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ARTICLE

Cooperative adsorption behavior of phosphopeptides on TiO₂ leads to biased enrichment, detection and quantification

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A. I. K. Eriksson, K. Edwards and V. Agmo Hernández

The adsorption behavior of phosphopeptides onto TiO₂ surfaces was studied using the quartz crystal microbalance with dissipation monitoring (QCM-D) as the main experimental technique. The main focus is the characterization of the emergence of positive cooperativity under conditions where the peptides have a positively charged C-term. It is shown that when carrying no net charge, small water-soluble peptides as a rule develop positive cooperativity. The impact of the adsorption mechanism on the outcome of TiO₂ based enrichment methods was investigated with the help of matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS). The data presented illustrate how the phosphopeptide profile in the enriched material may deviate from that in the native sample, as cooperative phosphopeptides are overrepresented in the former. Furthermore, commonly employed washing and elution solutions may facilitate preferential release of certain peptides, leading to further bias in the recovered sample. Taken together, the results of the present study demonstrate that thorough understanding of the mechanisms behind the adsorption of phosphopeptides on the enrichment material is necessary in order to develop reliable qualitative and quantitative methods for phosphoproteomics.

Introduction

The field of phosphoproteomics has grown rapidly in recent decades and mapping of the phosphoproteome is now recognized as essential for the complete characterization of proteins. Although the main focus to date has been the identification of new phosphorylation sites, recent research has also focused on characterizing the activity of already identified sites. The latter requires, in contrast to pure identification, accurate quantification of the amount of phosphorylated peptides both before and after triggering of the activation. As a result, quantitative phosphoprotein analysis has become essential for the field.¹⁻³

The most crucial part of any phosphopeptide analysis, be it qualitative or quantitative, is the specific enrichment step, where the relatively low abundant phosphopeptides are isolated from the native protein digest in order to make their detection possible. There is a variety of different enrichment methods to choose from at the present time.⁴⁻¹¹ However, poor overlap between the results obtained from different methods still remains a major problem.¹²⁻¹⁴ Some approaches seem to favor, for example, the enrichment of mono-phosphorylated peptides while others favor multi-phosphorylated.^{12, 15, 16} In order to help

devise an enrichment method that accurately reflects the phosphoproteome composition, it is first necessary to elucidate the mechanisms leading to enrichment in the methods most commonly used today.

In a recently published study we characterized the physical chemistry behind the phosphopeptide interaction with TiO₂,¹⁷ which is one of the most commonly used enrichment materials (e.g. in metal oxide affinity chromatography (MOAC)^{15, 18-20} and on-target methods^{10, 11, 13, 21-32} for matrix assisted laser desorption-ionization mass spectrometry (MALDI-MS) analysis). In this study¹⁷, the very interesting phenomenon of positive cooperativity was observed in the adsorption behavior of certain peptides. This means that, for the cited cases, there exists an attractive interaction between the peptides themselves, which implies that they can adsorb in multiple layers.³³ The source of the cooperativity was determined to be electrostatic interactions between positive amino acids in at least one of the peptide end terms and negative phosphoryl groups. The electrostatic nature of the interaction suggests that the phenomena might be quite common in the digested samples normally used for analysis. The reason is that one of the most commonly used enzymes for protein digestion, trypsin, cleaves the amino acid chains C-terminally to lysine, K, or arginine, R,

both of which are positively charged under the conditions that are normally used during the enrichment procedure. Phosphorylated peptides recovered after trypsination would therefore fulfill the condition previously observed for cooperativity to emerge.

In the present work, the adsorption behavior of synthetic monophosphorylated peptides in which cooperativity can be expected is studied with the help of the quartz crystal microbalance with dissipation monitoring (QCM-D) technique. The conditions under which cooperativity emerges are discussed. The impact that cooperativity may have on the reliability of the results obtained with common TiO₂-based enrichment methods is also investigated and exemplified by studying on-target binding and results obtained from MALDI-MS. Even though it is virtually impossible to consider all possible variations in the structure and properties of phosphorylated peptides, this report illustrates phenomena which are likely to affect the output of most TiO₂-based enrichment methods in use today. It also establishes a starting point from which future investigations on phosphopeptide enrichment behavior can benefit.

Materials and methods

Chemicals

Custom designed phosphopeptides were purchased from Thermo Scientific (Ulm, Germany). Trifluoroacetic acid (TFA >99%), sulfuric acid, hydrogen peroxide, acetonitrile (ACN), 2,5-dihydrobenzoic acid (DHB), salicylic acid (SA), ammonium dihydrogen phosphate (98%), ammonium hydroxide solution (25-28%), phosphoric acid (85%), acetic acid (HAc), terpineol, sodium phosphate (mono- and dibasic), and sodium chloride were obtained from Sigma-Aldrich (Steinheim, Germany). All aqueous solutions were prepared in deionized water (18.4 MΩ cm) obtained from a Milli-Q system (Millipore, Bedford, USA). Indium-tin oxide (ITO) coated glass slides were obtained from Hudson Surface Technology (Fort Lee, NJ, USA). TiO₂ paste containing nanoparticles with a diameter of 18 nm and pure anatase crystal structure was purchased from Dyesol (New South Wales, Australia). The paste was further diluted with four parts of terpenol before use. Titanium coated QCM-D sensors were purchased from Q-Sense (Gothenburg, Sweden).

Peptides

Four custom designed phosphopeptides were used. These peptides are modifications of the EGFR peptide (DADE-pY-LIPQQG), whose interaction with TiO₂ was characterized in our previous study¹⁷. The peptides were designed so that they would fulfill the conditions that give rise to cooperativity according to our previous paper. First, the C-term amino acid (G) in all four custom peptides was changed to arginine (R), to simulate a tryptic digestion product. The N-term amino acid (D) was also changed to arginine (R) in two of the peptides. Finally, the phosphorylated amino acid was changed from tyrosine (Y)

to serine (S) in two of the peptides. In this way all combinations of either tyrosine or serine phosphorylated peptides with either one or two R-substitutions were obtained. The sequences, mass and expected charge of all peptides at the pH used (2.6) are shown in Table 1. All peptide solutions were prepared by dilution with 0.01% TFA (aq) from a stock solution of 1 mg/mL in TFA buffer (ACN/water/TFA 50/49.9/0.1 (v/v)).

