Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Hierarchically templated beads with tailored pore structure for phosphopeptide capture and phosphoproteomics

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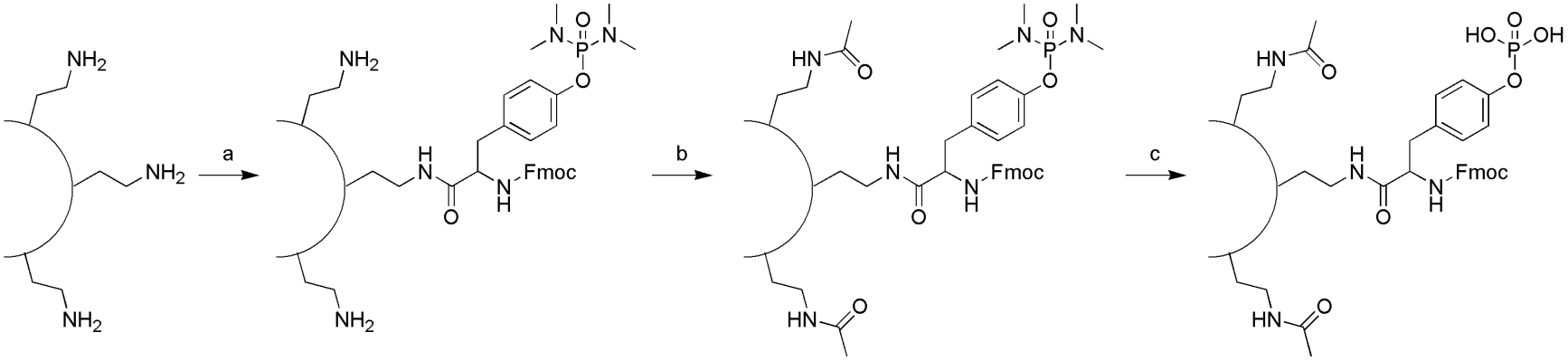


Figure S1. Template immobilization on silica surface, (a) PyBOP, HOBt, DIEA, Fmoc-Tyr($PO(NMe_2)_2$)-OH, DMF, room temperature, 24 h; (b) Ac_2O, DMF, 2 h; (c) TFA/H_2O 9/1 (v/v), room temperature, overnight. See experimental part for details.

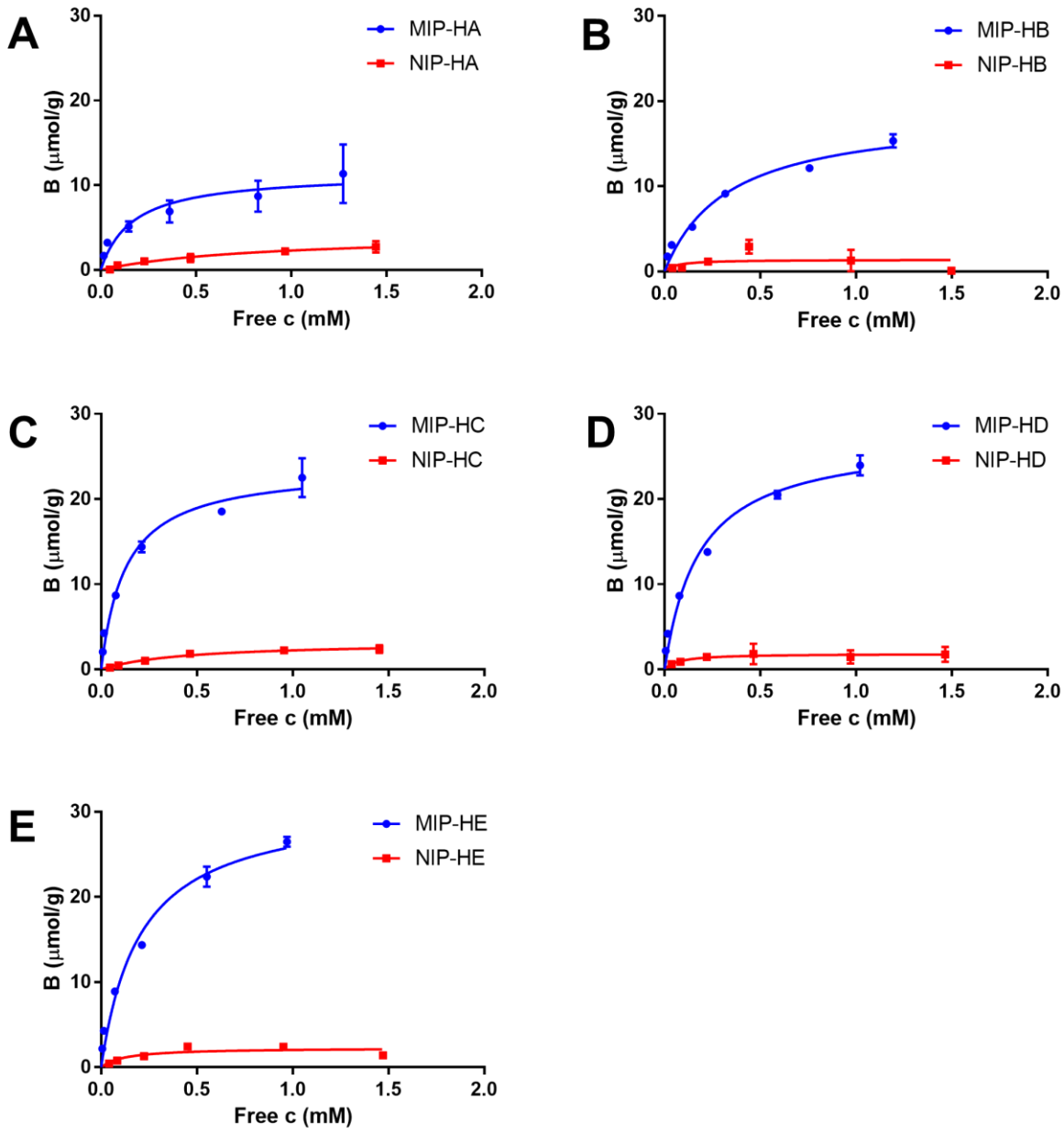


Figure S2. Binding isotherms of Fmoc-pTyr-OH (concentration range 0.05 mM-1.5 mM) for surface imprinted polymers and corresponding non-imprinted polymers. (A) MIP-HA/NIP-HA, (B) MIP-HB/NIP-HB, (C) MIP-HC/NIP-HC, (D) MIP-HD/NIP-HD, (E) MIP-HE/NIP-HE. Points show an average of three replicas with the error bars representing standard deviation.

