Amino Acids (2011) 41:311–320
DOI 10.1007/s00726-010-0647-7

ORIGINAL ARTICLE

Metal ion-mobilizing additives for comprehensive detection of femtomole amounts of phosphopeptides by reversed phase LC-MS

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Received: 29 March 2010 / Accepted: 31 May 2010 / Published online: 16 June 2010

Abstract It is hypothesized that metal ion-mediated adsorption of phosphorylated peptides on stationary phases of LC-columns is the major cause for their frequently observed poor detection efficiency in LC-MS. To study this phenomenon in more detail, sample solutions spiked with metal ion-mobilizing additives were analyzed by reversed phase μLC-ICP-MS or nanoLC-ESI-MS. Using μLC-ICP-MS, metal ions were analyzed directly as atomic ions. Using electrospray ionization, either metal ion chelates or phosphopeptide standard mixtures injected in subpicomole amounts were analyzed. Deferoxamine, imidazole, ascorbate, citrate, EDTA, and the tetrapeptide pSpSpSpS were tested as sample additives for the interlinked purposes of metal ion-mobilization and improvement of phosphopeptide recovery. Iron probably represents the major metal ion contamination of reversed phase columns. Based on the certified iron level in LC-grade solvents, a daily metal ion load of >10 pmol was estimated for typical nanoLC flow rates. In addition, phosphopeptide fractions from IMAC columns were identified as source for metal ion contamination of the LC column, as demonstrated for Ga^{3+}-IMAC. The three metal ion-chelating additives, EDTA, citrate and pSpSpSpS, were found to perform best for improving the LC recovery of multiply phosphorylated peptides injected at subpicomole amounts. The benefits of metal ion-mobilizing LC (mimLC) characterized by metal ion complexing sample additives is demonstrated for three different instrumental setups comprising (a) a nanoUPLC-system with direct injection on the analytical column, (b) a nanoLC system with inclusion of a trapping column, and (c) the use of a HPLC-Chip system with integrated trapping and analytical column.

Keywords Phosphopeptides · Metal ion mobilization · Metal ion chelators · Metal-free LC · Phosphopeptide recovery · Multiply phosphorylated peptides

Electronic supplementary material The online version of this article (doi:10.1007/s00726-010-0647-7) contains supplementary material, which is available to authorized users.

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Abbreviations

MS	Mass spectrometry
EDTA	Ethylendiaminetetraacetic acid
RP-LC	Reversed phase liquid chromatography
capLC	Capillary liquid chromatography
DFO	Deferoxamine
IMAC	Immobilized metal ion affinity chromatography
FA	Formic acid
Ni-NTA	Nickel nitrilotriacetic acid
ESI	Electrospray ionization
ICP	Inductively coupled plasma
mimLC	Metal ion-mobilizing LC

Introduction

Gradient liquid chromatography (LC) on reversed phase material directly coupled to electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) has developed into the most powerful technique in analytical proteomics. Due to the outstanding biological impact of reversible protein phosphorylation, phosphopeptide analysis by LC-ESI-MS/MS has attracted particular methodological interest. This field is challenging, since frequently low and erratic recoveries of phosphopeptides in LC-ESI-MS/MS analysis are observed, especially in connection with the analysis of multiply phosphorylated peptides. This phenomenon is also valid for histidine-containing peptides, in particular, for those carrying a typical His tag with a stretch of six His residues, which usually cannot be observed in reversed phase LC-MS due to their strong adsorption. In analogy to the intentional extraction of His-tagged proteins by Ni^{2+}-IMAC columns, the interaction of tryptic His-tag peptides with reversed phase columns is probably also mediated by metal ions. Metal ions can be immobilized at the reversed phase surface, e.g. attached to other adsorbed molecules with polar functional groups, such as peptides.

Metal ion-complexing sample additives have been tested for improvement of phosphopeptide analysis in MALDI-MS (Asara and Allison 1999; Yang et al. 2004; Kjellstrom and Jensen 2004). In LC-ESI-MS of RNA, an EDTA additive to the samples was shown to improve the quality of the corresponding ESI spectra, an effect mainly ascribed to an improvement of the ionization conditions during the ESI process due to the metal ion-masking properties of EDTA (Hölzl et al. 2005). For LC-ESI-MS analyses addition of phosphoric acid (Kim et al. 2004), EDTA (Liu et al. 2005) or a combination of both compounds (Qing et al. 2006) has been demonstrated to improve the detection of phosphopeptides. Upon repeated injection of samples in the presence of 50 mM EDTA as recommended (Qing et al. 2006), we observed precipitations on the reversed phase column and clogging of the ESI spray needle. This limitation could be overcome by selecting 50 mM citrate as additive which exhibited a similarly positive effect as EDTA but without its side effects (Winter et al. 2009). Citrate addition was found to particularly improve the detection of highly phosphorylated peptides with three or more phosphate groups as demonstrated by the detection of up to five times phosphorylated tryptic peptides of SET-DB1, which were not visible without citrate additive (Winter et al. 2009). In a very recent study, EDTA at a concentration of 0.1 mM was demonstrated to improve phosphopeptide detection avoiding solubility problems by the low level of the chelator additive (Nakamura et al. 2010). In the following, the mechanism by which chelators increase phosphopeptide recovery in LC-MS is investigated and discussed and new additives for metal ion complexation are tested.

Materials and methods

Chemicals

Water, acetonitrile, and formic acid were in 'ULC'-quality from Biosolve (Valkenswaard, The Netherlands). Citric acid monohydrate was from Calbiochem (Darmstadt, Germany). Phosphopeptides were from a commercially available standard mixture (MassPREP phosphopeptide standard) from Waters (Milford, MA) or synthesized in-house using Fmoc chemistry. Imidazole, deferoxamine (DFO), ethylenediaminetetraacetic acid (EDTA), ascorbate and all other chemicals were from Sigma (Deisenhofen, Germany) in at least analytical grade quality.

