

# Enrichment of Serum Phosphopeptides by Nanoparticles to Identify Lung Cancer Biomarkers

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## ABSTRACT

The role of kinases in transformation and cancer progression is established, though isolation and characterization of low abundance serum phosphoproteins remains a challenge. The existing technology lacks the specificity, efficiency and flexibility necessary to analyze larger sample volumes. To overcome these problems we have used nanoparticles to enrich phosphopeptide from serum and then analyze by mass spectrometry (MS). Nanoparticles provide a very large surface area, specificity and ability to analyze larger serum samples. We have developed and characterized Fe<sub>3</sub>O<sub>4</sub>-SiO<sub>2</sub>-TiO<sub>2</sub> core/shell/shell nanoparticles to specifically enrich phosphopeptides. Preliminary data indicates that particles bind specifically to phosphopeptides, and can be analyzed directly by MALDI-MS. The ongoing work will determine the efficiency of phosphopeptide enrichment from serum.

**Keywords:** nanoparticle, serum, phosphopeptide, titanium

## 1 INTRODUCTION

Lung cancer is a devastating illness with an overall poor prognosis. In order to effectively address this disease, early detection is urgently needed. Early detection of lung cancer is challenging, in part due to the lack of methodology to practically and reliably identify serum biomarkers. Moreover, the existing detection techniques do not have the ease and accessibility associated with the successful identification of a serum biomarker. Large-scale serum proteomics studies using mass spectrometry (MS) to identify proteins present differentially in patient serum met with very limited success. Also, MS-based methods are not suitable for quantitative estimation. This has led the field to the gradual change towards a more specific targeted proteomics approach for biomarker isolation and their characterization.

The human genome encodes an estimated 518 kinases. The role of kinases in transformation and cancer progression is established, including lung cancer. The c-Kit and c-Met receptor tyrosine kinases and their ligand are over-expressed in lung cancer, and it has been shown that the SCF/c-Kit pathway is functional in an autocrine and paracrine fashion in SCLC. Liotta and colleagues [1] have proposed molecular stratification of tumors based on type of

phosphoproteins. They have indicated that the activation state of kinase-driven signal networks contains important information relative to cancer pathogenesis and therapeutic target selection. Specifically, phospho-Akt over-expression has been shown to confer significant stage-independent survival disadvantage in non-small cell lung cancer.

Using anti-phosphotyrosine immunoprecipitation and anti-phosphotyrosine immunoblotting we have recently identified two phosphoproteins (60kDa and 20 kDa) differentially expressed in SCLC patient serum. However, the existing technology to isolate and enrich these phosphopeptides in large enough amounts from serum to enable characterization with MS lacks the specificity, efficiency and flexibility necessary to analyze larger sample volumes. We propose the use of an innovative magnetic nanoparticle-based technology to enrich phosphopeptides from serum.

The synthesis and functionalization of nanoparticles will ensure specificity, enhance efficiency (at least 1000-fold due to large surface area compared to macro-particles currently used) and will be suitable to analyze large volumes (up to 10 ml) serum samples. Using these nanoparticles, we will be able to enrich two 60 and 20 kDa differentially expressed phosphoproteins. The enrichment efficiency will be significantly higher, and we will be able to characterize the phosphoproteins by MS analysis. Two different groups have shown very promising results of phosphopeptide enrichment using magnetic nanoparticles [2][3].

## 2 MATERIALS AND METHODS

### 2.1 Nanoparticle Synthesis

Iron oxide nanoparticles were functionalized with silica and titanium oxide to get Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> core/shell nanoparticles. Briefly, 5 ml of 12nm or 250 nm iron oxide nanoparticles (approx. 40 mg/ml) were suspended in 8.95 ml of 25% ammonia. 150 µl of TEOS was added drop-wise to the solution while magnetically stirring and maintaining a temperature of 40°C for 2 hours. The solution was then sonicated for 1 hour, washed with ethanol three times and re-suspended in 30 ml of ethanol, followed by 12 hours of

heating at 60°C under reflux conditions to strengthen the silica coating. The resulting particles were then washed with ethanol three times, and re-suspended in 80 ml of ethanol for 1 hour of sonication. Titanium butoxide (0.6 ml) was added to the solution, and stirred vigorously while heating at 45°C for 4 hours. The particles were once again washed in ethanol three times, followed by washing with water twice. The nanoparticles were then re-suspended in 20 ml of water and heated to 60°C under reflux conditions for 12 hours to strengthen the titanium dioxide shell. The particles were then washed three times in water, and re-suspended in 5 ml of water for future use.

