pH=3.5, 50 mM Tris-HCl, 300 mM NaCl solution for 1 h at 4 ºC to remove the Ibio-His-tag and then dialyzed twice against 2 L buffer A for at least 6 h to remove the imidazole. The samples were concentrated using a centrifugal filtration devices (10 kDa molecule weight cutoff) for further studies. Similarly, 10 μM SA_1F_1 was mixed with 15 μM Ibio-His-tag and 15 μM D (doxorubicin-biotin) to prepare $SA_1F_1D_1$. As described before the different species ($S(Ibio\text{-}His\text{-}tag)_1A_1F_1D_1$, $S(Ibio\text{-}His\text{-}tag)_2A_1F_1$) were separated with Cu^{2+}-NTA column using a linear imidazole gradient and the Ibio-His-tag was removed under acidic conditions and dialysis. Then, $SA_1F_1D_1$ (2 μM) was mixed with C (3 μM, nucleus penetration peptide: biotin-NH-PKKKRKVC-COOH) and incubated for 20 min at room temperature. $SA_1F_1D_1C_1$ was dialyzed (10 kDa molecule weight cutoff) against 2 L buffer A twice for at least 6 h to remove excess C.

Cellular uptake of streptavidin conjugates: For cellular uptake studies, MDA-MB-231 or MCF-7 cells were seeded at $5x10^4$ cell/well on glass coverslips (VWR, diameter 18 mm) in 12-well cell culture plates (Greiner bio-one, F-bottom) and were cultured in RPMI-1640 medium without folic acid supplemented with 10 % heat inactivated fetal bovine serum (FBS, 10%) and 1 % penicillin/streptomycin (P/S) at 37 ºC, 5% CO_2 overnight. The next day, the cells were washed twice with PBS and 500 μL of RPMI-1640 medium without folic acid + 10 % FBS + 1 % P/S containing 1 μM of different streptavidin conjugates (SA_1, SA_1F_1, SA_1F_2, SA_1F_3, $SA_1F_1D_1$ or $SA_1F_1D_1C_1$, cells without any streptavidin conjugates were set as blank) was added to each well. The cells were incubated at 37 ºC, 5% CO_2 for 4 h, washed twice with PBS and fixed with 4 % PFA in PBS for 15 min at room temperature. Subsequently, the cells were washed twice with PBS and mounted on a glass slide (ROTH, 24x60 mm) with 40 μL Mowiol-488 containing 1 μg/ml DAPI. The cells were imaged in the DAPI and atto-565 channels using a confocal laser scanning microscope (Leica TCS SP8) equipped with 405 nm and 552 nm laser lines through a 63x H_2O objective. Images were analyzed by Fiji ImageJ. For the analysis, the average fluorescence intensities of single cell in the atto-565 channel were measured by encircling single cells and mseasuring their average intensities. 20 cells were analyzed per sample and fluorescence intensities were background corrected.

MTT assay: MDA-MB-231 or MCF-7 cells were seeded at $5x10^3$ cell/well in 96-well cell culture plates (Greiner bio-one, F-bottom) and were cultured in RPMI-1640 medium without folic acid + 10 % FBS + 1 % P/S at 37 ºC, 5% CO_2 overnight. The next day, the cells were washed twice with PBS and cultured in 200 μL of RPMI-1640 medium without folic acid + 10 % FBS + 1 % P/S containing different concentrations (0 - 2.0 μM) of different streptavidin conjugates (D, $SA_1F_1D_1$, $SA_1F_1D_1C_1$ or statistical mixture of streptavidin conjugates) at 37 ºC, 5% CO_2 for 72 h. The statistical mixture of streptavidin conjugates was prepared by mixing streptavidin with A, F, D and C in a ratio of 1:1:1:1 for 20 minutes. Subsequently, the cells were washed twice with PBS and 200 μL of RPMI-1640 medium without folic acid + 10 % FBS + 1 % P/S containing 1.2 mM MTT was added to each well and the cells were incubated at 37 ºC, 5% CO_2 for 4 h. Then, the culture medium was discarded and 200 μL of DMSO was added to the cells to dissolve the dark blue formazan crystals. After 10 minutes, absorbance at 550 nm of each well was measured using a plate reader (TECAN, infinite M1000). The experiment was repeated three times. The cell viability was expressed as the percentages of viable cells compared to the survival of the control cells not incubated with a streptavidin conjugate. The IC_{50} (50% inhibitory concentration) was determined by log dosage versus concentration curve. For Fig. S15, the concentration of $SA_1F_1D_1$, $SA_1F_1D_1C_1$ or mixture was set as 0.5 μM and repeat the MTT test, also a control experiment was conducted by adding 20 μM extra free folic acid in the process.

