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Supporting Information

Multifunctional streptavidin-biotin conjugates with precise stoichiometries

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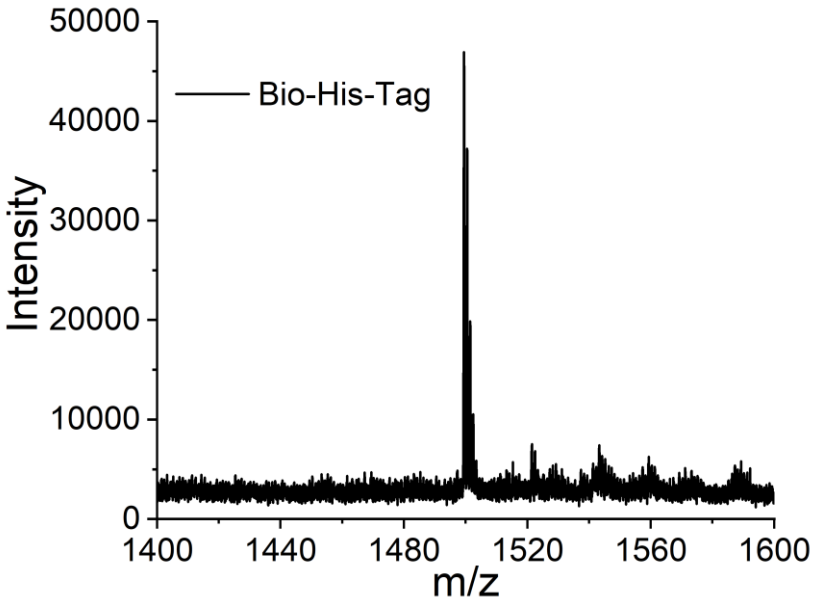

Fig. S1 MALDI-TOF mass spectrum of biotin-$(His)_6$ (Bio-His-Tag). The molecular weight is 1500 g/mol.

