Electronic Supplementary Material (ESI) for Analyst
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Electronic Supplementary Information for

Bifunctional polymer brushes for low-bias enrichment of mono- and multi-phosphorylated peptides prior to mass spectrometry analysis

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1. Experimental details

Materials

Most chemicals and reagents were obtained from Sigma-Aldrich. Other chemicals and materials included *N*-hydroxysulfosuccinimide sodium salt (sulfo-NHS, ProteoChem), $FeCl_3$ (anhydrous, 98%, Spectrum), dimethylsulfoxide (DMSO, EMD), and acetonitrile (ACN, EMD). Tetrahydrofuran (THF, Mallinckrodt Baker) was distilled before use, and deionized water was obtained from a Millipore ion-exchange system (Milli-Q, 18 MΩ cm). Si wafers were Au-coated (200 nm of sputtered Au on 20 nm of sputtered Cr on Si wafers) by LGA Thin Films (Santa Clara, CA). Materials for protein sample preparation included sequencing grade modified porcine trypsin (treated with L-1-tosylamide-2-phenylethyl chloromethyl ketone, Promega), Tris-HCl (Invitrogen), urea (J.T. Baker), DL-dithiothreitol (Fluka), ammonium bicarbonate (Columbus Chemical) and trifluoroacetic acid (TFA, EMD).

