Supporting Information

Functionalization of clinically approved MRI contrast agents for the delivery of VEGF

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1. Materials and methods

N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC, BioXtra purity grade) and N-hydroxysuccinimide (NHS, 98% purity grade) used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany) and used without further purification. Gibco^{TM} Dulbecco's Phosphate buffered Salines (DPBS, no calcium or magnesium) was used from ThermoFisher Scientific (Germany). Mouse VEGF 165 recombinant protein was purchased from (Thermo Fischer, USA). Rienso (Ferumoxytol) was bought from Takeda Pharmaceutical (Tokio, Japan). Amicon centrifuge filters (cut-off 30 kDa) were purcharsed from Merck (Germany). Basal HUVEC medium (EBM-2) and medium supplements (EGM-2 SingleQuot Kit) were purchased from Lonza Walkersville Inc. (Walkersville, Maryland, USA).

TEM analyses were performed using an EM-410 transmission electron microscope (Philips, Eindhoven, Netherlands) at 60 kV. Electron micrographs were taken using DITABISTM imaging plate technology with a pixel size of 2250x2000 (80x90 mm effective area size). The samples were prepared on TEM grids (Plano GmbH, Wetzlar, Germany) with a carbon film (thickness ~5-10 nm) on a cupper grid. The software ImageJ (National Institutes of Health, USA, version 1.39u, Java 1.6.0_02) was used to analyze the nanoparticles.

For DLS measurements the Zetasizer Nano ZS (Malvern Instruments Ltd., Worcestershire, UK) was used with 1.5 ml low-volume disposable PMMA cuvettes for DLS measurements (Brand GmbH & Co KG, Wertheim). The measurements were performed at a constant temperature of 25 °C. Data analysis was carried out using OriginPro 2017G (64-bit) b9.4.0.220 (OriginLab Corporation, Northampton, USA).

IR spectra were recorded by use of a Varian Type 310 Fourier transformation IR spectrometer. The background was subtracted and the spectra were characterized using the software Resolution Pro.

X-ray photoelectron spectra of nanoparticle samples immobilized onto silicon substrates were recorded with a Kratos Axis Ultra (Kratos Analytical Ltd, UK). A monochromatic Al Kα source (hv = 1486.6 eV) at 10 mA filament current and 12 kV filament voltage source energies was used. The pass energy was set to 20 eV for high resolution and to 160 eV for survey scans. The obtained data were analyzed with CasaXPS Software Suite v2.3.15. All spectra were calibrated to the binding energy of the C-1s-orbital in aliphatic carbon-carbon chains (285 eV).

Cell growth was documented with the help of an inverted microscope Eclipse TE2000 from Nikon Instruments (Nikon Instruments, The Netherlands).

T2*-mapping was performed on a clinical 1.5 T MRI (Philips Ingenia, Best, The Netherlands). Sequence acquisition parameters were set to: Fast Field Echo, TR = 151 ms, TE = 1.54 ms, N(TE) = 60, voxel size 1x1x10 mm^3. Subsequent regression analysis for a pixelwise T2*-quantification was performed with Matlab (The MathWorks Inc., Natick, Massachusetts, USA).

2. VEGF-functionalized ferumoxytol nanoparticles

2.1 Synthesis

A solution of ferumoxytol nanoparticles (3.5 mg) in PBS buffer (3 ml, pH 7.4) was incubated with EDC (1.5 ml, 1 mg/ml EDC in PBS, pH 7.4) and NHS (1.5 mL, 1 mg/mL NHS in PBS, pH 7.4) at room temperature while agitating for 5 h. To remove unreacted EDC and NHS, the nanoparticles were centrifuged through a centrifuge filter (7500 rcf, 10 min, 4°C) and washed three times with PBS buffer (1.0 ml, pH 7.4). The activated nanoparticles were in incubated with 50 µl of VEGF (5 µg) for 2 h at room temperature and stored overnight at 4°C. The resulting VEGF-functionalized nanoparticles were centrifuged through a centrifuge filter (7500 rcf, 10 min, 4°C) and washed three times with PBS buffer (1 ml, pH 7.4). Finally, the conjugated nanoparticles were dissolved in 1 ml buffer solution.

