Protein Nanoconjugation

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# Site-Specific Conjugation of ScFvs Antibodies to Nanoparticles by Bioorthogonal Strain-Promoted Alkyne–Nitrone Cycloaddition**

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Hybrid multifunctional nanoparticles (MFN), which combine unique superparamagnetic properties and fluorescence emission, have been envisaged as promising bimodal tracers for noninvasive diagnosis of cancer both in vitro and in vivo.[1–4] The design of ideal targeted MFN (TMFN) needs careful optimization of fundamental features including uniform size and shape,[5] surface charge,[6] optical and magnetic properties,[7,8] and efficient functionalization with suitable homing ligands[9–11] to improve the signal amplification and target selectivity toward malignant cells. When the ligands are complex molecules, such as proteins, their proper orientation on the surface of nanoparticles becomes a crucial factor for maximizing the affinity for their molecular counterparts.[12–14] In this context, copper-catalyzed azide-alkyne cycloaddition (CuAAC), is gaining attention as a versatile strategy for protein immobilization on iron oxide nanoparticles.[15] However, CuAAC requires metal catalysts and these are toxic to cells and unsuited for several kinds of proteins.[16] In light of this severe limitation, the bioorthogonal conjugation, which aims at avoiding the use of unsafe promoters, is rapidly becoming popular.[17–19] The main advantages of this approach are speed, efficiency, and biocompatibility. The strain-promoted azide–alkyne cycloaddition (SPAAC) modification of CuAAC, in which the terminal alkyne is replaced by a highly reactive cyclooctyne, does not require $Cu^{I}$ catalysis and has provided excellent results both in solution and in living cells.[20–22] The kinetics of the reaction can be improved by introducing electron-withdrawing substituents adjacent to the triple bond of the ring-strained cyclooctyne.[23] However, problems still arise when a site-specific attack to a selected amino acid residue under physiological conditions is desired. The attempts so far have mainly followed genetic encoding or metabolic labeling strategies, in which non-natural amino acids bearing an alkyne or azide functionality can be introduced artificially in the peptide sequence.[24,25] However, these methods suffer from poor generality and involve complicated and laborious procedures, which are not accessible in most laboratories.

Recently, an elegant variant to SPAAC has been proposed, which replaces the azido functionality with a nitrone group.[26] Termed strain-promoted azide-nitrone cycloaddition (SPANC), this reaction was fast and high-yielding with several molecular species, including peptides,[27] and entire cells,[28] provided that an accessible nitrone group could be incorporated in the molecular architecture.

We have recently reported the production of a scFv variant (scFv800E6) of the anti-HER2 antibody in *Pichia pastoris*.[29] There is a growing interest for scFvs due to the high target selectivity and reduced immunogenicity compared to whole antibodies.[30] Herein, we explore the potential of the SPANC reaction for site-specific bioengineering of MFN with recombinant scFv bioligands and demonstrate the potential of the resulting targeted MFN (TMFN) in selectively binding to HER2 breast cancer cell receptors.

In principle, the SPANC reaction seems particularly well suited for the immobilization of scFv antibodies on nanoparticles, as 1) it prevents homodimer formation, which is common in thiol-based ligations; 2) the introduction of a serine at the peptide N-terminus, which can be easily accomplished by genetic engineering, leads to one single nitrone species; 3) the conjugation through the N-terminal residue is expected not to interfere with the affinity toward HER2. Indeed, N-terminal serine is located sufficiently far from the antigen-binding site of the peptide. Details on homology modeling of the N-terminal serine mutant of scFv800E6 (scFv1) are included in the Supporting Information. Serine was inserted during gene amplification by the polymerase chain reaction (PCR), cloned in pPICZ$\alpha$ and transformed in *P. pastoris*. ScFv1 was obtained with a C-terminal c-myc epitope, 6 × His-tag, and purified by a single-step purification method on a Ni-NTA agarose affinity column in 2.5 mg $L^{-1}$ yields.