Table 1. Phosphopeptide information, including amino acid sequence, mass and net charge at the pH used.

Name	Sequence*	Mass (Da)	Net charge (pH = 2.6)
EGFR	DADE-pY-LIPQQG	1328.6	-1
mod-S-R	DADE-pS-LIPQQR	1349.6	0
mod-S-2R	RADE-pS-LIPQQR	1390.6	+1
mod-Y-R	DADE-pY-LIPQQR	1425.6	0
mod-Y-2R	RADE-pY-LIPQQR	1466.6	+1

*Phosphorylations are marked with a -p - and all charged amino acids are shown in bold.

QCM-D

The quartz crystal microbalance with dissipation monitoring (QCM-D) is a technique that allows both the mass and viscoelastic properties of an adsorbed layer to be determined. The technique has been used to study the interactions, structure and kinetics of many different biological systems (lipids, DNA, proteins, cells, etc).³⁴⁻⁴⁰ The core of the technique is a thin, circular quartz crystal with one of its faces (the sensing face) coated with the substrate material of interest. The other face is connected to an alternate potential which causes the crystal to oscillate due to the piezoelectric properties of quartz. During the QCM-D measurement, two parameters are monitored: the oscillation frequency (f) of the quartz crystal and the dissipation factor (D). Negative shifts in the oscillation frequency are related to the adsorption of material on the surface of the sensor (the more mass adsorbed, the larger the shift). Changes in the dissipation factor, on the other hand, are related to the viscoelastic properties of the adsorbed film. For rigid films ($\Delta D \approx 0$), the frequency change obtained (Δf) can be directly related to the adsorbed mass (Δm) through the Sauerbrey relationship⁴¹

$$\Delta m = -x A \Delta f_n n^{-1} \quad (1)$$

where x is the mass sensitivity constant (17.7 ng cm⁻² Hz⁻¹ at $f_0 = 5$ MHz), A the active area of the sensor, and n the overtone number.

All experiments were performed as described previously by Eriksson et al¹⁷. Briefly, an E1 QCM-D model (Q-Sense, Gothenburg, Sweden) was used with a pump connected downstream in the flow system. The temperature was set to 21 °C and the pump flow to 100 μL/min. Data was collected from the fundamental sensor oscillation frequency (5 MHz) as well as the 3rd, 5th, 7th, 9th, 11th and 13th overtones. The obtained data was used to build adsorption isotherms for the peptides studied.

PRE-EXPERIMENTAL TREATMENTS. The purchased titanium coated QCM-D sensors were first cleaned thoroughly with 95% ethanol, dried with nitrogen gas and then treated in hot piranha

1 solution (5:1 sulfuric acid:hydrogen peroxide) for 10-15 s in
2 order to oxidize the surface. The clean sensors were then rinsed
3 with water and left in 99% ethanol for at least 5 min to wash
4 away any chemical residues. The sensors were dried with a
5 gentle nitrogen flow before being mounted in the instrument.

6 The QCM-D flow system (excluding the sensor) was incubated
7 in a PBS buffer (10 mM phosphate, 150 mM NaCl, pH 5.5) for
8 30 min before the experiment in order to saturate all surfaces
9 with PO_4^- and avoid phosphopeptide loss in the loop. After
10 mounting of the sensor, the system was loaded with the
11 working solution (0.01% TFA) and allowed to stabilize.

12 **ADSORPTION EXPERIMENTS.** The adsorption experiments were
13 made with solutions of the pure peptides in 0.01% TFA (aq, pH
14 2.6). To build adsorption isotherms, 7-8 different peptide
15 concentrations, ranging from 0.01 to 3.2 μM were sequentially
16 loaded onto the system. The measurements began with the
17 lowest peptide concentration and, once the recorded signal was
18 stable, the system was rinsed with the loading buffer until
19 stability was reached again. The difference between the value
20 of the baseline and the value after rinsing gives a frequency
21 change proportional to the surface concentration of adsorbed
22 peptide. The procedure was then repeated for the next lowest
23 concentration, and so on until no significant frequency change
24 upon increasing concentration was obtained. All experiments
25 were performed at least twice, but using different
26 concentrations in order to obtain a detailed adsorption isotherm.
27 All frequency data was then normalized against the shift
28 observed at saturation.

29 Since the equilibrium is reached very slowly, the peptide
30 solution was recirculated to avoid the use of large sample
31 volumes. The equilibrium concentration was then calculated by
32 subtracting the amount of adsorbed molecules from the total
33 amount of molecules in the loading solution.

34 Phosphopeptide binding to TiO_2 -modified MALDI-MS targets

35 A 1:1:1:1 (0.25 μM each) mixture of the four peptides studied
36 was loaded onto MALDI-MS targets modified with TiO_2 spots
37 according to the protocol for phosphopeptide enrichment
38 reported by Eriksson et al.²⁴. MALDI-MS experiments were
39 performed after loading of the phosphopeptides on the substrate
40 as well as after washing with different buffers/solutions that are
41 often used in the washing or elution steps of common
42 enrichment methods. The solutions used in this study were: i)
43 SA buffer (18 mg/mL SA in ACN/water/TFA 50/49.9/0.1
44 (v/v)), aqueous ammonium acetate ii) pH 8, and iii) pH 9, iv)
45 aqueous TFA (50 mM, pH 1.33), and v) aqueous phosphate (10
46 mM $\text{NH}_4\text{H}_2\text{PO}_4$, pH 2.6). Finally, the mixture was also
47 analyzed with the combined enrichment and separation method
48 (stripe target), developed by Eriksson et al.¹³

49 **STEEL TARGET.** 0.5 μL of the mixture were first loaded on a
50 normal steel target, left to dry before 0.5 μL of DHB matrix (20
51 mg/mL DHB in ACN/water/ H_3PO_4 50/49/1 (v/v)) were added,
52 and again left to dry before proceeding with the MALDI-MS
53 measurement. Phosphoric acid is included in the matrix in order
54 to increase the ion response of the phosphorylated peptides⁴².