The ferric concentration of functionalized ferumoxytol was assessed with spectrophotometric methods [1]. In brief, UV/vis absorbance in a serial dilution of 1-100 µg non-functionalized ferumoxytol was measured (U-3010, Hitachi, Japan). In line with the published protocol by Dadashzadeh et al. the optimal wavelength of the standard curve, related absorbance and ferric content, was found to be 370 nm. Thus, in order to determine the unknown iron concentration after functionalization, the absorbance was measured at 370 nm.

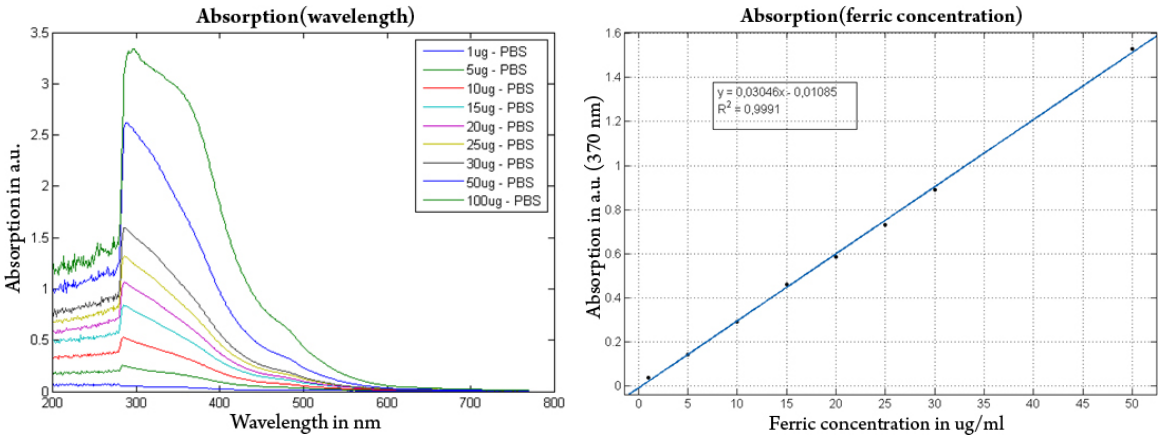


Figure S1. Absorption measurements in UV-vis spectra (left) and respective absorption at 370 nm in relation to the ferric concentration (right).

2.2 Nanoparticle characterization

2.2.1 Dynamic light scattering (DLS)

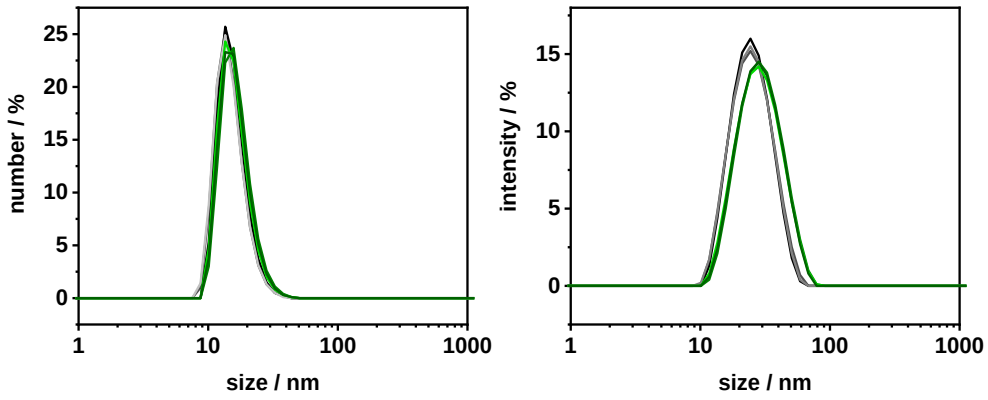


Figure S2. DLS measurements in PBS buffer of the unfunctionalized (black/grey) and VEGF-functionalized (green) nanoparticles.