Preparation of a bifunctional polymer and control polymers

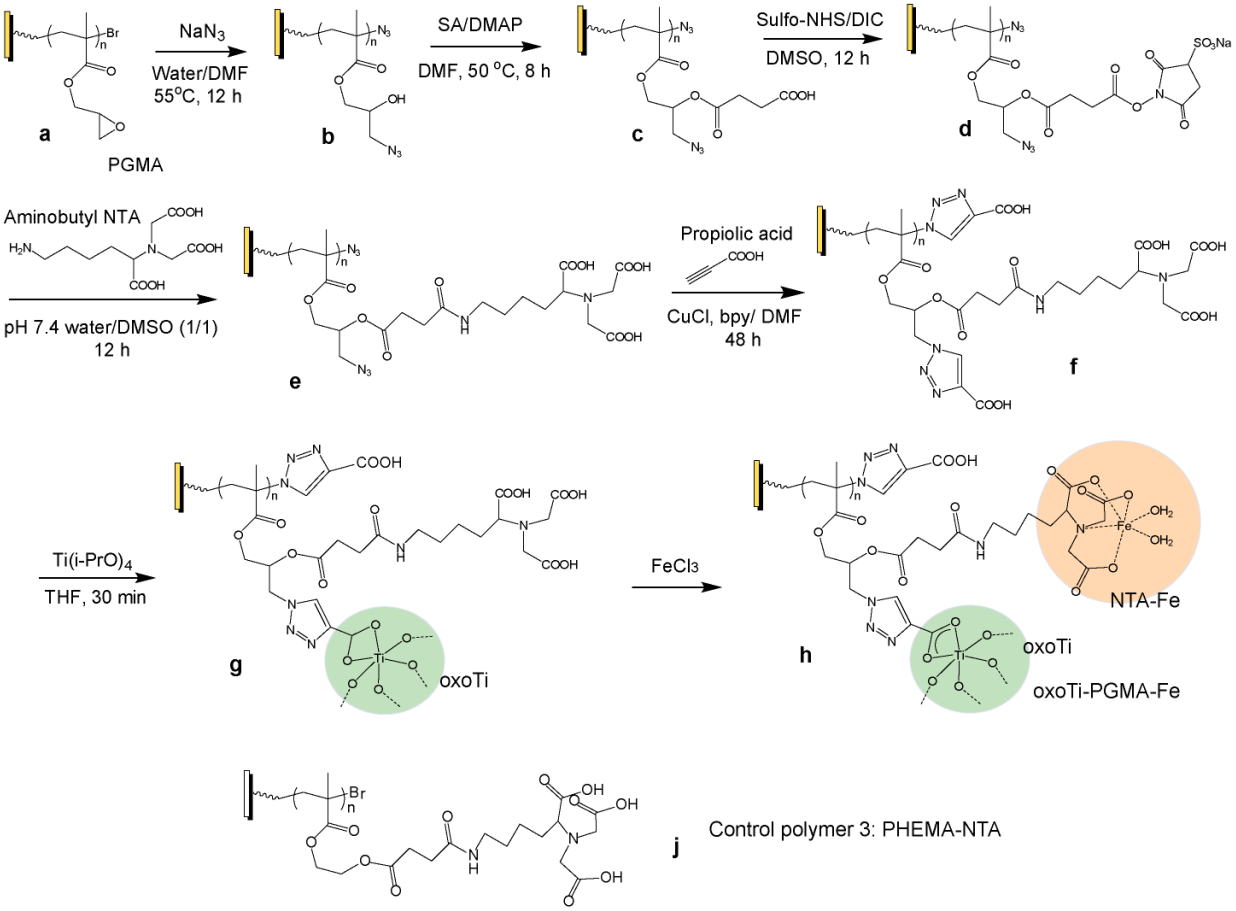

Fig. S1. (**a**-**h**) Scheme for orthogonal modifications of PGMA brushes to obtain a bifunctional structure, oxoTi-PGMA-Fe. (**j**) The structure of control polymer 3, PHEMA-NTA.

A disulfide initiator monolayer was deposited on a Au-coated Si wafer by immersing a cleaned wafer (UV/O_3 for 15 min) in an ethanol solution containing 1 mM $(BrC(CH_3)_2COO(CH_2)_{10}S)_2$ for 24 h followed by rinsing with ethanol (the disulfide initiator synthesis was described previously[1]) and drying. ATRP of glycidyl methacrylate (GMA) from the immobilized initiator followed a literature procedure.[2] Typically, a solution containing GMA (5.00 mL, 5.21 g, 36.7 mmol), methanol (4 mL), water (1 mL) and 2,2'-dipyridyl (141 mg, 0.904 mmol) was degassed with three freeze-pump-thaw cycles. CuCl (36.4 mg, 0.368 mmol) and

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$CuBr_2$ (3.9 mg, 0.017 mmol) were added to the frozen solution prior to three additional freeze-pump-thaw cycles. In a N_2-filled glove bag, the initiator-modified wafers were immersed in the degassed monomer/catalyst solution for 1 to 4 h to yield PGMA brushes with different thicknesses on the Au surface. The wafers were finally removed from the monomer/catalyst solution, rinsed with ethanol, and dried with N_2.

Fig. S1**a**-**h** shows the PGMA functionalization steps, which were accomplished by sequentially immersing the Au-PGMA wafer in the following solutions: (1) 1.5 M NaN_3 in water/DMF (1:1, v/v) at 50 °C for 24 h; (2) 0.1 M succinic anhydride (SA) and 0.16 M 4-dimethylaminopyridine (DMAP) in anhydrous DMF at 50 °C for 8 h; (3) 0.1 M sulfo-NHS and 0.1 M *N,N'*-diisopropylcarbodiimide (DIC) in DMSO for 12 h; (4) 0.1 M N_α,N_α-bis(carboxymethyl)-L-lysine hydrate (aminobutyl-NTA) in pH 7.4 water/DMSO (1:1, v/v) for 4 h (The solution was prepared by mixing aminobutyl-NTA with water and adding 0.1 M NaOH until the powder dissolved. Subsequently 0.1 M HCl was added to lower the solution pH to 7.4 prior to dilution with water and DMSO.); (5) DMF containing 0.1 M propiolic acid, 5 mM CuBr and 8.75 mM 2,2'-dipyridyl (the solution was degassed with three freeze-pump-thaw cycles) for 48 h, while stirring with a small magnetic bar near the wafer to promote mass transfer; (6) 0.1 M pH 7.4 EDTA for 30 min to remove any chelated Cu (I) or Cu (II) ions in the polymer followed by rinsing with 0.1% TFA containing 50% ACN to acidify the carboxylic acid; (7) 0.1 M Ti(*i*-PrO)$_4$ in anhydrous THF for 30 min followed by rinsing with anhydrous THF in the N_2-filled glove bag; (8) 0.1 M $FeCl_3$ for 30 min. Unless otherwise stated, each of the above steps ended with rinsing the wafer with the solvent employed for reaction and then with ethanol. The wafer was always dried with N_2 before the next step.

