Functional Magnetic Nanoparticle-Based Label Free Fluorescence Detection of Phosphorylated Species

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Electronic Supplementary Information

Experimental Section

Materials and Reagents. Acetonitrile and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Adenosine, adenosine 5'-triphosphate disodium salt, angiotensin I, bovine serum albumin (BSA), bradykinin, carbonic anhydrase, α-casein (from bovine milk), β-casein (from bovine milk), cytochrome C, dithiothreitol (DTT), D-glucose-6-phosphate dipotassium salt hydrate, IgG (from human serum), iodoacetic acid (IAA), monophosphopeptide (from β-casein), O-phospho-L-serine, O-phospho-L-threonine, O-phospho-L-tyrosine, riboflavin 5'-monophosphate sodium salt, L-serine, L-threonine, tetraphosphopeptide (from β-casein), trypsin (from bovine pancreas, TPCK treated), tetramethylammonium hydroxide pentahydrate, trypsinogen, L-tyrosine, and ubiquitin were obtained from Sigma (St. Louis, MO). Aluminum isopropoxide, α-cyano-4-hydroxycinnamic acid, and 2,5-dihydroxybenzoic acid were obtained from Aldrich (Steinheim, Germany). Ammonium hydrogen carbonate, D-glucose, iron (III) chloride hexahydrate, phosphoric acid, sodium sulfite, trifluoroacetic acid, and urea were obtained from Riedel-de Haën (Seelze, Germany). Ammonium hydroxide was purchased from Fluka (Buchs, Switzerland), and sodium silicate was obtained from J. T. Baker (Phillipburg, NJ). Human serum samples donated by healthy individuals were obtained from Mackay Memorial Hospital (Hsinchu, Taiwan).

Preparation of the Fe_3O_4 MNPs. The preparation of Fe_3O_4 MNPs has been

described elsewhere. Briefly, iron(III) chloride ($FeCl_3 \cdot 6H_2O$, 6.48 g) was dissolved in hydrochloric acid (2 M, 12 mL) and then diluted with 100 mL water. The mixture was degassed using a vacuum pump. Sodium sulfite (0.08M, 50 mL) was slowly added to the above solution under nitrogen with stirring at room temperature. Subsequently, ammonia (5%, 45 mL) was slowly added to the reaction solution with vigorous stirring at room temperature. The mixture was left to react for 30 min in an oil bath maintained at 70° C. The MNPs were rinsed twice with deionized water (40 mL), followed by resuspension in aqueous tetramethyl ammonium hydroxide (25 mg/mL, 40 mL) under stirring for 1 h at room temperature. The MNPs were then magnetically isolated from the solution, rinsed twice with deionized water (40 mL), and re-suspended in deionized water (40 mL). The suspension was stored in a 4 °C refrigerator prior to use.

Preparation of the Silica-Immobilized Fe_3O_4 MNPs. The Fe_3O_4 MNP suspension (5 mg/mL, 40 mL) was sonicated for 1 h under nitrogen protection. Sodium silicate aqueous solution (0.6%, 40 mL, pH ca. 9) was added to the solution, which was then vortex-mixed for 24 h at 35° C. The MNPs were rinsed three times with deionized water (40 mL), re-suspended in deionized water (40 mL), and sonicated for 30 min.

Preparation of the Fe_3O_4 MNPs Coated with Alumina (Fe_3O_4@Al_2O_3 MNPs). Aluminum isopropoxide (15 mg) was added to the above solution (40 mL), followed by sonication (300 W, 40 kHz) for 30 min at room temperature. The mixture was allowed to react in an oil bath maintained at 80 °C with vigorous stirring for 1 h, and then the cap of the vial was opened to remove the generated gas (*i.e.,* 2-propanol). The mixture was reacted at 90 °C for 30 min and then refluxed at 90 °C for another 2 h. After the mixture was cooled to room temperature, the alumina-coated Fe_3O_4

MNPs (Fe_3O_4@Al_2O_3 MNPs) were isolated, rinsed with water (40 mL × 3), re-suspended in water, and stored in a 4 °C refrigerator before use.

