One-step bioengineering of magnetic nanoparticles *via* a surface diazo transfer/azide–alkyne click reaction sequence†

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Received (in Cambridge, UK) 18th October 2007, Accepted 20th November 2007
First published as an Advance Article on the web 4th December 2007
DOI: 10.1039/b716113a

We have developed an efficient conversion of amino iron oxides to carbohydrate and protein derived nanoparticles with highly conserved bioactivity through a combination of diazo transfer and azide–alkyne click technology.

Magnetic nanoparticles (MNP) based on iron oxides have attracted much attention for their eclectic applications in biotechnology and medicine. These applications include use as magnetic resonance imaging contrast agents,[1] heating mediators for cancer thermotherapy,[2] magnetic force-based gene delivery,[3] and selective separation and detection of biomolecules.[4] The appeal of MNP with diameters below 30–40 nm is due to their exceptional magnetic properties and biocompatibility. In recent years, the growing need of high-quality hybrid MNP for biomedical purposes has stimulated many efforts towards the development of general methods to achieve bioactive surface engineered MNP. Current strategies mostly involve noncovalent approaches such as biotin–streptavidin technology[5] or substrate passive adsorption.[6] Alternatively, the formation of more stable covalent connections between hydroxyl groups on the outer particle surface and a suitable anchoring agent located in a specific position of the target molecule has received broad attention.[7,8] The latter approach is definitely more specific, thus improving the quality of the hybrid nanoparticles, but it suffers from the necessity to effect a great deal of hard-working chemical modifications, that could be impracticable with complex biomolecules, such as proteins.

A convenient route is represented by the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), the "click" reaction.[9] This reaction has several advantages: 1) it may be performed in water, buffered media or mixtures of aqueous/organic solvents; 2) usually it is high yielding and it does not require temperature and pressure control; 3) the two active functionalities (azide and alkyne groups) are easily accessible and they are located orthogonally on the respective coupling entities, thus avoiding homodimer formation, such as in the case of disulfide bridges;[10] 4) the reaction is irreversible and the resulting linkage is very stable owing to the formation of a triazole ring. Although click reaction is rapidly spreading in all areas of surface chemistry as a valuable method for the efficient immobilization of organic molecules, the application to MNP is still in its infancy.[11,12] This concept has the potential to open the way to a reliable labeling of bioactive molecules. However, the practical limitation of the current strategies is that they require multiple reactions with time consuming purification and characterization steps. Hence, a simple and general protocol for the efficient bioconjugation of MNP is still desired. In the present paper, we propose a straightforward, one-step route to site-specific immobilization of biologically relevant molecules onto MNP. By exploiting a convenient *in situ* tandem combination of diazo transfer and CuAAC reactions, we demonstrate that biomolecules such as carbohydrates and proteins can be easily immobilized onto the surface of iron oxide nanoparticles retaining their biological activity.

We used commercial γ-Fe_2O_3 nanoparticles (Alpha Aesar) with a size dispersion of 10 ± 4 nm and approximately spherical in shape, as confirmed by TEM. Frequently, an initial step to modified iron oxide nanoparticles involves their sonochemical alteration with γ-aminopropyl triethoxysilane in toluene or water, leading to aminated nanoparticles **MNP1**. NH_2-MNP may also be easily accessed from dextran coated Fe_3O_4.[13] Our first objective was to turn NH_2-MNP into N_3-MNP, necessary for CuAAC reaction.

A high-yielding method alternative to traditional S_N2 approaches consists in the generation of azido functionalities from the corresponding amines *via* the Cu(II)-catalyzed diazo transfer reaction.[14] We considered this strategy and applied it successfully to **MNP1**, which were easily converted into azido MNP (**MNP2**). **MNP1** were suspended in a H_2O/MeOH solution and treated with freshly prepared trifluoromethanesulfonyl (triflic) azide dissolved in the minimum amount of dichloromethane, in the presence of triethylamine and catalytic $CuSO_4$. Upon addition of the catalyst, the surface amino groups rapidly turned into the corresponding azides. The progress of the reaction can be monitored by ninhydrin assay and the formation of the azide was observed by reflection anisotropy spectroscopy (RAS) FTIR, which showed the appearance of a strong absorbance at 2095 cm^{-1} due to N=N=N antisymmetric stretching.

