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SUPPLEMENTARY INFORMATION

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Supplementary Information

Bioorthogonal chemistry amplifies nanoparticle binding and enhances the sensitivity of cell detection

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Methods

Synthesis of magnetic nanoparticles (CLIO). Cross-linked iron oxide (CLIO) nanoparticles were prepared as described elsewhere[1]. Briefly, 3 nm monocrystalline cores composed of $(Fe_2O_3)_n(Fe_3O_4)_m$ were covered with a layer of 10 kDa dextran, which was cross-linked with epichlorohydrin and aminated to produce CLIO with primary amine groups (amino-CLIO). The number of amines per CLIO was approximately 89 primary amines, as determined by sequential reaction with *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Thermo Fisher) and dithiothreitol to liberate pyridine-2-thione[2]. The hydrodynamic diameter was 28.8 nm as measured by dynamic light scattering (Zetasizer 1000HS; Malvern Instruments). The r_1 and r_2 relaxivities were 25.2 mM^{-1} s^{-1} and 64.5 mM^{-1} s^{-1}, respectively, at 40°C and 0.47 T (Minispec MQ20; Bruker).

Preparation of amine-reactive tetrazine (Tz-NHS). 2,5-dioxopyrrolidin-1-yl 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoate (Tz-NHS) was prepared from 3-(4-benzylamino)-1,2,4,5-tetrazine (Tz-benzylamine) that was synthesised as previously described[3]. Tz-benzylamine (10 mg) was added to a solution of methylene chloride containing 6 mg glutaric

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anhydride and reacted overnight at 50°C while stirring. The methylene chloride was removed by rotary evaporation and the crude mixture purified by column chromatography resulting in 5-(4-(1,2,4,5-tetrazin-3-yl)benzylamino)-5-oxopentanoic acid (Tz-acid) in quantitative yield. This acid was then immediately introduced to an acetonitrile (2 mL) solution of N, N'-disuccinimidyl carbonate (68 mg) and triethylamine (30 mg) and allowed to stir at room temperature until the reaction reached completion (monitored by TLC). The acetonitrile was removed by rotary evaporation and the crude mixture purified by column chromatography yielding 17 mg (80% yield) of the desired amine-reactive Tz (Tz-NHS). ^{1}HNMR (400 MHz $CDCl_3$): δ 10.3-10.2 (s, 1H), 8.7-8.5 (d, 2H), 7.6-7.4 (d, 2H), 6.6-6.2 (br, 1H), 4.7-4.4 (m, 2H), 3.1-2 (m, 10H). LR-MS [M+H]+ calc mass 399.1 found mass 399.2.

MALDI-TOF mass spectrometry for determination of TCO and biotin valencies on antibodies. Sinapinic acid (Thermo Fisher) was used as the MALDI matrix, and was prepared by dissolving 1 mg in 0.1 mL 70/30 acetonitrile/water with 0.1% trifluoroacetic acid. Unmodified, TCO-modified, and biotinylated antibodies were diluted to 150 µg/mL with PBS, combined at a 2:1 ratio with sinapinic acid solution, and 1 µL was dried onto a MALDI sample plate. The sample plate was loaded into a Voyager-DE BioSpectrometry Workstation (Applied Biosystems) MALDI-TOF mass spectrometer, and 500 shots were obtained per spectrum. The anti-EpCAM and TCO-modified anti-EGFR antibodies did not ionise well, requiring increased laser power that resulted in higher background levels. Data was acquired using Voyager Control Panel software, and were then exported to a custom MatLab program (MathWorks) for analysis. All measurements were performed in triplicate, and the average molecular weight for each replicate was averaged. The number of TCO and biotin modifications per antibody was

then calculated based on the difference in molecular weight compared to the unmodified antibody and assuming 152.2 and 226.1 dalton net mass added, respectively. The data are presented in Supplementary Figs. S1 and S2. The degree of TCO modification varied somewhat for the different antibodies despite identical reaction conditions. This effect was also observed for biotinylation, and was most likely caused by differences in the number or availability of modification sites (primary amines).

