Supporting Information

Low-cost iron oxide magnetic nanoclusters affinity probe for the enrichment of endogenous phosphopeptides in human saliva

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Part 1. Supplementary figures

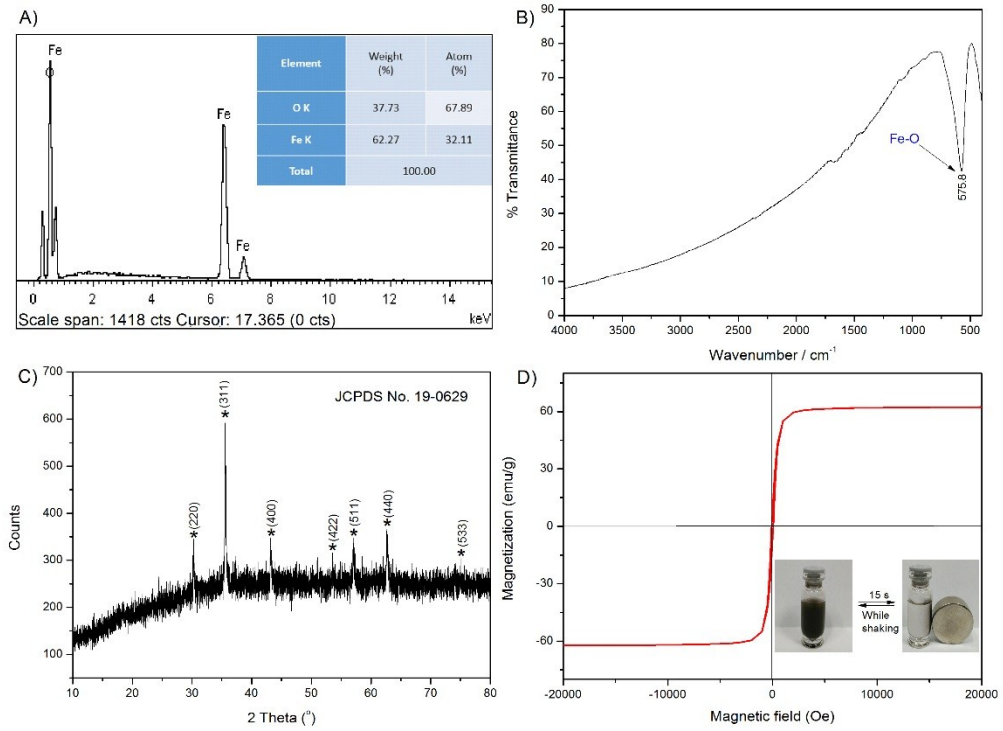


Fig. S1. Additional characterization of Fe_3O_4 MNCs affinity probe. A. EDX; B. FT-IR; C. XRD; D. Hysteresis loop (Inset shows magnetic apparent behavior).

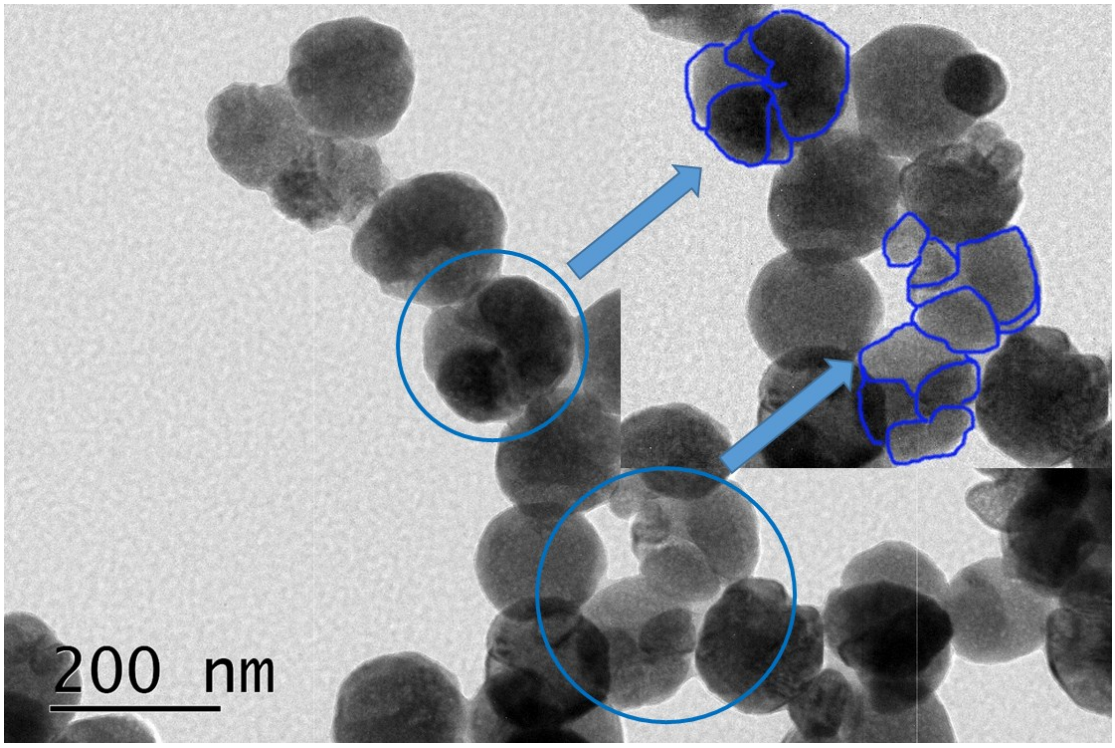


Fig. S2. Graphic illustration Fe_3O_4 MNCs composed of several monocrystallines.

