Quantitative Mass Spectrometry Combined with Separation and Enrichment of Phosphopeptides by Titania Coated Magnetic Mesoporous Silica Microspheres for Screening of Protein Kinase Inhibitors

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Supporting Information

1. Experimental Section
2. Figures S1-S3: Schematic illustration of the synthesis strategy and characterization of TiO_2/MHMSS materials
3. Figure S4: Mass spectrum for a S4 for a peptide mixture containing SP2, PS2 and IS2 at a molar ratio of 20:1:1
4. Figure S5: calibration curve for MS quantification

EXPERIMENTAL SECTION

Synthesis of EGFR inhibitors. Three potential EGFR inhibitors were synthesized in this work. The quinazoline derivative 6-(2-(1H-imidazol-1-yl)ethoxy)-4-(3'-chloro-4'-fluoroanilino)-7-methoxy-quinazoline (**1**) was synthesized following a procedure described in literature (Gibson, K. H.; Barker, A. J.; Grundy, W.; Godfrey, A. A.; Barlow, J. J.; Healy, M. P.; Woodburn, J. R.; Ashton, S. E.; Curry, B. J.; Scarlett, L.; Henthorn, L.; Richards, L. *Bioorg. Med. Chem. Lett.* 2001, *11*, 1911-1914) with slight modification. A mixture of 0.319 g (4.68 mmol) of imidazole, 18.9 mg (0.059 mmol) of tetrabuthylammonium bromide (TBAB), 0.281 g (7.03 mmol) of NaOH, and 20 mL of acetonitrile was heated under reflux with vigorous stirring for 1 h. Then the 6-(2-bromoethoxy)-4-(3'-chloro-4'-fluoroanilino)-7-methoxyquinazoline (1 g, 2.34 mmol), partially dissolved in 10 mL acetonitrile, was added dropwise to the mixture over a period of 30 min at 353-358 K with vigorous stirring. After the mixture had been heated at 358 K with vigorous stirring for 3 h, it was cooled to room temperature. The mixture was evaporated, and the residue was partitioned between ethyl acetate and water. Then the triturated residue was isolated, recrystallised from a 1:1 mixture of isopropanol and water and dried. There was thus obtained compound **1** (0.669 g, yield: 69%). The elemental analysis and spectroscopic data correspond to those reported in the literature.

The second quaniazoline derivative 6-(2-(2-aminoethylamino)ethoxy)-4-(3'-chloro -4'-fluoroanilino)-7-methoxy-quinazoline (**2**) was synthesized *via* a two-step pathway.

Firstly, a precursor compound 6-(2-bromoethoxy)-4-(3'-chloro-4'- fluoroanilino)-7-methoxyquinazoline (**L0**) was synthesized as follows. 4-(3'-chloro-4'-fluoroanilino)-6, 7-dimethoxyquinazoline (2.0 g, 6.3 mmol) and potassium carbonate (5.0g, 36mmol) were mixed together in DMF (30 mL). Then 1,2-dibromoethane (2 mL, 23 mmol) was added and the mixture was heated at 353 K for 4.5 h. After cooling down to room temperature, the mixture was filtered in vacuum and the filtrate was collected and poured into water (130 mL). Then the mixture was extracted using ethyl acetate (30 mL × 4), organic layers were combined and dried by magnesium sulfate. After concentration, the residue was separated by flash chromatography on Silica gel using ethyl acetate/petroleum (1:5, v/v) as eluent to give compound **L0** (0.45 g, yield: 16.7%) as white power.

Secondly, the precursor 6-(2-bromoethoxy)-4-(3'-chloro-4'-fluoroanilino)-7-methoxyquinazoline (0.8 g, 1.87 mmol) and ethylenediamine (1 mL, 15 mmol) were dissolved in acetonitrile (30 mL).The solution was heated to 353 K and refluxed for 2.5 h. Then the solvent was evaporated in vacuum and the residue was recrystallized from mixture of water and ethanol to give white powder of compound **2** (0.5 g). Yield: 65.8%. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.74 (s, 1H), 8.63 (s, 1H), 8.13 (m, 1H), 7.92 (s, 1H), 7.80 (m, 1H), 7.51 (t, 1H), 7.26 (s, 1H), 5.02 (m, 4H), 4.23 (m, 4H), 3.36 (m, 6H). ESI-MS *m/z* calculated 405.9, found 406.9 for $[M+H]^+$. Elemental analysis (%) calcd for $C_{38}H_{31}N_3O_2$: C 81.26, H 5.56, N 7.48; found: C 81.59, H 5.53, N 7.51.