Measurement of functional TCO loadings by reacting with a Tz conjugated fluorophore. Unmodified and TCO loaded Herceptin (0.5 mg/mL) was reacted with an excess (100 μM) of near-IR Tz probe (Tz-VT680, synthesised as previously reported[3] at room temperature for 6 hours. After reaction, the antibody was purified by centrifuge filtration using 50,000 dalton molecular weight cutoff filters (Amicon) and stored in PBS. The number of fluorochromes per antibody (which corresponds to the number of functional TCO sites) was determined by spectrophotometric analysis, and are indicated in Supplementary Fig. S3.

Measurement of functional biotin loadings using HABA assay. Functional biotin valency on the anti-HER2 and anti-EpCAM antibodies was determined using the HABA assay (Thermo Fisher). HABA reagent (20 μL) was combined with antibody (180 μL at 10 μg/mL) in a 96 well plate (in triplicate), and reacted for 1 hour at room temperature. Biotin concentration was then determined based on the absorbance at 500 nm (Safire 2, Tecan), extinction coefficient of 34,000 M^{-1} cm^{-1}, and 0.5 cm path length. Biotin valencies (molecules/antibody) were calculated based on the ratio of molar concentrations, and are indicated in Supplementary Fig. S3.

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Direct conjugation of MFNP. MFNP immuno-conjugates were prepared using two different coupling chemistries, the Tz/TCO cycloaddition (BOND-1) and maleimide/thiol reaction. For BOND-1, antibodies were modified with 10 equivalents of TCO-NHS as described in the main text. The TCO-antibodies were then reacted with 0.25 mg Tz-MFNP for 3 hours at room temperature. For maleimide/thiol conjugations, antibody fragments were produced via disulfide reduction and attached to maleimide-functionalised nanoparticles, as described elsewhere[4]. Antibody reduction was performed using cysteamine, a mild reducing agent that selectively cleaves the disulfide bond connecting the IgG heavy chains[5]. Briefly, cysteamine and ethylenediamine tetraacetic acid (EDTA, Thermo Fisher) were added to antibody samples at final concentrations of 25 and 10 mM, respectively, and incubated at 37°C for 4 hours. The reduced antibodies were then purified using 2 mL Zeba columns. Maleimide-MFNP was prepared by reacting 1 mg amino-MFNP with 1 mg sulfosuccinimidyl-(4-*N*-maleimidom-ethyl)cyclohexane-1-carboxylate (sulfo-SMCC, Thermo Fisher) in PBS for 3 hours at room temperature. Excess sulfo-SMCC was removed using Sephadex G-50, and the maleimide-MFNP was reacted with reduced antibody for 3 hours at room temperature. For both BOND-1 and maleimide/thiol cases, the immuno-conjugates were purified using Sephadex G-1000 (GE Healthcare) and the MFNP concentration was determined by absorbance measurement at 410 nm using the amino-MFNP stock sample for calibration. Avidin-MFNP was generated using the BOND-1 coupling technique to attach Neutravidin protein (Thermo Fisher) in a similar fashion.

Measurement of antibody and avidin valencies on MFNP. Protein concentration was measured for the antibody-MFNP and avidin-MFNP conjugates using the Micro BCA assay (Thermo Fisher). BCA reagent (200 µL) was combined with MFNP (200 µL at 10 nM) in a 96

well plate (in triplicate), and reacted for 2 hours at 37°C. The samples were then cooled to room temperature and absorbance was measured at 564 nm (Safire 2, Tecan). Absorbance was converted to protein concentration using calibration curves prepared with stock solutions of the three antibodies and Neutravidin. The number of antibodies attached per MFNP using the two coupling techniques (BOND-1; maleimide/thiol) were as follows: anti-HER2 (3.1 ± 0.6; 3.5 ± 0.4), anti-EpCAM (2.0 ± 0.5; 3.2 ± 0.5), and anti-EGFR (2.3 ± 0.2; 2.7 ± 0.2). The number of Neutravidin proteins attached per MFNP was 8.4 ± 0.7.

Kinetic analysis of TCO-modified anti-HER2 antibodies. SK-BR-3 cells (250,000) were incubated for 1 hour in PBS^+ with unmodified and TCO-loaded anti-HER2 antibody at concentrations ranging from 0.07 to 200 nM. Samples were then washed twice by centrifugation with ice-cold PBS^+ and resuspended with a saturating concentration (5 µg/mL) of FITC-conjugated anti-human IgG_1 polyclonal antibody. Following incubation for 1 hour on ice, samples were washed three times with PBS^+ by centrifugation and the fluorescence signal was quantified by flow cytometry using an LSRII flow cytometer (Becton Dickinson).

