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Cyclic acetals as cleavable linkers for affinity capture

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Labeling proteins with biotin is a widely used method to identify target proteins due to biotin's strong binding affinity for streptavidin. Combined with alkyne-azide cycloaddition, which enables the coupling of probes to targeted proteins, biotin tags linked to an alkyne or azide have become a powerful tool for purification and analysis of proteins in proteomics. However, biotin requires harsh elution conditions to release the captured protein from the bead matrix. Use of these conditions reduces signal to noise and complicates the analysis. To improve affinity capture, cleavable linkers have been introduced. Here, we demonstrate the use of a cyclic acetal biotin probe that is prepared easily from commercially available starting materials, is stable to cell lysates, yet is cleaved under mildly acidic conditions, and which provides an aldehyde for further elaboration of the protein, if desired.

Introduction

The azide moiety has been used as a functional group for bioorthogonal reaction because an azide and its reacting partner are not present in biological systems.[1, 2] In a reaction termed a "click" reaction, an azide undergoes cycloaddition with an alkyne in the presence of Cu(I)[3, 4] or with a highly ring strained alkyne, such as difluorinated cyclooctyne.[4, 5] Once an azide and an alkyne form a triazole, the resulting triazole is stable to further reaction conditions such as reduction, oxidation, and hydrolysis.[6, 7] Due to its fast reaction kinetics and exceptional functional group tolerance, the azide-alkyne click reaction has been chosen for many biological studies, e.g. selective protein modification *in vitro* and *in vivo,*[8, 9] and activity-based protein profiling.[10]

Biotin is often used to capture the targeted protein due to its strong binding affinity for the egg-white glycoprotein avidin or to the bacterial protein streptavidin. Enrichment of biotinylated proteins from a complex mixture is efficiently achieved using a streptavidin-coated solid support such as an agarose resin. However, conventional methods to release biotinylated proteins from streptavidin bead matrices are harsh because of the strong binding interaction. For example, 2% SDS/6M urea,[11] boiling in 2% SDS, or on-bead tryptic digestion are required to release the targeted protein. These non-selective conditions release streptavidin monomer and the proteins that are non-specifically bound to streptavidin bead matrices. Similarly, digested peptides of strepavidin from on-bead trypsin treatment contaminate the protein to be analysed. Even though the signal from streptavidin can be easily subtracted from the LC-MS/MS experiment for bioinformatics searches, the signals from non-specifically bound proteins will hamper the target protein search.

Recently, several biotin probes containing cleavable linkers have been developed to avoid such harsh elution conditions. Disulfide linkers have been widely used due to their rapid cleavage under mild reducing conditions. However, a disulfide linker is unstable to electrophilic and nucleophilic polar reagents, and thiol exchange with thiols in biological fluids can occur. Long-wave UV light can be used to release photocleavable linkers, but in some conditions, illumination of the sample is limited.[11-13] There is also an

acid labile linker from Pierce (proprietary structure) that is cleaved in 95% TFA. Another alternative is a dialkoxydiphenylsilane linker invented by Szychoswski et al. that is reported to be efficiently cleaved upon treatment with 10% formic acid for 0.5 h.[14] Other types of cleavable linkers[15, 16] have been developed such as an enzymatically (TEV) cleaved linker,[17] diazobenzene-derived linkers that are cleaved with $Na_2S_2O_4$,[18-20] vicinal diol linkers cleaved with $NaIO_4$[21] and linkers that are released upon reactions with nucleophiles including levulinoyl ester[22] and nitrobenzenesulfonamide.[23]

In this report, we exploited the cyclic acetal moiety as an acid-sensitive linker. Orthoesters, ketals, and acetals are accepted cleavable linkers for drug delivery in vivo.[24] In consideration of long term storage needs in combination with the requirements for stability in physiological conditions and fast cleavage for target identification, we designed cyclic acetals as acid-cleavable linkers (Figure 1). They are readily prepared by simple chemistry from commercially available starting materials. In addition, the resulting aldehyde after hydrolysis of the acetal can serve as a chemical reporter via further modification of the purified protein. Here we introduce the synthesis and capture utility of cyclic acetal linkers in two model systems.

