Electronic Supplementary Material (ESI) for Dalton Transactions.

Multivalency in CXCR4 chemokine receptor targeted iron oxide nanoparticles

Neazar E. Baghdadi[a,b], Benjamin P. Burke[c], Tahani Alresheedi[b,c], Shubhanchi Nigam[b,c], Abdu Saeed[d], Farooq Almutairi[b,e], Juozas Domarkas[b,c], Abid Khan[c,f] and Stephen J. Archibald[*b,c,]

Affiliations:

[a]Centre of Nanotechnology, King Abdul-Aziz University, Jeddah, Saudi Arabia.

[b]Department of Chemistry, University of Hull, Cottingham Road, Hull, HU6 7RX, UK

[c]Department of Biomedical Sciences and PET Research Centre, University of Hull Cottingham Road, Hull, HU6 7RX, UK

[d]Department of Physics, Faculty of Science, King Abdul-Aziz University, Jeddah 21589, Saudi Arabia

[e]Biomedical Sciences Research Centre, Deanship of Scientific Research, Taif University, Taif, Saudi Arabia

[f]Division of Pharmacy and Optometry, School of Health Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, UK.

* Corresponding author: s.j.archibald@hull.ac.uk

Supplementary Information

S1. Materials and characterisation techniques

S1.1 Materials

1,4,8,11-Tetraazacyclotetradecane (cyclam) and the other chemicals listed below were purchased from Sigma Aldrich, Acros, or Alfa Aesar. The external rare earth magnet (N42, NdFeB, 30 × 30 × 40 mm) was purchased from MagnetExpert Ltd.

S1.2 Characterisation techniques

^{1}H NMR and ^{13}C NMR were recorded on a Jeol JNMLA400 spectrometer at 400 and 101 MHz, respectively. All NMR spectra were referenced to non-deuterated solvents or against an internal standard TMS signal. The chemical shifts (δ) were recorded in parts per million (ppm) and, in the data below, the splitting patterns are designated as s (singlet), td (triple doublet) or m (multiplet). The NMR spectra were processed using MestrReNova software (Mestrelab Research, version 6.0.2-2575).

Low-resolution mass spectrometry data were recorded using a Varian 500 ion trap mass spectrometry system, a Finnegan MAT 900 XLT mass spectrometer or an Advion, Expression compact Mass Spectrometer. The samples were diluted with methanol before direct injection.

The size distribution of the nanoparticles was measured by Nanoparticle tracking analysis (NTA) using a NanoSight® with an LM10-HS microscope and a laser power of 75 mW at 532 nm (green).

Fourier transform infra-red (FTIR) spectra were measured using a Perkin Elmer 20 FTIR spectrometer (model Spectrum RXI).

The elemental analysis to measure the carbon, hydrogen and nitrogen content was performed using an elemental analyser (CE Instruments 1108 CHN, UK). The sample results were measured as weight percentages.

ICP-OES analysis (Inductively Coupled Optical Emission Spectroscopy) was performed using a Perkin Elmer Optima 5300DV. The freeze dried powder samples were digested by nitric acid, other samples containing silica needed a mixture of hydrofluoric acid and nitric acid in 1:3 ratio.

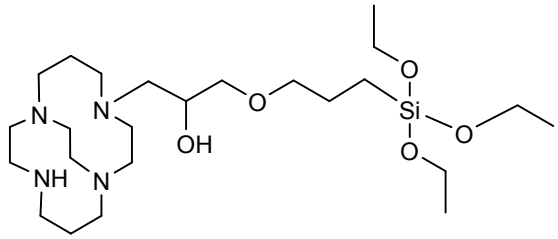
For transmission electron microscopy (TEM) and energy dispersive x-ray (EDX), the diluted samples in ethanol were collected on carbon –coated copper grid and allowed to dry in air. The images were obtained using a Gatan US4000 digital camera (Gatan UK, Abingdon, Oxford) mounted onto a JEOL 2010 transmission electron microscope (Jeol UK) running at 200 kV.

Zeta potential was measured using a Zeta Nanosizer (Malvern Instruments, ZEN3600, UK) at 25°C using a ca. 0.1 mM concentration of nanoparticles. The Smoluchowski equation was used for the conversion of the electrophoretic mobility (μ) into zeta potential (ZP).

