Azide-Transfer Probes for Affinity-Guided Labeling of Metal-Binding Proteins

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1 Supporting information

Figure S1: Reaction time analysis of the conjugation reactions for the probes. Reducing SDS-PAGE (4-12%) analyses of the reaction time dependence of the probes in the conjugation to cetuximab in presence (left gels) or absence (right gels) of CuSO₄. The analyses indicate that the conjugation reaction is done after 8-10 hours for both probes. Two conjugation reactions were performed in HEPES (50 mM, pH 7.5) containing NaCl (100 mM), cetuximab (2 µM), probe (2 eq.) and either CuSO₄ (4-6 eq., left gels) or EDTA (2 mM, right gels). An aliquot of the reaction mixtures were stopped by addition of EDTA (2 mM – final concentration) at the indicated times – the samples were stored at -20 °C until the labeling reaction was performed. After the last time point, all reactions were subjected to DBCO-Cy5 (10 eq.) in PBS at rt. for 2 hours before analysis. Lane R: control of unspecific labeling in the SPAAC reaction – no probe or cations were added during the conjugation. Lane M: SeeBlue Plus2 protein weight marker (kDa). The same gels were scanned for Cy5-fluorescence (top) and stained for protein using SimpleBlue SafeStain (bottom).
Figure S2: Optimization of the pH in the conjugation reaction. Reducing SDS-PAGE (4-12%) analysis of the labeling of the antibody cetuximab using both the trisNTA and bisNTA probes performed at different pH. The analyses indicate that pH 7.0 – 8.0 is optimal for the conjugation reaction. The conjugation reactions were performed overnight at room temperature in buffer (50 mM) containing NaCl (100 mM), cetuximab (2 µM), probe (5 eq.), and CuSO$_4$ (10-15 eq., 5 eq. per NTA of the probe). The conjugation reactions were washed in PBS and concentrated by ultracentrifugation. The labeling reactions were performed in PBS for 2 h at room temperature with DBCO-Cy5 (10 eq.). Lane 1: MES-buffer (pH 5.0, 50 mM), cetuximab (2 µM), trisNTA probe (5 eq.), CuSO$_4$ (15 eq.). Lane 2: MES-buffer (pH 5.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 3: MES-buffer (pH 6.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), CuSO$_4$ (15 eq.). Lane 4: MES-buffer (pH 6.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 5: HEPES-buffer (pH 7.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), CuSO$_4$ (15 eq.). Lane 6: HEPES-buffer (pH 7.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 7: HEPES-buffer (pH 8.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), CuSO$_4$ (15 eq.). Lane 8: EPPS-buffer (pH 8.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 9: EPPS-buffer (pH 9.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), CuSO$_4$ (15 eq.). Lane 10: EPPS-buffer (pH 9.0, 50 mM), cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 11: MES-buffer (pH 5.0, 50 mM), cetuximab (2 µM), bisNTA probe (5 eq.), CuSO$_4$ (10 eq.). Lane 12: MES-buffer (pH 5.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 13: MES-buffer (pH 6.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.). Lane 14: MES-buffer (pH 6.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 15: HEPES-buffer (pH 7.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.). Lane 16: HEPES-buffer (pH 7.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 17: HEPES-buffer (pH 8.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.). Lane 18: EPPS-buffer (pH 8.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 19: EPPS-buffer (pH 9.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.). Lane 20: EPPS-buffer (pH 9.0, 50 mM), cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). The same gels were scanned for Cy5-fluorescence (top) and stained for protein using SimpleBlue SafeStain (bottom).
**Figure S3:** The dependency of the labeling efficiency on the amount of the probes. Reducing SDS-PAGE (4-12%) analysis of the labeling of the antibody cetuximab using increasing amounts of both the trisNTA and bisNTA probes. The analyses indicate that 20 eq. of the trisNTA probe can be used without significant undirected labeling occurring, while the bisNTA probe suffers from undirected conjugation. The conjugation reactions were performed overnight at room temperature in HEPES (50 mM, pH 7.0) containing NaCl (100 mM), cetuximab (2 µM), probe (X eq.), and CuSO$_4$ (2-3X eq., X eq. per NTA of the probe). The conjugation reactions were washed in PBS and concentrated by ultracentrifugation. The labeling reactions were performed in PBS for 2 h at room temperature with DBCO-Cy5 (10 eq.). Lane 1: cetuximab (2 µM), trisNTA probe (2 eq.), CuSO$_4$ (6 eq.). Lane 2: cetuximab (2 µM), trisNTA (2 eq.), EDTA (2 mM). Lane 3: cetuximab (2 µM), trisNTA (5 eq.), CuSO$_4$ (15 eq.). Lane 4: cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 5: cetuximab (2 µM), trisNTA (10 eq.), CuSO$_4$ (30 eq.). Lane 6: cetuximab (2 µM), trisNTA (10 eq.), EDTA (2 mM). Lane 7: cetuximab (2 µM), trisNTA (20 eq.), CuSO$_4$ (60 eq.). Lane 8: cetuximab (2 µM), trisNTA (20 eq.), EDTA (2 mM). Lane 9: cetuximab (2 µM), trisNTA (0 eq.), CuSO$_4$ (0 eq.). Lane 10: cetuximab (2 µM), trisNTA (2 eq.), CuSO$_4$ (4 eq.). Lane 11: cetuximab (2 µM), bisNTA probe (2 eq.), EDTA (2 mM). Lane 12: cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.). Lane 13: cetuximab (2 µM), bisNTA (5 eq.), CuSO$_4$ (10 eq.).
bisNTA (10 eq.), EDTA (2 mM). Lane 14: cetuximab (2 µM), bisNTA (10 eq.), CuSO₄ (20 eq.). Lane 15: cetuximab (2 µM), bisNTA (10 eq.), EDTA (2 mM). Lane 16: cetuximab (2 µM), bisNTA (20 eq.), CuSO₄ (40 eq.). Lane 17: cetuximab (2 µM), bisNTA (20 eq.), EDTA (2 mM). Lane 18: cetuximab (2 µM), bisNTA (0 eq.), CuSO₄ (0 eq.). Lane M: SeeBlue Plus2 protein weight marker (kDa). The same gels were scanned for Cy5-fluorescence (left) and stained for protein using SimpleBlue SafeStain (right).