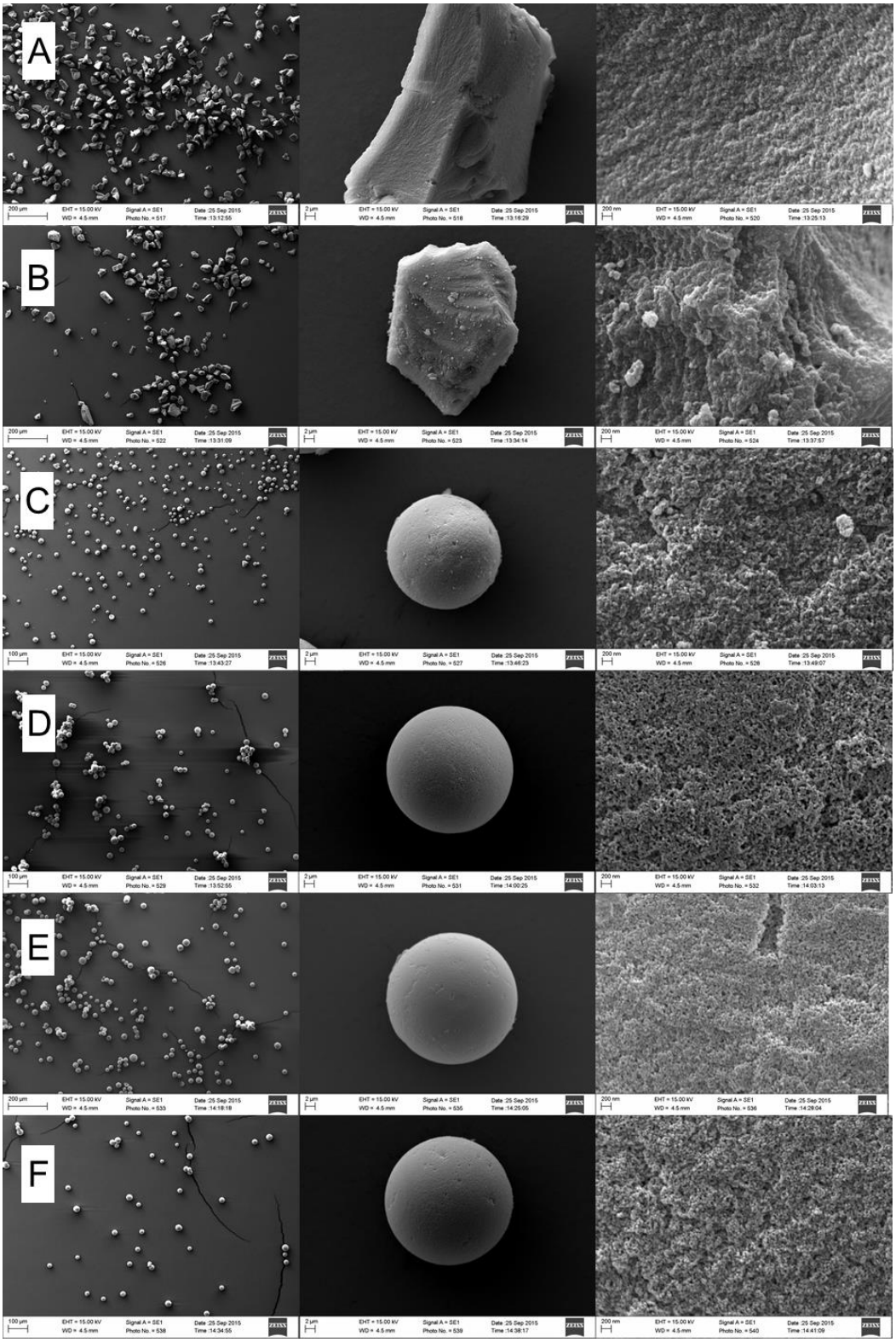


Figure S3. SEM images of (A) MIP-B, (B) NIP-B, (C) MIP-M, (D) NIP-M, (E) MIP-HE, and (F) NIP-HE at different magnifications.