Magnetic resonance measurements. Human tumor cell lines (SK-BR-3, HCT 116, A549, A431, NCI-H1650, and SK-OV-3) and control NIH/3T3 cells were maintained as described in the main text. Blood leukocytes were obtained from fresh peripheral blood by venipuncture into EDTA-coated VacuTainer collection tubes (BD Biosciences). Red blood cells were lysed using PharmLyse (BD Biosciences) and the remaining leukocytes were washed twice with PBS+ by centrifugation. In all cases, cells were labelled with 10 µg/mL TCO-antibody (control; anti-HER2, EpCAM, EGFR, Mucin1, or CD45) and 100 nM MFNP, washed twice with PBS+ to

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remove unbound MFNP, and counted using a hemacytometer. Transverse relaxation rate (R_2) values were measured using the miniaturised nuclear magnetic resonance system previously reported[6]. The sample volume per measurement was 1 µl, and contained approximately 1,000 cells (or as indicated in Supplementary Fig. S2). We used Carr-Purcell-Meiboom-Gill pulse sequences with the following parameters: echo time (TE): 4 ms; repetition time (TR): 6 s; the number of 180° pulses per scan: 500; the number of scans: 8. All measurements were performed in triplicate and the data are presented as mean ± standard error. The R_2 values were converted to the marker expression level per cell as described elsewhere[6]. This involved subtracting the R_2 value for unlabelled cells to obtain ΔR_2, and dividing the marker specific ΔR_2 (ΔR_2^+) by the control (ΔR_2^θ).

Magnetic Resonance Imaging. SK-BR-3 and control NIH/3T3 cells (10^6 each) were labelled using each of the targeting strategies with 100 nM MFNP as described for flow cytometry and diagnostic magnetic resonance experiments, washed twice with PBS+ to remove unbound MFNP, and counted using a hemacytometer. The labelled cells were then lysed and embedded in agar before imaging on a Bruker Pharmascan MR scanner at 4.7T.

Measurement of biomarker expression levels on cells. The expression level of HER2, EpCAM, EGFR, Mucin1, and CD45 on tumor cells (SK-BR-3, HCT 116, A549, A431, H1650, and SK-OV-3) and control cells (NIH/3T3 and peripheral blood leukocytes) was measured by flow cytometry using fluorescent antibody staining. 10^6 cells per sample were incubated in 0.1 ml PBS+ containing 1 µg/mL primary antibody for 15 minutes at room temperature. The primary antibodies employed were unmodified forms of the antibodies used for targeting

MNFP, with the exception of HER2 and EGFR. Mouse antibodies (HER2: clone 191924, R&D Systems; EGFR: clone LA1, Millipore) were used to target these markers for calibration purposes rather than the humanised (trastuzumab) or chimeric (cetuximab) antibodies. Following a centrifugal wash with ice-cold PBS+, appropriate fluorescein-conjugated secondary antibodies were added at 1 μg/mL and incubated for 30 minutes on ice. Cells were then washed twice with ice-cold PBS+ and analyzed on a LSRII flow cytometer. Fluorescence intensities were converted to markers per cell using Quantum Simply Cellular anti-mouse antibody beads (Bangs Labs), which were similarly incubated with each primary and secondary antibody pair and analyzed by flow cytometry to determine fluorescence levels for a known number of binding sites. Expression levels are listed in Supplementary Table S1.