Reference:

1. D. Xu and S. V. Wegner, *Chem. Sci.*, 2020, **11**, 4422-4429.
2. J. Callan, A. Mchale, S. Kamila, K. Loagan, WIPO Patentscope, 2018, Application No. PCT/GB2018/051481.

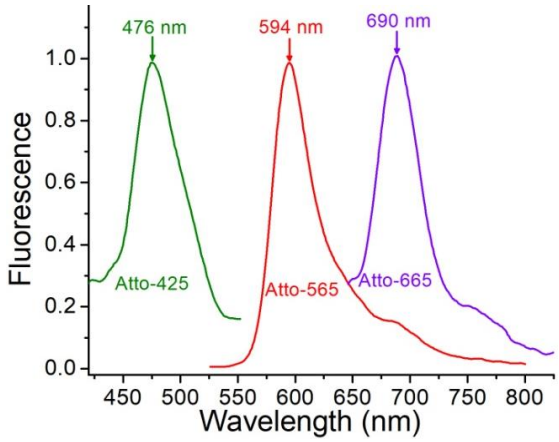


Fig. S1 Emission spectra of the used fluorescent biotin conjugates (atto-425-biotin (excitation at 400 nm), atto-565-biotin (excitation at 505 nm) and atto-665-biotin (excitation at 625 nm)).

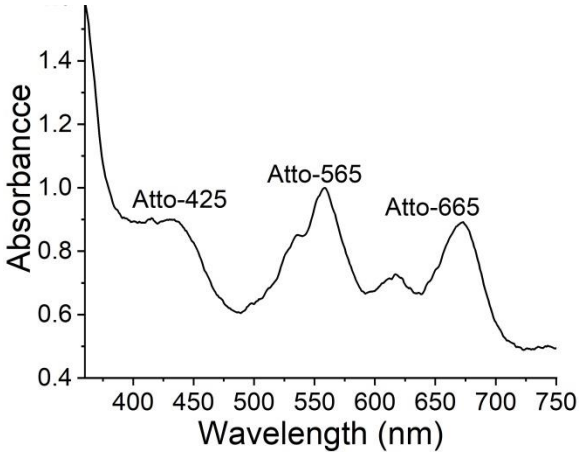


Fig. S2 Absorbance spectrum of $SA_1B_1C_1$ (S: streptavidin, A: atto-565-biotin, B: atto-425-biotin and C: atto-665-biotin). The ratio of A:B:C was determined to be 1.0: 1.2: 1.0 based on the extinction coefficients of the pure A, B and C at 535 nm, 440 nm and 665 nm, respectively.

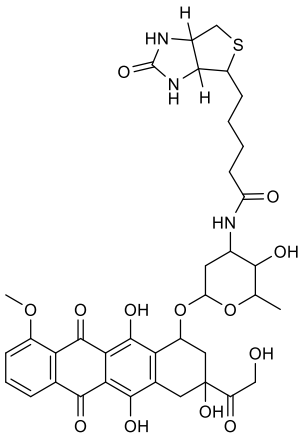


Fig. S3 The chemical structure of doxorubicin-biotin.