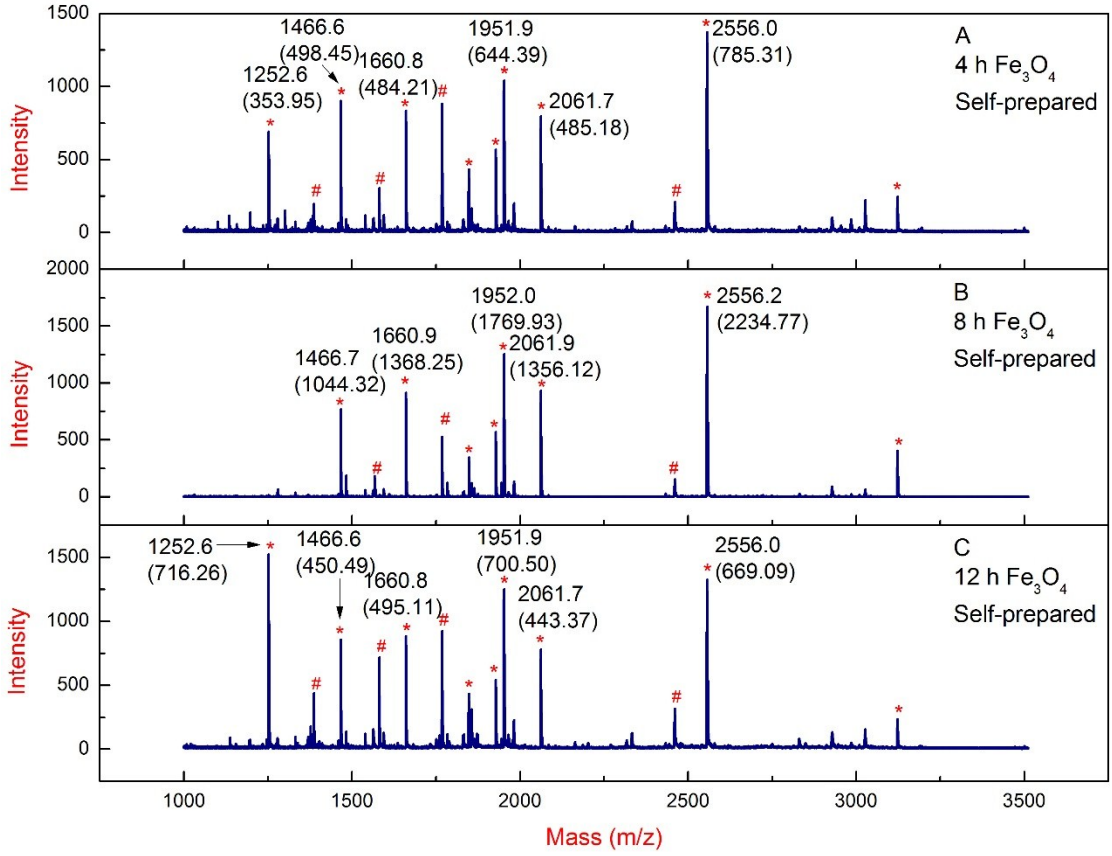


Fig. S3. MALDI-TOF MS spectra of mixture of bovine α-casein digest (10 pmol) and β-casein digest (10 pmol) enriched by Fe_3O_4 MNCs affinity probes prepared in different synthesis time. A. 4 h; B. 8 h; C. 12 h.