SPOT TARGET. Spots of TiO_2 (~5 mm in diameter and ~1-3 μm
thickness) were sintered onto ITO coated glass slides. A
volume of 1 μL of the peptide mixture was loaded on each spot
and the binding of the phosphopeptides to the surface was
allowed to proceed for 30 min. The glass slide was then either
i) dipped into 0.01% TFA (aq, pH 2.6) for 10 seconds, in order
to remove non-bound peptides, or ii) immersed for 10 minutes
with gentle shaking in the desired washing solution, followed
by a 10-second immersion in a 0.01% TFA bath. The spots
were then left to dry before 0.5 μL 0.4 M NH_4OH was added
and the spots left to dry again. 0.3 μL 2% (v/v) TFA (aq) was
then added and the spots were left to dry once more. Finally,
each spot was covered with 2 x 0.5 μL DHB matrix before
analysis with MALDI-MS. Each experiment was repeated at
least 8 times.

STRIPE TARGET. The binding surface was created by sintering a
thin stripe (1 mm x 75 mm and ~1.25 μm thickness) of
mesoporous TiO_2 onto an ITO-coated conductive glass slide.
1 μL of the peptide mixture was loaded at one of the ends of the
stripe, ~1 cm from the edge, and allowed to equilibrate for 30
min. The stripe was then bathed in 0.01% TFA (aq, pH 2.6) for
10 seconds and left to dry. The glass slide was then held
vertically, with the sample end of the stripe pointing
downwards, and put in an elution chamber filled to a level of ~5
mm with the elution buffer, 0.1 M $\text{NH}_4\text{H}_2\text{PO}_4$ (aq, pH 4.6).
With time, the buffer is slowly drawn up the stripe by means of
capillary action. The presence of phosphate causes the release
of the phosphorylated peptides from the surface and, as the
liquid migrates up through the stripe, the peptides become
separated according to their relative affinity for TiO_2 . The
elution was allowed to proceed for a total of 15 h at room
temperature. After the elution, the stripe was first left to dry,
then wetted with 2% (v/v) TFA (aq), left to dry again and
finally covered with DHB buffer (20 mg/mL DHB in
ACN/water/TFA 50/49.9/0.1 (v/v)) and dried prior to analysis.

MALDI TOF/TOF MS. Mass data was acquired with an
Ultraflex II MALDI-TOF/TOF (Bruker Daltonics) in reflector
positive mode. A mass range of 1000–2000 Da was analyzed
for the spots and of 1200–1700 Da for the stripe. The power of
the 337 nm nitrogen laser was adjusted to a level where the
signal to noise ratio was optimized. The steel and spot targets
were irradiated with 800 shots, randomly over the surface, and
a single spectrum was obtained. This was then repeated twice
and the mean values of peak area from all measurements,
obtained from the Flex Analysis 3.0 software, were used for
further analyses. The stripe target, on the other hand, was
divided into fifteen 5 mm sections. Each section was irradiated
randomly with 800 shots and a spectrum obtained for the
specific section.

55 Results and discussion

56 Emergence of cooperativity

57 Adsorption isotherms were constructed from the QCM-D data
58 by plotting the fraction of surface coverage (θ) versus the
59

equilibrium bulk concentration (C). In agreement with our previous report¹⁷, the dissipation changes were in all cases negligible and a proportional dependence of the adsorbed mass on the frequency shift can therefore be assumed (according to the Sauerbrey relationship given in equation 1 above). The value of θ for each of the loaded concentrations can therefore be calculated directly as the ratio of the frequency shift Δf measured for each point divided by the frequency shift observed at saturation (Δf_{max}). With the obtained data, Scatchard plots (θ/C vs θ) were built to better visualize any cooperativity. The different shapes obtained in the Scatchard plots are shown in Fig. 1. The initial positive slope of the Scatchard plots observed for the mod-Y-R (a) and mod-S-R (b) peptides (resulting in a characteristic “sad-mouth” shape of the curve), corresponds to the increase in surface affinity of the analyte upon increasing surface coverage expected for positive cooperativity⁴³. On the other hand, the Scatchard plots obtained for mod-S-2R (c) and mod-Y-2R (d) can be fitted to a straight line, indicating a Langmuir-like behavior.

The two latter peptides follow an almost ideal Langmuir behavior, as suggested by the linear Scatchard plots obtained.

Unfortunately, the QCM-D technique is not sensitive enough to resolve the adsorption behavior at surface coverage values lower than those shown in the figure. However, the observed wide linear range recorded for mod-S-2R and mod-Y-2R serves as a reliable indication of a Langmuir-like behavior. It is noteworthy that, for these samples, only the peptides substituted with a single arginine (R) show unequivocally positive cooperativity. Notably, in our previous work¹⁷ we demonstrated that IR (a tri-phosphorylated peptide derived from the insulin receptor and with three positive residues at or close to the ends) also shows cooperativity in spite of the multiple positive residues. The common characteristic of all peptides showing cooperative behavior, besides the presence of positive residues at the end of the sequence, is a net zero-charge of the molecule. Therefore, it can be speculated that electrostatic attraction forces between negative phosphoryl groups and positive amino acid residues alone are not enough for cooperativity to emerge. It also appears to be important that electrostatic repulsive forces between the peptides are absent or minimal.