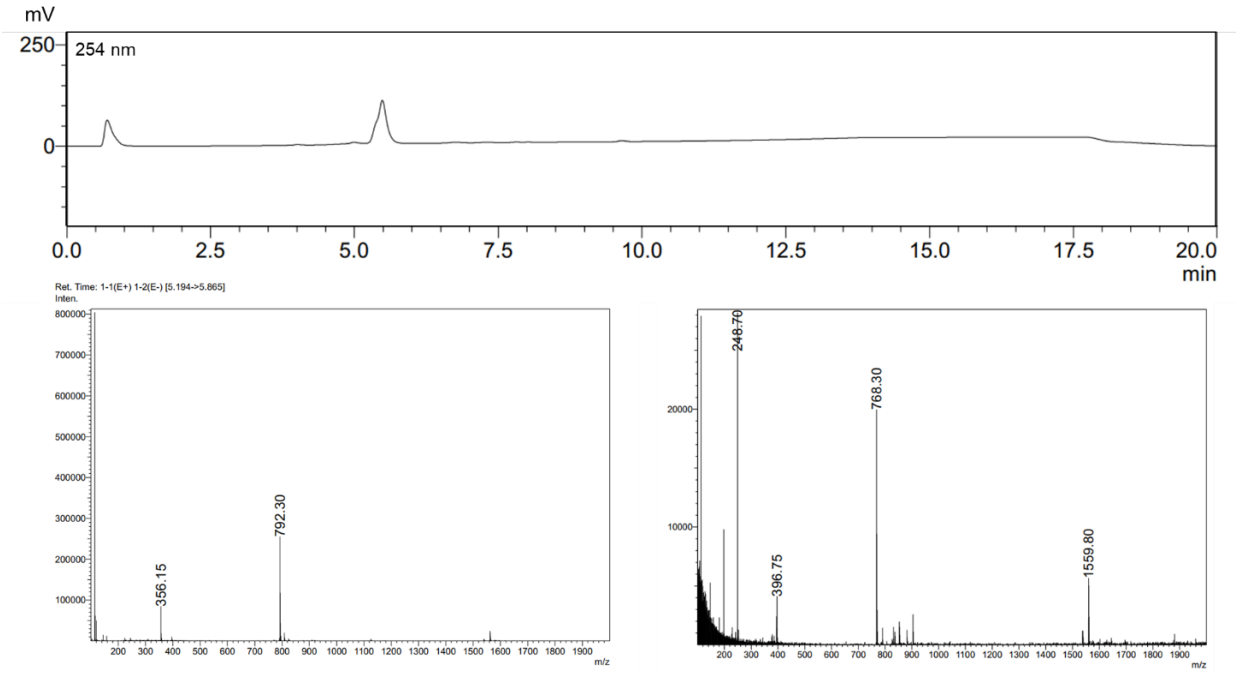


Fig. S4 LC-Spectrum, T_R= 0.75 DMF, T_R= 5.5 Doxorubicin-biotin. ESI(+) left and ESI (-) right.

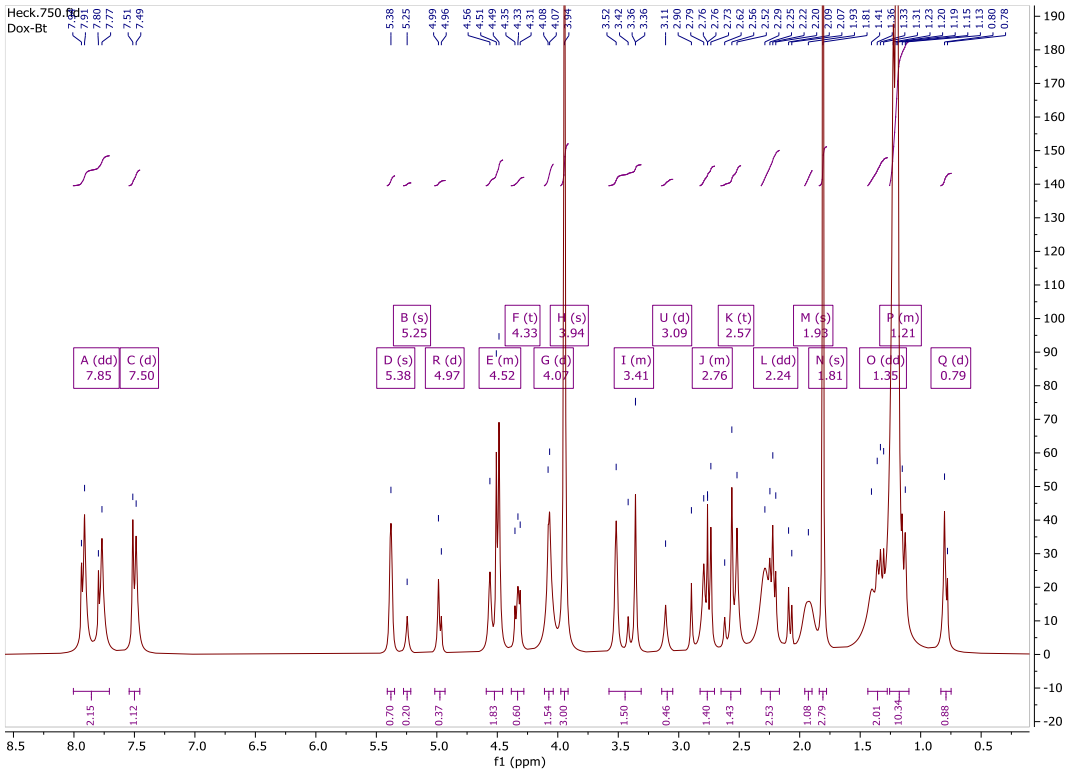
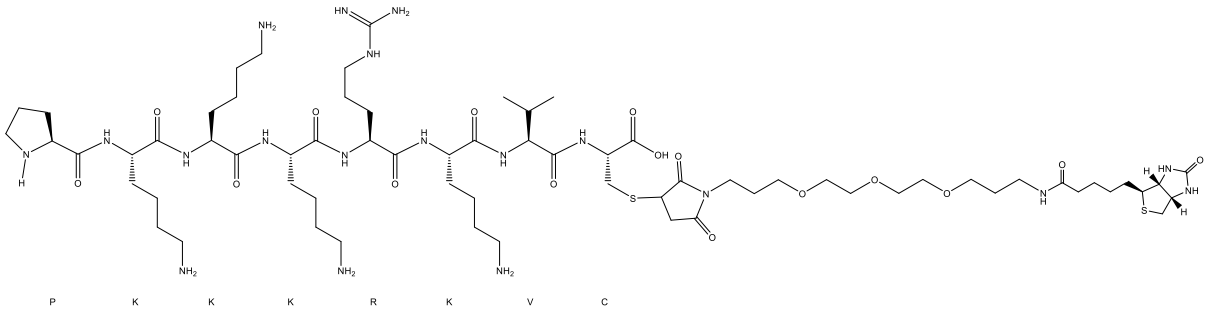