Culture media were purchased from Scientific Laboratories Supplies, Trypsin replacement enzyme (TrypLE Express Enzyme) was purchased from Life Technologies, heat inactivated foetal bovine serum (FBS) and phosphate-buffered saline (PBS) tablets were purchased from Thermofisher Scientific. 1X PBS was prepared according to supplier's recommendations (1 tablet/100 mL of water). Glassware and heat stable solutions were autoclaved prior to use; sterile disposable equipment was purchased from Sarstedt or Starlab.

S2. Experimental procedures and analytical data

S2.1. Synthesis of siloxane cross-bridged (CB) cyclam 4



4

The synthesis of the siloxane derivative to the macrocycle was carried out following the methods developed in our previous work.[1]

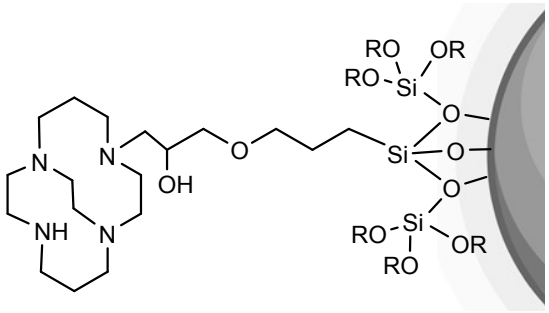
A solution of cross-bridged cyclam (0.20 g, 0.88 mmol) in chloroform (16 ml) was placed in a microwave tube. In a separate vessel, (0.24 g, 0.88 mmol) of GPTES was dissolved in chloroform (16 ml) and added in a dropwise manner to the macrocyclic solution. The mixture was heated in a microwave reactor at 90^{o}C for 30 minutes with some formation of the bis-substituted compound (ca. 10% by NMR). The solvent was removed to form a dark yellow oil and used as crude product (0.4 g, 89%). Attempts at purification resulted in polymerization.

The dark yellow oil also polymerises over time to slowly form a white solid, hence, it was synthesised in fresh batches for use in nanoparticle functionalization. It was redissolved and filtered before use.

^{1}H NMR ($CDCl_3$): δ 3.84 – 3.73 (m, 6H), 3.73 – 3.64 (m, 2H), 3.63 – 3.54 (m, 1H), 3.52 – 3.31 (m, 5H), 3.27 – 3.17 (m,2H), 3.15 – 3.03 (m, 3H), 2.88 – 2.56 (m, 14H), 1.74 – 1.63 (m, 4H), 1.24 – 1.12 (m, 11H), 0.67 – 0.55 (m, 2H) ^{13}C NMR ($CDCl_3$): δ 77.24, 73.83, 71.42, 58.43, 56.74, 53.69, 51.95, 51.04, 47.19, 45.29, 44.30, 25.70, 23.05, 18.36, 6.50

HR ES-MS (m/z): Calculated $[M+H]^+$ = 505.3785 Found $[M+H]^+$ 505.3784

S2.2. Synthesis of iron oxide nanoparticles with Si-CB cyclam (NPs-Si-CB cyclam)



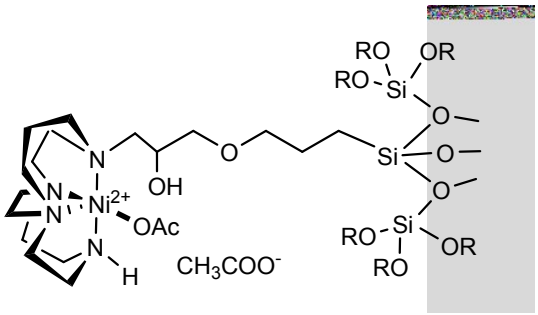
Synthesis of iron oxide nanoparticles was carried out using a methodology based on that reported by Larsen *et al*.[2] The synthesis procedure for attachment of the SPIONs with the siloxane macrocycle was carried out following modified version of Barreto's method.[3] See Figure S2.1 for FT-IR spectrum.