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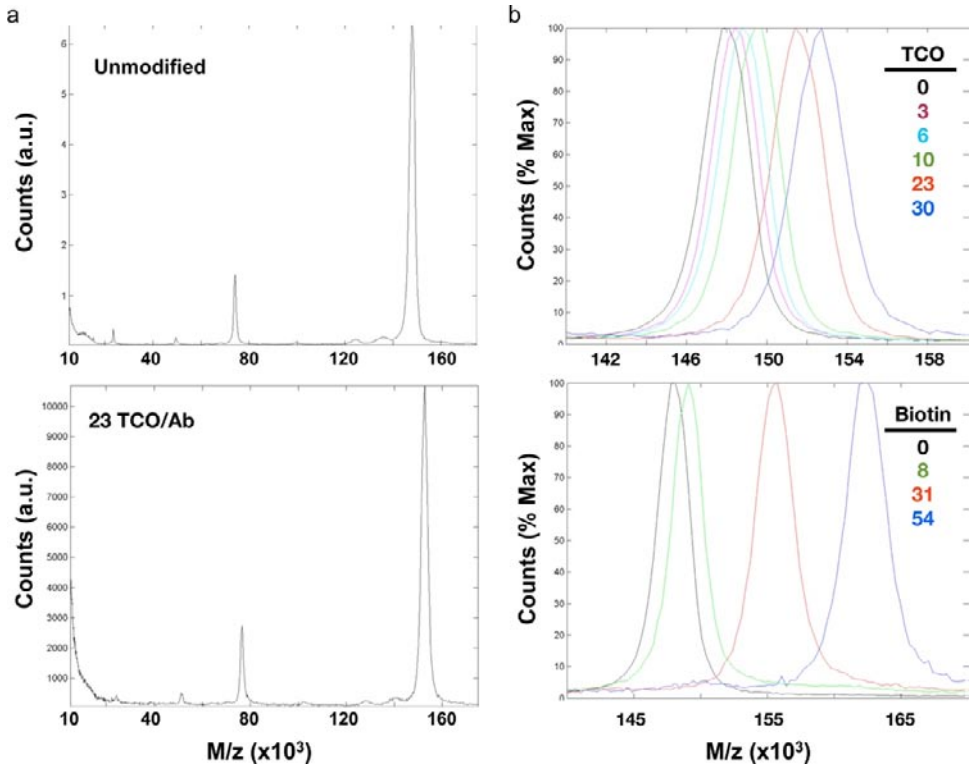

Figure S1: Mass spectrometry to quantitate antibody modifications with TCO or biotin for anti-HER2 antibody. TCO and biotin loadings on the anti-HER2 antibody were determined by MALDI-TOF mass spectrometry based on changes in molecular weight. a, Representative molecular weight/ioniziation (M/z) histograms for the unmodified anti-HER2 antibody, and following reaction with 1000-fold excess of TCO-NHS. The singly ionised peak (z=1) has an average molecular weight of ~148,000 dalton for the unmodified antibody, with a doubly ionised peak (z=2) also detectable at ~75,000 dalton. The approximately 3500 dalton change in molecular weight with addition of the TCO corresponds to ~23 TCO/antibody based on 152.2 dalton mass added. b, Merged data for TCO and biotin modifications to the anti-HER2 antibody. Biotin valencies were determined based on 226.1 dalton mass added. Average molecular weight and data merges were performed using MatLab.

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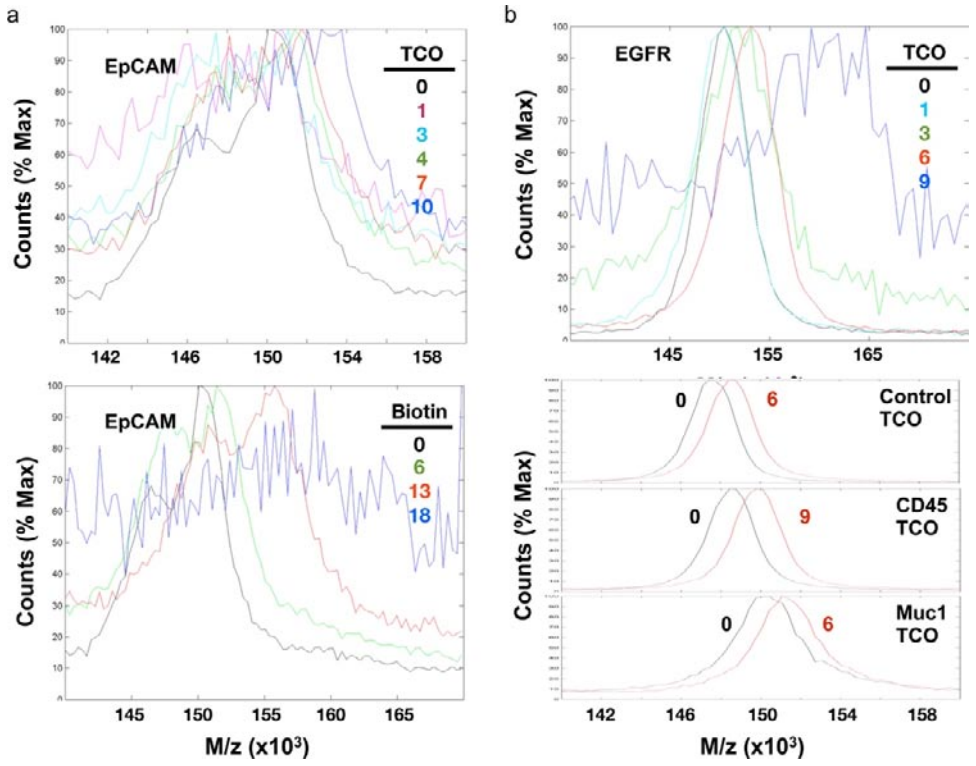