To synthesize the ruthenium(II) complex $[Ru^{II}Cl_2(DMSO)_2(L)]$ (**3**, L= **2**), the as-prepared quinazoline derivative **2** (40.58 mg, 0.1 mmol) was added to

cis-$Ru^{II}C1_2(DMSO)_4$ (48.5 mg, 0.1 mmol) in dry ethanol (10 mL), and the mixture was heated at 353 K for 6 h. A good quantity of precipitate formed. The precipitate was filtered off, washed with ethanol and ether, and dried in a vacuum to give rise to compound **3**. Yield: 40 mg (55%). ESI-MS (positive): *m/z* 736.21 for $[Ru^{II}Cl_2(DMSO)_2(L)]^+$, 658.17 for $[Ru^{II}Cl_2(DMSO)(L)]^+$, 580.14 for $[Ru^{II}Cl_2(L)]^+$, 542.15 for $[Ru^{II}Cl(L)]^+$ and 506.17 for $[Ru^{II}(L)]^+$. ^{1}H NMR: (DMSO-d_6) δ(ppm): 9.49 (s, 1H), 8.48 (s, 1H), 8.11-8.09 (m, 1H), 7.85 (s, 1H), 7.81-7.77 (m, 1H), 7.45-7.40 (t, 1H), 7.20 (s, 1H), 4.42-4.38 (m, 2H), 4.33-4.30 (m, 1H), 4.24-4.21 (m, 1H), 3.92 (s, 3H), 3.88-3.83 (m, 1H), 3.44-3.37 (m, 1H), 3.29 (s, 6H), 3.23 (s, 3H), 3.12-3.11 (m, 8H). X-ray diffraction-quality crystals were grown by slow diffusion of diethyl ether into the DMSO/acetone (1:5) solutions of **2**, and will be published elsewhere.

Sample Preparation. For optimization of separation and enrichment conditions, 10 μL substrate peptide (SP) (100 μM) was mixed with 5 μL internal standard (IS) (10 μM) and 5 μL phosphorylated substrate (PS) (10 μM), and then 80 μL acidic solution (pH 2.5) was added to the mixture, such that the final concentrations of peptides were 10.0 (SP), 0.5 (IS) and 0.5 (PS) μM, respectively.

To generate the calibration curve for quantification of phosphorylated substrate, an aliquot (10 μL) of SP solution (180 μM) was mixed with 5 μL IS (3 μM) and 15 μL of different concentrations of PS, and then 20 μL 20% ACN/79.9%H_2O/0.1%TFA (v/v/v) was added to give rise to a series of standard samples containing 36 μM SP

and having a gradient molar ratio of PS to IS (0.3 μM): 0.1, 0.3, 0.5, 1, 2, 3, and 4 for enrichment of phosphopeptide using TiO_2/MHMSS followed by MS analysis.

For determination of IC_{50} values of EGFR inhibitors, an aliquot (12.5 μL) of solutions (H_2O/DMSO: 99%/1% (v/v)) containing various concentrations of a screened inhibitor was added to 12.5 μL DTT/kinase buffer (pH 7.5) containing EGFR kinase (7.8 U) and 26.5 ng EGF, and then incubated at room temperature (298 K) for 5 min, followed by addition of 25 μL ATP (0.4 mM)/substrate (SP2, 100 μM) solutions. The resulting mixture was left for reaction at 310 K for 1 h, and then incubated at 333 K for 15 min to stop the enzymatic reaction. Finally 20 μL internal standard (1.05 μM) in 90%ACN/9%H_2O/1%TFA were added to the reaction mixture for subsequent enrichment of phosphopeptide using TiO_2/MHMSS followed by MS analysis.

