Element mass spectrometry as a tool for high-resolution temporal dynamics investigations of peptide phosphorylation†

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The great potential of capillary HPLC (capHPLC) coupled to element mass spectrometry (ICPMS) to study peptide phosphorylation dynamics was evaluated, and the high precision associated with the absolute quantification of the phosphopeptides provided the most detailed phosphorylation time profile ever reported.

Reversible protein phosphorylation is a post-translational modification that regulates many critical biological events,[1,2] forming the basis of intracellular signalling networks. Cells continuously receive stimulus from outside to which they have to respond. The dynamics of signalling reactions occurring in the cell are of paramount importance as such reactions take place step by step in an ordered fashion. On the other hand, it is well recognized today that phosphorylation plays a pivotal role in such responses so it is necessary to quantify the phosphorylation dynamics of each protein in order to clarify the relationship between signalling reactions, cascades and eventually observed biological responses.[2] Furthermore a single protein can contain multiple phosphorylation sites playing different roles and showing different kinetics and phosphorylation degrees. For such reasons, among others, new strategies for temporal analysis of phosphorylation within cellular signalling networks are urgently needed.[3–5]

Phosphorylation analysis has been traditionally performed by radioactive phosphorus isotopes and antibody-based technologies.[6] These approaches are very sensitive, but not very suitable for quantitative kinetic studies due to the large variability associated with the classical gel electrophoresis techniques usually required to appropriately separate the proteins. Molecular mass spectrometry is currently established as the most powerful tool for the study of phosphorylation temporal changes because of its versatility and sensitivity. MS-based approaches of studying the temporal dynamics of cellular signalling are mainly based on the use of stable isotope labelling of peptides differing in isotope composition and incorporation strategy.[7,8] Stable isotope labels can be incorporated *in vivo* in cell culture (SILAC) allowing samples from different time points to be compared in a single analysis and the temporal profiles of phosphorylation to be built up in a global manner.[3] On the other hand, *in vitro* labelling methods involve incorporation of the stable isotopic tags onto selective sites of peptides *via* chemical reactions such as ICAT,[9] iTRAQ[10] or ^{18}O incorporation.[11] All these label-based approaches only allow the relative quantification of protein phosphorylation and the time resolution achievable is always limited by the number of different isotopic tags used and, above all, by the relatively high variability of quantitative molecular MS (10–20% RSD).[3] Recently, an absolute quantification method was reported[12] based on the used of heavy-isotope labelled synthetic peptides introduced into a sample as internal standards (AQUA) for each peptide sought.

ICPMS has also been reported for the determination of protein phosphorylation.[13,14] The elemental response by ICPMS, which is species and matrix independent, allows the absolute and site specific quantification of every phosphorylated protein (unknown or not) after its enzymatic digestion. Another advantage of ICPMS is its wide linear dynamic range (over 5–6 orders of magnitude), which allows simultaneous quantifications of major and very minor phosphopeptides. It also offers exceptional accuracy and precision ($<5\%$ RSD),[15] far superior to those available using current molecular MS methods, making this technique a very promising alternative to investigate and assess small quantitative changes occurring in kinetic phosphorylation studies.

Herein, we evaluate for the first time the potential use of elemental MS detection to generate the highest temporal resolution data of peptide phosphorylation dynamics ever published. We use an analytical strategy recently proposed[15] based just on the use of a commercially-available certified phosphorus standard to obtain a species-independent quantification for each different phosphorylation state. The high accuracy and precision achievable with ICPMS lead to the reliable discrimination of a large number of temporal points in peptide phosphorylation dynamics investigations. Such a method was used here to monitor the progression of the phosphorylation degree of several phosphopeptide standards simultaneously. The proposed approach used a post-column sheath flow with defined acetonitrile content (40%) to buffer the gradient composition changes at the exit of the capillary column. In such conditions the phosphorus sensitivity remains constant along the capHPLC–ICPMS gradient, necessary to separate the different phosphopeptides originally present in the sample and also the spiked P-containing standard, BNPP (bis(4-nitrophenyl)phosphate). The sensitivity factor obtained for BNPP (peak area units per pmol of P injected) is easily calculated and accurate and precise quantification of every phosphopeptide present in absolute terms is enabled.[15]

