SUPPLEMENTARY INFORMATION

Monodispersity of magnetic immuno-nanoprobes enhances the detection sensitivity of low abundance biomarkers in one drop of serum

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1. Materials and Methods

1.1 Reagents and Materials

All chemicals were used as received. 1,2-hexadecanediol (97%), Oleic Acid (99%), 2-aminoethanethiol hydrochloride (98%), cyclohexane (99%), IGEPAL CO-520 (M_n 441), Suberic acid bis(N-hydroxy-succinimide ester (DSS, ≥ 95%), and Chloroform (≥99%) were obtained from Sigma; NH_4OH (28-30%), Dextran (M_r 15,000-25,000), Hexane (95%), from Sigma-Aldrich; (3-aminopropyl)trimethoxysilane (APS, 97%), ethanolamine (≥ 99.5%) from Aldrich; tetraethyl orthosilicate (TEOS, ≥ 98%), Phenyl ether (99%), Benzyl ether (99%), oleylamine (80-90%), 3-aminophenylboronic acid monohydrate (98%) from Acros Organics; dimethyl sulfoxide (DMSO) from Thermo Scientific; Gold Acetate ($Au(OOCH_3)_3$, 99.9%) from Alfa Aesar and iron(III) acetylacetonate ($Fe(acac)_3$ 99%) from Strem Chemicals Inc. Methoxyethyleneglycol (MEG) was provided by Chun-Cheng Lin of National Tsing Hua University. Standard C-Reactive Protein (CRP) was obtained from Calbiochem and monoclonal Anti-CRP antibody was obtained from Meridian Life Science.

All aqueous solutions and reagents used in the synthesis were prepared using deionized water purified by Milli-Q® reverse osmosis system unless otherwise stated.

1.2 Synthesis of Gold-coated Magnetic Nanoparticles (MNP@Au)

Gold-coated magnetic nanoparticles (*MNP@Au*) were synthesized based on a modified protocol.[1,2] Briefly, a mixture composed of $Fe(acac)_3$ (0.71 g) in phenyl ether (20 mL), oleic acid (2 mL) and oleylamine (2 mL) was stirred under nitrogen environment. Addition of 1,2-hexadecanediol (2.58 g) was carried out and the mixture was heated to reflux (210 °C) for 2 hours. After cooling to room temperature, the phenyl ether reaction solution containing Fe_3O_4

was subsequently used without any purification as seeds for the gold coating process. The nanoparticle mixture (10 mL, ~0.33 mmol Fe_3O_4) was mixed with $Au(OOCH_3)_3$ (0.83 g), 1,2-hexadecanediol (3.1 g), oleic acid (0.5 mL), oleylamine (3 mL) and phenyl ether (30 mL) and was heated to 190^{o}C for 1.5 hours. After cooling to room temperature, the mixture was precipitated by the addition of ~20 mL ethanol and isolated by centrifugation. The particles were washed with ethanol and re-dispersed in hexane. This process was repeated two times. After final washing with ethanol, the gold-coated magnetic nanoparticles was separated from the non-magnetic gold nanoparticles using a magnet and the final pellet was dispersed in hexane with ~75 mM oleic acid and oleylamine to produce MNP@Au.

1.3 Synthesis of NH_2-MNP@Au

NH_2-MNP@Au nanoparticles were fabricated based on the modified protocol.[4,5] An ethanol solution of 2-aminoethanethiol was prepared by dissolving 2-aminoethanethiol (200 mg) in 90% ethanol (6 mL). To this, MNP@Au (10 mg) dispersed in chloroform (5 mL) was added and was stirred for 2 hours. The nanoparticles were magnetically isolated and washed with 80% ethanol three times. The final nanoparticles were dried in vacuum and stored until needed.

