Supporting Information

Investigating the structural biofunctionality of antibodies conjugated to magnetic nanoparticles

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General Materials and Instrument details.

All reagents and solvents were purchased from Sigma-Aldrich (St. Louis, MO), Fluka (St. Gallen, Switzerland) and Riedel-de Haën (Seelze, Germany) and used as received without further purification. Trastuzumab was received from Roche Products Ltd. (UK) as a powder lyophilized for intravenous administration; before reactions it was purified from additives by dialysis (membrane, MW cutoff 12-14000 Da) in phosphate buffer solution, pH 7.4, 300 mM NaCl, 2.7 mM KCl, 10 mM phosphate. Water was deionized and ultrafiltered by a MilliQ apparatus (Millipore Corporation, Billerica, MA). Unless otherwise specified, all of the reactions were performed in an inert atmosphere of argon under dry conditions. Ultrasounds were generated by S15H Elmasonic Apparatus (Elma, Singen, Germany). TEM images of nanoparticles were obtained by a Zeiss EM-109 microscope (Oberkhochen, Germany) operating at 80 kV. T_2 relaxation times were performed at a temperature of 313 K using a Bruker Minispec mq20 system (Ettlingen, Gerrmany) working with ^{1}H at 20 MHz magnetic field with the following parameters: Carr-Purcell-Meiboom-Gill pulse sequence, 1000 echoes with a 20 ms echo times and 2 s repetition time. Samples were introduced using a 10 mm NMR spectroscopy tubes prewarmed and sonicated at 40 °C. Dynamic Light Scattering (DLS) measurements were performed at 90° with a 90 Plus Particle Size Analyzer from Brookhaven Instrument Corporation (Holtsville, NY) working at 15 mW of a solid-state laser (λ = 661 nm). Zeta-potential measurements were elaborated on the same instrument equipped with AQ-809 electrode and data were processed by ZetaPlus Software. UV-vis spectra were recorded by using a Uvikon 930 UV/Vis Spectrophotometer (Kontron Instruments) in a range of wavelenghts from 190 nm to 600 nm. The FTIR absorption spectra were acquired using the Varian 610-IR infrared microscope coupled to a Varian 670-IR spectrometer (Varian Australia Pty Ltd, Mulgrave

VIC, Australia) and equipped with a nitrogen-cooled, mercury–cadmium–tellurium (MCT) detector. Spectra were collected in transmission under the following conditions: 2 cm^{-1} spectral resolution, 25 kHz scan speed, 512 scan co-additions, and triangular apodization. Measured spectra were smoothed by a binomial function (11 points) and the second-derivatives were obtained by the Savitsky–Golay method (3rd grade polynomial, 5 smoothing points) using the GRAMS/32 software (Galactic Industries Corporation, Salem, NH, USA). For FTIR measurements, 2 μL of the sample solution were deposited on a BaF_2 infrared window and dried at room temperature for 30 min. For measurements in heavy water solution, samples were lyophilized and resuspended in D_2O PBS, pH 7.4. A BaF_2 transmission cell (Wilmad, Buena, NJ, U.S.A.) with path length of 100 μm was employed and the spectra were collected under the same conditions reported above.

Synthesis of MNP1 and MNP2

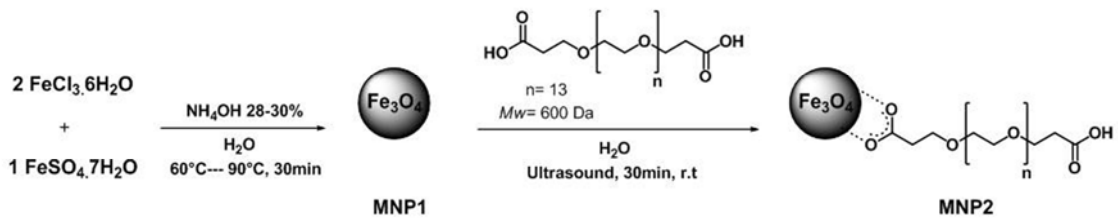

Fig. S1 Synthesis of MNP1 and of pegylated MNP2.

Synthesis of Fe_3O_4 nanoparticles (MNP1) by coprecipitation method: Monodisperse magnetic nanoparticles were obtained as previously described in our previous work with minor modification.[1] Briefly, to a solution of distilled water (173 mL) and NH_4OH 28-30% (14 mL), prewarmed at 60 °C under vigorous stirring, was added a mixture of 1 M $FeCl_3 \cdot 6H_2O$ in distilled water (20 mL) and 2 M $FeSO_4 \cdot 7H_2O$ in 2 N HCl (5 mL); the solution turned dark instantaneously and a black precipitate was formed. The reaction suspension was heated immediately at 90 °C under vigorous magnetic stirring for 30 min. At the end of the reaction, the product was collected from the suspension with a rare-hearth permanent magnet while the supernatant was discarded. The particulate was washed several times with distilled water until neutral pH of the washings. Finally obtained MNP1 were redispersed in distilled water (220 mL) at a concentration of 7.5 mg_{Fe3O4} mL^{-1}.