Ligand **L1** (Scheme 1), containing a 4-dibenzocyclooctynol derivatized with a SH-terminated poly(ethylene glycol) [with a molecular weight of 5000] ($PEG_{5000}$) carbamate linker, was synthesized in seven steps according to the procedure

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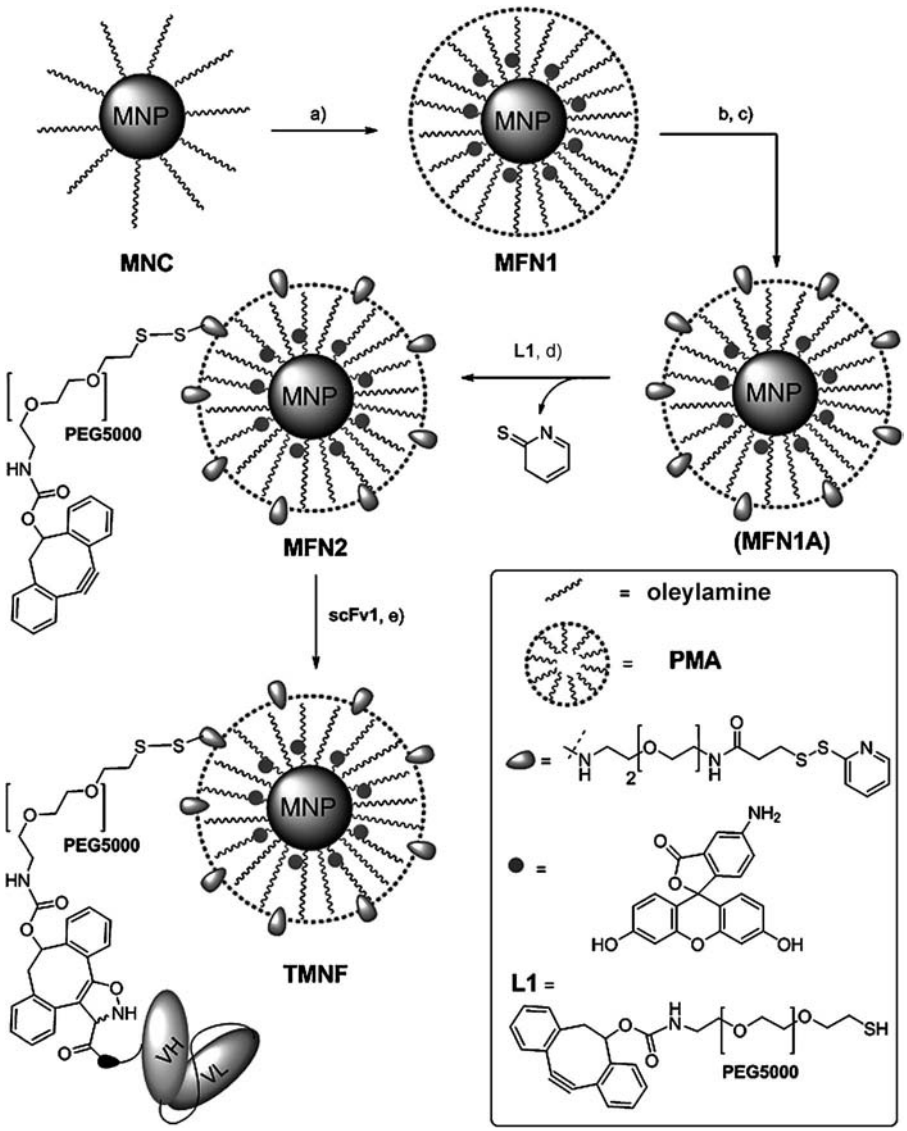