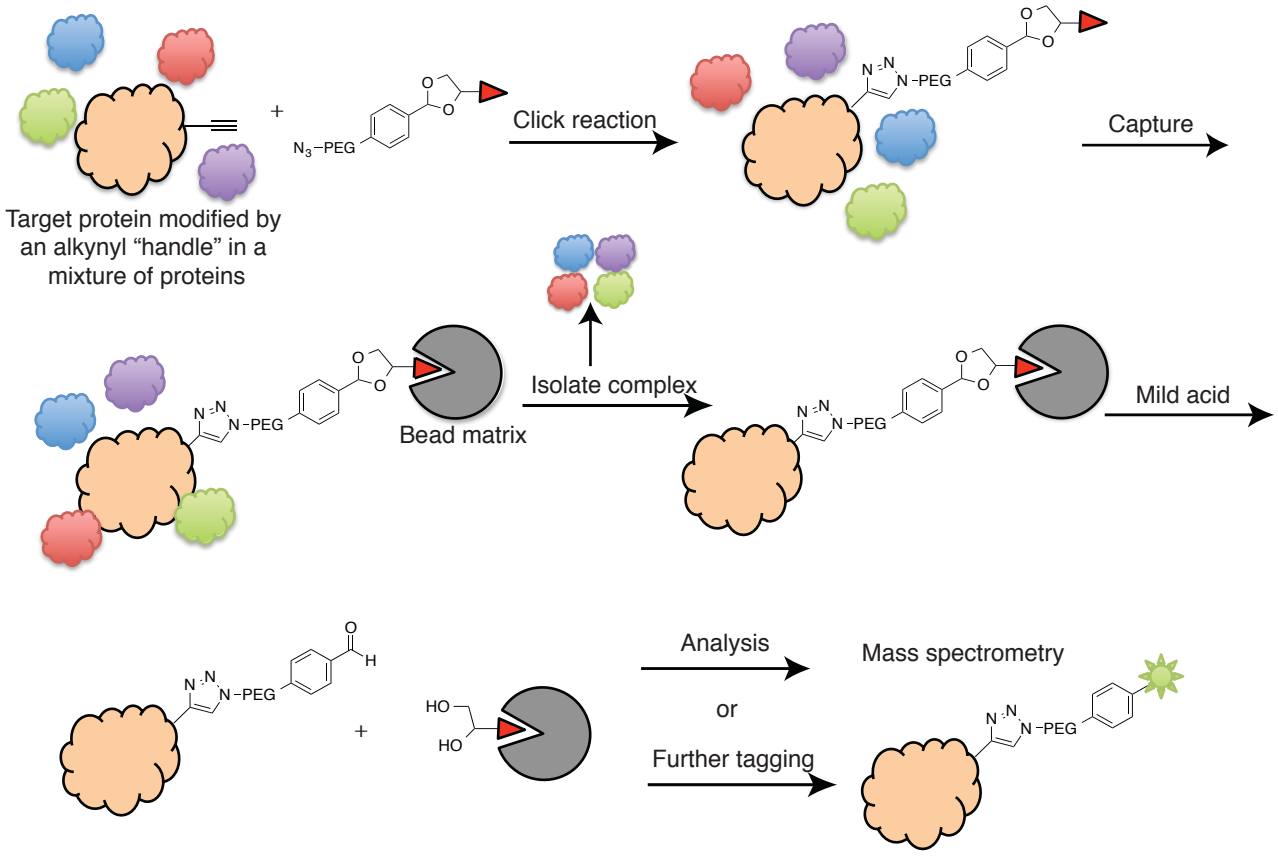
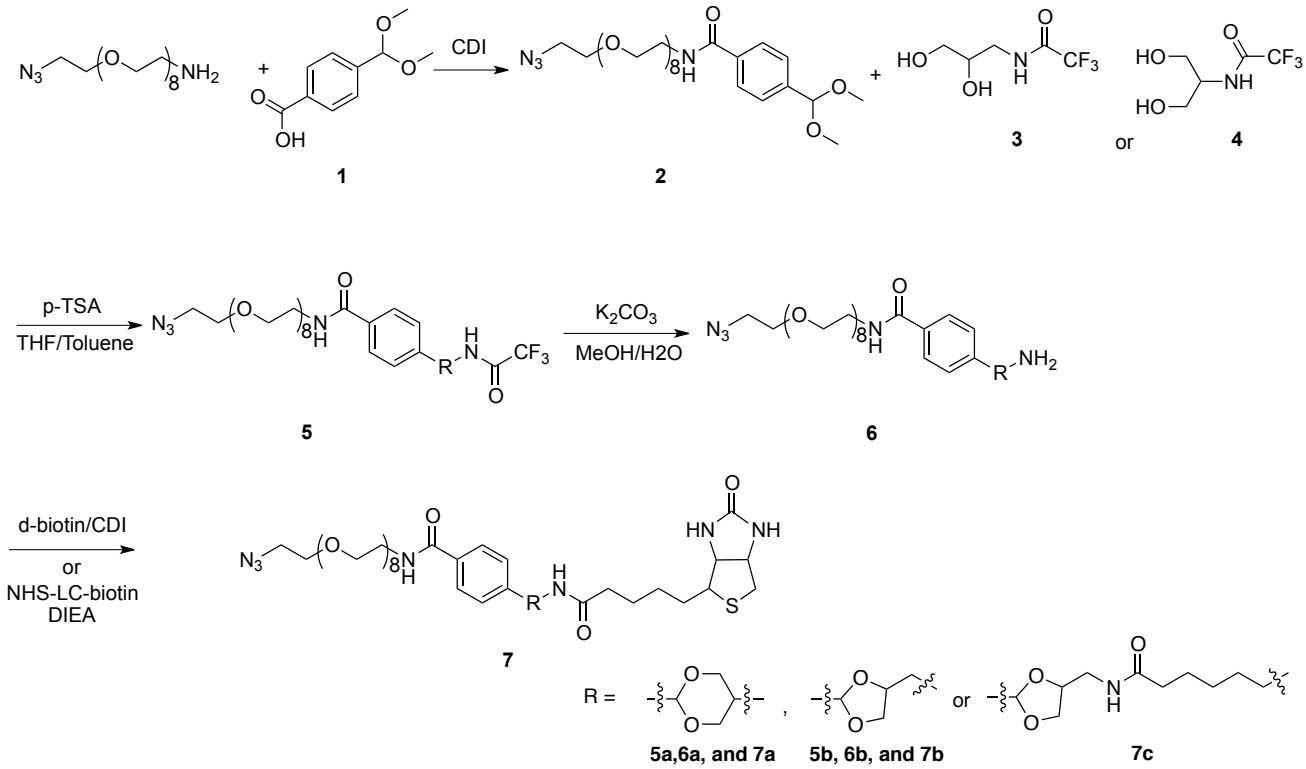


Figure 1. Schematic representation of target isolation using a cyclic acetal biotin probe.

Results and Discussion

Synthesis of cleavable biotin probe. To prepare the acetal-based cleavable biotin probe, azide-PEG_8-amine was coupled to dimethoxy acetal **1** using carbonyl diimidazole as a coupling reagent to generate acetal **2** (Scheme 1). A dimethoxy acetal was used to generate the cyclic acetals rather than forming the cyclic acetal directly from the aldehyde because the kinetics of formation were more favorable. Basic alumina was employed for purification since the dimethoxy acetal is very sensitive to acid.

In order to generate penta or hexacyclic acetal, protected 3-amine-1,2-diol or serinol, respectively, was coupled to acetal **2**. The free amines in the diols were protected as their trifluoracetamides**, 3** or **4**. The cyclic acetal was formed using *p*-toluene sulfonic acid as a catalyst in THF/toluene to generate trifluoroacetamide **5**. THF was used to dissolve diol **3** or **4** and dry toluene was used to remove water by azeotrope formation to drive the equilibrium toward cyclic acetal formation. The reaction was monitored by thin layer chromatography. Trifluoroacetamide **5** was obtained in 64% - 72% yield. The trifluoroacetamide moiety was removed to generate free amine for the following coupling reaction to form **6**. Cyclic acetal **6** was purified on basic alumina in 82% yield. Finally, the amine was coupled to biotin activated with CDI to produce the final product **7a**, **7b or 7c** in 50% - 70% yield after gravity column chromatography (neutral alumina).