Enzyme-linked Immunosorbent Assay. Enzyme-linked immunosorbent assay (ELISA) was performed following the instruction provide by the supplier of the assay kits (Cell Signaling Technology, USA). An aliquot (12.5 μL) of solution of a screened inhibitor was mixed with EGFR solution (12.5 μL) and incubated at room temperature for 5 minutes, followed by addition of 25 μL of ATP/substrate mixture, and then the resulting mixture was incubated at 310 K for 1 h. The phosphorylation reaction was terminated by the addition of 50 μL/well stop buffer (50 mM EDTA, pH 8).

Each well of a microtitre plate was coated with 100 μL of 10 μg mL^{-1} streptavidin in carbonate-bicarbonate buffer and incubated overnight at 277 K, and then blocked

with 1.5% bovine serum albumin (BSA) in PBST solution at 310 K for 2 h, followed by three times of washing with PBST prior to use. Next, 25 μL/well of each enzymatic reaction mixture and 75 μL/well of D_2O were added to the wells (in triplicate) for incubation at 310 K for 1 h. Following three times of washing with PBST, 100 μL of primary antibody (Phospho-Tyrosine Mouse mAb, 1:1000 in PBST with 1.5% BSA) was added to each well and the plate was incubated at 310 K for another 1 h. The plate was again washed three times with PBST, then 100 μL of secondary antibody (HRP-labeled Goat Anti-Mouse lgG, 1:1000 in PBST with 1.5% BSA) was added to each well for 45 min of incubation at 310 K, followed by three times of washing with PBST. Finally, 100 μL of TMB substrate system was added to each well and the plate was incubated at 310 K for 15 min, and then the reaction was stopped by addition of 100 μL of 2 M H_2SO_4 to each well, and the plate was read on an ELISA plate reader (SpectraMaxM5, Molecular Devices Corporation, USA) at 450 nm to determine the OD values.

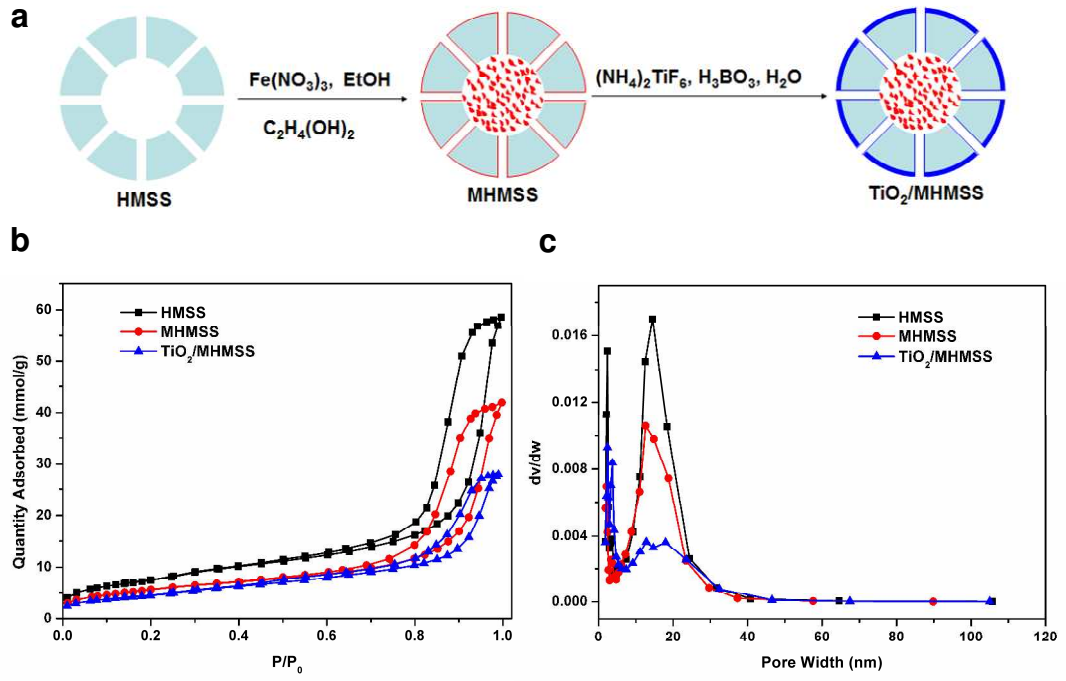

Figure S1. a) Schematic illustration of the synthesis strategy of TiO2/MHMSS; b) N2 adsorption-desorption isotherms and c) the pore diameter distributions of HMSS, MHMSS and TiO2/MHMSS materials.

