Nanostructured Silicon Coupled with Iron Oxide Magnetic Nanoparticles as Enhanced Desorption/Ionization on Silicon Mass Spectrometry for Phosphopeptide Analysis

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ABSTRACT

In this paper, we demonstrate using nanostructured silicon (nSi) as desorption/ionization on silicon (DIOS) substrate and coupled with functionalized ioron oxide magnetic nanoparticles (MNPs) for phosphopeptide mass spectrometry (MS) analysis. Phospholyted peptide has been successfully selectively detected by nSi incorporated with TiO_2 and ZnO MNPs. Results show that MNPs coupled nSi MS technique (MNPs/nSi-MS) is a high performance approach for phosphopeptide analysis where nSi service as DIOS-MS target substrate for high sensitivity MS detection and MNPs can selectively capture the phosphopeptide for enhanced MS detection selectivity.

Keywords: desorption/ionization on silicon, nanostructured silicon, magnetic nanoparticle, mass spectrometry, Phosphopeptide

1 INTRODUCTION

Stem cells can differentiated into various cell types which can be used to repair damage organs in regenerative medicine applications. The cell regulation process such as cell division, differentiation, and apoptosis are deeply related to protein phosphorylation in cell transduction pathway. However, low abundance of phosphorylated peptides and proteins in cell makes identification of phosphorylation sites difficult. Therefore, developing a rapid, high performance analytical tool to analysis phosphopeptide is important in tissue engineering as well as other related biolgofical fields.

MS is a widely used analytical tool to analysis biomolecules. Recently, DIOS-MS have been proposed as matrix free and high sensitivity MS analysis approach. DIOS-MS have shown its applications in various fields like forensic polymer analysis, drug abuse screening, peptide sequencing, and MS imaging[1-3] with high detection sensitivity. However, for phosphopeptide analysis, DIOS-MS only showed two-fold MS intensity enhancement on immobilized metal ion -Ni^{2+} modificated DIOS-MS surface[4]. In this study, we first demonstrate using nSi as DIOS substrate coupled with functionalized MNPs for MNPs/nSi-MS analysis. TiO_2 and ZnO functionalized MNPs (TiO_2@MNPs and ZnO@MNPs) were used to selectively concentrate phosphopeptide. Performance of phosphopeptide analysis by TiO_2@MNPs/nSi-MS and TiO_2@MNPs/nSi-MS methods were detail reported.

2 EXPERIMENTAL

2.1 Nanostructured Silicon Chip Fabrication

The nSi chip was fabricated by electrochemical etching process. P-type <100> silicon wafer with 0.001 ~ 0.02 Ω·cm resistivity was first cleaned with acetone in an ultrasonic cleaner and then rinse with deionized water and nitrogen blow dry. After wafer cleaning, the silicon wafer was then deposited with 3nm Au catalyst layer by E-beam deposition (ULVAC EVA-E500) with deposition rate of 0.1~0.2 Å/s. After wafer deposition, the 3nm Au-coated silicon wafer was then immerse in a HF/ EtOH/ H_2O_2 (1:1:1, v/v/v) mixture for silicon etching for 300 seconds. After etching, the nSi chip was clean with methanol, DI water followed by nitrogen blow dry and stored in a N_2 filled container to prevent surface oxidation. The nSi surface SEM image was shown in Figure 1.

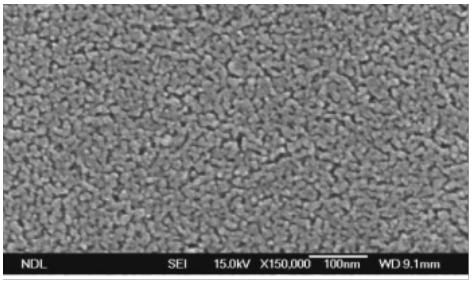

Figure 1: SEM image of nSi surface

2.2 TiO_2@MNPs and ZnO@MNPs Synthesis

The TiO_2@MNPs and ZnO@MNPs synthesis procedure is shown in Figure 2. First, MNPs was prepared by mixing 2g of $FeCl_2 \cdot 4H_2O$ and 5.4g $FeCl_3 \cdot 6H_2O$ with 25 mL 2 M HCl solution and degassed by ultrasonication. Add 25 mL of 25% (v/v) ammonia and stirred for 30 min at room temperature. Place a magnet under the beaker to concentrate the Fe_3O_4 particles and fully rinsed with deionized water and ethanol. Then, the MNPs are coated with a thin SiO_2 layer by adding the MNPs into the 17.9 mL 25% ammonia, 150 mL 99.5% ethanol, 0.3 mL TEOS and 15 mL mass-grade water mixture. Heat the MNPs-added mixture to 45 ˚C and maintaining for 2 hour. The MNPs are

rinse with ethanol then resuspended in a 150 mL 99.5% ethanol and heated under 45 ˚C for 12 hours to strengthen the Si-O-Fe cross-linking on the MNPs surfaces.