Standard mixture preparation

The MassPREP phosphopeptide standard was dissolved in water to 200 pmol/μL and spiked with three multiply phosphorylated peptides synthesized in-house in concentrations of 400–800 pmol/μL. This stock solution was diluted in two subsequent 1:1,000 steps to achieve a final dilution by 10^6 directly before analysis with a solution containing 50 mM citrate, 50 mM ascorbate, 250 μM EDTA, 50 μM pSpSpSpS or 500 μM imidazole before LC-MS analysis, respectively.

nanoESI-MS

Experiments were carried out on a QTOF2 mass spectrometer (Waters, Micromass, Manchester, UK). Solutions were sprayed from in-house produced gold-coated spray needles. Static electrospray was established by applying a capillary voltage of 1,000 V.

UPLC-ESI-MS

Analyses were performed using two different systems. A nanoAcquity UPLC System (Waters, Milford, MA) was used either without (Fig. 1a) or with (Fig. 1b) trapping column in combination with a QTOF2 mass spectrometer (Waters, UK) or a LTQ Orbitrap (Thermo, Bremen, Germany). The analytical column used was a 150 mm × 75 μm BEH C18 column packed with 1.7 μm particles with a pore width of 130 Å. LC-MS coupling was performed using a Pico Tip sprayer (Waters, Manchester, UK) operated using PicoTips (New Objective, Woburn, MA) with an inner diameter of 10 μm. UPLC-MS/MS analyses were carried out at a flow rate of 400 nL/min and a column

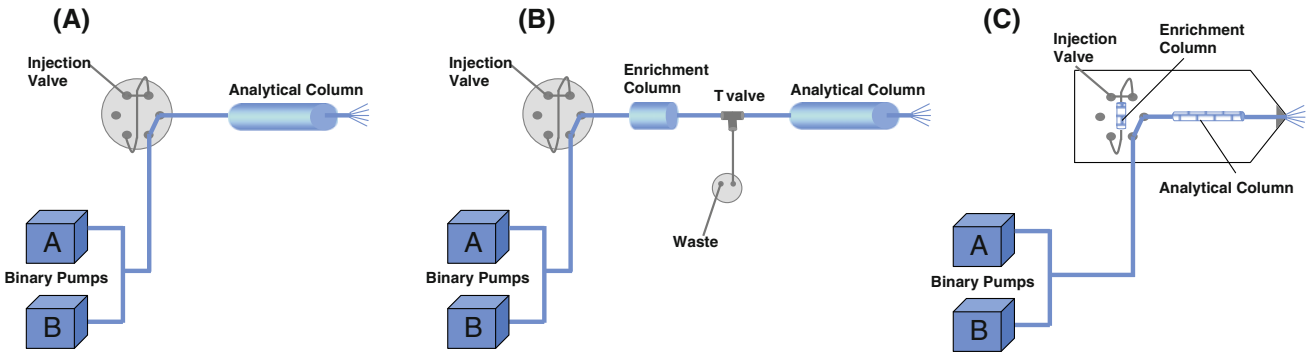

Fig. 1 Schematic display of LC set-ups used in this study. **a** nanoUPLC without trapping column, **b** nanoUPLC with separate trapping and analytical column and **c** nanoLC-ESI chip with integrated trapping column

temperature of 35°C. When samples were loaded directly onto the analytical column, constant elution with 99% A, 1% B (A: H_2O + 0.1% FA; B: ACN, 0.1% formic acid) for 24 min was used, followed by a linear gradient from 99% A to 70% A in 30 min. Using a set-up with trapping column (Symmetry C18, 5 µm, 180 µm × 20 mm, Waters), samples were loaded at a flow rate of 10 µL/min. Then samples were desalted for 11 min using 1% B, eluted onto the analytical column, and separated using the gradient described above. Both mass spectrometers were operated in positive ion mode with a capillary voltage of 2,400 V and a cone voltage of 35 V.

HPLC-Chip/MS

Analyses were performed on a QTOF mass spectrometer (Agilent 6520, Agilent Technologies, Waldbronn, Germany) coupled to an 1200 Agilent nanoflow system via a HPLC-Chip cube ESI interface (Fig. 1c). Peptides were separated on a HPLC-Chip with an analytical column of 75 µm i.d. and 150 mm length and a 40-nL trap column both packed with Zorbax 300SB C-18 (5 µm particle size). Peptides were eluted with a linear acetonitrile gradient with 1%/min at a flow rate of 300 nL/min (starting with 3% acetonitrile). The QTOF was operated in the 2 GHz extended dynamic range mode. Internal calibration was performed by one-point calibration.

µLC-element MS (µLC-ICP-MS)

A capillary LC system (Waters, Milford, MA) equipped with a 300 µm × 150 mm capillary column (Waters, Milford, MA) packed with C18 particles of 5 µm and a pore size of 300 Å was coupled online to a sector field ICP-MS type Element 2 (Thermo, Bremen, Germany) through a low-flow microconcentric nebulizer CEI-100 (CETAC, Omaha, USA) at a flow rate of 5 µL/min. A standard injection volume of 5 µL was applied. The ICP-MS was operated with the following conditions: sample gas, 1.0 L/min; auxiliary gas, 0.8 L/min; plasma power, 1,300 W. ^{31}P and ^{56}Fe were monitored with a mass spectrometric resolution of 1,500 or 4,000, respectively. Each selected isotope was recorded by a set of 20 data points with a dwell time of 10 ms per data point.