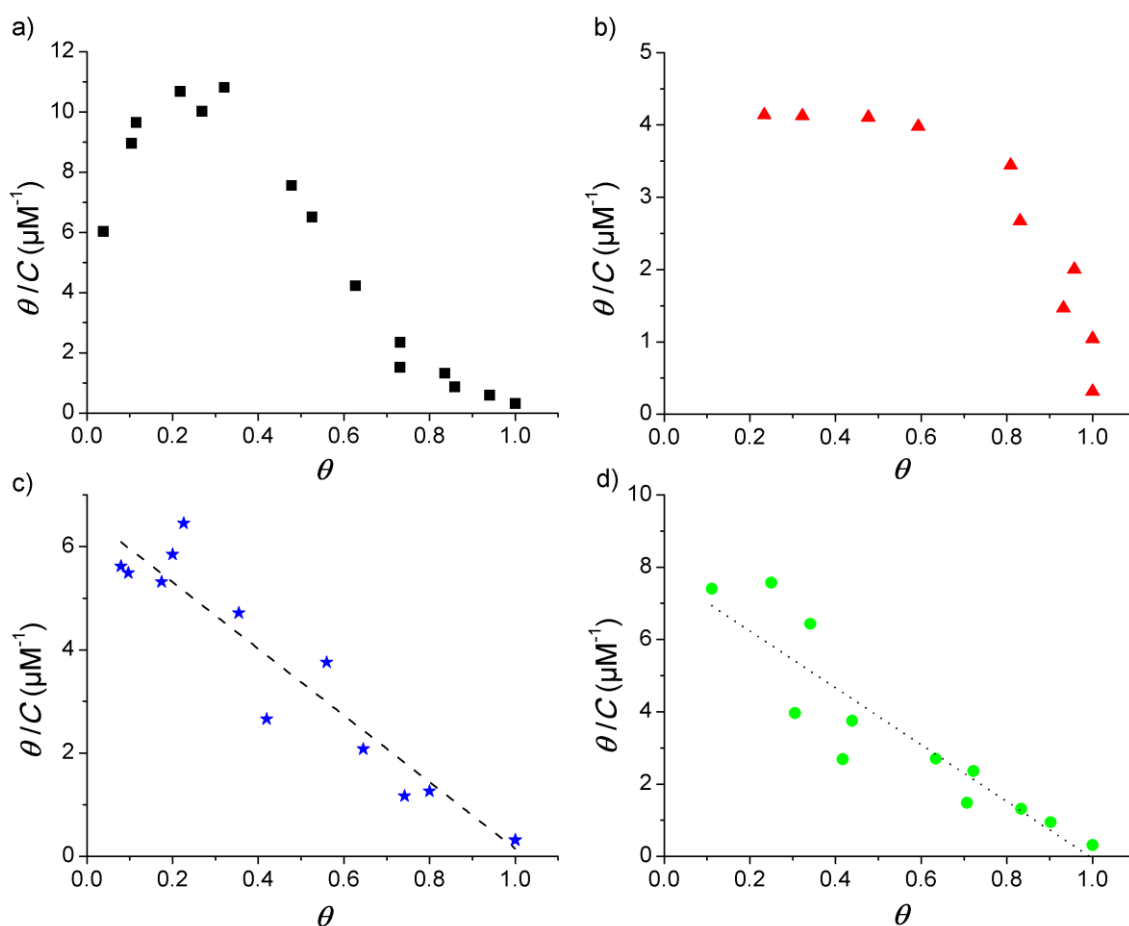


Fig. 1. Scatchard plots obtained from the adsorption isotherms of the modified peptides. a) mod-Y-R, b) mod-S-R, c) mod-Y-2R, and d) mod-S-2R. The lines represent the linear fitting of the plots in c and d: c) y -intercept = $-\text{slope} = K = 6.5 \pm 0.33 \mu\text{M}^{-1}$, $R^2 = 0.920$; d) $K = 7.8 \pm 0.65 \mu\text{M}^{-1}$, $R^2 = 0.849$

The plots in Fig. 1 further suggest that the cooperativity appears irrespective of whether the phosphorylated amino acid residue

is tyrosine or serine. However, the overall shapes of the Scatchard plots obtained for the two cooperative peptides are

quite different, which indicates that even though both are cooperative, the parameters describing the interaction are different. It is beyond the scope of this report to study the mechanisms behind this variance, but it is an interesting observation for future studies.

It is also necessary to point out that, although all the peptides in the present study contain an arginine end; other positively charged C-term amino acids may give rise to cooperative adsorption behavior. As an example, we previously showed that the IR peptide¹⁷, in which lysine is found at the C-term, presents a distinct cooperative behavior. Given the evidence gathered up to this point, it would appear that a positive charge at the C-term and an overall neutral charge of the peptide are the only requirements for cooperativity to emerge. Whether the same would be observed if the positive charge is located in the N-term, and whether cooperativity would arise from a positive charge just right next to the end-term amino acids, are matters for future research.

In summary, the observations described above suggest that positive cooperativity might be a common phenomenon during phosphopeptide enrichment from biologically relevant samples, since most monophosphorylated tryptic peptides (considering no missed cleavages) will present a positively charged C-term and display a zero-net charge under the conditions commonly used in these studies. This may bias the analysis, as cooperative phosphopeptides can be overrepresented in the enriched sample, as will be shown in the following sections.

Impact of cooperativity on TiO₂-based enrichment methods PHOSPHOPEPTIDE ADSORPTION. Fig. 2 shows the MALDI mass spectrum obtained from a 1:1:1:1 mixture of the four custom modified peptides on a normal steel target (Fig. 2a) and after equilibration on a TiO₂ spot (Fig. 2b). The figure is the result of adding several spectra obtained at different random positions on different spots.

In order to be able to estimate the efficiency of the phosphopeptide binding, i.e. the proportion of phosphopeptides in the sample that bind to the TiO₂ surface, a known concentration (1 μM) of native phosphorylated EGFR peptide was added to the DHB matrix in both experiments. This serves as a standard to monitor the extent of binding. Interestingly, the cooperative peptides present a higher signal intensity in both measurements, despite the results of earlier research which have shown that the ionization efficiency is related to the amount of R residues.⁴⁴ The MS spectrum obtained on the steel target is used as a reference to monitor any bias during the adsorption on TiO₂. Fig. 2b presents the mean mass spectrum obtained after equilibration on the TiO₂ spots, which shows a clear decrease in all phosphorylated signals with respect to the EGFR standard. This decrease in the relative signal may arise from complete saturation of the surface with phosphopeptides and subsequent removal of the excess peptides during the 10 seconds immersion in the 0.01% TFA bath described in the methods.