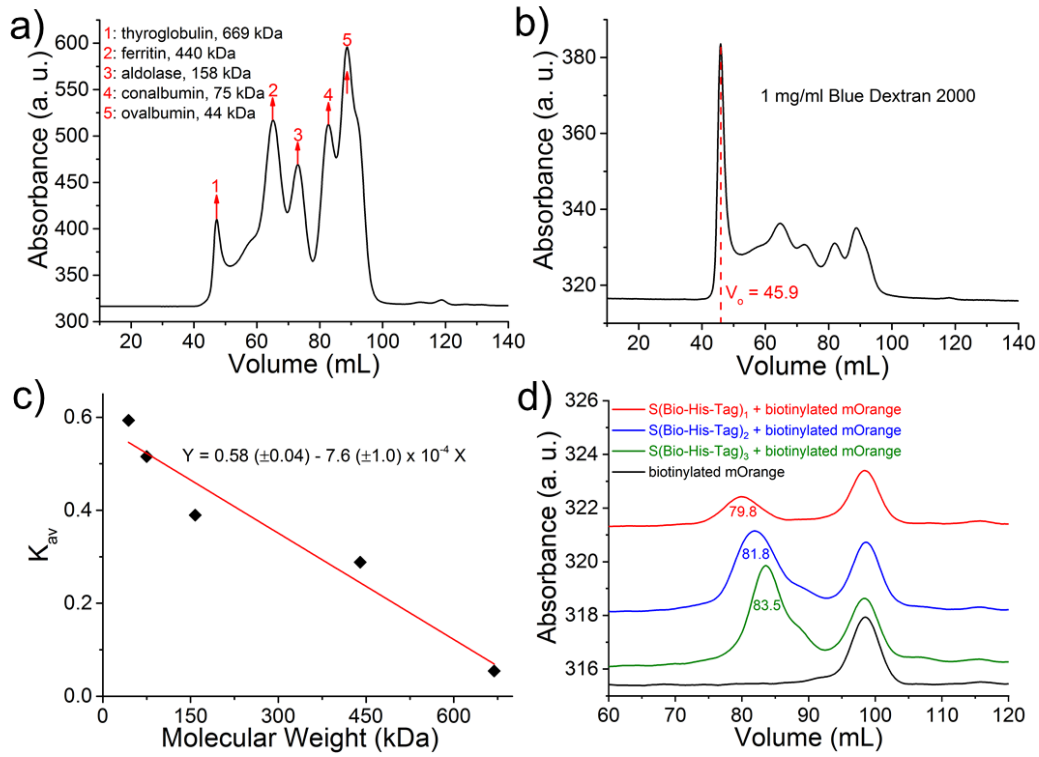

Fig. S2 a) Chromatogram of standard protein mixture and b) blue dextran 2000 on HiLoad™ 16/600, Superdex™ 200 pg size exclusion column. Elution volumes (V_e) for each protein were determined as maximum peak height: peak 1 at 47.2 mL for thyroglobulin (669 kDa), peak 2 at 65.2 mL for ferritin (440 kDa), peak at 73.0 mL for aldolase (158 kDa), peak 4 at 82.7 mL for conalbumin (75 kDa) and peak 5 at 88.7 mL for ovalbumin (44 kDa). The void volume (V_o) is the first eluted peak in b) at 45.9 mL. c) Standardization curve of K_{av} versus protein molecule weight. d) Chromatogram of different streptavidin conjugates ($S(Bio\text{-}His\text{-}Tag)_1$ (trivalent), $S(Bio\text{-}His\text{-}Tag)_2$ (divalent) and $S(Bio\text{-}His\text{-}Tag)_3$ (monovalent)) reacted with biotinylated mOrange. The molecular weights: mOrange (31.3 kDa) streptavidin (53 kDa), Bio-His-Tag (1.5 kDa). The peaks in the chromatogram were for the reaction of biotinylated mOrange with $S(Bio\text{-}His\text{-}Tag)_1$, $S(Bio\text{-}His\text{-}Tag)_2$ and $S(Bio\text{-}His\text{-}Tag)_3$ were at 79.8 mL, 81.8 mL and 83.5 mL, respectively. The theoretical molecular weight was calculated assuming that all remaining open binding pockets were occupied by biotinylated mOrange. $S(Bio\text{-}His\text{-}Tag)_1O_3$ (theo. 148.4 kDa, exp. 141.0 kDa), $S(Bio\text{-}His\text{-}Tag)_2O_2$ (theo. 118.6 kDa, exp. 108.6 kDa) and $S(Bio\text{-}His\text{-}Tag)_3O_1$ (theo. 88.8 kDa, exp. 81.3 kDa). The theoretical and experimental molecular weights were in

agreement showing that the open binding pockets in the streptavidin Bio-His-Tag conjugates were indeed occupied by biotinylated mOrange.