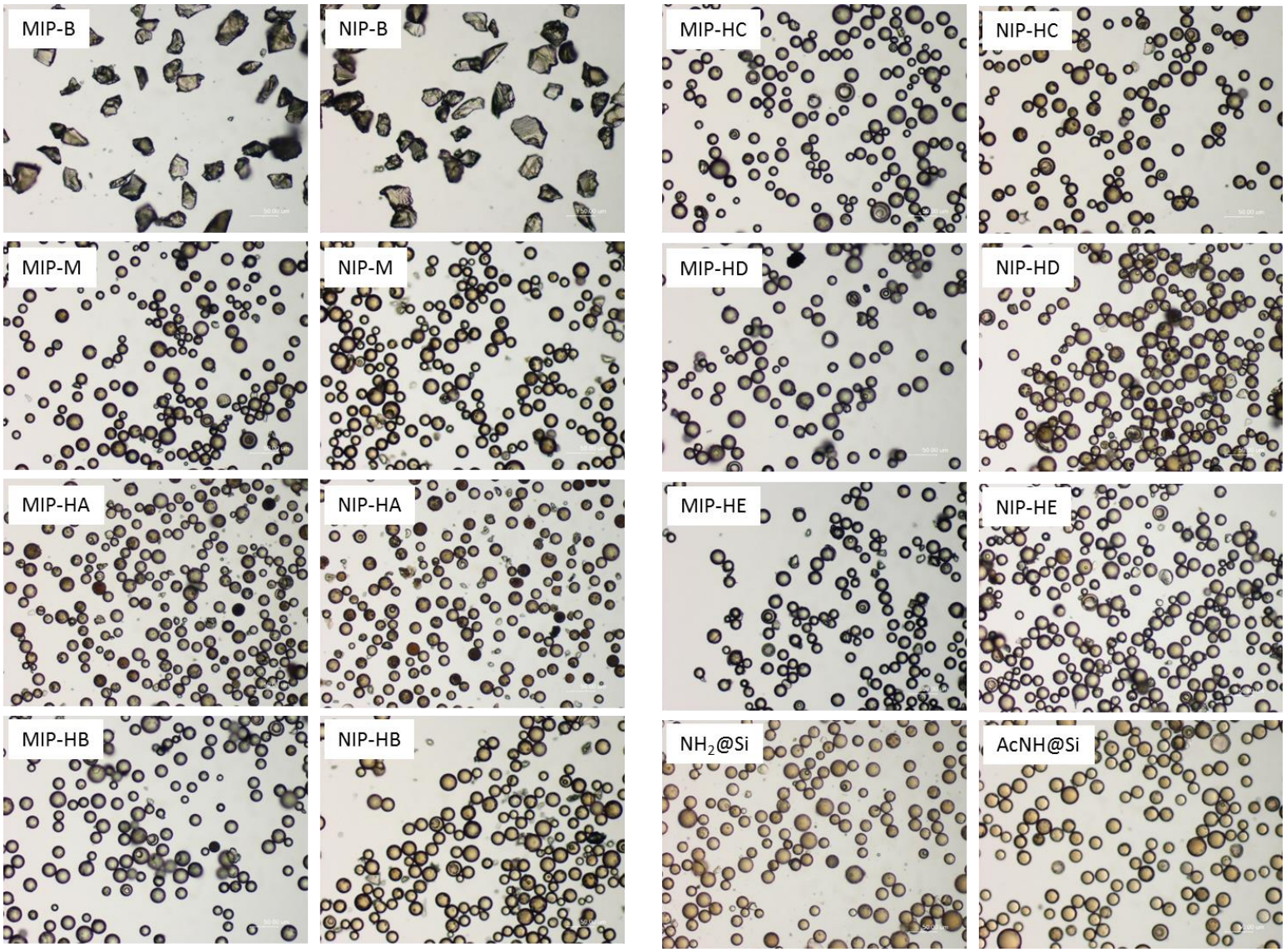


Figure S4. Optical micrographs of synthesized polymers and silica microparticles.

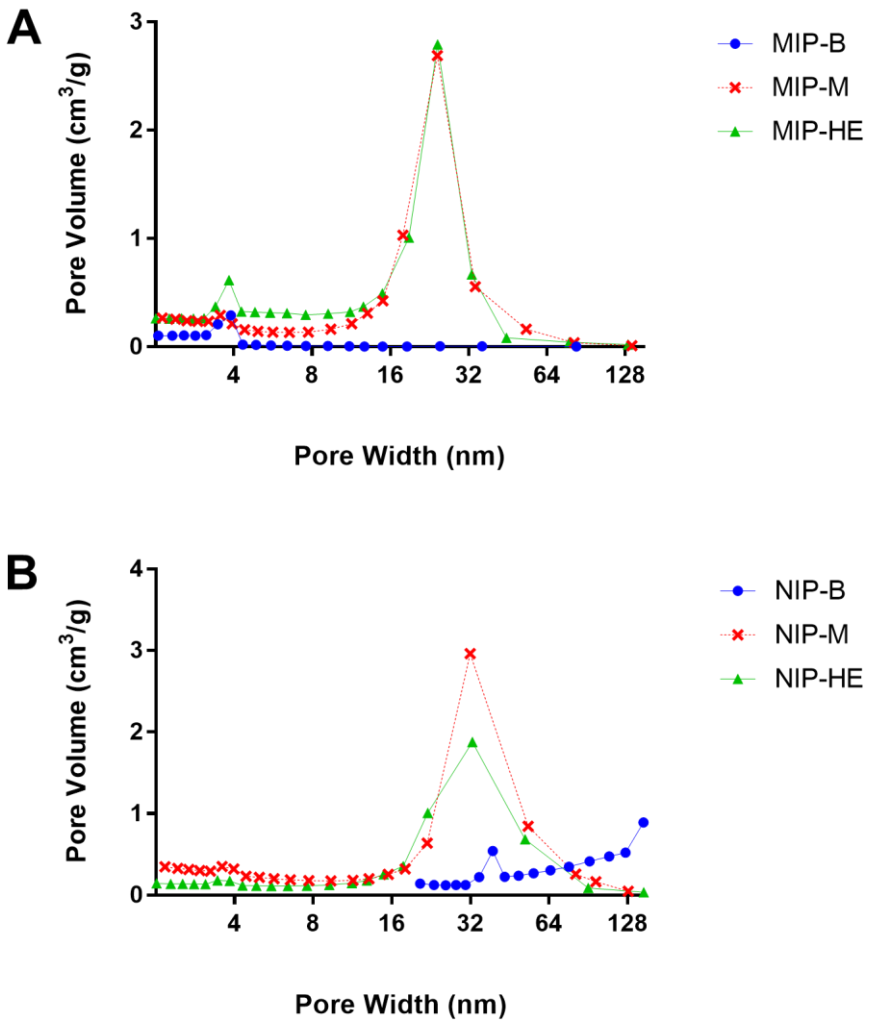
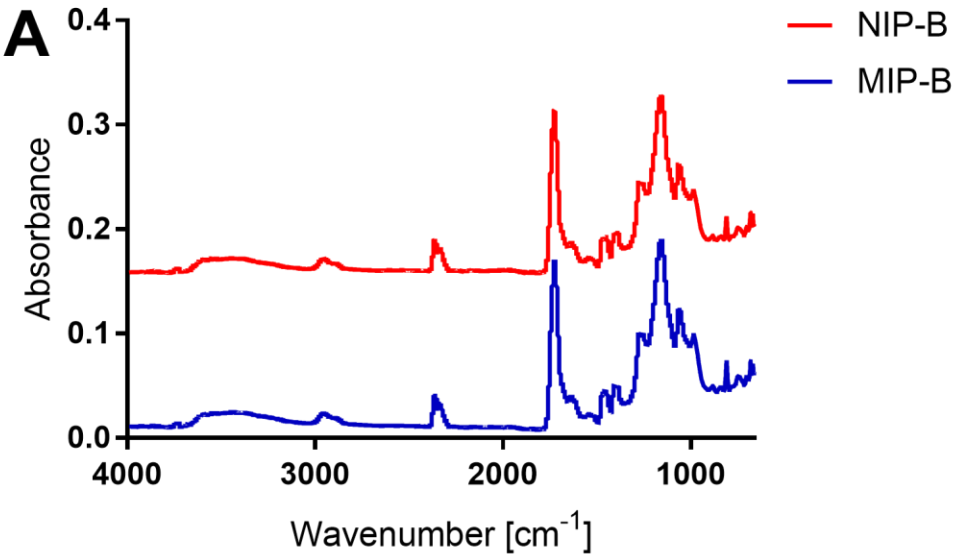
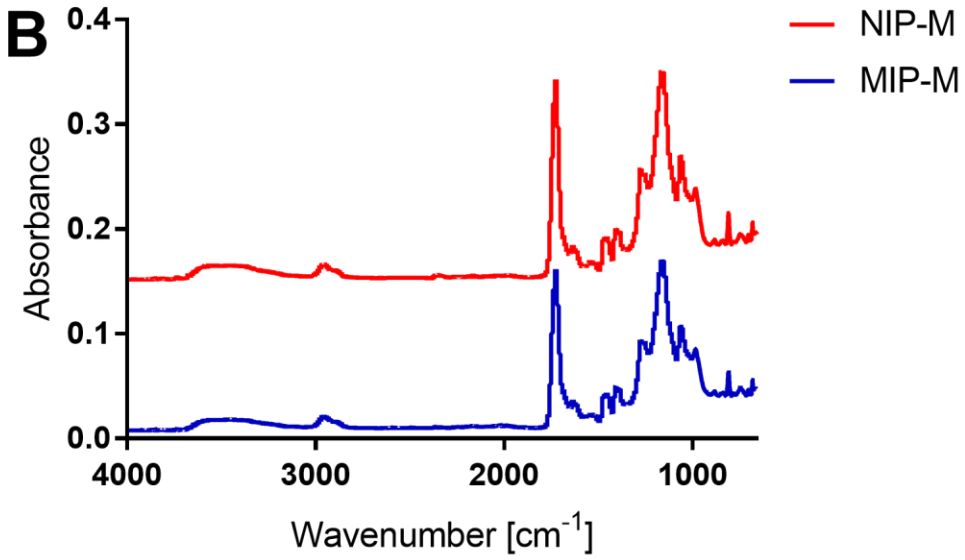


