This item was submitted to [Loughborough's Research Repository](#) by the author.
Items in Figshare are protected by copyright, with all rights reserved, unless otherwise indicated.

An ICP-MS, ESI-MS and molecular modelling investigation of homogeneous gallium affinity tagging (HMAT) of phosphopeptides

PLEASE CITE THE PUBLISHED VERSION

<http://dx.doi.org/10.1016/j.ijms.2013.03.007>

PUBLISHER

© Elsevier

VERSION

AM (Accepted Manuscript)

LICENCE

CC BY-NC-ND 4.0

REPOSITORY RECORD

Camp, Claire L., Eslam M. Moustafa, Helen J. Reid, Barry L. Sharp, and Tamer Shoeib. 2019. "An ICP-MS, ESI-MS and Molecular Modelling Investigation of Homogeneous Gallium Affinity Tagging (HMAT) of Phosphopeptides". figshare. <https://hdl.handle.net/2134/12992>.

This item was submitted to Loughborough's Institutional Repository (<https://dspace.lboro.ac.uk/>) by the author and is made available under the following Creative Commons Licence conditions.

For the full text of this licence, please go to:
<http://creativecommons.org/licenses/by-nc-nd/2.5/>

An ICP-MS, ESI-MS and molecular modelling investigation of homogeneous
gallium affinity tagging (HMAT) of phosphopeptides

Claire L. Camp[#], Eslam M. Moustafa[†], Helen J. Reid[#], Barry L. Sharp[#] and Tamer Shoeib^{#†*}

[#] Centre for Analytical Science, Department of Chemistry, Loughborough University,
Loughborough, Leicestershire, LE11 3TU, UK.

[†] The Chemistry Department, the American University in Cairo, New Cairo, 11835, Egypt.

* T.Shoeib@aucegypt.edu

Abstract

Protein phosphorylation and de-phosphorylation is one of the most common signalling pathways within cells, it is involved in regulating cellular processes, mediating enzyme inhibition, protein-protein recognition and protein degradation. Compared with normal proteomics, phosphoproteomics poses some additional challenges requiring more initial separation and additional sensitivity to detect and quantify potentially ultra-low abundance species. In this work, the selective detection of phosphopeptides is described based on the incorporation of a metal tag, gallium–N,N-biscarboxymethyl lysine (Ga-LysNTA), in solution before separation and detection by liquid chromatography coupled to inductively coupled plasma mass spectrometry (LC-ICP-MS). Experimental and theoretical characterisation of the resulting Ga-phosphopeptide complex is presented based on linear ion trap electrospray ionisation mass spectrometry (ESI-MS), Fourier transform mass spectrometry (FT-MS) and molecular modelling data. Linear ion trap electrospray ionisation mass spectrometry (ESI-MS) was employed to study the interaction of the gallium tag with platelet derived growth factor beta receptor (β -PDGF), a small phosphopeptide. In addition high resolution Fourier transform mass spectrometry (FT-MS) was used for accurate mass determination and multistage tandem mass spectrometry of the gallium- β -PDGF complex identified the fragmentation pathway. Finally, molecular modelling was used to investigate the energetically favoured structures of both the Ga-LysNTA material and the β -PDGF-Ga-LysNTA complex.

Keywords: Phosphopeptides, metal tagging, ICP-MS, Ion-trap ESI-MS, FTMS, DFT, ONIOM

Introduction

Protein phosphorylation is an important post-translational modification and plays an important role in the regulation of many systems within organisms. Irregular protein phosphorylation can result in diseases such as chronic myelomonocytic leukaemia and papillary renal cancer.[1] The characterisation of phosphorylation sites has been widely investigated by the use of tandem mass spectroscopy.[2-11] A typical phosphoproteome work-flow involves the purification of the protein of interest by gel electrophoresis or reversed phase chromatography, tryptic digestion, enrichment of phosphopeptides followed by analysis using liquid chromatography coupled to tandem mass spectrometry. Phosphopeptide identification by triple quadrupole mass spectrometry is achieved by two methods; neutral loss scans that identify phosphopeptides from the loss of H_3PO_4 or HPO_3 on collisional activation from phosphorylated serine and threonine residues or parent ion scans for the common product ions H_2PO_4^- or PO_3^- . [12-14]

It is often necessary to enrich phosphopeptides following digestion of the target protein, due to their low concentrations and poor ionisation efficiencies in positive mode mass spectrometry.[15-29] Many different analytical techniques have been developed to specifically enrich phosphopeptides including Immobilised Metal Affinity Chromatography (IMAC), utilizing iron, gallium, and more recently titanium and zirconium. [15, 18, 29-47] The efficiency of each of these metals in selectively binding phosphopeptides has been extensively investigated many times with varying success. This has been attributed to the non-specific binding of the acidic amino acid residues asparagine and glutamine to the metal ions, the binding of hydrophobic residues to the gel bed and the elution of multiply phosphorylated peptides and impurities in the metal oxide material. [48, 49] Research suggests that selectivity can also be dependent on the combination of resin bed and metal ion,

with several studies showing that nitrilotriacetic acid (NTA) combined with Fe^{3+} gave higher specificity for phosphopeptides than iminodiacetic acid (IDA) with Fe^{3+} [50, 51] however, when Ga^{3+} was used the reverse was found to be true and IDA gave better specificity than NTA, [52] although Ga^{3+} was found to have better selectivity overall. [52-55] Selectivity can also be improved by blocking acidic amino acid residues by methyl-esterification, [56] however there were two problems reported with this approach these being incomplete esterification [57] and increased complexity due to side reactions. [39] Additionally, selectivity for mono- and multiply-phosphorylated peptides is dependent on both the metal ion and the pH of the loading and elution buffers. Reports have shown that Ti^{2+} and Fe^{3+} have a higher selectivity for mono-phosphorylated peptides. [58, 59] Peptides containing phosphorylated serine and threonine residues can be enriched and modified by methods based on Isotope Coded Affinity Tags, [10, 60] in which the phosphate group is eliminated by base hydrolysis and a biotin or mercaptoethanol group is introduced by Michael addition, then selectively detected by capture/elution with streptavidin or with sulphur specific antibodies. [12] Several metals have been used as tags for phosphopeptide detection and quantitation especially chelating agents containing zinc and manganese di-coordinate centres. [61-65] The advantage of using these metal tags is that the metal chelate binds to the phosphate group regardless of which amino acid residue is phosphorylated. [63] Precipitation of phosphopeptides and phosphoproteins by metal ions including calcium [66] and trivalent europium, terbium and erbium [67] have also been used for enrichment from complex samples such as milk and egg whites. Homogeneous metal based assays have been previously reported, including polymer-based methods [68] in which titanium dioxide was used to identify phosphopeptides in complex samples and antibody metal labelled assays [69] which have utilised mass cytometry and single cell analysis to provide system-wide views of immune signalling.