Figure S4: Investigation of the labeling efficiency on the concentration of protein. Reducing SDS-PAGE (4-12%) analysis of the labeling of the antibody cetuximab at different protein concentrations. The analyses indicate that high protein concentrations could be used for the trisNTA probe without observing significant unguided labeling, while the bisNTA probe suffers from undirected conjugation at high concentrations. The conjugation reactions were performed overnight at room temperature in HEPES (50 mM, pH 7.0) containing NaCl (100 mM), cetuximab (X µM), probe (5 eq.), and CuSO₄ (10-15 eq., 5 eq. per NTA of the probe). The conjugation reactions were washed in PBS and concentrated by ultracentrifugation. The labeling reactions were performed in PBS for 2 h at room temperature with DBCO-Cy5 (10 eq.). Lane 1: cetuximab (2 µM), trisNTA probe (5 eq.), CuSO₄ (15 eq.). Lane 2: cetuximab (2 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 3: cetuximab (5 µM), trisNTA (5 eq.), CuSO₄ (15 eq.). Lane 4: cetuximab (5 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 5: cetuximab (10 µM), trisNTA (5 eq.), CuSO₄ (15 eq.). Lane 6: cetuximab (10 µM), trisNTA (5 eq.), EDTA (2 mM). Lane 7: cetuximab (2 µM), bisNTA (5 eq.), CuSO₄ (10 eq.). Lane 8: cetuximab (2 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 9: cetuximab (5 µM), bisNTA (5 eq.), CuSO₄ (10 eq.). Lane 10: cetuximab (5 µM), bisNTA (5 eq.), EDTA (2 mM). Lane 11: cetuximab (10 µM), bisNTA probe (5 eq.), CuSO₄ (10 eq.). Lane 12: cetuximab (10 µM), bisNTA (5 eq.), EDTA (2 mM). Lane M: SeeBlue Plus2 protein weight marker (kDa). The same gels were scanned for Cy5-fluorescence (left) and stained for protein using SimpleBlue SafeStain (right).
Figure S5: Labeling of various metal-binding proteins using both probes. Non-reducing SDS (4-12%) analysis of the conjugation and subsequent labeling of metal-binding proteins with the trisNTA and bisNTA probes. The reactions in lanes 1-5 were performed in HEPES-buffer (pH 7.5, 50 mM) with NaCl (100 mM) containing the protein (2 µM). All reactions were washed with PBS by ultrafiltration and reacted with DBCO-Cy5 (10 eq.). Trastuzumab, rituximab and transferrin: Lane 1: protein, CuSO₄ (6 eq.), trisNTA (2 eq.). Lane 2: protein, trisNTA (2 eq.), EDTA (2 mM). Lane 3: protein, CuSO₄ (4 eq), bisNTA (2 eq.). Lane 4: protein, bisNTA (2 eq.), EDTA (2 mM). Lane 5: protein, EDTA (2 mM). Lane M: protein weight marker SeeBlue Plus2 (kDa). Carboxypeptidase B: Lane 1: protein, CuSO₄ (30 eq.), trisNTA (10 eq.). Lane 2: protein, trisNTA (10 eq.), EDTA (2 mM). Lane 3: protein, CuSO₄ (10 eq), bisNTA (5 eq.). Lane 4: protein, bisNTA (5 eq.), EDTA (2 mM). Lane 5: protein, EDTA (2 mM). Lane M: protein weight marker SeeBlue Plus2 (kDa). The same gels were stained for proteins by SimpleBlue SafeStain (right) and scanned for Cy5 fluorescence (left).
Figure S6: MALDI-TOF MS of transferrin conjugation reactions. The conjugation reactions was performed according to the general procedure with the indicated equivalents of the probes. The spectra for transferrin conjugation contain the peak from unmodified transferrin (the first peak indicated with a red line) and the conjugate (the second peak indicated with a red line). Due to the small size of the modification, only a small shift is observed in mass resulting in the peaks appearing as shoulders in the spectra. The modification has an expected mass of 245 Da.
Figure S7: MALDI-TOF MS of nanobody EgA1-His$_6$ conjugation reactions. The conjugation reactions was performed according to the general procedure with the indicated equivalents of the probes. The spectra for EgA1 conjugation contain the peak from unmodified EgA1 (the first peak indicated with a line) and the conjugate (the second peak indicated with a line). The modification has an expected mass of 245 Da. Surprisingly, the bisNTA probe is more efficient at labeling the nanobody even using fewer equivalents of probe.
Figure S8: MALDI-TOF MS of a GFP enhancer nanobody conjugation reactions. The conjugation reactions was performed according to the general procedure with the indicated equivalents of the probes. The spectra for GFP enhancer nanobody (GFP-NB) conjugation contain the peak from unmodified GFP-NB (the first peak indicated with a line) or the conjugate (the second peak indicated with a line). The modification has an expected mass of 245 Da. Surprisingly, the bisNTA probe is more efficient at labeling the nanobody even using fewer equivalents of probe.
Figure S9: MALDI-TOF MS of a GFP enhancer nanobody conjugation reactions. The conjugation reactions was performed according to the general procedure with the indicated equivalents of the probes. The large mass of the antibody cetuximab results in the spectra not resolving the peaks for cetuximab and conjugate, however, the peaks move toward higher masses in the conjugations indicating modification of the proteins has been performed. The fact that the peaks do not move the expected 245 Da of the modification could be an indication that full conversion of the protein is not obtained.
Figure S10: MALDI-TOF MS of His$_6$-GFP conjugation reactions. The conjugation reactions were performed according to the general procedure with the indicated equivalents of the probes. The spectra for GFP conjugation contain the peak from unmodified GFP (the first peak indicated with a line) or the conjugate (the second peak indicated with a line). Unfortunately, the second peak is positioned at a shoulder peak for unmodified GFP, however, a clear trend toward higher masses is observed indicating the conjugation of the probe. The peak for unmodified GFP is still observed in the conjugations indicating that full conversion of the unmodified protein is not obtained. The modification has an expected mass of 245 Da.
Estimations of conversion for the conjugation reactions using different proteins. Non-reducing SDS-PAGE (4-12%) analysis of the labeling efficiency of the conjugation reactions for metal-binding proteins with the trisNTA and bisNTA probes. Conjugation reactions were performed overnight at room temperature in HEPES-buffer (pH 7.5, 50 mM) with NaCl (100 mM) containing the protein (2 μM), probe (5 eq. bisNTA and 10 eq. (gel B) - 20 eq. (gel A) trisNTA), and CuSO\textsubscript{4} (2-3X eq., X eq. per NTA of the probe). After the reaction, EDTA (2.5 mM final concentration) was added and the reactions was washed with PBS containing 0.02% tween and concentrated by ultracentrifugation. The concentration was determined by UV absorbance. An aliquot of 30 pmol protein of each reaction mixture were subjected to DBCO-Cy5 (10 eq.) in PBS containing 0.02% tween at rt. for 2 hours. The gel loading buffer was added and the reactions were left for additional 30 min. before analysis. The same gels were stained for proteins by SimpleBlue SafeStain (right) and scanned for Cy5 fluorescence (left). B shows lanes from the same visualization of the same gel. Lane M: SeeBlue Plus2 protein weight marker (kDa).