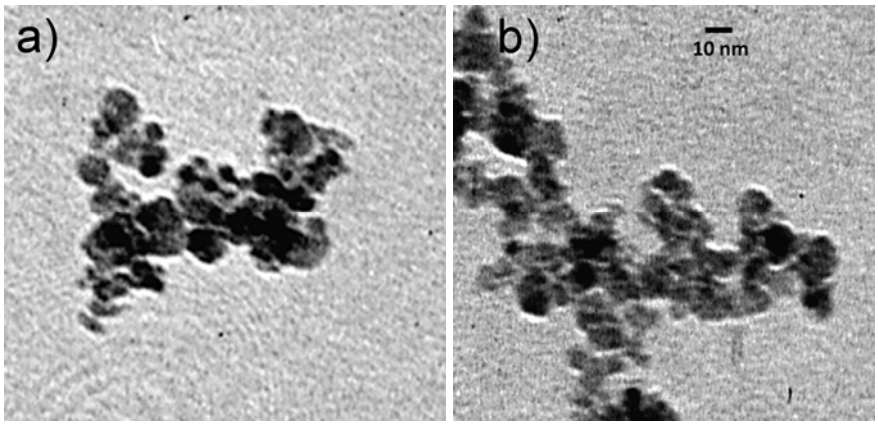

Fig. S2 TEM images of a) MNP1 synthesized by the coprecipitation method and b) MNP2 obtained by reaction of MNP1 with polyethylene glycol 600 diacid. For TEM analyses, the nanoparticles were dispersed under sonication in ethanol (50 μg mL^{-1}) and a drop of the resulting solution was placed on a Formvar/carbon-coated copper grid and air-dried.

Synthesis of PEG-coated Fe_3O_4 nanoparticles (MNP2): A water suspension of MNP1 (3 mL, 7.5 mg mL^{-1}) was sonicated 30 min at r.t.; subsequently, polyethylene glycol 600 diacid (57 μL, 0.112 mmol) was added to the suspension and the resultant mixture was sonicated for 30 min at r.t. At the end of the reaction, nanoparticle suspension was transferred in a centrifuge tube and unreacted nanoparticles were centrifuged at 10000 rpm min^{-1} for 5 min. In this way, supernatant solution, which appeared as a brown clear solution, was separated from the precipitates, which were discarded. To remove the excess of the unreacted reagent, the solution was purified by dialysis membrane (*MW CutOff* 12-14000 Da) overnight; finally MNP2 were diluted with double distilled water to a final concentration of 1 mg mL^{-1}. Under these conditions, nanoparticle suspension was stable for several months at r.t.

Measurement of proton transverse relaxation times (T_2) of MNP2: Before T_2 measurements, the tubes were pre-warmed at this temperature for 10 min in order to obtain thermal equilibration and T_2 values were acquired on the samples at this stage. Relaxivity was determined as the slope of a $1/T_2$ plot as a function of iron concentration expressed in mM.

Dynamic Light Scattering and zeta-potential measurements: Viscosity and refractive index of pure water were used to characterize the solvent. Nanoparticles were dispersed in water under sonication for several minutes before analyses; sporadically, to avoid the formation of large aggregates, the suspension was filtered on a 0.45 μm cellulose acetate filter. The final sample concentration used for measurements was typically 0.01 mg mL^{-1}.