Blacken et al. [12, 59] reported the use of post separation column tagging of phosphopeptides with N,N-bis(carboxymethyl)-lysine ligand (Ga-LysNTA, $C_{10}H_{16}N_2O_6Ga$) prior to analysis by tandem mass spectrometry. We hypothesized that this tag could be used homogeneously in solution to selectively tag phosphopeptides followed by separation and analysis employing the very high selectivity and sensitivity of ICP-MS to quantify the Ga. For this approach to become a viable analytical method the requirement is for a stable stoichiometry between the Ga and the phosphopeptide (notionally a 1:1 complex) and for high selectivity to the phosphate group. Binding to the phosphate group was reported to be independent of the amino acid to which the phosphate group is bound. [12, 59] Due to the potential complexity of working with mixtures of phosphopeptides derived from tryptic digests, a model system was chosen that would lend itself to careful analysis by mass spectrometry supported by molecular modelling. Conventional structural analysis techniques such as XRD or NMR were not considered here because in the analytical context this method has to work at the nmol level if it is to be useful and therefore we have restricted the experimental work to techniques that can function at that level.

The feasibility of using this homogeneous metal affinity tagging 'HMAT' of phosphopeptides in solution was investigated. HPLC coupled to high resolution, sector-field ICP-MS was used for the separation of the resultant complexes and detection of both ^{69}Ga and ^{31}P signals. Elemental mass spectrometry is preferred over other techniques as it offers lower detection limits, a larger dynamic range, a response that is to a first approximation independent of molecular form, and potentially more accurate quantification at low concentrations by isotope dilution analysis. Linear ion trap electrospray ionisation mass spectrometry (ESI-MS) was employed to study the interaction of the gallium tag with platelet derived growth factor beta-receptor (β -PDGF) which is a cell surface receptor that is

important in the regulation of cell proliferation, growth and development and is linked to many disease states including cancer. In addition high resolution Fourier transform mass spectrometry (FT-MS) was used for accurate mass determination and multistage tandem mass spectrometry of the gallium- β -PDGF complex formation and determination of the fragment pathway. Finally, molecular modelling was used to complement the experimental results and to obtain more detailed structural and thermodynamic data for the complexes formed.

Materials and methods

Instrumentation

Electrospray-MS. An LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose, USA) and a high resolution Q-Exactive Fourier transform mass spectrometer (Thermo Scientific, San Jose, USA) were used in this study for mass spectrometric determinations. The LTQ-MS was equipped with an electrospray ionisation (ESI) source operated in positive mode at 5.5 kV and 280 °C for the capillary temperature. Nitrogen was used as the nebulising gas with sheath, auxiliary and sweep gas flows set at 50, 10, and 10 arbitrary units, respectively. The Q-Exactive was equipped with a Tri Versa NanoMate ESI chip nanospray (Advion, New York, USA). Both instruments were calibrated using Ultramark[®] 1621, caffeine and Met-Arg-Phe-Ala (MFRA) in accordance with the manufacturer's recommendations.

ICP-MS. Thermo Element 2 XR (Thermo Scientific, Hemel Hempstead, UK), with operating conditions as follows, an operating power of 1.25 kW, plasma gas flow of 15.50 L min⁻¹, nebuliser gas flow of 1.18 L min⁻¹ and an auxiliary flow of 0.85 L min⁻¹. The instrument was optimised with a standard solution containing 1 ng mL⁻¹ of In, Li and Pb. For LC-ICP-MS analysis, the column outlet was directly connected via a 0.005'' peek tube to a

poly(fluoroalkoxy), (PFA) LC nebuliser (Elemental Scientific, Omaha, USA). A cyclonic spray chamber (Elemental Scientific, Omaha, USA) was used throughout all analyses.

Reversed-Phase HPLC system. Reversed-phase separations were carried out using a Thermo Surveyor MS Pump Plus (San Jose, Ca, USA) and sample injections were performed by a Thermo Micro AS (San Jose, Ca, USA). The HPLC column used for the separation was a Supelco Ascentis Express Peptide C18 column (150 x 2.1mm, 2.7 μ m).

Materials

N_{α},N_{α} -Bis(carboxymethyl)-L-lysine hydrate (LysNTA, $C_{10}H_{18}N_2O_6 \cdot xH_2O$ > 97%), Gallium (III) chloride (anhydrous, 99.99% trace metals free), ammonium acetate (99.99% trace metals free) and acetic acid were purchased from Sigma Aldrich (Dorset, England). Trypsin Gold (mass spectrometry grade) was purchased from Promega (Southampton, UK). Methanol (HPLC grade) was sourced from Thermo Fisher (Loughborough, UK). Water was purified to 18 M Ω using an Elix and Element System (Millipore, Billerica, Massachusetts, USA).

Sample Preparation and Procedures

Formation of β -PDGF-Ga-LysNTA: LysNTA (5.2 mg), Ga_2Cl_6 (4.7 mg) and β -PDGF (0.5 mg) were dissolved in 5 mL of 50:50 (v/v) methanol:water solution to give a final concentration of 0.1 mg mL⁻¹ of β -PDGF-Ga-LysNTA with a 10 fold excess of LysNTA and Ga_2Cl_6 . For ESI-MSⁿ analysis further dilution was not necessary, for LC-ICP-MS analysis the sample was diluted in 50:50 (v/v) methanol:water solution to a concentration of 10 μ g mL⁻¹ of β -PDGF-Ga-LysNTA assuming a 100% tagging efficiency.

Confirmation of β -PDGF-Ga-LysNTA formation by ESI-MSⁿ. To optimise signal intensity for the ions of interest, the LTQ Ion Max auto-tune optimisation was performed to set the

values for the lens, quadrupole and octapole voltages. Helium gas was used as the collision gas for collision-induced dissociation (CID) with the ion-trap held at a pressure of approximately 10^{-3} Torr. Fragmentation experiments were conducted by selecting the ion of interest with an isolation width of 8 Daltons, collisional activation was applied by setting the activation amplitude (the amplitude of the radiofrequency (RF)) at 15 – 25 % of the maximum voltage available (determined empirically) and an activation Q set at 0.25 units. Sample solutions were infused at a flow rate of $10 \mu\text{L min}^{-1}$ through an electrospray probe using nitrogen as the nebulising gas, with sheath gas flows set to 10 arbitrary units.

Confirmation of β -PDGF-Ga-LysNTA formation by LC-ICP-MS. To confirm successful binding of Ga-LysNTA to β -PDGF, HPLC separation coupled to ICP-MS detection was performed employing a linear gradient over 15 mins from 5 % aqueous (Eluent A: 100 % H_2O with 0.05 % acetic acid) to 70 % organic (Eluent B: 100 % CH_3OH with 0.05 % acetic acid) at a total flow rate of $100 \mu\text{L min}^{-1}$. The column was allowed to re-equilibrate for 5 minutes between injections. Both mobile phases were spiked with 500 ppt of Mo to act as an internal standard to monitor changes in sensitivity due to the increase in organic solvent. The sample injection volume was $5 \mu\text{L}$, which gave 50 ng of β -PDGF-Ga-LysNTA (34 ng of β -PDGF) on column. The column was heated to 30°C for the length of the run. To decrease the concentration of organic solvent in the plasma, an additional flow of $700 \mu\text{L min}^{-1}$ of H_2O was added post-column. For the ICP-MS analysis; the power was set to 1.25 kW, nebuliser, auxiliary and plasma gases to 1.18, 0.85 and 15.50 L min^{-1} respectively and operated in ‘speed mode’ in medium resolution.