Results and discussion

Concept

This study is based on the concept that irreversible adsorption on reversed phase columns is a major mechanism responsible for incomplete recovery of peptides, in particular, when they carry multiple residues of phosphoamino acids, histidine, glutamic acid or aspartic acid. As explanation for this phenomenon, a three-component system is considered, consisting of the column surface, a multivalent surface-immobilized metal ion, and the adsorbed peptide which is coordinated to the surface-bound metal ion by one of the residues listed above. This model is schematically outlined for phosphopeptide adsorption in Fig. 2.

Destruction of the three-component system outlined in Fig. 2 in an acidic LC solvent can be accomplished by displacement of phosphopeptides by an excess of free metal ions, capping of metal ions by an excess of ligands, or mobilizing of metal ions by complexing agents. These three strategies will be shortly discussed. Using a Mg^{2+} gradient, phosphopeptide elution from IMAC columns has been demonstrated (Muszyńska et al. 1986). This strategy cannot be recommended for trace analysis by LC-ESI, due to the detrimental effects of salt contaminations on the efficiency of ESI. The addition of 0.1% phosphoric acid to the sample improved the detection efficiency for the tetraphosphorylated β-casein fragment in LC-MS (Kim et al. 2004). However, the corresponding singly phosphorylated tryptic fragment was still detected at much higher sensitivity compared to the quadruply phosphorylated tryptic

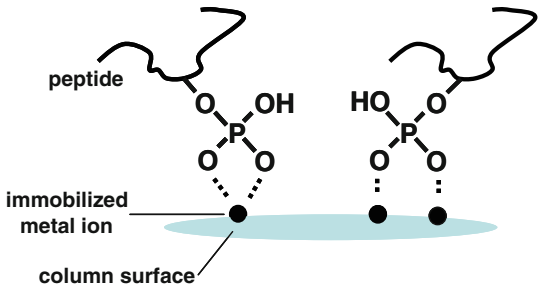

Fig. 2 Model for explaining adsorption of phosphorylated peptides on the stationary phase of an LC column. The system consists of the three components column surface, immobilized metal ion, and phosphopeptide. Peptides containing histidine groups or acidic groups may interact similarly with surface-immobilized multivalent metal ions

fragment. In addition, phosphate ions probably form precipitates on the analytical column such as of iron(III) phosphate, leading to alterations of the column properties and an increased back pressure. Addition of metal ion-mobilizing ligands to the samples appears to be a more promising strategy compared to metal ion addition or phosphate addition. The acronym mimLC (for metal ion-mobilizing LC) is introduced for LC performed with addition of metal ion-complexing sample additives. In mimLC, the complete LC system including the stationary phase is depleted from metal ions. In this way, metal ion-mediated adsorption of polar analytes such as phosphopeptides is minimized since adsorbed metal ions are eluted as metal ion complexes.

Metal ion mobilization from reversed phase columns

We first selected μLC-ICP-MS to directly monitor a presumed chelator-induced metal ion mobilization from reversed phase columns. Repeated injections were applied as tool to monitor metal ion mobilization from the LC column. The general observation was that repeated injections of identical samples containing a metal ion complexing agent led to a decreasing metal ion mobilization indicating a gradual depletion of column-bound metal ions. As an example, Fig. 3 shows the elution of Fe, Al, Ti and P for two subsequent injections of samples containing 50 mM citrate. The peak areas for all elements in the second injection are lower compared to the first injection indicating that the elements in part stem from the analytical column. The elution of phosphorus shows the same behavior, supporting the three-component model for phosphopeptide adsorption as outlined in Fig. 2.

The use of mimLC-ICP-MS provides a direct insight into the amount and nature of mobilized metal ions. However, this specialized equipment is only rarely available in analytical proteomics laboratories. Therefore, the possibility to monitor the effect of mimLC columns using LC-ESI-MS is an attractive alternative. For this purpose, we selected the siderophore deferoxamine (DFO), which is a natural trivalent metal ion chelator clinically applied for iron depletion in patients with beta-thalassemia (Giardina and Grady 1995) or hereditary hemochromatosis (Nielsen et al. 2003), diseases characterized by accumulation of excessive amounts of iron mainly in the liver. Removal of excess iron by administration of DFO is based on the extremely high affinity of deferoxamine to trivalent metal ions such as Fe^{3+}, Ga^{3+}, or Al^{3+} ($K_d = 10^{-31}$ M for Fe^{3+}) (Rosebrough 1993) in combination with good water solubility of the deferoxamine–Fe^{3+} complex. To test the suitability of ESI-MS for detection of DFO in free and iron-loaded form, DFO at 1 pmol/μL was incubated with Fe^{3+} at pH 2 for 15 min at molar DFO/Fe ratios from 1/0 to 1/1 and analyzed by positive ion nanoESI-MS. Pure solutions of DFO only showed a signal for free protonated DFO at *m/z* 561, whereas the equimolar DFO + Fe^{3+} mixture only showed a signal for the protonated intact DFO–Fe^{3+} complex at *m/z* 614. The mixtures with DFO and Fe^{3+} at molar ratios between 0 and 1 showed signals for both free and Fe^{3+}-loaded DFO with an intensity ratio corresponding roughly to the gravimetric molar DFO to Fe^{3+} ratio (see Fig. 4).

In the next step we attempted to demonstrate iron adsorption on and its mobilization from a nanoLC column. For this purpose nine consecutive injections of 100 pmol of free DFO were performed. The result in Fig. 5 shows that a substantial amount of the DFO–Fe^{3+} complex is eluted after each injection and that this amount is exponentially decreasing over the series of injections. This time course indicates a gradual mobilization of iron from the nanoLC system. The elution of the DFO–Fe^{3+} complex extends over 7–8 min, compared to about 1.5 min for the peak of free DFO. This difference in elution behavior may indicate a mobilization of iron from various regions of the nanoLC system. Due to the high water solubility of DFO only a negligible spillover to consecutive injections is observed as demonstrated in Figure S1.

