Electronic Supporting Information

Multiparametric Modulation of Magnetic Transduction for Biomolecular Sensing in Liquids

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Table S1.- Translational diffusion coefficient and standard deviation values measured by Nanosight NS300 at 25°C for MNPs and b-MNPs (1$g_{Fe/Fe+Co}$ /L) dispersed in PB 0.1x in absence (0 μM) and presence of 2 μM monovalent analyte after incubation (1h at 25ºC).

Magnetic suspensión	Diffusion coefficient ($nm^2/\mu s$)
bare CoFeNFs	10.3 ±0.1
b-CoFeNFs 0 μM	9.4 ±0.1
b-CoFeNFs 2 μM	9.1 ±0.2
bare IONFμ	8.9 ± 0.5
b-IONFs 0 μM	8.7 ± 0.3
b-IONFs 2 μM	7.7 ± 0.4

Table S2.- Experimental parameters employed for the simulations of AC magnetization cycles.

MNP parameter	bare CoFeNFs	bare IONFs
M_{sat}	222 kA/m	190 kA/m
K	$1x10^5$ J/m^3	$3x10^3$ J/m
D_C	27.2 nm	29 nm
ΔDc	0 nm	3 nm

Table S3.- Comparison of the mean hydrodynamic size ($\langle D_h \rangle$) and the standard deviation (s) between the experimental (number weighted) and the fitted (obtained from simulation) values in nm.

	bare CoFeNFs	**b-CoFeNFs 0 μM**	**b-CoFeNFs 2 μM**
Experimental $\langle D_h \rangle$	46.6 ±0.4	54 ± 3	56.9 ± 0.9
Fitting $\langle D_h \rangle$	48.0 ±0.5	50.0 ± 0.5	57.0 ± 0.5
Experimental σ	16.7 ± 1.1	21 ± 7	20.5 ± 2.4
Fitting σ	12.5 ± 0.5	14.5 ± 0.5	20.5 ± 0.5

Table S4.- D_H and PDI values measured by DLS for b-IONFs (1g_{Fe}/L) in absence (0 μM) and presence (1 and 2 μM) of divalent analytes after incubation (for 1 h at 25°C) in PB 0.1x.

[Analyte] (μM)	**D_H intensity (nm)**	**D_H number (nm)**	**D_H volume (nm)**	**PDI**
0	53.4 ± 0.1	41.1 ± 0.4	47.2 ± 0.2	0.05
1	98.1 ± 13.3	38.2 ± 1.9	66.1 ± 3.7	0.32
2	226.0 ± 69.3	34.3 ± 2.3	83.4 ± 22.1	0.37

Table S5.- Input parameters for single molecule spectroscopy data analysis.

Electronic Supporting Information

Setup Rapid Storm	
Pixel Size (nm)	107
Amplitude Threshold	3000
Initial FWHM (nm)	260
Setup SPT analysis	
Astigmatism Filter	0.75 - 1.25
FWHM Filter (nm)	180 - 280
Intensity Filter	3000
Localization Radius (nm)	400
Gap Closing for non-consecutive Frames	Infinity