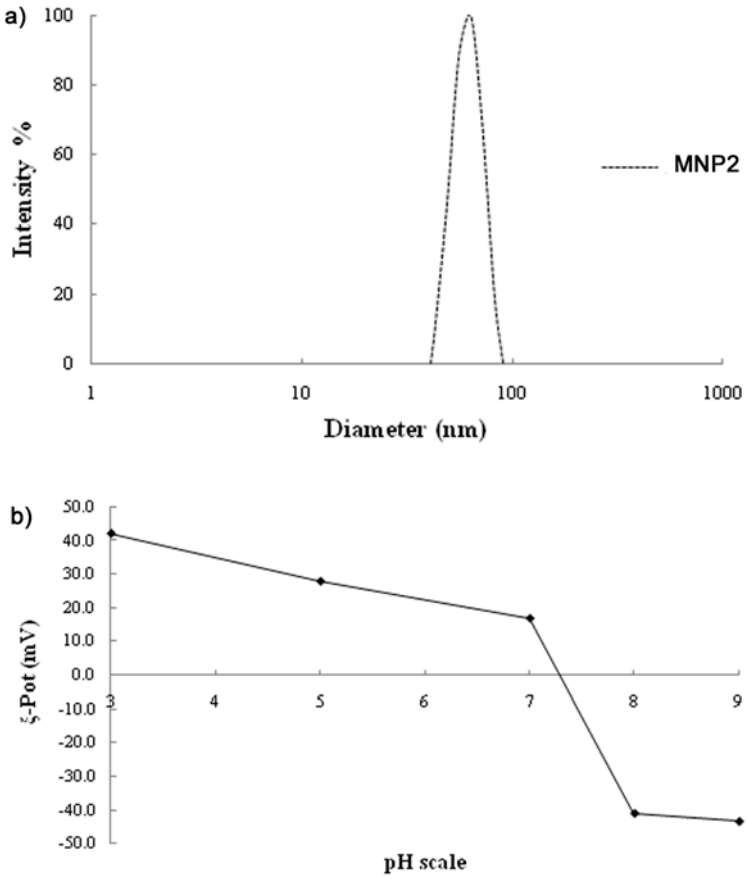

Fig. S3 a) Hydrodynamic size distribution histogram of MNP2 in pure water (the horizontal bare scale is semi-logarithmic). b) Behavior of ζ-Potential of MNP2 in pure water by varying the pH value in the 3 to 9 range. Acidic pHs were gained by dropwise adding 0.02 N HCl, alkaline pHs were obtained by adding 0.02 N NaOH.

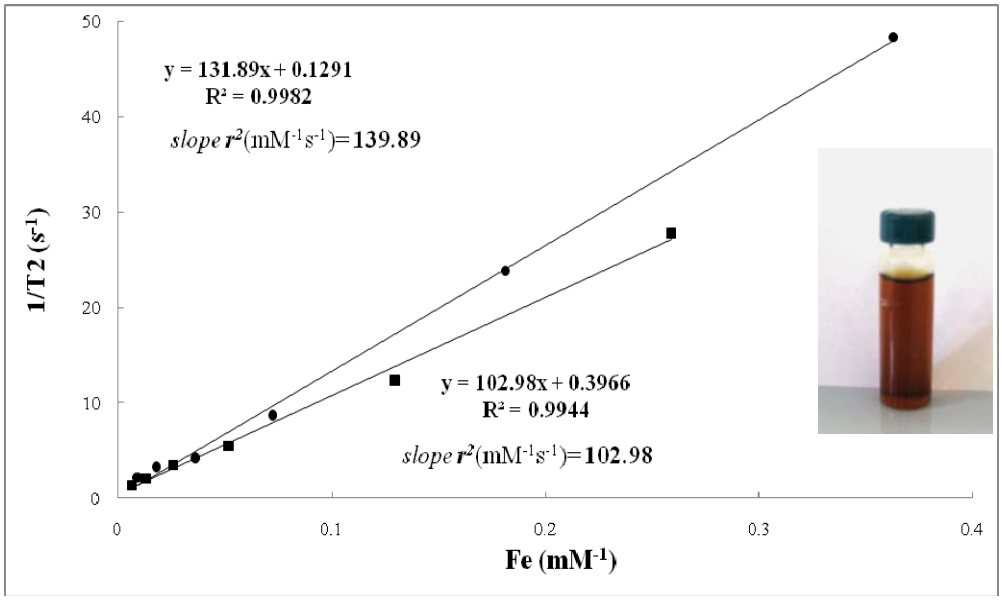

Fig. S4 T_2 relaxometry analyses of as-synthesized MNP2 (dots) and TMNP (squares) in phosphate buffer saline (PBS). The inverse of experimental T_2 values obtained at different MNP2 concentration were plotted vs. iron concentration. Experimental data were fitted by a line. Inset: photographic view of the MNP2 suspension at 1 mg mL^{-1} in pure water.

Dependence of MNP2 stability on pH: different vials with MNP2 suspension (200 μL) in double distilled water (1.8 mL) were prepared at different pH (C_{NPs} = 0.1 mg mL^{-1}). Initial pH value of **MNP2** was around 5. The acidic pH values were obtained adding 0.5 M HCl dropwise in a range of pH from 1 to 5. The alkalinity was raised above this value by adding 0.5 M NaOH dropwise in the range between 7 and 11. In this way, the stability of MNP2 was controlled by monitoring the decreasing of UV-vis spectra of the PEG absorption from 0 to 90 min (in the range of λ from 200 and 600 nm).