We suspected that phosphopeptide fractions generated by immobilized metal ion chromatography (IMAC) could represent an important source of metal ion contamination for analytical LC columns, since these fractions may be contaminated with the 'immobilized' metal ions from the IMAC columns. Phosphopeptide-loaded Ga^{3+}- or Fe^{3+}-IMAC are frequently eluted at basic pH using, for example, 400 mM NH_4OH at pH 11.5 (Kokubu et al. 2005), which can also effect a mobilization of the metal ions. Phosphopeptide fractions often are directly injected into the LC system without a desalting step to guarantee optimal phosphopeptide recovery. Hence, it is likely that in such a

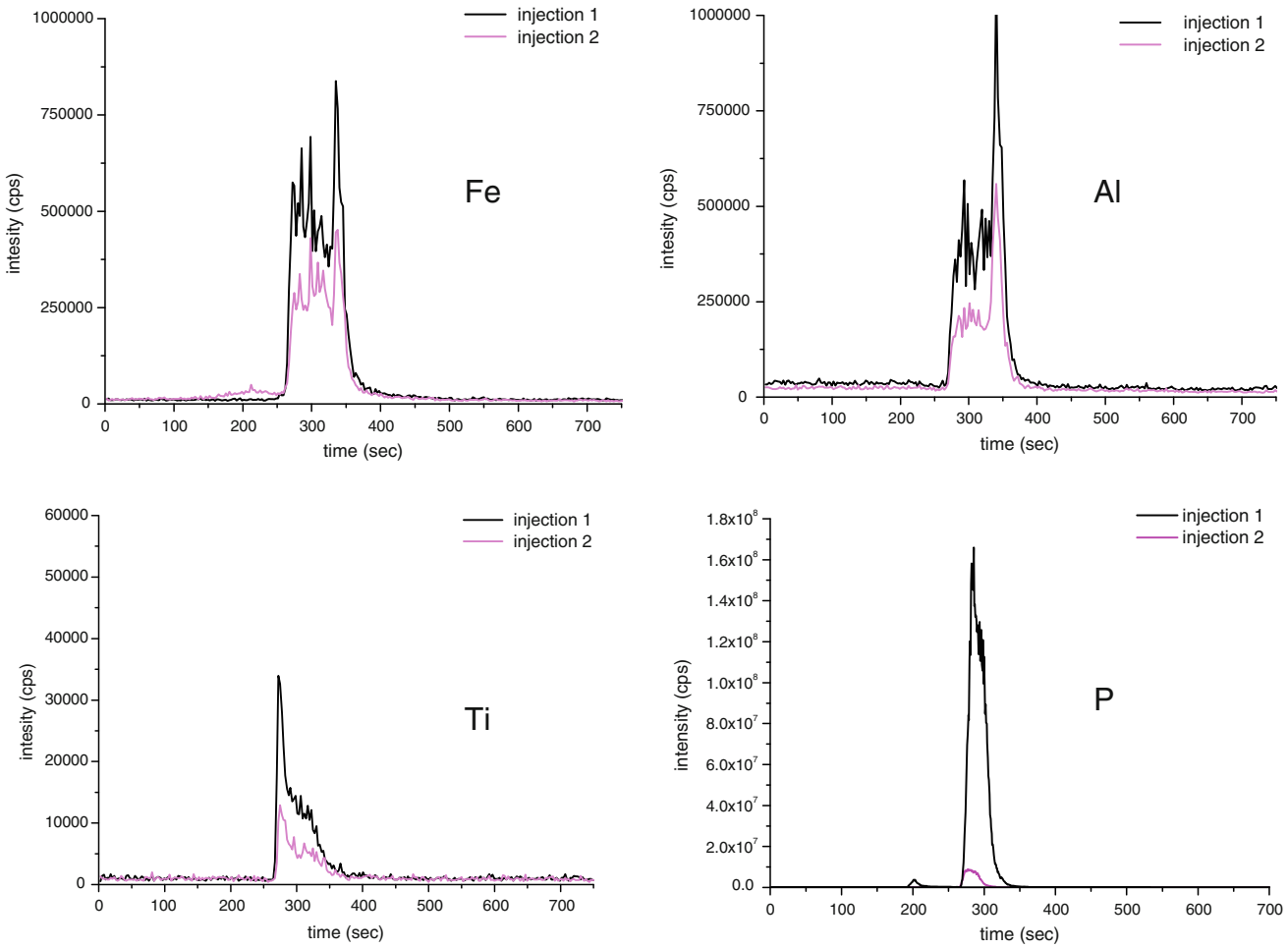

Fig. 3 Detection of Fe, Al, Ti and P via µLC-ICP-MS in two subsequent injections of 50 mM citrate. The second injection always shows the elution of a reduced element amount, indicating their mobilization from the reversed phase column. The findings support the model shown in Fig. 2

straightforward analysis, mobilized metal ions are transferred onto the analytical column. We subjected digests of autophosphorylated cAMP-dependent protein kinase A (PKA) to Ga^{3+}-IMAC phosphopeptide enrichment and analyzed the phosphopeptide fractions by nanoUPLC-MS using on-column injection (Seidler et al. 2009). Before and after analysis of these phosphopeptide fractions, 1 pmol of DFO was injected in the nanoUPLC-MS system. As shown in Fig. 6, the DFO mimLC before analysis of the IMAC fractions showed the elution of DFO–Fe^{3+} and DFO–Al^{3+} complexes at *m/z* 614 and *m/z* 585, respectively (Fig. 6a). After analysis of three phosphopeptide IMAC fractions, DFO mimLC resulted in the elution of the DFO–Ga^{3+} complex at *m/z* 627/629 in an amount similar to the DFO–Fe^{3+} complex (Fig. 6b). The characteristic isotope distribution of gallium is visible in the DFO–Ga^{3+} complex and supports its annotation (Figure S2). It is concluded that metal ions eluting from IMAC columns in the phosphopeptide elution step contribute significantly to metal ion loading of analytical LC columns. It is hypothesized that these metal ion contaminations convert reversed phase columns into 'low performance IMAC' columns, resulting in phosphopeptide adsorption.

