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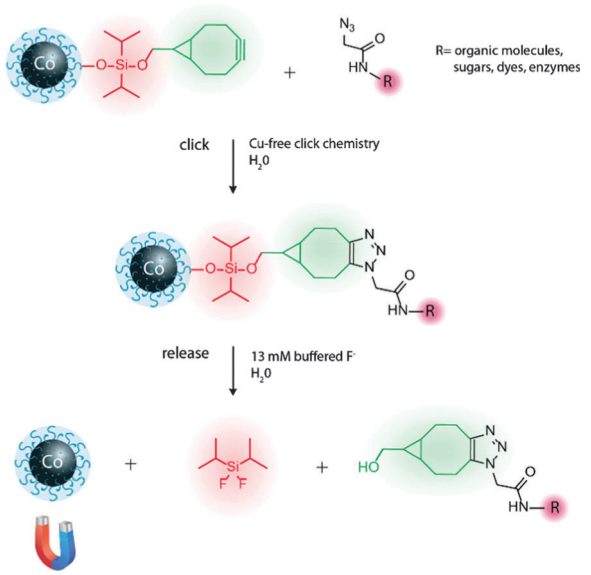
Click and release: fluoride cleavable linker for mild bioorthogonal separation†

Elia M. Schneider, Martin Zeltner, Vladimir Zlateski, Robert N. Grass and Wendelin J. Stark*

Herein, we present a water dispersable, magnetic nanoparticle supported "click and release" system. The cleavable linker has been synthesized by using a strain-promoted copper-free "click" reagent to establish the specific link and a fluoride cleavable silane moiety for mild cleavage. Small organic molecules, azide-bearing dyes and functionalized enzymes have been bound to the magnetic particle and released in a bioorthogonal way.

Bioorthogonal chemistry[1] opened the way for a detailed description and understanding of many biological processes, namely by the possibility to label and track specific biomolecules such as enzymes, lipids and sugars *in vivo*.[2] However, these kind of reactions, such as the Staudinger ligation or the 1,3-dipolar Huisgen cycloaddition of azides and alkynes ("click"),[3] are not reversible and cannot be cleaved in a specific and bio-compatible way (*i.e.* low temperatures, at neutral pH and in water). This would be beneficial for a large set of applications, for example native protein analysis,[4] protein glycosylation analysis[5] and analysis of protein complexes[6] *via* mass spectroscopy. There exist only a few reports of such linker systems;[7] however, the cleaving conditions are often quite harsh or not fully bioorthogonal. Our idea was to implement a cleavable silane moiety, one of the most used protecting groups for alcohols in organic chemistry.[8] The main advantage of this system is the cleavage *via* fluoride ions, which has already been used in solid peptide synthesis.[9] However, instead of using anhydrous HF we opted for buffered oxide etch (mild buffered fluoride solution) which has been used to uncage DNA and RNA from silica without damaging the biomolecules.[10] Our aim was to combine this mature technique to the modern copper-free "click" chemistry. Since the Huisgen-cycloaddition usually needs a high amount of copper catalyst, which hinders proper cell function,[11] a variety of strain-promoted copper-free click reagents have been developed.[12] These reagents promote the relatively fast dipolar cycloaddition without the need of any copper catalyst, thus leading to bio-compatible chemistry. By combining the mature and biorthogonal silane-chemistry with the state-of-the-art copper-free "click"-chemistry, creation of a cleavable "click and release" system is anticipated. Additionally, a suitable way to get in and out of a biological system has to be considered. A promising approach is provided by magnetic nanoparticles, since they can be separated quickly from any solution by using a permanent magnet. Recently, a plethora of magnetic nano-sized reagents has been developed,[13] based on magnetic carbon-coated metal nanoparticles. Advantages of these nanoparticles are a higher saturation magnetization and thus faster separation compared to silica coated iron oxide nanoparticles.[13a] Furthermore, the graphene layer protects the particle from degradation by



Scheme 1 Copper-free dipolar cycloaddition (click) on the magnetic nanoparticle followed by the fluorine-mediated release.

Institute for Chemical and Bioengineering, ETH Zurich, Vladimir-Prelog-Weg 1, 8093 Zurich, Switzerland. E-mail: Wendelin.stark@chem.ethz.ch

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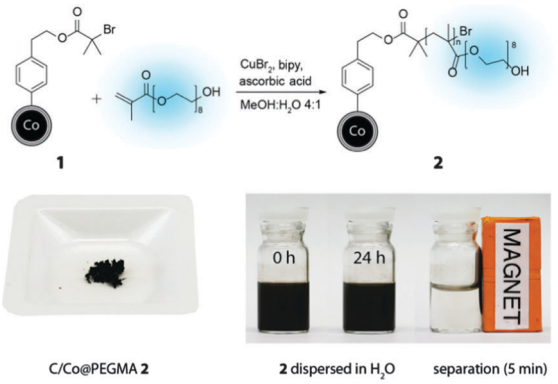


Fig. 1 PEG-methacrylate polymerisation *via* SI-ATRP. Sufficient dispersion stability in aqueous media is obtained while magnetic separation is still possible. Amount of solid (left) 150 mg, concentration (right) 10 mg mL^{-1}.