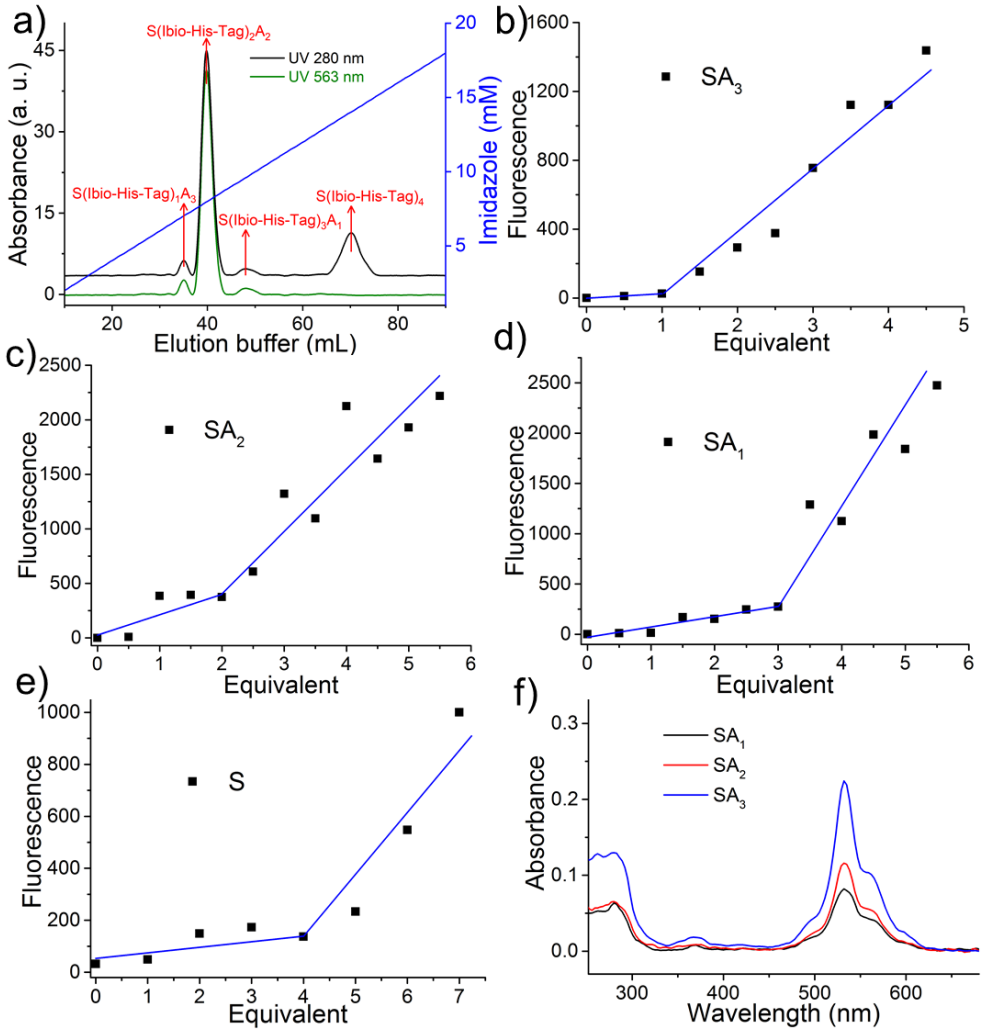

Fig. S3 a) Chromatogram of the reaction mixture of streptavidin (S) (30 µM), which was first reacted with Ibio-His-Tag (90 µM) for 15 min and then with atto-565-biotin (A) (50 µM) for 15 min, separated on a Cu^{2+}-NTA column using an imidazole gradient. The elution of different species was monitored through the absorbance 280 nm (both streptavidin and atto-565-biotin absorb) and 563 nm (only atto-565-biotin absorbs). The different $S(Ibio\text{-}His\text{-}Tag)_nA_{4-n}$ species eluted at increasing imidazole concentrations as the numbers of Ibio-His-Tags increases from 1 to 4. First peak at 6.9 mM imidazole was $S(Ibio\text{-}His\text{-}Tag)_1A_3$, second peak at 8.0 mM

imidazole was S(Ibio-His-Tag)$_2$A$_2$, third peak at 9.6 mM imidazole was S(Ibio-His-Tag)$_3$A$_1$ and the fourth peak at 14.0 mM imidazole S(Ibio-His-Tag)$_4$ (only observed in the absorbance at 280 nm since it does not contain atto-565-biotin). The relative areas of the peaks are: 5.1% 1st peak, 42.6% 2nd peak, 26.7% 3rd peak and 25.6% 4th peak. b-e) The Ibio-His-Tag was removed from the different S(Ibio-His-Tag)$_n$A$_{4-n}$ species through acidification to yield the corresponding SA$_{4-n}$. The open biotin binding pockets of b) SA$_3$, c) SA$_2$, d) SA$_1$ and e) S were titrated with the fluorophore biotin-5-fluorescein, which is quenched upon binding to streptavidin. SA$_3$, SA$_2$, SA$_1$ and S required 1, 2, 3 and 4 equivalents the dye to saturate all biotin binding sites, respectively. (f) The UV-vis absorption spectra of SA$_1$, SA$_2$ and SA$_3$ measured by Nanodrop (ND 8000, 8-sample spectrophotometer, light path= 1 mm). The absorbance at 280 nm and 532 nm were used confirm the stoichiometry of these streptavidin conjugates, as shown in Table S1.