Estimation of labeling efficiency: the labeling efficiency of transferrin was estimated to 50% from the MS spectrum of the conjugation using the trisNTA probe (Figure S6) in 10 or 20 equivalents. While quantification of the conversion is generally not possible for these type of MS experiments, we approximate that transferrin and conjugated transferrin will behave similar in the MS experiments due to the small size of the modification and the large size of the protein that means it have several groups that can be protonated for ionization. The remaining conjugation reactions were evaluated based on the relative fluorescence from the conjugations compared to the trisNTA coupling of transferrin. Surprisingly, the nanobodies show very poor conversion, and they even show higher conversion for the bisNTA using only 5 equivalents of probe.
**bisNTA probe**

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**trisNTA probe**

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_Figure S12. Conclusion on LC-MS/MS analysis of cetuximab modified using the trisNTA or bisNTA probes._ The conclusion is based on the analyses shown in Figures S13-S16. LC-MS/MS fragmentation: the presence of the modification was based on the fragmentation spectra of modified peptides. Chromatographic presence: the presence of modified peptides was compared between the trisNTA, bisNTA and unmodified samples, which indicated that some of the modifications found in the bisNTA sample was also physically present in the trisNTA sample even though they were not identified by MS/MS fragmentation.
Sequence map of the heavy chain modifications from LC-MS/MS analysis. Red: matched peptides, Green: modified peptides from bisNTA probe, Blue: Modified by both the trisNTA and bisNTA probes. Both the bisNTA and trisNTA have 70% sequence coverage.

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1   QVQLKQSGPG LVQPSQLSLI TCTVSGLSLT NYGVHWVRQS PGKGLEWLGV
51  IWSSGNTDYN TPFTSRLSN KDNSSQVFF KMNSLQSNDD AIYYCARALT
101  YDYEFAYWG QGTLVTSSAA STKGFSVFPL APSSKSTSGG TAALGCLVKD
151  YFPEPVTVSW NSGALTSGVH TFPAVLQSST LYSLSVVTVV PSSSSLGTQTY
201  ICNVNHKPSN TKVDKKVEFK SCDKHTCPP CPAPELLGGP SVFLPPPKPK
251  DTLMSRTEP UTCVVVDSVH EDPEVKFNWY VGDVENVNAY TKPREEQYNS
301  TYRVVSVLTV LHQDWLNGKE YKCKVSNKAL PAPIEKISK AKGQPREPQV
351  YTLPPSRDEL TKNQVSLTCL VKGFYPGDI AVEWESNGQPE NNYKTTPVVL
401  DSGSSFLYLS KLTVDKSRWQ QGNVFSCSVM HEALRHNYTQ KSLSLSPGKS
451  PXQXXXXXXXXX ETUXIMABLI GHTCHAINXG OTHELFHEJE DILTQTSPVI
501  LSVPSPGERSV FSCRASQSIG TNHIWYQQRT NGSPLLKY ASESISGIPS
551  SFSGSSTGDT FTLSINSVES EDIADYYYCQQ NNNWPTTFGA GTRLKLEKRTV
601  AASVFIIFPP SDEQLKSGTA SVVCLNNFY PREAKVQWVK DNLASGNSNQ
651  ESUETQDSKD STYSLSTLT LSKADYEHK K YACEVTHQG LSSPVKTSFN
701  RGEQ
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Fragmentation spectra of the modified heavy chain peptides from LC-MS/MS analysis.
Figure S13. Sequence map and fragmentation spectra from the tandem MS analysis of the heavy chain peptides of cetuximab. Heavy chain of cetuximab was analysed by LS-MS/MS and a protein sequence coverage of 70% was obtained. Matched peptides shown in bold red in the sequence map. In sample the bisNTA sample, four peptides were identified (p<0.002) to be modified by the reduced modification on lysine residues, depicted by green color in the sequence, with modified lysine residue in gray. The peptide KDNSKSQVFFK was also identified in the trisNTA sample, while the remaining peptides were exclusively observed in the bisNTA sample.
Sequence map of the light chain modifications from tandem MS analysis. Red: matched peptides, Green: modified peptides from bisNTA probe. No modified peptides were found in the trisNTA sample. Both the bisNTA and trisNTA samples showed 90% sequence coverage.