Figure S2: Mass spectrometry to quantitate antibody modifications with TCO or biotin for other antibodies. A similar analysis to Supplementary Fig. S1 was perfomed for the a, TCO and biotin modifications of the anti-EpCAM antibody and b, TCO modification of the anti-EGFR, control, anti-CD45, and anti-Mucin1 antibodies. The anti-EpCAM antibody and -EGFR antibody (at high TCO loadings) did not ionise well, resulting in histograms with significantly more noise.

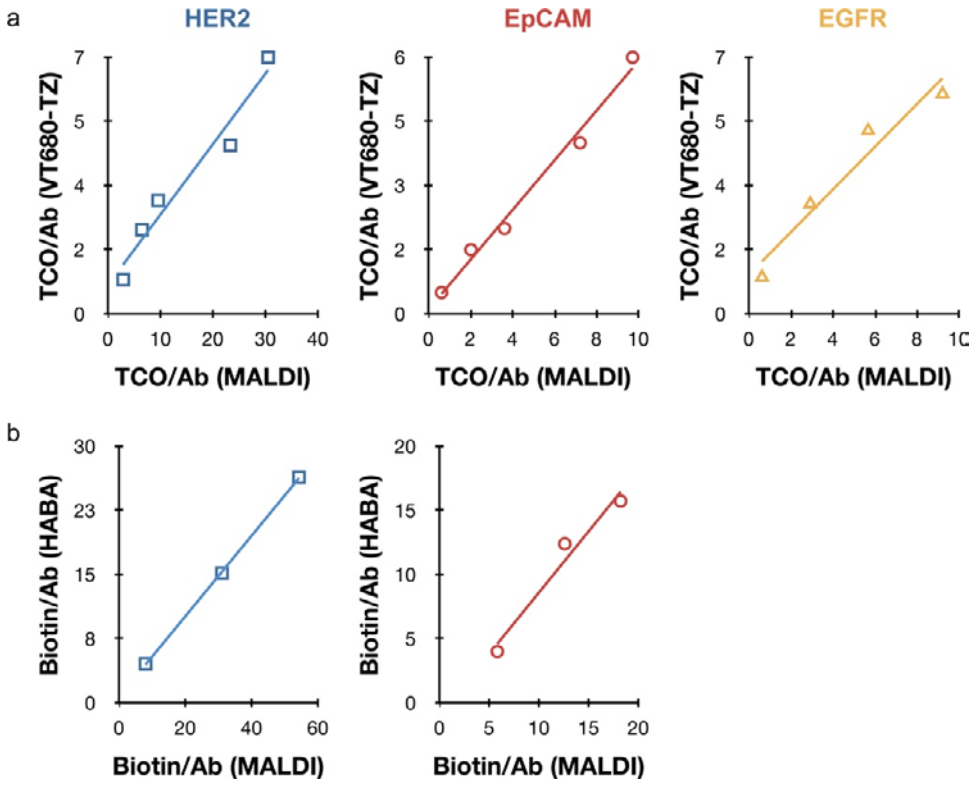

Figure S3: Correlation of TCO and biotin valencies between the MALDI-MS measurements and functional assays. a, TCO valencies correlated well between mass measurements and functional assays performed using at Tz-fluorophore (VT680) for each of the antibodies tested. However, the functional values were considerably lower than the mass values, presumably because certain TCO sites could not be accessed or due to physical interaction between the large, highly-charged VT680 molecules. b, Similar results were obtained for biotin valencies, this time where the functional metric was avidin binding using the HABA assay. TCO and biotin valencies used throughout the text reflect the MALDI-MS results so that the TCO and biotin species can be compared directly.