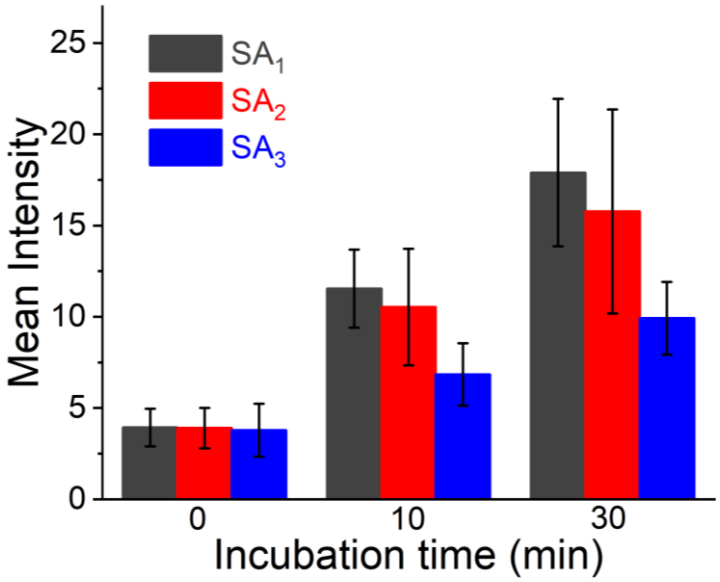

Fig. S4 Intracellular fluorescence intensity of cells with biotinylated surface proteins and labelled with different streptavidin conjugates. 25 cells were analysed per data point. The error bars represent the standard error.

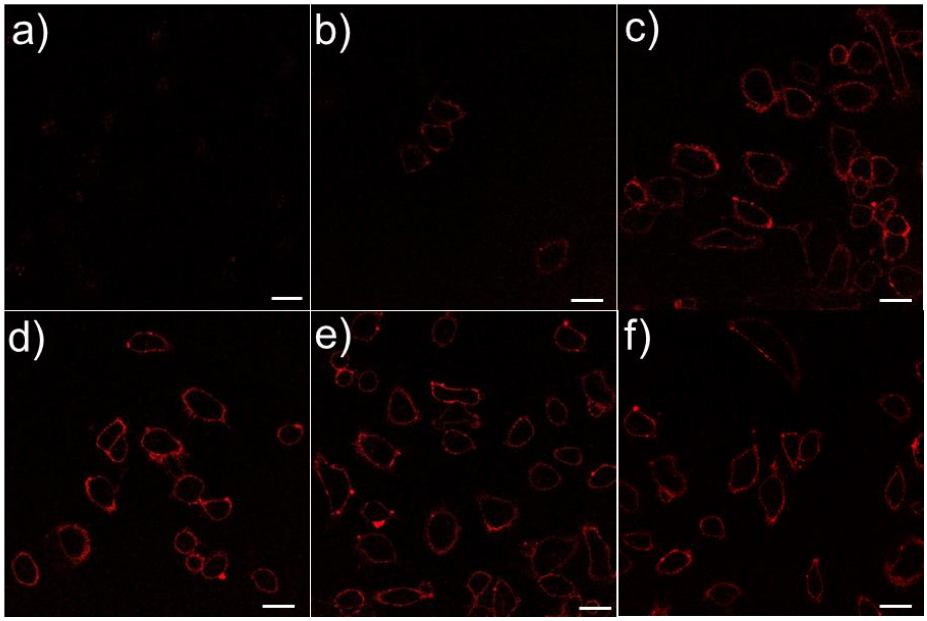

Fig. S5 Fluorescent images in the atto-565 channel of cells with biotinylated surfaces, which were incubated with different concentrations of SA_3. a) 0 µM b) 0.05 µM. c) 0.1 µM. d) 0.5 µM. e) 1.0 µM and f) 1.5 µM. Scale bar: 25 µm.