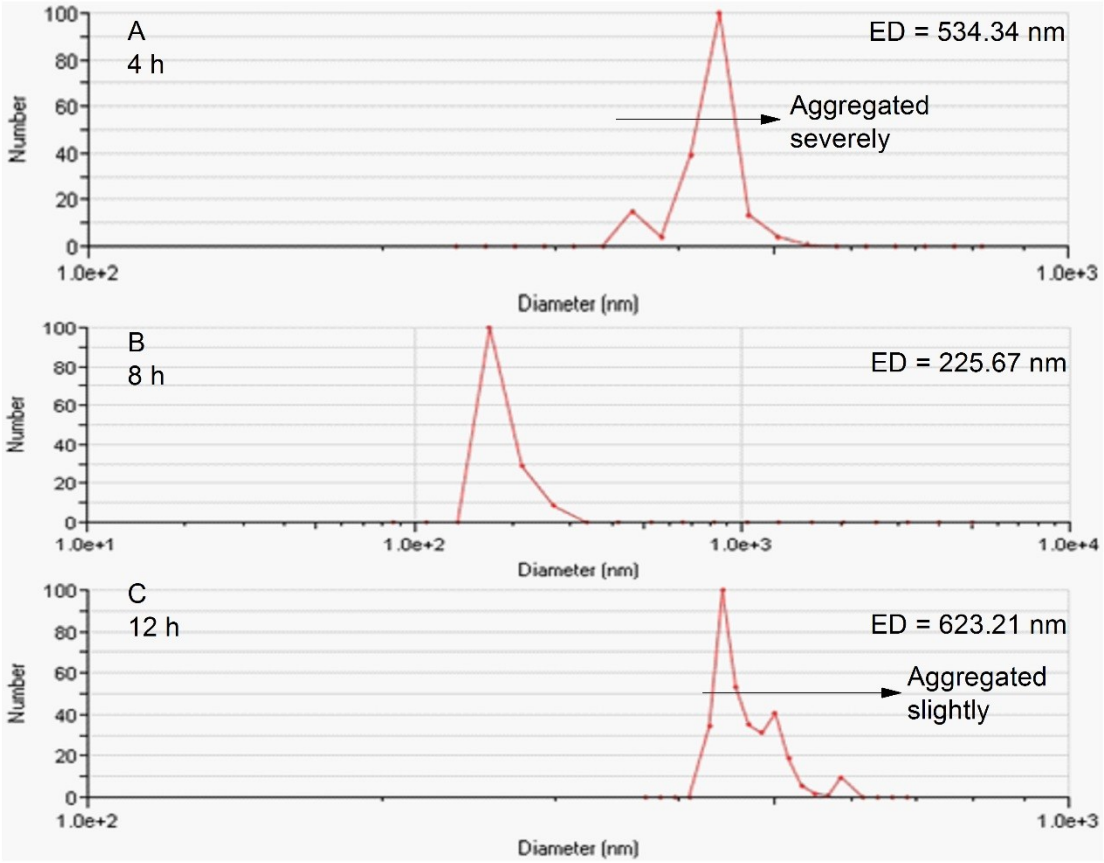


Fig. S4. The hydrodynamic diameters of Fe_3O_4 MNCs in different synthesis time. A. 4 h, aggregated severely; B. 8 h, larger hydrodynamic diameter than SEM/TEM measurement; C. 12 h, aggregated slightly. ED: Efficient diameter.

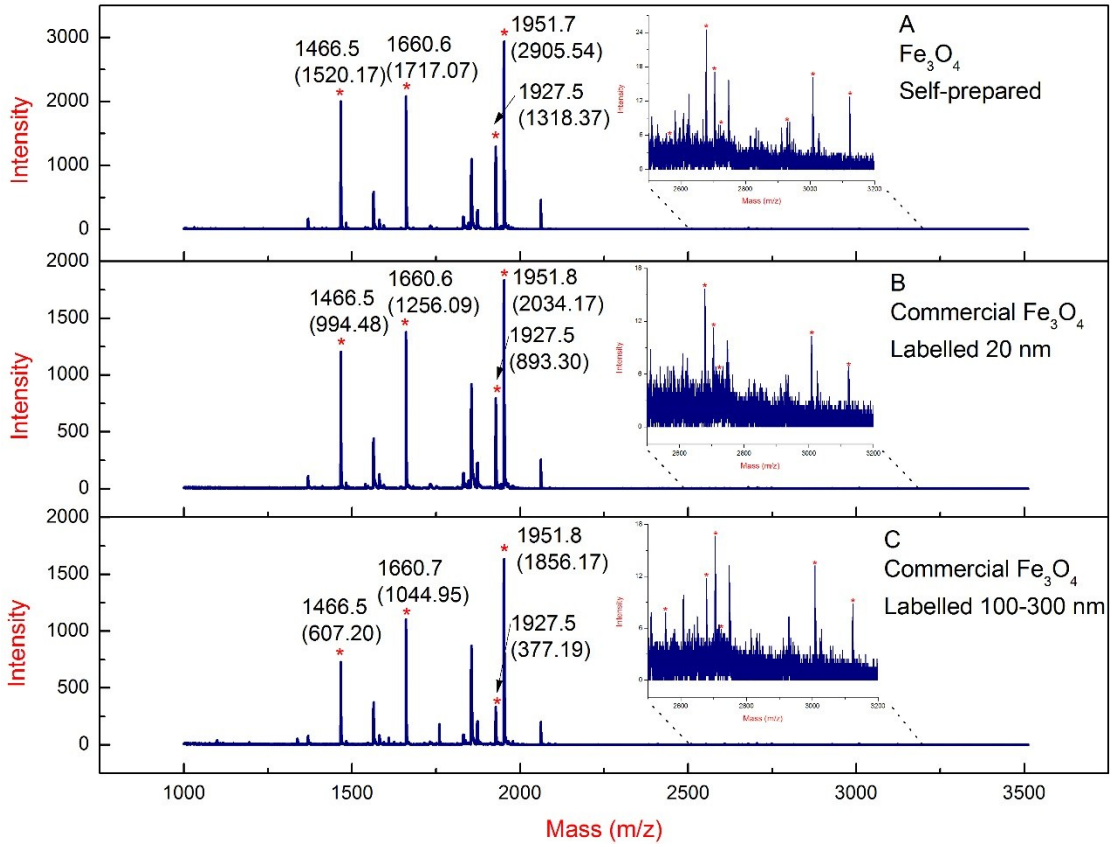


Fig. S5. MALDI-TOF MS spectra of bovine α-casein digest (10 pmol) enriched by different Fe_3O_4 NPs affinity materials. A. Self-prepared Fe_3O_4 MNCs; B. Commercial Fe_3O_4 NPs (20 nm); C. Commercial Fe_3O_4 NPs (100-300 nm).