Figure S5. The BJH differential pore volume (dV/dlog(w)) distribution plots against pore width for (A) MIP-B, MIP-M, MIP-HE and (B) NIP-B, NIP-M, NIP-HE.


A
0.4
0.3
0.2
0.1
0.0
Absorbance
4000
3000
2000
1000
Wavenumber [cm-1]
NIP-B
MIP-B


B
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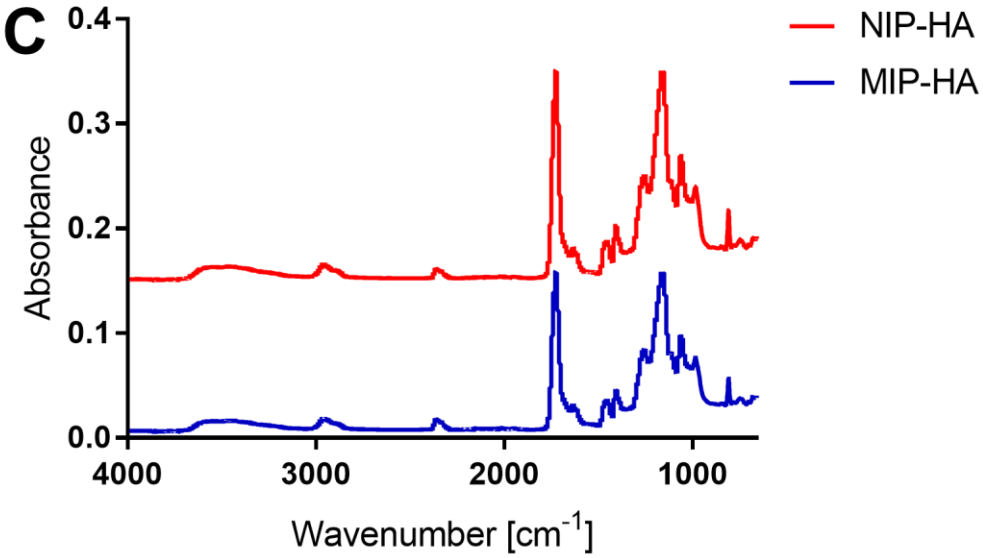
0.3
0.2
0.1
0.0
Absorbance
4000
3000
2000
1000
Wavenumber [cm-1]
NIP-M
MIP-M