1.4 Synthesis of NH_2-MNP@IGEPAL

Amine-modified nanoparticles using the IGEPAL CO-520 surfactant were prepared by adopting the reported protocol with some modifications.[3] The crude phenyl ether Fe_3O_4 mixture obtained in *1.2* was purified by precipitating with ethanol (~20 mL) and isolated via centrifugation (7500 rpm, 15 minutes). The nanoparticle pellets were dispersed with hexane in the presence of oleic acid (0.05 mL) and oleylamine (0.05 mL) and subsequent centrifugation (6000 rpm, 10 minutes)

was carried out to remove any undispersed residue. Washing and centrifugation was repeated two more times and final dispersion of the nanoparticle with hexane (~10 mL) was carried out. Then, an equivalent amount (1 mg) of dried nanoparticle was dispersed in cyclohexane (1 mL) and was added to a previously sonicated (30 minutes) IGEPAL-cyclohexane mixture (1 mmol/10 mL cyclohexane). The mixture was stirred for 4 hours at room temperature. Then, NH_4OH (80 μL) and TEOS (60 μL) was sequentially added and the mixture was continuously stirred for 30 hours at room temperature. APS (50 μL) was added thereafter and stirring was continued for another 18 hours. The particles were then precipitated and washed with ethanol several times and then dried under vacuum until needed.

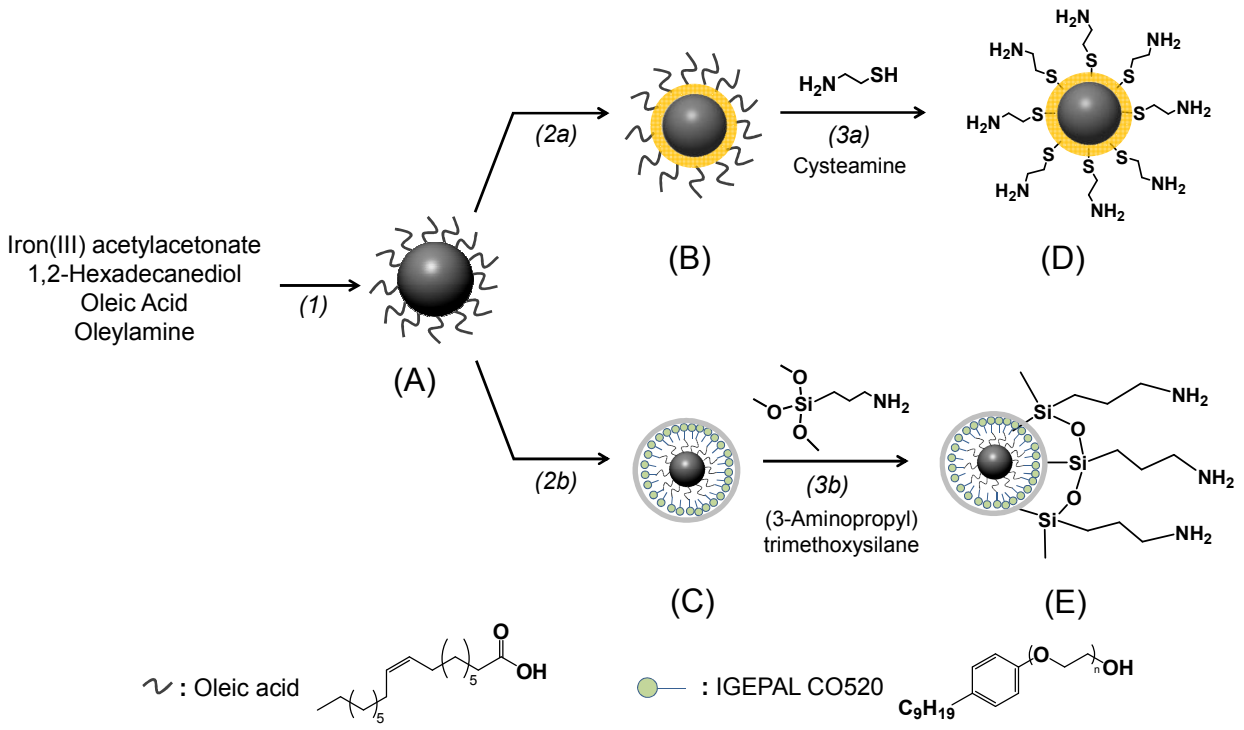

Figure S1. Synthesis of well-dispersed magnetic nanoparticles. (A) Core MNP (in hexane). (B) MNP@Au (in hexane). (C) MNP@IGEPAL. (D) NH_2-MNP@Au. (E) NH_2-MNP@IGEPAL. The steps are as follows: (1) Iron precursor together with the reducing agent and the capping ligand were decomposed at higher temperature (210^{o}C) via thermal decomposition method. The nanoparticles obtained (dispersible in hexane) were coated with both Au metal and surfactant (IGEPAL) in 2a and 2b, respectively. Lastly, amine functionality was afforded by ligand exchange with cysteamine for MNP@Au (3a) and functionalizing with aminosilane for MNP@IGEPAL (3b).