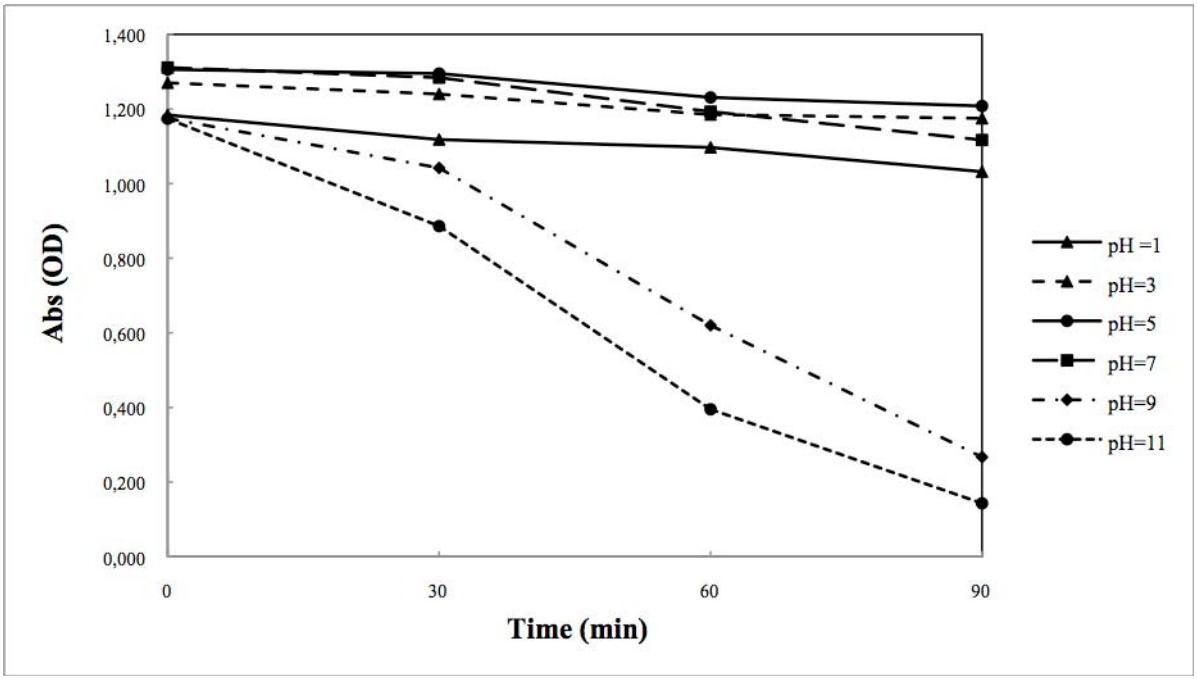

Fig. S5 Stability of Uv-Vis absorbance values at λ_{max} = 290 nm for MNP2 after 90 min incubation as a function of pH.

Table S1. Summary of the ΔAbs of MNP2 at λ_{max} = 290 nm after 90 min incubation.

	Initial Abs (λ = 290nm)	**Abs after 90 min (λ = 290nm)**	**Δ Abs**
pH=1	1.184	1.032	-0.152
pH=3	1.271	1.175	-0.096
pH=5	1.306	1.208	-0.098
pH=7	1.311	1.117	-0.194
pH=9	1.175	0.267	-0.908
pH=11	1.173	0.143	-1.030

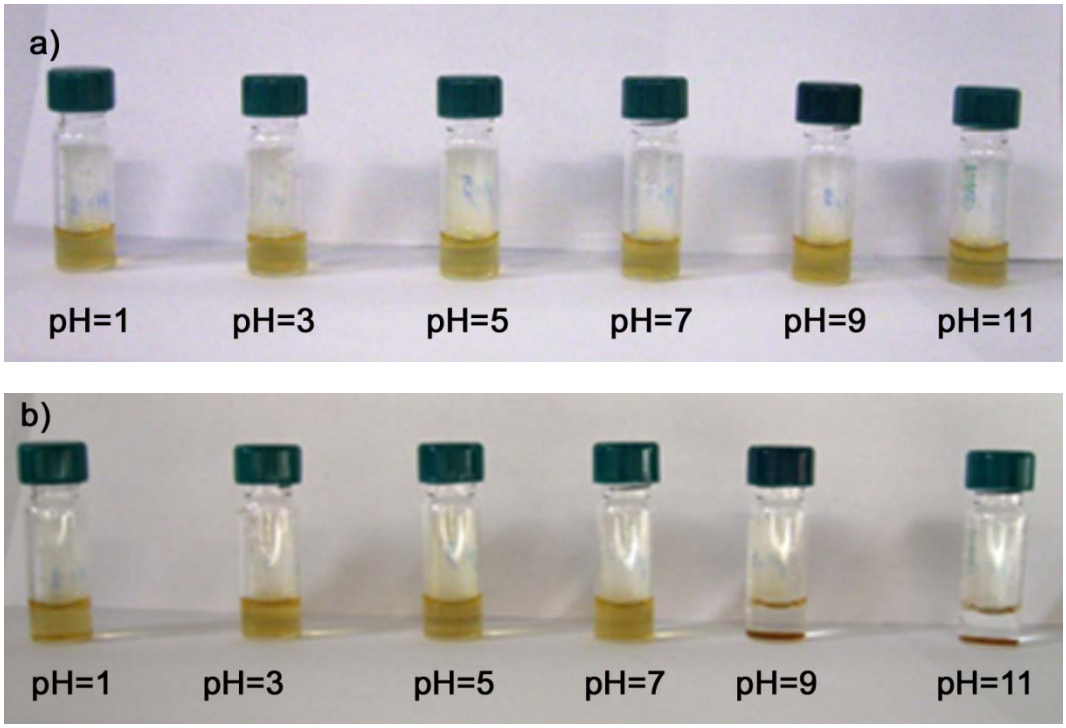

Fig. S6 MNP2 solutions (0.1 mg mL^{-1}) in deionized water at different pH values. a) Freshly prepared solutions and b) after 90 min.