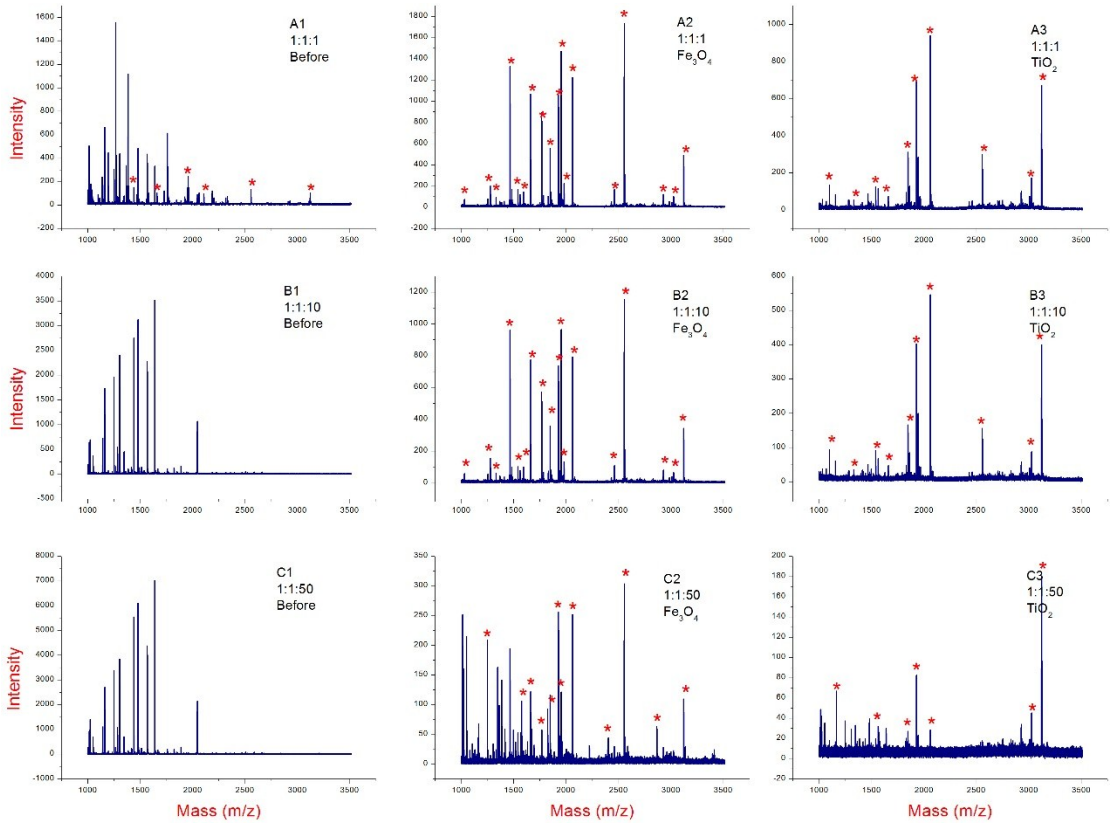


Fig. S6. MALDI-TOF MS spectra of phosphopeptides from tryptic digests of bovine α-casein, β-casein, and BSA with different molar ratios enriched by Fe_3O_4 MNCs and commercial TiO_2 NP_S. Left: Without enrichment; Middle: After enrichment by Fe_3O_4 MNCs affinity materials; Right: After enrichment by commercial TiO_2 NP_S. Analytes: Bovine α-casein:β-casein:BSA: A. 1:1:1; B. 1:1:10; C. 1:1:50. "*" indicates phosphopeptides.