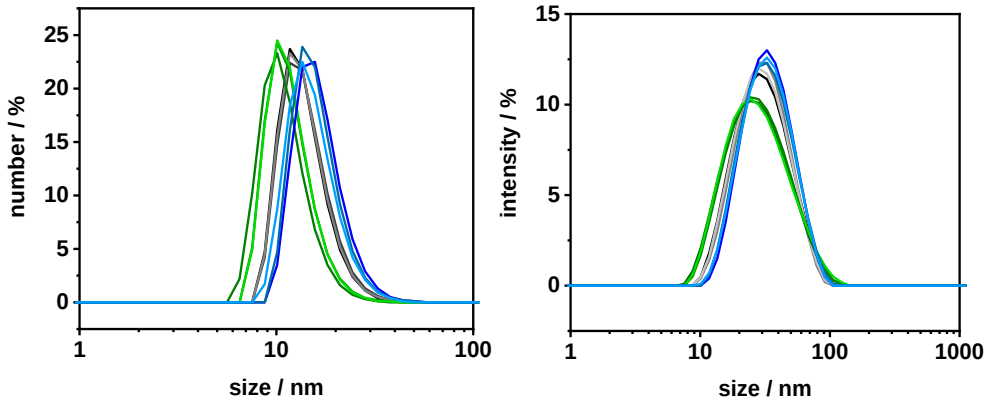


Figure S3. DLS measurements in milli Q water of the unfunctionalized (black/grey), activated (blue) and functionalized (green) nanoparticles.