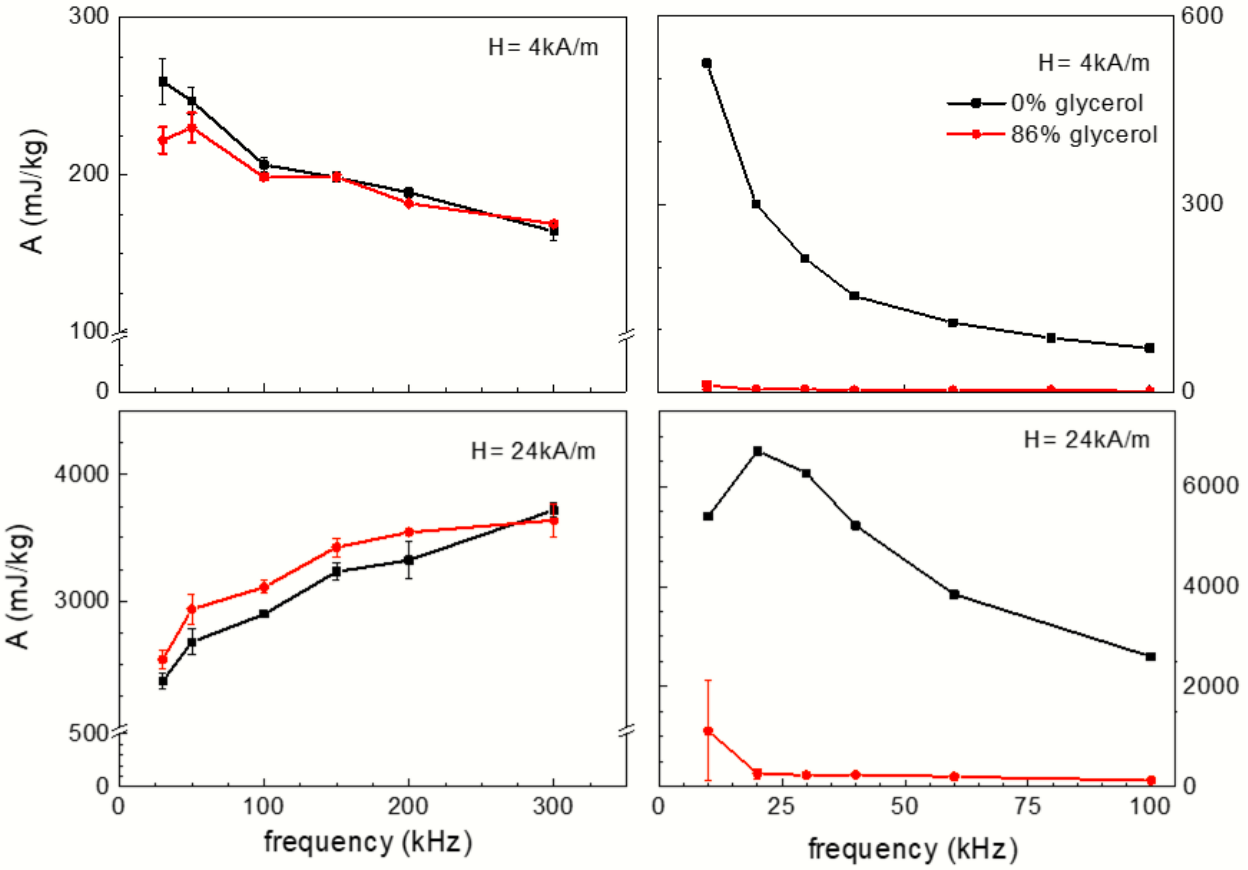

Figure S1.- Frequency dependence of normalised AC magnetic hysteresis areas at two field intensities, 4 kA/m (top panels) and 24 kA/m (bottom panels), extracted from AC hysteresis loops of b-MNPs measured at $1g_{Fe/Fe+Co}/L$ dispersed in 0 and 86 % glycerol fraction solutions. Left panels: b-IONFs; Right panels: b-CoFeNFs.

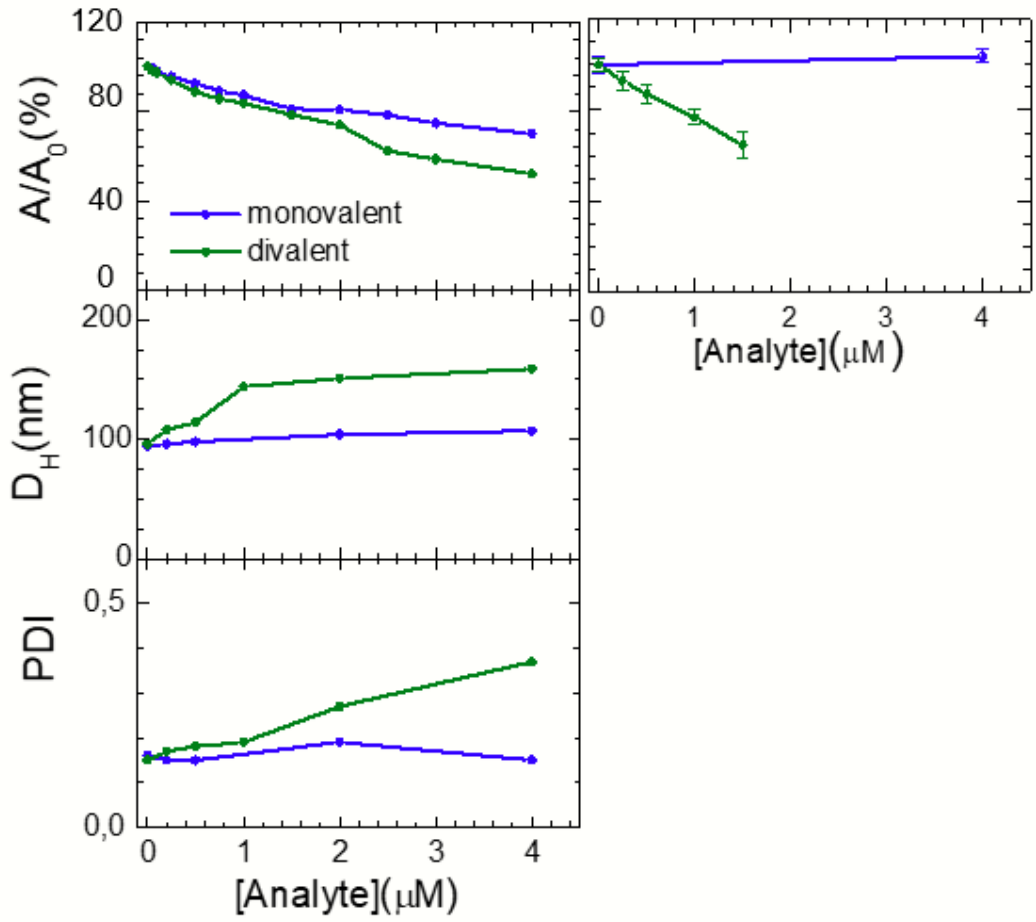

Figure S2.- Analyte concentration dependence of normalized AC magnetic area (*A/A₀*) extracted from AC hysteresis loops measured for : Left) b-CoFeNCs at 50 kHz and 24 kA/m; Right) b-IONPs at 100 kHz and 24 kA/m. Incubation conditions: 1 hour incubation at 25ºC on increasing monovalent (blue color) and divalent (green color) concentrations in 0.1xPB at $1g_{Fe/Fe+Co}$/L. D_H and PDI values of b-CoFeNC suspensions after 1 hour incubation at 25ºC on increasing analyte mono (blue color) and divalent (green color) concentrations in 0,1xPB at $1g_{Fe/Fe+Co}$/L.