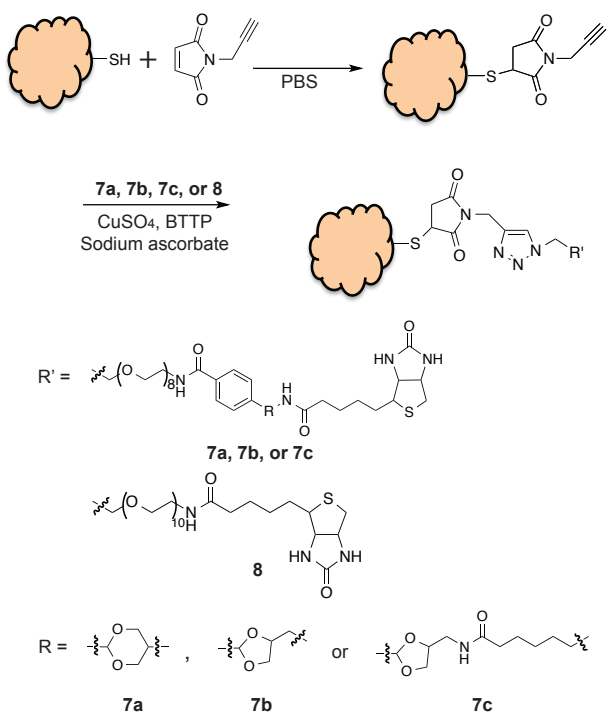


Scheme 1. Synthesis of cyclic acetal biotin probes.

Preparation of biotinylated BSA and evaluation of the cleavable acetal probes. To examine the efficiency of capture with the acetal biotin probes, BSA was used as a model protein. BSA has one cysteine on the surface and an alkyne was installed on the thiol through N-propynyl-maleimide coupling. To facilitate complete coupling, 100 eq of N-propynyl-maleimide was used. After 12 hours, the remaining N- propynyl -maleimide was removed by precipitation of BSA using cold acetone. The alkyne-functionalized BSA was subjected to azide-alkyne cycloaddition with each of the biotin probes (Scheme 2). After 1 hour, excess click reagents were removed using a microconcentrator with a molecular weight cut off of 3 kDa.

In order to find effective cleavage conditions, each of the biotinylated BSA conjugates was incubated in 1% TFA at 37 °C with gentle agitation and aliquots were removed at 30 minutes, 1 hour, and 2 hours. The quantity of biotin remaining on the BSA was detected by streptavidin blot. The acetal BSA-**7b** was successfully cleaved in 30 minutes. On the other hand, non-acetal BSA-**8** and acetal BSA-**7a** were stable to the cleavage conditions (Figure 2A). Upon treatment with higher concentrations of TFA, non-acetal probe BSA-**8** was released from the resin (data not shown). Therefore, a higher concentration of TFA could not be used to cleave the more stable six-membered ring acetal BSA-**7a** selectively.

Acetal BSA-**7b** was further tested to evaluate the efficiency of cleavage when bound to streptavidin beads. BSA-**8** was used as a negative control to confirm that the elution of the streptavidin protein is not responsible for cleavage. The BSA conjugated to probe **7b** or **8** was captured on streptavidin ultralink resin for 1 hour at room temperature, and the beads were washed sequentially with 1% SDS in PBS, 6M urea in 250 mM ammonium bicarbonate, 1 M NaCl in PBS to remove as much non-specifically bound protein as possible, and finally, washed two times with water. The loaded beads were incubated in 1% TFA at 37 °C with gentle agitation. After 1 hour, the supernatant was collected, the resin was washed with 0.1% SDS in PBS and PBS, and all eluate and wash fractions were combined. After washing, the beads were boiled to release BSA that was not eluted during the cleavage procedure. As shown in Figure 2B, BSA-**7b** was successfully released from the streptavidin resin under the cleavage conditions. However, the cleavage of BSA-**7b** did not go to completion. In addition, a small amount of BSA-**8** was released under the cleavage conditions, although the probe linker remained intact as evidenced by the biotin signal in the due to limited solvent access to the acetal because the short linker between acetal and biotin places the acetal in close proximity to



Scheme 2. Preparation of BSA or RNase A labeled with each biotin probe. BTTP: 3-[4-({bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]propanol.[25]

the biotin-binding pocket on the streptavidin. We investigated addition of various additives, e.g. SDS and guanidinium hydrochloride, to increase cleavage efficiency with limited success. Therefore, an extended linker with an additional seven atoms was introduced between the acetal and biotin by coupling NHS-LC-biotin with compound **6** (Scheme 1) to provide probe **7c**.

We repeated the capture/cleavage procedures with streptavidin-ultralink resin to compare cleavage of BSA-**7c** to BSA-**7b**. However, the cleavage efficiencies of BSA-**7b** and BSA-**7c** were similar (data not shown). Because the pore size of the bead can also affect solvent access to acetal and dissociation of the product aldehyde, we tried a different capture medium, streptavidin-agarose beads. As shown in Figure 3A, the probe with the extended linker, **7c**, was released more efficiently from the streptavidin-agarose bead complex than probe **7b** which has a shorter linker. This result suggests that the combination of the extended linker and larger pore size are required to favor