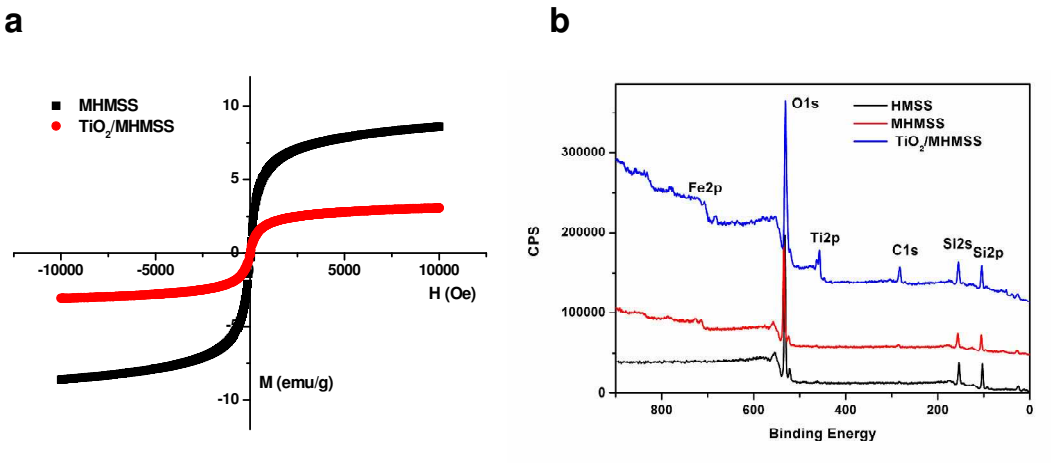

Figure S2. a) The magnetization curves of MHMSS and TiO2/MHMSS materials. b) XPS pattern of HMSS, MHMSS and TiO2/MHMSS materials.

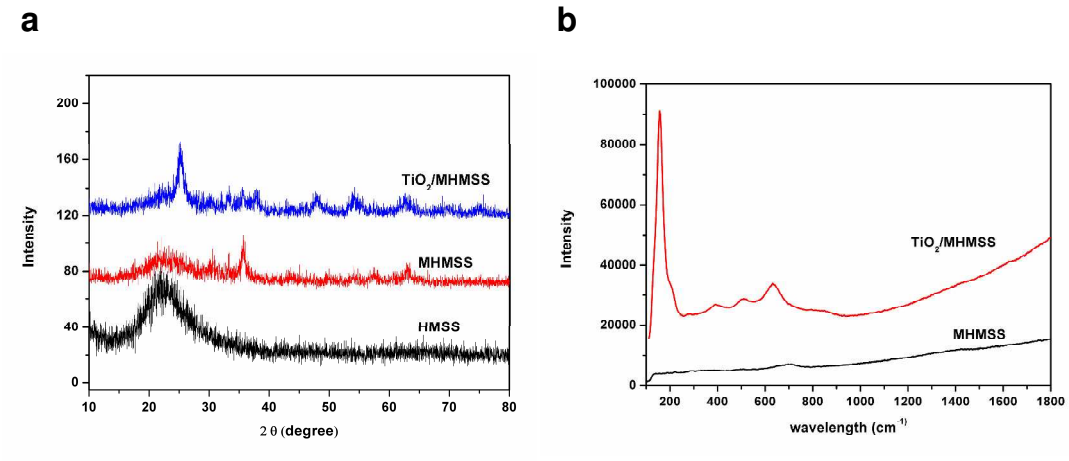

Figure S3. a) XRD profiles of HMSS, MHMSS and TiO2/MHMSS materials; b) Raman spectra of MHMSS and TiO2/MHMSS materials.