Examination of the Binding Constant of RFMP toward Fe_3O_4@Al_2O_3 MNPs. Fe_3O_4@Al_2O_3 MNPs and RFMP (10^{-7} ~ 2 × 10^{-5} M) were prepared in a buffer *A* (pH 4.5) containing TFA (0.075%) and ammonium hydrogen bicarbonate (50 mM) with a volume ratio of 9/1. The MNPs in the suspension (10 mg/mL, 5 μL) were isolated by magnetic separation, followed by the addition of RFMP prepared in buffer *A* (1 mL). The mixture was vortex-mixed for 2 h and then the MNPs were isolated by magnetic separation. The binding capacity of the MNPs toward RFMP was estimated by measuring the fluorescence intensity ($\lambda_{excitation}$= 450 nm, $\lambda_{emission}$= 530 nm) of the supernatant before and after magnetic trapping. Furthermore, to reduce the possibility of over-estimating the binding capacity, the isolated MNP–RFMP conjugates were rinsed repeatedly with buffer *A* (1 mL × 2). The fluorescence intensity at 530 nm ($\lambda_{excitation}$= 450 nm) of the rinse solution was recorded and taken into consideration as a nonspecific binding. The binding constant of RFMP toward the MNPs was determined by SigmaPlot (version 10) based on Langmuir adsorption equation.

Determination of Binding Capacity of RFMP toward Fe_3O_4@Al_2O_3 MNPs (RFMP–Fe_3O_4@Al_2O_3 MNPs). To accelerate the binding of RFMP onto the surfaces of Fe_3O_4@Al_2O_3 MNPs, the binding process was performed in a microwave oven (power: 450 W). The MNPs (10 mg/mL, 5 μL) prepared in buffer *A* were isolated by magnetic separation and then added with RFMP prepared in buffer *A* (1 mL) under microwave-irradiation for 60 s, while the cap of the sample vial was kept open. The binding amount of RFMP onto the MNPs was estimated by measuring the fluorescence intensity ($\lambda_{excitation}$= 450 nm, $\lambda_{emission}$= 530 nm) of the RFMP solution

obtained before and after interacting with the MNPs. To reduce the possibility of over-estimating the binding amount, the isolated MNP–RFMP conjugates were repeatedly rinsed with buffer *A* (1 mL × 2). The fluorescence intensity at 530 nm ($\lambda_{excitation}$= 450 nm) of the rinse solution was recorded and taken into consideration as nonspecific binding. The binding amount was estimated based on an RFMP calibration curve.

Preparation of Human Serum Samples. Serum samples were obtained from healthy individuals and stored in a freezer at -80 ^{o}C before use. When performing the experiment, the serum samples were diluted 10-fold by aqueous ammonium hydrogen bicarbonate (50 mM) and then mixed with 0.075% TFA with a ratio of 1/9 (v/v).

Using RFMP–Fe_3O_4@Al_2O_3 MNPs as Sensing Probes. The RFMP–Fe_3O_4@Al_2O_3 NPs (50 μg) obtained above were re-suspended in buffer *A* (50 μL) and placed in a microwave oven (power: 450 W) for 60 s. After cooling to room temperature, 3 μL of the suspension was mixed well with sample solutions (50 μL). The cap of the sample vial was kept open during incubation in a microwave oven (power: 900 W) for a given time (10~60 s). The fluorescence intensity at 530 nm ($\lambda_{excitation}$= 450 nm) of the solution isolated from the suspension was recorded. Blank control experiments were performed in parallel by reacting the RFMP–Fe_3O_4@Al_2O_3 NP suspension (3 μL) with buffer *A* solution (50 μL) alone without containing any samples. Adenosine, adenosine 5'-triphosphate disodium salt, D-glucose, D-glucose-6-phosphate dipotassium, O-phospho-L-serine, O-phospho-L-threonine, L-tyrosine, O-phospho-L-tyrosine, L-serine, L-threonine, monophosphopeptide (FQsEEQQQTEDELQDK, "s" standards for phosphorylated serine), tetraphosphopeptide (RELEELNVPGEIVEsLsssEESITR), trypsinogen, angiotensin I,