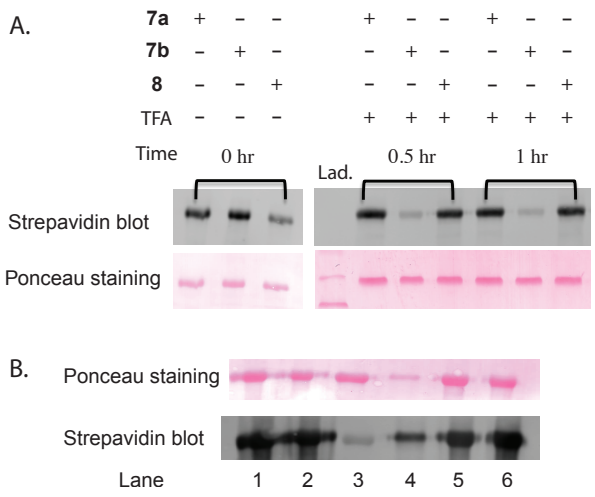


Figure 2. Evaluation of biotin probes **7a** and **7b** cleavage and comparison to non-cleavable biotin probe **8**. (A) BSA (20 μg) conjugated to each probe, **7a**, **7b**, or **8**, was incubated in 1% TFA at 37 °C in the absence of streptavidin resin for the indicated time. (B) Test of BSA-**7b** linker cleavage. Lane 1, BSA-**7b** (20 μg) captured on streptavidin-ultralink beads; Lane 2, BSA-**8** (20 μg) captured on streptavidin-ultralink beads; lane 3, supernatant from BSA-**7b** (20 μg) bead sample in lane 1 incubated in 1% TFA at 37 °C for 1 hour; lane 4, supernatant from BSA-**8** (20 μg) bead sample in lane 2 incubated in 1% TFA at 37 °C for 1 hour; lane 5, streptavidin-ultralink beads with captured BSA-**7b** from lane 3 after TFA incubation; lane 6, streptavidin-ultralink beads with captured BSA-**8** from lane 4 after TFA incubation.

Cleavage and capture in cell lysates. Since the linker should be stable to physiological conditions and allow efficient capture from a complex mixture, the cleavage test was performed in the presence of bacterial cell lysates. Whole bacterial cell lysates (1 mg) mixed with BSA-**7c** (100 μg) was incubated with streptavidin beads. After washing as described above, the loaded beads were treated with 1% TFA at 37 °C with gentle agitation. After 1 hour, the supernatant was collected, the resin washed and protein eluted as described above for BSA-**7b**. The cyclic acetal linker **7c** remained intact in the cell lysate, and allowed successful capture of the BSA conjugate on the bead matrix. Subsequent release with mild acid treatment efficiently yielded the cleaved BSA with little protein remaining on the bead (Figure 3B).

To further establish the optimal cleavage conditions, we tested capture of another protein, RNase A that has a low molecular weight, 13.7 kDa. RNase A has two free cysteines that were used to conjugate an alkyne handle via maleimide chemistry as described above for BSA. The alkyne was further conjugated with cleavable biotin probe **7c** through azide-alkyne 4A). However, the eluted protein was contaminated with streptavidin monomer that was released from the bead matrix during the cleavage step (Figure 4A).

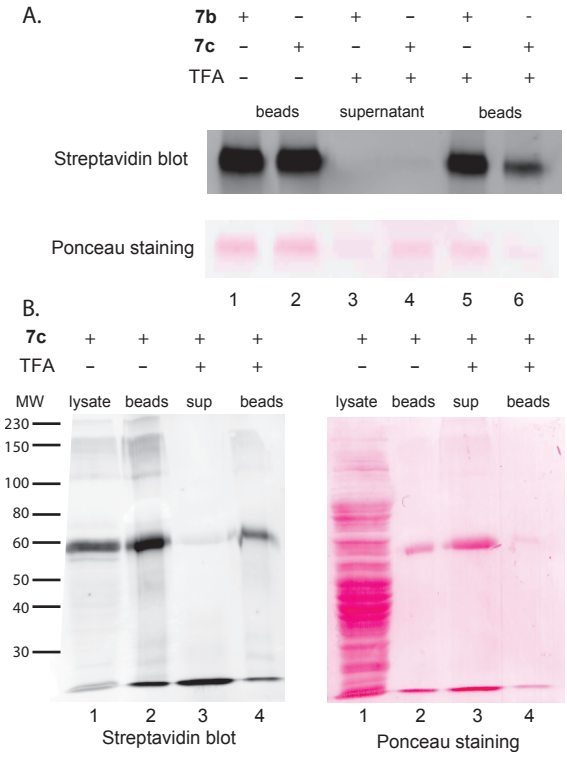


Figure 3. Comparison of BSA-**7b** and BSA-**7c** linker cleavage (A). Lane 1, BSA-**7b** (20 μg) captured on streptavidin-agarose beads ; lane 2, BSA-**7c** (20 μg) captured on streptavidin-agarose beads; lane 3, supernatant from BSA-**7b** bead sample in lane 1 incubated in 1% TFA at 37 °C for 1 hour; lane 4, supernatant from BSA-**7c** bead sample in lane 2 incubated in 1% TFA at 37 °C for 1 hour; lane 5, streptavidin-agarose beads with captured BSA-**7b** from lane 1 after TFA incubation; lane 6, streptavidin-agarose beads with captured BSA-**7c** from lane 2 after TFA incubation. (B) Capture and cleavage of BSA-**7c** in the presence of bacterial whole cell lysates; lane 1, cell lysate + BSA-**7c** (20 μg); lane 2, BSA-**7c** captured on streptavidin-agarose beads from cell lysate sample in lane 1; lane 3, supernatant from BSA-**7c** bead sample in lane 2 incubated in 1% TFA at 37 °C for 1 hour; lane 4, streptavidin-agarose beads with captured BSA-**7c** from lane 2 after TFA incubation.