C
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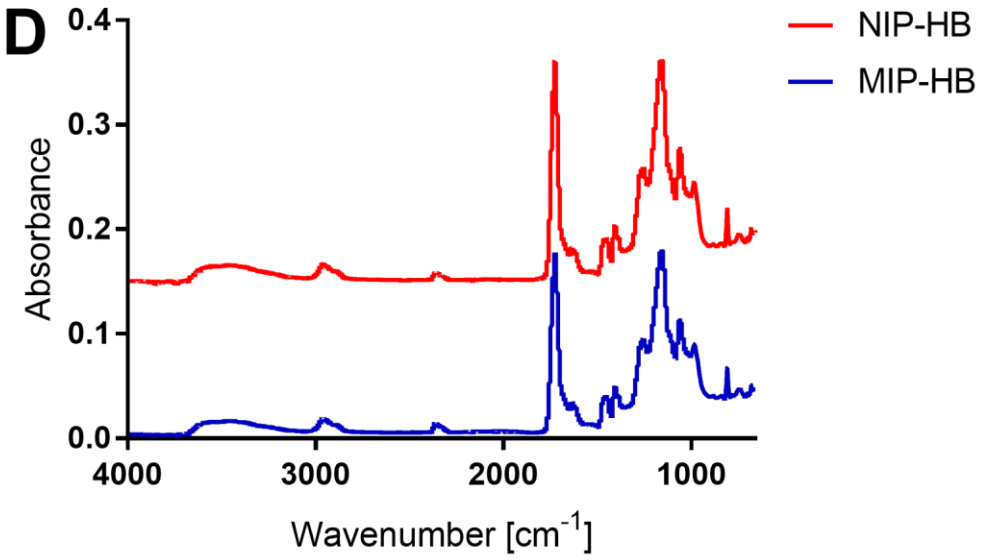
0.3
0.2
0.1
0.0
Absorbance
4000
3000
2000
1000
Wavenumber [cm-1]
NIP-HA
MIP-HA


D
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Absorbance
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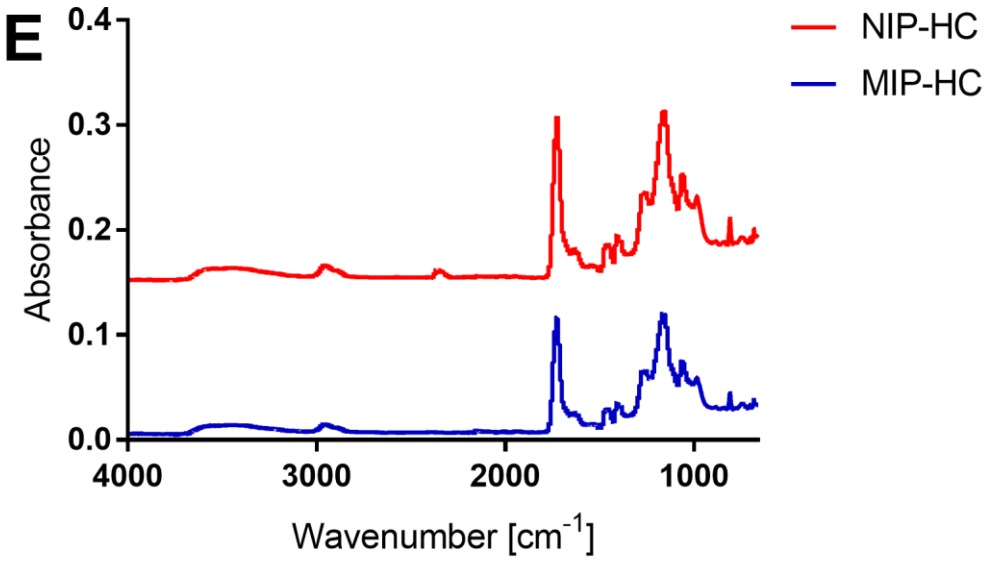
2000
1000
Wavenumber [cm-1]
NIP-HB
MIP-HB


E
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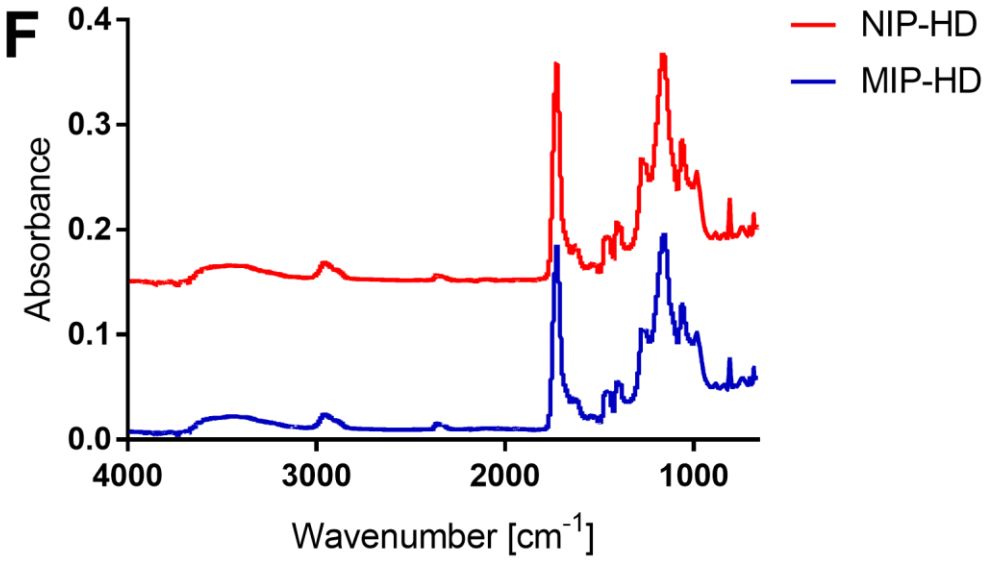
0.1
0.0
Absorbance
4000
3000
2000
1000
Wavenumber [cm-1]
NIP-HC
MIP-HC


F
0.4
0.3
0.2
0.1
0.0
Absorbance
4000
3000
2000
1000
Wavenumber [cm-1]
NIP-HD
MIP-HD