1  DILLTQSPVI  LSVSPGERS  FSCRASQSIG  TNIHWYQRT  NGSPPLILIY
51  ASEISGIPS  RFSGSGSGTD  FTLSINSVES  EDIADYYCQQ  NNNWPTTFGA
101  GTKLELKRTV  AAPSVFIFPP  SDEQLKSCTA  SVVCLNNFY  PREAKVQWKV
151  DNALQSGNSQ  ESVTEQDSKD  STYSLSTLT  LSKADYEKHK  VYACEVTHQG
201  LSSPVTKSFN  RGEC

Fragmentation spectra of the modified light chain peptides from tandem MS analysis.
Figure S14. Sequence map and fragmentation spectra from the tandem MS analysis of the light chain peptides of cetuximab. Light chain of cetuximab was analysed by LS-MS/MS and a protein sequence coverage of 90% was obtained. Matched peptides are shown in bold red in the peptide map. In the bisNTA sample, four peptides were identified (p<0.002) to be modified by the reduced modification on a lysine residue, depicted by green color in the sequence, with modified lysine residues in gray. No statistically significant modifications were obtained for the trisNTA sample.
Figure S15. Chromatographic verification of light chain tandem MS. Extracted Ion Chromatograms (XIC) for the peptides identified to be modified in light chain samples. In each graph the scale of the three figures were the same. All four peptides (A-D) had MS/MS evidence (p<0.002) for the bisNTA probe. For the trisNTA probe no peptide modification passed statistical criteria, although one of the peptides (A) had XIC signal for the trisNTA probe, and two peptides (Fig B and C) had trace amounts in trisNTA sample. Blue color
indicate position of the identified peptide, for which the indicated m/z value was extracted. The total ion intensity of the three samples are shown in in Fig E.

Figure S16. Chromatographic verification of heavy chain modification from tandem MS analysis. Extracted Ion Chromatograms (XIC) for the peptides identified to be modified in heavy chain samples. In each graph the scale of the three figures were the same. Peptide DNSKSQVFFK (Fig A) had MS/MS evidence (p<0.002) in both sample bisNTA and trisNTA, whereas the other three only had MS/MS evidence in the bisNTA sample. Fig A and B indicate presence of modification in both bisNTA and trisNTA sample. Blue color indicate position of the identified peptide peak, for which the indicated m/z value was extracted. The total ion intensity (E) were similar for the three samples.
2 General information

In general, the chemicals were purchased from Sigma-Aldrich. EDC•HCl was purchased from Iris Biotech GmbH. Organic reactions for small molecule synthesis were monitored by thin-layer chromatography using Merck® silica gel 60 F254 TLC plates. The solvents for small molecule synthesis were purchased as HPLC grade, and dry solvents were purified and dried by a MBRAUN MB SDS-800 solvent purification system (dry DMF was purchased from Sigma-Aldrich). Small molecules were analyzed with NMR (1H-NMR 400 MHz and 13C-NMR 100 MHz) on a Bruker Ascend 400 spectrometer. The NMR spectra are calibrated to the residual solvent peak, and the following abbreviations have been utilized for the reports: singlet (s), doublet (d), triplet (t), quartet (q), double doublet (dd) and multiplet (m). High-resolution mass spectrometry (HRMS) was performed for small molecules on a Bruker Daltonics MicroTOF using electrospray ionization (ESI).

Proteins were generally used without further purification except antibodies purchased as pharmaceutical. These were washed repeatedly (10x) in pH 6.8 phosphate buffer using Amicon Spin filters. Apo-serotransferrin (Product number: T1147), Alkaline phosphatase (Product number: 10713023001) and carboxypeptidase B (Product number: 8039852001) were purchased from Sigma-Aldrich. His6-tagged GFP (Product number GFPG8965-10E) was purchased from US Biological. Herceptin™ (Trastuzumab), MabThera™ (Rituximab) and Erbitux™ (Cetuximab) were purchased as the pharmaceutical at the pharmacy of Aarhus University Hospital. The EgA1 nanobody had been expressed using a standard protocol, and the gene purchased from Genscript.