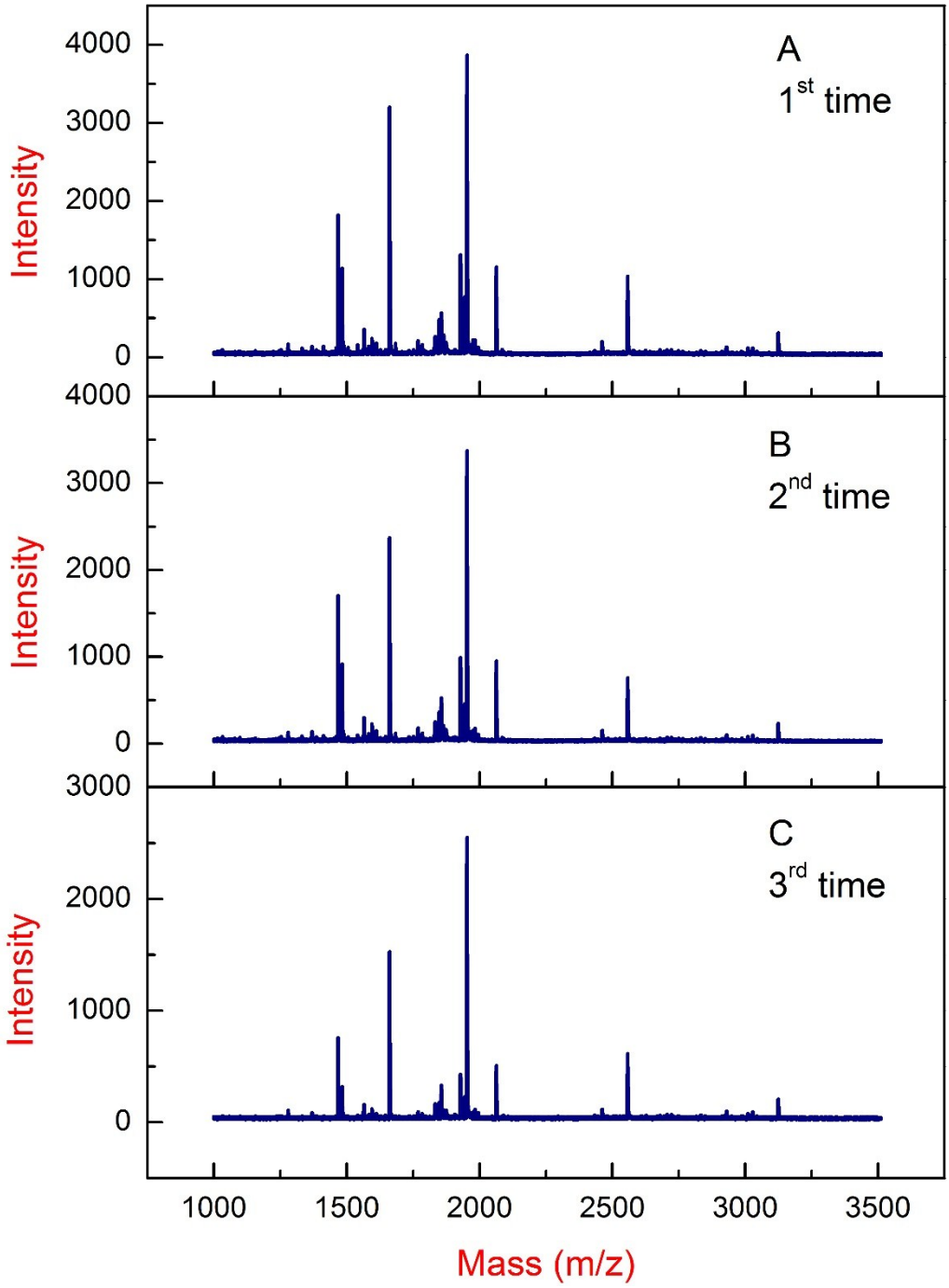


Fig. S7. Recyclability test of Fe_3O_4 MNCs affinity probe in enrichment of the mixture of bovine α-casein digest (10 pmol) and