Due to its strong complex formation with trivalent metal ions, DFO can be used to study the contamination of LC columns, e.g. by iron, aluminum or gallium. However, free DFO and DFO–Me^{3+} complexes exhibit retention times on LC columns in the same region as peptide retention times. Therefore, the use of DFO is recommended for cleaning and monitoring of the metal ion contamination status of an LC column, rather than as general sample additive. For the latter purpose, metal ion mobilizing agents eluting during the wash phase of a gradient LC run are preferred, as outlined in the following.

Novel metal ion-mobilizing additives

In contrast to the DFO–Me^{3+} chelates, many other metal complexes are not directly amenable to ESI-MS analysis in

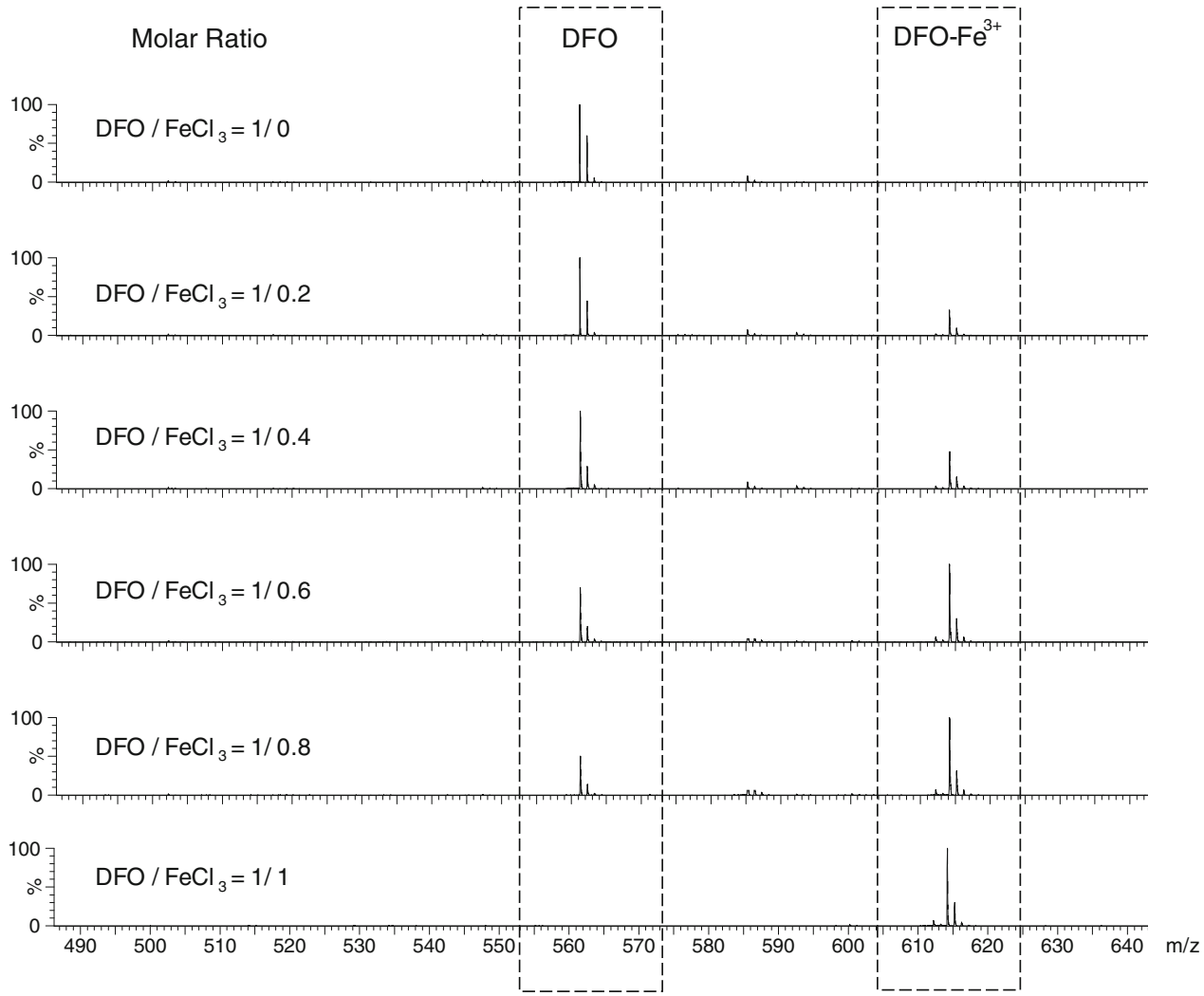

Fig. 4 NanoESI-MS analysis of DFO/Fe^{3+} mixtures with the indicated DFO/Fe^{3+} molar ratios. The observed intensity ratios of the molecular ion signals of free DFO (*m/z* 561) and of the DFO–Fe^{3+} complex (*m/z* 614) correspond roughly to the molar ratios of DFO and $FeCl_3$

intact form. Thus, their efficiency in mimLC can be tested either directly using LC-ICP-MS or indirectly via the beneficial effect on the recovery of multiply phosphorylated peptides. Since the comprehensive and optimal LC-recovery of phosphopeptides is a major goal in protein phosphorylation analysis, we used a seven-component phosphopeptide standard mixture (see Table 1) for testing the metal ion-mobilizing capacity of new sample additives in comparison with the established additives EDTA (Nakamura et al. 2010) and citrate (Winter et al. 2009).

