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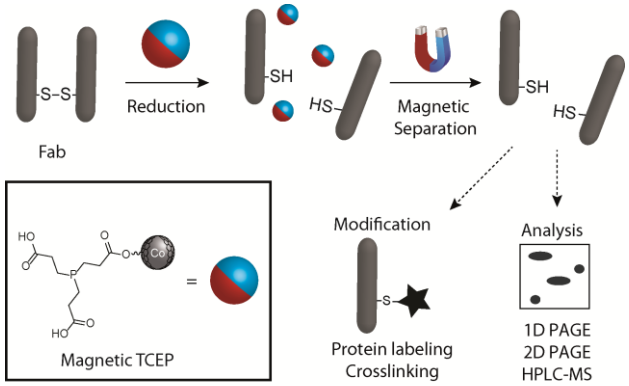
Protein Reduction and Dialysis-Free Work-Up Through Phosphines Immobilized on a Magnetic Support: TCEP-Functionalized Carbon-Coated Cobalt Nanoparticles

Adrian Zwyssig,[a] Elia M. Schneider,[a] Martin Zeltner,[a] Balder Rebmann,[b] Vladimir Zlateski,[a] Robert N. Grass,[a] and Wendelin J. Stark*[a]

Tris(2-carboxyethyl)phosphine (TCEP) is an often used reducing agent in biochemistry due to its selectivity towards disulfide bonds. As TCEP causes undesired consecutive side reactions in various analysis methods (*i.e.* gel electrophoresis, protein labeling), it is usually removed *via* dialysis or gel filtration. Herein, an alternative method of separation is presented, namely the immobilization of TCEP on magnetic nanoparticles. This magnetic reagent provides a simple and rapid approach to remove the reducing agent after successful reduction. A reduction capacity of 70 µmol per gramm of particles was achieved by using surface-initiated atom transfer polymerization.

Polyacrylamide gel electrophoresis (PAGE) is a frequently used analytic method in biochemistry. For the majority of those PAGE-type methods, bio-molecules (*e.g.* proteins) have to be denatured or unfolded. For denaturation of proteins sodium dodecyl sulfate (SDS) or lithium dodecyl sulfate (LDS) are often used (especially for denaturation of secondary and non-disulfide-containing tertiary structures).[1] Trialkylphosphines on the other hand are highly effective agents for the selective reduction of disulfide bonds in proteins, peptides and other disulfide bond-containing molecules.[2] The solubility issue of alkylphosphines has been tackled by the development of tris(2-carboxyethyl)phosphine (TCEP) which is active over a broad pH-range (pH 1.5 – 8.5).[3] TCEP is stable in aqueous solutions and is not prone to rapid oxidation that often occurs with other common disulfide reducing agents, such as dithiotreitol (DTT) and β-mercaptoethanol (BME).[2a] Residual TCEP causes loss of focus in two-dimensional electrophoresis[4] and interferes with protein labeling and modification agents (*e.g.* maleimides and iodoacetic acid) under conditions relevant for protein chemistry.[5] Additionally, as TCEP can lead to cleavage of the protein backbone (in case of exposure over prolonged time),[6] the demand for reliable removal methods of TCEP before subsequent modification reactions (*e.g.* labeling, bio-conjugate crosslinking) is high.[7] Dialysis, the conventional TCEP removal procedure, is time-consuming, has to be performed under non-oxidating conditions and causes loss of valuable proteins. Therefore, efforts for alternative solid support removal methods were considered: TCEP has previously been immobilized on polyethylene glycol (PEG) beads, but has only been shown effective for small peptides (*e.g.* mesethericin 3.8 kDa) and under microwave irradiation (at potentially problematic higher temperatures).[8] The commercially available TCEP bound on agarose Gel (*Pierce™ Immobilized TCEP Disulfide Reducing Gel*)[9] has recently been introduced as a promising reducing agent also for larger proteins (at room temperature, using excess amounts of reducing agent). However, the loading capacity was relatively low (> 8 µmol mL^{-1}) and the necessity of using centrifuges or spin-columns resulted in loss of sample (especially positively charged proteins)[10] or dilution of the sample due to washing of the gel. Here, we investigate an alternative support, based on magnetic nanoparticles. They exhibit a beneficial surface-to-volume ratio, and less diffusion limitations than resins (*e.g.* polyethylene based resins). The magnetism permits simple separation by application of a commercial permanent magnet (see Fig. 5).[11] Therefore, this kind of supporting material avoids common work-up procedures (*e.g.* filtration, centrifugation), resulting in a separation step that is faster overall and does not result in loss or dilution of the sample. Thus, combination of the reducing capacities of TCEP with the attributes of functionalized magnetic nanoparticles (magnetic separation[12] and high possible loading capacity[13]) offers a substantial improvement in the field of immobilized reducing agents.