β-casein digest (10 pmol). (a-c) represent the 1st to 3rd reuse of the material.

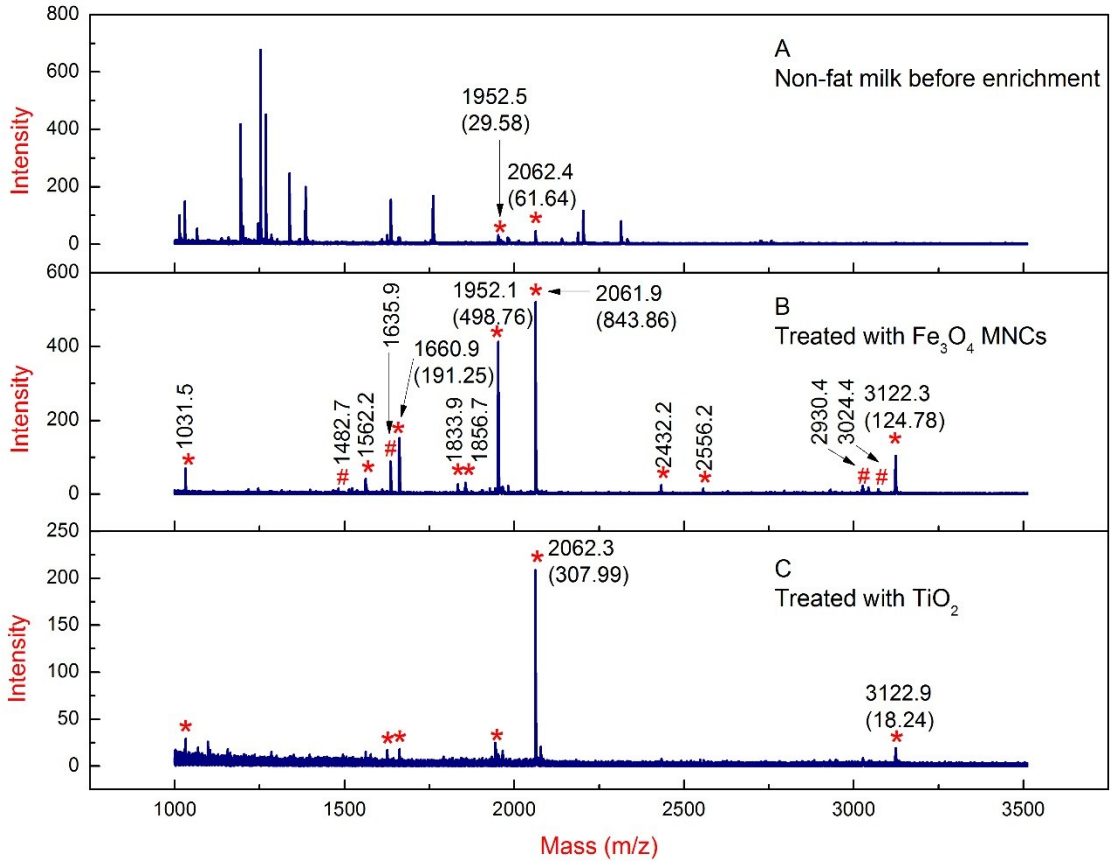
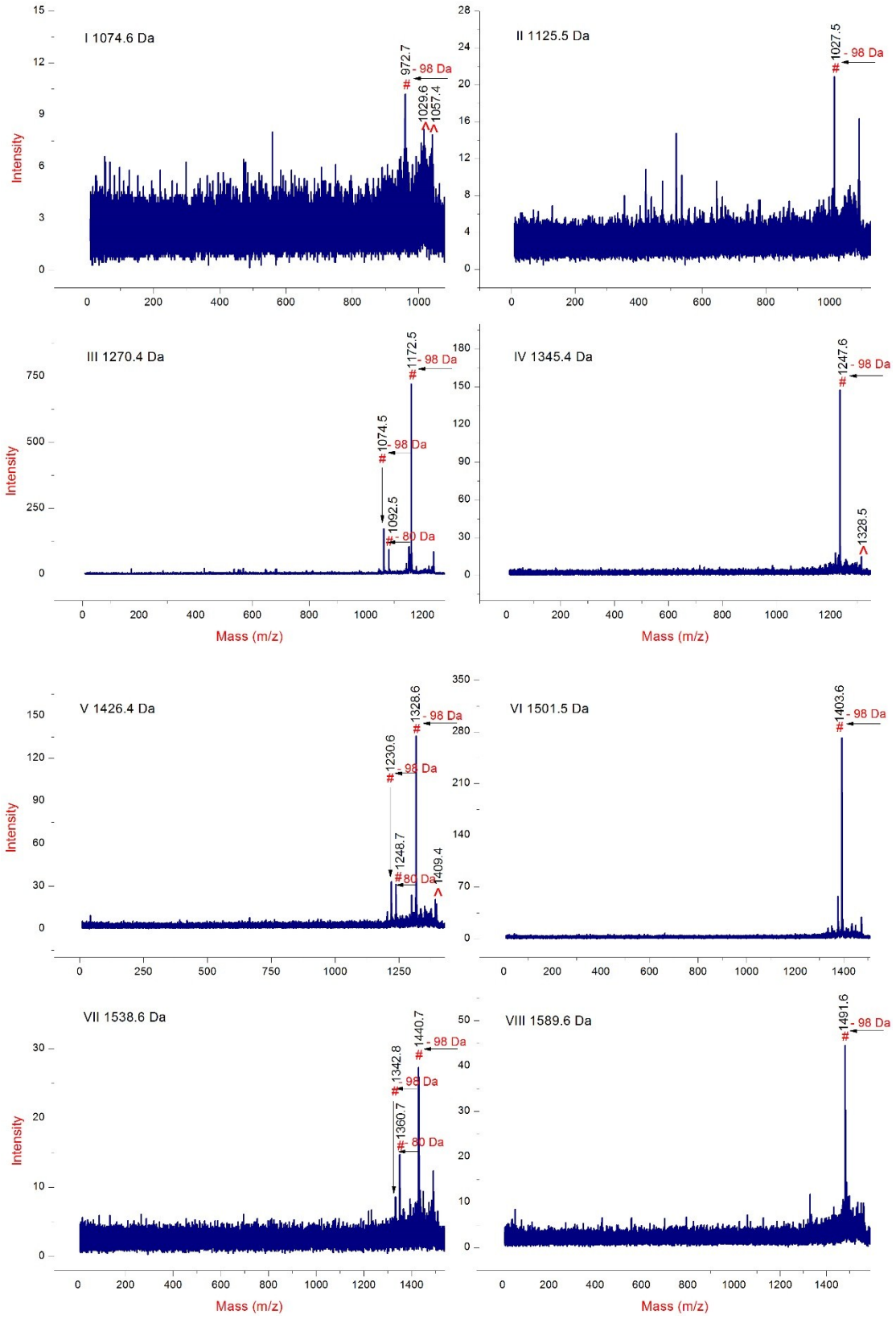