Synthesis of TMNP

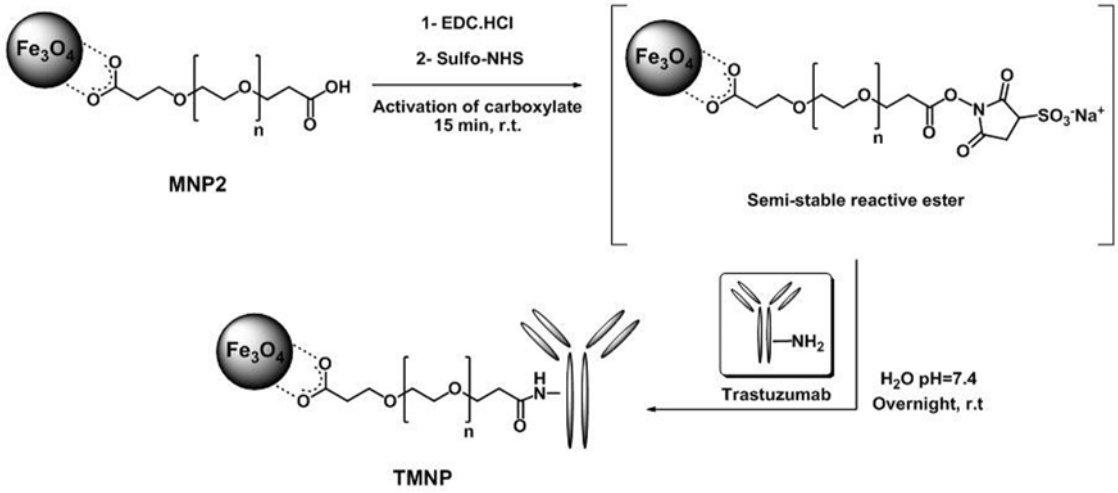

Fig. S7 Schematic synthesis of trastuzumab-modified pegylated nanoparticles (TMNP).

Amide coupling of MNP2 with TZ: The conjugation reaction was performed at room temperature as follows. EDC·HCl (1.9 mg, 10 μmol) and Sulfo-NHS (5.42 mg, 25 μmol) were dissolved in MNP2 water suspension (800 μg, 0.8 mg mL^{-1}, pH 5) under vigorously magnetic stirring. After the activation of the carboxylic acid (approximately 15 min), purified TZ (48 μL, 33.3 mg mL^{-1}) was

added to this solution. Next, the pH of the reaction system was adjusted to 7.4 using 1 M NaOH. The reaction lasted overnight at r.t under magnetic stirring. At the end of the reaction, the particulate was precipitated by centrifugation at 6000 rpm min^{-1} for 10 min and the supernatant was discarded; in order to remove the unbound antibodies, the resultant particulate was washed three times (3 × 1 mL) with phosphate buffered saline solution (PBS: 10 mM phosphate, 300 mM NaCl, 2.7 mM KCl, pH 7.4). Finally, the product was redispersed in the same PBS (1 mL) and stored at 4 °C before further experiments.

Determining TZ loading on nanoparticles surface: Fe_3O_4 nanoparticles were spherical in shape with an average radius of about 5 nm; the average volume of nanoparticles was 5.23 10^{-25} m^3 and the density of magnetite was 5 10^6 g m^{-3}; the mass of a single nanoparticle that is 2.615 10^{-18} g. Hence, 1 mg of Fe_3O_4 contains 3.824 10^{14} nanoparticles. The amount of antibodies appended on nanoparticles surface was determined by using a Bradford Protein Assay. According this method and the curve calibration standard elaborated at different concentation of the antibody, we estabilished that 217 μg of TZ were loaded on 1 mg of MNP2. In 217 μg of TZ (*MW*= 150 KDa) there are 8.71 10^{14} molecules of antibody for 1 mg of nanoparticles. In conclusion, 2 molecules of TZ per nanoparticle were statistically determined.

Protein assay onto nanoparticles: The functionalized nanoparticles were magnetically separated, washed with PBS to remove unbound antibodies and resuspended at the final concentrations of 1 mg mL^{-1}. The amount of antibody bound to magnetic nanoparticles was determined by using a Bradford Protein Assay. We used unreacted nanoparticles at the same concentrations as a blank. The concentration of antibodies needed to gain optimal signal from the surface-modified nanoparticles was determined to be 50 μg mL^{-1}.