Herein, we present the synthesis and use of a magnetically recoverable TCEP reagent (Scheme 1). Carbon-coated magnetic nanoparticles (*e.g.* graphene-coated cobalt nanoparticles (C/Co)) have already been modified with numerous desired moieties such



Scheme 1. Magnetic tris(2-carboxyethyl)phosphine (TCEP) for a facile reduction of disulfide bonds in biomolecules is presented. This magnetic reagent enables site selective modification or analysis of the biomolecule without tedious reagent removal procedures.

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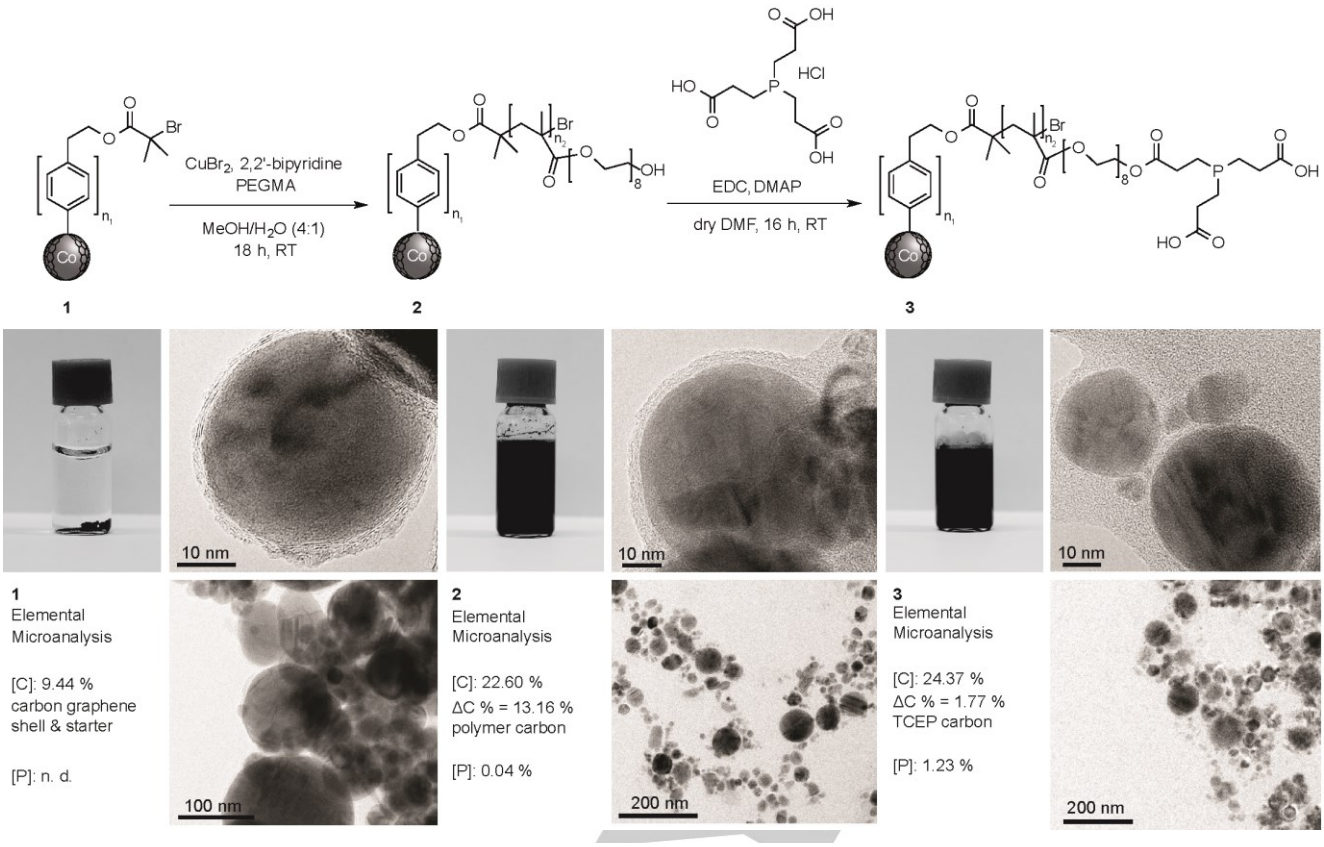

