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

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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 $\text{H}_3\text{PO}_4$ or $\text{HPO}_3$ on collisional activation from phosphorylated serine and threonine residues or parent ion scans for the common product ions $\text{H}_2\text{PO}_4^-$ or $\text{PO}_3^-$.\cite{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.\cite{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. \cite{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.\cite{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_{2}O_{6}Ga) 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 ($\beta$-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 ºC 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 µm).

Materials

Nα,Nα-Bis(carboxymethyl)-L-lysine hydrate (LysNTA, C16H18N2O6 .xH2O > 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 MΩ using an Elix and Element System (Millipore, Billerica, Massachusetts, USA).

Sample Preparation and Procedures

Formation of β-PDGF-Ga-LysNTA: LysNTA (5.2 mg), Ga2Cl6 (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 Ga2Cl6. 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 µg 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 µL 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$_2$O with 0.05 % acetic acid) to 70 % organic (Eluent B: 100 % CH$_3$OH with 0.05 % acetic acid) at a total flow rate of 100 µL 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 µL, 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 µl min$^{-1}$ of H$_2$O 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 $\beta$-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:
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.

\[
ppm = \left( \frac{M_{exp} - M_{calc}}{M_{calc}} \right) \times 10^6 \quad (eq.2)
\]

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 LystNTAGa•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 ³¹P signal trace at a retention time of 3.89 mins which corresponded to an exact retention time match to that of the complexed ⁶⁹Ga signal peak, confirming the formation of Ga-LysNTA-β-PDGF, see Figure 7. The earlier eluting ⁶⁹Ga 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 μl injection equates to 5 pg Ga or ~50 pg of PDGF. The ⁹⁸Mo 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-MS$^6$ 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.

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**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/Δm.

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 [Ga-LysNTA.H2O]+ 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 H2O and CH3OH from LTQ Ion Trap MS, Panel B theoretically predicted composite spectrum of a 1:2 ratio of [Ga-LysNTA.2H2O]+ and [Ga-LysNTA.CH3OH]+ at a resolving power of 1000 m/Δm.

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 of [Ga-LysNTAPDGF]+) at a resolving power of 1700 m/Δm.

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

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

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.
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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/Δm.
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 (in kcal per mol) are indicated in parenthesis.
Figure 3: Formation of [Ga-LysNTA.H$_2$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$_2$O and CH$_3$OH from LTQ Ion Trap MS, Panel B theoretically predicted composite spectrum of a 1:2 ratio of [Ga-LysNTA.2H$_2$O]$^+$ and [Ga-LysNTA.CH$_3$OH]$^+$ at a resolving power of 1000 m/Δm.
Figure 5: Full scan spectra of reaction mixture from LTQ Ion Trap MS showing evidence of [Ga-LysNTAPDGFl]⁺ 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 \([\text{Ga-LysNTAPDG}]^+\) at m/z 1030 from LTQ Ion Trap MS.
Figure 7: LC-ICP-MS chromatogram of [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.

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<th>( \Delta H_0^{298} - H_0^0 ) (kcal mol⁻¹)</th>
<th>Entropy (cal mol⁻¹ K⁻¹)</th>
<th>Relative free energy at 298 K (kcal mol⁻¹)</th>
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ONIOM: PM6 model system energy: -0.612107102528
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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.

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