2.2.2 X-ray photoelectron spectroscopy (XPS)

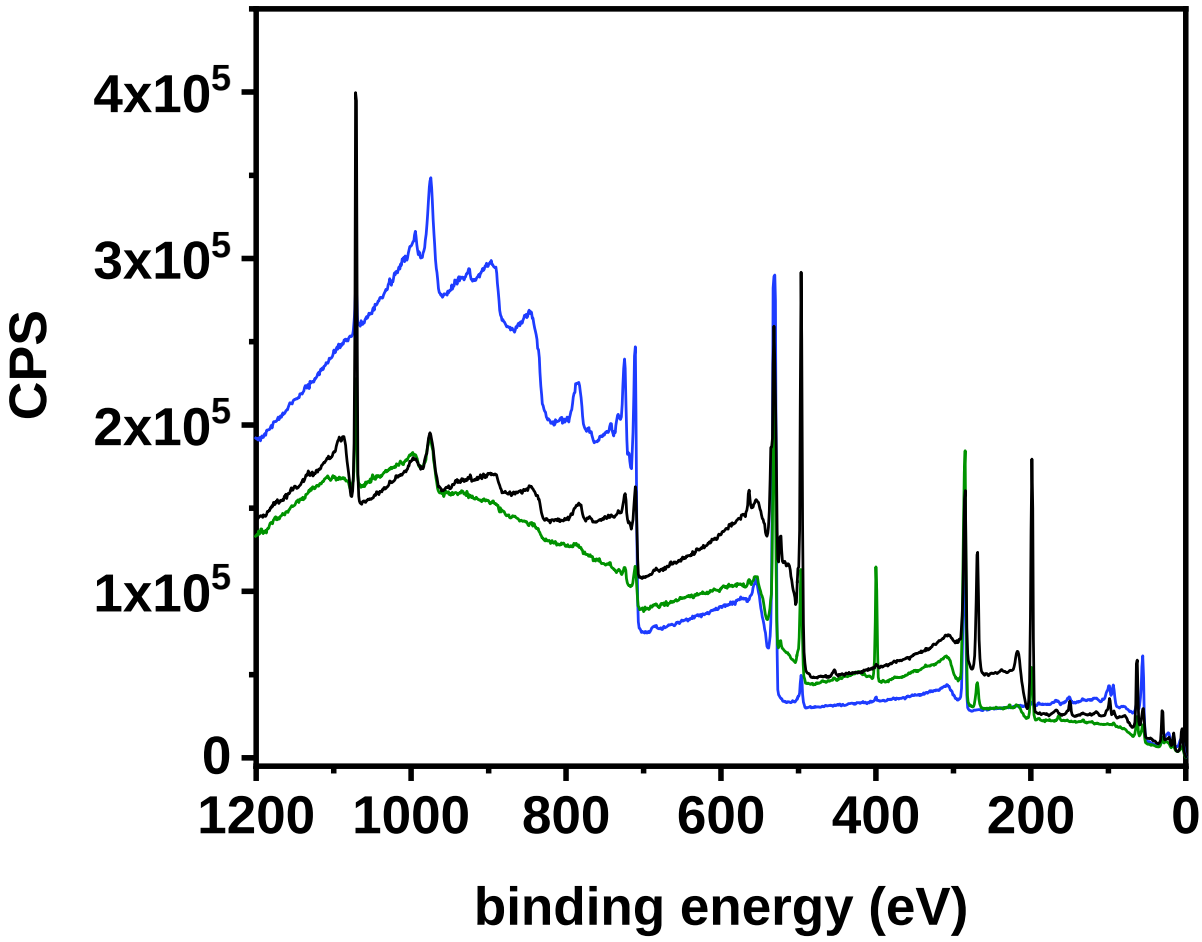
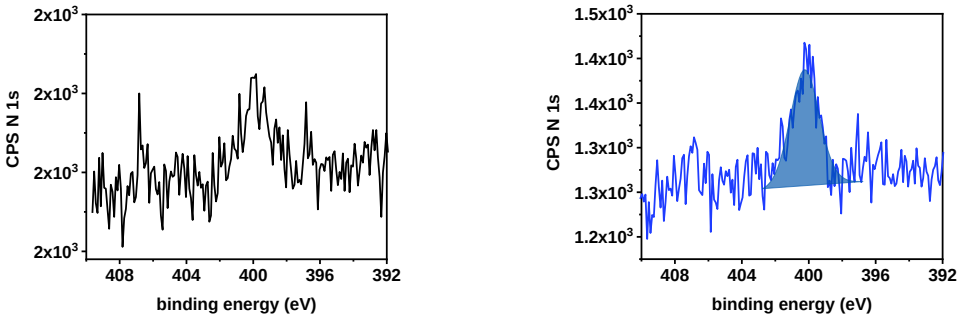


Figure S4. XPS survey spectrum of the unfunctionalized (black), activated (blue) and VEGF-functionalized ferumoxytol nanoparticles.



FigureS5. XPS spectra of the N1s-signal of a) the unfunctionalized and b) of the activated nanoparticles.

2.2.3 Infrared spectroscopy

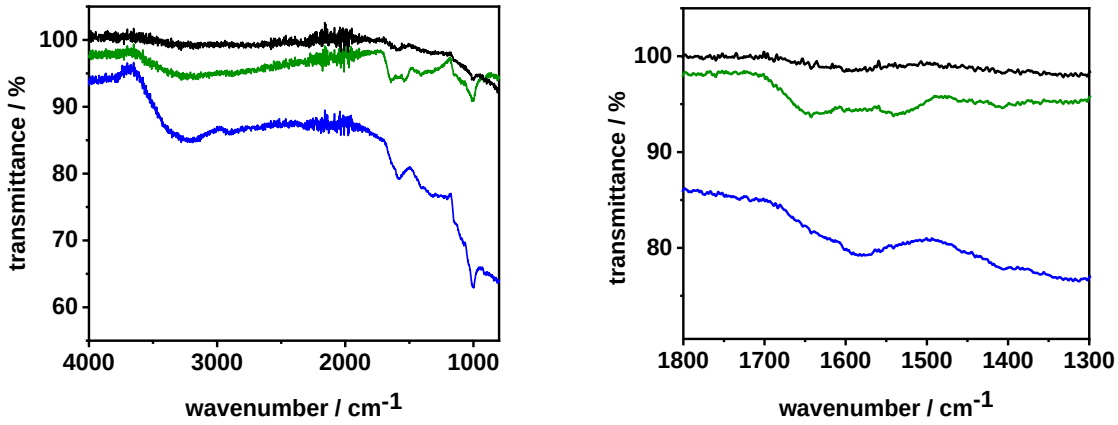


Figure S6. Infrared spectra of the unfunctionalized (black), activated (blue) and VEGF-functionalized (green) nanoparticles and zoom into the region of the characteristic signals for the peptide bonds.