In the course of this work, Beckmann *et al.* have reported a one-pot procedure for diazo transfer and click reaction in solution.[15] In light of the above observations, we explored the possibility to tether biomolecules to **MNP1** *via* triazole formation without recovering the intermediate azido derivative. The key point of our method is the *in situ* generation of Cu(I) species required for the Hüisgen cycloaddition, deriving it directly from Cu(II) salts necessary for diazo transfer. Indeed, the addition of sodium ascorbate after diazo transfer completion causes the reduction of

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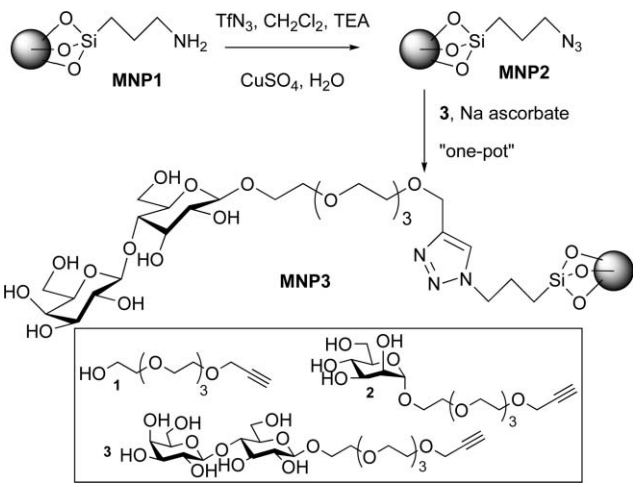
Cu(II) to Cu(I), which, once formed, becomes immediately available to promote the subsequent cross-reaction with the complementary alkyne. The organic counterparts required for CuAAC reaction were provided with an appropriate acetylenic functionality. As a general strategy, we introduced a propargyl group through a tetraethylene glycol chain (**1**), commonly employed as a soluble bioinert spacer for bioconjugation.

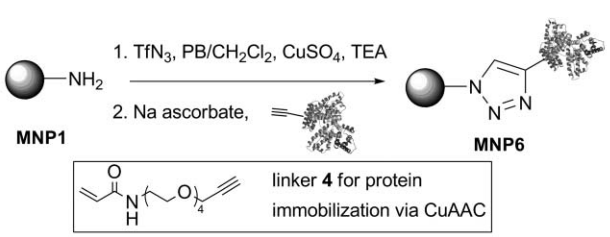
A representative reaction protocol for a sequential one-pot procedure is reported in Scheme 1 for the lactose derivative **3**. **MNP1** were sonicated under above mentioned diazo transfer conditions, affording **MNP2**, which accumulated within the aqueous layer of a biphasic H_2O–CH_2Cl_2 mixture. Sodium ascorbate was then added to the mixture, followed by a solution of previously synthesized precursor **3** (see ESI† for the synthesis of precursors **1–3**). The mixture was sonicated for 2 h, resulting in a water soluble dispersion of **MNP3**. **MNP3** was recovered after centrifugation, washed with water several times and dried under vacuum. Elemental analysis and ICP-AES indicated that an average of 835 ligands were clicked to each particle, corresponding to 2.7 ligands per nm^2. The same procedure was reproduced with **1** and **2**, obtaining the corresponding **MNP4** and **MNP5**, with surface densities of 3.2 ligands per nm^2 and 2.8 ligands per nm^2, respectively.