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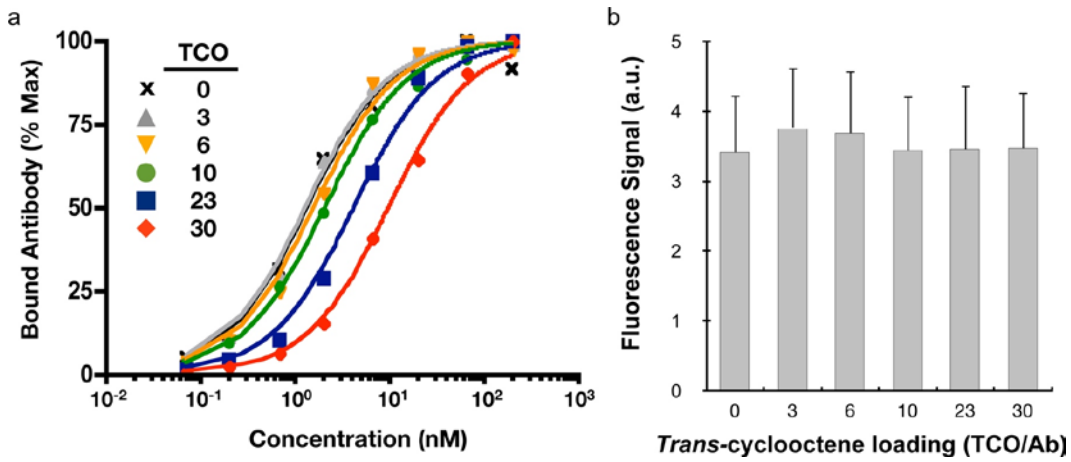

Figure S4: Effect of TCO loading on antibody affinity and background nanoparticle adhesion. a, Modification of the anti-HER2 antibody with TCO had negligible effect on binding affinity at low loadings, consistently exhibiting a K_D = ~1-2 nM up to approximately 10 TCO/Ab. Affinity decreased by approximately 3-fold at 23 TCO/Ab (K_D = ~4 nM) and 10-fold at 30 TCO/Ab (K_D = ~10 nM). These results also indicate that antibody binding is saturated at the concentration employed for cell labelling studies (10 μg/mL, or ~67 nM). b, Modification of the anti-HER2 antibody with TCO did not affect the level of MFNP binding to control NIH/3T3 fibroblast cells under the BOND-2 scheme. This result demonstrates that the TCO does not make the antibody more “sticky” to cells, even at high TCO loadings, and further confirms the specificity of the BOND-2 technique. Fluorescence values are comparable to controls for HER2-positive SK-BR-3 cells (see Fig. 2).