2.2.4 Transmission electron microscopy (TEM)

Small aliquots of VEGF-coated and non-coated ferumoxytol were absorbed to Formvar/carbon coated copper grids for 10 min. For immunogold electron microscopy, samples were washed with PBS and blocked with 2% (w/v) BSA in PBS. Afterwards, grids were incubated for 1 h at room temperature with monoclonal anti-VEGF antibodies (165b, clone 56/1, Millipore) diluted 1:25 in PBS containing 2% (v/v) BSA-c (Aurion, The Netherlands) and 0.025% (v/v) Tween 20. After washing, with the same solution, samples were incubated with 18 nm gold conjugated mouse immunoglobulins. In some control experiments, grids were only absorbed with 18 nm gold conjugated immunoglobulins. After washing with distilled water, ultrathin sections were negatively stained with 2% (w/v) uranyl acetate for 15 min.

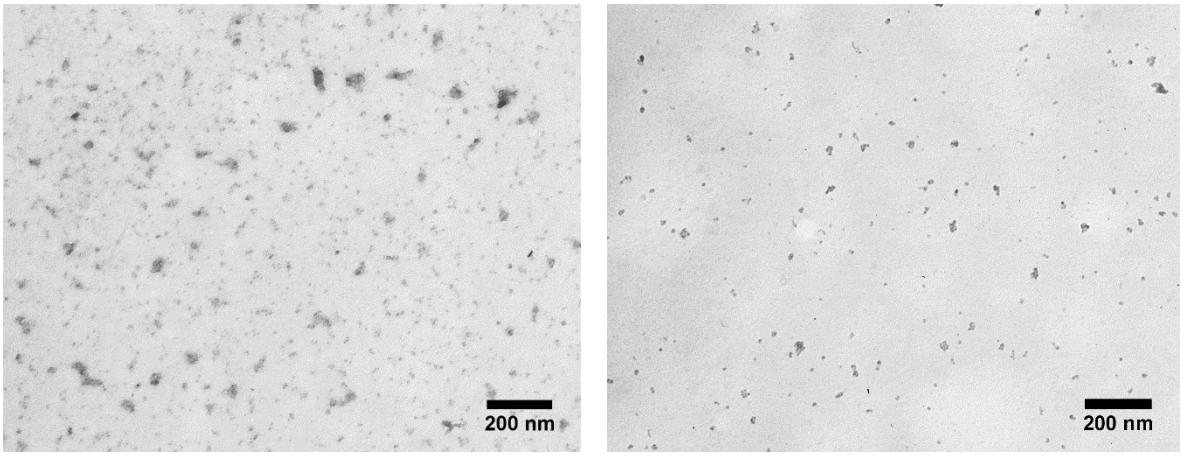


Figure S7. TEM images of ferumoxytol a) before and b) after functionalization with VEGF-165.