1.5 Synthesis of NH_2-MNP_{CP}

Core magnetic nanoparticles were prepared via co-precipitation method using $FeCl_2$ and $FeCl_3$ under basic condition. Amine-modified magnetic nanoparticles were produced as described from our previous report.[6-8] Briefly, the nanoparticles (50 mg) were dissolved in 25% 1-propanol solution (10 mL) and sonicated for 30 minutes. The mixture was transferred to a pre-heated silicon bath (~55 ^oC) and NH_4OH (20.8 mmol) and TEOS (1 mmol) was sequentially added and stirred continuously for 4 hours. APS (1 mmol) was then added and incubated overnight. The particles were then isolated by centrifugation, washed with 1-propanol three times, then with water three times, and the residue dried in vacuum and stored for further use.

1.6 Fabrication of boronic acid-oriented antibody nanoprobe (Ab@MNP)

Amine-functionalized magnetic nanoparticles (NH_2-MNP@Au, NH_2-MNP@IGEPAL, NH_2-MNP_{CP}, 2 mg), DSS (10 mg) and DMSO (250 μL) were mixed and sonicated for 30 minutes with occasional shaking every 10 minutes. Vigorous vortexing for 30 minutes and incubation for 6 hours was carried out. The nanoparticle mixture was then washed with DMSO three times. 3-aminophenylboronic acid (200 μL, 50 mM) was then added and incubated for 12 hours at 4^{o}C. The supernatant was removed by magnetic separation before the addition of ethanolamine (200 μL, 100 mM). The mixture was then further incubated for additional 6 hours at 4°C and washed five times with water. Then, antibody (200 μg) dissolved in PBS buffer (200 μL) was added and was incubated further for 12 hours at 4^{o}C. The nanoparticles were then washed with PBS buffer three times followed by the addition of dextran (40 μL, 2.5 mg/mL) and incubation for 6 hours at 4°C. Washing with PBS was finally carried out and the nanoparticles were dispersed to a final concentration of 10 mg/mL.