Dynamic Light Scattering and zeta-potential measurements of TMNP: TMNP were dispersed in PBS buffer, pH 7.4, under sonication for 1 minute before analyses; the final sample concentration used for measurements was typically 0.01 mg mL^{-1}. For TMNP the average hydrodynamic radius was 224.9 nm (± 0.194). By ζ-potential analysis we found that TMNP were slightly negatively charged, $\zeta = -7.8 \pm 1.1$ mV.

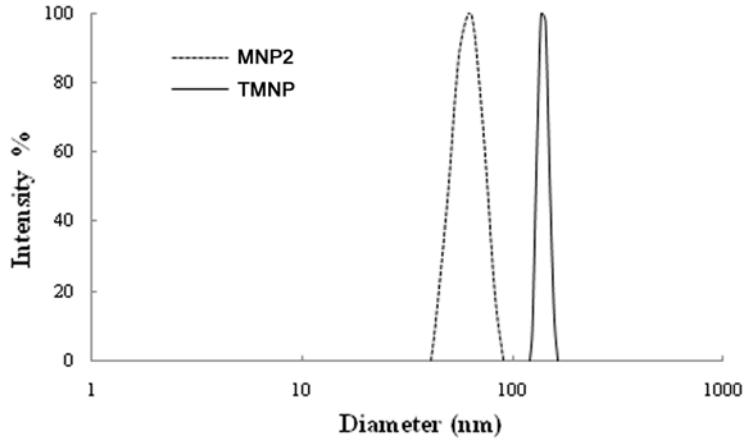

Fig. S8 Hydrodynamic size distribution histograms of MNP2 (dashed line) and TMNP (continuous line). As we expected, there was a great difference in diameter before the conjugation of the antibody (MNP2) and after the reaction (TMNP). However, the narrow peaks showed the high stability of the suspension in both cases, without formation of large clusters of nanoparticles.

Table S2. ζ-Potential behaviour of MNP2 and TMNP in physiological buffer. There was a great difference in the charge (11.7 mV) before the conjugation of the antibody and after the reaction.

	ζ-Potential (mV) (PBS buffer pH=7.4)	**Δ mV**
MNP2	-19.5 ± 0.5	11.7
TMNP	-7.8 ± 1.1	

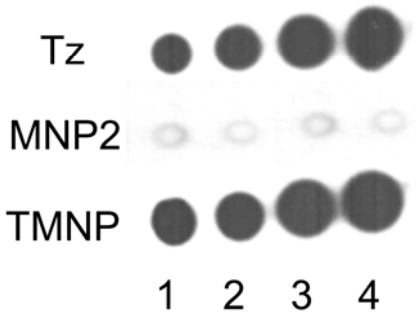

Fig. S9 Immunodot blot assays carried out in parallel with increasing amount (1, 50 ng; 2, 100 ng; 3, 200 ng; 4, 300 ng) of soluble and immobilized (TMNP) trastuzumab. Pegylated nanoparticles (MNP2) were the negative control. TMNP were incubated with anti-human antibodies conjugated with horseradish peroxidase (HRP-rabbit) at a 1:1000 dilution in PBS buffer. The immunoreaction was revealed by a strong signal using ECL western blotting as reagent.