0.3 g (1.29 mmol) of freshly prepared SPIONs were suspended in 75 ml of 60% ethanol and sonicated for 15 minutes. The black suspension was placed under argon and 10 ml triethylamine was added under continuous stirring over 15 minutes. To this mixture, 0.3 g of siloxane modified CB cyclam (Si-CB cyclam) was dissolved in 3 ml of ethanol and added to SPIONs dropwise followed by deionised water (4 ml). The mixture was stirred for 48 hours at room temperature (RT) under Ar.

The product was separated first by a rare earth metal magnet to get rid of any large particles, and the black supernatant was centrifuged at 12,000 rpm for 20 minutes, then washed successively with ethanol (20 ml x 2) and with deionised water (20 ml x 2) and centrifuged at the same speed and time on each occasion. Finally, 12 ml of water was added and centrifuged at 3000 rpm for 5 minutes, a dark stable suspension was formed. The isolated product was characterised by NTA, CHN, ICP-OES and TEM. See Figure S2.2 for NTA plot and TEM.

NTA: Mode 25 nm, mean 47 nm.
Found: C, 21.7; H, 5.3; N, 3.45; Fe, 47.4; Si, 3.3; Ni 0.0%

S2.3. Complex formation on nanoparticle surface with nickel(II) on NPs-Si-CB cyclam



NPs with the attached siloxane macrocycle ligands (50 mg) were suspended in ethanol (30 ml) with sonication for 20 minutes and shaken for a further 20 minutes. A solution of $Ni(CH_3CO_2)_2$ (0.05 mmol, 9 mg) in dry ethanol (10 ml) was added to the prepared NPs suspension dropwise over 5 mins and shaken for 24 hours at R.T. The product (47 mg) was isolated with a rare earth metal magnet and washed with absolute ethanol (20 ml x 2) and de-ionised water (20 ml x 2). Finally, 12 ml of water was added and centrifuged at 3000 rpm 5 minutes, a dark stable suspension was formed. The isolated product was characterised by FTIR, NTA, CHN, ICP-OES and TEM. See Figure 2 (main paper) for NTA plot and TEM. See Figure S2.1 for the FTIR spectrum.

NTA: Mode 72 nm, mean 92 nm.
Found: C, 21.0; H, 5.0; N, 3.4; Fe, 44.8; Si, 3.1; Ni 0.6%

Table S1. Calculation of atomic ratio based on % of nitrogen by mass from elemental analysis/ICP-OES of the coated nanostructure (assumed all N is included in the macrocycle and fixed 4 N atoms).

	Fe	Si	C	H	N	Ni
Mass %	44.77	3.41	20.99	5.02	3.13	0.61
Atom ratio (1% = 17.90 a.m.u.)	14.35	2.17	31.28	89.15	4	0.19

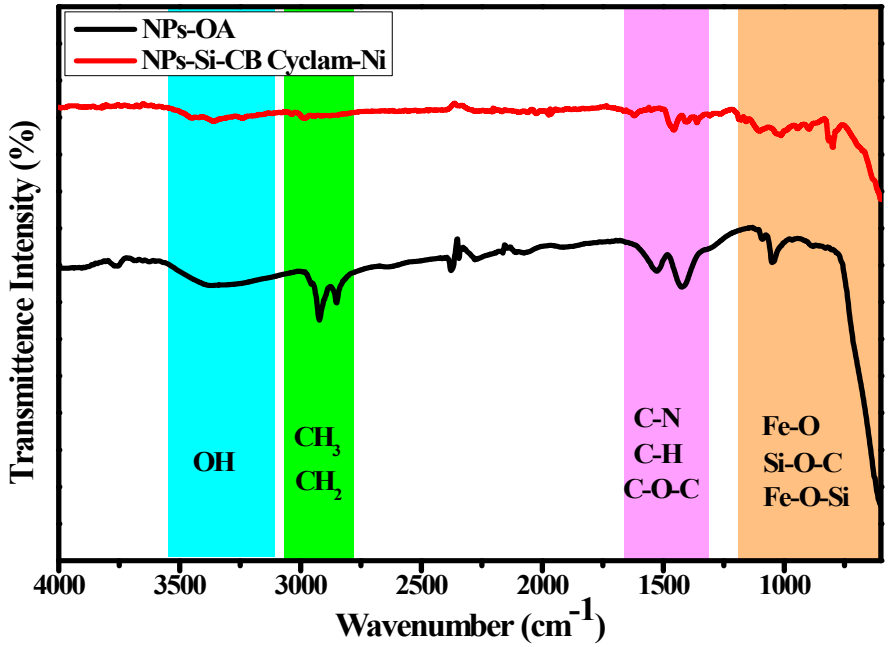


Figure S2.1 FTIR of starting material nanoparticles NPs-OA and product NPs-Si-CB cyclam-Ni