In order to synthesize Fe<sub>3</sub>O<sub>4</sub>/C/TiO<sub>2</sub> nanoparticles, a different procedure was used. 5 ml of 250 nm iron oxide particles (approx. 40 mg/ml) was sonicated in 20 ml of 0.1 M nitric acid, followed by washing with water three times. The particles were then re-suspended in a 0.5 M aqueous glucose solution and stirred vigorously for 10 minutes. The solution was then placed in a pressure vessel such that it could be heated to 180°C for 4 hours in an oven without evaporation. The resultant black, carbon coated nanoparticles were magnetically isolated and washed with water and ethanol three times each. The samples were then dried in an oven for 4 hours at 80°C in an oven, and re-suspended in 10 ml of ethanol. The particles were washed three times in ethanol, then re-suspended in 80 ml of ethanol and sonicated for 1 hour. The titanium shell was synthesized via the same methods as written earlier. In order to create a titanium dioxide shell with a consistent pore diameter, the nanoparticles were calcinated at 500°C for 1 hour in a nitrogen furnace.

## 2.2 Phosphopeptide Binding

β-casein (10 nmol) was dissolved in 20 mM ammonium bicarbonate and digested with 12 mM trypsin at 37 °C overnight. Different concentrations of β-casein were then incubated for 10 minutes with the iron-silica-titanium or iron-carbon-titanium nanoparticles (10 μl at approx. 40 mg/ml) in 0.1% acetic acid for a total volume of 250 μl.

Following incubation, the nanoparticles were washed three times with 0.1% acetic acid via magnetic separation. The nanoparticles were re-suspended in MALDI matrix (α-cyanol) and directly analyzed by MS.

## 2.3 Mass Spectrometry

Analysis of phosphopeptide enrichment efficiency was done using MALDI-TOF-TOF-MS (ABI 4800).

# 3 RESULTS

## 2.4 Nanoparticle Characterization

Characterization of the synthesized nanoparticles was done by dynamic light scattering (DLS), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR) (figure 1).

Average particle size was determined using DLS. Particles were analyzed in water using 500-fold averaging and 1 second integration time. A single mass-weighted value was used to characterize the average size of all particles in each sample. Histograms of particle radius distribution were generated, displaying sharp peaks representative of particles. The determined particle sizes for iron, iron-silicate and iron-silicate-titanium oxide particles were 10.9 nm, 13.5 nm and 15.5 nm respectively (Fig 1, top panel).

To confirm that the nanoparticles were functionalized with TiO<sub>2</sub> and SiO<sub>2</sub>, FTIR spectra were taken of both species in addition to the unfunctionalized Fe<sub>3</sub>O<sub>4</sub> (Fig 1, Lower panel, a). This method of verification has been used previously [2] and relies on distinct Si-O and Ti-O vibrational modes. Figure 1 (lower panel, b) shows the SiO<sub>2</sub> functionalized sample with bands at 1095 and 950 cm<sup>-1</sup> very close to the earlier reported values of 1091 and 956 cm<sup>-1</sup>. Upon addition of a thin layer of TiO<sub>2</sub> (Figure 1, lower panel, c), the dominant peaks shift to 1079 and 962 cm<sup>-1</sup>, again consistent with the reported values of 1086 and 971 cm<sup>-1</sup>. This data demonstrates functionalization of the nanoparticles. The peaks at 3500 and 1630 cm<sup>-1</sup> prominent in the TiO<sub>2</sub> sample are due to water and likely represent incomplete dehydration of the sample.

The particles were also analyzed by TEM. The particles suspended in water were placed on grid and directly observed by TEM. Figure 1 (middle panel) demonstrates the features of the particles. As part of specific aim 1, more efficient routes to iron oxide particles with improved morphology and phosphopeptide binding ability will be developed. Left panel represents core iron particles, middle panel Fe<sub>2</sub>O<sub>3</sub>/SiO<sub>2</sub> and right panel Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> functionalization.

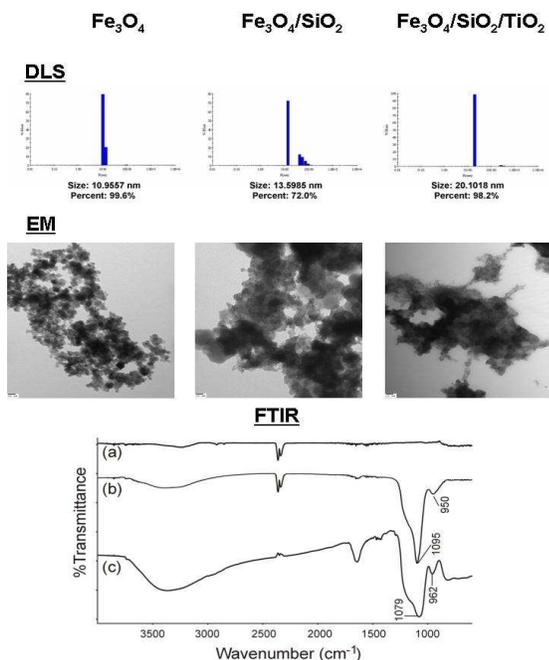


Figure 1: Characterization of nanoparticles.