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Characterization of bifunctional polymers

Reflectance FTIR spectra (Fig. 2 in the paper) provide evidence for the synthesis and derivatization of PGMA to form bifunctional brushes. The ester carbonyl absorbance at 1740 cm^{-1} in Fig. 2a confirms the formation of PGMA (**a**, Fig. S1) on the Au-coated wafer. The antisymmetric N=N=N stretch at 2100 cm^{-1} and the broad hydroxyl stretch at 3100-3650 cm^{-1} in Fig. 2b reflect the generation of azide and hydroxyl groups (**b**, Fig. S1) after the reaction of **a** with NaN_3. The increases in the ester carbonyl absorbance at 1740 cm^{-1} and the appearance of an antisymmetric COO^- stretch at 1590 cm^{-1} (Fig. 2c, the film was deprotonated with 1 mM NaOH followed by rinsing with acetone prior to obtaining the spectrum) confirm the formation of a polyacid (**c**, Fig. S1) after reaction of SA with the hydroxyl group of **b**. We monitored the completeness of this step by occasionally removing the wafer from the reaction solution and examining the intensity of the antisymmetric COO^- stretch at 1590 cm^{-1}. We stopped the reaction when this absorbance ceased to increase with reaction time. The NHS ester peaks at 1825 cm^{-1} and 1793 cm^{-1} in Fig. 2d demonstrate the activation of the polyacid to **d**, and the disappearance of the succinimide ester peaks and the appearance of a broad signal around 1685 cm^{-1} in Fig. 2e provide evidence for derivatization with aminobutyl NTA (**e**, Fig. S1). The absorbance around 1685 cm^{-1} and its broad tail likely stem from the newly formed amide bond and the partially deprotonated carboxylic acids of NTA (the film was deprotonated in 0.1 mM NaOH and rinsed with acetone prior to obtaining the spectrum). The minimal residual N=N=N stretch at 2100 cm^{-1} in Fig. 2f suggests a nearly quantitative yield for the azide-alkyne cycloaddition (**f**, Fig. S1) between **e** and propiolic acid. When **d** is below 100 nm thick, the N=N=N stretch completely disappears after 24 h of reaction. Unfortunately, IR spectra do not

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show evident changes upon reaction of **f** with Ti(*i*-PrO)$_4$ and Fe^{3+} (data not shown). However, elemental analysis confirms the presence of Ti and Fe in the film (see below).

Ellipsometry was performed using a rotating analyzer spectroscopic ellipsometer (J. A. Woollam, M-44, assuming a film refractive index of 1.5) to determine the polymer film thickness (dry form). The film thickness generally increases after each derivatization step, and thickness values are noted in Fig. 2 of the paper. The only exception is the substitution of sulfo-NHS with aminobutyl NTA, which leads to a slight drop in thickness. However, the thickness of structure **e** (Fig. S1) is still 50% greater than that of **c**. Reaction of **f** with Ti(*i*-PrO)$_4$ to form **g** results in a ~10% thickness increase, while further chelation of Fe^{3+} by **g** gives no change in thickness. To verify if part of the modified oxoTi is due to the reaction of NTA carboxylic acids with Ti(*i*-PrO)$_4$, we immersed a wafer coated with PHEMA-NTA (control polymer 3 in Fig. S1**j**) in Ti(*i*-PrO)$_4$/THF for 30 min. The resulting polymer film showed almost no thickness change (<1 nm). The above ellipsometry measurements were performed on three different regions of each wafer to obtain an average thickness, and the standard deviations of the three measurements were always within ±3 nm.

Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was performed to determine the amounts of Fe and Ti in oxoTi-PGMA-Fe (**h**, Fig. S1). Each polymer-modified Au-coated Si wafer was immersed in 4 mL of aqua regia for 5 min to remove the entire polymer along with Au from the Si wafer, and the aqua regia was diluted to 50 mL with water. To prepare standard solutions in the same matrix as the sample solution, 20 pieces of 2.4 × 1.1 cm Au-coated wafers were immersed in 80 mL of aqua regia to form the Au/aqua regia solution, of which 4 mL was added to a series of 50-mL volumetric flasks. Specific volumes of 998 ppm Ti and 100 ppm Fe stock solutions were added to each flask prior to dilution to 50 mL with water.

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Using these standards, calibration curves for Ti and Fe were obtained with ICP-OES, and the amounts of Ti and Fe in different sample solutions were determined (three times for each) using the calibration curve. Table S1 shows that surface coverages of Fe and Ti generally increase with the thickness of the base polymer film (**f**, Fig. S1). The metal coverages normalized to base film thickness are 0.28±0.02 nmol/(cm^2-nm) Fe and 0.40±0.06 nmol/(cm^2-nm) Ti in oxoTi-PGMA-Fe films. Assuming the density of the film is 1 g/cm^3 and using a repeating-unit mass of 599.6 g/mol (structure **f** in Fig. S1 prior to reaction with Ti(*i*-PrO)$_4$ and Fe^{3+}), these metal densities correspond to 1.6±0.1 Fe atoms and 2.4±0.4 Ti atoms per repeating unit. The excess of Fe atoms may stem from some polymerization on the backside of the wafer,[3, 4] which was not coated with Au. The amount of Ti in polymer films shows a nonlinear increase with the film thickens, perhaps because crowding restricts the formation of oxotitanium clusters in deeper regions of the thick films to some extent.