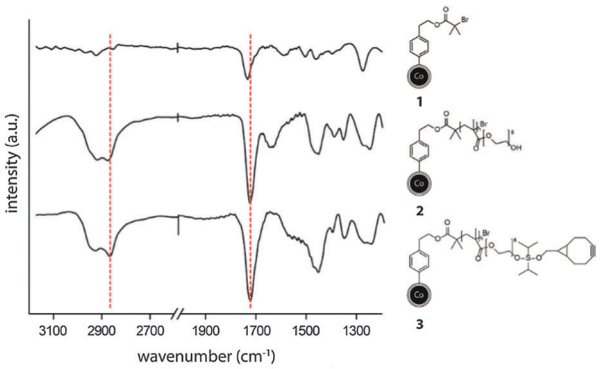
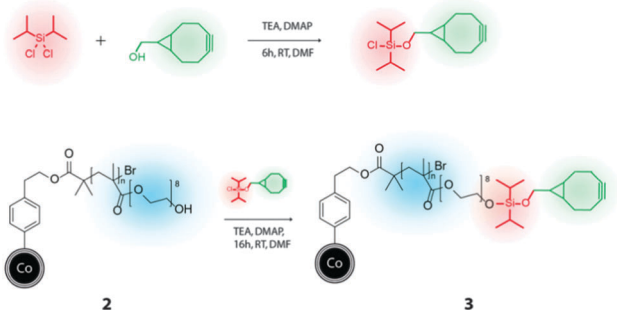


Fig. 2 FT-IR spectra of the magnetic compounds **1–3**. Red lines highlight the following vibrations: 2876 cm^{-1} C–H stretch, 1725 cm^{-1} carbonyl stretch.

buffered oxide etch and can be functionalized in a covalent and stable manner.[13d] However, one of the main drawbacks of this material is the unspecific protein adsorption (''fouling'') on the highly hydrophobic, graphene-like surface.

To provide a solution for this problem, defined coatings have been developed, often PEG-chains or analogues.[14] A practical route to coat nanoparticles in an uniform and defined way is surface initiated atom transfer radical polymerization (SI-ATRP), a ''grafting from'' approach.[15] Herein, we present the synthesis of a bioorthogonal ''click'' and release system (Scheme 1) on a magnetic, PEG-coated support.

The preparation of the PEG-coated magnetic nanoparticles was conducted *via* standard surface initiated atom transfer radical polymerization using $CuBr_2$ and bipyridine (bipy) as catalyst and ligand, respectively (Fig. 1). First, the magnetic carbon-coated cobalt nanoparticles (C/Co) were functionalized according to literature,[14b] yielding covalently bound starter particles **1**. Subsequently, **1** was polymerized using ascorbic acid to reduce Cu(II) to the active Cu(I) form (known as activator regenerated by electron transfer (ARGET) ATRP),[16] resulting in the novel PEG-coated magnetic nanoparticles C/Co@PEGMA **2** (Fig. 2). Using this polymerization technique, control of the polymer length and thus the desired dispersion stability could be obtained.[14a] Indeed, the so obtained polymer-coated particles were sufficiently dispersible in an aqueous solution and the formation of a polymer layer around the magnetic particles was confirmed by transmission electron microscopy (TEM, see Fig S1 in ESI†). Comparing the unfunctionalized C/Co particles to the polymerized C/Co@PEGMA **2**, the first stack while the latter do not due to steric repulsion of the transparent polymer layer. Compound **2** was characterized *via* elemental microanalysis (ΔC = 24.4%, see Table S1 in the ESI†) and IR (Fig. 2). Since an extensive polymer layer can significantly lower the saturation magnetization, a vibrating sample magnetometer measurement (VSM) was executed. 151 emu g^{-1} was obtained for non-functionalized carbon coated cobalt nanoparticles C/Co, while for **2** 61.1 emu g^{-1} was measured. Despite the lower magnetization and high dispersion stability, fast separation by using a permanent magnet was possible (Fig. 1). To introduce the cleavable silyl linker, dichloro-diisopropylsilane was reacted with a hydroxyl-bearing moiety for 6 h, and subsequently added to well-dispersed C/Co@PEGMA particles **2** thus affording the fluoride-cleavable linker system (Scheme 2). In order to verify that the silane linking system is indeed cleavable with dilute F^- solution (buffered oxide etch, BOE) and to determine the ideal cleaving conditions, test reactions were run using fluorescent 1-naphthol as the leaving group (see Fig. S2 in the ESI†). The linker system was stable in water, 5 mM HCl solution and pH 4 sodium phosphate buffer (SPB). Notably, 13 mM of BOE pH 4 proved to be an optimal amount of fluoride to cleave the silane bond in quantitative yields. The formation of 1-naphthol and difluoro-diisopropylsilane upon treatment with BOE was confirmed by LC-MS and GC-MS, respectively. Subsequently, the more sophisticated copper free click reagent ((1*R*,8*S*,9*S*)-bicyclo-[6.1.0]non-4-yn-9-ylmethanol), which contains an accessible hydroxyl group, was reacted with dichloro-diisopropylsilane and *in situ* added to C/Co@PEGMA **2** to afford a magnetic, cleavable copper-free click reagent C/Co@PEGMA-Si-octyne **3** (Scheme 2). Characterization *via* elemental microanalysis and IR (esp. C–H stretch at 2876 cm^{-1}) confirmed the establishment of the desired linker system. The loading was determined by clicking the commercially available azide functionalized dye carboxy-tetramethylrhodamine (TAMRA-azide) to **3** (Fig. 3). Therefore the dye and **3** were shaken for 6 h, resulting in a 1,3-Huisgen dipolar cycloaddition. Subsequently, the absorbance of the supernatant