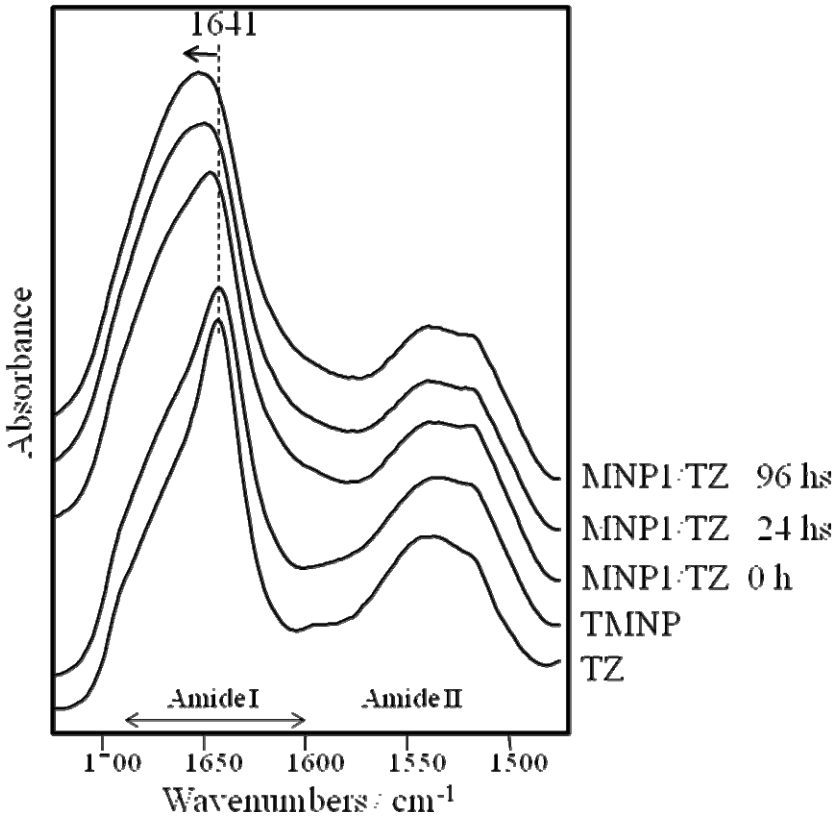

Fig. S10 FTIR absorption spectra of free TZ, of TMNP, and of TZ adsorbed to MNP1 at different times of incubation at 37 °C (0, 24, and 96 hours). The maximum of the Amide I band shifts from 1641 cm^{-1} in the free TZ to 1650 cm^{-1} in the adsorbed TZ at 96 hours of incubation, consistent with a gradual unfolding of protein secondary structure.

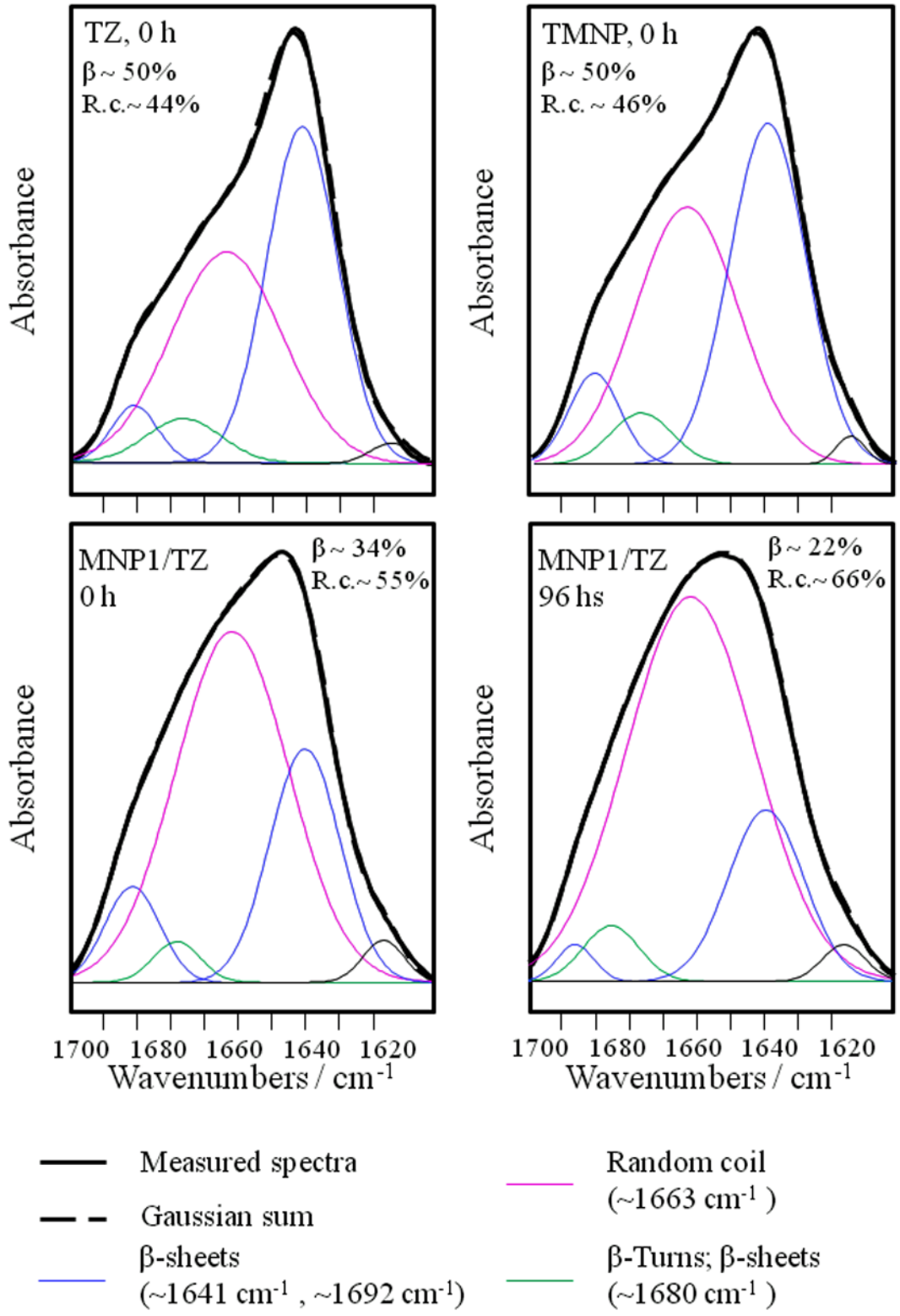

Fig. S11 Gaussian curve fitting of the absorption spectra in the Amide I region of free TZ, of TMNP, and of TZ adsorbed to MNP1 immediately after preparation and after 96 h of incubation at 37 °C. The β-sheet components are reported in blue and their percentage weights over the total Amide I band are indicated. The component peaked at ~1663 cm^{-1} (reported in pink) is mainly due to random coil structures (R.c.) with possible contribution of β-turns.[2] The curve fitting of the measured spectra into Gaussian function was performed following the method previously described.[2,3]