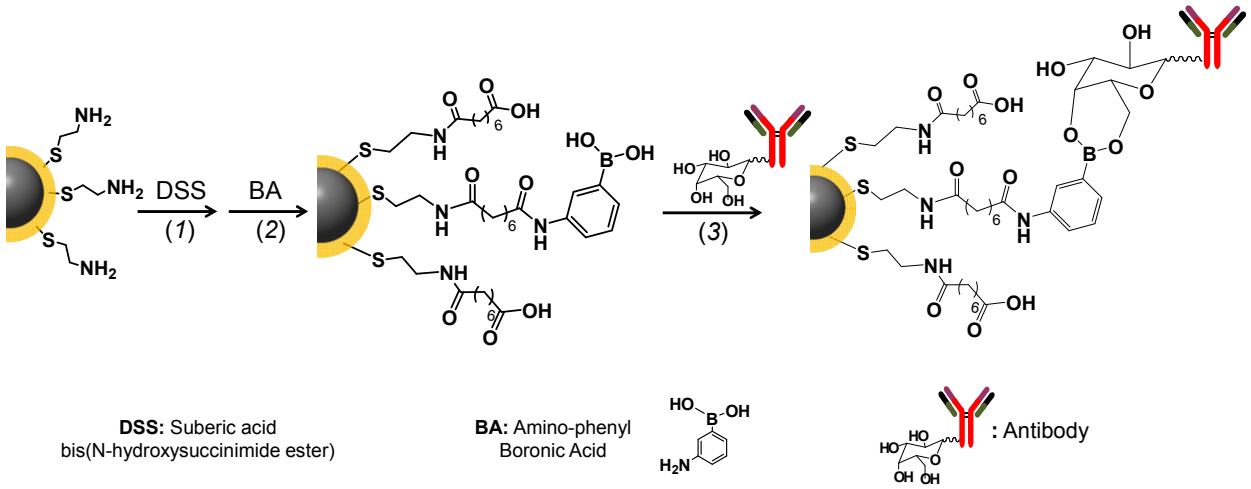

Figure S2. Fabrication of boronic acid-oriented antibody-conjugated nanoprobe: (1) amine-modified MNP (2 mg), DSS (10 mg), DMSO (250 µL), 4°C, 6 hours; (2) BA (200 µL, 50 mM), 4°C, 12 hours followed by ethanolamine (200 µL, 100 mM), 4°C, 6 hours; (3) CRP-antibody (200 µg in 200 µL PBS), 4°C, 12 hours. The boronic acid moiety is expected to bind the carbohydrate chain in the antibody as shown.

1.7 Antibody density measurement

The amount of protein conjugated in the nanoparticle was measured using Bicinchoninic acid (BCA) protein assay kit (Pierce^{TM}). BSA standards and known amount of Ab@MNP were prepared and reacted with the BCA reagents at 60°C for 30 minutes. Protein quantification was carried out by constructing a calibration curve based on absorbance (at 562 nm wavelength) of various amounts of BSA standards. The nanoparticles were magnetically isolated before UV measurement of the supernatant was conducted. Calculation of the protein amount was obtained after subtracting with the blank sample (MNP without antibody) and comparing the measured absorbance with the standard curve.

1.8 Characterization

Samples for transmission electron microscopy (TEM) analysis were prepared by depositing the dispersion (dilute hexane dispersion for the core Au@MNP) in an amorphous carbon-coated copper grid. Particle structure and selected area diffraction pattern (SAED) were scanned using High Resolution TEM (Philips Tecnai F20 G2 FEI-TEM, 400 kV). Magnetic property of the nanoparticles was measured using the Superconducting Quantum Interference Device (SQUID-VSM, Quantum Design, USA) and Infrared spectra by PerkinElmer Spectrum 100 (FTIR-Spectrometer). Malvern Zetasizer NanoZS Dynamic Light Scattering (DLS) was used to measure the hydrodynamic diameter and the polydispersity index (PDI). Elemental analysis of the core and amine-functionalized nanoparticles was conducted using Energy Dispersive X-Ray Spectroscopy (EDX, Oxford Instruments, X-Max) by depositing a small amount of sample in a carbon tape-covered sample holder. Likewise, crystal structure of the nanoparticles was

monitored by Multi Function High Power X-ray Diffractometer (Bruker R8 Discover SSS) by scanning from (2θ) 20 to 80 degrees.

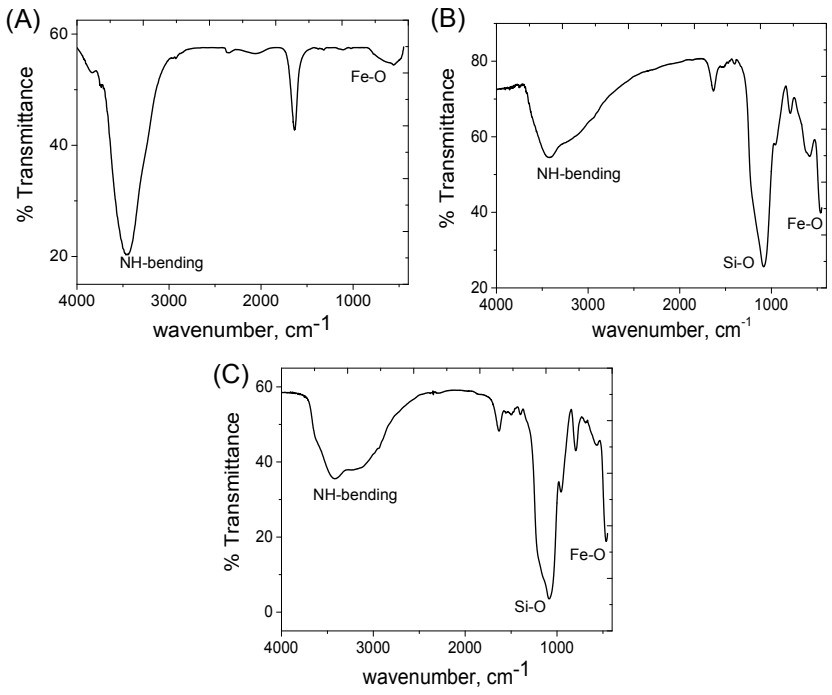

Figure S3. IR spectra of NH_2-modified magnetic nanoparticles (A) NH_2-MNP@Au. (B) NH_2-MNP@IGEPAL. (C) NH_2-MNP_{CP}.