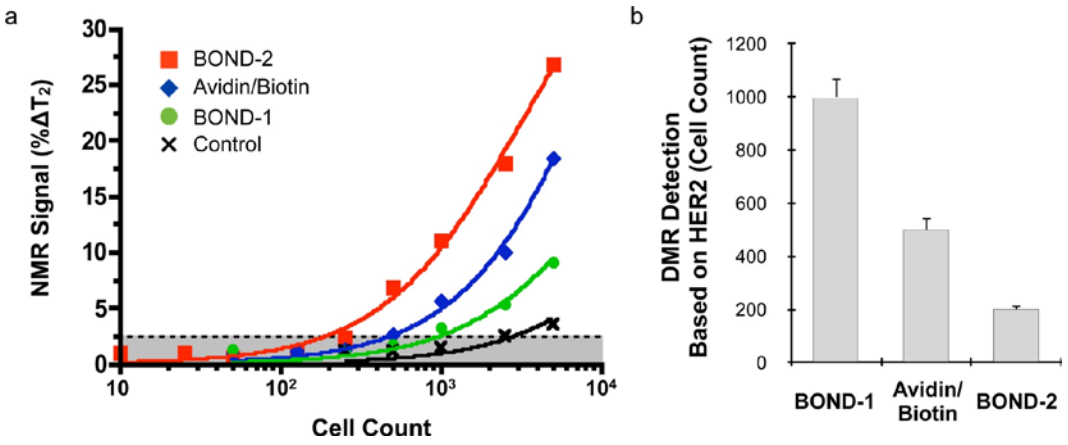

Figure S5: Diagnostic magnetic resonance detection of the different nanoparticle targeting strategies. (a) Magnetic resonance detection sensitivity for cancer cells based on HER2. BOND-2 outperformed the other approaches, with a detection threshold of approximately 200 cells in comparison to > 1000 cells for direct immuno-conjugates. (b) Detection thresholds for each technique, which was determined based on the number of cells required to register a 2.5% change in transverse relaxation time (T_2). Note that using more magnetic nanoparticles can significantly improve detection thresholds to near single cell levels[6].

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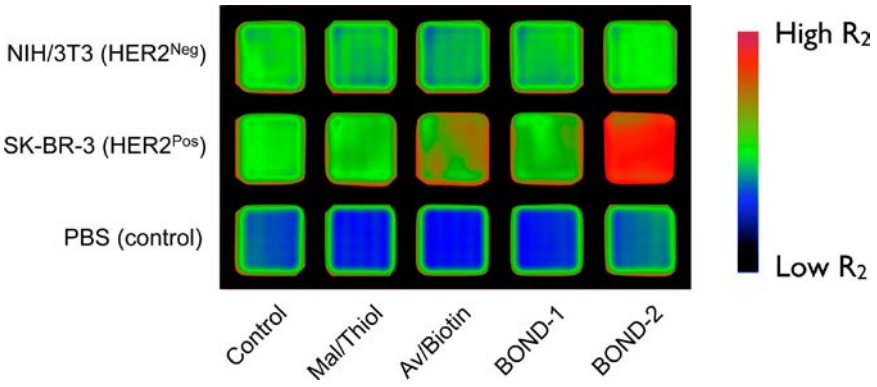

Figure S6: Magnetic resonance imaging of the different nanoparticle targeting strategies. Magnetic resonance imaging of targeted cells. Cancer (SK-BR-3) and normal (NIH/3T3) cells were labelled using the different targeting strategies and imaged by a magnetic resonance scanner (4.7 T; Bruker Pharmascan). Consistent with fluorescence and diagnostic magnetic resonance measurements, BOND-2 yielded the most pronounced T_2 changes due to higher nanoparticle binding. Prior to imaging, labelled cells (10^6) were lysed and embedded in agar.

Supplementary Table S1: Biomarker expression levels for the cell lines used for magnetic resonance profiling (Fig. 4). The number of biomarkers per cell was determined by flow cytometry using fluorescent antibody staining and calibration beads (see Supplementary Methods).

	EpCAM	HER2	EGFR	Mucin1	CD45
Fibroblast	ND*	ND	ND	ND	ND
Leukocyte	ND	ND	ND	ND	**1.60 ± 0.04 x10^6**
A431	**0.67 ± 0.02 x10^6**	0.02 ± 0.01 x10^6	**1.23 ± 0.03 x10^6**	0.05 ± 0.02 x10^6	ND
A549	0.01 ± 0.01 x10^6	ND	**0.40 ± 0.01 x10^6**	0.02 ± 0.01 x10^6	ND
NCI-H1650	**1.270 ± 0.03 x10^6**	0.05 ± 0.01 x10^6	0.10 ± 0.01 x10^6	**3.45 ± 0.01 x10^6**	ND
HCT 116	**3.43 ± 0.02 x10^6**	ND	0.08 ± 0.01 x10^6	ND	ND
SK-BR-3	**1.32 ± 0.03 x10^6**	**2.00 ± 0.07 x10^6**	0.08 ± 0.01 x106	0.09 ± 0.01 x10^6	ND
SK-OV-3	**1.03 ± 0.02 x10^6**	**2.60 ± 0.01 x10^6**	0.13 ± 0.01 x10^6	**1.80 ± 0.05 x10^6**	ND

*ND: Not detected, or < 10,000 markers/cell