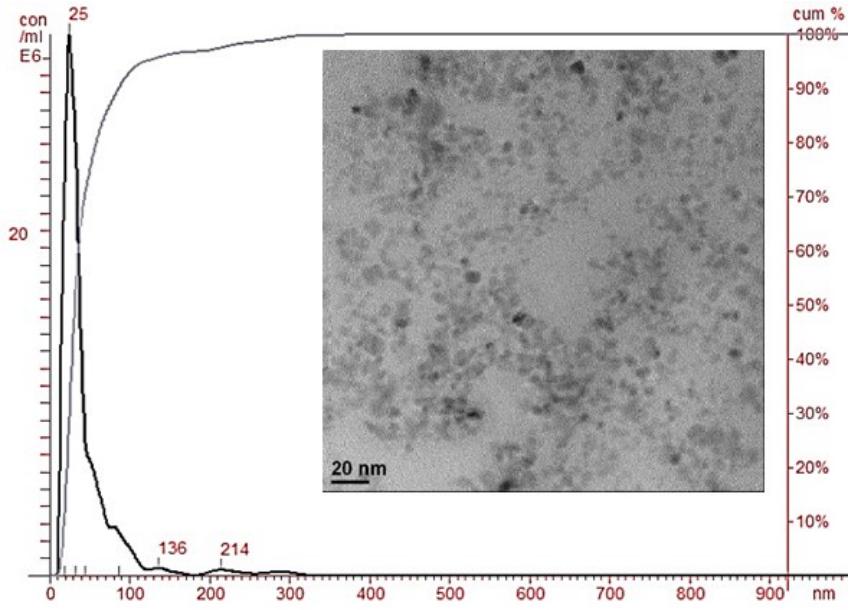


Figure S2.2 Nanoparticle tracking analysis (NTA) of the initially prepared NPs with oleic acid coating suspended in toluene; the TEM image is inset.

(a)

(b)

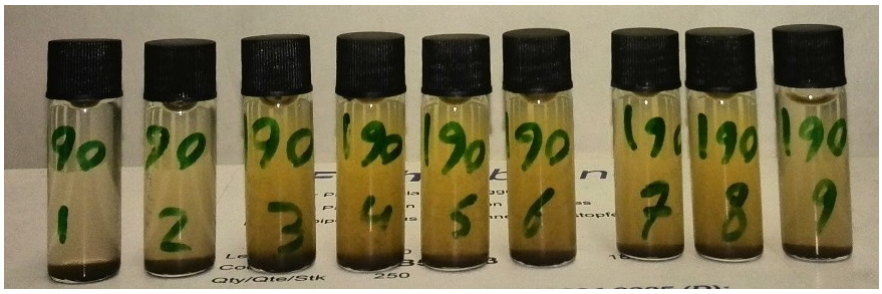


Figure S2.3: Qualitative observation of stability of NPs-Si-CB cyclam-Ni. Examples: (a) pH stability (1 -12) iron concentration 7.5 mM NPs-Si-CB cyclam-Ni, observation after 6 hours. (B) salt stability test, salt concentration range from 0.1 to 0.001 M, samples have iron concentration of 7.5 mM, observation after 6 hours

S2.4. Synthesis of 1-[4-aminomethylbenzyl]-8-[methyl]-1,4,8,11-tetraazabicyclo[6.6.2]hexadecane nickel(II) acetate $[Ni2]^{2+}$

1-[4-Aminomethylbenzyl]-7-[methyl]-1,4,7,10-tetraazabicyclo[5.5.2] dodecane (**2**) was produced by our previously published procedure.[4] **2** (200 mg, 0.56 mmol) was dissolved in degassed anhydrous MeOH (10 ml), an anhydrous methanolic (5 ml) solution of nickel(II) acetate (108 mg, 0.61 mmol) was added dropwise and the mixture was refluxed under argon for 24 hours. Solvent was removed *in vacuo* to ~5 ml then purified *via* size exclusion chromatography (sephadex LH20), solvent removed and dried to yield a green solid (187 mg, 63%).