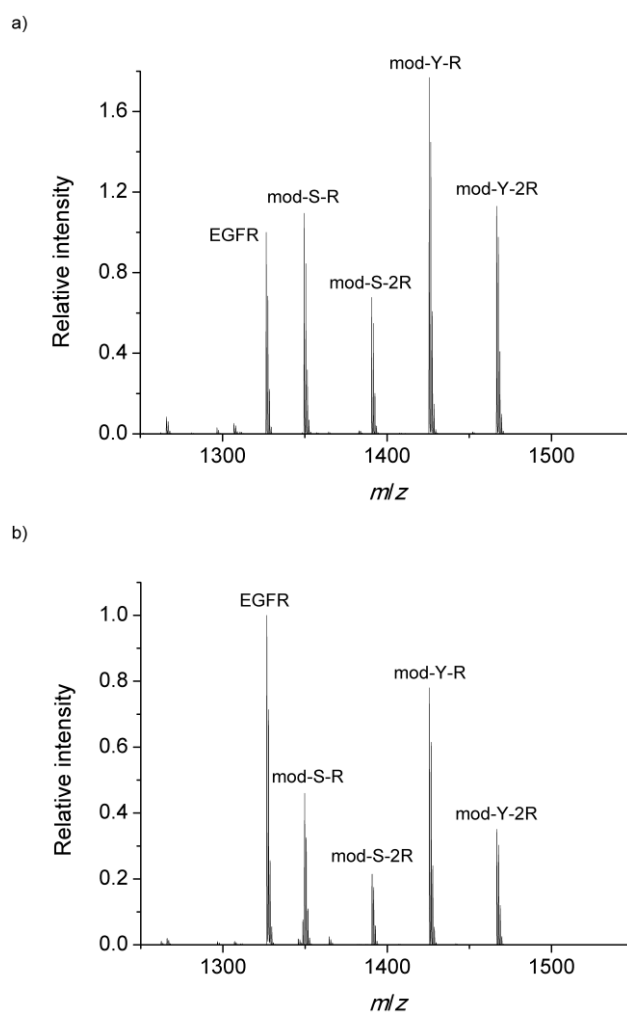


Fig. 2. Internal standard normalized MALDI-MS spectrum obtained a) on the steel target and b) after binding on TiO₂.

However, even though EGFR fulfills the requirements for a suitable standard (similar MW, structure, etc)^{45, 46}, the results should be treated with caution as MALDI-MS is not completely reliable as a quantitative technique⁴⁵ given the irregular distribution of the analyte and matrix on the spots. The trend observed is merely an indication of a likely adsorption behavior. More interestingly, the spectra obtained after equilibration on the TiO₂-modified target show a pronounced decrease in the signal of the non-cooperative peptides with respect to the cooperative peptides. This is clearly appreciated in Fig. 3, which shows the signal from phosphopeptides adsorbed on TiO₂ normalized against the signals on the steel target.

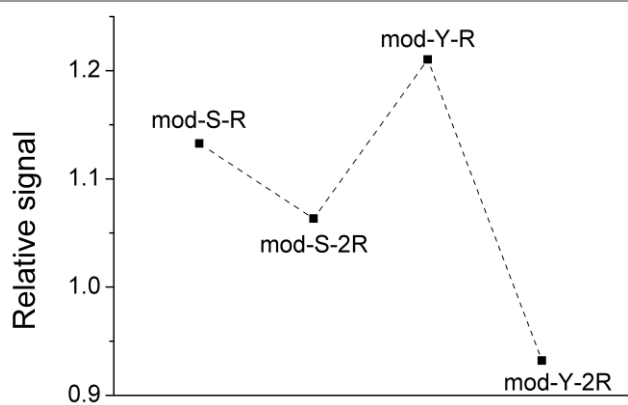


Fig. 3. Diagram showing the average of the ratio of the signal areas obtained on TiO_2 spots and on steel. The data presented is the average of the ratios calculated for every single pair of experiments ($y\text{-axis} = \langle (\text{Signal TiO}_2) / (\text{Signal steel}) \rangle$) and comprises 18 spectra obtained on 10 spots on steel and 23 spectra obtained on 10 TiO_2 spots (total = $18 \times 23 = 414$ ratios were calculated for each spot).

The same trend was observed in all repetitions of the experiment. In case of a non-biased adsorption, all points in the figure should appear at similar y -values. However, here it is apparent that the signals from the non-cooperative peptides decrease significantly more than those from the cooperative peptides, meaning that the binding under these conditions would be biased towards the latter. Paired t -tests corroborated that the relative signals of mod-Y-R and mod-S-R are indeed significantly larger than those of mod-Y-2R ($p \ll 0.001$) and mod-S-2R ($p = 0.007$), respectively. This result agrees with the notion of multiple adsorbed layers being formed in the case of cooperative peptides, which in principle can adsorb even when there are no sites left on the TiO_2 surface (a condition that is likely fulfilled given that most of the loaded phosphopeptides remain in solution). Both the raw experimental data and the supporting statistical analysis are included as Appendix A in the supporting information.

These findings clearly show that phosphopeptide enrichment methods may be intrinsically biased, as cooperative phosphopeptides will be overrepresented in the enriched sample. In qualitative studies, in which the aim is to determine the amino acid residues that can be found in a phosphorylated protein, the non-cooperative peptides may be missed as their concentration will be lower. In quantitative phosphoproteomics, the consequences may be even more severe, as, even if the peptides are successfully detected, the quantification will only reflect the relative composition of what was enriched, and not necessarily that of what was originally present in the sample. The co-enrichment of known concentrations of isotopically labeled peptides could in principle serve as an internal standard to reflect and correct for this bias. However, this would only be reliable if the concentrations of labeled and unlabeled peptides are in the same range or if the adsorption of the peptide follows pseudo-first-order kinetics. The first condition requires previous knowledge concerning the concentration in the sample, which makes the quantification experiment pointless. The second condition may be achieved if samples with

sufficient peptide concentration are used, or when the number of binding sites on the surface is excessively large. The former option requires a highly concentrated sample while the latter may result in a highly diluted sample for analysis. Even though the right conditions could be achieved to account for the preferential enrichment of cooperative peptides, it is in any case necessary to, first and foremost, characterize the adsorption mechanism of the peptide.