Scheme 2 Synthesis of C/Co@PEGMA-Si-octyne.

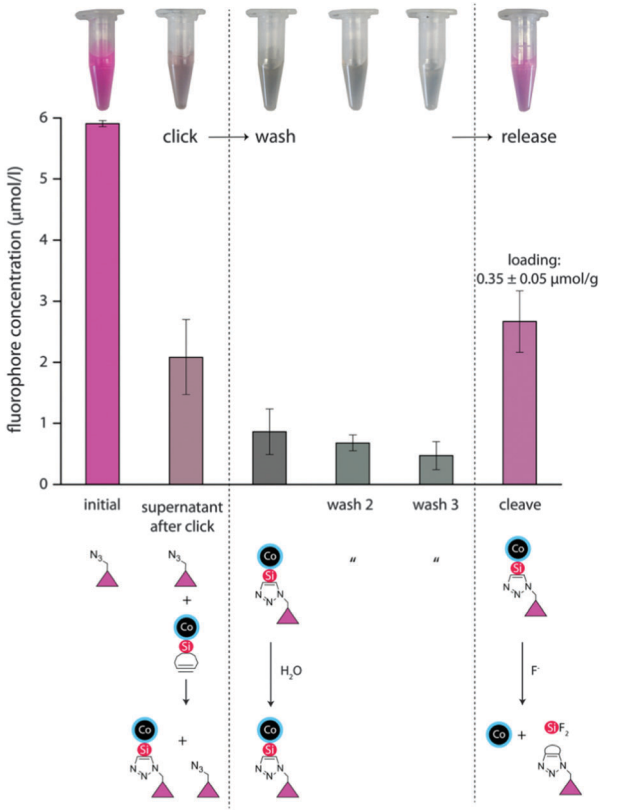


Fig. 3 Click and release of an azide-functionalized dye (TAMRA-azide). Conditions: in water, click for 3 h at RT, wash with water (3×, 1 h), cleave for 16 h using 13 mM BOE.