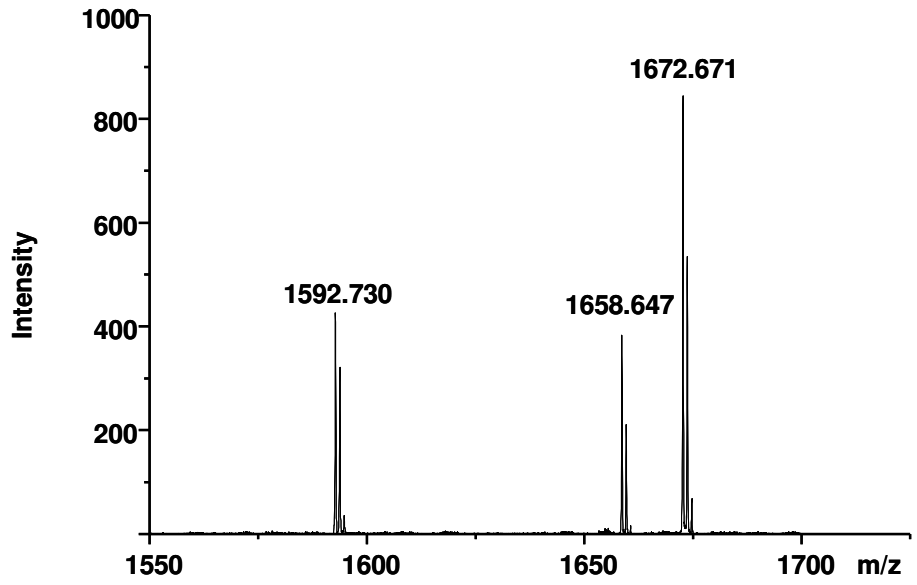

Figure S4. Mass spectrum for a peptide mixture containing EGFR substrate peptide SP2 at *m/z* 1592.730, the phosphorylated substrate PS2 at *m/z* 1672.671 and the internal standard phosphopeptide IS2 at m/z 1658.647 after pre-separation and enrichment using TiO2/MHMSS with loading in aqueous solution at pH 1.0 and washing by 20% ACN/79.9%H_2O/0.1%TFA at pH 1.0. [SP2] : [PS2] : [IS2] = 10 : 0.5 : 0.5 μM. It indicates that although non-specific binding of acidic groups in SP2 to TiO_2 was eliminated further as evidenced by the lower signal intensity of SP2 when the pH values of loading and washing solutions decrease from 2.5 down to 1.0, the MS signal intensity of the phosphopeptides PS2 and IS2 also slightly decreases and the TiO_2/MHMSS material shows more discrimination between PS2 and IS2.

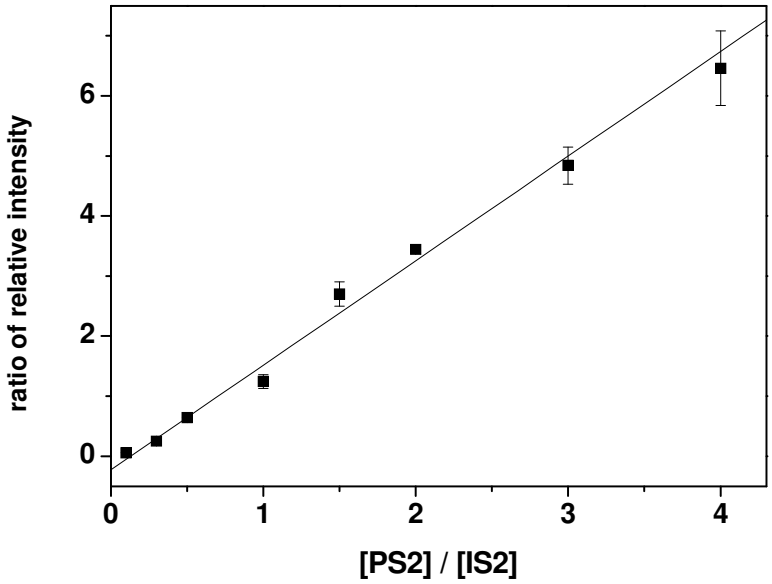

Figure S5. Calibration curve by plotting the ratio of signal intensities of phosphorylated substrate (PS2) to internal standard phophopeptide (IS2, 0.3μM) as function of the molar ratio of PS2 to IS2.