Protein concentrations were determined using A ND-1000 NanoDrop® spectrophotometer for the UV-vis measurements. Water was purified by Milli-Q Biocell System before use in protein containing experiments. Samples for SDS-PAGE analysis were prepared by addition of NuPAGE™ LDS Sample buffer (4X) to a concentration of 1X followed by heating to 70-90°C for 5-10 min prior to loading of the gel. For reducing gels: to the protein containing samples were added TCEP (2 mM) or DTT (2 mM) with the loading buffer. SDS-PAGE analysis was performed using precast gels (NuPAGE®; Novex™ 4-12% Bis-Tris protein gel 1.0 mm) according to the manufacturer's instructions. Native PAGE was performed using precast gels (Novex™ TBE gels, 4-20%) with a sucrose loading buffer (6x) containing Orange G and performed according to manufacturer’s instructions. Gels were stained for protein using SimplyBlue™ SafeStain (Life Technologies™) and imaged with a Gel Doc™ EZ (Bio-Rad). The images were analyzed with ImageLab software. Gels containing fluorescent proteins or conjugates were imaged on a Typhoon Trio scanner from GE Healthcare and analyzed using ImageQuant TL software.
3 Protocols

Bioconjugation with the imidazole carbamate probes and SPAAC reaction
Stock solutions of the probes (1 mM in DMSO) were made and stored at -20 °C. An aliquot of the stock was diluted (10 times) in water and used to perform the conjugation reaction. The metal binding protein (2 µM) was added to a solution of crude probe (4-10 µM), CuSO$_4$ (bisNTA: 8-20 µM or trisNTA: 12-30 µM) and NaCl (100 mM) in HEPES buffer (50 mM, pH 7.5). The reaction mixture was left at rt for 4 h to overnight, followed by removal of excess probe by washing (3xPBS buffer) using Amicon® Ultra centrifugal filters with appropriate MWCO (3K-30K MWCO, 14100 g for 30-10 min). DBCO-Cy5 (10 eq. to protein) was added to the solution and the reaction mixture was left at rt for 2 h. The samples were washed with PBS (3x) using Amicon® Ultra centrifugal filters with appropriate MWCO (3K-30K MWCO, 14100 g for 30-10 min) and were analyzed by SDS-PAGE (4-12%).

Protein analysis by nano-LC-MS/MS
Proteins were identified and quantified by a nano-LC-MS/MS (nanoscale-liquid chromatography tandem mass spectrometry) label-free proteomics approach. Each gel piece was cut into 1×1 mm pieces and cysteine residues were reduced by 50 mM tris(2-carboxyethyl)phosphine and subsequently blocked by 50 mM iodoacetamide. In-gel digestion was performed using trypsin (0.01 µg/µL Trypsin Gold, Promega, Madison, WI, USA) and the resulting peptides were extracted from the gel pieces using acetonitrile and trifluoroacetic acid and finally purified on PepClean C-18 Spin columns according to manufacturer's instructions (Thermo Scientific). Nano-LC-MS/MS was performed on an EASY nanoLC coupled to a Q Exactive Plus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Bremen, Germany). Peptide samples were separated on a C18 reverse phase column (PepMap from Thermo Scientific with 25 cm length, 75 µm inner diameter, and 2 µm particle size) by using a 62 minutes linear gradient of acetonitrile (4–40%) containing 0.1% formic acid. The mass spectrometer was operated in data dependent mode, automatically switching between MS survey scans (391-1400 m/z) and MS/MS fragmentation scans, with mass resolution of 70,000 and 17,500, respectively. 15 seconds of dynamic exclusion was applied. Up to ten most intense ions were fragmented per every full MS scan, by higher-energy collisional dissociation. Ions with single charge or unassigned charge states were excluded from fragmentation.

Proteomics database searches
Proteome Discoverer 2.2 (Thermo Scientific) equipped with the Mascot algorithm (www.matrixscience.com) was used for identification of peptides and peptide modifications. Manual quality control of the MS data, i.e. retention times, charges and fragmentation of peptides was performed in Qual-browser (Thermo Scientific). The MS raw files were searched against a database consisting of an in-house constructed database containing the two target Cetuximab sequences, together with the Bos taurus proteome (downloaded from UniProt.org) to minimize false discovery identification rates. Carbamidomethylation of cysteines was set as dynamic modification together with the specific modification of the probe; mass addition of 219.110673 from the adduct C$_9$H$_{17}$NO$_5$.

The adduct corresponds to the reduced azide, since the reduction of the disulfides with TCEP also reduces the azide to the amine. The search was performed for all nucleophilic amino acid residues, however, only modification on lysine was statistically significant.
Cell experiments
Cell culture
The MDA-MB-231 cells were maintained in DMEM with high glucose (Sigma). The cell cultures were supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (50 µg/mL), and they were kept at 37 °C in humidified air containing 5% CO₂.

Immunofluorescence analysis
For microscopy analysis, 80,000 MDA-MB-231 cells expressing EGFR were seeded in 8-well chamber slides and incubated overnight in growth medium. The cells were incubated with 100 nM Cy5-conjugated cetuximab in fresh growth medium at 37 °C for 1 h. In the competition experiments, cells were charged with 1 µM unmodified cetuximab 5 min before addition of the fluorescent conjugates. The cells were subsequently washed in PBS (3x), and fixed with 4% PFA in PBS for 15 min followed by two washes with PBS. The slide was dried and mounted with ProLong Gold antifade mountant with DAPI (Thermo Fischer). The mounted slide was allowed to settle by storage at 4 °C overnight before microscopy.