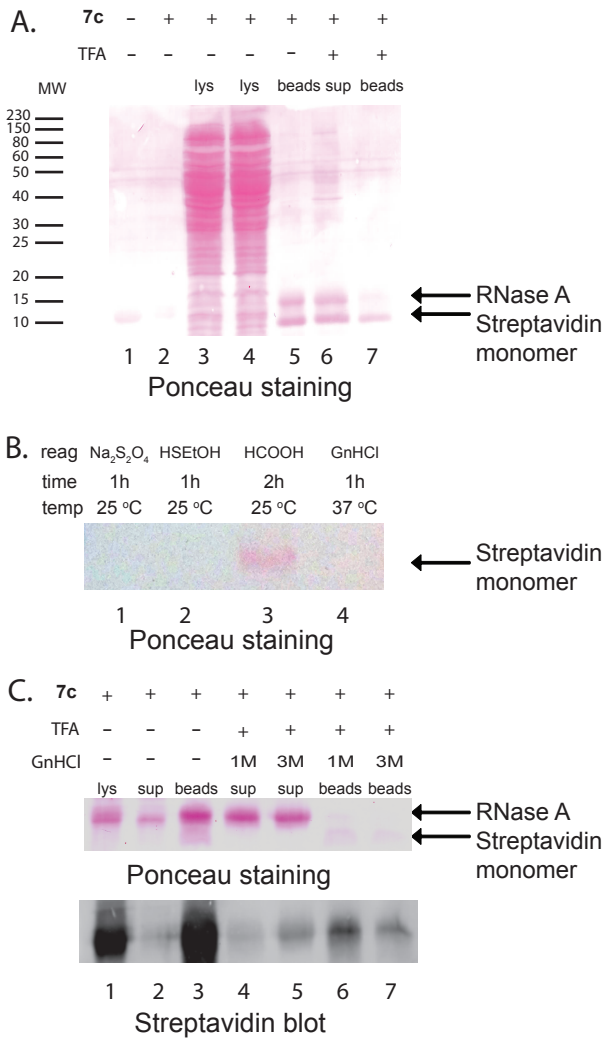


Figure 4. RNase A-**7c** capture and elution. (A) Capture of RNase A-**7c** in the presence of bacterial whole cell lysate and release; lane 1, RNase A-maleimide alkyne; lane 2, RNase A-**7c**; lane 3, cell lysate + RNase A-**7c** (20 μg); lane 4, supernatant after RNase A-**7c** streptavidin capture from sample in lane 3; lane 5, RNase A-**7c** captured on streptavidin-agarose beads from cell lysate sample in lane 3; lane 6, supernatant from RNase A-**7c** bead sample in lane 5 incubated in 1% TFA at 37 °C for 1 hour; lane 7, streptavidin-agarose beads with captured RNase A-**7c** from lane 5 after TFA incubation. (B) Test of streptavidin monomer release under cleavage conditions used for other cleavable linkers. Supernatant after treating streptavidin beads: lane 1, $Na_2S_2O_4$ for 1 hour at 25 °C; lane 2, 2% 2-mercaptoethanol for 1 hour at 25 °C; lane 3, 5% formic acid for 2 hours at 25 °C; lane 4, 1M guanidine hydrochloride in 1% TFA at 37°C for 1 hour. (C) Effect of guanidine concentration on streptavidin monomer release from the beads. Lane 1, cell lysate + RNase A-**7c**; lane 2, supernatant after RNase A-**7c** streptavidin capture. Note: the remaining RNase A is from incomplete reaction with maleimide incubated in 1M guanidine/1% TFA at 37 °C for 1 hour; lane 5, supernatant from RNase A-**7c** incubated in 3 M guanidine/1% TFA at 37 °C for 1 hour; lane 6, streptavidin-agarose beads with captured RNase A-**7c** after 1M guanidine/TFA incubation; lane 7, streptavidin-agarose beads with captured RNase A-**7c** after 3M guanidine/TFA incubation. Exact amounts used in each step are given in the Materials and Methods.

Suppression of streptavidin monomer release. When the amount of a targeted protein to be eluted is low, co-elution of the streptavidin monomer should be minimized in order to avoid signal suppression and elevated noise in the subsequent mass spectral analysis. We observed during the course of our investigations to improve cleavage efficiency that 1M guanidinium hydrochloride improved the efficiency of cleaved protein release and suppressed the release of streptavidin. Therefore, we analyzed the use of guanidinium hydrochloride during cleavage and compared its use to cleavage conditions for other cleavable biotin probes in order to determine whether the problem of streptavidin monomer release is widespread.

Streptavidin agarose beads were incubated separately under the following cleavage conditions: 5% $Na_2S_2O_4$ for 1 hour at 25°C, 2% of 2-mercaptoethanol for 1 hour at 25 °C, 5% formic acid for 2 hours at 25 °C, and 1M guanidine in 1% TFA at 37 °C for 1 hour. As shown in Figure 4B, formic acid treatment also resulted in the release of streptavidin from agarose beads, whereas, reducing conditions did not. Gratifyingly, inclusion of 1 M guanidine in the 1% TFA cleavage mixture suppressed non-specific release of the streptavidin monomer (Figure 4A, lane 6 vs Figure 4B, lane 4).

Next we tested the effect of guanidine concentration on release and elution. Two different concentrations, 1 M or 3 M guanidine hydrochloride, in combination with 1% TFA were tested. In both samples, suppression of streptavidin monomer release was observed. However, 3 M guanidine also releases some RNase A with the biotin probe still attached indicating that the RNase A release is due to protein denaturation rather than acetal cleavage (Figure 4C, lane 5), which results in reduced retention of biotinylated RNase A on the beads (Figure 4C, lane 7). We tested cleavage efficiency with BSA since other proteins can be sensitive to 1 M guanidine. 1 M guanidine did not affect the release of BSA from the streptavidin resin (Figure S1). Therefore, 1 M guanidine in 1% TFA is the preferred elution

Further labeling of the aldehyde tag on protein after cleavage. Aldehyde tags can be used to modify cell surface proteins specifically since the aldehyde functionality is not typically present in proteins.[26] Aldehydes readily react with a variety of aminooxy or hydrazide-functionalized molecules.[27, 28] The use of a capture linker that unmasks an aldehyde upon release can prove useful for further functionalization.[29] Cleavage of the cyclic acetal linker **7** generates an aldehyde functionality on the tagged protein after purification. Therefore, we tested if the aldehyde is available for further modification of the protein. The cleaved BSA-**7c** was incubated with alkoxyamine-PEG-biotin for 4 hours, and the reaction mixture was directly analyzed by SDS-PAGE and streptavidin blot. After cleavage of the BSA-**7c** acetal, no BSA biotin signal remained (Figure 5, Lane 3). Upon reaction of the cleaved BSA with alkoxyamine-PEG-biotin, the biotin signal was restored (Figure 5, Lane 4). Likewise, RNase A-**7c** underwent the analogous reaction sequence (Figure S3). Therefore, the aldehyde funtionality generated through cleavage can be successfully conjugated with nucleophilic labeling reagents.