***Scheme 1.*** Synthesis of TMFN, see text for details. a) FITC-PMA; b) EDBE, EDC·HCl, 2 h, room temperature; c) SPDP, 4 h, room temperature; d) **L1**, 2 h, room temperature; e) scFv1, 1 h, room temperature, 14 h at 4 °C. FITC = fluoresceine isothiocyanate, SPDP = *N*-succinimidyl-3-[2-pyridyldithio] propionate, VL = variable light chain, VH = variable heavy chain (VH and VL are variable heavy and light region of IgG molecules, respectively: VH + VL + linker = scFv).

described in the Supporting Information. Highly uniform magnetite nanocrystals (MNC, $7.5 \pm 0.5$ nm) coated with oleylamine surfactant were obtained by solvothermal decomposition in organic solvents.[31] Nanoparticles were transferred to the water phase by mixing with an amphiphilic polymer, obtained by condensation of poly(isobutylene-*alt*-maleic anhydride) and dodecylamine (PMA),[32] which was previously reacted with fluoresceinamine, resulting in the highly stable, dispersible, and fluorescent MFN1. MFN1 were functionalized with amino groups using the bifunctional linker 2,2-(ethylenedioxy)bis(ethylamine) (EDBE). **L1** was linked to the amines generated on the polymer envelop by activation with thiol-reactive *N*-succinimidyl-3-[2-pyridyldithio] propionate to give MFN2 (Scheme 1). We estimated an average of 20 **L1** molecules per nanoparticle by UV measurement of pyridine-2-thione released upon binding. MFN2 were $60 \pm 3$ nm in size, as measured by dynamic light scattering (DLS) in phosphate buffer saline (PBS, pH 7.4) at 5 $\mu g\,mL^{-1}$, with a $\zeta$ potential of $-38 \pm 6$ mV, and exhibited a maximal fluorescence emission at 515 nm. Even after reaction with **L1**, MFN2 were completely stable in PBS giving a clean solution. MFN2 were subjected to a one-pot SPANC reaction sequence. Unfortunately, the original procedure caused nanoparticle agglomeration, which forced us to adjust the reaction conditions. ScFv1 (2.0 equiv) was diluted 1:1 in 0.1 M ammonium acetate buffer, pH 6.9, and incubated at 25 °C for 1 h with sodium periodate (2.0 equiv), leading to the oxidation of the N-terminal serine. The resulting aldehyde was immediately treated with excess *p*-methoxybenzenethiol (13.0 equiv, 1 h) followed by *p*-anisidine (18.6 equiv, 15 min). Next, MFN2 (1.0 equiv) and *N*-methylhydroxylamine hydrochloride (18.6 equiv) were added, incubated 1 h at room temperature and left overnight at 4 °C, leading to TMFN. The excess scFv1 was removed by centrifuge-assisted dialysis using 100 kDa pore Amicon filters at 3500 rpm. The concentrated TMFN solution was washed two times in the same way, then recovered and diluted to a 7 $mg\,mL^{-1}$ stock solution in PBS. An aliquot was drawn, and the presence of scFv1 on TMFN was cross-checked by dot-blot analysis. The final hydrodynamic size of TMFN in PBS was $158 \pm 7$ nm with a $\zeta$ potential of $-47 \pm 4$ mV, confirming an increase in size after protein conjugation.

TMFN were assessed by flow cytometry to evaluate their affinity and target selectivity in labeling MCF7 breast cancer cells expressing HER2 membrane receptor. TMFN were incubated in parallel for 1 h with HER2-positive ($HER2^+$) MCF7 cells at two different concentrations (20 $\mu g\,mL^{-1}$ and 100 $\mu g\,mL^{-1}$, respectively) and with HER2-negative ($HER2^-$) MDA cells (100 $\mu g\,mL^{-1}$). Flow cytometry showed a remarkable right shift of fluorescence signal for both TMFN concentrations with $HER2^+$ cells, which was not observed with MDA cells (Figure 1). Values in Figure 1 show a remarkable increase in mean fluorescence intensity of 100 $\mu g\,mL^{-1}$ TMFN in comparison with the 20 $\mu g\,mL^{-1}$ sample, while the latter exhibits a population at low intensities, which is accounted for by the presence of residual