The derivatized particles **MNP3-5** were characterized by FTIR, high resolution magic angle spinning (HRMAS) NMR and TEM. We observed our samples by attenuated total reflection (ATR) and RAS FTIR. Infrared spectra of **MNP3-5** showed a diagnostic peak at 1550 cm^{-1} in agreement with the literature value for a 1,2,3-triazole. Strong absorbance due to Si–O and Fe–O bonds were evident at 1010–1032 cm^{-1} and 453–533 cm^{-1}, respectively. Characteristic bands owing to C–H were also identified at 2850 cm^{-1} (stretching) and 1400–1500 cm^{-1} (bending). ^1H NMR resolved spectra of ligands bound to a paramagnetic nanocrystal are difficult to perform due to large broadening effects caused by paramagnetic features.[16] Since we have experienced HRMAS NMR as a powerful spectroscopic technique for the characterization of supported molecules, we explored the possibility to analyse our MNP by HRMAS NMR. Actually, this method proved very valuable for structure-resolved analysis of the organic layer of our hybrid nanoparticles. Signals are sharp and well-resolved. The presence of a peak at *ca.* 8 ppm in **MNP3-5** NMR spectra due to the triazole proton confirmed that 1,3 dipolar cycloaddition was successful. The comparison with ^1H NMR of **2** and **3** not only confirmed the presence of sugar moieties on **MNP3** and **MNP5**, but also allowed us to attribute the respective carbohydrate structures. Anomeric protons for lactose in **MNP3** are located at 4.41 ppm (*d*, J = 10 Hz, β-galacto) and 4.48 ppm (*d*, J = 10 Hz, β-gluco), while α-mannoside H-1 in **MNP5** was shifted to 4.95 ppm (*s*). HRMAS NMR spectra of **MNP3** and **MNP5** are included in the ESI†.

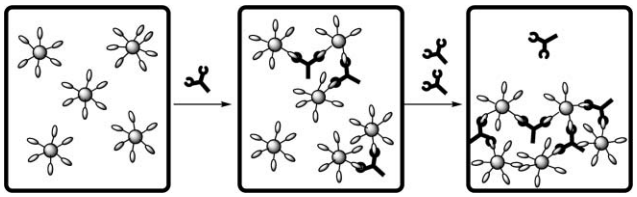
As a proof of principle that our strategy is readily adaptable to complex biomolecules, MNP were clicked to human serum albumin (HSA). This plasma protein consisting of 585 amino acids is involved in maintenance of osmotic pressure and transport of endogenous and exogenous (drugs) substances. HSA includes 35 cysteine residues, 34 of which are involved in 17 disulfide bonds, while the unpaired Cys34 is present as a free thiol.[17] Therefore, Cys34 is available for site-specific alkylation *via* Michael addition by acrylamides. This labeling step is commonly used with any protein provided with accessible cysteines without significant loss of biological activity. We synthesized the alkyne-bearing acrylamide **4**, in which tetraethylene glycol linker was introduced to space out the protein from the MNP surface. HSA was reacted with **4** at 37 °C in deionized water for 12 h, then dialyzed to remove alkyne excess, and lyophilized. The amount of alkylated protein was determined by titrating residual cysteine thiols with Ellman's reagent.[18] We quantified that about 50% of the overall HSA molecules were modified and we used this mixture in the next experiments without further purification. The propargyl-conjugated protein was successfully attached to MNP by following our protocol (Scheme 2). In order to avoid possible protein denaturation by contact with organic solvent, dichloromethane was accurately removed by syringe before adding the protein. An azido particle suspension in 0.1 M phosphate buffer, pH 7.0 (PB) and Cu(II) from diazo transfer were incubated at 37 °C with propargyl–HSA and sodium ascorbate and collected after 24 h by means of a permanent magnet, obtaining the HSA–MNP (**MNP6**). Several particle centrifugation–redispersion cycles were effected to remove unreacted proteins until the supernatant was protein-free (by ninhydrin test). Particles were purified by filtering them through 0.45 μm pore septa, and characterized by ATR-FTIR. The IR profile of **MNP6** was similar to that of native protein, confirming the presence of HSA molecules on the particle surface. The filtered suspension was stable for months. As a control, we attempted the same reaction with native HSA, but we didn't observe any reactivity, suggesting that **MNP2** were substantially inert towards protein passive adsorption. After magnetic recovery of **MNP6**, we determined the amount of loaded protein



Scheme 1 One-step synthesis of saccharide–MNP.



Scheme 2 Immobilization of HSA onto MNP.



Scheme 3 Schematic antibody-induced **MNP6** agglomeration.