Figure 1. Synthetic route for the production of magnetic tris(2-carboxyethyl)phosphine (TCEP). The Initiator is attached *via* diazonium chemistry, followed by esterification (C/Co@Initiator **1,** n_1= 0.1 mmol g^{-1} particles). Poly(ethylene glycol) methacrylate is polymerized using surface-initiated ATRP (C/Co@PEGMA **2,** n_2 = 6 PEGMA units on average). TCEP is linked *via* a modified Steglich esterification (C/Co@PEGMA-TCEP **3**). The successful modification was monitored by microanalysis (bottom) and transmission electron microscopy (middle). The dispersion stability in water changes during modification due to the surface alternation (pictures of capped vials).

as polymers, catalysts, ligands, chelating agents or enzymes.[14] The graphene-like coating protects the magnetic metal core from oxidation and allows chemical modification of the surface (*i.e.* covalent attachment of various chemical functional groups).[15] If these particles are applied in aqueous solution, the modification of the hydrophobic graphene is necessary in bifocal perspective. Turning the intrinsically hydrophobic surface into a hydrophilic surface leads to improved dispersion stability of the particles in aqueous solutions, hence optimal exploitation of the large surface area.[16] Furthermore, the hydrophobic graphene surface is prone to non-selective physisorption of complex, hydrophobic structures (bio-fouling),[17] that is tremendously undesired in most biochemical uses. Altering the surface properties to avoid bio-fouling (*i.e.* unspecific adsorption of proteins) is an irrevocable requirement of such a supporting material.[18] Well-established neutral non-biofouling polymer brushes comprise 2-hydroxyethyl methacrylate (HEMA) or poly(ethylene glycol) methacrylate (PEGMA).[19] Therefore, the initiator (**1**, Figure 1) and the poly(ethylene glycol) methacrylate (PEGMA) brushes were covalently attached to the graphene-like surface as described in previous work *via* surface-initiated atom transfer radical polymerization (SI-ATRP),[16] providing magnetic nanoparticles with non-biofouling properties.[12a, 20] After polymerization, the graphene layers were still intact (see Figure S3). The TCEP-moiety was attached by a modified Steglich esterification reaction with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and *N*,*N*-dimethylpyridine-4-amine (DMAP), where only one carboxylic group of the TCEP is activated and bound to **2**, yielding TCEP-bearing magnetic nanoparticles (**3**, Figure 1). Cross-linking of more than one carboxylic group had to be avoided, as this negatively influenced the dispersion stability of the particles (see Figure S1 and S2 in the ESI). For detailed

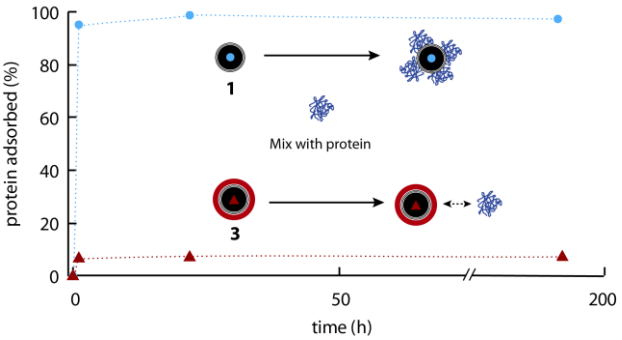

Figure 2. Non-biofouling control using tetramethylrhodamine attached to albumin from bovine serum (BSA, 0.007 mg of labeled protein with 0.7 mg of particles in 1 ml water). Unfunctionalized C/Co particles **1** (max. loading 0.03 mg protein mg^{-1} particles): blue circles, magnetic reduction agent **3**: red triangles.