Colloidal properties related to specific and non-specific interaction

In order to identify the specific interaction recognition between nanoparticles and divalent analytes, we have assessed the hydrodynamic size and colloidal stability of b-MNPs/MNPs were assessed for comparison after incubation with analytes under similar conditions as shown in **Figure S3**. Thus, 100 mL of b-IONFs and IONFs suspensions at 1 g_{Fe}/L were separately incubated in PB 0.1x buffer for 1h at room temperature (25 ^{o}C) with divalent analytes at different concentrations (0, 0,5, 1,5 and 4 μM). A reduction of colloidal stability was observed by eye-inspection for IONFs. Nanoparticle sedimentation was observed in IONFs for all analyte concentrations for incubation times shorter than 30 min (see **Figure S3b**). Contrary, b-IONF suspensions only showed sedimentation for analyte concentrations higher than 2 μM and incubation times larger than 1h. The different sedimentation dynamics observed for IONFs and b-IONFs is a fingerprint of specific interactions.

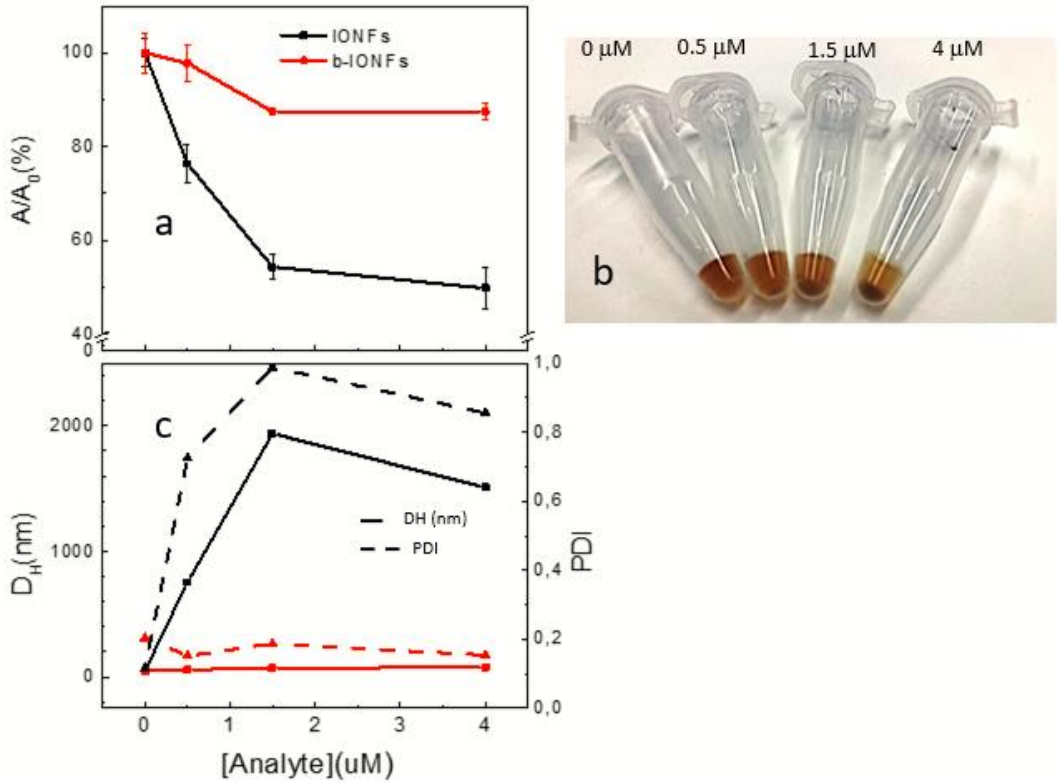

Figure S3.- a) Divalent analyte concentration dependence of normalized magnetic area extracted from AC hysteresis loops measured at 100 kHz and 24 kA/m in IONFs (black colour) and b-IONFs (red colour) dispersed at 1 g_{Fe}/Lin PB 0.1x buffer incubated 30 minutes at 25^{o}C with different divalent analyte concentrations

ranging from 0 up to 4 μM (on the right); b) image of the Eppendorf tubes containing IONFs suspensions after 15 minutes of incubation time; c) D_H and PDI values of the above mentioned IONFs and b-IONF suspensions.