Fig. S5 ^{1}H NMR of doxorubicin-biotin (MeOD-d4, 300 MHz).



Chemical Formula: $C_{67}H_{121}N_{19}O_{16}S_2$
Exact Mass: 1511,87

Fig. S6 The chemical structure of biotin-NH-PKKKRKVC-COOH.

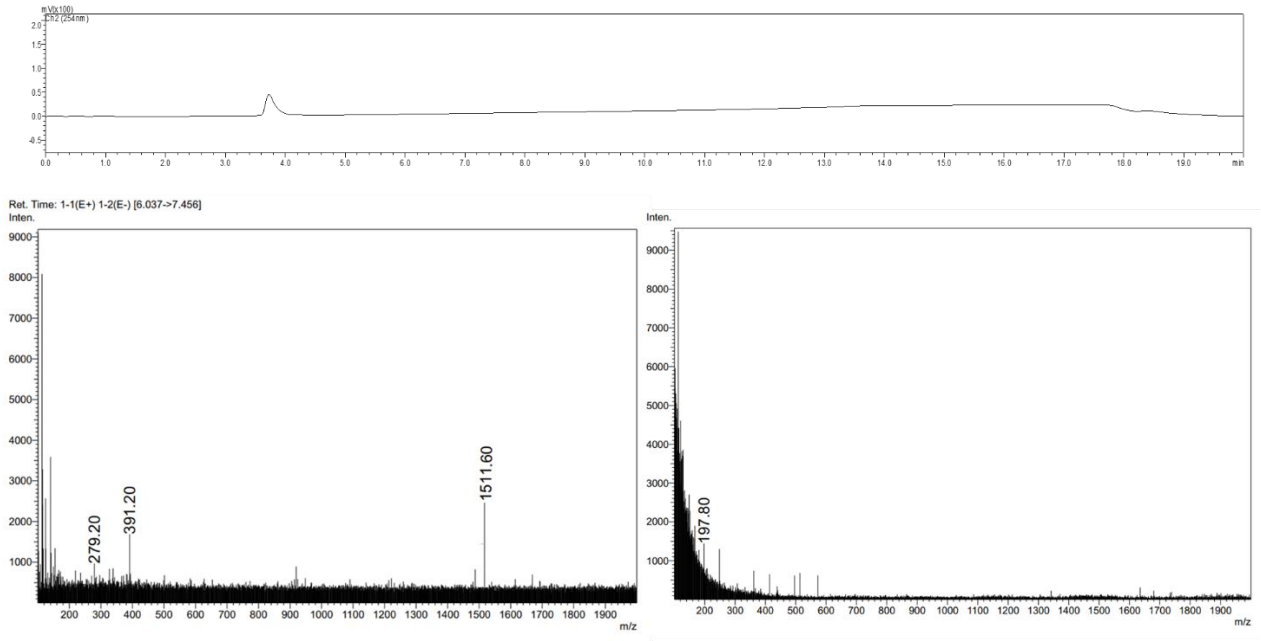


Fig. S7 LC-Spectrum, T_R= 3.91, Biotin-PKKKRKVC. ESI (+) left and ESI (-) right.