Fig. S8. MALDI-TOF MS spectra of phosphopeptides from tryptic digests of non-fat milk treated with different affinity materials. A. Without enrichment; B. Fe_3O_4 MNCs; C. Commercial TiO_2 NPs. "*" indicates phosphopeptides or the corresponding doubly charged ions peaks; "#" demonstrates the metastable ion losses of phosphoric acid or dephosphorylate fragments of phosphopeptide. The data in parentheses represent *S*/*N* ratios.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972.7
- 98 Da
1029.6
1057.4
II 1125.5 Da
1027.5
- 98 Da
III 1270.4 Da
1172.5
- 98 Da
1074.5
- 98 Da
1092.5
- 80 Da
IV 1345.4 Da
1247.6
- 98 Da
1328.5
V 1426.4 Da
1328.6
- 98 Da
1230.6
- 98 Da
1248.7
- 80 Da
1409.4
VI 1501.5 Da
1403.6
- 98 Da
VII 1538.6 Da
1440.7
- 98 Da
1342.8
- 98 Da
1360.7
- 80 Da
VIII 1589.6 Da
1491.6
- 98 Da
Intensity
Mass (m/z)

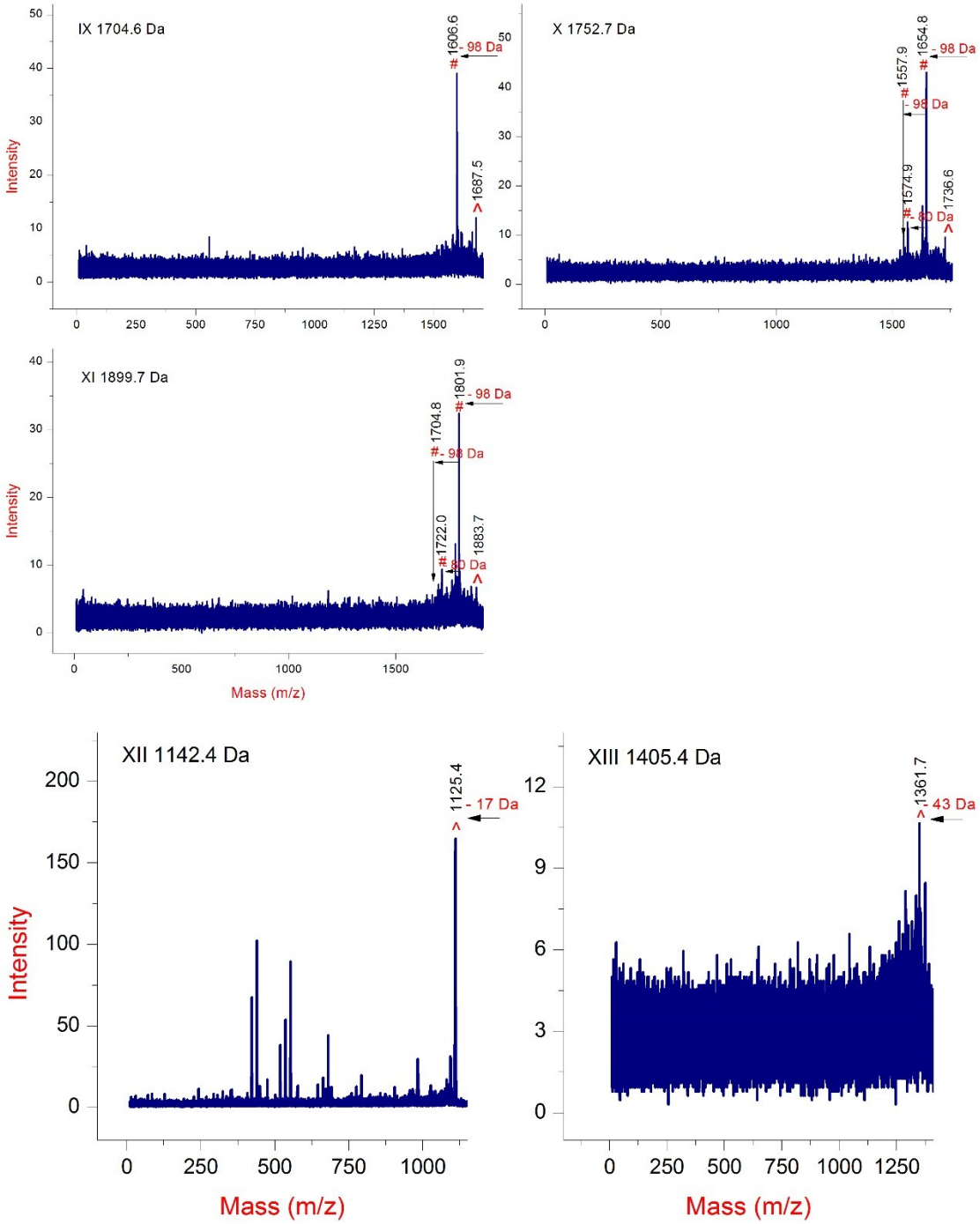
IX 1704.6 Da
1606.6
- 98 Da
1687.5
X 1752.7 Da
1654.8
- 98 Da
1557.9
- 98 Da
1574.9
- 80 Da
1736.6
XI 1899.7 Da
1801.9
- 98 Da
1704.8
- 98 Da
1722.0
- 80 Da
1883.7
Intensity
Mass (m/z)
XII 1142.4 Da
1125.4
- 17 Da
XIII 1405.4 Da
1361.7
- 43 Da
Intensity
Mass (m/z)

Fig. S9. MALDI-TOF/TOF mass spectra of human saliva treated with Fe_3O_4 MNCs affinity probe for the precursor ion at *m*/*z* of 1074.6 (I), 1125.5 (II), 1270.4 (III), 1345.5 (IV), 1426.5 (V), 1501.6 (VI), 1539.6 (VII), 1589.5 (VIII), 1704.5 (IX), 1752.6 (X), 1898.7 (XI), 1142.4 (XII), and 1405.4 (XIII). The intensity is very low in MS spectrum, but MS/MS experiment shows the characteristic loss of 98 Da, and also provides unambiguous identification, *e.g.* the peptide at *m*/*z* 1270.4 is phosphopeptide due to the presence of the fragment ion at *m*/*z* 1172.5 adjacent to the parent ion with a mass difference of 98 Da in MS/MS spectrum. "#" indicates fragment ion peak with a loss of 98 Da; "^" represents fragment ion peaks with losses of 43 Da, 17 Da, or 16 Da.

Part 2. Supplementary tables

Table S1. The single Fe_3O_4 mono-crystalline nanoparticle size calculated by different planes (*hkl*) directions*

hkl	2θ (°)	β (°)	β (rad)	κ	λ (nm)	D (nm)	$\bar{D}$ (nm)
220	30.2	0.1299	0.002266			63.0	
311	35.6	0.1299	0.002266			64.0	
400	43.2	0.2165	0.003777	0.89	0.154056	39.5	50.0
511	57.1	0.3464	0.006043			26.0	
440	62.6	0.1732	0.003021			53.5	

* Calculated by Eq. 1 (Scherrer equation). *h*, *k*, *l*: Crystal face directions; θ: Bragg diffraction angle, degree; β: Half height width of the diffraction peak, rad; κ: Constant, 0.89; λ: The incident X-ray wavelength, 0.154 056 nm; *D*: Particle size of crystalline, nm; $\bar{D}$: The mean particle size of crystalline, nm.

$$D = \kappa\lambda/(\beta\cos\theta) \quad (1)$$

Table S2. The *S*/*N* values of representative phosphopeptides adsorbed by Fe_3O_4 MNCs prepared in different synthesis time

Synthesis time (h)	Representative phosphopeptides $[M+H]^+$ (Da)				
	1466.6	1660.8	1951.9	2061.7	2556.0
	S/*N*				
4	498.45	484.21	644.39	485.18	798.31
8	1044.32	1368.25	1769.93	1356.12	2234.77
12	450.49	495.11	700.50	443.37	669.09