The considerations for selecting the new additives are shortly given in the following.

Ascorbate

Iron is mainly occurring as Fe^{3+} which forms salts of low solubility with multivalent anions such as phosphate. Ascorbate is capable to reduce Fe^{3+} to Fe^{2+}, the salts of which exhibit higher general water solubility. Accordingly, immobilized metal ion chelators used in IMAC form stable complexes with Fe^{3+} but not with Fe^{2+}. This phenomenon has been used for phosphopeptide elution from Fe^{3+}-IMAC columns by reduction of Fe^{3+} to Fe^{2+} using ascorbate (Zarling et al. 2006) and in analogy mobilization of Fe^{3+} upon reduction to Fe^{2+} is expected.

Imidazole

Due to its high affinity to divalent cations, imidazole is the typical buffer used for the elution of His-tagged proteins from Ni^{2+}-NTA columns during their purification. Smaller amounts of imidazole are thereby often used in the washing buffers for the elution of unspecific binding-partners from the divalent cation columns. In addition to the affinity to divalent cations, imidazole also has high affinity to trivalent ones. For example, an imidazole moiety is able to play

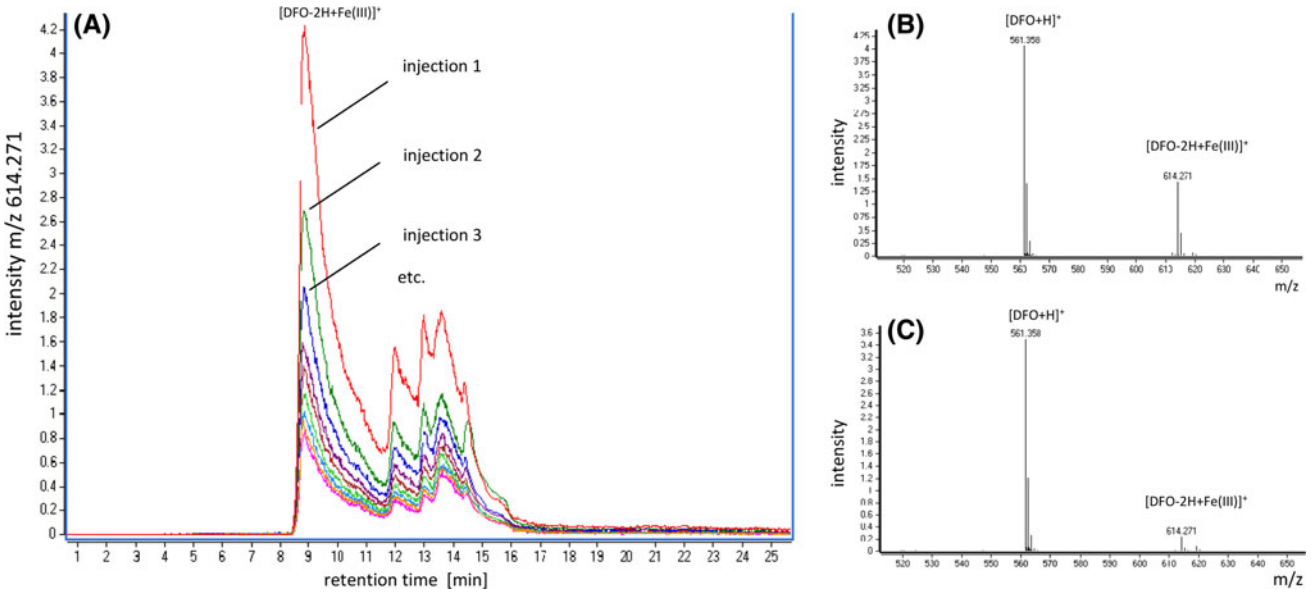

Fig. 5 Deferoxamine mimLC using a HPLC-Chip MS system. Repeated injections of 100 pmol of free DFO were performed; **a** extracted ion chromatograms for *m/z* 614.27 representing the [DFO – 2H + Fe(III)]$^{+}$ ion for injections 1–9; **b** ESI spectrum of injection 1 showing both free and iron-loaded DFO (spectra were accumulated from 8 to 16 min retention time); **c** corresponding spectrum of injection 9. Repeated injections of DFO result in an exponentially decreasing mobilization of iron (**a**). The decreasing amount of mobilized Fe is also mirrored in the increasing ion intensity ratio of free over iron-loaded DFO as demonstrated for injection 1 (**b**) and 9 (**c**)

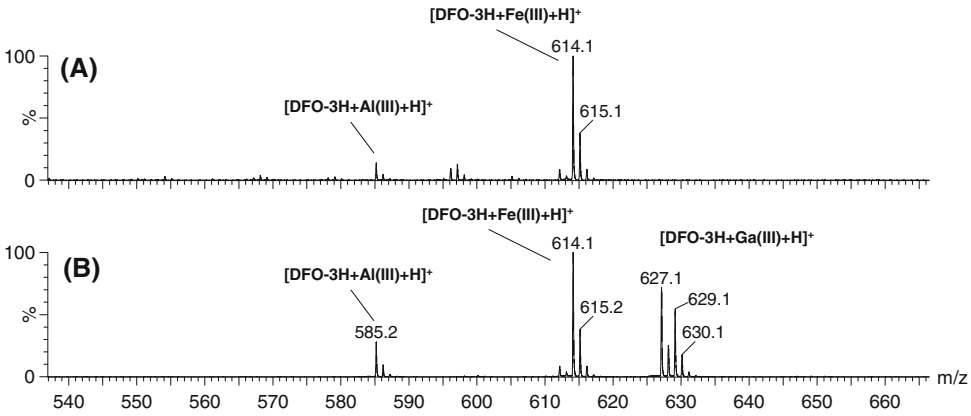

Fig. 6 DFO mimLC-MS showing Ga^{3+} contamination of a nanoUPLC column by injection of 1 pmol DFO. **a** Metal ion–DFO complexes before analysis of Ga^{3+}-IMAC phosphopeptide fractions, **b** metal ion–DFO complexes after analysis of three Ga^{3+}-IMAC phosphopeptide fractions