tryptic digests of proteins, BSA, bradykinin, carbonic anhydrase, α-casein, β-casein, cytochrome C, IgG, ubiquitin, and human serum prepared in aqueous ammonium hydrogen bicarbonate (50 mM) were used as the samples for examining the feasibility of this approach. The samples were diluted with 0.075% TFA at a ratio of 1/9 (v/v) before analysis. All the proteins were denatured prior to analysis. That is, proteins (2×10^{-5} M) were initially prepared in aqueous ammonium hydrogen bicarbonate (50 mM, 0.1 mL) containing urea (8 M). The protein samples were placed at 38 °C for 30 min followed by reacting with DTT (50 μL, 100 mM) in aqueous ammonium hydrogen bicarbonate (50 mM) at 50 °C for 1 h. After cooling to room temperature, the mixture was added with IAA (50 μL, 200 mM) prepared in aqueous ammonium hydrogen bicarbonate (50 mM) in an ice bath for 2.5 h. The sample vial was wrapped with aluminum foil during reaction. After reaction, the protein samples were diluted by 0.075% TFA with a volume ratio of 1/9. Blank control experiment was performed by reacting the RFMP–Fe_3O_4@Al_2O_3 MNPs with the mixture of denatured buffer, which did not contain protein/0.075% TFA (1/9, v/v), under microwave-irradiation (power: 900 W) for 60 s.

MALDI-MS Analysis. Target species trapped by RFMP-Fe_3O_4@Al_2O_3 MNPs obtained above were characterized by MALDI-MS by mixing with the matrix containing 2,5-DHB (15 mg/mL) and CHCA (15 mg/mL) prepared in acetonitrile/deionized water (2/1, v/v) containing 1% phosphoric acid. After evaporation of solvent, the samples were ready for MALDI MS analysis.

Instrumentation. Fluorescence spectra were obtained using a Horiba Jobin Yvon FluoroMax-3 spectrophotometer (NJ, USA). Mass spectra were obtained using a Bruker Daltonics Autoflex III MALDI mass spectrometer (Germany) equipped with a 355-nm Nd:YAG laser.