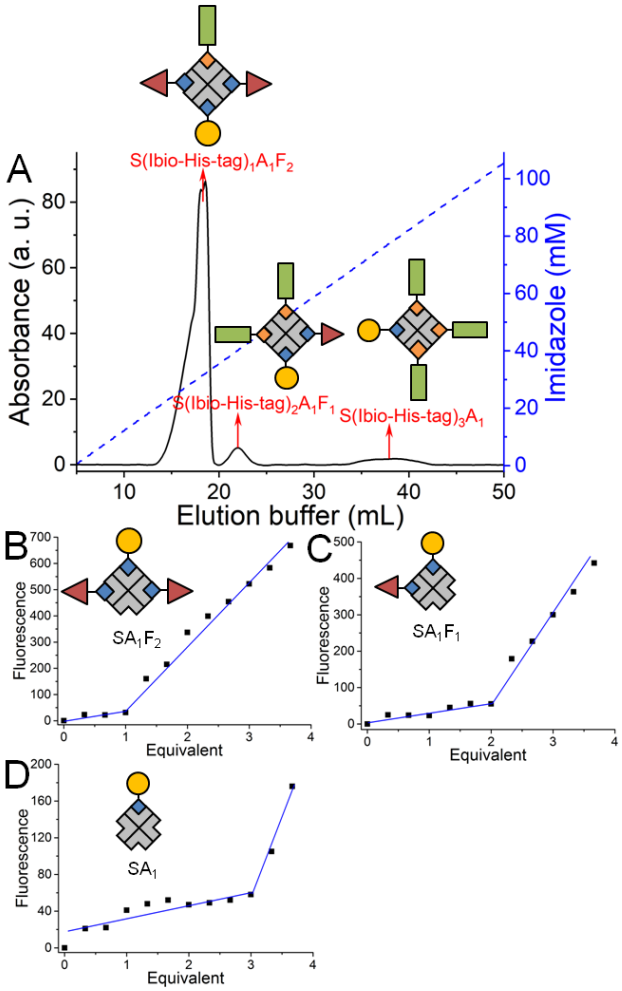


Fig. S8 Preparation and characterization of streptavidin conjugates with fluorophore and folic acid functionality, (SA_1F_2 and SA_1F_1). (A) A statistical mixture of products formed by mixing 10 μM SA_1 first with 20 μM Ibio-His-tag for 15 min, then 20 μM F (folic acid-biotin) was separated with a linear imidazole gradient on a Cu^{2+}-NTA column. S(Ibio-His-tag)$_1A_1F_2$ (the first peak, 83.1%) eluted at 28.4 mM imidazole, S(Ibio-His-tag)$_2A_1F_1$ (the second peak, 12.1%) eluted at 38.7 mM imidazole and S(Ibio-His-tag)$_3A_1$ (third peak, 4.8%) eluted at 74.2 mM imidazole. The Ibio-His-tag was removed from the different S(Ibio-His-tag)$_nA_1F_{3-n}$ species through acidification to yield the corresponding SA_1F_{3-n}. The open biotin binding pockets of SA_1F_2 (B), SA_1F_1 (C) and SA_1 (D) were titrated with biotin-5-fluorescein, where the fluorescence of biotin-5-fluorescein is quenched upon binding to streptavidin. The conjugates SA_1F_2, SA_1F_1 and SA_1 required one, two and three equivalents of biotin-5-fluorescein to saturate all biotin binding sites, respectively.