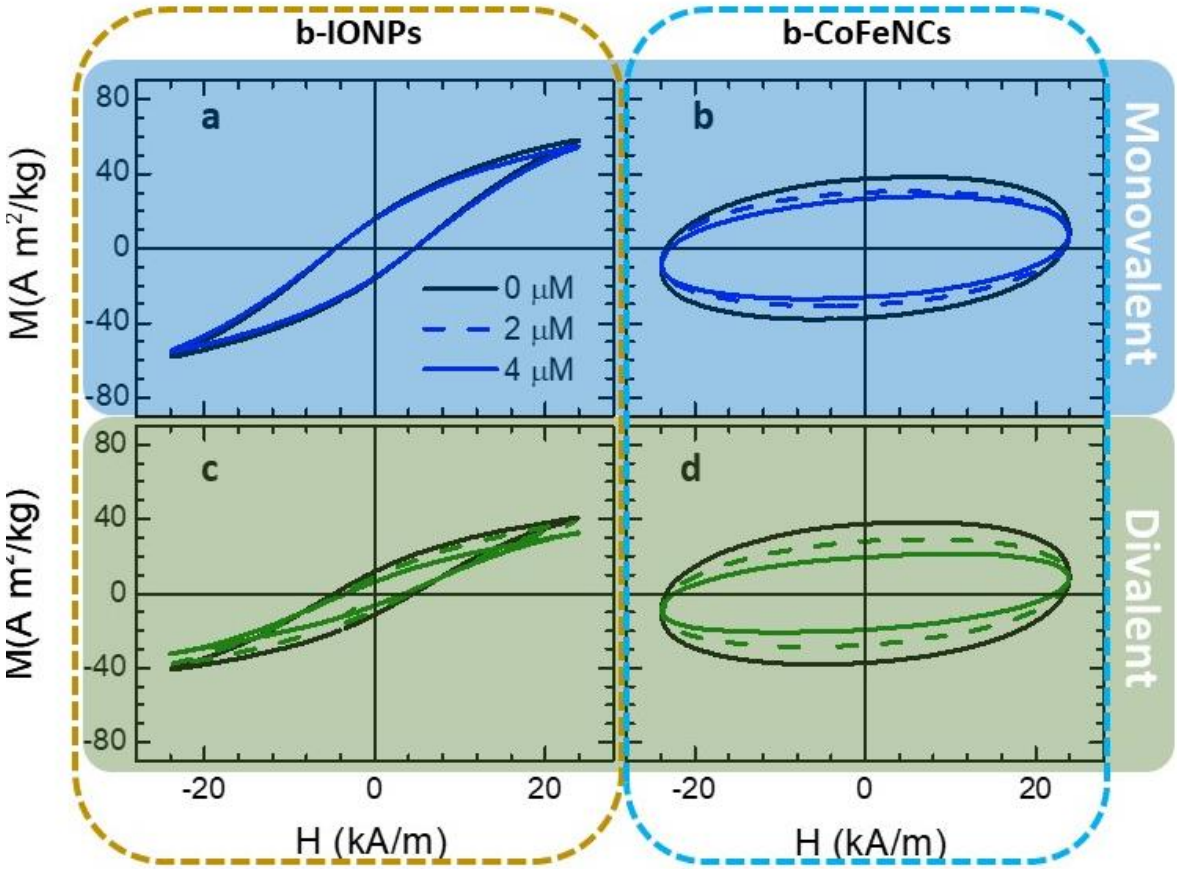

Figure S4. AC hysteresis loops of the b-IONPs and b-CoFeNCs on increasing concentrations of monovalent and divalent analytes up to 4 μM. Measurements were performed at room temperature under the following AC field conditions: (a,c) 100 kHz and 24 kA/m; (b,d) 50 kHz and 24 kA/m. Incubation conditions: b-MNPs ($1g_{Fe/Fe+Co/L}$) dispersed in PB 0.1x buffer for 1h at 25°C.

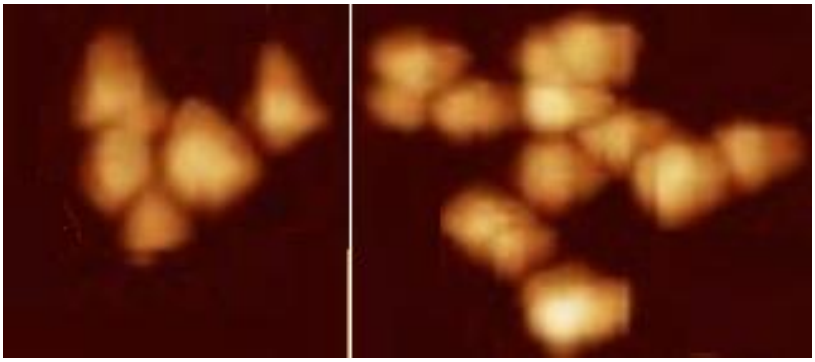

Figure S5.- AFM images of b-IONF nanoclusters formed after 1 hour incubation at 25ºC in 0.1xPB at 0.75 mM divalent analyte and 1g_{Fe}/L b-IONFs content.