Confocal laser scanning microscopy
Confocal laser scanning microscopy was performed on a LSM 700; Zeiss, Jena, Germany. The instrument contains 4 solid state lasers (405, 488, 543 and 639 nm), and the light was collected with an 63 x 1.4 DIC M27 oil immersion objective (Zeiss). DAPI was excited with the 405 nm laser, and Cy5 was excited with the 639 nm laser. Image recording and analysis were performed in ZEN 2011 software, version 7.0 (Zeiss).

Synthesis of the probes
Tetra-tert-butyl 10-(2-(1H-imidazol-4-yl)acetamido)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,14,20-tetraazahenicosane-1,3,19,21-tetracarboxylate (1)

DMAP (0.156 g, 1.28 mmol, 2.5 eq.), EDC•HCl (0.296 g, 4.54 mmol, 3 eq.) and 4-imidazoleacetic acid hydrochloride (0.169 g, 1.05 mmol, 2 eq.) were added to a stirring solution of the bisNTA amine² (0.500 g, 0.514 mmol, 1 eq.) in dry DMF (40 mL). The reaction was stirred overnight at rt. under an argon atmosphere. The solvent was removed under reduced pressure, and the residue was dissolved in DCM (40 mL) and washed with a 5% NaHCO₃ solution (40 mL), water (40 mL) and brine (40 mL). The organic phase was dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure. The residue was purified by flash column chromatography (DCM with 0% to 8.5% MeOH) yielding the product 1 (0.260 g, 0.260 mmol, 50%) as a colorless oil. Rₜ = 0.32 (DCM:MeOH, 9:1): ¹H NMR (100 MHz, CDCl₃) δ 7.79 (d, J = 7.8 Hz, 1H), 7.63 (s, 1H), 7.03 (t, J = 5.7 Hz, 1H), 6.92 (s, 1H), 6.54-6.50 (m, 1H), 4.37 (td, J = 8.2, 4.7 Hz, 1H), 3.68-3.04 (m, 3.68-3.62, 16H), 3.35-2.20
(m, 2H), 2.16-2.08 (m, 1H), 2.08-1.96 (m, 1H), 1.65 – 1.30 (m, 69H).^1\textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) δ 172.8, 172.4, 172.3, 171.3, 171., 170.9, 170.8, 135.8, 81.3, 81.2, 81.1, 81.0, 65.3, 64.9, 54.2, 54.0, 53.1, 39.3, 39.1, 36.0, 32.6, 30.6, 29.9, 29.0, 28.4, 28.2, 28.1, 27.5, 23.3, 23.1.

HRMS (ESI): calc. for C\textsubscript{54}H\textsubscript{95}N\textsubscript{7}O\textsubscript{15}\textsuperscript{2+} ([M+2H]\textsuperscript{2+}) 540.8438, found: 540.8445.\textsuperscript{†} Integral off due to water.

2-(2-(2-(2-Azidoethoxy)ethoxy)ethoxy)ethyl (2,5-dioxopyrrolidin-1-yl) carbonate (2)

Azide-modified tetraethylene glycol was synthesized according to a literature procedure.\textsuperscript{3} N,N’-disuccinimidyl carbonate (438 mg; 1.71 mmol; 2.5 eq.) was added to a solution of azide-modified tetraethylene glycol (150 mg; 0.684 mmol; 1 eq.) and K\textsubscript{2}CO\textsubscript{3} (52 mg; 0.376 mmol; 0.55 eq.) in MeCN (5 mL) at 0 °C. The reaction mixture was stirred for 2 h at 0 °C, before the solvent was evaporated under reduced pressure. The residue was dissolved in EtOAc, and the organic phase was washed with 10% eq. NaHCO\textsubscript{3}. The aqueous phase was extracted with DCM and EtOAc. The combined organic phases were dried over Na\textsubscript{2}SO\textsubscript{4}, filtered and concentrated \textit{in vacuo}. The crude product 2 was used without further purification.\textsuperscript{1H} NMR (400 MHz, CDCl\textsubscript{3}) δ 4.46 (t, \(J = 4.7\) Hz, 2H), 3.79 (t, \(J = 4.7\) Hz, 2H), 3.72 – 3.65 (m, 10H), 3.39 (t, \(J = 5.1\) Hz, 2H), 3.30-3.13 (m, 6H), 2.41-2.34, 2.23-2.11 (m, 4H), 1.68-1.48 (m, 10H)\textsuperscript{†}.

tetra-tert-buty 10-(2-(1-(13-azido-2,5,8,11-tetraoxatridecanoyl)-1H-imidazol-4-yl)acetamido)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,14,20-tetraazahenicosane-1,3,19,21-tetracarboxylate (3)