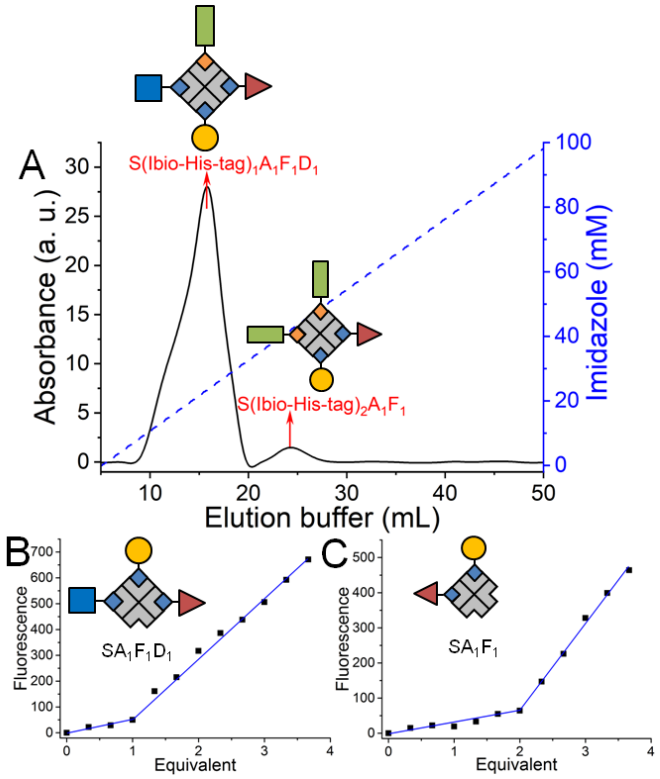


Fig. S9 Preparation and characterization of streptavidin conjugates with fluorophore, folic acid and doxorubicin functionality, ($SA_1F_1D_1$). (A) A statistical mixture of products formed by mixing 10 μM SA_1F_1 first with 15 μM Ibio-His-tag for 15 min, then 15 μM D (doxorubicin-biotin) was separated with a linear imidazole gradient on a Cu^{2+}-NTA column. $S(Ibio\text{-}His\text{-}tag)_1A_1F_1D_1$ (the first peak, 96.2%) eluted at 23.1 mM imidazole and $S(Ibio\text{-}His\text{-}tag)_2A_1F_1$ (the second peak, 3.8%) eluted at 41.5 mM imidazole. The Ibio-His-tag was removed from the different conjugates through acidification to yield the corresponding $SA_1F_1D_1$ and SA_1F_1. The open biotin binding pockets of $SA_1F_1D_1$ (B) and SA_1F_1 (C) were titrated with biotin-5-fluorescein, where the fluorescence of biotin-5-fluorescein is quenched upon binding to streptavidin. The conjugates $SA_1F_1D_1$ and SA_1F_1 required one and two equivalents of biotin-5-fluorescein to saturate all biotin binding sites, respectively.

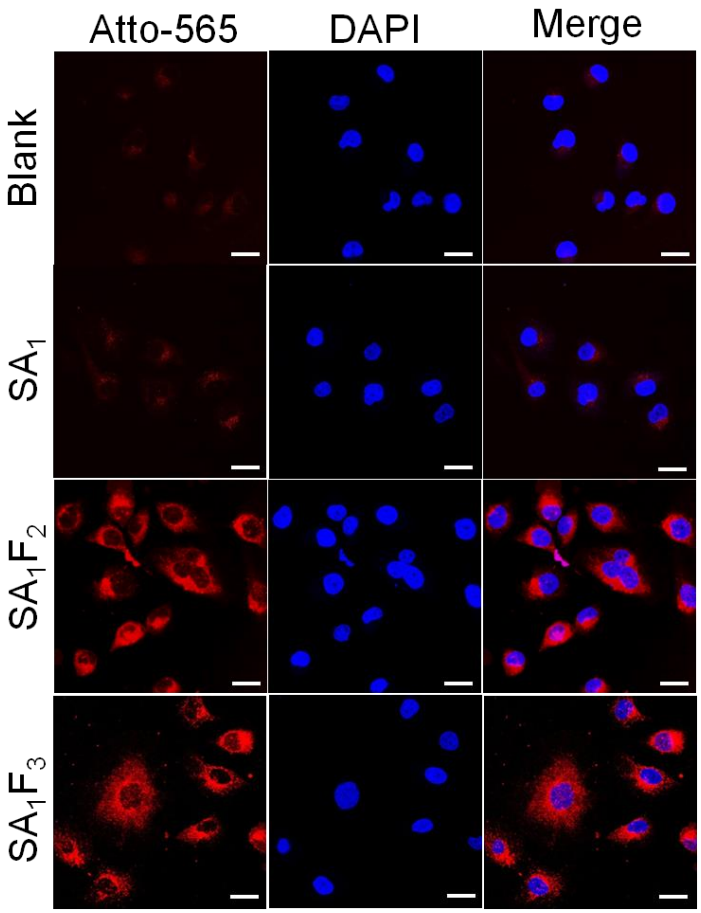


Fig. S10 Confocal microscopy images of MDA-MB-231 cells incubated with different streptavidin-biotin conjugates. Cells were incubated with 1 μM SA_1, SA_1F_2 or SA_1F_3 in RPMI-1640 (no folic acid) medium for 4 hours at 37 ºC, 5% CO_2. Atto-565 fluorescence from the streptavidin conjugates is shown in red and cell nuclei stained with DAPI are shown blue. Cells not incubated with any streptavidin conjugate were used as blank. Scale bars are 25 μm.