was measured at 550 nm, containing the remaining dye species. The particles were washed with water three times for one hour, resulting in no absorbance at 550 nm, *i.e.* no color. However, if dispersed in 13 mM BOE, a clear peak at 550 nm appeared, also visible as a purple coloring. It should be noted that 13 mM buffered oxide etch contains a relatively low amount of fluoride (247 ppm), comparable to common toothpaste (1000–1500 ppm).[17] The amount of cleaved dye was used to calculate the loading, determined as 0.35 ± 0.05 µmol g^{-1}. The loading was intentionally set lower (by controlling the amount of mono-chlorinated species added to C/Co@PEGMA) than in the case of 1-naphthol (49 ± 0.4 µmol g^{-1}) because many biomolecules are large in diameter (1–6 nm)[18] and therefore hinder a high loading onto nanoparticles due to size-constraints. It should be mentioned that a higher loading could possibly be achieved, but since copper-free click reagents are expensive in large quantities (*e.g.* grams) and the resulting benefit (*i.e.* higher biomolecule loading) is low, this was not further pursued. Furthermore, unspecific protein binding onto the nanoparticles (“fouling”) was investigated, since the aim of this work is to catch and release specific (*i.e.* azide-labelled) compounds. It is crucial that non-labelled proteins do not adhere to the particle surface and that bound proteins can be released effectively. This means that there is no unspecific interaction between the particle and a bound protein. Therefore, rhodamine labelled BSA was dissolved in water (0.5 nmol mL^{-1}) and the absorption at 555 nm was measured at t = 0 h, then after 2, 18, 32 h using naked particles, **2** or **3** (20 mg each, see Fig. S3 in the ESI†). While the naked C/Co particles irreversibly adsorbed over 90% of the protein over time, the PEG-functionalized particles **2** and **3** showed much lower unspecific protein adsorption (*i.e.* <5% after 32 h). Since PEG-coating has been known to prevent unspecific protein adsorption, these results are in line with recent literature.[19] To demonstrate applicability in the biological field, the protease enzyme α-chymotrypsin (from bovine pancreas) has been functionalized with an azide group by using a commercially available azide–PEG_4–NHS reagent. The well-studied reaction of NHS-ester with unprotonated, free amine groups of proteins (*e.g.* lysine) proceeded smoothly in basic buffer solution. The activity of the azide-functionalized enzyme was then compared to the immobilized enzyme (*i.e.* bound by click-reaction to the magnetic particle) using a standard enzymatic assay (Fig. 4).

As anticipated, the immobilized enzyme exhibited a lower specific activity (*i.e.* <5% of the free enzyme). Compared to other reports of covalent[20] or biospecific[21] immobilization of enzymes on magnetic nanoparticles (15–35%, resp. 106% retained activity) the herein reported retained activity is low. A possible explanation for this drop can be the random orientation of the immobilized enzyme. Also, the covalent linking strategy might negatively influence the enzyme activity.

However, if the clicked enzyme was released by the use of 13 mM BOE, the activity of the released enzyme was substantially increased (*i.e.* to 67%, compared to the free enzyme). It should be noted that the destructive effect of buffered oxide etch on chymotrpysin is marginal (93 ± 3.1% retained activity after 16 h in 13 mM BOE at RT). As mentioned before, the here used dilute F^- solution proved to be harmless to DNA and even RNA.[10] Therefore buffered oxide etch can be assumed to

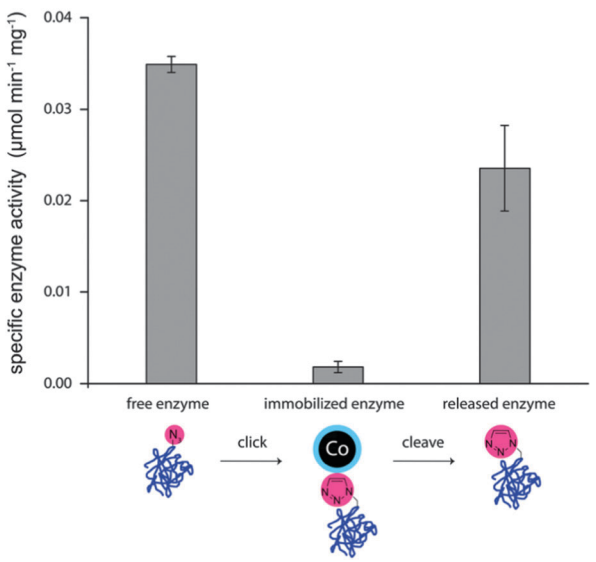


Fig. 4 Specific activity of azide-functionalized α-chymotrypsin. Enzymatic assay was performed measuring the formation of nitroaniline with UV spectroscopy at 390 nm, using *N*-benzoyl-L-tyrosine *p*-nitroanilide as a substrate. Amount of bound enzyme was determined by elemental analysis.

be bio-orthogonal and mild, making it a suitable reagent for a wide use in biochemistry. However, there are also limitations to this cleavage system: pH 4 for 16 h can be too harsh for certain proteins, resulting in denaturing. The long time-span could be reduced by optimizing the conditions (*e.g.* higher fluoride concentration), if necessary. Furthermore, fluoride can interact with certain metal ions, (*e.g.* calcium, forming CaF_2) forming stable compound and thus deactivating an enzyme by binding the cofactor.

In summary, a well-dispersed, magnetic and cleavable copper-free click-nano-reagent has been synthesized and characterized. An azide-functionalized dye has been clicked to the magnetic particle and released using 13 mM dilute F^- solution. Furthermore it was shown that also enzymes could be bound and released without a substantial loss of activity.

The authors declare the following competing financial interest(s): two authors (W. J. S. and R. N. G.) declare financial interests, as they are shareholders of Turbobeads LLC, a company producing magnetic nanoparticles.

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