WASHING. The effect of the five different washing solutions, i.e. i) SA buffer, ii) pH 8, iii) pH 9, iv) pH 1.33 (TFA, aq) and v) 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$ (pH 2.6), is illustrated in Fig. 4. Due to clearly improved experiment reproducibility, the data shown in this figure is based on peak area instead of peak intensity values. The data has also been normalized against the corresponding data obtained after loading of the TiO_2 surface, but before washing. This means that what is observed in Fig. 4 is the relative change in the bound peptides' composition upon washing (bias due to washing). The raw experimental data, together with the statistical analyses on which our discussion is based, can be found in the supporting information as Appendixes B-F for the different washing solutions.

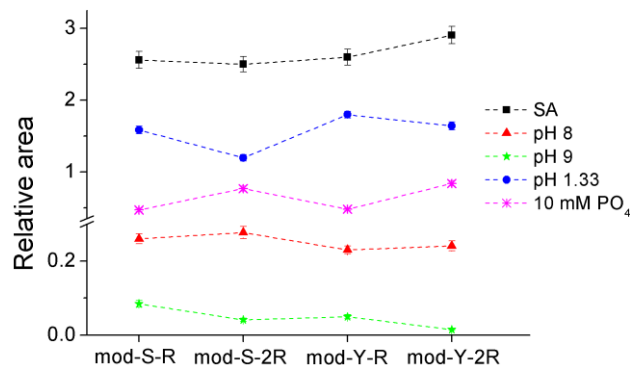


Fig. 4. Diagram showing the average of the ratio of the signal areas obtained after washing on a TiO_2 spot and on the spot without washing treatment. The washing solutions used are indicated in the legend. The data presented is the average of the ratios calculated for every single pair of experiments ($y\text{-axis} = \langle (\text{Signal after washing}) / (\text{Signal on TiO}_2) \rangle$) and comprises 23 spectra obtained on 10 TiO_2 spots and at least 9 spectra obtained on at least 3 washed spots. The error bars represent the standard error.

SA BUFFER. Small organic acids like SA are commonly used in the washing steps after the enrichment, with the purpose of removing potential bound acidic peptides from the surface.^{19, 47} The pattern obtained from the SA washing (Fig. 4, black squares) approaches a flat straight line with the exception of the data obtained for mod-Y-2R. The analysis included as Appendix B in the supporting information indicates that this peptide is slightly but significantly overrepresented ($p \ll 0.001$) while there is no significant difference between the other peptides ($p \gg 0.05$). The more likely interpretation of the data is that the SA buffer only washes away a very small proportion of the bound peptides, with the likely exception of mod-Y-2R, which may not be washed away at all. The composition and acidity of the solution is very similar to that of the loading buffer (with DHB being replaced by SA) and, therefore, the established phosphopeptide- TiO_2 interaction is not significantly

1 disturbed. One can conclude from this that washing with SA,
2 which is a common procedure used in TiO₂-based enrichment
3 methods^{13, 14, 19, 47}, will only modify the original enriched
4 composition marginally. In the present case, it is noteworthy
5 that the signals obtained are significantly larger than without
6 the washing step, as illustrated in Fig. 4, suggesting that the SA
7 treatment may facilitate the ionization by MALDI, making the
8 overall peptide identification easier.

9 **EFFECT OF pH.** The most common way to desorb/elute the
10 enriched phosphopeptides, both in on-target methods and in
11 MOAC, is to change the pH of the solution in contact with the
12 TiO₂ surface.²⁰ Basic eluents (pH 9-11) are mainly used, though
13 acidic eluents (pH ~1) should theoretically also work, since in
14 this case the phosphoryl groups (expected pK_a ~1.2^{48, 49}) would
15 be at least partially protonated, thus decreasing the electrostatic
16 interaction with TiO₂. We chose to study the washing effect of
17 two basic solutions (pH 8 and 9), as well as one acidic buffer
18 (pH 1.33). The reason that we chose not to go above pH 9 on
19 the basic side, was that higher pH values wash away essentially
20 all bound peptides.

21 The results from the treatment with pH 8 (Fig. 4, red triangles)
22 show that the serine substituted peptides are slightly
23 overrepresented with respect to the tyrosine substituted peptides
24 ($p < 0.05$). On the other hand, cooperativity does not seem to
25 play a role (raw data and the results from the paired t-test can
26 be found in Appendix C in the supporting information). At pH
27 9 (green stars), the excess of serine substituted peptides is still
28 present, and a significant excess of cooperative peptides is
29 observed (raw data and paired t-tests are provided as Appendix
30 D in the supporting information). In fact, non-cooperative
31 peptides were not observed at all in 80% of the experiments
32 performed. It can be speculated that the preferential release of
33 non-cooperative peptides arises due to the lower pK_a of TiO₂
34 (~5.8)⁵⁰ as compared to that of the side chain of the arginine
35 residues (~12.8), meaning that the interaction assumed to give
36 rise to cooperativity is much less affected than the one keeping
37 the peptides bound to the surface. It may seem counterintuitive
38 that a non-affected peptide-peptide interaction can prevent the
39 release of peptides from the TiO₂ surface when the peptide-
40 TiO₂ interaction is decreased. However, it is necessary to take
41 into account the fact that the peptide-TiO₂ and the peptide-
42 peptide binding reactions are simultaneous and reversible and
43 therefore codependent. It can be demonstrated from the
44 extended 2-layer BET-isotherm model, which describes the
45 cooperative behavior¹⁷, that the amount of cooperative peptides
46 that would remain at the surface is in fact larger than that of
47 non-cooperative if only, or primarily, the TiO₂-peptide
48 interaction is reduced.