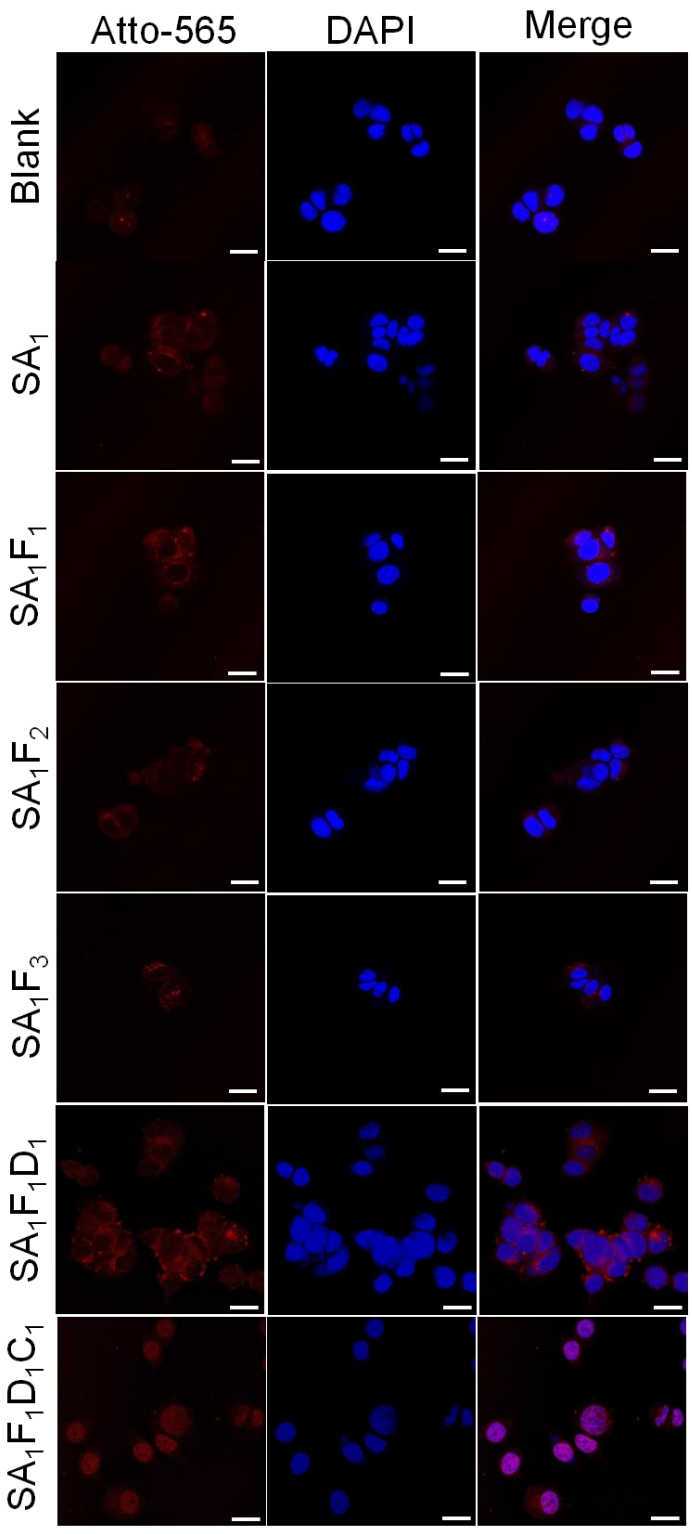


Fig. S11 Confocal microscopy images of MCF-7 cells incubated with multifunctional streptavidin-biotin conjugates. Cells were incubated with 1 μM SA_1, SA_1F_2 or SA_1F_3 in RPMI-1640 (no folic acid) medium for 4 hours at 37 ºC, 5% CO_2. Atto-565 fluorescence from the streptavidin conjugates is shown in red and cell nuclei stained with DAPI are shown blue. Cells not incubated with any streptavidin conjugate were used as blank. Scale bars are 25 μm.

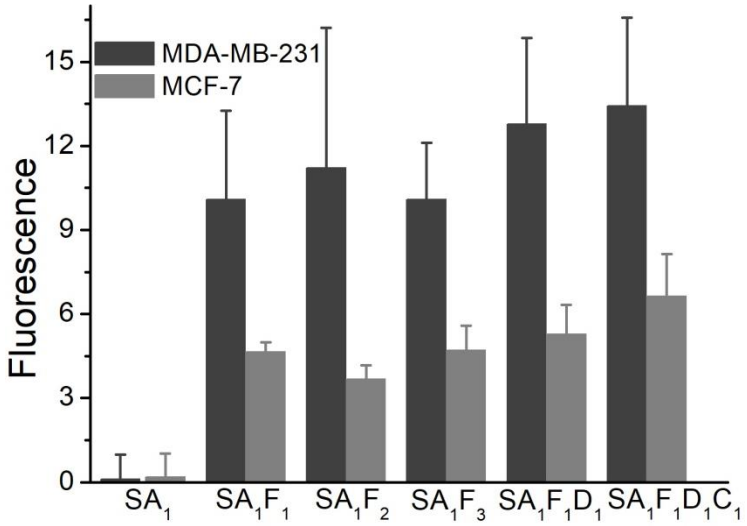


Fig. S12 Average intracellular fluorescence intensities of MDA-MB-231 and MCF-7 cells incubated by different streptavidin conjugates. The fluorescence in the atto-565 channel was measured by encircling single cells and measuring their average intensities and the background fluorescence determined from the blank sample was subtracted. 20 cells were analyzed per sample and the error bars represent the standard error of the mean.