Computational Methods

All calculations were performed with Gaussian 09. [70] Initially structures were optimized without symmetry constraints by means of Hartree–Fock (HF) calculations using the STO3G basis set. These initial optimisations were then used as starting geometries for higher level calculations employing density functional theory (DFT) at the B3LYP level, [71] in conjunction with the electron core potential (ECP) double-zeta LANL2DZ basis set. [72, 73] All critical points were characterized by harmonic frequency calculations and were shown to be at minima. Total energies, zero point energies, thermal corrections and entropies are given in supplementary material. The relative computational tractability of this theoretical method makes it suitable for calculations of systems containing relatively large numbers of heavy atoms such as those under study here. Much more importantly however are the results of DFT calculations in systems involving transition metals, which are in many cases in better agreement with experimental data than those obtained from Hartree–Fock (HF) calculations. [74] A two-layer ONIOM (Quantum Mechanical (QM):Semi-Empirical (SE)) calculation at the (B3LYP/LANL2DZ:PM6) level was conducted on the lowest energy species found for the β -PDGF-Ga-LysNTA complex. In a two-layered calculation the complex under study is separated into *model* and *real* regions. The *real* region contains all the atoms of the complex and is thus calculated only at the lower level being the PM6 semi-empirical method. The PM6 method was chosen here as it has been shown to have suitable parameterization for biochemical systems and the capabilities of carrying out calculations on molecules containing transition metals such as the complex investigated here. [75] The *model* region on the other hand contains the reactive centre and any other part of the system that is of particular interest. Both higher level of theory employing the B3LYP/LANL2DZ method and lower level calculations using PM6 were carried out for the *model* region. The total energy of this system was obtained from these three independent calculations:

$$E^{ONIOM} = E^{real,SE} + E^{model,QM} - E^{model,SE} \quad (eq. 1)$$

When dividing the complex into the *real* and *model* regions care was exercised not to cut through multiple covalent bonds or aromatic systems. However, because there are single covalently bonded interactions between the two regions, hydrogen link atoms were chosen to saturate the open valence created.

Results and Discussion

Evidence for Ga-LysNTA tag formation

Evidence for the formation of Ga-LysNTA can be observed in the experimental data shown in Panel A of Figure 1. This experimental isotopic pattern compares very favourably with the theoretical isotopic pattern of Ga-LysNTA shown in Panel B of Figure 1. The formation of Ga-LysNTA was further confirmed by means of accurate mass FT-MS where the average mass deviation was only 0.08 ppm (See Table 1) (as calculated according to equation 2 shown below) for the observed isotopic peaks that make up the experimental pattern for Ga-LysNTA.

$$ppm = \left(\frac{M_{exp} - M_{calc}}{M_{calc}} \right) \times 1E^6 \quad (eq. 2)$$

Calculations on the Ga-LysNTA⁺ species were initially performed at HF/STO3G, the lowest five energy species from these calculations were re-optimised at the B3LYP/LANL2DZ level of theory. The lowest energy conformation calculated for this species is structure **1A** as shown in Figure 2. In this structure the gallium metal is tetra-coordinated, binding to three carbonyl oxygen atoms as well as to the backbone nitrogen of the LysNTA substrate. This

binding arrangement, while serving to dissipate the large positive charge on the metal centre, also results in the formation of three distinct five membered heterocyclic rings with the gallium metal being at their intersection. Highlighted in Structure **1A** is a strong hydrogen bond (1.595 Å as calculated at B3LYP/LANL2DZ) where the hydrogen atom formally resides on the terminal amino nitrogen and is shared with the closest carbonyl oxygen atom. This hydrogen bonding results in the formation of a pseudo-nine membered ring along the LysNTA backbone. The large number of rings that result due to this conformational geometry is responsible for one of the lowest entropy terms calculated out of all conformations considered for the Ga-LysNTA species as listed in supplementary materials (see Table S1). The next lowest energy species on this surface is Structure **1B** being 10.3 kcal mol⁻¹ higher in free energy, in this structure the gallium centre is also tetra-coordinated and is also at the intersection of three distinct heterocyclic rings. The coordinating sites to the gallium metal in Structure **1B** are the terminal amino nitrogen, the backbone nitrogen as well as two of the carbonyl oxygen atoms of the LysNTA substrate. This geometric arrangement results in the formation of two of the smaller five membered heterocyclic rings and one larger eight membered heterocyclic ring. Structure **1C** is calculated to be 37.6 kcal mol⁻¹ higher in free energy on the potential energy surface relative to Structure **1A**. This higher energy species shows a similar gallium metal coordination as that of Structure **1A** being tetra-coordinated and binding to three carbonyl oxygen atoms as well as to the backbone nitrogen of the LysNTA substrate. The lack of formal coordination and the absence of any hydrogen bonding at the amino nitrogen in Structure **1C** allows for a much more extended backbone arrangement relative to that of Structure **1A**. This is evident in the larger entropy term listed in supplementary materials for Structure **1C**. Structure **1D** is nearly identical to Structure **1C** with the former having a gallium hydroxyl oxygen coordination substituting for a stronger coordination of the metal to one of carbonyl oxygen atoms of the

LysNTA substrate in the latter. This difference in coordination makes Structure **1D** seven kcal mol⁻¹ higher in free energy relative to Structure **1C**. The lowest energy structure calculated on this surface is Structure **1E**, where the gallium metal is tri-coordinated, the metal centre binding to two carbonyl oxygen atoms and the backbone nitrogen of the LysNTA substrate. The gallium-carbonyl oxygen bonds in Structure **1E** are calculated to be 1.758 and 1.754 Å which are much shorter and thus stronger than those found in Structure **1A** being 1.787, 1.794 and 1.841 Å respectively. Structure **1E** also features a strong hydrogen bond (1.493 Å as calculated at B3LYP/LANL2DZ) between a hydrogen atom which formally resides on the terminal amino nitrogen and a carbonyl oxygen atom. This hydrogen bonding helps to stabilise Structure **1E**, however, the lower coordination number of gallium in this structure makes it nearly 60 kcal mol⁻¹ higher in energy relative to Structure **1A**. The remaining structures **1F**, **1G** and **1H** shown in Figure 2 were the highest energy species calculated at HF/STO3G being 48.8, 57.0 and 127.3 kcal mol⁻¹ higher in free energy respectively relative to Structure **1A** and were thus not considered at the higher level of theory.

The presence of a water adduct to Ga-LysNTA was identified and confirmed by comparison of the experimental signal with the theoretical isotope pattern model and was found to be in excellent agreement with an average mass accuracy of 0.08 ppm (see Figure 3 and Table 1). An interesting cluster was observed at m/z 361-370, which is proposed to be due to the signals of LysNTAGa•CH₃OH and Ga-LysNTA•2H₂O adducts (see Figure 4). Closer inspection of this cluster and matching to modelled theoretical isotope patterns indicated that it consisted of a 2:1 ratio of [Ga-LysNTA•MeOH]⁺: [Ga-LysNTA•2H₂O]⁺. This suggests that the two acidic carbonyl groups have lost a hydrogen atom in order to form two ionic bonds to

the gallium cation in the Ga-LysNTA complex; therefore the Ga-LysNTA is unlikely to accept lone pairs from remaining water molecules in the gas phase.