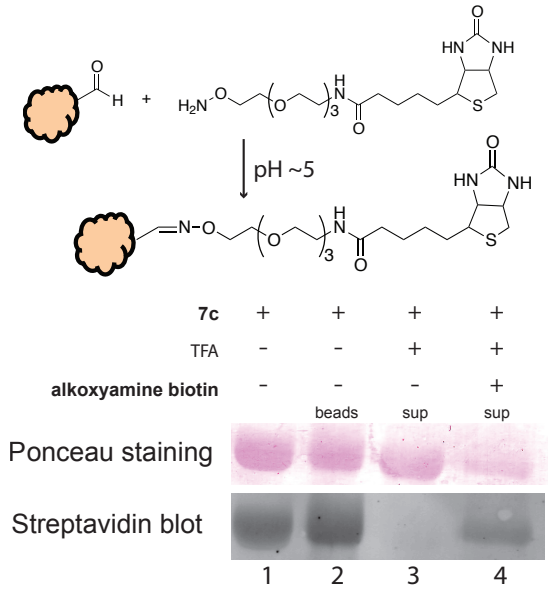


Figure 5. Further modification of the BSA aldehyde tag. Lane 1, BSA-**7c** (20 μg); lane 2, BSA-**7c** sample from lane 1 captured on streptavidin-agarose beads; lane 3, supernatant from BSA-**7c** bead sample in lane 2 incubated in 1% TFA at 37 °C for 1 hour; lane 4, BSA-aldehyde from sample in lane 3 after reaction with alkoxyamine-PEG-biotin at pH 5, 37 °C for 4 hours. Exact amounts used in each step are given in the Materials and Methods.

Conclusions

Existing methods to release biotinylated proteins from cleaved under mildly acidic conditions, yet is sufficiently stable for use in cell lysates. Moreover, addition of guanidinium hydrochloride to the cleavage mixture suppresses release of monomeric streptavidin from the capture matrix under the acidic cleavage conditions. Lastly, cleavage of the cyclic acetal provides an aldehyde functionality on the captured protein, which conveniently can undergo further reaction to provide specific labeling of the captured protein.

Experimental Section

General. Coupling reactions were performed under an Ar atmosphere using dry solvents. All commercially available reagents were purchased from Sigma-Aldrich and were used as received. NHS-LC-biotin and streptavidin agarose beads were purchased from Thermo Scientific. Spin ultrafilters (vivaspin 500) were purchased from GE Healthcare. ^{1}H and ^{13}C NMR spectra were recorded on Bruker instruments (400 or 500 MHz for ^{1}H and 100 or 125 MHz for ^{13}C). MS data were collected with AQUITY UPLC from Waters.

Acetal 1.[30] To a solution of 4-carboxybenzaldehyde (2.00 g, 13.3 mmol) in dry MeOH (40 mL) was added ammonium chloride (4.00 g, 74.8 mmol). The mixture was heated under reflux for 20 h. The solvent was evaporated under reduced pressure and the product was recrystallized from boiling hexane (2.0 g, 77 %): 199.5-200.0 °C, which was identical to the literature. ^{1}H NMR (500 MHz, CD_3OD) δ = 7.81 (d, J = 8.4 Hz, 2H), 7.51 (d, J = 8.1 Hz, 2H), 5.43 (s, 1H), 3.32 (s, 6H). ^{13}C NMR (126 MHz, $CDCl_3$) δ = 171.94, 143.86, 130.21, 129.61, 126.98, 102.27, 52.70; MS (m/z) $[M-H]^-$ calcd for $C_{10}H_{11}O_4$: 195.07, found: 195.1.

Acetal 2. Carbonyldiimidazole (88.7 mg, 0.54 mmol) and **1** (107.4 mg, 0.54 mmol) were dissolved in DCM. The mixture was stirred for 30 min at rt. To the solution was added azido-PEG_8-amine (200 mg, 0.45 mmol). After 5 h, the solvent was evaporated under reduced pressure. Product **2** was obtained by gravity column chromatography (basic alumina, 0% - 5% MeOH/DCM) as an oil (210 mg, 75 %): ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.78 (2H, d, J = 8.6 Hz, 2H), 7.47 (d, J = 9.3 Hz, 2H), 6.92 (b, 1H), 5.43 (s, 1H), 3.73 – 3.56 (m, 34H), 3.42 – 3.35 (m, 2H), 3.32 (s, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 167.21, 141.34, 134.71, 127.05, 126.92, 102.39, 70.72, 70.69, 70.66, 70.61, 70.59, 70.58, 70.55, 70.31, 70.07, 69.81, 52.65, 50.70, 39.83; MS (m/z) $[M+H]^+$ calcd for $C_{28}H_{49}N_4O_{11}$: 617.34, found:

Trifluoroacetamide 3. To a solution of 3-amino-1,2-propanediol (250 mg, 2.74 mmol) in THF, ethyl trifluoroacetate (2.33 g, 16.46 mmol) was added drop-wise. After 4 h, the solvent was evaporated. DCM was added to the oil and evaporated. This step was repeated two more times. Benzene was added and evaporated. This step was also repeated two more times. The resulting product was used without further purification to yield compound **3**: ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.85-4.7 (m, 1H); 3.6-3.3 (m, 4H); MS (m/z): $[M-H]^-$ calcd for $C_5H_7F_3NO_3$: 186.04, found: 185.98.

Trifluoroacetamide 4. Trifluoracetamide **4** was prepared from serinol (250 mg, 2.74 mmol) and ethyl trifluoroacetate (2.33 g, 16.46 mmol) as described for **3** to yield compound **4**: ^{1}H NMR (500 MHz, DMSO-d_6) δ = 8.99 (m, 1H), , 4.75 (t, J = 7.1 Hz, 1H), 3.86-3.477 (m, 1H), 3.52-3.38 (m, 4H); MS (m/z): $[M-H]^-$ calcd for $C_5H_7F_3NO_3$: 186.04, found: 185.98.