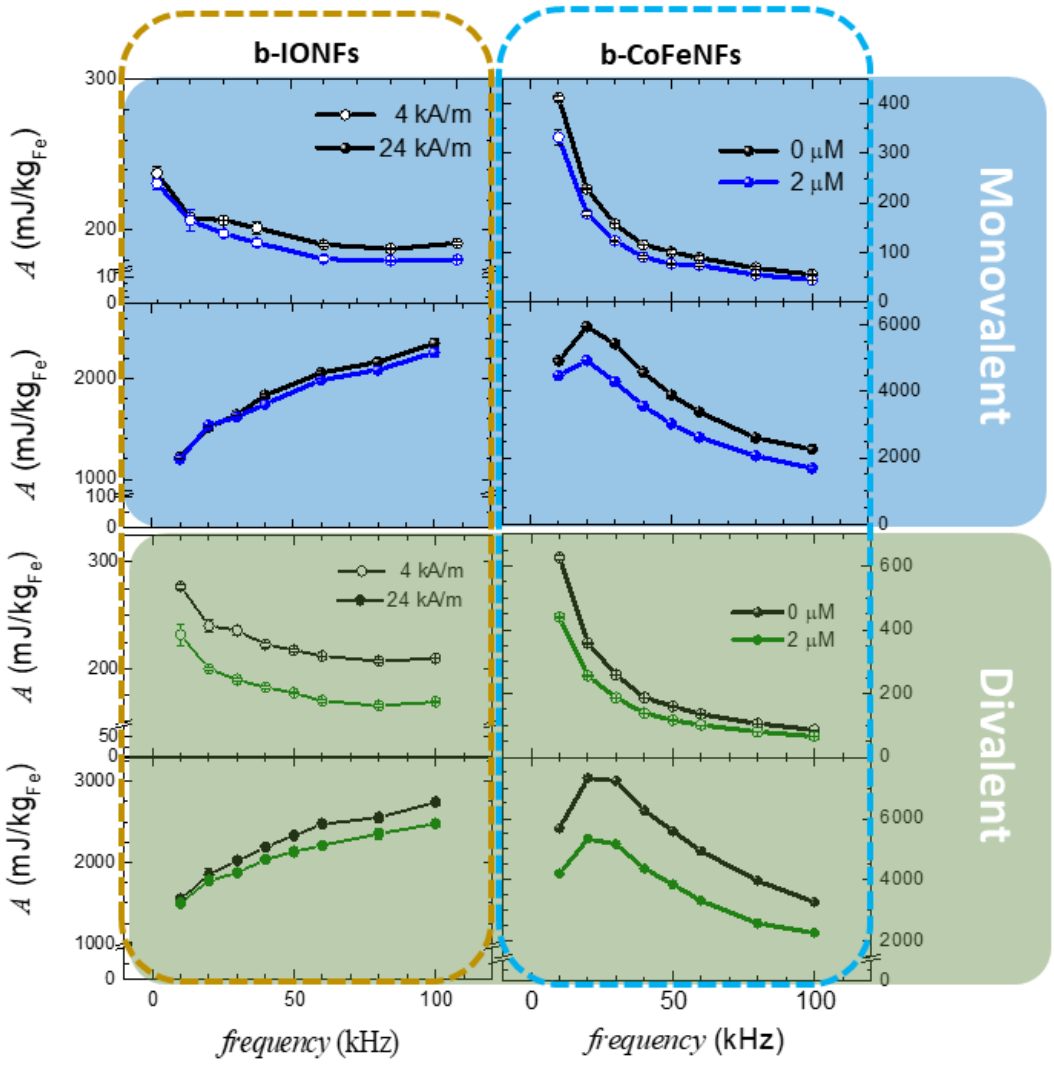

Figure S6. Frequency dependence of AC magnetic hysteresis area at two field intensities (4 and 24 kA/m) from b-IONFs (left columns) and b-CoFeNFs (right columns) in absence (black dots) and in presence of 2 µM monovalent (blue dots) and divalent (green dots) analytes. Incubation conditions: b-MNFs (1$g_{Fe\ or\ Fe+Co}$/L) dispersed in PB 0.1x buffer for 1h at 25°C.

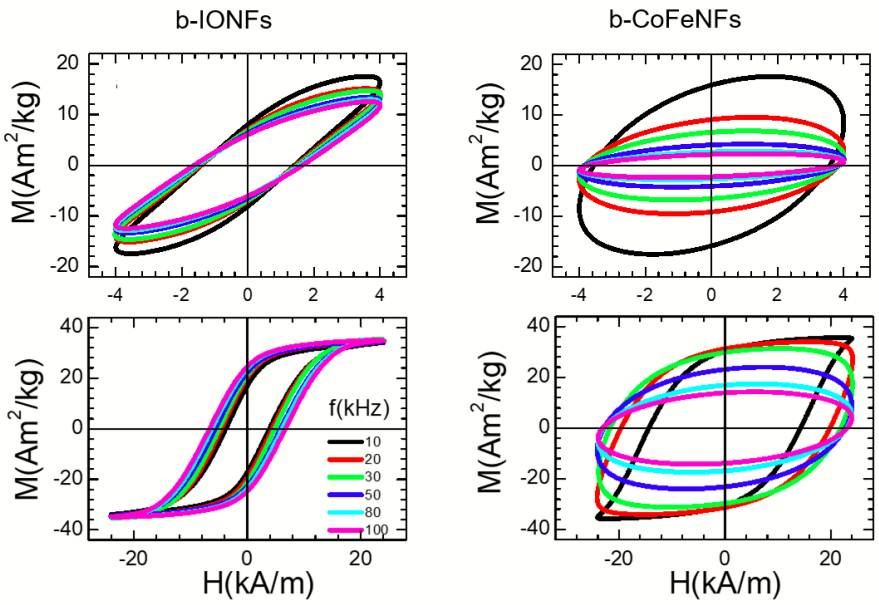

Figure S7.- Frequency dependence of AC hysteresis loops at two field intensities 4 kA/m (top panels), and 24 kA/m (bottom panels) for b-IONFs (left panels) and b-CoFeNFs (right panels) dispersed in PB 0.1x at $1g_{Fe/Fe+Co}/L$ after 1 h incubation at 25ºC in absence of analyte.