Table 1 Components of the seven phosphopeptide standard

No.	Peptide	Relative concentration
A	HLADLpSK	1
B	NVPLpYK	1
C	VNQIGTLpSESIK	1
D	VNQIGpTLSEpSIK	1
E	VVEEINGNNpYVpYIDPTQLPYDHK	2
F	FpSIAPSpSLDPpSNR	3
G	FpSIAPpSpSLDPpSNR	4

The letters represent the indication in the chromatograms

an important role in the interaction with Fe^{3+}, e.g. even in a hydroxamate surrounding that is known to have an especially high affinity to Fe^{3+} (Farkas et al. 2007).

pSer-pSer-pSer-pSer (pSpSpSpS)

An often used model analyte in phosphopeptide analysis by LC-MS is a tryptic β-casein digest, in particular, because of its quadruply phosphorylated peptide RELEELNVPGE-IVE-pS-L-pS-pS-pS-EESITR (Ding et al. 1994; Beck et al. 2001; Tholey et al. 2005; Winter et al. 2009). This peptide is particularly in the subpicomole level difficult to detect (Wind et al. 2001), probably because of the close vicinity of the phosphate groups forming highly stable complexes with metal ions (Gaucheron et al. 1995). To mimic the binding properties of this peptide, a tetrapeptide containing four pSer in a row has been synthesized and added to the sample. Although pSpSpSpS is of peptide nature, due to its highly hydrophilic properties it has no retention on a

standard RP-LC column and elutes with the injection peek (see Figure S3). Thus, it does not interfere with any peptides eluting from the column. Furthermore, it has identical chemical properties as the desired analytes and is therefore likely to exactly tackle those binding sites responsible for the trapping.

The established additives have been used at concentrations of 250 µM for EDTA (Nakamura et al. 2010) or 50 mM for citrate (Winter et al. 2009). The additives tested in this study have been added at concentrations between 50 µM and 50 mM, as indicated in the individual chromatograms (see Supplement). Detailed concentration-dependent studies to find 'optimal' concentration for each additive were not performed in this study.

Comparative evaluation of chelating additives using a 7 phosphopeptide mix

A standard mixture with seven phosphopeptides containing one to four phosphorylated peptides was created to validate the performance of the sample additives discussed above (see Table 1). In a first step the standard mixture was analyzed with a nanoUPLC system whereby the additive containing samples were injected directly onto the analytical column as outlined in Fig. 1a. Before each standard analysis, four runs of a commercially available protein digest followed by one run of a Ga^{3+}-IMAC-enriched fraction were performed without additive to establish a "standardized" contamination status. Then the selected additive was injected three times with the aim to mobilize metal contaminations from the column. Subsequently, the standard was injected in water, EDTA, citrate, ascorbate, imidazole mix or pSpSpSpS, respectively. In comparison to the injection in water, mimLC with all additives except imidazole showed a significant increase in the recovery of phosphopeptides. The signal intensities of the phosphopeptides are summarized in Fig. 7. The corresponding chromatograms are available as Figures S4 A–F. Using imidazole, the detection of the singly and doubly phosphorylated peptides was possible, but detection of the triply and quadruply phosphorylated species was not possible. Ascorbate improves the recovery of all seven phosphopeptides. However, compared to EDTA, citrate and pSpSpSpS, the performance of ascorbate appears to be lower. In agreement with previous studies, citrate and EDTA showed superior performance for the detection of phosphopeptides when combined with on-column injection. Besides, also pSpSpSpS showed this superior performance by detecting all seven components although a concentration of only 50 µM of pSpSpSpS was applied.

mimLC combined with different instrumental LC-MS set-ups

To further characterize the best performing additives EDTA, citrate and pSpSpSpS, mimLC with these additives was tested on alternative LC systems with trapping columns. Due to its high robustness, the use of trapping columns currently represents the standard setup in routine LC-MS systems. One system had a conventional trapping/analytical column setup and one contained the HPLC-Chip technology (see Fig. 1b, c).

nanoUPLC-ESI-MS

In principle, citrate, EDTA and pSpSpSpS injected with the sample travel through the trapping column but do not enter the analytical column as they have no retention on the

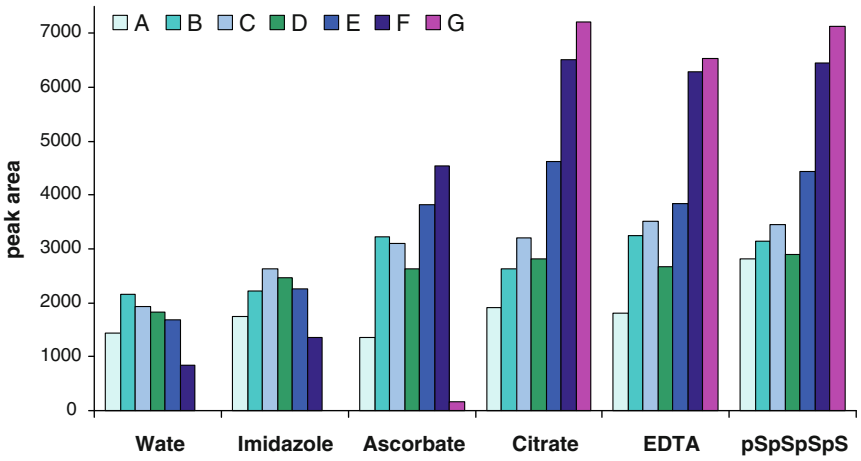

Fig. 7 Recovery of phosphopeptides by mimLC with different additives performed on a nanoUPLC-MS system with on-column injection. **a** HLADLpSK, **b** NVPLpYK, **c** VNQIGTLpSESIK, **d** VNQIGpTLSEpSIK (100 fmol each), **e** VVEEINGNNpYVpYIDPTQ LPYDHK (200 fmol), **f** FpSIAPSpSLDPpSNR (300 fmol), **g** FpSIAP pSpSLDPpSNR (400 fmol)