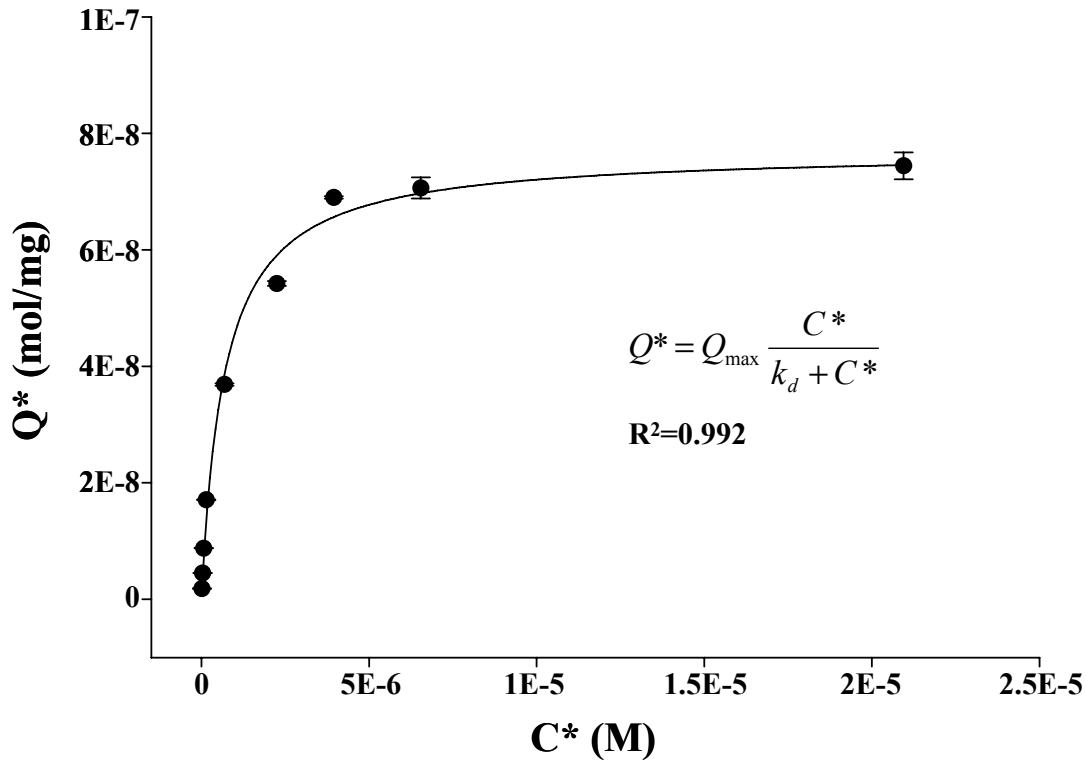

Figure S1. Langmuir adsorption plot obtained from the binding behavior of RFMP toward the Fe_3O_4@Al_2O_3 MNPs. C* stands for the initial concentration of RFMP when incubating with the MNPs, while Q* stands for the binding amount of RFMP on the surface of Fe_3O_4@Al_2O_3 MNPs at different initial concentrations of RFMP.