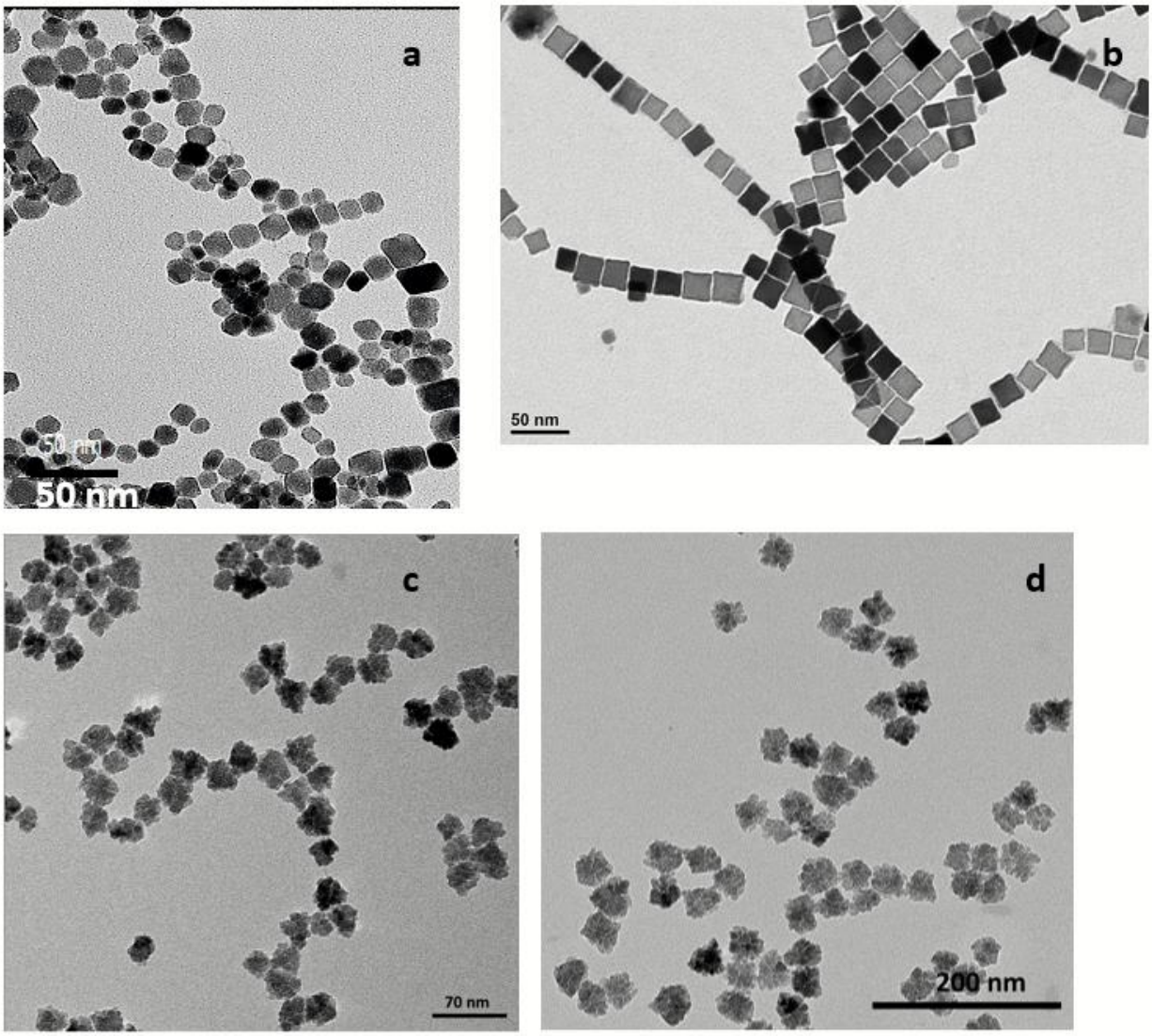

Figure S8.-Transmission Electron Microscopy images of the studied MNPs: a) IONPs; b) CoFeNCs; c) IONFs; d) CoFeNFs.