HRMS (*m/z*): $[M - CH_3CO_2^-]^+$ calcd for $C_{23}H_{40}N_5NiO_2$, 476.2532; found, 476.2530. Elemental anal. calcd for $C_{21}H_{37}N_5Ni.2CH_3CO_2.CH_3OH.H_2O$: C, 53.25; H, 8.42; N, 11.94. Found: C, 52.98; H, 8.80; N, 12.10. UV-vis (MeOH) λmax, nm (ε): 623 (194 M^{-1} cm^{-1}).

S2.5. Cell culture and Flow cytometry studies[5, 6]

Cell culture was carried out using aseptic techniques in a laminar flow cabinet. Cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Cells were incubated at 37°C, in a humidified 5% CO_2 controlled atmosphere. To maintain cell growth, cells were sub-cultured three times a week. Culture media was removed from the flask and cells were washed with 1 X PBS. Trypsin was added and the flask incubated at 37°C until complete detachment was observed. Trypsin was neutralised by addition of an equal volume of culture media and the cell suspension was centrifuged for 5 min at 200 x g. The supernatant was removed and the cell pellet was resuspended in fresh media. To ensure constant seeding densities, live cells were counted before being seeded. To a sample of cells suspension, an equal volume of Trypan blue and live cells were counted using a Neubauer haemocytometer.

To determine CXCR4 expression by flow cytometry, 70-80% confluent cells were harvested following the same protocol as described above for sub-culturing. Cells were aliquoted at 3 x 10^5 cells per sample and centrifuged for 5 min at 200 x g. The supernatant was removed and the cell pellet was resuspended in 97 µL of FACS buffer (1X PBS, 5 mM $MgCl_2$, 1 mM $CaCl_2$) supplemented with 2% FBS. Following the addition of 3 µL of the relevant antibody (for a final volume of 100 µL), cells were incubated for 1 h at RT and in the dark. Cells were incubated with either phycoerythrin (PE)-conjugated anti-human CXCR4 monoclonal antibody (clone 12G5, R&D Systems) for determination of CXCR4 expression. To account for non-specific binding, negative controls were also prepared where cells were incubated with the PE-conjugated mouse IgG_{2A} (R&D Systems) isotype control antibody. After the incubation, unbound antibody was removed with three washes (200 µL) with FACS buffer. After the last wash, the cell pellet was resuspended in 400 µL of FACS buffer supplemented with 1% formaldehyde and transferred into FACS tubes. Acquisition was carried out on a BD FACSCalibur™ flow cytometer. For each sample, a gate was manually drawn around the population of interest and 10000 events were acquired. The data was analysed with BD CellQuest™ Pro.

Competition binding experiment

The binding affinity of compounds was determined by flow cytometry through competition binding experiments. A similar protocol as the one described above for receptor expression was followed. Cells were aliquoted at 3 x 10^5 cells per sample and centrifuged for 5 min at 200 x g. The supernatant was removed and the cell pellet was resuspended in 90 µL of FACS buffer supplemented with 2% FBS. 10 µL of nanoparticle stock solution (20 mg/mL) was added and the samples were incubated for 1 h at RT. Unbound nanoparticles were removed with three washes (200 µL) of FACS buffer. The cells were then incubated with the relevant antibody (anti-human CXCR4) as described above for 1 h at RT and in the dark. Unbound antibody was removed with three washes (200 µL) of FACS buffer. The cell pellet was resuspended in 400 µL of FACS buffer supplemented with 1% formaldehyde and transferred into FACS tubes. For each experiment, two controls were included: (1) a positive control where only the anti-CXCR4 antibody was added to the cells and (2) a negative control with unlabelled cells where no antibody was added. The acquisition was carried out using a BD FACSCalibur™ flow cytometer. For each sample, a gate was manually drawn around the population of interest and 10000 events were acquired. The data was analysed with BD CellQuest™ Pro. The geometric mean (GeoMean) of the histograms was used to calculate the percentage inhibition of antibody binding using Equation 2.

$$\%\ mAb\ inhibition = 100 - \left(\frac{GeoMean\ cells\ with\ compound - GeoMean\ cells\ with\ isotype\ mAb}{GeoMean\ cells\ positive\ control - GeoMean\ cells\ with\ isotype\ mAb} \times 100\right)$$

Equation 2: Determination of the percentage of antibody inhibition.