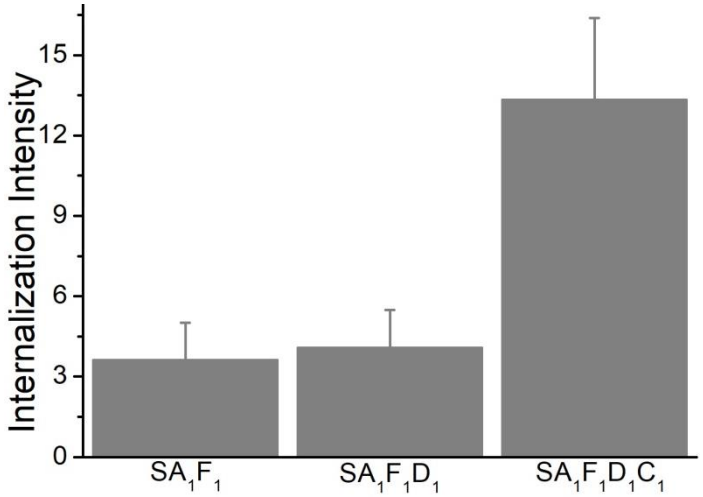


Fig. S13 Average nuclear fluoresce intensity of different streptavidin conjugates in MDA-MB-231 cells. The extend of nuclear localization of the different streptavidin conjugates was quantified by measuring fluorescence intensity in the atto-565 channel inside the nucleus of MDA-MB-231 cell. 20 cells were analysed per sample and the error bars represent the standard error of the mean.

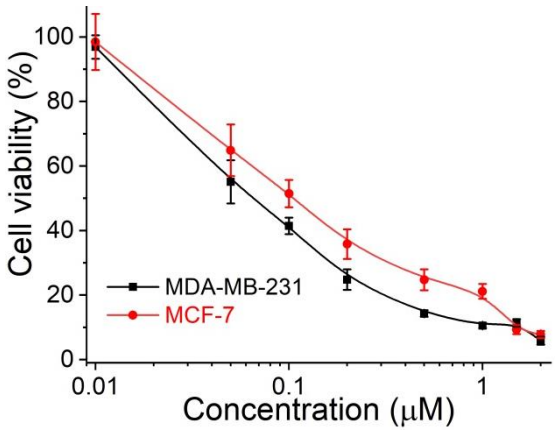


Fig. S14 Cytotoxicity of free doxorubicin-biotin against MDA-MB-231 and MCF-7 cell as measure with the MTT assay. Cells were incubated by RPMI-1640 medium (without folic acid) containing 0.01, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 2.0 μM of doxorubicin-biotin at 37 ºC, 5% CO_2 for 72 hours. The IC_{50} is 0.07 μM and 0.10 μM for MDA-MB-231 and MCF-7 cells, respectively. Data is expressed as the percentages of viable cells relative to a sample without doxorubicin.

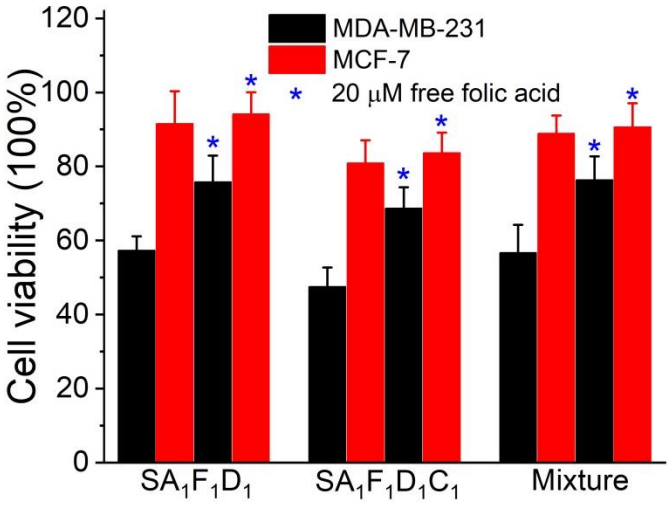


Fig. S15 Cell viability of MDA-MB-231 and MCF-7 cells incubated with 0.5 μM $SA_1F_1D_1$, 0.5 μM $SA_1F_1D_1C_1$ or 0.5 μM of a statistical mixture of streptavidin conjugates (0.5 μM, S was mixed with one equivalent of A, F, D and C) for 72 hours without or with 20 μM extra free folic acid (shown with a blue star), as measured using the MTT assay. S: streptavidin, A: atto-565-biotin, F: folic acid-biotin, D: doxorubicin-biotin, C: nucleus penetrating peptide-biotin.