In order to evaluate the suitability of such an approach to provide detailed time course studies of peptide (de)phosphorylation, we made a model phosphopeptide mixture to subject it to phosphatase treatment for different time periods. Two

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peptide standards phosphorylated at tyrosine (TSTEPQ-pYQPGENL, pA and N-Acetyl-DpYVPML-NH_2, pB) and one peptide phosphorylated at serine (FQpSEEQQQTE-DELQDK, pC) were selected. Reaction was then stopped by bringing pH to below 5. Each of those "time point" samples was then spiked with BNPP and analyzed by capHPLC–ICPMS. Absolute quantitative data are so obtained for each phosphopeptide allowing, taking the zero point as reference, the determination of its phosphorylation degree at the different incubation times studied. As an example, Fig. 1a and b show the ICPMS chromatograms obtained for the model mixture of pA, pB and pC at time 0 and at 3.5 min, respectively. As can be observed in Fig. 1b, a significant ^{31}P peak appears at the column void volume corresponding to the phosphate groups (inorganic P) removed during this phosphatase treatment (which are not retained in standard reversed-phase columns). By direct comparison of Fig. 1a with 1b, it can be seen clearly that the P signal for pA and pB decreased very quickly while the P signal corresponding to pC hardly diminished after 3.5 min of reaction with phosphatase. This differential P signal trend demonstrated that, as expected, dephosphorylation kinetics are completely different for each studied phosphopeptide (and, therefore sequence dependent).

Fig. 2 summarizes the phosphorylation degree of each phosphopeptide *versus* time of phosphatase treatment (raw data are given in the ESI as Table S1†). In this plot the different time evolution of phosphorylation degrees for each studied phosphopeptide can be clearly observed. The rate of dephosphorylation kinetics is pB > pA > pC. For example,

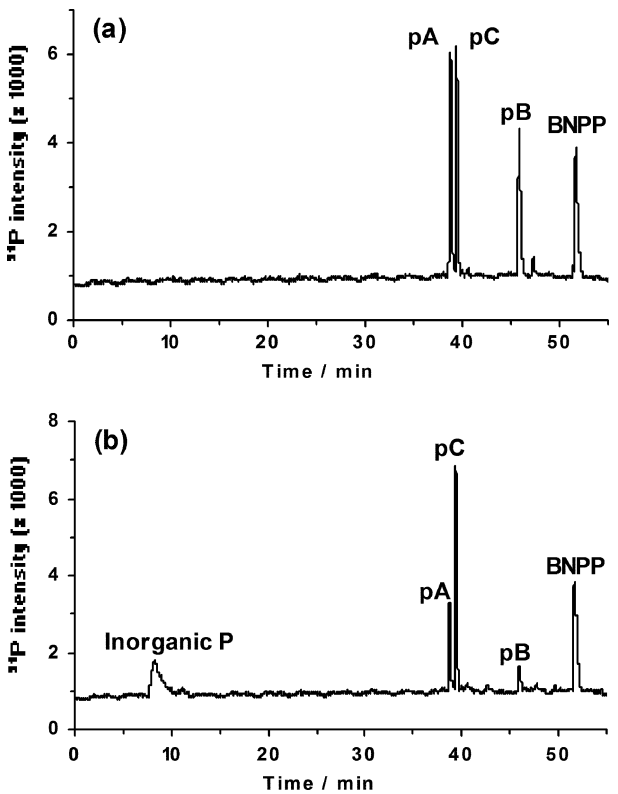

Fig. 1 Reversed-phase capHPLC–ICPMS chromatograms of a mixture of pA, pB and pC: (a) time 0 min, before phosphatase treatment, and (b) after 3.5 min of incubation with phosphatase.