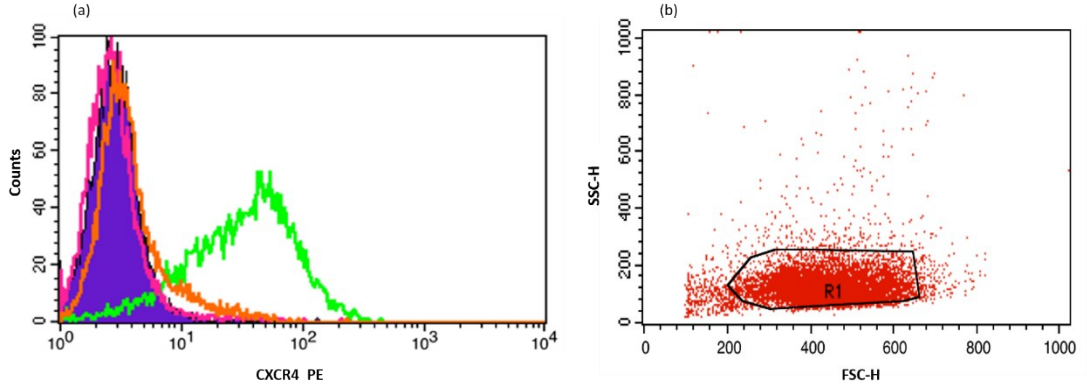


Figure S2.4: (a) Flow cytometry histogram of the binding of anti-CXCR4-PE (12G5) on the cell surface of Jurkat cells. Negative (solid purple), Anti-CXCR4-PE mAb only (green), and competition with NPs (Pink, NZ192, 100 times dilution and Orange, NZ192, 10 times dilution) and (b) FSC/SSC dot plot of the manual gate drawn around cell population of interest.

Negative isotype

Histogram Statistics

File: sn.001
Log Data Units: Linear Values
Sample ID:
Acquisition Date: 04-Aug-14
Gate: G1
Gated Events: 8125
Total Events: 10000
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	8125	100.00	81.25	4.17	3.35	623.05	3.43	3

Positive control

Histogram Statistics

File: sn.002
Log Data Units: Linear Values
Sample ID:
Acquisition Date: 04-Aug-14
Gate: G1
Gated Events: 8195
Total Events: 10000
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	8195	100.00	81.95	15.99	11.09	157.98	10.94	12

NPs-Si-CB cyclam- Ni (NZ 192)

Histogram Statistics

File: sn.003
Log Data Units: Linear Values
Sample ID:
Patient ID:
Tube: Untitled
Panel: Untitled Acquisition Tube List
Acquisition Date: 04-Aug-14
Gate: G1
Gated Events: 8126
Total Events: 10000
X Parameter: FL2-H (Log)

Marker	Left, Right	Events	% Gated	% Total	Mean	Geo Mean	CV	Median	Peak Ch
All	1, 991(	8126	100.00	81.26	4.16	3.52	175.17	3.43	3

Calculation of % binding using Equation 2

negative	3.09
Positive	43.98
NZ192(Ni) 10 fold dilution	3.08
NZ192(Ni) 100 fold dilution	4.47

Sample ID	MFI(c)-MFI(-ve)	MFI(+ve)-MFI(-ve)	C/D	% Inhibition
NZ192(Ni) 10 fold dilution	-0.01	40.89	-0.02446	100.0
NZ192(Ni) 100 fold dilution	1.38	40.89	3.37491	96.62

Competition binding of control compounds $[Ni2]^{2+}$ and nanoparticles with no macrocycle