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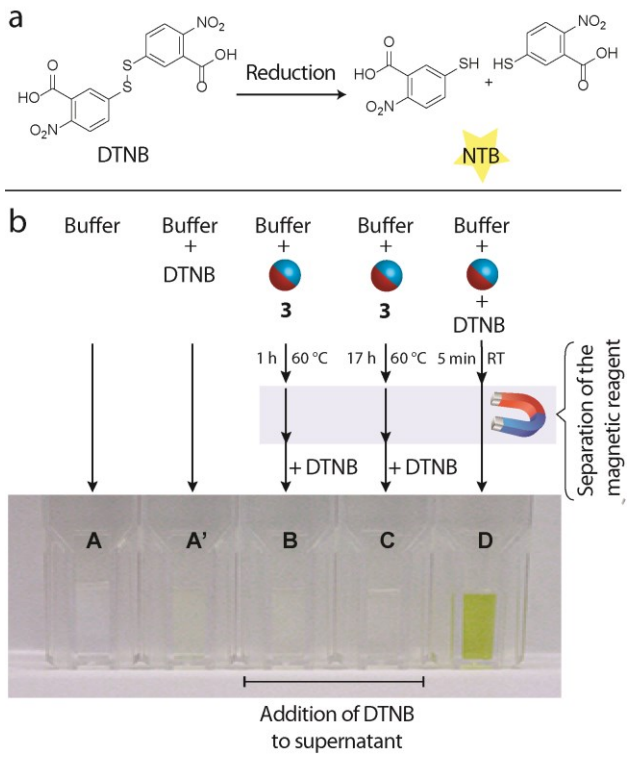

Figure 3. a) Reduction of dithionitrobenzoic acid to nitrothiobenzoic acid to determine the reducing capacity of nanoparticles C/Co@PEGMA-TCEP **3**. b) Washing protocol to ensure covalent connection of TCEP and the magnetic support. No DTNB reduction occurred in the supernatant of buffer exposed to the magnetic reagent (control experiments B and C), while the reagent **3** itself reduces DTNB in 5 minutes (positive control).

experimental conditions concerning the synthesis, see supporting information. The successful modification of the carbon-coated magnetic nanoparticles was monitored by elemental microanalysis (EMA, Figure 1, bottom), infrared spectroscopy (see Figure S4 and S5 in the ESI) and transmission electron microscopy (TEM), where for PEGMA modified nanoparticles **2** the polymer shell is visible (Figure 1, middle). Dynamic light scattering (DLS) and Lumisizer particle-size distribution measurement C/Co@PEGMA **2** resulted in an average particle diameter of 558 nm (Figure S11) and 466 nm (Table S1), respectively. Compared to the TEM micrographs, the difference in size is most likely due to agglomeration of particles and the hydrophilic nature of the polymer brushes, which increase the hydrothermal radius in aqueous solution substantially. The polymerization led to a significant increment of the carbon content (ΔC = 13.16 %), which translates to an average of six PEGMA units per starter moiety. The phosphor content was then increased by attachment of TCEP (from 0.04 % for **2** up to 1.23 % for **3**). Thermogravimetric analysis before and after polymerization (Figure S12) confirmed successful polymerization. Generally, a polymer content of between 12 to 20 [C] % on the particles proved to be ideal for optimal dispersion-stability, non-biofouling properties and short separation time. The non-biofouling property of the final magnetic reagent **3** was tested *via* adsorption of commercially available tetramethylrhodamine labeled albumin from bovine serum (0.007 mg of labeled protein with 0.7 mg of particles in 1 ml water). 6.7 % of protein adsorbed after 8 days stirring with **3** (Figure 2), while non-modified particles already adsorb 95 % after 1 hour. Besides the proper characterization of the material (see ESI for full characterization), the performance of magnetic TCEP (*i.e.* reducing activity) was of eminent importance. Therefore, a standard performance test was chosen, namely the cleavage of dithionitrobenzoic acid (DTNB; Ellman's reagent), which can be observed by UV/VIS-spectroscopy (Figure 3a).[21] For this test, the nearly transparent DTNB (5 mM) was dissolved in Tris-HCl buffer and 0.1 mg of C/Co@PEGMA-TCEP was added and dispersed by ultrasonication for seven minutes at room temperature. After separation of magnetic TCEP, the newly generated yellow nitrothiobenzoic acid (NTB) was detected by measuring the absorbance at 412 nm. The test showed a discrepancy between the theoretical loading, which was calculated by phosphor content and the reduction capacity towards DTNB (400 µmol g^{-1} particles