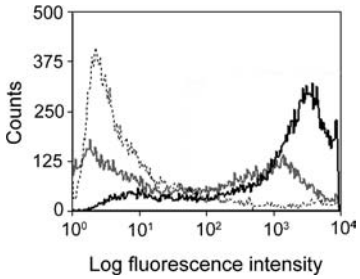


***Figure 1.*** MCF7 cells were incubated 1 h at 37 °C with 20 μg mL$^{-1}$ (gray line) and 100 μg mL$^{-1}$ (black line), respectively, of TMFN. As a negative control, MDA cells were treated with TMFN (dashed line).

unlabeled cells. These results suggest that TMFN accumulate selectively on MCF7 cells and that TMFN-HER2 recognition is concentration dependent. The specificity of binding between TMFN and HER2 was validated by confocal laser scanning microscopy. MCF7 and MDA cells were both treated with TMFN (100 μg mL$^{-1}$) and, in parallel, with dye-labeled scFv1 (20 μg mL$^{-1}$). As HER2 is a transmembrane receptor, we observed accumulation of both TMFN and scFv1 at the cell membranes of HER2$^+$ cells only (Figure 2 A and C, respectively, green). Figure 2 panels N and O showed membrane colocalization (orange), which confirmed that nanoparticle capture by cells was actually mediated by specific membrane receptor interaction). HER2$^-$ cells showed only membrane staining (B and D). TMFN bound to MCF7 membrane could not be displaced by free scFv1 even at threefold concentration, confirming our previous evidence that multiple scFv presentation on spherical nanoparticles improves significantly the antigen recognition.[33]

To evaluate the potential of TMFN as magnetic contrast agents in living cells, we performed a set of $T_2$ relaxation experiments on MCF7 and MDA cells. Two MCF7 and two MDA cell cultures (1 mL) were treated with 20 μg and 100 μg TMFN, respectively, for 3 h at 37 °C. The cells were washed with PBS, fixed with 2.5% buffered glutaraldehyde, and eventually resuspended in PBS, at a final concentration of $5\times10^5$ cells mL$^{-1}$. The treated samples as well as unlabeled MCF7 and MDA cells (controls) were analyzed by relaxometric measurements. All the treated MCF7 cells exhibited a significant dose-dependent fall in $T_2$ compared to the control, confirming the capture of paramagnetic iron by cultured cells (Figure 3). As expected, cells treated with 100 μg TMFN exhibited greater fall in $T_2$ (relaxivity enhancement) compared to 20 μg TMFN, suggesting a dose-dependent nanoparticle capture. In contrast, MDA cells did not provide evidence of $T_2$ decrease attributable to the interaction with TMFN at all the concentrations tested.

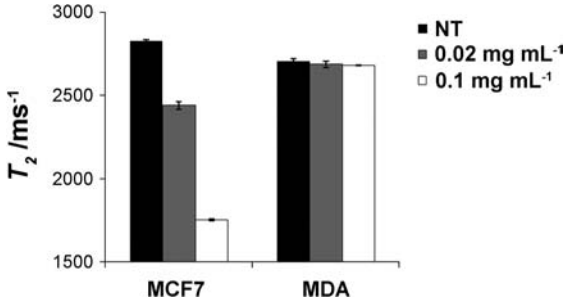


***Figure 3.*** Relaxivity measurements on treated and untreated (NT, controls) MCF7 and MDA cells. Data are the mean ± S.E. (standard error) of three different relaxivity measurements. $T_2$ values refer to $5\times10^5$ cells after incubation with different concentrations of TMFN.