Evidence for β -PDGF Ga-LysNTA complex formation

The complexation of Ga-LysNTA with the phosphopeptide PDGF β -receptor, which has a primary structure of H-pTyr-Val-Pro-Met-Leu-OH, to form the Ga-LysNTA- β -PDGF adduct at m/z of 1030.3, was confirmed by comparison with the theoretical isotope pattern (see Figure 5) and by accurate mass FT-MS with an average mass accuracy of 4 ppm (see Table 1). The Ga-LysNTA and β -PDGF reactants are also visible in the mass spectrum (see Figure 5). Several other species which were observed in the mass spectrum were identified and annotated in Figure 5. To further confirm the formation of Ga-LysNTA- β -PDGF, the resulting entire isotopic pattern was selected at an isolation width of 8 Da and fragmented at collision energy of 15 eV, resulting in the MS/MS spectrum shown in Figure 6. Fragmentation of the complex resulted in daughter ions representing β -PDGF and the cluster at m/z 361-370 as described above.

Complementary evidence for the formation of Ga-LysNTA- β -PDGF was obtained by means of HPLC-ICP-MS, using the conditions outlined in the methods section. The chromatogram obtained shows a single peak in the ^{31}P signal trace at a retention time of 3.89 mins which corresponded to an exact retention time match to that of the complexed ^{69}Ga signal peak, confirming the formation of Ga-LysNTA- β -PDGF, see Figure 7. The earlier eluting ^{69}Ga peak at 3.13 mins, was most likely due to unbound Ga-LysNTA as confirmed from a separate LC-ICP-MS run of Ga-LysNTA in the absence of β -PDGF (data not shown). The sample introduction system was not optimised for sensitivity and so an 8 fold post column dilution was employed to reduce plasma instability due to the high concentration of methanol in the

mobile phase. The detection limit for Ga (calculated from three times the standard error in the curve) was 0.5 ng ml^{-1} which for a $10 \text{ }\mu\text{l}$ injection equates to 5 pg Ga or $\sim 50 \text{ pg}$ of PDGF. The ^{98}Mo signal remained constant throughout the chromatographic run as would be expected given that the gradient eluent was diluted 8 fold post column.

Computational modelling of this complex between the Ga-LysNTA species and the phosphopeptide PDGF β -receptor was performed in order to gain insight into the structure. Six conformations of this complex were calculated at HF/STO3G (see the results listed in Table S1). Due to the size of the Ga-LysNTA- β -PDGF system it was computationally prohibitive to employ higher levels of theory for their calculations, however, as observed before for the Ga-LysNTA species calculated in this work, the relative free energy ordering of the conformers calculated did not change from the lower HF method to the higher level of theory employed here. The most favoured of the six structures energetically is Structure **2A** as shown in Figure 8 in which the gallium metal is a penta-coordinate centre. In this structure the gallium centre binds to two carbonyl oxygen atoms of the LysNTA substrate which does not exhibit any formal binding or hydrogen bonding at its terminal amino nitrogen resulting in an extended open arrangement for backbone of the LysNTA substrate in the formed complex. Structure **2A** also shows the gallium centre binding to the two carbonyl oxygen atoms of the valine and methionine residues of the phosphopeptide PDGF β -receptor while the last coordinating site to the gallium metal is one of the oxygen atoms of the phosphate moiety which replaces the hydrogen of the hydroxyl group of the tyrosine residue. This observation is extremely important as it throws some light on why the literature offers diverse and sometimes contradictory information about the efficiency and selectivity of IMAC (and in this case the related HMAT) techniques. Whilst the Ga certainly does target the phosphate group, it only uses one of the phosphate oxygens with the other analyte derived coordination

sites being provided by electron donating centres on the amino acid residues of the peptide. Structure **2B** (not shown, see Table S1) is the lowest energy conformer calculated here where the Ga does not involve coordination to the phosphate group of the dipeptide ligand. In this structure the Ga is bound to three carbonyl oxygen atoms of the LysNTA substrate and a carbonyl oxygen atom of the valine residue. Structure **2B** is calculated to be 17.1 kcal mol⁻¹ higher in free energy relative to structure **2A** indicating a preference for Ga binding to the phosphate group. The inference here (which would take a much larger study to verify) is that Ga tagging complexes, such as the one described here, can successfully target the phosphate group, but that the stability of the complex will also depend on the residue sequence of the peptide. It is also possible that weaker complexes could form with peptides that do not contain phosphate. This was investigated by comparing the enthalpies of two reactions, the first being the reaction between the Ga-LysNTA substrate and phosphopeptide PDGF β -receptor to produce the complex Ga-LysNTA- β -PDGF, structure **2A** as shown in Figure 8, while the second is the equivalent reaction between the Ga-LysNTA substrate and same dipeptide where the phosphate group was removed and replaced with a phenolic OH group. The enthalpies for these reactions at 298 K were calculated to be -113.7 and -51.5 kcal mol⁻¹ respectively; indicating weaker complex formation in the absence of the phosphate group and at the same time a strong preference for the Ga binding to the phosphate group. This would be revealed by an increased complexity in the elemental chromatogram, but could be controlled by varying the experimental conditions or chemical blocking of competing groups. A group of five internal hydrogen bonds help not only to stabilize Structure **2A** but also results in a somewhat constricted configuration. Although we expect 6 coordination for Ga, efforts to obtain structures with higher coordination at the gallium centre were not successful; in fact, only one structure of the other five conformers calculated for this complex exhibited gallium penta-coordination whereas others involved either tri- or tetra-coordinated gallium

metal and were shown (see HF data listed in Table S1) not to be very energetically competitive. This is most likely due to steric crowding given the bulky ligands that are surrounding the Ga. Finally, a two layer ONIOM method was used to model Structure **2A** shown in Figure 8, predominantly in order to determine the feasibility of this method for examining larger transition metal containing complexes to be investigated in future works. The selection of the high layer, modelled at B3LYP/LANL2DZ, included the active portion of the system the gallium centre and its coordinating sites, the phosphate group as well as all atoms involved in hydrogen bonding, the remainder of the complex on the other hand was treated using semi-empirical techniques employing the PM6 method. Of major interest are the ONIOM obtained geometries which were shown in Figure 8 to be very similar to those resulting from the B3LYP/LANL2DZ calculations. This provides us with good indications that such two layered calculations may be used in the future to obtain relative energies of different conformers of larger transition metal-containing complexes provided that adequate care is taken to include exactly the same numbers and types of atoms in the high layer in each of the conformers examined.

Conclusions

This work demonstrates that Ga-LysNTA can be used to tag the phosphate moiety in a model phosphopeptide thereby enabling the detection of the peptide through the Ga atom by ICP-MS. LC-ICP-MS data showed the co-elution of the ^{31}P of the phosphorylated peptide and the ^{69}Ga of the label, indicating successful Ga-LysNTA- β -PDGF formation. Unambiguous identification of the labelled complex was achieved through ESI-MSⁿ and accurate mass FT-MS data. Excellent correlation with the theoretically predicted spectra were shown (see Figure 5). Molecular modelling supported the MS data and revealed that just one of the phosphate oxygen atoms was targeted by the Ga centre, the other analyte derived coordination sites being provided by electron donating groups on the amino acid residues.

This suggests that complex stability will vary from peptide to peptide and may go some way to explaining why there are diverse and sometimes contradictory reports in the literature regarding the efficiency and selectivity of metal affinity techniques.

Future work will aim to investigate further the issue of selectivity using different peptide sequences and tryptic digests of phosphopeptides. The ultimate aim is to quantify phosphopeptides by elemental mass spectrometry using isotope dilution analysis with spiked gallium.