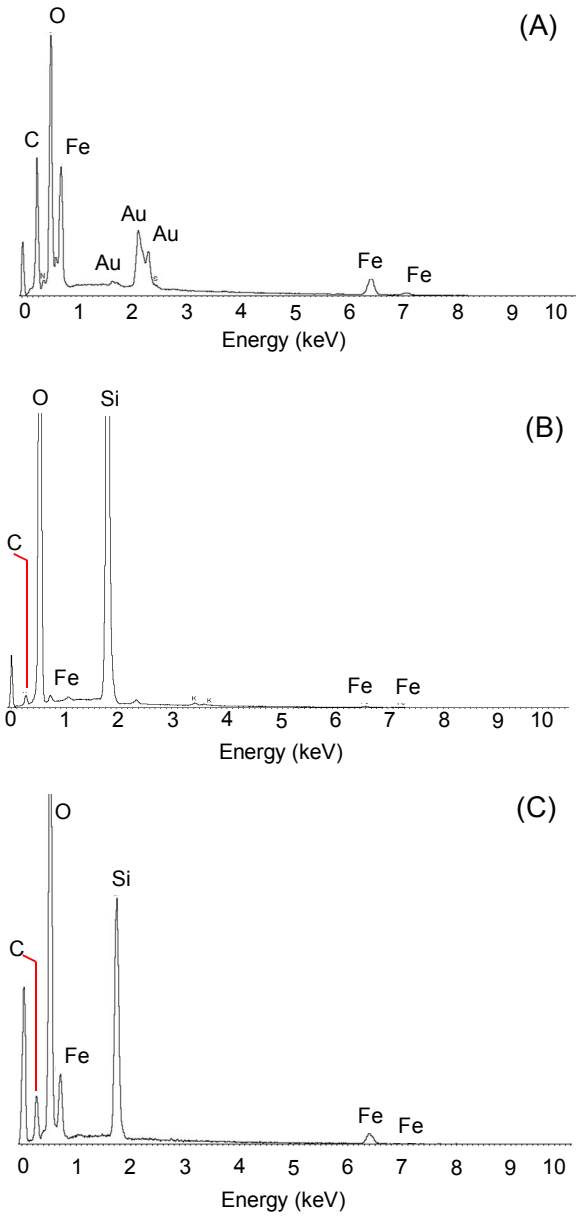

Figure S4. Energy Dispersive X-ray spectra of NH_2-modified magnetic nanoparticles (A) NH_2-MNP@Au. (B) NH_2-MNP@IGEPAL. (C) NH_2-MNP_{CP}. The results suggest that all of the expected elements were detected in all of the three nanoparticles. Silica (Si) peak for both MNP@IGEPAL and MNP_{CP} showed the highest count, as a confirmation of the formation of a silica shell on the surface of the nanoparticles.