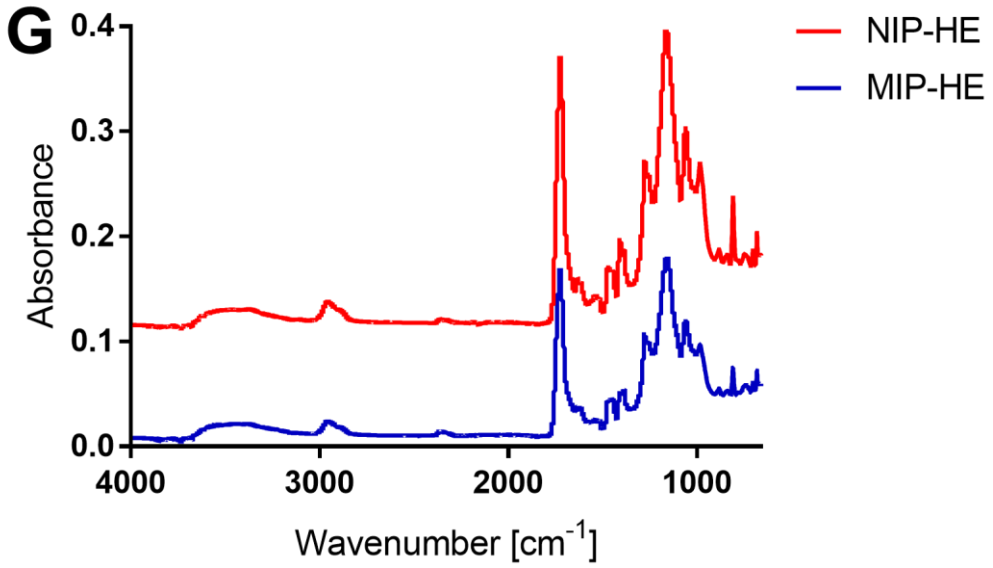


Figure S6. ATR-FTIR spectra of imprinted and non-imprinted polymers (A) MIP-B/NIP-B, (B) MIP-M/NIP-M, (C) MIP-HA/NIP-HA, (D) MIP-HB/NIP-HB, (E) MIP-HC/NIP-HC, (F) MIP-HD/NIP-HD, (G) MIP-HE/NIP-HE.

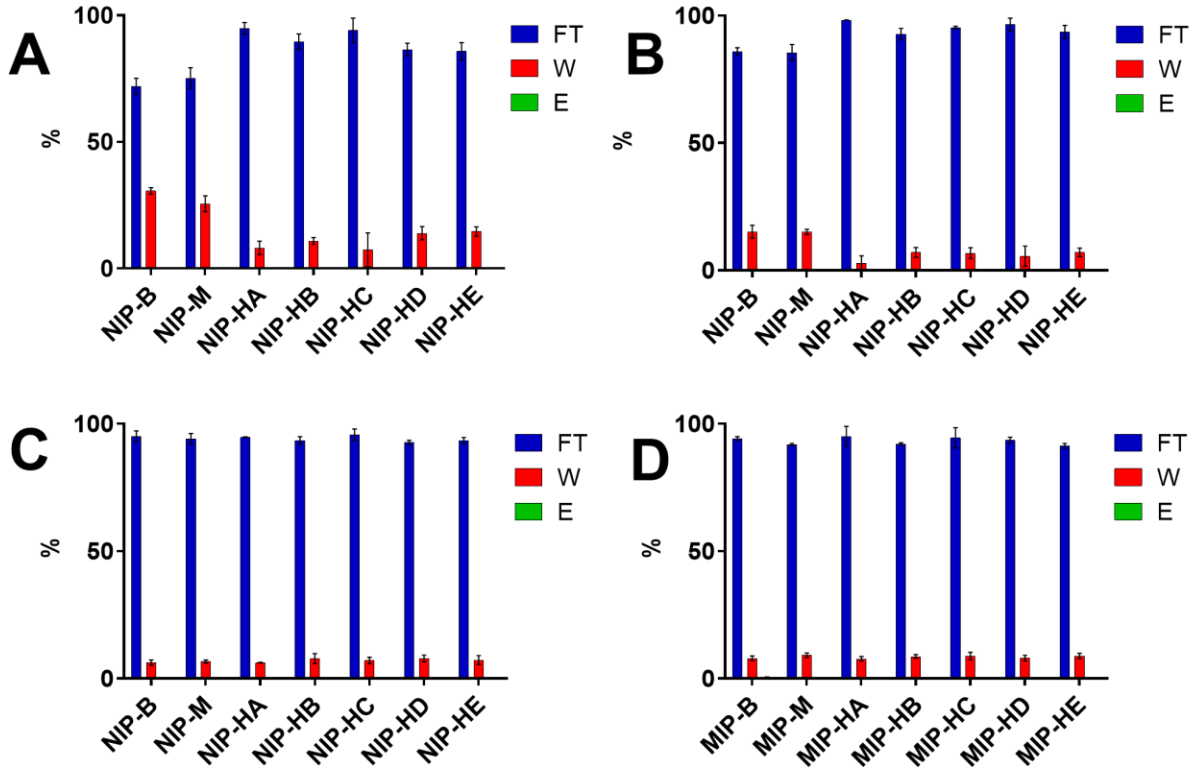


Figure S7. SPE test results Fmoc-pTyr-OH (A), and Fmoc-pSer-OH (B) and Fmoc-Tyr-OH (C, D) showing % of each analyte in flow through (FT), washing (W) and elution (E) fractions. The following conditions were applied: loading 95% ACN + 0.1% FA, washing 80% ACN + 0.1% FA, elution 80% MeOH + 1% TFA. The bars show the average of three replicas and the error bars represent standard deviation.