Trifluoroacetamide 5. To a solution of **3** or **4** (191 mg, 1.022 mmol) in THF/toluene (3/7), **2** (210 mg, 0.34 mmol) and *p*-toluene sulfonic acid•H_2O (13 mg, 0.068 mmol) were added. The mixture was heated to 100 °C. The solvent was distilled to remove H_2O generated during the reaction and toluene added to maintain reaction volume as the reaction proceeded. After 4 h, the reaction was quenched with 50 µl of TEA. Product **5** was obtained by column chromatography (basic alumina, 0%-5% MeOH/DCM) as an oil: **5a** (from **4,** 180 mg, 72 %)**:** ^{1}H NMR (500 MHz, CD_3OD) δ = 7.86 – 7.83 (m, 2H), 7.62 – 7.55 (m, 2H), 5.69, 5.55 (s, 1H, two isomers), 4.30 – 4.21 (m, 4H), 3.87 – 3.81 (m, 1H), 3.68 – 3.56 (m, 34H), 3.36 (t, J = 5.0 Hz, 2H); ^{13}C NMR (126 MHz, CD_3OD) δ = 169.81, 169.78, 159.14, 159.12, 158.84, 142.88, 142.49, 136.15, 130.66, 129.15, 128.41, 128.33, 128.28, 128.25, 128.21, 128.03, 127.99, 127.97, 127.55, 127.53, 127.51, 127.44, 127.42, 118.56, 116.29, 102.13, 102.02, 101.79, 71.63, 71.57, 71.56, 71.55, 71.51, 71.33, 71.13, 70.51, 70.50, 69.98, 69.95, 69.67, 61.64, 57.32, 51.77, 47.06, 45.12, 44.29, 41.03, 36.51, 27.10, 26.50; MS (m/z): $[M+H]^+$ calcd for $C_{31}H_{49}F_3N_5O_{12}$: 740.36, found: 740.66**. 5b** (from **3,** 160 mg, 64 %)**:** ^{1}H NMR (500 MHz, CD_3OD) δ = 7.87 – 7.84 (m, 2H), 7.61 – 7.54 (m, 2H), 5.99, 5.82 (1H, two isomers), 4.48 – 4.43 (m, 1H), 4.22 4.12 (dd, J = 8.6, 6.4 Hz, 1H, two isomers), 3.94, 3.78 (dd, J = 8.5, 5.0 Hz, 1H, two isomers), 3.68 – 3.58 (m, 34H), 3.55 – 3.49 (m, 2H), 3.40 – 3.33 (m, 2H); ^{13}C NMR (126 MHz, CD_3OD) δ = 169.78, 169.75, 159.55, 159.50, 159.26, 159.20, 142.96, 142.11, 136.58, 136.39, 130.65, 129.14, 128.41, 128.37, 128.06, 127.73, 118.65, 116.37, 104.97, 104.10, 76.04, $[M+NH_4]^+$ calcd for $C_{31}H_{52}F_3N_6O_{12}$: 757.36, found: 757.55.

Amine 6. To a solution of **5a or 5b** (160 mg, 0.22 mmol) in MeOH/H_2O (7/3) K_2CO_3 (209.35 mg, 1.5149 mmol) was added. The reaction was heated at reflux for 2 h. After evaporating all the solvent, the product was purified by gravity column chromatography (basic alumina, 2%-10% MeOH/DCM) to yield **6** as an oil. **6a** (from **5a**, 134 mg, 82 %): ^{1}H NMR (500 MHz, $CDCl_3$) δ = 7.88 – 7.75 (m, 2H), 7.58 – 7.48 (m, 2H), 5.52, 5.40 (s, 1H, two isomers), 4.37 – 4.00 (m, 4H), 3.67 – 3.55 (m, 34H), 3.35 (q, J = 4.6 Hz, 2H), 3.30 – 3.10 (m, 1H); $[M+H]^+$ calcd for $C_{29}H_{50}N_5O_{11}$: 644.34, found: 644.49. **6b** (from **5b,** 105 mg, 80 %): ^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.82 – 7.78 (m, 2H), 7.57 – 7.46 (m, 2H), 6.90 (b, 1H), 5.95, 5.82 (s, 1H, two isomers), 4.32, 4.16 (m, 1H, two isomers), 4.09 (t, J = 10.0 Hz 1H), 3.89, 3.69 (m, 1H, two isomers), 3.66 – 3.57 (m, 34H), 3.35 (t, J = 5.0 Hz, 2H), 3.01 – 2.78 (m, 2H). MS (m/z): $[M+H]^+$ calcd for $C_{29}H_{50}N_5O_{11}$: 644.34, found: 644.57.

Amide 7. d-Biotin or NHS-LC-biotin (50 mg, 0.205 mmol) and carbonydiimidazole (33 mg, 0.205 mmol) were dissolved in dried DMF. The mixture was stirred for 30 min. To the mixture, **6** was added and the reaction was stirred for 12 h at rt. The product was purified by gravity column chromatography (neutral alumina, 3%-7% MeOH/DCM) to yield **7** as an oil. Compound **7a** (90 mg, 50 %): ^{1}H NMR (400 MHz, CD_3OD) δ = 7.86 – 7.83 (m, 2H), 7.66 – 7.53 (m, 2H), 5.67, 5.52 (s, 1H, two isomers), 4.64 – 4.38 (m, 1H), 4.33 – 4.10 (m, 4H), 3.81 (s, 1H), 3.55 – 3.70 (m, 35H), 3.36 (t, J = 4.9 Hz, 2H), 3.26 –3.12 (m, 1H), 2.96 – 2.85 (m, 1H), 2.70 (t, J = 12.4 Hz, 1H), 2.40 – 2.19 (m, 2H), 1.78 – 1.25 (m, 6H); ^{13}C NMR (126 MHz, CD_3OD) δ = 197.12, 176.01, 169.77, 143.12, 142.70, 142.43, 136.06, 128.21, 128.17, 127.54, 127.51, 102.09, 101.72, 71.61, 71.55, 71.53, 71.52, 71.49, 71.32, 71.30, 71.12, 70.96, 70.93, 70.51, 63.25, 61.57, 56.97, 54.82, 51.77, 41.02, 36.44, 29.47, 26.92; HRMS ESI (m/z): $[M+H]^+$ calcd for $C_{39}H_{64}N_7O_{13}S$: 870.4277, found: 870.4295. Compound **7b** (100 mg, 67%): ^{1}H NMR (400 MHz, CD_3OD) δ = 7.88 – 7.84 (m, 2H), 7.62 – 7.55 (m, 2H), 5.98, 5.81 (s, 1H, two isomers), 4.48 – 4.32 (m, 2H), 4.25 – 4.20 (m, 1H), 4.12 – 4.07 (m, 1H), 3.92 – 3.86 (m, 1H), 3.78 – 3.72 (m, 1H), 3.69 – 3.56 (m, 34H), 3.51 – 3.41 (m, 2H), 3.37 (t, J = 4.9 Hz, 2H), 3.19 – 3.09 (m, 1H), 2.92 – 2.84 (m, 1H), 2.70 (d, J = 12.8 Hz, 1H), 2.30 – 2.18 (m, 2H), 1.78 – 1.35 (m, 6H); ^{13}C NMR (126 MHz, CD_3OD) δ = 176.41, 169.69, 166.04, 143.15, 142.38, 136.54, 136.39, 128.41, 128.12, 127.81, 122.64, 104.78, 104.09, 76.91, 76.71, 71.63, 71.57, 71.56, 71.54, 71.33, 71.13, 70.52,