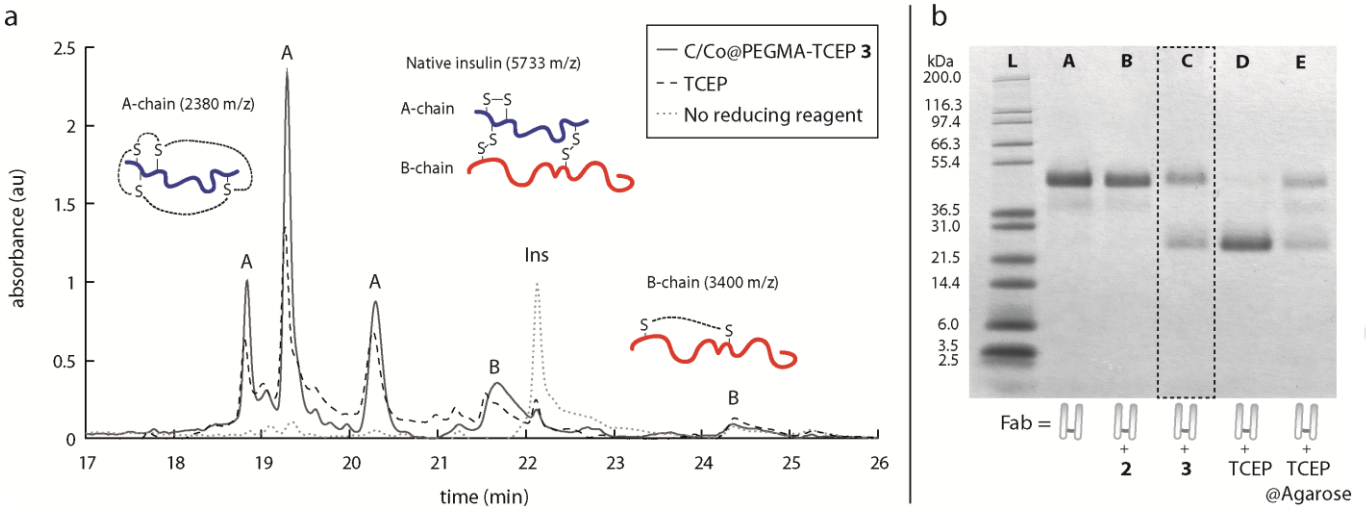

Figure 4. a) Reduction of bovine insulin using an excess of reducing agent (3-fold) and prolonged reaction times (17 hours) at 60°C. UV-spectra of LC-MS measurement of the reduction using C/Co@PEGMA-TCEP **3** (gray line), TCEP (black dashes) and no reducing agent (grey dots). A and B-chains were identified using MS. b) Reductive cleavage of human IgG Fab fragment; Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS PAGE). L = Mark12TM unstained standard, A = Fab fragment from human IgG, B = Fab + C/Co@PEGMA, C = Fab + 720-fold excess of C/Co@PEGMA-TCEP, D = Fab + TCEP 470-fold excess, E = Fab + Agarose-TCEP 942-fold excess.

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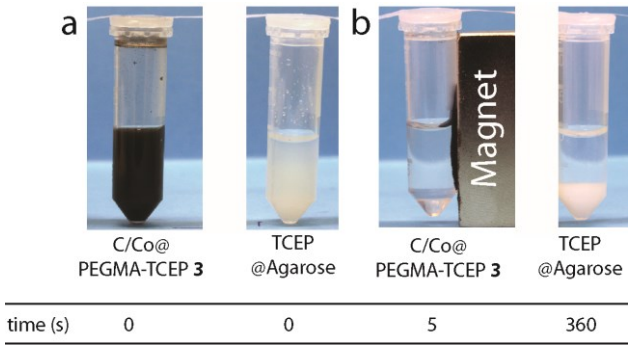

Figure 5. Magnetic tris(2-carboxyethyl)phosphine **3** (left, black) and agarose tris(2-carboxyethyl)phosphine (right, white) in dispersion respectively suspension (a). Fast separation (5 s) from the solution by use of a permanent magnet for **3** and centrifugation (1 min at 10k RPM & 5 min handling) for the agarose gel (b).