The NHS-carbonate 2 (0.082 g, 0.227 mmol, 3.5 eq) was dissolved in dry DMF (0.5 mL) and added to a stirring solution of the bisNTA-imidazole 1 (0.068 g, 0.063 mmol, 1 eq.) and DIPEA (0.030 mL, 0.172 mmol, 2.7 eq.) in dry DMF (1 mL). The mixture was stirred overnight at rt under an argon atmosphere. The solvent was removed under reduced pressure at 60 °C, and the residue was purified by flash column chromatography (DCM with 0% to 5% MeOH) yielding 3 (0.054 g, 0.063 mmol, 65%) as a colorless oil. \(R_t = 0.47\) (DCM:MeOH, 9:1): \textsuperscript{1H} NMR (100 MHz, CDCl\textsubscript{3}) δ 8.13 (s, 1H), 7.74 (d, \(J = 7.2\) Hz, 1H), 7.34 (s, 1H), 7.23 (t, \(J = 5.6\) Hz, 1H), 6.61 (t, \(J = 5.4\) Hz, 1H), 4.55-4.52 (m, 2H), 4.40 (td, \(J = 7.5, 5.3\) Hz, 1H), 3.83 – 3.81 (m, 2H), 3.67-3.35 (m, 10H), 3.53-3.36 (m, 12H), 3.30-3.13 (m, 6H), 2.41-2.34, 2.23-2.11 (m, 4H), 1.68-1.48 (m, 10H)\textsuperscript{13}C NMR (100 MHz, CDCl\textsubscript{3}) δ 173.2, 172.5, 172.4, 171.1, 170.9, 170.8, 169.7, 148.5, 137.6, 137.4, 115.2, 81.3, 81.2, 80.9, 80.8, 70.9, 70.8, 70.2, 68.7, 67.3, 65.3, 65.1, 54.1,
53.9, 53.1, 50.8, 39.6, 39.5, 36.1, 32.8, 30.4, 30.0, 29.2, 29.0, 28.6, 28.3, 28.3, 23.5, 23.1. HRMS (ESI): calc. for C_{63}H_{110}N_{10}O_{20}^{2+} ([M+2H]^{2+}) 663.3943, found: 663.3983. \(^\dagger\) Integral off due to water.

10-(2-(1-(13-azido-2,5,8,11-tetraoxatridecanoyl)-1H-imidazol-4-yl)acetamido)-2,20-bis(carboxymethyl)-9,13-dioxo-2,8,14,20-tetraazahenicosane-1,3,19,21-tetracarboxylic acid (bisNTA probe)

Compound 3 (29 mg; 0.022 mmol; 1 eq.) was dissolved in DCM/TFA 1:1 (4 mL) and the reaction mixture was stirred at rt. overnight under an argon atmosphere. The volatiles were removed by evaporation, and the residue was triturated with diisopropyl ether (x3). The remaining white solid was dried under high vacuum to afford the bisNTA probe (62% NMR yield\(^*\)) as a salt. HRMS (ESI): calc. for C_{39}H_{59}N_{10}O_{20}^{-} ([M-H]^{-}) 987.3913, found 987.3914. \(^*\)\(^{1}H\)-NMR (400 MHz, D_{2}O) is in the NMR spectra section and shows 20% hydrolysis of the bisNTA probe.

Tetra-tert-butyl 10-(4-(((benzyloxy)carbonyl)amino)butyl)-11-(2-((5-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-6-(tert-butoxy)-6-oxohexyl)amino)-2-oxoethyl)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,11,14,20-pentaazahenicosane-1,3,19,21-tetracarboxylate (4)

To a solution of Cbz-protected NTA acid\(^4\) (500; 1.26 mmol; 1 eq.), EDC•HCl (1.12 g; 6.31 mmol; 5 eq.) and DMAP (524 mg; 4.29 mmol; 3.4 eq.) in dry DCM (200 mL) was added tert-butyl protected monoNTA-amine\(^5\) (2.39 g; 5.55 mmol; 4.4 eq.). The reaction mixture was stirred under an argon atmosphere at rt overnight. The mixture was diluted with DCM, washed with water (x2) and brine, dried over Na_{2}SO_{4}, filtered and concentrated \textit{in vacuo}. Flash column chromatography (0% to 10% MeOH in DCM) afforded the desired product 4 (1.61 g; 78%) as a colorless oil. R\(_f\) = 0.39 (1:9
MeOH:DCM); ¹H-NMR (400 MHz, CDCl₃) δ 7.50 – 7.45 (m, 3H), 7.34 – 7.27 (m, 6H), 5.05 (s, 2H), 3.50 – 3.30 (m, 16H), 3.29 – 3.11 (m, 3H), 1.69 – 1.23 (m, 3H); ¹³C NMR (100 MHz, CDCl₃) δ 172.4, 172.3, 171.6, 170.9, 170.8, 170.5, 166.8, 164.8, 128.4, 128.1*, 81.2, 81.1, 80.8*, 77.4, 66.5, 65.9, 65.3, 65.1, 56.4, 53.9, 53.8, 40.7, 39.3, 30.3, 30.0, 29.7, 29.9, 28.8, 28.3, 28.2, 24.4, 23.5, 23.2; HRMS (ESI) calc. for C₁₈₄H₃₄₈N₈O₂₃ ([M+Na+H]²⁺) 828.5155, found 828.5153. †integral off due to water. *two peaks.

Tetra-tert-butyl 10-(4-aminobutyl)-11-(2-(5-(bis(2-tert-butoxy)-2-oxoethyl)amino)-6-(tert-butoxy)-6-oxohexyl)amino)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,11,14,20-pentaazahenicosane-1,3,19,21-tetracarboxylate (5)

To a solution of Pd/C (50 mg; 0.047 mmol (Pd); 0.4 eq. (Pd)) in MeOH (10 mL) was added Cbz-protected amine 4 (200 mg; 0.122 mmol; 1 eq.). The reaction mixture was stirred under an atmosphere of H₂ (balloon, 1 atm) at rt overnight. The reaction mixture was filtered through Celite®, and the solvent was evaporated under reduced pressure yielding 5 (173 mg; 94%) as a colorless oil. Rf = 0.71 (1:1 MeOH:DCM); ¹H-NMR (400 MHz, CDCl₃) δ 7.87 – 7.74 (m, 1H), 7.59 – 7.45 (m, 2H), 3.56 – 3.02 (m, 26H), 2.88 – 2.74 (m, 1H), 2.39 – 2.29 (m, 1H), 1.90 – 1.24 (m, 10H); ¹³C-NMR (100 MHz, CDCl₃) δ 172.6, 172.4#, 172.3, 171.6, 171.5, 170.9, 170.8*, 81.3*, 81.2, 81.1*, 81.0, 80.9, 80.8*, 80.7, 77.4, 65.4*, 65.3, 65.2, 53.9*, 53.8, 39.3, 30.4, 30.2, 30.0, 29.5, 28.9, 28.3, 28.2, 23.6, 23.2.; HRMS (ESI) calc. for C₇₆H₃₄₈N₈O₂₁ ([M+3H]³⁺) 500.6732, found 500.6731. †integral off due to water. *two peaks. #three peaks.