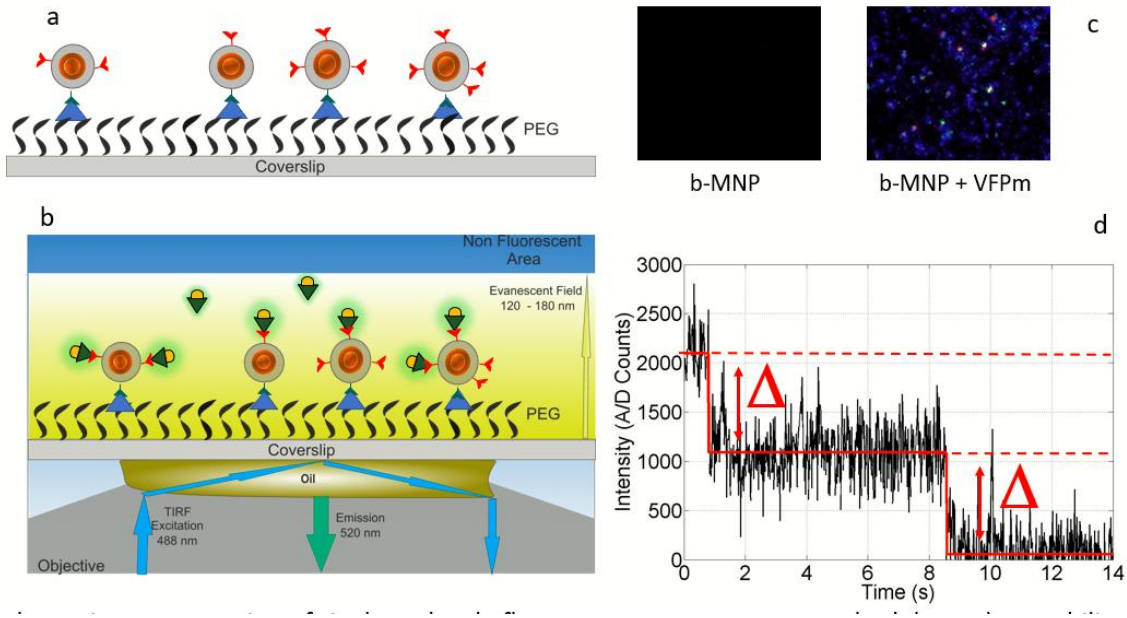

Figure S9.- Schematic representation of single molecule fluorescence spectroscopy methodology: a) Immobilization of biotinylated b-MNPs on streptavidin sites located onto PEGylated coverslip; b) home-made total internal reflection fluorescence setup with 488 nm laser excitation via evanescent field and collect the $VFP_{monomer}$-TRP2-MMY analyte fluorescence at 520 nm; c) fluorescence map of b-MNP in absence and in presence of $VFP_{monomer}$-TRP2-MMY analyte bound to the immobilized b-MNPs; d) time dynamics of analyte fluorescence intensity from single b-MNP during optical excitation. Δ depicts the discrete fluorescence intensity steps.

Electronic Supporting Information

Procedure for biotinylated b-MNP immobilization and single molecule fluorescent preparation.

Coverslip cleaning

1. Immerse the coverslips in piranha solution (3 parts of concentrated H_2SO_4 and 1 part of 30 wt. % H_2O_2 solution), leaving them five minutes.
2. Wash coverslips with Mili-Q water 5-7 times.
3. Store coverslips in Mili-Q water until functionalization.

Derivatization process

4. Dry the coverslips using N_2.
5. Prepare PEG5000 solution that contains 300 *mg* of NHS-PEG in 1500 *µL* of sodium borate 100 *mM pH =* 8.5.
6. Prepare PEG10000-Biotin solution that contains 1 mg of Biotin-PEG in 1000 *µL*.
7. Mix 71.4 *µL* of PEG10000-Biotin solution with 1428.6 *µL* of PEG5000 solution to get a ratio of PEG-Biotin:PEG ~ 1:10000.
8. Choose a side of your coverslip; place it in a glass dish over a falcon tap full of water. This is to avoid evaporation of the functionalization solution.
9. Cover one side of the glass coverslip around 600 *µL* of the PEG mixture until you have a water film covering the whole coverslip surface.
10. Close the glass dish to avoid evaporation.
11. Leave for 3 h, verifying every hour the volume of water film to check evaporation.
12. Wash coverslips with water, dry with N_2. Being careful not to mix the functionalized side.
13. Attach Ibidi-8 well stick slide to the functionalized site of the coverslip.
14. Add 300 µl of 500 pM solution of b-MNPs in each well and incubate for 90 minutes.
15. Remove all b-MNP solutions and wash from wells with PBS 0.1x. Repeat washing 10 times.
16. Add 300 µl of PBS 0.1x on each well.
17. Add 12 µl of a 0.9 µM monovalent analyte solution into the prepared wells. The final concentration of $VFP_{monomer}$-MMY on each well was 36 nM.

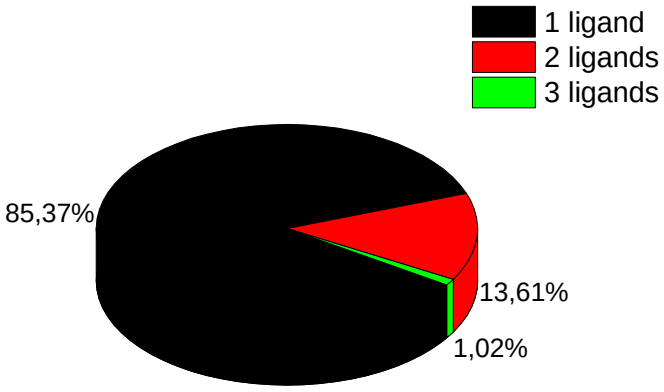

Figure S10.- Histogram of Photobleaching Step distribution analyzed for b-IONFs (1 receptor : 1 IONF ratio) after the incubation with monovalent analyte in PB 0.1x buffer. The histogram represents the % of individual b-IONFs containing 1 (black), 2 (red), and 3(green) receptors.