Table S1. Film thicknesses and surface coverages of Fe and Ti in functionalized polymer brushes synthesized on Au-coated wafers.

		oxoTi-PGMA-Fe					PHEMA-Fe-1**	PHEMA-Fe-2***
		1	2	3	4	5		
Base polymer* thickness (nm)		71.4	76.9	120	151	193	148	143
Fe	μg/cm^2	1.15± 0.02	1.13± 0.08	1.73± 0.20	2.43± 0.41	3.32± 0.28	2.68± 0.05	2.90± 0.02
	nmol/cm^2-nm	Average 0.28±0.02					0.32±0.01	0.36±0.00
Ti	μg/cm^2	1.45± 0.03	1.50± 0.07	2.67± 0.06	2.76± 0.16	2.89± 0.06	0.04± 0.00	0.56± 0.00
	nmol/cm^2-nm	Average 0.40±0.06					0.01±0.00	0.08±0.00

* Base polymer is the polymer structure before modification with either Ti(IV) or Fe(III) species.
** The NTA-derivatized PHEMA film was directly immersed in 0.1 M $FeCl_3$.
*** The NTA-derivatized PHEMA film was immersed in Ti(*i*-PrO)$_4$/THF solution for 30 min followed by rinsing, 30-min immersion in 0.1 M $FeCl_3$, and rinsing.

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We also examined the extent of reaction of NTA groups with Ti(*i*-PrO)$_4$. Immersing an NTA-derivatized PHEMA brush (Fig. S1**j**)[5] in a stirred Ti(*i*-PrO)$_4$/THF solution for 30 min followed by Fe(III) chelation (PHEMA-Fe-2 in Table S1) leads to a low loading of Ti in the film (0.08 nmol Ti atoms/cm^2-nm) compared to the loading of Fe in the same film (0.36 nmol Fe atoms/cm^2-nm) or the loading of Ti in the bifunctional polymer (0.40 nmol Ti atoms/cm^2-nm). Elemental analysis of a PHEMA-Fe film that was not exposed to Ti(*i*-PrO)$_4$ (PHEMA-Fe-1 in Table S1) shows 0.1 nmol Ti/cm^2-nm, which is essentially the experimental uncertainty of the measurement.

Protein digestion and MALDI-MS analysis

The digestion of standard proteins followed a literature procedure.[6] Briefly, 100 μg of each phosphoprotein (α-casein, β-casein, ovalbumin and BSA) was dissolved in 20 μL of 6 M urea containing 50 mM Tris-HCl. Addition of 5 μL of 10 mM DL-dithiothreitol to the protein followed by heating in a water bath at 56 °C for 1 h cleaved any disulfide bonds. Subsequently 160 μL of 50 mM ammonium bicarbonate and 10 μL of 100 mM iodoacetamide were added to the protein solutions, which were then placed in the dark for 1 h to alkylate any Cys residues. Each solution was mixed with 10 μL of 0.5 μg/μL trypsin and incubated at 37 ˚C for 16 h. Finally the digestion was quenched by addition of 11 μL of glacial acetic acid. All the digests were dried with a Speed Vac (Savant SC110) and dissolved in TFA solutions (0.1 or 1 % TFA). The standard protein digests mixtures were prepared by mixing different amount of each protein digest. A 1-μL mixture of digests of α-casein, β-casein and ovalbumin (200 fmol of each) contains 0.028 M urea and 1.8 mM NH_4HCO_3; whereas introducing 2 pmol of BSA digest into such a 1-μL mixture results in 0.19 M urea and 12 mM NH_4HCO_3.

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MS analysis was performed using an LTQ XL ion trap mass spectrometer equipped with a vMALDI source (Thermo Fisher Scientific, San Jose, CA). Table S2 lists the detected-phosphopeptide sequence information, which was confirmed previously on the same instrument using CID-MS/MS and a Mascot database search.[7]

2. Phosphopeptide sequences and additional mass spectra after phosphopeptide enrichment

Table S2. Phosphopeptides detected in positive-ion mode MALDI-MS spectra. The list contains the phosphopeptide sequence* (if identified), the range of amino acids, the number of phosphoryl groups, and the monoisotopic m/z value for $[M+H]^+$.