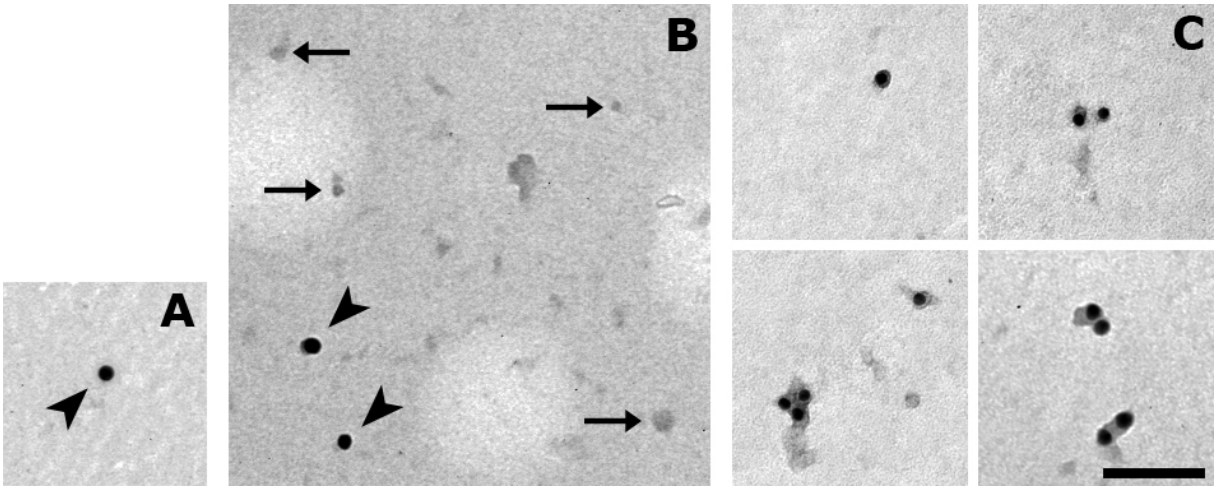


Figure S8: A) Single immunogold particle (18 nm) coated with monoclonal VEGF-165 antibodies; B) blind sample showing no unspecific binding / aggregation between immunogold particles (arrowheads) and pure ferumoxytol (arrows); C) specific binding of immunogold and VEGF-165 functionalized ferumoxytol.

3. Cell experiments

Human umbilical vein endothelial cells (HUVECs) were plated at 4000 cell/cm² per 6 wells coated with 0.2% gelatin in EBM-2 basal medium supplemented with grow-factor containing EGM-SingleQuot-Kit (Lonza Walkersville Inc., USA). All experiments were carried out between passages 2 and 5. Cultures were allowed to grown for 24 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2, before serum starvation in basal medium containing only 0.5% FCS. After 6 h the cell medium was exchanged again, now supplemented with a reduced concentration of grow-factors (1/3 R3-IGF, 1/3 hEGF, 1/8 hFGF-B, no FCS or VEGF) and the respective test substance (N = 3): 300 µg/ml ferumoxytol, 300 µg/ml VEGF-coated ferumoxytol, 10 ng/ml VEGF or 50 ng/ml VEGF (Mouse VEGF 165 Recombinant Protein, Thermo Fisher, USA). While cell grow was documented every 24 h (Eclipse TE2000, Nikon Instruments, The Netherlands), living cells were collected and counted in a hemocytometer after 72 h.

Table S1: Proliferation rates of HUVEC cultures in grow factor reduced media plus different supplements - expressed as multiples of the initial seeding cell number.

#	Supplements	Mean cell doubling after 72 h	N (after 72 h)	Cell doubling after 72 per well
a)	Grow factor reduced media only	4.2x	6	5.1x, 3.3x, 4.3x, 4.5x, 4.2x, 3.5x
b)	+ 300 µg/ml functionalized ferumoxytol	6.2x	3	6.2x, 6.4x, 6.2x
c)	+ 300 µg/ml ferumoxytol	2.7x	2	3.2x, 2.1x
d)	+ 10 ng/ml VEGF-165	3.7x	3	3.9x, 4.0x, 3.1x
e)	+ 50 ng/ml VEGF-165	5.7x	3	5.8x, 5.6x, 5.6x

4. References

[1] Dadashzadeh, E. R., Hobson, M., Henry Bryant, L., Dean, D. D., and Frank, J. A. (2013) Rapid spectrophotometric technique for quantifying iron in cells labeled with superparamagnetic iron oxide nanoparticles: potential translation to the clinic. *Contrast Media Mol. Imaging 8*, 50–56.