Acknowledgements

The authors would like to thank the EPSRC, LGC Ltd., the Centre for Analytical Science at Loughborough University, the American University in Cairo and the Centre for Innovative and Collaborative Engineering at Loughborough University for the funding sponsorship and provision of resources for the project.

Figure and Table Captions

Figure 1: Signal due to Ga-LysNTA tag from LTQ Ion Trap MS; Panel A experimental data, Panel B theoretically predicted spectra at a resolving power of 1500 m/ Δ m.

Figure 2: Structures for the Ga-LysNTA singly charged positive ions as calculated at the HF/STO3G and B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.

Figure 3: Formation of $[\text{Ga-LysNTA.H}_2\text{O}]^+$ from Q-Exactive FTMS; Panel A experimental data, Panel B theoretically predicted spectra.

Figure 4: Panel A experimental signal due to $[\text{Ga-LysNTA}]^+$ adducts with H_2O and CH_3OH from LTQ Ion Trap MS, Panel B theoretically predicted composite spectrum of a 1:2 ratio of $[\text{Ga-LysNTA.2H}_2\text{O}]^+$ and $[\text{Ga-LysNTA.CH}_3\text{OH}]^+$ at a resolving power of 1000 m/ Δ m.

Figure 5: Full scan spectra of reaction mixture from LTQ Ion Trap MS showing evidence of $[\text{Ga-LysNTAPDGF}]^+$ formation (main figure, insert A experimental data, insert B theoretically predicted spectra of $[\text{Ga-LysNTAPDGF}]^+$) at a resolving power of 1700 m/ Δ m.

Figure 6: Product ion scan of $[\text{Ga-LysNTAPDGF}]^+$ at m/z 1030 from LTQ Ion Trap MS.

Figure 7: LC-ICP-MS chromatogram of $[\text{Ga-LysNTAPDGF}]^+$ from Element 2XR; panel A signal from ^{31}P , panel B signal from ^{69}Ga .

Figure 8: the lowest energy structure 2A calculated for the Ga-LysNTA- β -PDGF singly charged positive ions as calculated using the ONIOM method at B3LYP/LANL2DZ:PM6 and the B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.

Table 1: Summary of the mass accuracy for all proposed assignments of Ga-LysNTA with methanol and water adducts and PDGF.

Supplementary Material

Table S1: Electronic energies, Zero-Point Vibrational Energies, Thermal Energies, Entropies and relative free energies for species calculated at HF/STO3G and B3LYP/LANL2DZ (italicized values). ONIOM values for species **2A** are listed in bold within and underneath the table.

References

1. P. Cohen, The role of protein phosphorylation in human health and disease, *Eur. J. Biochem.*, 268, (2001), 5001-5010.
2. L. E. B. Taylor, A. Traweger, B. Larsen, D. Cox, S. A. Tate and T. Pawson, Monitoring Phosphorylation Sites Using LC/MRM/MS/MS: Trials and Tribulations of Targeting Biologically Important but Poorly Ionized Phosphopeptides, *Proceedings ASMS conference on mass spectrometry and allied topics*, 2008.
3. C. Wu, J. Hsu, S. Huang and S. Chen, Mapping N-Terminus Phosphorylation Sites and Quantitation by Stable Isotope Dimethyl Labeling, *J. Am. Soc. Mass Spectr.*, 21, (2010), 460-471.
4. D. Pflieger, M. Junger, M. Muller, O. Rinner, H. Lee, P. Gehrig, M. Gstaiger and R. Aebersold, Quantitative proteomic analysis of protein complexes: Concurrent identification of interactors and their state of phosphorylation, *Mol. Cell. Proteomics*, 7, (2007), 326-346.
5. F. Yang, N. Jaitly, H. Jayachandran, Q. Luo, M. Monroe, X. Du, M. Gritsenko, R. Zhang, D. Anderson, S. Purvine, J. Adkins, R. Moore, H. Mottaz, S. Ding, M. Lipton, D. 2. Camp, H. Udseth, R. Smith and S. Rossie, Applying a Targeted Label-Free Approach Using LC-MS AMT Tags to Evaluate Changes in Protein Phosphorylation Following Phosphatase Inhibition, *J. Proteome Res.*, 6, (2007), 4489-97.
6. J. Wang, Y. Zhang, H. Jiang, Y. Cai and X. Qian, Phosphopeptide detection using automated online IMAC-capillary LC-ESI-MS/MS, *Proteomics*, 6, (2006), 404-11.
7. A. Sano and H. Nakamura, Chemo-affinity of titania for the column-switching HPLC analysis of phosphopeptides, *Anal. Sci.*, 20, (2004), 861-864.
8. S. H. Payne, M. Yau, M. B. Smolka, S. Tanner, H. L. Zhou and V. Bafna, Phosphorylation-specific MS, *J. Proteome Res.*, 7, (2008), 3373-3381.

9. H. K. Kweon and K. Hakansson, Metal oxide-based enrichment combined with gas-phase ion-electron reactions for improved mass spectrometric characterization of protein phosphorylation, *J. Proteome Res.*, 7, (2008),749-755.
10. A. P. Navaza, J. R. Encinar, M. Carrascal, J. Abian and A. Sanz-Medel, Absolute and site-specific quantification of protein phosphorylation using integrated elemental and molecular mass spectrometry: Its potential to assess phosphopeptide enrichment procedures, *Anal. Chem.*,80, (2008),1777-1787.
11. E. M. Woo, D. Fenyo, B. H. Kwok, H. Funabiki and B. Chait, Efficient Identification of Phosphorylation by Mass Spectrometric Phosphopeptide Fingerfrinting, *Anal. Chem.*, 80, (2008),2419-2425.
12. G. R. Blacken, M. H. Gelb and F. Turecek, Metal Affinity Capture Tandem Mass Spectrometry for the Selective Detection of Phosphopeptides, *Anal. Chem.*, 78, (2006),6065-6073.
13. S. A. Carr, M. J. Huddleston and R. S. Annan, Selective detection and sequencing of phosphopeptides at the femtomole level by mass spectrometry, *Anal. Biochem.*,239,(1996), 180-192.
14. M. J. Schroeder, J. Shabanowitz, J. C. Schwartz, D. F. Hunt and J. J. Coon, A neutral loss activation method for improved phosphopeptide sequence analysis by quadrupole ion trap mass spectrometry, *Anal. Chem.*, 76, (2004),3590-3598.
15. U. K. Aryal and A. R. S. Ross, Enrichment and analysis of phosphopeptides under different experimental conditions using titanium dioxide affinity chromatography and mass spectrometry, *Rapid Commun. Mass Spectrom.*,24,(2010),219-231.
16. S. Choi, J. Kim, K. Cho, G. Park, J. H. Yoon, S. Park, J. S. Yoo, S. H. Ryu, Y. H. Kim and J. Kim, Sequential Fe₃O₄/TiO₂ enrichment for phosphopeptide analysis by liquid