Peptide #		Phosphopeptide sequence	Sequence Number**	# $-PO_3H_2$	m/z MH^+
α-casein	1	K.TVDME**pS**TEVFTK.K M(cam)-105	S2 153-164	1	1418.6
	2	K.VPQLEIVPN**pS**AEER.L	S1 121-134	1	1660.8
	3	K._VPQLEIVPN**pS**AEER.L Carbamyl (N-term)	S1 121-134	1	1703.8
	4	K.VPQLEIVPN**pS**AEER.L E(cam)	S1 121-134	1	1717.8
	5	YLGEYLIVPN**pS**AEER	S1***	1	1832.8
	6	K.DIG**pS**E**pS**TEDQAMEDIK.Q M(cam)-105	S1 58-73	2	1879.7
	7	K.DIG**pS**E**pS**TEDQAMEDIK.Q sodium adduct, M(cam)-105	S1 58-73	2	1901.7

	8	K._DIG**pS**E**pS**TEDQAMEDIK.Q Carbamyl (N-term), M(cam)-105	S1 58-73	2	1922.7
	9	K.DIG**pS**E**pS**TEDQAMEDIK.Q	S1 58-73	2	1927.7
	10	K.DIG**pS**E**pS**TEDQAMEDIK.Q M(cam)-105, D(cam)	S1 58-73	2	1936.7
	11	K.DIG**pS**E**pS**TEDQAMEDIK.Q M(ox)	S1 58-73	2	1943.7
	12	K.YKVPQLEIVPN**pS**AEER.L	S1 119-134	1	1952.0
	13	K.DIG**pS**E**pS**TEDQAMEDIK.Q Carbamyl (K)	S1 58-73	2	1970.7
	14	K.DIG**pS**E**pS**TEDQAMEDIK.Q D(cam)	S1 58-73	2	1984.7
	15	K._YKVPQLEIVPN**pS**AEER.L Carbamyl (N-term)	S1 119-134	1	1995.0
	16	N/A		1	2027
	17	N/A		1	2037
	18	N/A		5	2575
	19	K.QMEAE**pS**I**pS**p**S**p**S**EEIVPN**pS**VEQK.H M(cam)-105, pyro-glu from Q	S1 74-94	5	2655.9
	20	N/A		≥2	2672
	21	K.QMEAE**pS**I**pS**p**S**p**S**EEIVPN**pS**VEQK.H Pyro-glu from Q	S1 74-94	5	2702.9

	22	K.QMEAE**pS**l**pSpSpS**EEIVPN**pS**VEQK.H	S1 74-94	5	2720.9
	23	N/A		4	2760
	24	N/A		≥3	2819
	25	R.NANEEEYSIG**pSpSpS**EE**pS**AEVATEEVK.I	S2 61-85	4	3008.0
	26	N/A	S2 61-85****	5	3088.0
β-casein	27	K.FQ**pS**EEQQQTEDELQDK.I	48-63	1	2061.8
	28	K.FQ**pS**EEQQQTEDELQDK.I Sodium adduct	48-63	1	2083.8
	29	K._FQ**pS**EEQQQTEDELQDK.I Carbamyl (N-term)	48-63	1	2104.8
	30	K.FQ**pS**EEQQQTEDELQDK.I E(cam)	48-63	1	2118.8
	31	R.ELEELNVPGEIVE**pS**L**pSpSpS**EESITR.I	17-40	4	2966.2
	32	A.RELEELNVPGEIVE**pS**L**pSpSpS**EESITR.I	16-40	4	3122.3
ovalbumin	33	R.EVVG**pS**AEAGVDAASVSEEFR.A	341-360	1	2088.9

* Some phosphopeptides share the same sequence, but are modified differently during protein denaturing, alkylation, or digestion. "Carbamyl" corresponds to carbamylation, (cam) corresponds to carbamidomethylation, M(cam-105) corresponds to the neutral loss of S-(methylthio)acetamide from M(cam), and M(ox) corresponds to methionine sulfoxide.