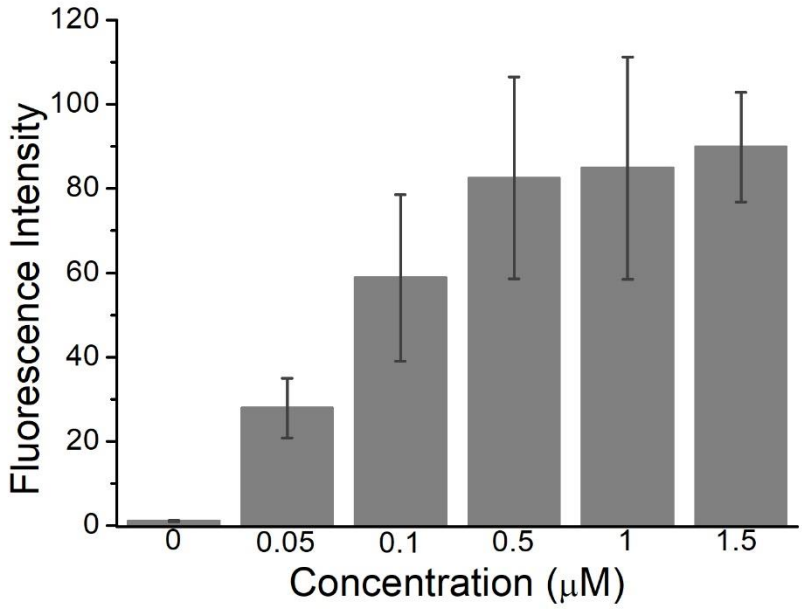

Fig. S6 Fluorescence intensity at the cell membrane of cells in Fig. S5. For each concentration 15 cells were analysed. The error bars represent the standard error.

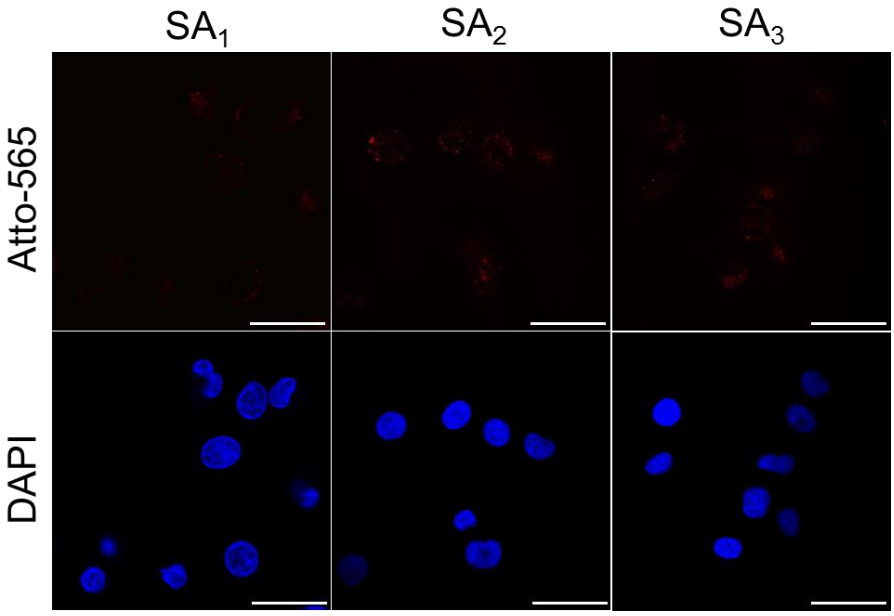

Fig. S7 Confocal microscopy images of folate-receptor positive MDA-MB-231 cells incubated with atto-565 (A) (shown red) streptavidin conjugates with different stoichiometries. Cells were incubated with 1μM SA_1, SA_2 or SA_3 with RPMI-1640 (no folic acid) medium for 4h at 37 ˚C. Cell nuclei were stained with DAPI (shown blue). Scale bars are 50 μm.