- chromatography/tandem mass spectrometry, *Rapid Comm. Mass Spec.*, 24, (2010), 1467-1474.
17. A. Leitner, M. Sturm, O. Hudecz, M. Mazanek, J. H. Smatt, M. Linden, W. Lindner and K. Mechtler, Probing the Phosphoproteome of HeLa Cells Using Nanocast Metal Oxide Microspheres for Phosphopeptide Enrichment, *Anal. Chem.*, 82, (2010), 2726-2733.
 18. M. Mazanek, E. Roitinger, O. Hudecz, J. R. A. Hutchins, B. Hegemann, G. Mitulovic, T. Taus, C. Stingl, J. M. Peters and K. Mechtler, A new acid mix enhances phosphopeptide enrichment on titanium- and zirconium dioxide for mapping of phosphorylation sites on protein complexes, *J. Chromatogr. B*, 878, (2010), 515-524.
 19. J. Wu, P. Warren, Q. Shakey, E. Sousa, A. Hill, T. E. Ryan and T. He, Integrating titania enrichment, iTRAQ labeling, and Orbitrap CID-HCD for global identification and quantitative analysis of phosphopeptides, *Proteomics*, 10, (2010), 2224-34.
 20. J. Tang, P. Yin, X. H. Lu, D. W. Qi, Y. Mao, C. H. Deng, P. Y. Yang and X. M. Zhang, Development of mesoporous TiO₂ microspheres with high specific surface area for selective enrichment of phosphopeptides by mass spectrometric analysis, *J. Chromatogr. A*, 1217, (2010), 2197-2205.
 21. D. Qi, Y. Mao, J. Lu, C. Deng and X. Zhang, Phosphate-functionalized magnetic microspheres for immobilization of Zr⁴⁺ ions for selective enrichment of the phosphopeptides, *J. Chromatogr. A*, 1217, (2010), 2606-2617.
 22. C. T. Chen and Y. C. Chen, Trapping performance of Fe₃O₄ vs Al₂O₃ and Fe₃O₄ vs TiO₂ magnetic nanoparticles in the selective enrichment of phosphopeptides from human serum, *J. Biomed. Nanotech.*, 4, (2008), 73-79.
 23. G. Han, M. Ye and H. Zou, Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography, *Analyst*, 133, (2008), 1128-38.

24. G. Han, M. Ye, H. Zhou, X. Jiang, S. Feng, X. Jiang, R. Tian, D. Wan, H. Zou and J. Gu, Large-scale phosphoproteome analysis of human liver tissue by enrichment and fractionation of phosphopeptides with strong anion exchange chromatography, *Proteomics*, 8, (2008),1346-61.
25. Y. Li, H. Q. Lin, C. H. Deng, P. Y. Yang and X. M. Zhang, Highly selective and rapid enrichment of phosphorylated peptides using gallium oxide-coated magnetic microspheres for MALDI-TOF-MS and nano-LC-ESI-MS/MS/MS analysis, *Proteomics*, 8, (2008),238-249.
26. B. Lin, T. Li, Y. Zhao, F. K. Huang, L. Guo and Y. Q. Feng,Preparation of a TiO₂ nanoparticle-deposited capillary column by liquid phase deposition and its application in phosphopeptide analysis,*J. Chromatogr. A*, 1192, (2008), 95-102.
27. S. Mohammed, K. Kraiczek, M. Pinkse, S. Lemeer, J. Benschoop and A. Heck, Chip-Based Enrichment and NanoLC-MS/MS Analysis of Phosphopeptides from Whole Lysates, *J. Proteome Res.*, 7, (2008), 1565-1571.
28. Y. Li, D. W. Qi, C. H. Deng, P. Y. Yang and X. M. Zhang, Cerium ion-chelated magnetic silica microspheres for enrichment and direct determination of phosphopeptides by matrix-assisted laser desorption ionization mass spectrometry, *J. Proteome Res.*, 7, (2008),1767-1777.
29. E. S. Simon, M. Young, A. Chan, Z. Q. Bao and P. C. Andrews,Improved enrichment strategies for phosphorylated peptides on titanium dioxide using methyl esterification and pH gradient elution,*Anal. Biochem.*,377,(2008), 234-242.
30. S. Park and S. Maudsley, Discontinuous pH gradient-mediated separation of TiO₂-enriched phosphopeptides, *Anal. Biochem.*,409, (2011),81-88.
31. M. Pinkse, S. Mohammed, J. Gouw, B. B. van, H. Vos and A. Heck, Highly robust, automated, and sensitive online TiO₂-based phosphoproteomics applied to study

- endogenous phosphorylation in *Drosophila melanogaster*, *J. Proteome Res.*, 7, (2008),687-97.
32. H. Hsieh, C. Sheu, F. Shi and D. Li, Development of a titanium dioxide nanoparticle pipette-tip for the selective enrichment of phosphorylated peptides, *J. Chromatogr. A*, 1165, (2007),128-35.
33. M. Larsen, S. Jensen, L. Jakobsen and N. Heegaard, Exploring the sialome using titanium dioxide chromatography and mass spectrometry, *Mol Cell Proteomics*, 6, (2007),1778-87.
34. M. Schirle, M. Bantscheff, M. Raida, B. Kuster, Quantitative mass spectrometry in proteomics: a critical review, *Anal. Bioanal. Chem.*, 389, (2007),1017-1031.
35. C. Xu, H. Wang, D. Li, X-P. Kong, T. A. Neubert, Selective enrichment and fractionation of phosphopeptides from Peptide Mixtures by Isoelectric Focusing after Methyl Esterification, *Anal. Chem.*, 95, (2007),2007-2014.
36. L. Yu, Z. Zhu, K. C. Chan, H. J. Issaq, D. S. Dimitrov and T. D. Veenstra, Improved Titanium Dioxide Enrichment of Phosphopeptides from HeLa Cells and High Confident Phosphopeptide Identification by Cross-Validation of MS/MS and MS/MS/MS Spectra, *J. Proteome Res.*, 6, (2007),4150-4162.
37. ZirChromSeparationsInc., Comparison of Titanium Dioxide & Zirconium Dioxide SPE Tips for Phosphopeptide Enrichment, *LC GC North America*, 25, (2007),30.
38. C. Klemm, S. Otto, C. Wolf, R. Haseloff, M. Beyermann and E. Krause, *J. Mass Spectr.*, 41, (2006),1623-32.
39. M. R. Larsen, T. E. Thingholm, O. N. Jensen, P. Roepstorff and T. J. D. Jorgensen, Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns, *Mol. Cell. Proteomics*, 4, (2005),873-886.