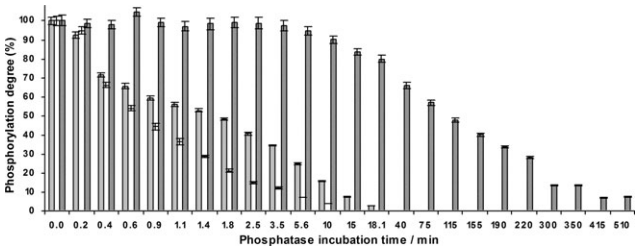

Fig. 2 Time course quantitative data corresponding to phosphorylation degree of pA (light grey bars), pB (white bars) and pC (dark grey bars) after alkaline phosphatase treatment. Uncertainty bars corresponds to one standard deviation, $n = 2$.

after 10 min of incubation, pB is almost completely dephosphorylated (3.9 ± 0.1% phosphorylated), while 15.8 ± 0.3% and 90 ± 2% of pA and pC, respectively, remain phosphorylated. After 15 min of incubation, pB is already completely dephosphorylated. However, pA requires slightly longer to be dephosphorylated (after 18 min there is only 2.88 ± 0.05% left which is gone after 40 min). In contrast, more than 8 h of phosphatase incubation (510 min) are required to reach an almost complete dephosphorylation of pC (7 ± 0.2% phosphorylation degree).

The outstanding precision of this quantification method[15] demonstrates a high discrimination potential between very close phosphorylation degrees. As can be seen in Fig. 2, fourteen "time points" statistically distinguishable were obtained in the phosphorylation time course study for pA. Precision associated to such measurements of absolute phosphopeptide and phosphorylation degree ranged from 0.6 to 4.1% RSD and from 1.5 to 4.3% RSD, respectively. In the case of pB these values ranged from 0.4 to 3.8% RSD and from 1.9 to 4.2% RSD, respectively. Similarly, the highly precise absolute quantitative data obtained for pC at each time point (0.2–2.4% RSD) allowed a clear discrimination between 15 different phosphorylation levels along its long dephosphorylation process (from 3.5 to 510 min, see Fig. 2). These results confirmed that the developed strategy could be used equally well for phosphorylation quantification occurring at serine, threonine or tyrosine residues. Moreover, it could also be applied to any phosphorylation peptide, independently of its number of phosphorylation sites, since quantification of multiply phosphorylated peptides by capHPLC–ICPMS has been already demonstrated.[16]

To the best of our knowledge, the exceptional capability to discriminate between close phosphorylation levels demonstrated here is the most powerful one reported in the literature. So far, effective experimental methods for assessing in depth the dynamics of activation/deactivation of specific phosphorylation sites (phosphopeptides) are lacking. The most powerful methods reported so far make use of metabolic isotope labelling (SILAC)[3,4] to identify and quantify a vast amount of phosphorylation events *in vivo* (cell culture) as a function of time after stimulation. However, time resolution is limited by the isotope labelling technique to three time points (a combination of two experiments can be done to generate five-point profiles).[4] Chemical isotope labelling (iTRAQ)[5] has also been applied for such purpose. Again, duplication of the experiments, while

maintaining a time point sample in both subsets of experiments as a normalization point, allowed to obtain a seven-point time course study. The already higher temporal resolution data obtained in the last study[5] allowed differentiation of temporal phosphorylation dynamics of phosphorylation sites that had been considered to be the same when using a lower resolution approach.[17] It is therefore expected that the so-far unrivalled temporal resolution obtained using the approach proposed in this work could further discriminate different phosphorylation sites (even those previously showing similar dynamic profiles). As a matter of fact, we have demonstrated that very similar dephosphorylation dynamic profiles, such as those shown by pA and pB, can be distinguished clearly. As can be seen in Fig. 2, any alternative experimental method of lower resolution and precision would not allow such clear discrimination between the dephosphorylation dynamics of those two phosphopeptides (*i.e.* phosphorylation sites).

In conclusion, the great potential of element mass spectrometry for assessing dynamics and specificity of phosphorylation-related enzymes is demonstrated here for the first time. It is expected that this method could be applied in the near future to study phosphorylation dynamics at individual sites of active proteins and experiments in this direction are currently underway in our laboratory. This approach is complementary to the MS global phosphorylation dynamic studies using isotopic labelling:[4] once phosphorylation dynamics of a protein or a subset of proteins have been identified as a key parameter in the response of a cell to a specific stimulus by MS isotopic encoding techniques, such dynamics could be studied in greater detail using ICPMS (of course, after proper isolation and perhaps preconcentration using previously validated enrichment procedures, such as high quality antibodies or IMAC- and TiO_2-based approaches).[16] Such higher resolution data could provide a deeper insight into the temporal involvement of such protein(s) in the whole signalling process. Additionally, it is worth stressing that this approach provides the absolute amount of each separated phosphopeptide at any given time studied so it can be applied to clinical samples.

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