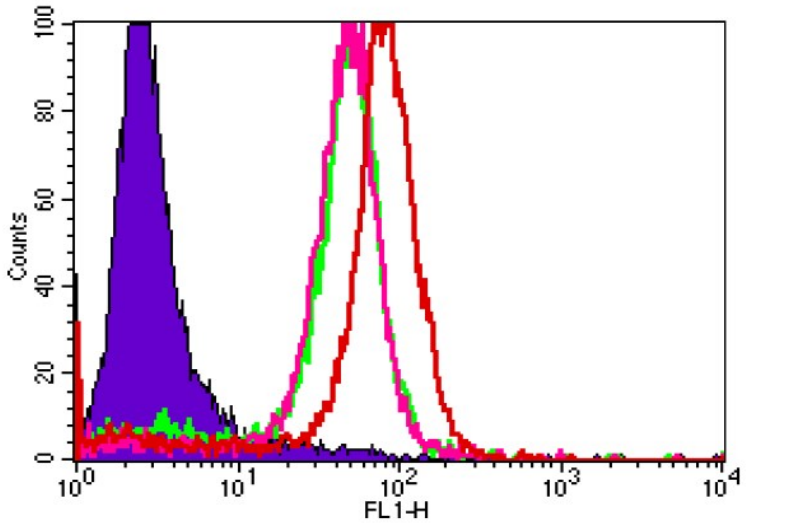


Figure S2.5: Flow cytometry histogram showing <40% binding inhibition of the 12G5 antibody with monovalent control compounds [Ni**2**]$^{2+}$

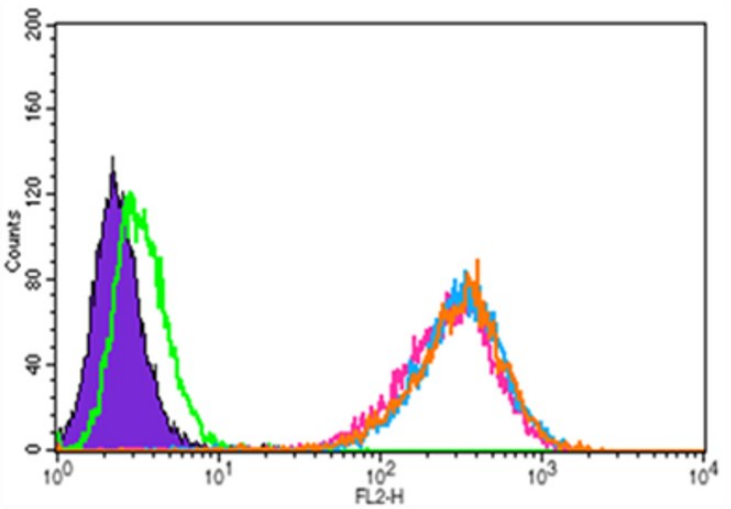


Figure S2.6: Flow cytometry histogram showing no observable 12G5 mAb binding inhibition with uncoated nanoparticles. Some precipitation occurred in the cell culture media despite efforts to suspend the nanoparticles in compatible solvents/ buffers. No binding was observed for any of the suspensions prepared.

References:

1. B. P. Burke, N. Baghdadi, A. E. Kownacka, S. Nigam, G. S. Clemente, M. M. Al-Yassiry, J. Domarkas, M. Lorch, M. Pickles, P. Gibbs, R. Tripier, C. Cawthorne and S. J. Archibald, *Nanoscale*, 2015, **7**, 14889-14896.
2. B. A. Larsen, M. A. Haag, N. J. Serkova, K. R. Shroyer and C. R. Stoldt, *Nanotechnology*, 2008, **19**.
3. J. A. Barreto, M. Matterna, B. Graham, H. Stephan and L. Spiccia, *New J. Chem.*, 2011, **35**, 2705-2712.
4. R. Smith, D. Huskens, D. Daelemans, R. E. Mewis, C. D. Garcia, A. N. Cain, T. N. C. Freeman, C. Pannecouque, E. D. Clercq, D. Schols, T. J. Hubin and S. J. Archibald, *Dalton Trans.*, 2012, **41**, 11369-11377.
5. A. Khan, J. D. Silversides, L. Madden, J. Greenman and S. J. Archibald, *Chem. Commun.*, 2007, 416-418.
6. A. van Hout, T. D'Huys, M. Oeyen, D. Schols and T. Van Loy, *PLoS One*, 2017, **12**.