40. M. Pinkse, P. Uitto, M. Hilhorst, B. Ooms and A. Heck, Selective isolation at the femtomole level of phosphopeptides from proteolytic digests using 2D-NanoLC-ESI-MS/MS and titanium oxide precolumns, *Anal. Chem.*, 76, (2004),3935-43.
41. G. T. Cantin, W. Yi, B. W. Lu, S. K. Park, T. Xu, J. D. Lee and J. R. Yates,Combining protein-based IMAC, peptide-based IMAC, and MudPIT for efficient phosphoproteomic analysis,*J. Proteome Res.*, 7, (2008),1346-1351.
42. C. Gan, T. Guo, H. Zhang, S. Lim and S. Sze, A Comparative Study of Electrostatic Repulsion-Hydrophilic Interaction Chromatography (ERLIC) versus SCX-IMAC-Based Methods for Phosphopeptide Isolation/Enrichment, *J. ProteomeRes.*,7, (2008),4869-4877.
43. X. Liang, G. Fonnum, M. Hajivandi, T. Stene, N. H. Kjus, E. Ragnhildstveit, J. W. Amshey, P. Predki and R. M. Pope,Quantitative Comparison of IMAC and TiO₂ Surfaces Used in the Study of Regulated, Dynamic Protein Phosphorylation,*J. Am. Soc. Mass Spectr.*, 18, (2007),1932-1944.
44. T. S. Nuhse, A. Stensballe, O. N. Jensen and S. C. Peck,Large-scale Analysis of in Vivo Phosphorylated Membrane Proteins by Immobilized Metal Ion Affinity Chromatography and Mass Spectrometry,*Mol.Cell. Proteomics*, 2, (2003), 1234-1243.
45. T. Hoang, U. Roth, K. Kowalewski, C. Belisle, K. Steinert and M. Karas,Highly specific capture and direct MALDI MS analysis of phosphopeptides by zirconium phosphonate on self-assembled monolayers,*Anal. Chem.*, 82, (2010),219-28.
46. Y. Xue, J. Wei, H. Han, L. Zhao, D. Cao, J. Wang, X. Yang, Y. Zhang and X. Qian, Application of open tubular capillary columns coated with zirconium phosphonate for enrichment of phosphopeptides, *J. Chromatogr. B*, 877, (2009), 757-764.

47. L. Zhang, J. Xu, L. Sun, J. Ma, K. Yang, Z. Liang, L. Zhang, Y. Zhang, Zirconium oxide aerogel for effective enrichment of phosphopeptides with high binding capacity, *Anal. Bioanal. Chem.*, 399, (2011), 3399-3405
48. M. R. Larsen, S. S. Jensen, Evaluation of the impact of some experimental procedures on different phosphopeptide enrichment techniques, *Rapid Commun. Mass Spectr.*, 21, (2007), 3635-3645.
49. J. Ye, X. Zhang, C. Young, X. Zhao, Q. Hao, L. Cheng and O. N. Jensen, Optimized IMAC-IMAC protocol for phosphopeptide recovery from complex biological samples, *J. Proteome Res.*, 9, (2010), 3561-3573.
50. D. C. Neville, C. R. Rozanas, E. M. Price, D. B. Gruis, A. S. Verkman and R. R. Townsend, Evidence for phosphorylation of serine 753 in CFTR using a novel metal-ion affinity resin and matrix-assisted laser desorption mass spectrometry, *Protein Sci.*, 6, (1997), 2436-2445.
51. A. Dubrovskaya and S. Souchelnytskyi, Efficient enrichment of intact phosphorylated proteins by modified immobilized metal-affinity chromatography, *Proteomics*, 18, (2005), 4678-4683.
52. M. C. Posewitz and P. Tempst, Immobilized gallium(III) affinity chromatography of phosphopeptides, *Anal. Chem.*, 71, (1999), 2883-2892.
53. M. B. Gates, K. B. Tomer and L. J. Deterding, Comparison of Metal and Metal Oxide Media for Phosphopeptide Enrichment Prior to Mass Spectrometric Analyses, *J. Am. Soc. Mass Spectrom.*, 10, (2010), 1649-1659.
54. M. O. Collins, L. Yu, H. Husi, W. P. Blackstock, J. S. Choudhary and S. G. Grant, Robust Enrichment of Phosphorylated Species in Complex Mixtures by Sequential Protein and Peptide Metal-Affinity Chromatography and Analysis by Tandem Mass Spectrometry, *Science. STKE*, 298, (2005), 16.

55. M. Machida, H. Kosako, K. Shirakabe, M. Kobayashi, M. Ushiyama, J. Inagawa, J. Hirano, T. Nakano, Y. Bando, E. Nishida and S. Hattori, Purification of phosphoproteins by immobilized metal affinity chromatography and its application to phosphoproteome analysis, *FEBS J.*, 274, (2007),1576-1587
56. S. B. Ficarro, M. L. McClelland, P. T. Stukenberg, D. J. Burke, M. M. Ross, J. Shabanowitz, D. F. Hunt and F. M. White, Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*, *Nat. Biotechnol.*, 20, (2002),301-305.
57. J. C. Trinidad, C. G. Specht, A. Thalhammer, R. Schoepfer and A. L. Burlingame, Comprehensive identification of phosphorylation sites in postsynaptic density preparations, *Mol. Cell. Proteomics*, 5, (2006),914-922.
58. S. P. Gygi, B. Rist, S. A. Gerber, F. Turecek, M. H. Gelb and R. Aebersold, Quantitative analysis of complex protein mixtures using isotope-coded affinity tags, *Nat. Biotechnol.*, 17,(1999),994-999.
59. G. R. Blacken, M. Sadilek and F. Turecek, Gallium metal affinity capture tandem mass spectrometry for the selective detection of phosphopeptides in complex mixtures, *J. Mass Spectr.*, 43, (2008),1072-1080.
60. M. Wind, M. Edler, N. Jakubowski, M. Linscheid, H. Wesch and W. D. Lehmann, Protein phosphorylation degree: Determination by capillary liquid chromatography and inductively coupled plasma mass spectrometry, *Anal. Chem.*, 73, (2001),29-35.
61. H. Takeda, A. Kawasaki, M. Takahashi, A. Yamada, T. Koike, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of phosphorylated compounds using a novel phosphate capture molecule, *Rapid Commun. Mass Spectrom.*, 17 (2003), 2075–2081

62. T. Nakanishi, E. Ando, E. Kinoshita, E. Kinoshita-Kikuta, T. Koike, S. Tsunasawa, O. Nishimura, Identification on Membrane and Characterization of Phosphoproteins Using an Alkoxide-Bridged Dinuclear Metal Complex as a Phosphate-Binding Tag Molecule, *J. Biomol. Tech.*, 18, (2007), 278-286.
63. K. Inamori, M. Kyo, Y. Nishiya, Y. Inoue, T. Sonoda, E. Kinoshita, T. Koike T, Y. Katayama, Detection and Quantification of On-Chip Phosphorylated Peptides by Surface Plasmon Resonance Imaging Techniques Using a Phosphate Capture Molecule, *Anal. Chem.*, 77, (2005), 3979-3985.
64. M. Prudent, J. Rossier, N. Lion, H. Girault, Microfabricated Dual Sprayer for On-Line Mass Tagging of Phosphopeptides, *Anal. Chem.*, 80 (2008), 2531-2538.
65. E. Kinoshita, E. Kinoshita-Kikuta, K. Takiyama, T. Koike, Phosphate-binding Tag, a New Tool to Visualize Phosphorylated Proteins, *Mol. Cell. Prot.*, 5, (2006), 749-757.
66. X. Zhang, J. Ye, O. L. Jenson, P. Roepstorff, Highly Efficient Phosphopeptide Enrichment by Calcium Phosphate Precipitation Combined with Subsequent IMAC Enrichment, *Mol. Cell. Prot.* 6, (2007), 2032-2042.
67. Y. Güzel, M. Rainer, M. Mirza, G. Bonn, Highly efficient precipitation of phosphoproteins using trivalent europium, terbium, and erbium ions, *Anal. Bioanal. Chem.* , 403, (2012), 1323-1331.
68. A. Iliuk, V. Martin, B. Alicie, R. Geahlen, W. Tao, In-depth Analyses of Kinase-dependent Tyrosine Phosphoproteomes Based on Metal Ion-functionalized Soluble Nanopolymers, *Mol. Cell. Proteomics*, 10, (2010), 2162–2172
69. S. C. Bendall, E. F. Simonds, P. Qiu, E. D. Amir, P. O. Krutzik, R. Finck, R.V. Bruggner, R. Melamed, A. Trejo, O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Pe'er, S. D. Tanner, G.P. Nolan, Single-Cell Mass Cytometry of

Differential Immune and Drug Responses Across a Human Hematopoietic Continuum, *Science*, 332, (2011), 687-695.