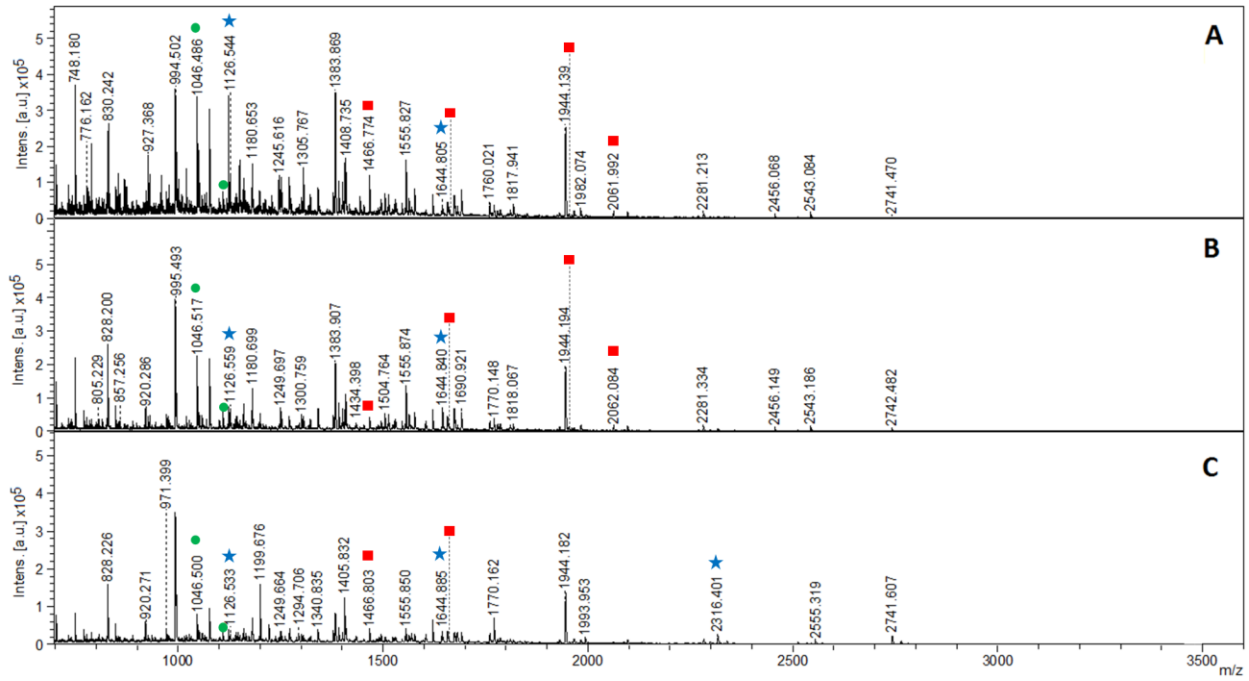


Figure S8. MALDI mass spectra obtained for combined flow through and washing fraction (FTW) from (A) MIP-B, (B) MIP-M and (C) MIP-HE. Marked are spiked phosphotyrosine peptides (asterisk), phosphoserine peptides (square) and spiked phosphoserine and non-phosphorylated peptides (circle).

Table S1. Characterization of silica with immobilized template.

Sample	Nominal template coverage		Real template coverage[a]		Mass loss [%][b]
	[µmol/m^2]	[µmol/g]	[µmol/m^2]	[µmol/g]	
Fmoc-pTyr@Si-A	0.44	20	0.23	10	3.1
Fmoc-pTyr@Si-B	0.60	2	0.40	18	3.5
Fmoc-pTyr@Si-C	0.76	34	0.73	33	3.8
Fmoc-pTyr@Si-D	0.90	40	0.86	39	4.1
Fmoc-pTyr@Si-E	1.20	54	1.22	55	4.5

[a] Calculated by Fmoc-cleavage test; [b] Determined by thermogravimetric analysis.

Table S2. Characterization of polymeric materials.[a]

Sample	Silica used for polymerization	Mass loss [%][a]
MIP-B	-	94.3
NIP-B	-	94.4
MIP-M	AcNH@Si	93.9
NIP-M	AcNH@Si	93.1
MIP-HA	Fmoc-pTyr@Si-A	94.9
NIP-HA	AcNH@Si	94.7
MIP-HB	Fmoc-pTyr@Si-B	94.2
NIP-HB	AcNH@Si	94.9
MIP-HC	Fmoc-pTyr@Si-C	93.2
NIP-HC	AcNH@Si	94.4
MIP-HD	Fmoc-pTyr@Si-D	93.5
NIP-HD	AcNH@Si	95.6
MIP-HE	Fmoc-pTyr@Si-E	95.2
NIP-HE	AcNH@Si	97.3

[a] The mass loss was determined by thermogravimetric analysis (TGA). The mass loss of the composites before etching was roughly 30%.

Table S3. Elemental composition of imprinted and non-imprinted polymers.[a]

Polymer	Theoretical			Experimental		
	%C	%H	%N	% C	% H	% N
MIP-B	56.30	5.91	1.06	56.36	5.80	0.98
NIP-B	56.30	5.91	1.06	55.88	6.94	0.96
MIP-M	56.30	5.91	1.06	56.28	6.89	0.99
NIP-M	56.30	5.91	1.06	55.78	6.28	0.92
MIP-HA	56.42	6.09	0.62	56.24	6.94	0.50
NIP-HA	56.42	6.09	0.62	56.08	6.82	0.49
MIP-HB	56.39	6.04	0.74	56.16	6.13	0.65
NIP-HB	56.39	6.04	0.74	56.05	5.81	0.69
MIP-HC	56.33	5.95	0.95	56.22	6.88	0.92
NIP-HC	56.33	5.95	0.95	56.08	6.80	0.82
MIP-HD	56.31	5.92	1.03	56.14	5.75	0.96
NIP-HD	56.31	5.92	1.03	55.78	5.77	0.96
MIP-HE	56.25	5.84	1.24	56.13	5.69	1.16
NIP-HE	56.25	5.84	1.24	56.20	5.53	1.14

[a] The theoretical nitrogen content for polymers prepared in absence of urea monomer **1** was 0.47%.

Table S4. Binding constants (K_a) maximum binding capacity (B_{max}) and imprinting efficiency (IE) for MIPs and NIPs.