Table S3. Relaxivity measurements on labeled MCF7 cells.[a]

Concentration (cells/mL)	T_2 (ms) (untreated)	T_2 (ms) (TMNP)[b]
1.0×10^6	2809 ± 34	294 ± 9
5.0×10^5	3185 ± 31	712 ± 13

[a] Data are the mean ± SD of three different relaxivity measurements. [b] Cells were treated with TMNP at a concentration of 50 μg mL^{-1}. The remarkable decrease in T_2 confirms the presence of TMNP on cells, thus demonstrating that fluorescently labeled antibodies localized on cell membrane (see Fig. 3) are still bound to magnetic nanoparticles.

Dot Blot Assay: Standard Minifold I dot blot apparatus were used in this assay. TZ or purified nanoparticle suspension were spotted onto PVDF membrane. After all samples were fixed the membrane was incubated in blocking solution (5% skim milk in PBS) for 60 min at RT. To evaluate TZ activity, membranes were then probed for 60 min at 25 °C using horseradish peroxidase-rabbit anti-human antibodies at a 1:20000 dilution in PBS buffer. Immunoreactive spots were revealed using ECL Western blotting reagent.

Cell cultures: MCF-7 cells were grown in 50% Dulbecco's Modified Eagle's Medium (DMEM) and 50% F12 (EuroClone Celbio, Milan, Italy), supplemented with 10% fetal bovine serum (HycloneCelbio, Milan, Italy), L-glutamine (2 mM), penicillin (50 UI mL^{-1}), and streptomycin (50 mg mL^{-1}, culture medium) at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured prior to confluence using trypsin/EDTA.

Immunoprecipitation assay: MCF7 cells were lysed with lysis buffer containing 20 mM Tris HCl, 150 mM NaCl, 10 mM EGTA, 10% glycerol and 1% Triton X-100, pH 7.4 for 30 min at 4 °C and then centrifuged at 8500 rpm for 10 min to remove membranes and cell debris from protein fraction. Bradford assay was used to determine protein content of whole cell extract. Preclearing step was performed with 200 μL of MNP2 (1 mg mL^{-1}) for 1 h on wheel at 4 °C. The immunoprecipitation was carried out with 1 mg of pre-cleared lysate and TMNP (200 μL, 1 mg mL^{-1}, corresponding to 3.75 μg of linked-antibody) for 16 h at 4 °C on wheel. Samples were washed three times in lysis buffer and boiled in sodium dodecyl sulfate sample buffer containing 5% 2-mercaptoethanol to cleave the binding to the beads. Samples were run on 8% poliacrilammide gel. Proteins were blotted onto PVDF membranes and incubated in blocking solution (5% skim milk in TBS, 0.05% Tween) for 1 h at r.t.. For HER-2 detection, membranes were then probed for 1 h at r.t. using anti-HER2 polyclonal antibodies (1:500 Millipore, Billerica, MA) in blocking solution. Specificity of the interaction between the HER2 and TMNP was verified with immunoblot

of immune-precipitated samples with unrelated proteins such as anti-Clnx pAb (1:500 Genetex) at r.t. for 1 h in TBS + 0.05% tween + 5% BSA. Specific HRP-conjugated secondary-antibodies were used always 1 h at r.t. (anti-mouse 1:10000 BioRad Cell Signaling, Danvers, MA). Immunoreactive bands were revealed using ECL Western blotting reagent (GE Healthcare).

Confocal Laser Scanning Microscopy: MCF7 cells were cultured on collagene (Sigma) pre-coated coverglass slides until 90% of confluence and incubated for 20 min with TMNP at a concentration of 100 μg mL^{-1} (corresponding to 11 μg of Tz) or 11 μg Tz in culture medium. Tz and TMNP were revealed by labeling with secondary antibodies anti-human Alexa fluor 488 (Invitrogen, Carlsbad, CA) at a 1:500 dilution. Cultures were washed with PBS, fixed for 10 min with 4% paraformaldehyde (Sigma) and then treated for 10 min with 0.1 M glycine (Sigma) in PBS. A blocking step was performed 1 h at RT with a solution containing 5% BSA (Sigma) and 0.1% Saponin (Sigma) in PBS, followed by staining with 33 μg of DiD oil (Invitrogen) in PBS for 30 min at 37 °C. Microscopy analysis was performed with a Leica SP2 AOBs microscope confocal system. Images were acquired with 63× magnification oil immersion lenses at 1024×1024 pixel resolution.

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[3] A. Natalello, D. Ami, S. Brocca, M. Lotti and S. M. Doglia, *Biochem. J.*, 2005, **385**, 511-517.