70. R. N. Perutz and J. J. Turner, Pulsed matrix isolation. A comparative study, *J.Chem.Soc. Faraday Trans.2*, 69, (1973), 452-461.
71. C. Lee, W. Yang and R. G. Parr, Development of the Colle-Salvetti correlation-energy formula into a functional of the electron density, *Phys. Rev. B*, 37, (1988), 785-789.
72. P. J. Hay and W. R. Wadt, Ab initio effective core potentials for molecular calculations. Potentials for the transition metal atoms Sc to Hg, *J. Chem. Phys.*, 82, (1985), 270-283.
73. W. R. Wadt and P. J. Hay, Ab initio effective core potentials for molecular calculations. Potentials for main group elements Na to Bi, *J. Chem. Phys.*, 82, (1985), 284-298.
74. T. Ziegler, Approximate density functional theory as a practical tool in molecular energetics and dynamics, *Chem. Rev.*, 91, (1991), 651-667.
75. J. Stewart, Optimization of parameters for semiempirical methods V: Modification of NDDO approximations and application to 70 elements, *J. Mol. Model.*, 12, (2007), 1173-1213.

Figure 1: Signal due to Ga-LysNTA tag from LTQ Ion Trap MS; Panel A experimental data, Panel B theoretically predicted spectrum at a resolving power of 1500 m/ Δ m.

1A (0.0, 0.0)

1B (7.8, 10.3)

1C (12.8, 37.6)

1D (17.4, 44.7)

Figure 2: Structures for the Ga-LysNTAsingly charged positive ions as calculated at the HF/STO3G and B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies (in kcal per mol) are indicated in parenthesis.

Figure 3: Formation of $[\text{Ga-LysNTA.H}_2\text{O}]^+$ from Q-Exactive FTMS; Panel A experimental data, Panel B theoretically predicted spectra.

Figure 4: Panel A experimental signal due to $[Ga-LysNTA]^+$ adducts with H_2O and CH_3OH from LTQ Ion Trap MS, Panel B theoretically predicted composite spectrum of a 1:2 ratio of $[Ga-LysNTA.2H_2O]^+$ and $[Ga-LysNTA.CH_3OH]^+$ at a resolving power of 1000 $m/\Delta m$.

Figure 5: Full scan spectra of reaction mixture from LTQ Ion Trap MS showing evidence of [Ga-LysNTAPDGF]⁺ formation (main figure, insert A experimental data, insert B theoretically predicted spectra) at a resolving power of 1700 m/Δm.

Figure 6: Product ion scan of $[\text{Ga-LysNTAPDGF}]^+$ at m/z 1030 from LTQ Ion Trap MS.

Figure 7: LC-ICP-MS chromatogram of $[\text{Ga-LysNTAPDGF}]^+$ from Element 2XR; Panel A signal from ^{31}P , Panel B signal from ^{69}Ga .

Figure 8: the lowest energy structure **2A** calculated for the Ga-LysNTA- β -PDGF singly charged positive ions as calculated using the ONIOM method at B3LYP/LANL2DZ:PM6 and the B3LYP/LANL2DZ (italicized numbers) levels of theory. Bond lengths are in Angstroms, relative free energies are indicated in parenthesis.

Table S1: Electronic energies, Zero-Point Vibrational Energies, Thermal Energies, Entropies and relative free energies for species calculated at HF/STO3G and B3LYP/LANL2DZ (italicized values). ONIOM values for species 2A are listed in bold within and underneath the table.

Structure	Electronic energy (Hartrees)	ZPE (kcal mol ⁻¹)	$H_{298}^0 - H_0^0$ (kcal mol ⁻¹)	Entropy (cal mol ⁻¹ K ⁻¹)	Relative free energy at 298 K (kcal mol ⁻¹)
1A	-2834.8816135 <i>-953.3907574</i>	204.4 <i>175.7</i>	10.8 <i>12.2</i>	130.3 <i>141.1</i>	0.0 <i>0.0</i>
1B	-2834.8678164 <i>-953.3728666</i>	204.6 <i>175.0</i>	11.6 <i>12.4</i>	136.5 <i>142.3</i>	7.8 <i>10.3</i>
1C	-2834.8555209 <i>-953.3229606</i>	203.2 <i>172.5</i>	11.9 <i>13.1</i>	141.9 <i>149.7</i>	12.8 <i>37.6</i>
1D	-2834.8480638 <i>-953.3104025</i>	203.1 <i>171.9</i>	12.0 <i>13.3</i>	142.4 <i>150.6</i>	17.4 <i>44.7</i>
1E	-2834.8099823 <i>-953.2955212</i>	204.8 <i>174.3</i>	11.5 <i>12.4</i>	136.0 <i>140.7</i>	44.4 <i>58.6</i>
1F	-2834.7960201 ---	202.4 --	12.3 --	145.2 --	48.8 --
1G	-2834.7911950 ---	204.7 --	11.1 --	131.8 --	57.0 --
1H	-2834.6674103 ---	201.4 --	12.6 --	150.0 --	127.3 --
2A	-5722.4171784 <i>-3167.9951469</i> -1334.8454825	787.1 <i>673.6</i> 624.0	42.6 <i>45.6</i> 47.2	376.7 <i>390.7</i> 408.8	0.0 -- --
2B	-5722.3869160	785.7	42.8	378.6	17.1
2C	-5722.3712784	786.0	43.1	380.1	21.7
2D	-5722.3510408	785.5	43.2	384.8	38.0
2E	-5722.1362298	786.4	43.8	399.2	170.0
2F	-5722.1339100	785.8	44.7	424.2	164.3

ONIOM: PM6 *model* system energy: -0.612107102528

ONIOM: B3LYP/LANL2DZ *model* system energy: -1334.375450261843

ONIOM: PM6 *real* system energy: -1.082139387972

ONIOM: extrapolated energy: -1334.845482547287

Table 1: Summary of the mass accuracy for all proposed assignments of LysNTAGa with methanol and water adducts and PDGF.

Ion or cluster (m/z)	Experimental m/z	Theoretical m/z	Mass Accuracy (ppm)
329.0259	329.0259	329.0259	0.0
	330.0292	330.0292	0.0
	331.0250	331.0250	0.0
	332.0284	332.0283	0.3
347.0364	347.0364	347.0364	0.0
	348.0398	348.0398	0.0
	349.0355	349.0356	-0.3
	350.0389	350.0389	0.0
361.0520	361.0520	361.0521	-0.3
	362.0554	362.0554	0.0
	363.0513	363.0512	0.3
	364.0556	364.0546	2.7
1030.3118	1030.3118	1030.3109	0.9
	1031.3152	1031.3118	3.3
	1032.3109	1032.3196	-8.4
	1033.3143	1033.3105	3.7