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

Laura Polito, a Diego Monti, b Enrico Caneva, †a Eleonora Denevo, §a Giovanni Russo a and Davide Prosperi †b

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,\textsuperscript{1} heating mediators for cancer thermotherapy,\textsuperscript{2} magnetic force-based gene delivery,\textsuperscript{3} and selective separation and detection of biomolecules.\textsuperscript{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\textsuperscript{5} or substrate passive adsorption.\textsuperscript{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.\textsuperscript{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(II)-catalyzed azide–alkyne cycloaddition (CuAAC), the “click” reaction.\textsuperscript{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;\textsuperscript{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.\textsuperscript{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 \textit{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 $\gamma$-Fe$_2$O$_3$ 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 $\gamma$-aminopropyl triethoxysilane in toluene or water, leading to aminated nanoparticles MNP1. NH$_2$-MNP may also be easily accessed from dextran coated Fe$_3$O$_4$.\textsuperscript{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 Staudinger approaches consists in the generation of azido functionalities from the corresponding amines via the Cu(II)-catalyzed diazo transfer reaction.\textsuperscript{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$_2$O/MEOH solution and treated with freshly prepared trifluoromethanesulfonfyl (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 \textit{et al.} have reported a one-pot procedure for diazo transfer and click reaction in solution.\textsuperscript{15} In light of the above observations, we explored the possibility to tether biomolecules to MNP1 \textit{via} triazole formation without recovering the intermediate azido derivative. The key point of our method is the \textit{in situ} generation of Cu(II) species required for the Hüsgen 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
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 H₂O–CH₂Cl₂ 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 nm². The same procedure was reproduced with MNP2, obtaining the corresponding MNP4 and MNP5, with surface densities of 3.2 ligands per nm² and 2.8 ligands per nm², 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).¹¹HN MRM spectra of 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 alkyn-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.
by accurately measuring the residual intensity of Trp214 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 evidenced in Table 1, relaxometric measurements after antiserum addition of the same volumes of PB solution had a minimal effect on the measured relaxivities due to dilution. As contrary, additions of the same volumes of PB solution had a 0.01 mg ml⁻¹ anti-HSA; C) added 0.10 mg ml⁻¹ anti-HSA.

Notes and references