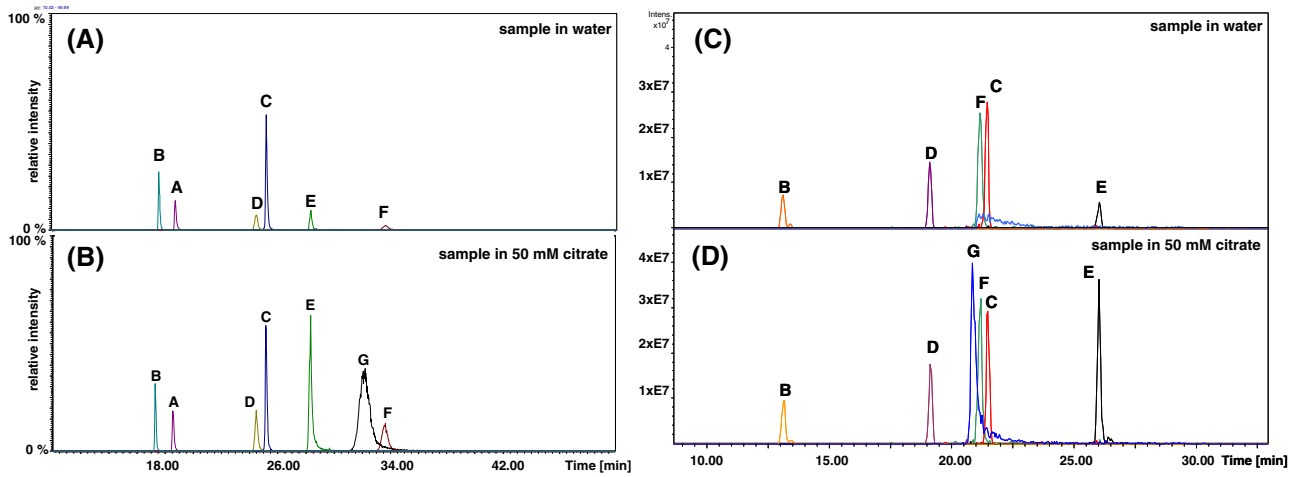

Fig. 8 LC versus mimLC in the analysis of the seven phosphopeptide standard. *Left part* Use of a nanoUPLC-MS system with trapping column and **a** injection in water and **b** injection with 50 mM citrate. *Right part* Use of a Chip-HPLC-MS system with **c** injection in water and **d** injection in 50 mM citrate. The absolute amounts of phosphopeptides injected were: *A* HLADLpSK, *B* NVPLpYK, *C* VNQIGTLpSESIK, *D* VNQIGpTLSEpSIK (20 fmol each), *E* VVEEI NGNNpYVpYIDPTQLPYDHK (40 fmol), *F* FpSIAPSpSLDPpSNR (60 fmol), *G* FpSIAPpSpSLDPpSNR (80 fmol)

trapping column. Nevertheless, all three additives showed a large beneficial effect on the phosphopeptide detection. Using this setup, the beneficial effect may be explained as the trapping column was loaded and eluted in direct flow with the analytical column and therefore a certain amount of the additive that was retained by valves and capillary surfaces enters the analytical column. In contrast to the analysis of the sample in water only (Fig. 8a), the analysis of samples containing citrate (Fig. 8b), EDTA and pSpSpSpS allowed the detection of all seven components including the quadruply phosphorylated peptide (Figure S5). It is noteworthy that the elution order has changed compared to the analysis without trapping column. The doubly pY phosphorylated peptide (E) elutes prior to the triply and quadruply phosphorylated peptide.

HPLC-Chip/MS

Citrate mimLC was also tested using a HPLC-Chip system. Prior to each analytical run, the system was cleaned by DFO, as DFO is retained by the trapping column and therefore is capable of mobilizing metal ions from both the trapping and the analytical column. Figure 8c shows the analysis of the phosphopeptide mixture injected in water, and Fig. 8d the corresponding analysis in 50 mM citrate. The positive influence of citrate is clearly visible since the intensities of the doubly and triply phosphorylated peptides are enhanced and the quadruply phosphorylated peptide is visible only with citrate additive. Using the HPLC-Chip system the elution order of the seven phosphopeptides also slightly differs from that of the other systems with VNQIGTLpSESIK eluting after the triply and quadruply phosphorylated peptide and the doubly pY phosphorylated peptide eluting as last component. The histidine containing peptide could not be detected at all. It may be speculated that it is not retained on the trapping column due to its low hydrophobicity.

Summary

Chelator additives mobilize metal ions from LC systems as can be visualized by LC-ICP-MS or by LC-ESI-MS in combination with DFO. Metal ion-depletion by chelators leads to an improved recovery of multiply phosphorylated from reversed phase LC columns. The sample additives EDTA, citrate, ascorbate, imidazole and the tetrapeptide pSpSpSpS have been tested for their potential to increase the recovery of phosphopeptides containing one to four phosphate groups. The analytical benefits of mimLC by chelator addition have been proven for LC systems with and without trapping column and for a HPLC-Chip system with integrated trapping column.

Acknowledgments We thank R. Pipkorn for expert peptide synthesis.

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