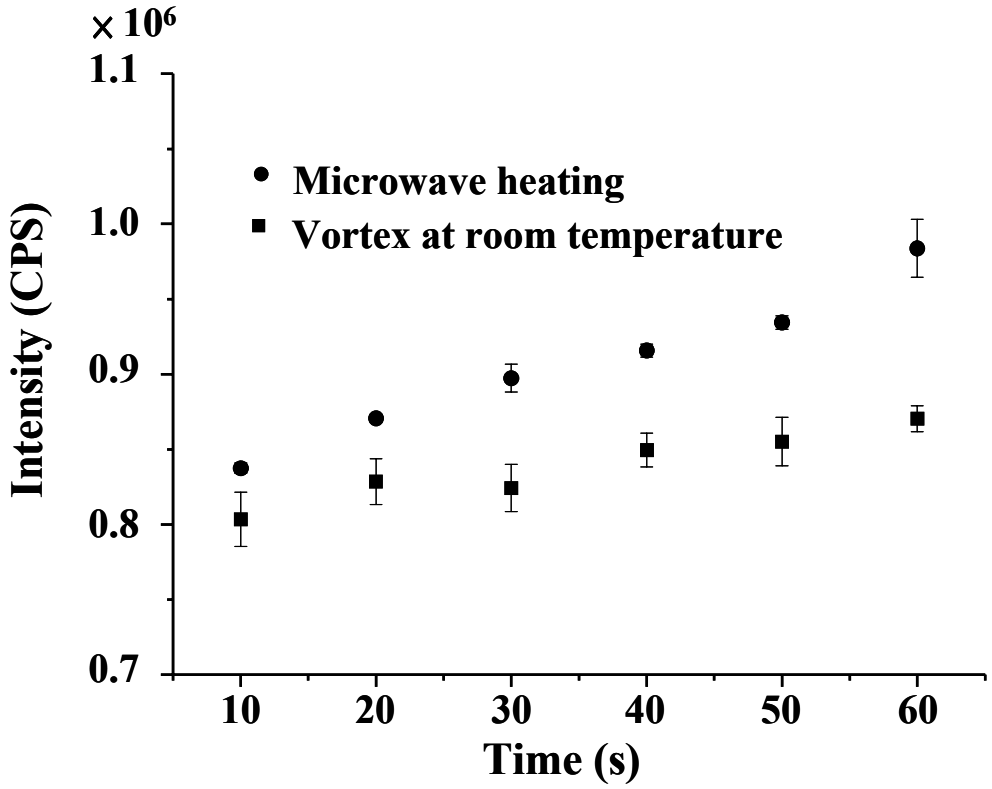

Figure S2. Plots of the fluorescence intensity at the wavelength of 530 nm ($\lambda_{excitation}$ = 450 nm) obtained from the samples (50 μL) containing monophosphopeptide sample (FQsEEQQQTEDELQDK) (10^{-6} M) incubated with the RFMP–Fe_3O_4@Al_2O_3 MNPs (1 mg/mL, 3 μL) under microwave-heating (power: 900 W) (marked with circles) and vortex-mixing at room temperature (marked with squares) as a function of time.