Tetra-tert-butyl 10-(4-(2-(1H-imidazol-5-yi)acetamido)butyl)-11-(2-(5-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-6-(tert-butoxy)-6-oxohexyl)amino)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,11,14,20-pentaazahenicosane-1,3,19,21-tetracarboxylate (6)
To a mixture of 4-imidazoleacetic acid hydrochloride (22 mg; 0.13 mmol; 2 eq.), DMAP (20 mg; 0.17 mmol; 2.5 eq.) and EDC-HCl (38 mg; 0.20 mmol; 3 eq.) in dry DMF (10 mL) was added the trisNTA-amine 5 (0.100 mg; 0.067 mmol; 1 eq.). The reaction mixture was stirred at rt overnight, and the solvent was removed under reduced pressure. The residue was taken up in DCM and washed with water (x2) and brine, dried over Na₂SO₄, filtered and concentrated in vacuo. Flash column chromatography (5% to 10% MeOH in DCM, then 10% MeOH in DCM containing 0.2% Et₃N) yielded 6 (46 mg; 43%) as a colorless oil. Rₓ = 0.34 (1:9 MeOH:DCM); ¹H NMR (400 MHz, CDCl₃) δ 8.12 (s, 1H), 7.77 – 7.38 (m, 4H), 7.04 (s, 1H), 3.69 (d, J = 15.7 Hz, 1H), 3.57 (d, J = 15.7 Hz, 1H), 3.51 – 2.97 (m, 38H), 1.85 – 0.99 (m, 105H); ¹³C NMR (100 MHz, CDCl₃) δ 172.4, 172.3, 171.9, 171.0, 170.9, 135.1, 81.5, 81.3, 81.2, 81.0, 65.4, 65.1, 56.3, 53.9, 46.0, 39.4, 39.3, 38.7, 34.6, 30.4, 30.3, 29.5, 29.0, 28.4, 28.3, 28.1, 23.7, 23.6, 23.4; HRMS (ESI) calc. for C₆₁H₁₄₂N₁₀O₂₂ ([M+2H]²⁺) 804.5223, found 804.5246. †Integral off due to water.

Tetra-tert-butyl 10-(4-(2-(1-(13-azido-2,5,8,11-tetraoxatridecanoyl)-1H-imidazol-4-yl)acetamido)butyl)-11-(2-((5-(bis(2-(tert-butoxy)-2-oxoethyl)amino)-6-(tert-butoxy)-6-oxohexyl)amino)-2-oxoethyl)-2,20-bis(2-(tert-butoxy)-2-oxoethyl)-9,13-dioxo-2,8,11,14,20-pentaazahenicosane-1,3,19,21-tetracarboxylate (7)
To a solution of trisNTA-imidazole 6 (50 mg; 0.031 mmol; 1 eq.) and DIPEA (0.02 mL; 0.104 mmol; 3.3 eq.) in dry DMF (1 mL) was added the NHS-carbonate 2 (13 mg; 0.037 mmol; 1.2 eq.). The reaction mixture was stirred under an argon atmosphere at rt for 3 h. The solvent was removed under reduced pressure, and the residue purified by flash column chromatography (0% to 10% MeOH in DCM) affording 7 (40 mg; 70%) as a colorless oil. Rf = 0.49 (1:9 MeOH:DCM); 1H NMR (400 MHz, CDCl3) δ 9.00 – 8.10 (m, 5H), 7.52 (s, 1H), 4.72 – 4.50 (m, 2H), 4.05 – 2.93 (m, 44H), 2.03 – 1.07 (m, 105H); HRMS (ESI) calc. for C90H157N13O27 ([M+H+Na]2+) 938.0638, found 938.0644. integral off due to water.

10-(4-(2-(1-(13-Azido-2,5,8,11-tetraoxatridecanoyl)-1H-imidazol-4-yl)acetamido)butyl)-11-(2-((5-(bis(carboxymethyl)amino)-5-carboxypentyl)amino)-2-oxoethyl)-2,20-bis(carboxymethyl)-9,13-dioxo-2,8,11,14,20-pentaazahenicosane-1,3,19,21-tetracarboxylic acid (trisNTA probe)

To a solution of 7 (20 mg; 0.011 mmol; 1 eq.) in dry DCM was added TFA (1.5 mL). The reaction mixture was stirred under an argon atmosphere at rt overnight. The volatiles were evaporated, and the residue triturated in diisopropyl ether (x2). The resulting solid was dried under reduced pressure forming the crude trisNTA probe (42% NMR-yield*) as a salt. The probe was not further purified prior to the bioconjugation investigations. HRMS (ESI) calc. for C54H85N13O27 ([M-3H+K]2-) 691.7546, found 691.7517. *1H-NMR (400 MHz, D2O) is in the NMR spectra section and shows 33% hydrolysis of the trisNTA probe.

4 References
5 NMR Spectra