Table 1 Activity of **MNP6** towards anti-HSA by T_2 measurement[a]

[Anti-HSA]/ mg ml^{-1}	0.00	0.03	0.10	Ab/PB[b]
T_2/ms (1 min)	2.14 ± 0.05	3.89 ± 0.05	6.06 ± 0.1	2788 ± 50
T_2/ms (10 min)	2.16 ± 0.05	4.19 ± 0.05	12.46 ± 0.2	2796 ± 50

[a] T_2 values were acquired at 37 °C in PB with a [**MNP6**] of 0.01 mg ml^{-1}. [b] This column reports T_2 measured in absence of **MNP6**.

by accurately measuring the residual intensity of Trp_{214} maximal wavelength in the supernatant mother solution. This value was then subtracted from the one relative to the initial HSA concentration. This step was necessary for accurate quantification of protein binding because scattering and absorption artifacts due to iron oxide prevented the direct quantification of tryptophan in the bound proteins. The amount of unreacted HSA was fluorometrically determined after having first established a standard calibration curve. On this basis, an average particle coating of 7.5 HSA ligands pet MNP was estimated, corresponding to about 84% coverage of the available surface area (details and calculations are summarized in the ESI†).

With **MNP6** in hand, we evaluated the bioactivity of HSA-decorated MNP obtained by our method. Actually, it is well documented that protein immobilization onto solid surfaces may lead to partial or even complete denaturation.[19] We tested our nanoparticles observing the behavior of a **MNP6** PB dispersion in the presence of anti-HSA polyclonal antibody (Sigma). The antiserum induces particle agglomeration by bridging couples of HSA molecules situated on the respective nanoparticles (Scheme 3). Such agglomeration was sensitively detected by accurate relaxometric analysis of the particle dispersion (Table 1). An effect of the formation of magnetic aggregates with more then 10–15 particles is the decrease in the overall relaxivity of water protons, corresponding to a detectable increment of spin–spin relaxation time T_2. In fact, owing to maghemite high density (4.9 g ml^{-1}), MNP clusters above 400–500 nm may induce a loss of the colloidal status of the particulate, thus sequestrating active contrast agent from water solution, which significantly influences the relaxation rates. Immunoprecipitation due to antigen–antibody interactions was unambiguously detected at anti-HSA femtomoles. On the contrary, additions of the same volumes of PB solution had a minimal effect on the measured relaxivities due to dilution. As evidenced in Table 1, relaxometric measurements after antiserum additions revealed a time- and dose-dependent response of **MNP6** to HSA-specific antibody, testifying to the retained biological

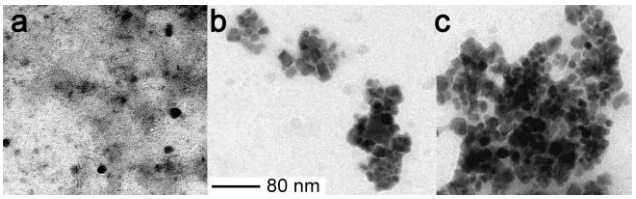


Fig. 1 TEM images of different stages of **MNP6** agglomeration in response to addition of anti-HSA: A) 0.02 mg ml^{-1} **MNP6** in PB; B) added 0.01 mg ml^{-1} anti-HSA; C) added 0.10 mg ml^{-1} anti-HSA.

activity by anchored HSA molecules. The formation of **MNP6** clusters was also confirmed by TEM analysis (Fig. 1).

In conclusion, we have reported a versatile, one-pot biofunctionalization of MNP *via* diazo transfer followed by *in situ* CuAAC reaction. In this context, we have also shown HRMAS as a valuable technique enabling a structure-resolved ^{1}H NMR surface characterization of MNP. Moreover, we demonstrate that our method is particularly suitable for protein immobilization, resulting in a site-specific anchorage onto the nanoparticle surface, which prevents loss of protein bioactivity. Exploiting the unique magnetic properties of MNP, this approach may favour the generation of novel diagnostic tools for active targeting.

We thank Dr P. S. Coghi for UV-visible spectra and Dr F. Tedoldi for help in relaxometric investigations. The work was supported by FIRB RBPR05NWWC, Cariplo foundation and MIUR-COFIN 39903.

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