(m/z): $[M+H]^+$ calcd for $C_{39}H_{64}N_7O_{13}S$: 870.4277, found: 870.4288. Compound **7c** (70 mg, 61%): ^{1}H NMR (500 MHz, CD_3OD) δ = 7.88 – 7.84 (m, 2H), 7.62 – 7.55 (m, 2H), 5.98, 5.82 (s, 1H, two isomers), 4.49 – 4.46 (m, 1H), 4.39 – 4.33 (m, 1H), 4.31 – 4.26 (m, 1H),), 4.22 – 4.07 (m, 1H, two isomers), 3.89 – 3.73 (m, 1H, two isomers), 3.70 – 3.55 (m, 34H), 3.51 – 3.35 (m, 4H), 3.22 – 3.10 (m, 3H), 2.93 – 2.90 (m, 1H), 2.70 (d, *J* = 12.8 1H), 2.25 – 2.17 (m, 4H), 1.75 – 1.31 (m, 15H); ^{13}C NMR (126 MHz, CD_3OD) δ = 176.50, 175.93, 169.78, 166.07, 143.16, 142.39, 136.53, 136.36, 128.41, 128.39, 128.08, 127.75, 104.78, 104.07, 76.89, 76.66, 71.63, 71.56, 71.54, 71.51, 71.33, 71.14, 70.51, 69.27, 69.20, 63.36, 61.60, 57.00, 51.77, 42.78, 42.13, 41.05, 40.19, 40.14, 36.87, 36.85, 36.81, 34.66, 30.13, 30.12, 30.09, 29.79, 29.50, 27.59, 27.46, 26.92, 26.63, 25.68; HRMS ESI (m/z): $[M+H]^+$ calcd for $C_{45}H_{75}N_8O_{14}S$: 983.5123, found: 983.5152.

Biotin-PEG$_{10}$-N$_3$, 8. Biotin-NHS (0.31 mmol, 107 mg) and *O*-(2-Aminoethyl)-*O′*-(2-azidoethyl)nonaethylene glycol (0.21 mmol, 110 mg) were dissolved in 1 mL dry DMF. DIEA (0.31 mmol, 56 µL) was added to the mixture, and the reaction was stirred for 16 h at rt. After evaporation of solvent, the product was precipitated with Et_2O. Chromatography (MeOH:EtOAc/1:1) yielded product **8**: ^{1}H NMR (500 MHz, d$_6$-DMSO) δ = 7.81 (t, *J* = 5.5, 1H), 6.40 (br s, 1H), 6.34 (br s, 1H), 4.30 (m, 1H), 4.12 (m, 1H), 3.60 (m, 2H), 3.53 (m, 38H), 3.39 (t, *J*= 5.1, 4H), 3.18 (q, *J*= 5.8, 2H), 3.09 (dd, *J*= 11.7, 7.3, 1H), 2.82 (dd, *J*= 12.4, 5.1, 1H), 2.58 (d, *J*= 12.4, 1H), 2.06 (t, *J*= 7.4, 2H), 1.62 (dd, *J*= 21.4, 7.9, 1H), 1.50 (dt, *J* = 14.4, 7.5, 3H), 1.30 (m, 2H); MS (m/z): $[M+H]^+$ calcd for $C_{32}H_{61}N_6O_{12}S$: 753.4, found: 753.4.

Alkyne functionalized bovine serum albumin. To a solution of bovine serum albumine (20 µM) in PBS, N-(1-propynyl)-maleimide (2 mM) was added. The mixture was gently agitated for 12 h in the dark. The excess maleimide was removed using cold acetone.

Preparation of bovine serum albumin labeled with biotin probe. Alkyne functionalized BSA (50 µM) was mixed with biotin probe **7a** or **7b** (100 µM), BTTP[25] (200 µM), $CuSO_4$ (100 µM), and sodium ascorbate (2.5 mM) for 1 h at rt. The reagents were removed using an ultrafiltration spin filter (MWCO = 3 kDa). The concentration of BSA was measured by Pierce Coomassie Plus protein assay, following the manufacturer's instructions.

Cleavage test. After coupling of BSA to probe, the mixture

PBS) for 1 h at rt. The beads loaded with biotinylated BSA were spun at 1000 g for 3 min. The pelleted beads were washed sequentially with 1% SDS in PBS, 6 M urea in 250 mM ammonium bicarbonate, 1 M NaCl in PBS, and two times with water. The beads were incubated in 1 mL 1% TFA for 1 h at 37 °C. The supernatant was collected by pelleting the beads. The beads were washed sequentially with 0.1% SDS/PBS, and two times with PBS and the supernatant was combined with the washes. The combined solutions were concentrated using an ultrafiltration spin filter (MWCO = 3 kDa) at 7000 g. After two more washes with PBS, the beads were boiled in sample loading buffer for 15 min.

Analysis of protein capture and release. Each protein sample (typically equivalent to about 20 µg BSA in the initial sample per lane) was separated by 12% or 15% SDS-PAGE gel and transferred onto a PDVF membrane (Bio Rad). The membrane was blocked with 4% BSA/TBST for 1 h at rt. After washing the membrane with TBST three times, streptavidin conjugated with Alexa-488 (20 mg/mL) was added and the solution was gently agitated for 1 h at 25°C. The membrane was washed with TBST three times and was visualized using a Typhoon 9400 scanner (GE Healthcare).

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Notes

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