calculated *via* P-content versus 70 µmol g^{-1} particles *via* reduction capacity). Thus, it could be estimated that a part of the immobilized TCEP was inactive due to oxidation or inaccessible due to agglomeration of the magnetic particles. However, 17.5 % of the calculated TCEP was active and remained active if appropriately stored (-20°C in EtOH under argon atmosphere, Figure S6, see ESI). Within this test series, it could also be shown that the non-biofouling layer is of huge importance, as TCEP directly attached to the carbon surface of C/Co (C/Co@TCEP) had no visible activity (see ESI, -1 µmol g^{-1}) at all because the hydrophobic surface immediately adsorbs the generated products. Covalent linking of TCEP to the magnetic support was proven by using an intensive washing protocol (Figure 3b). If TCEP was only physisorbed, one could detect reduced DTNB in samples B and C. However, not even trace amounts of free, unbound TCEP were detected. Now, the target proteins for PAGE exhibit far more complex structures than the small organic molecules such as Ellman's reagent (DTNB). For this reason, the magnetic TCEP was applied on more relevant substrates, namely on bovine insulin (5.8 kDa) and on the Fab fragment from human IgG (55 kDa). After optimization of the reduction conditions concerning the use of magnetic TCEP, bovine insulin could successfully be reduced using equivalent amounts of magnetic reducing agent (observed by LCMS, Figure 4a). After removal of the magnetic reagent partial intramolecular reoxidation occurred (possibly during the HPLC measurement) and several peaks corresponding to the A- and B-chain of insulin were detected (Figure 4a), implying different chain conformations. However, the use of unbound TCEP resulted in similar spectra. For a more detailed explanation of the subject, see ESI, Figure S7-S10. An even more challenging task involved the reduction of the more complex Fab fragment from human IgG, where for example the free thiol groups can be used for sophisticated antibody modification.[22] The performance within this setup was observed by LDS-Page (Figure 4b). As expected, C/Co@PEGMA without attached TCEP did not show any reducing capacity (Column B). Free TCEP fully reduced the protein (column D), while magnetic TCEP partially reduced the disulfide bonds of the Fab fragment from human IgG (column C). However, the magnetic approach showed promising potential, especially since partial reduction due to sterical hindrance is an important feature in biochemistry to obtain site selective coupling. In order to set the magnetic TCEP in a comparative context, an already applied and commercial available bound TCEP (*Pierce™ Immobilized TCEP Disulfide Reducing Gel*, loading capacity of > 8 µmol mL^{-1})[9] was also used in this experimental series (column E). The agarose bound TCEP was suspended in the protein/buffer solution. After the reaction, the sample had to be centrifuged and decanted or centrifuged using a spin column. The chemical performance of this commercial available TCEP is comparable to the magnetic TCEP. However, in contrast to the agarose species, magnetic TCEP could be separated within seconds by application of a permanent magnet (Figure 5), thus minimizing the time dependent reoxidation in air. Also, it should be noted that there is always loss of protein when conducting centrifugation or dialysis (after reduction with agarose TCEP), especially if no buffer is used (40-60 % loss compared to <10% with C/Co@PEGMA-TCEP).[10]

In conclusion, a promising and fast magnetic alternative to common and commercial methods for disulfide reduction is presented. Magnetic nanoparticles were modified with a non-biofouling PEGMA layer and TCEP. Performance of the TCEP functionalized particles was successfully tested for bovine insulin and the Fab fragment from human IgG. The former resulted in full reduction (and partial reoxidation after removal of the reducing agent) while the latter resulted in a partially reduction of the Fab fragment. Probable future applications of this magnetic reagent will be mainly in the field of partial reduction, where sterically hindered reducing agents are essential (*e.g.* site-specific modifications of antibodies).

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Keywords: nanoparticles • magnetic • reduction • separation • TCEP

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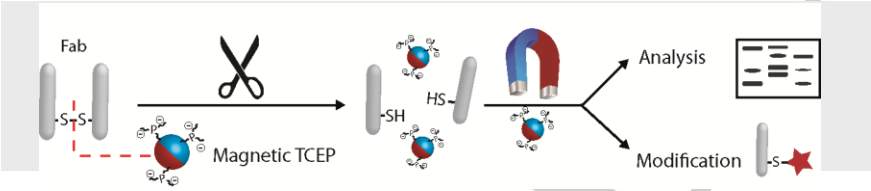

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