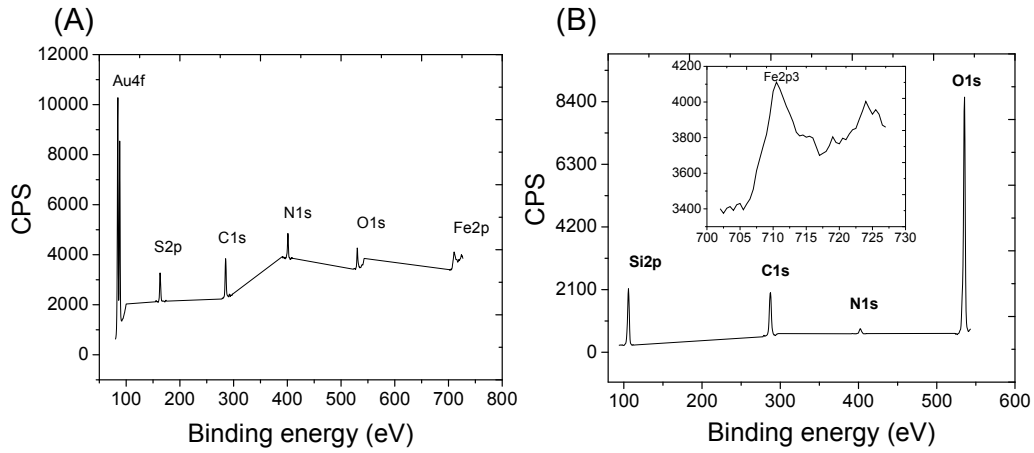

Figure S5. X-ray Photoelectron Spectra obtained from NH_2-modified magnetic nanoparticles: (A) NH_2-MNP@Au and (B) NH_2-MNP@IGEPAL. The spectra showed the peaks (in CPS) for the corresponding elements detected in the nanoparticles. Inset for (B): the spectrum for the Fe element showing a slight shift from the theoretical Fe peak value signifying the formation of Fe-O.

1.9 Aggregation and Sedimentation Experiment

The sedimentation profiles of nanoparticles in different media were obtained by measuring the optical absorbance as a function of time at wavelength of 508 nm.[9-13] Following approaches presented previously,[9,11] the different sedimentation rates were obtained by fitting the sedimentation profile to the exponential decay function:

$$A = A_o e^{-t/\tau}$$

where A is the concentration of the sedimenting phase at time t, A_0 is the initial particle concentration at the beginning of the sedimentation region and τ is the characteristic decay time.

The decay time was obtained after fitting the exponential decay curve using OriginLab® (V8) software. The obtained decay time (τ) was used for the quantitative evaluation of the settling behavior of the nanoparticle suspension; a large τ value signifies the slow drop in normalized absorbance (A/A_o) implying that the nanoparticles do not settle very fast. Table S1 below shows the detailed summary of the fitting parameters in the determination of the characteristic decay time (τ).

Table S1. Summary of fitting parameters in the determination of the estimated decay time (τ)

MNP	Concentration (mg/mL)		Decay time (τ, s)			
	PBS	Serum*	PBS	R^{2**}	Serum	R^{2**}
NH_2-MNP@Au	0.02	0.2	4095	0.9297	6590	0.9920
NH_2-MNP@IGEPAL	0.02	0.2	3195	0.8342	4506	0.9702
NH_2-MNP_{CP}	0.02	0.2	708	0.8854	570	0.9918

**0.2 mg MNP dispersed in a PBS-diluted serum (1:1 dilution) with total volume of 1 mL.*
***R^2 after fitting*

1.10 Human serum samples

Healthy human serum samples were obtained with informed consent from Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung, Taiwan.

1.11 Comparison of analytical performance of magnetic immuno-assays for CRP detection

Literature	Detection method	LOD	RSD	Recovery
Bor Fuh, C. *et al.* (*Analyst*, **2014**, *139*, 5576-5581)	Fluorescent magnetic immuno-assay	1.0 ng/mL	5.5% (average)	91.5% at 3.0×10^{-10}M
Bor Fuh, C. *et al*. (*Anal. Chem.* **2007**, *79*, 8416-8419)	Magnetic-immunoassay in a channel (particle counting)	0.12 μg/mL	1.2%	None
Liu, M. Y. *et al.* (*J. Chromatogr. A*, **2013**, *1315*, 188-194)	Capillary Zone Electrophoresis with Laser-Induced Detection	9.2 μg/mL	None	None
Chen, Y. J. *et al.* (*Anal. Chem.* **2005**, *77*, 5990-5997)	Nanoprobe-based Affinity Mass Spectrometry	None	None	None
This work				
MNP@Au	Nanoprobe-based Affinity Mass Spectrometry	1.0 ng/mL*	5.10%	61%
MNP@IGEPAL		4.2 ng/mL*	9.60%	49%
MNP_{CP}		2.85 ng/mL*	9.30%	48%

**Criteria : S/N=3*

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