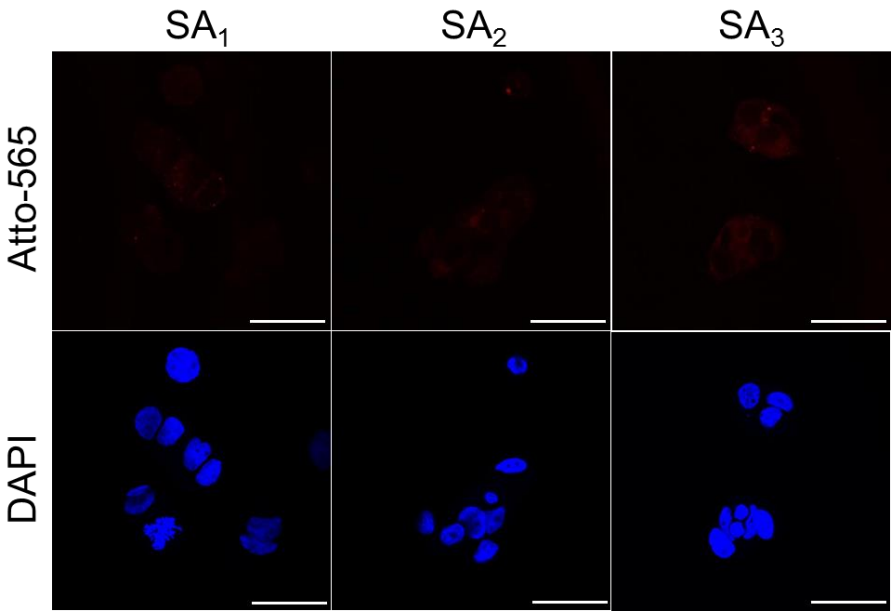

Fig. S8 Confocal microscopy images of folate-receptor negative MCF-7 cells incubated with atto-565 (A) (shown red) streptavidin conjugates with different stoichiometries. Cells were incubated with 1μM SA_1, SA_2 or SA_3 with RPMI-1640 (no folic acid) medium for 4h at 37 ˚C. Cell nuclei were stained with DAPI (shown blue). Scale bars are 50 μm.

Table S1 The concentrations of SA_1, SA_2 and SA_3 were calculated based on the extinction coefficients of pure streptavidin (41326 M^{-1} cm^{-1} at 280 nm) and atto-565-biotin (36734 M^{-1} cm^{-1} at 280 nm and 91835 M^{-1} cm^{-1} at 532 nm) and the stoichiometry of the streptavidin conjugate. Light path= 1 mm, ε_{280} (SA_n) = ε_{280} (S) + n x ε_{280} (A). ε_{532} (SA_n) = n x ε_{532} (A). The concentrations determined based on the absorbance at 280 nm and 532 nm were in agreement confirming the assumed stoichiometry of the SA_{1-3}.

	A_{280}	**ε_{280} (M^{-1} cm^{-1})**	**C_{280} (µM)**	**A_{532}**	**ε_{532} (M^{-1} cm^{-1})**	**C_{532} (µM)**
SA_1	0.0653	78060	8.37	0.0801	91835	8.71
SA_2	0.0664	114794	5.78	0.1108	183670	5.99
SA_3	0.1296	151528	8.55	0.2243	275505	8.14

Table S2 The relative fluorescence brightness's of SA_1F_3, SA_2F_2 and SA_3F_1 were measured in solution and used to normalize the fluorescence intensities measured in confocal images for quantification in Figure 4b.

	# A	**Cons. (nM)**	**Fluorescence**	**Fluorescence / nM**	**Relative fluorescence brightness**
SA_1F_3	1	466	2200	4.721	1
SA_2F_2	2	378	2414	6.386	1.353
SA_3F_1	3	319	2927	9.176	1.944