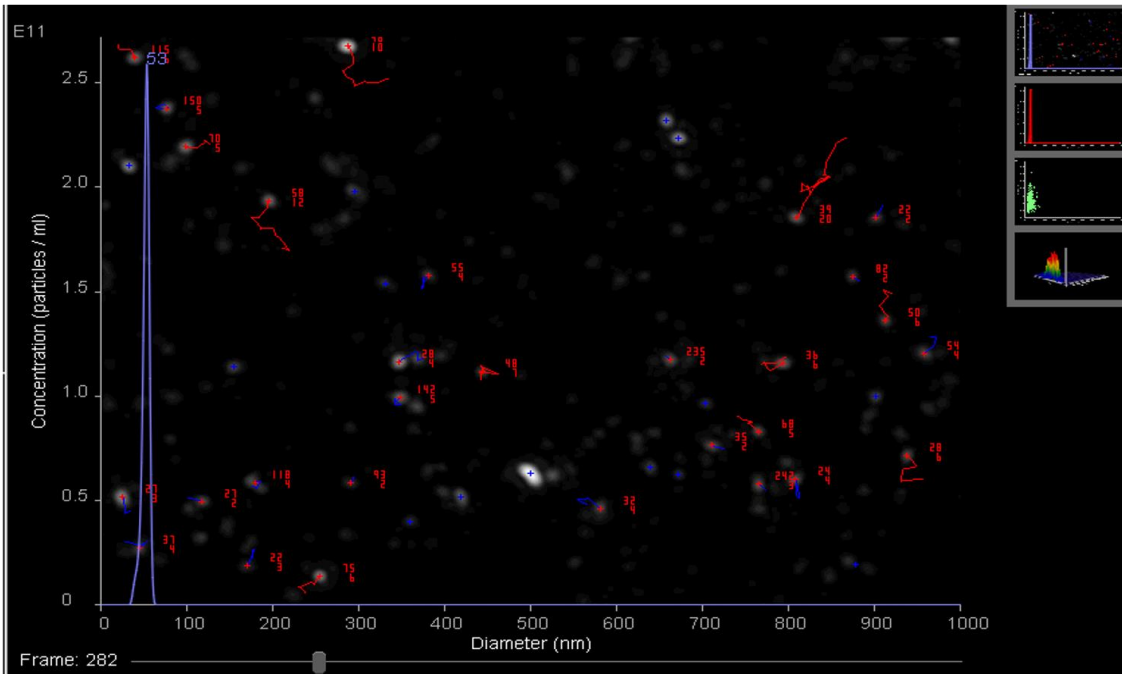

Figure S11.- Representative image of the individual nanoparticle tracking analysis for determining their translational diffusion coefficient of MNPs by Nanosight NS300. Red lines correspond to the individual Brownian pathways of each MNPs in the dilution PB 0.1x buffer.

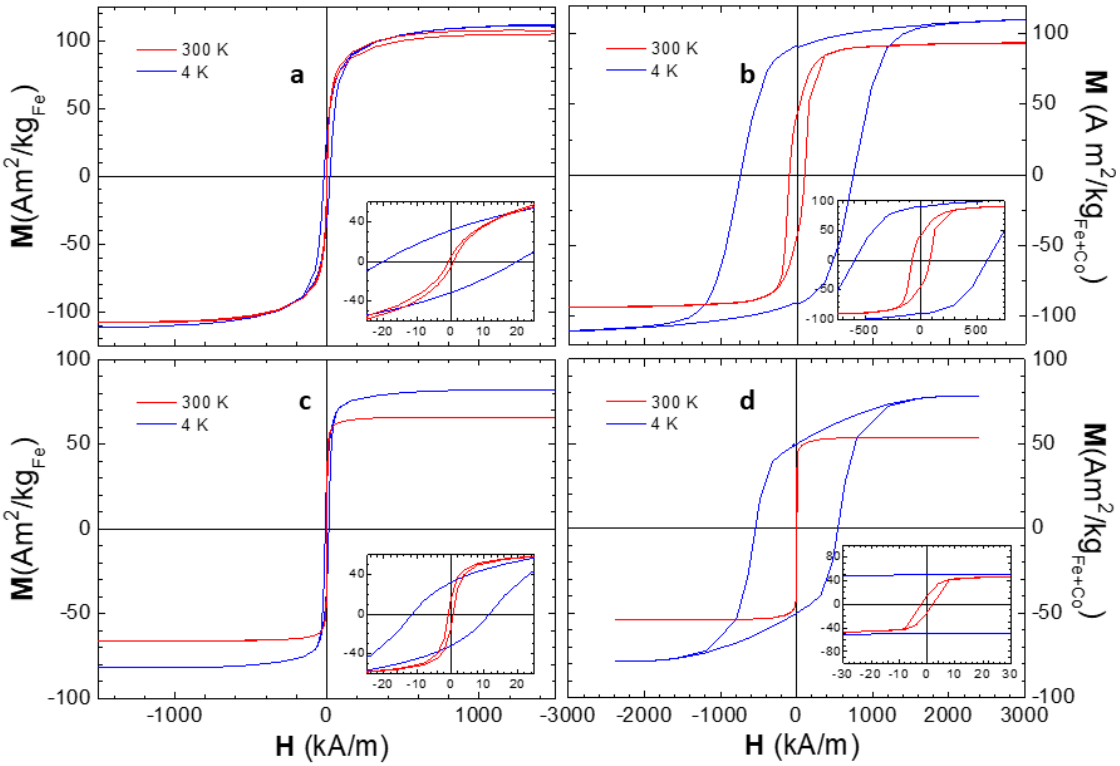

Figure S12.- Mass-normalized magnetization cycles the studied MNPs at different temperatures (4 K and 300 K) for a) IONPs; b) CoFeNCs; c) IONFs; d) CoFeNFs suspensions at 2 $g_{Fe/Fe+Co}$ /L dispersed in DDW.