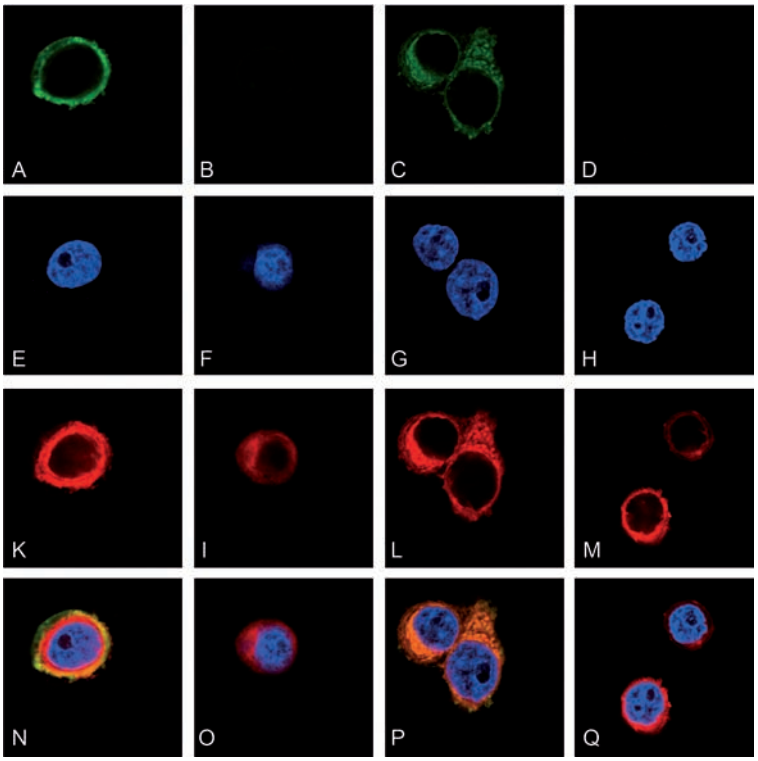


***Figure 2.*** HER2$^+$ cells (MCF7: A, E, K, N) and HER2$^-$ cells (MDA: B, F, I, O) were incubated for 1 h at 37 °C with TMFN (100 μg mL$^{-1}$). ScFv1 incubation with MCF7 and MDA cells was used as positive (C, G, L, P) and negative controls (D, H, M, Q), respectively. Then cells were fixed and ScFv1 was revealed by a FITC-conjugated antibody to whole murine IgG (C, D). Nuclei were stained with DAPI (blue: E, F, G, H) and membranes were stained with DiD oil (red: K, I, L, M). Merged images are shown in panels N, O, P, and Q. Scale bar = 10 μm.

Finally, we investigated the cellular toxicity and cell proliferation of TMFN. TMFN were found to be nontoxic at 20 and 50 μg mL$^{-1}$ in MCF7 after 24 h of exposure, while induced about 15% mortality at 100 μg mL$^{-1}$, probably due to the high particulate concentration in suspension. No differences were observed between untreated and TMFN-treated cells in terms of proliferation. Altogether, these experiments suggested a good profile of safety in cultures for TMFN.

In summary, we have demonstrated that strain-promoted azide–nitrone cycloaddition (SPANC) allowed for the rapid and effective conjugation of proteins on nanoparticles functionalized with

  

cyclooctyne ligands under mild conditions, provided that a N-terminal serine residue was introduced by molecular engineering. In particular, the application of SPANC to a scFv antibody against HER2 tumor marker resulted in its prompt immobilization on multifunctional nanoparticles (MFN), leading to water-stable bioengineered targeted MFN (TMFN), which exhibited a complete conservation of protein effectiveness in selectively targeting HER2 receptor in living cells. As the structural motif of scFv fragments is highly conserved,[34] and other kinds of nanoparticles can be modified identically with the same polymer used herein,[32] this approach is expected to be of general utility and may become a universal strategy for the development of a new generation of targeted nanoparticles.

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