Table S3. The *S*/*N* values of representative phosphopeptides adsorbed by self-prepared and commercial Fe_3O_4 MNCs (Labelled 20 nm and 100-300 nm)

Fe_3O_4 MNCs		Representative phosphopeptides $[M+H]^+$ (Da)			
		1466.5	1660.6	1927.5	1951.7
		S/*N* value			
Self-prepared		1520.17	1717.07	1318.37	2905.54
Commercial	Labelled 20 nm	994.48	1256.09	893.30	2034.17
	Labelled 100-300 nm	607.20	1044.95	377.19	1856.17

Table S4. The enrichment factor (*EF*) values obtained by comparing with the ratio of *S*/*N* for after/before enrichment phosphopeptides

$[M+H]^+$	*S*/*N* for before enrichment	*S*/*N* for after enrichment	*EF*	Average *EF*
1466.9	89.95	760.89	8.5	
1482.9	50.60	449.86	8.9	
1661.1	139.01	1094.82	8.0	
1952.3	195.96	1473.93	7.6	7.2
2062.2	61.49	439.19	7.2	
2556.6	41.68	272.16	6.6	
3122.8	23.07	68.85	3.0	

Table S5. Identified phosphopeptides from proteolytic digests of non-fat milk with Fe_3O_4 MNCs and commercial TiO_2 NPs affinity materials

Position	$[M+H]^+$	Number of phosphoryl group	Amino acid sequence	Fe_3O_4 MNCs	TiO_2
β-c/48-63	1031.5^	1	FQ[pS]EEQQQTEDELQDK	√	√
β-c/16-40	1562.2^	4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR	√	
αS_1/121-134	1660.9	1	VPQLEIVPN[pS]AEER	√	√
αS_1/104-119	1833.9	1	YLGEYLIVPN[pS]AEER	√	
αS_1/104-119	1856.7	1	YLGEYLIVPN[pS]AEER-Na	√	
αS_1/119-134	1952.1	1	YKVPQLEIVPN[pS]AEER	√	√
β-c/48-63	2061.9	1	FQ[pS]EEQQQTEDELQDK	√	√
β-c/45-63	2432.2	1	IEKFQ[pS]EEQQQTEDELQDK	√	
β-c/48-67	2556.2	1	FQ[pS]EEQQQTEDELQDKIHPF	√	
β-c/16-40	3122.3	4	RELEELNVPGEIVE[pS]L[pS][pS][pS]EESITR	√	√
The number of phosphopeptides obtained				10	5

"[pS]" shows phosphorylation on serine; "^" denotes doubly charged peak;

Table S6. Identification and characterization of the special information of the parent ions for capturing endogenous phosphopeptdies from human saliva

No.	$[M+H]^+$	Number of phosphoryl group	Losses (Da)	Characteristic
1	1074.6	1	98; 43; 17	[pS]; [Ac-]; [Q*]
2	1125.5	1	98	[pS]
3	1142.4	0	17	[Q*]
4	1270.4	2	98; 196	2[pS]
5	1345.5	1	98; 17	[pS]; [Q*]
6	1405.4	0	43	[Ac-]
7	1426.5	2	98; 196;17	2[pS]; [Q*]
8	1501.6	1	98	[pS]
9	1539.6	2	98; 196	2[pS]
10	1589.5	1	98	[pS]
11	1704.5	1	98; 17	[pS]; [Q*]
12	1752.6	2	98; 196;16	2[pS]; [Mo]
13	1898.7	2	98; 196;16	2[pS]; [Mo]

[pS]: Phosphoric acid group; [Ac-]: Acetylation; [Mo]: Oxidation on methionine; [Q*]: Deletion or truncation of N-terminal on glutamine.

Table S7. Comparisons of different types of metal oxides (MOs) nanoparticles (NPs) or functionalized magnetic-MOs affinity probes for phosphopeptides enrichment, in regards to their magnetic saturation (M_s), separation mode and time, BSA interference, detection limit, and the capability of capturing phosphopeptides from non-fat milk tryptic digests

Typical affinity probes	M_s (emu/g)	Separation mode / Time (min or s)	BSA interference	Detection limit (DL, fmol)	Number of phosphopeptides captured from non-fat milk tryptic digests	Refs.
Fe_3O_4@$LuPO_4$	18.6	MS (within 1 min)	50-fold	200	13	[S1]
NiO NPs	-		50-fold	100	15	[S2]
ZrO_2 aerogel	-	CS	100-fold	NM	17	[S3]
Octahedral SnO_2	-	(5 min)	100-fold	800	14	[S4]
SnO_2-$ZnSn(OH)_6$	-		NM	20	15	[S5]
SiO_2/TiO_2	-	MC (4 min)	100-fold	10	NM	[S6]
Fe_3O_4@Ti-mSiO_2	33.8		100-fold	10	16	[S7]
Fe_3O_4@mTiO_2	21.2	MS	1000-fold	NM	NM	[S8]
Fe_3O_4@ZrO_2	29.9	(NM)	100-fold	250	NM	[S9]
Fe_3O_4@Ga_2O_3	NM		50-fold	40	NM	[S10]

Fe_3O_4@Al_2O_3	NM		50-fold	NM	12	[S11]
Fe_3O_4@fTiO_2	30.1	MS (1 min)	1000-fold	50	NM	[S12]
Fe_3O_4 MNCs	62.4	MS (15 s)	50-fold	10	10	This work

MS: Magnetic separation; CS: Centrifugal separation; NM: Not mentioned; MC: Monolithic column.

References:

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