49 Another interesting feature of the pattern obtained at basic pH
50 values is the relative increase in the serine containing peptides
51 signals compared to the equivalent tyrosine containing versions.
52 This indicates that tyrosine phosphorylated peptides are more
53 easily washed away by basic pH than the corresponding serine

phosphorylated peptides. It should be considered, however,
that, as illustrated in Fig. 4 and shown in Appendix C, the
measured signals are very low, suggesting that the great
majority of the peptides are washed away at this moderately
high pH.

The results obtained at pH 1.33 (blue circles) show a similar
trend concerning cooperativity to that observed at pH 9, i.e.
cooperative peptides are significantly overrepresented after the
washing treatment. In this case, the results are far more
trustworthy given the high signal intensities (raw data and
paired t-test included in Appendix E in the supporting
information). Under the used acidic conditions, it is expected
that a significant proportion (~40%) of the phosphoryl groups
will be protonated, thus losing their negative charge, which
should affect both the binding to TiO₂ and the interaction
between peptides. The decrease in the signals of non-
cooperative relative to the cooperative peptides can be expected
from this assumption and is in line with the 2-layer BET
isotherm model used to describe the cooperative behavior.
Noteworthy, in contrast to the behavior found at basic pH
values, is the fact that the relative signal from serine substituted
peptides decreases with respect to the signal from the
equivalent tyrosine substituted versions. This observation
indicates that the binding of phosphorylated tyrosine is less
affected at acidic pH values, suggesting the possibility that the
phosphoryl group in phosphotyrosine has a lower pK_a than in
phosphorylated serine.

PHOSPHATE SOLUTION. Phosphate-containing solutions are
another group of common eluents. These exploit the possibility
to replace phosphopeptides on the surface with phosphate ions,
thus causing the peptide to be released. In this part of the study,
we used a 10 mM phosphate solution at the same pH as in the
loading buffer (2.6). Remarkably, the results (Fig. 4, pink
crosses) show a bias pattern that is very different from that
observed in the cases above. The signals from the cooperative
peptides are thus significantly ($p \ll 0.001$) reduced relative to
those of the non-cooperative peptides (raw data and paired t-
test are included in Appendix F in the supporting information).
This indicates that the phosphate solution mainly disrupts the
cooperative behavior, while its effect on the TiO₂-
phosphopeptide interaction is more limited. This effect on
cooperative behavior may not necessarily be related to the
presence of phosphate per se, but could instead be due to the
increased ionic strength of the solution. As discussed in our
previous publication¹⁷, cooperativity is likely to be affected by
the ionic strength of the media in a more pronounced way than
the TiO₂-phosphopeptide interaction.

In principle, the bias obtained from phosphate washing can help
compensate for the bias of the enrichment method itself as they
are opposite. However, it is not clear which of the effects will
be predominant.

ARTICLE

Table 2. Summary of the effect of different eluent/washing solutions on the recovery of adsorbed phosphopeptides.

Washing/elution solution	Preferent binding	Preferent release (bias* for column MOAC methods)
SA	None	None
Ammonium acetate, pH 8	None	None
Ammonium acetate, pH 9	Serine-phosphorylated peptides	Non-cooperative peptides
TFA, pH 1.33	Cooperative peptides	Non-cooperative peptides
Phosphate 10 mM, pH 2.6	Non-cooperative peptides	Cooperative peptides

*Not considering the bias from the adsorption.

SUMMARY. In conclusion, from the results shown above (summarized in Table 2) it is clear that both the enrichment and the washing/elution procedures may bias the results.

First, peptides that show cooperative adsorption will be overrepresented after TiO₂ enrichment, regardless of the experimental approach used (on-target or in-column enrichment). Secondly, the washing/elution solution may cause further bias. With regard to on-target methods, in which usually SA or similar buffers are used for washing, this second problem can be diminished or avoided. On the other hand, in the column-based MOAC approach, where pH changes are usually used to release the bound peptides, the results will be dependent on the eluent used. This is a problem already addressed by Aryal et al²⁰, who discovered that the phosphopeptides recovered from TiO₂-MOAC columns varied significantly with the eluent used, as the results in this report also show.

COMBINED LOADING AND SEPARATION. In order to test the predictions from the bias behavior summarized in Table 2, an experiment was performed using the combined enrichment and separation method, developed by Eriksson et al¹³, which is based on a thin stripe-shaped TiO₂ target, on which the peptides are loaded and posteriorly eluted with a phosphate solution. The separation itself works like ion-chromatography, where the individual affinity for the surface/eluent determines the distance each peptide moves on the stripe. The method has been shown to be capable of both separating multi- and monophosphorylated peptides and increasing the signal of less abundant peptides and is therefore clearly a method of interest evaluating cooperativity.

The results of the experiment are shown in Fig. 5, where the MALDI mass spectra obtained from different sections of the stripe (see methods section) are shown.