### 3.2 <sup>32</sup>P-Labeled Phosphopeptide Binding

In order to see which of our two functionalized nanoparticles, the Fe<sub>3</sub>O<sub>4</sub>/C/TiO<sub>2</sub> or Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub>, worked best for the binding and enrichment of phosphopeptides, myelin basic protein (MBP) was radioactively labeled with <sup>32</sup>P. 100 nmol of MBP was incubated with PKCδ (a kinase) in the presence of kinase buffer (50 mM Tris, 10 mM MgCl<sub>2</sub> and 4 mM DTT) and <sup>γ</sup>-<sup>32</sup>P ATP. Unincorporated ATP was removed by a Sephadex G-50 column and <sup>32</sup>P labeled MBP was used for binding experiments. Figure 2 shows the percentage of bound <sup>32</sup>P detected following the binding and three subsequent washings of the calcinated and uncalcinated Fe<sub>3</sub>O<sub>4</sub>/C/TiO<sub>2</sub> nanoparticles, as well as for the Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> nanoparticles. With the Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> nanoparticles showing a clear advantage in binding affinity for phosphopeptides, these were the nanoparticles chosen for our later experiments.

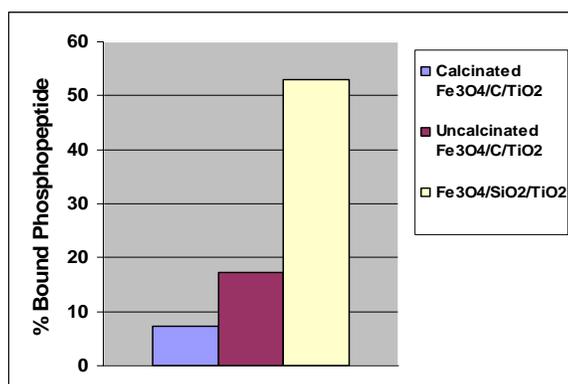


Figure 2: <sup>32</sup>P-labeled phosphopeptide binding to functionalized nanoparticles

### 3.3 Isolation of Phosphopeptide from β-casein Digest

β-casein digestion was performed by standard protocol using trypsin, and the trypsin digest was used to determine the enrichment efficiency of the nanoparticles.

Following the binding of digested β-casein to the functionalized nanoparticles, enrichment analysis was done via MALD-MS. Figure 3a shows the peptides found for trypsin digested β-casein alone, while figure 3b shows the digested β-casein bound to our functionalized Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> nanoparticles. The m/z 3122 phosphopeptide of β-casein can clearly be seen in the nanoparticles sample, but is absent in the β-casein only digest (figure 3a and 3b, red arrows). A MS/MS analysis was done in order to verify that this was indeed a phosphopeptide (figure 3c).

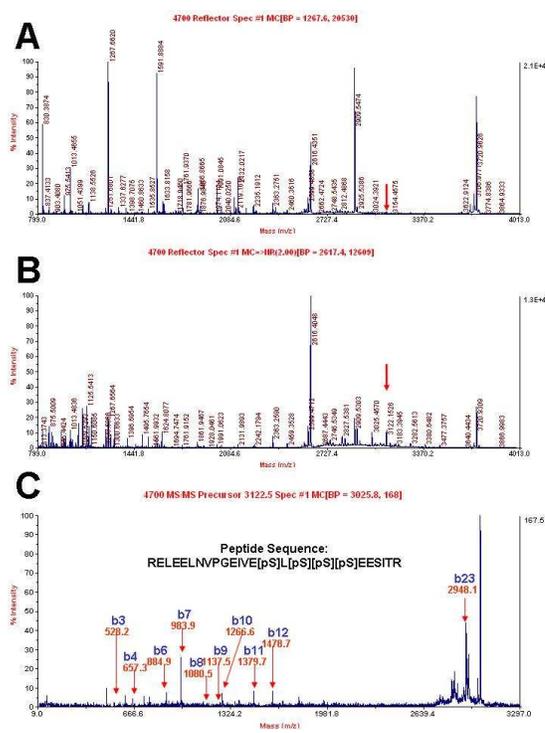


Figure 3: β-casein digest was analyzed by MALDI-TOF-TOF-MS. Mass spectrum indicated various tryptic masses of β-casein digest (A). The tryptic digest was incubated with Fe<sub>3</sub>O<sub>4</sub>/SiO<sub>2</sub>/TiO<sub>2</sub> nanoparticles, and the particle-bound peptides were analyzed by MS. The spectrum indicates a significant enrichment of a phosphopeptide (m/z 3122.1) indicated by the red arrow (B). The phosphopeptide with m/z 3122 was further analyzed by MS/MS. Collision induced fragment spectra of the peptide is shown in panel C. Significant b-ion coverage indicates the enrichment of the phosphopeptide.

## 4 SUMMARY

1. Nanoparticles offer a to enrich phosphoproteins from serum
2. Two different nanoparticles were functionalized,  $\text{Fe}_3\text{O}_4/\text{C}/\text{TiO}_2$  and  $\text{Fe}_3\text{O}_4/\text{SiO}_2/\text{TiO}_2$  core/shell/shell.
3.  $\text{Fe}_3\text{O}_4/\text{SiO}_2/\text{TiO}_2$  nanoparticles were found to better enrich phosphopeptides.
4. Experiments using human serum are currently underway.

## 5 REFERENCES

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