** S1 and S2 represent S1- and S2-chains of α-casein, respectively.

*** According to Larsen, M. R. et al., this is a new sequence variant of the α-S1 casein in the region 104–119.[8]

**** According to Larsen, M. R. et al., this is probably a penta-phosphorylated peptide.[8]

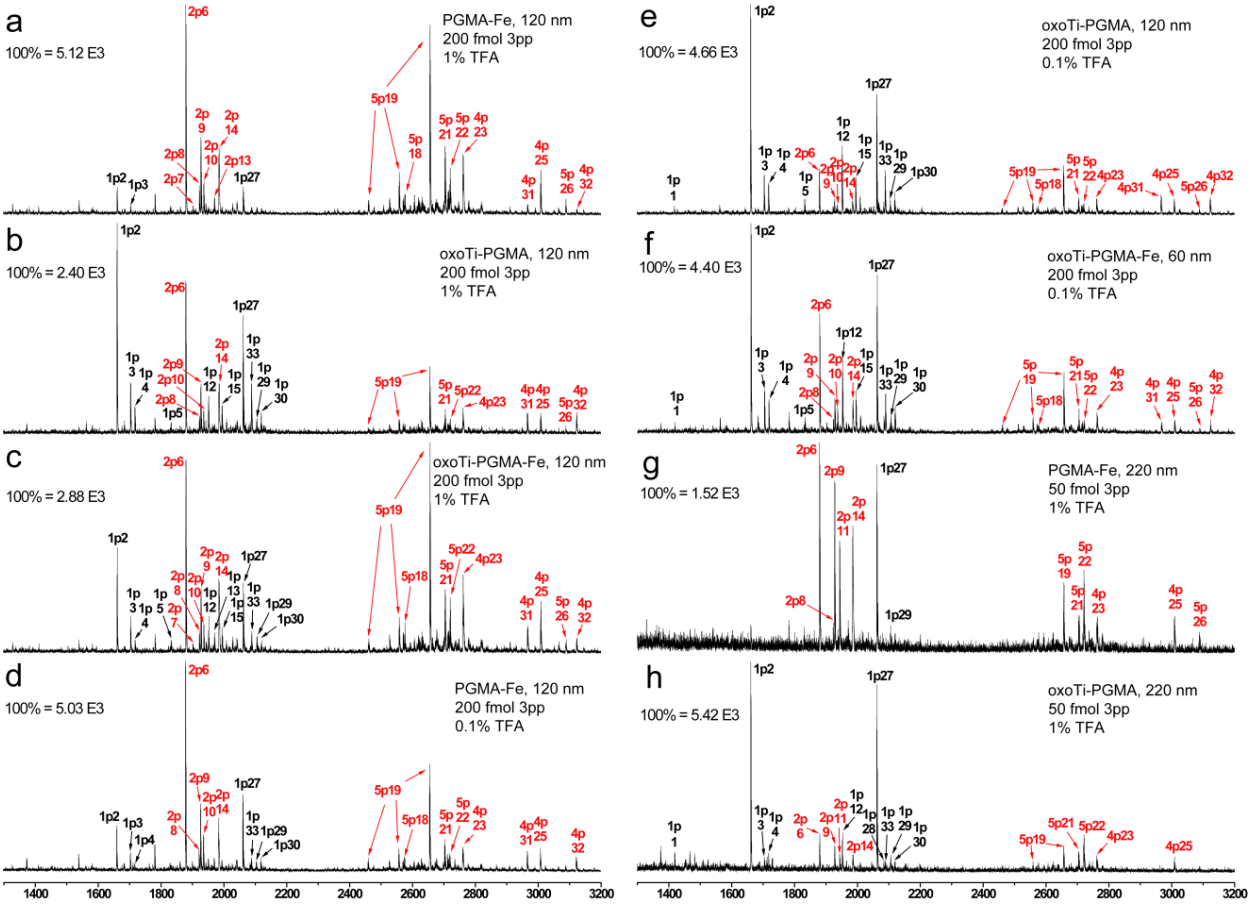

Fig. S2. MALDI mass spectra after on-plate phosphopeptide enrichment from a 1-μL solution containing three digested phosphoproteins (3pp), α-casein, β-casein and ovalbumin. The polymers employed for enrichment, their thicknesses (prior to addition of oxoTi or Fe(III)), the amounts of protein digests, and sample acidity are noted in each spectrum. Each phosphopeptide is labeled as #p#, where the first # denotes the number of phosphates in that peptide and the second # denotes the peptide number in Table S2. Black and red labels represent mono- and multiply phosphorylated peptides, respectively.

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3. References

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