Polymer	K_a x 10^3 [M^{-1}]	B_{max} [µmol/g]	IE (%)
MIP-B	16.5 ± 2.6	25.0 ± 1.0	23
MIP-M	16.7 ± 2.3	36.9 ± 1.3	33
MIP-HA	5.9 ± 2.6	11.4 ± 1.4	36
MIP-HB	3.0 ± 0.9	18.7 ± 2.0	33
MIP-HC	7.8 ± 1.9	23.8 ± 1.6	23
MIP-HD	5.5 ± 1.4	27.3 ± 2.2	22
MIP-HE	4.8 ± 1.3	31.2 ± 2.8	18
NIP-B	2.1 ± 0.5	7.8 ± 0.7	-
NIP-M	1.8 ± 0.5	6.5 ± 0.7	-
NIP-HA	1.2 ± 0.3	4.2 ± 0.6	-
NIP-HB	17.4 ± 49	1.4 ± 0.8	-
NIP-HC	2.2 ± 0.5	3.2 ± 0.2	-
NIP-HD	13.7 ± 4.7	1.8 ± 0.1	-
NIP-HE	8.2 ± 6.9	2.3 ± 0.5	-

Table S5. Phosphotyrosine (pY), phosphoserine (pS) and spiked tyrosine (Y) peptides found in the sample before enrichment and in combined flow through and washing (FTW) and elution (E) fractions.[a]

Peptide sequence	**$[M+H]^+$ (Da)**	**Origin**	**Sample**	**MIP-B**		**MIP-M**		**MIP-HE**	
				FTW	E	FTW	E	FTW	E
DRVYIHPF	1046.54	Spiked	+	+	+	+	+	+	+
DRV**pS**IHPF	1050.48	Spiked	+	-	+	-	+	-	+
GADDSYYTAR	1118.48	Spiked	+	+	+/-	+	+	+	+
DRV**pY**IHPF	1126.51	Spiked	+	+	+	+	+	+	+
GADDSY**pY**TAR	1198.44	Spiked	-	-	+	-	+	-	+
GADDS**pY**p**Y**TAR	1278.41	Spiked	-	-	+/-	-	+	-	+
GSTAENAE**pY**LR	1290.54	Spiked	-	-	+	-	+	-	+
TVDME**pS**TEVFTK	1466.61	CASA2[b]	+	+	+	+	+	+	+
MHLPSPTDSNF**pY**R	1644.69	Spiked	+	+	+	+	+	+	+
VPQLEIVPN**pS**AEER	1660.79	CASA1[c]	+	+	+	+	+	+	+
DIG**pS**E**pS**TEDQAMEDIK	1927.69	CASA1[c]	-	-	+	-	+	-	+
YKVPQLEIVPN**pS**AEER	1951.95	CASA1[c]	+	+	+	+	+	+/-	+
FQ**pS**EEQQQTEDELQDK	2061.83	CASB[d]	+	+	+	+	+	+/-	+
GSHQISLDNPD**pY**QQDFFPK	2315.99	Spiked	+	+/-	+	+/-	+	+	+
RPAGSVQNPV**pY**HNQPLNPAPSRD	2594.22	Spiked	+	-	+	-	+	-	+
YSSDPTGALTEDSIDDTFLPVPE**pY**INQSVPK[e]	3478.58	Spiked	+	-	+	-	+	+/-	+

[a] + = peptide signal found in the sample; - = peptide signal not found in the sample; +/- = peptide signal intensity below 9000 a. u. threshold limit; [b] α-S2-Casein; [c] α-S1-Casein; [d] β-Casein; [e] intensity threshold was 3000 a. u.

Table S6. Peak intensity analysis for samples before and after MIP enrichment.

Peptide sequence	$[M+H]^+$ (Da)	Origin	Intensity (a. u.)			
			Sample	MIP-B	MIP-M	MIP-HE
DRVYIHPF	1046.54	Spiked	363213	43437	35139	31570
DRV**pS**IHPF	1050.48	Spiked	144086	43153	100226	95112
GADDSYYTAR	1118.48	Spiked	42264	5691	11240	13426
DRV**pY**IHPF	1126.51	Spiked	224855	99533	213266	223734
GADDSY**pY**TAR	1198.44	Spiked	0	11759	371113	54946
GADDS**pY**p**Y**TAR	1278.41	Spiked	0	7799	21441	21148
GSTAENAE**pY**LR	1290.54	Spiked	0	24775	79378	97943
TVDME**pS**TEVFTK	1466.61	CASA2[a]	125076	12472	19664	29531
MHLPSPTDSNF**pY**R	1644.69	Spiked	207047	137941	281338	199752
VPQLEIVPN**pS**AEER	1660.79	CASA1[b]	66293	47317	114561	124710
DIG**pS**E**pS**TEDQAMEDIK	1927.69	CASA1[b]	0	9353	24299	24762
YKVPQLEIVPN**pS**AEER	1951.95	CASA1[b]	169969	109210	267326	202206
FQ**pS**EEQQQTEDELQDK	2061.83	CASB[c]	71904	60667	167517	123085
GSHQISLDNPD**pY**QQDFFPK	2315.99	Spiked	51887	34352	110974	65043
RPAGSVQNPV**pY**HNQPLNPAPSRD	2594.22	Spiked	35786	27254	99005	60164
YSSDPTGALTEDSIDDTFLPVPE**pY**INQSVPK	3478.58	Spiked	3982	3078	8240	3641
Total intensity of all peptides			1506362	677791	1924727	1370773
Total intensity of pY peptides			523557	346491	1184755	726371
Total intensity of pS peptides			577328	282172	693593	599406
Total intensity of Y peptides			405477	49128	46379	44996
% of pY peptides intensity			34.76	51.12	61.55	52.99
% of pS peptides intensity			38.33	41.63	36.04	43.73
% of Y peptides intensity			26.92	7.25	2.41	3.28
Normalized pY/pS ratio			0.91	1.35	1.88	1.34
Normalized pY/Y ratio			1.29	5.46	19.78	12.50

[a] α-S2-Casein; [b] α-S1-Casein; [c] β-Casein.