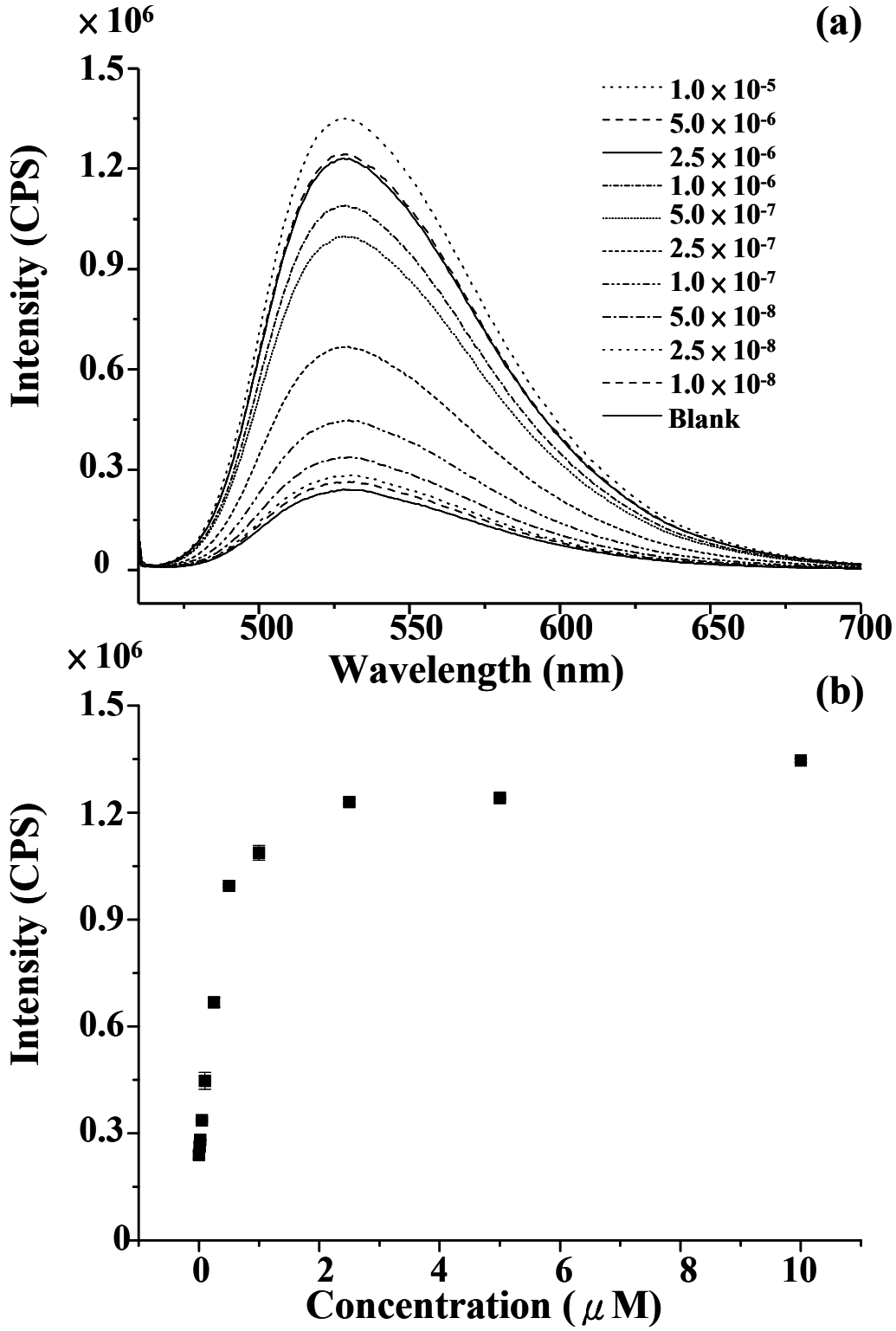

Figure S3. (a) Representative fluorescence spectra ($\lambda_{excitation}$= 450 nm) obtained from the solutions that were separated from the samples (50 μL) containing the monophosphopeptide (FQsEEQQQTEDELQDK) at different concentrations incubated with the RFMP–Fe_3O_4@Al_2O_3 MNPs (3 μL, 10 mg/mL) under microwave-heating (power: 900 W) for 60 s. (b) Plot of the fluorescence intensity at the wavelength of 530 nm as a function of the concentration of the monophosphopeptide obtained from the results shown in Panel a.

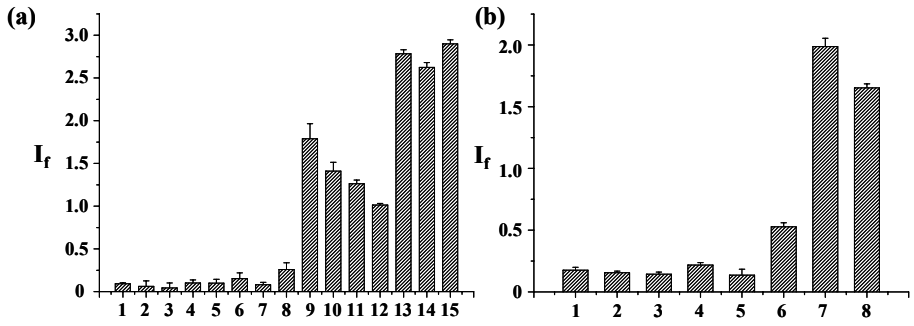

Figure S4. (a) Bar graph obtained from the fluorescence intensity at $\lambda_{emission}$= 530 nm ($\lambda_{excitation}$= 450 nm) contributed by RFMP in the solutions separated from the samples (10^{-6} M, 50 μL), including adenosine (1), L-serine (2), L-threonine (3), L-tyrosine (4), D-glucose (5), bradykinin (6), angiotensin I (7), the tryptic digest of cytochrome C (8), O-phospho-L-serine (9), O-phospho-L-threonine (10), O-phospho-L-tyrosine (11), D-glucose-6-phosphate (12), adenosine 5'-triphosphate (13), monophosphopeptide (14), and tetra-phosphopeptide (15) incubated with the RFMP–Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg/mL) under microwave-heating (power: 900 W) for 60 sec. I_f is equal to $(I_s-I_0)/I_0$. I_s stands for the intensity of the RFMP molecules released from the MNPs, while I_0 represents the fluorescence intensity of the background from the solution of the RFMP–Fe_3O_4@Al_2O_3 MNP suspension after isolation of the MNPs. (b) Bar graph obtained from the fluorescence intensity at $\lambda_{emission}$= 530 nm ($\lambda_{excitation}$= 450 nm) contributed by RFMP in the solutions separated from the protein samples (10^{-6} M, 50 μL) including ubiquitin (1), cytochrome C (2), trypsinogen (3), carbonic anhydrase (4), BSA (5), IgG (6), α-casein (7), and β-casein (8), incubated with the RFMP–Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg/mL) under microwave-heating (power: 900 W) for 60 sec.