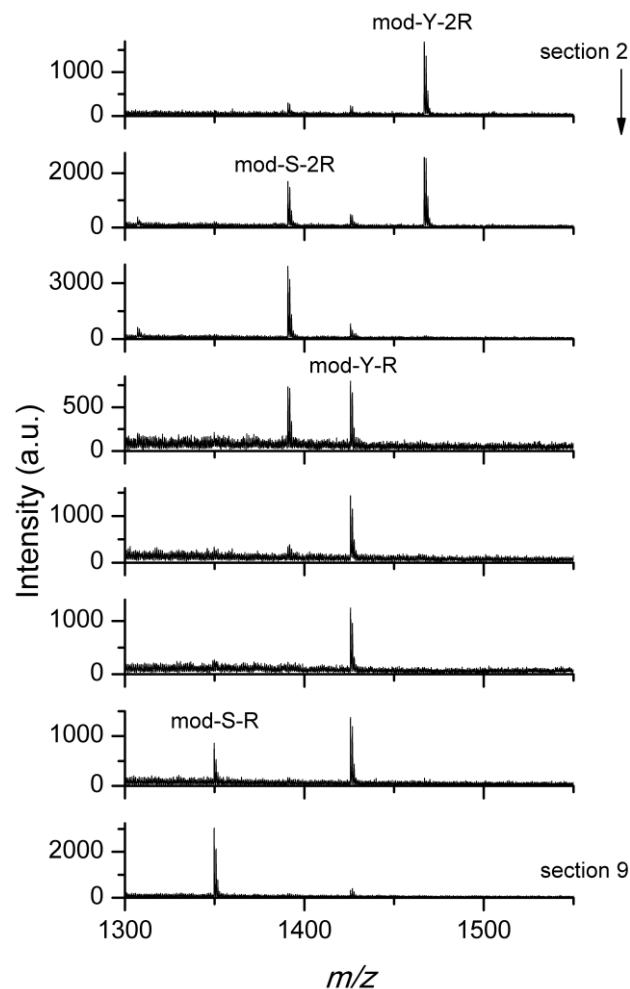


Fig. 5. MALDI mass spectra for sections 2 (start) – 9 (end) of the stripe target. The first occurrence of each peptide peak is indicated. The arrow indicates the direction of the elution.

As can be seen from the figure, the loaded peptides were successfully separated. In agreement with the results discussed in the previous section, the elution with phosphate anions favored the release of cooperative peptides, which are found

1 further up the stripe. The small difference between serine and
2 tyrosine substituted peptides observed in Fig. 4 also affects the
3 elution behavior, as the serine peptides are more easily eluted
4 than the corresponding tyrosine substituted versions. Similar
5 experiments were performed using basic buffers (pH value
6 between 6.5 and 9) as eluents instead of the phosphate solution
7 used above, with the purpose of confirming a behavior opposite
8 to that illustrated in Fig. 4. Unfortunately, the almost instant
9 release of the peptides at high pH is a problem, given the slow
10 elution rate. The peptides diffuse in the available volume and
11 are lost in the pool of eluent. The small traces detected in the
12 stripe do not allow for further analysis.

13 In any case, the good agreement between the QCM-D, the on-
14 target spot binding/washing, and, in the case of phosphate
15 washing, the stripe separation experiments, is remarkable.
16 Taken together, our results provide solid evidence concerning
17 the preferential binding/washing/elution of different sets of
18 peptides depending on their adsorption mechanism and the
19 eluent/washing solution used.

22 Conclusions

23 The presence of competing adsorption mechanisms can result in
24 significant overrepresentation of certain phosphopeptides in the
25 enriched sample. The results from this study suggest that
26 positive cooperativity, i.e. positive interaction between
27 adsorbed peptides and peptides in the bulk solution, might be a
28 common phenomenon during the enrichment of tryptic
29 peptides. It is, however, important to note that the peptides
30 studied here are rather small and water-soluble. Further
31 investigations are therefore needed to elucidate if the
32 predictions from the present study also hold for larger and more
33 hydrophobic peptides, where more complex interactions may
34 come into play. Furthermore, phosphopeptide enrichment from
35 complex samples (such as tryptic digests from large proteins) is
36 likely to involve several other adsorption mechanisms, and
37 whether cooperativity is significantly relevant in such a
38 scenario needs to be explored further.

39 However, it is clear that unless the adsorption mechanisms of
40 each peptide is known and taken into account, the analysis of
41 the eluted fraction in chromatography, or of the remaining
42 peptides bound in on-target methods, may give a skewed
43 picture of the distribution of phosphorylations. Even if the
44 purpose is to perform a comparative analysis, complex
45 adsorption behavior may lead to erroneous conclusions
46 concerning the extent of the increase or decrease in the
47 phosphopeptide concentration. Data collected in the present
48 study also illustrate that the washing and elution steps needed
49 to recover the phosphopeptides, or to wash away the remaining
50 non-phosphorylated peptides, may lead to further bias. These
51 observations may, at least partially, explain the lack of overlap
52 between the phosphoproteomes determined from different
53 methods. This problem clearly needs to be resolved in order to
54 obtain reliable quantitative phosphopeptide analysis methods.
55 Given the potential complexity of the adsorption behavior in a
56 relevant peptide mixture, it is likely that the only option to

perform a reliable quantitative analysis is to enrich or isolate
100% of all the phosphopeptides present.

In summary, it can be concluded that in-depth knowledge of the
various mechanisms governing the affinity of phosphopeptides
to the enrichment material is necessary for the further
development of reliable enrichment protocols. In the present
study we have clarified some important factors relevant to the
interaction between small, water-soluble phosphopeptides and
TiO₂-based materials.

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Notes

Department of Chemistry – BMC, Uppsala University, Uppsala, Sweden
victor.agmo@kemi.uu.se

*V. Agmo Hernández, Department of Chemistry - BMC, Uppsala
University, Husargatan 3, Box 579, 75123, Uppsala, Sweden. Phone: +46
(0) 18 471 3635. Fax: +46 (0) 18 471 3654. E-mail:
victor.agmo@kemi.uu.se

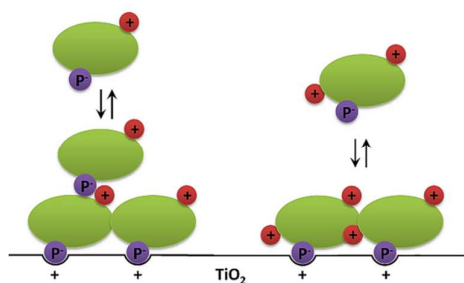
Electronic Supplementary Information (ESI) available: Both the raw
experimental data and the statistical analyses upon which the discussion
in the section "Impact of cooperativity on TiO₂-based enrichment
methods" is based can be found in the supporting information as
Appendixes A-F. See DOI: 10.1039/b000000x/

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Table of contents entry



Novel data show that anomalous adsorption behavior and common washing procedures can lead to biased results in TiO_2 -based phosphoproteomics.