For the TiO_2 coating [5], the SiO_2 coated MNPs immerse in a 1.2 mL titanium butoxide and 20 mL 99.5% ethanol solution followed by stirred heat in a mass-grade water at 45 ˚C for 4 hours allow sufficient reaction. Rinse clean the unreacted impurities with ethanol and water then heated under 60 ˚C for 12 hours to strengthen the cross-linking of TiO_2 on the SiO_2-coated MNPs surfaces to complete TiO_2@ MNPs. For the ZnO coating [6], prepare a 16 mL 40 mM zinc acetate and 16 mL 1.6 mM triethanolamine mixture and added SiO_2-coated MNPs in an oil bath at 90˚C for 12 hours followed by water and ethanol rinse clean to complete ZnO@MNPs.

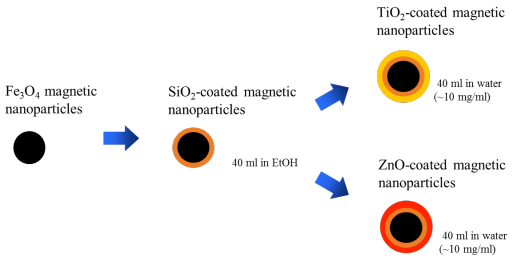

Figure 2: TiO_2@Fe_3O_4 and ZnO@Fe_3O_4 MNPs synthesis procedure

2.3 Mass Spectrometry Analysis

The MS analysis was performed on a SELDI mass spectrometer (Proteinchip SELDI System, Bio-rad laboratories, USA). In this study, MALDI-MS analysis was performed by mixing the matrix with analyte and pipette on the MALDI target substrate. The MALDI matrix was prepared by mixing 20 mg/ml 2,5-dihydroxybenzoic acid (DHB) with mass grade water and trifluoroacetic acid (TFA) with 50:50:1 volume ration. The nSi-MS analysis was performed by directly deposit 10 µL aqueous analyte on the nSi surface and staying for 30 seconds allow sufficient sample absorption. N_2 blow the droplet away from the nSi surface and then fixed to the custom-made target holder and loaded to the mass spectrometer for MS analysis.

The MS were evaluated by the mixture of the following peptides: histidine tag-containing bradykinin (His, RPPVFHHH, MW=1026), des-Arg^9-bradykinin (Bk, PPGFSPFR, MW= 904, hydrophobic peptide), positively charge-bradykinin (PC, RPPGSSPR, MW=852), extracellular signal-regulated kinase fragment (Erk, GFLTYVATR, MW=1156) and tyrosine phosphorylated extracellular signal-regulated kinase fragment (Erk_pY, GFLTE(pY)VATR, MW=1263). All MS spectra were recorded in the linear, positive ion mode and averaged over 50 laser pulses using a 337 nm N_2 laser. An optimized laser energy was set for the maximum signal-to-noise ratio (S/N) for all MS measurements. We define the MS peak is discriminable when S/N > 3 in this study.

3 RESULTS AND DISCUSSIONS

3.1 MNPs Coupled nSi-MS Analysis

The MNPs/nSi-MS procedure was shown in Figure 3a. First mix the MNPs with analyte in 1:7 volume ratio in a tube for 90 minutes. Place a magnet under the tube to concentrate the MMPs. Take the concentrated MNPs in citric buffer (diammonium hydrogen citrate (DAHC) and citric acid (CA)) and deposit 1µL sample on the nSi surface. In order to prevent MNPs aggregates and fully block the nSi surface pores for MS analysis, we deposit 1~2 µL acetonitrile to spread the MNPs on the nSi surface. The spotting size of MNPs on nSi is approximately 2~3 mm. Figure 3b and 3c shows SEM images of TiO_2 and ZnO coated MNPs on nSi surface. For both MNPs, the MNPs measured approximately 100~300 nm diameter. After the MNPs deposited on the nSi surface, the MNPs were aggregates into couple micron sized clusters on the nSi surface. The MNPs clusters were not fully block the nSi surface leaving the nSi nanopores open for MS analysis.

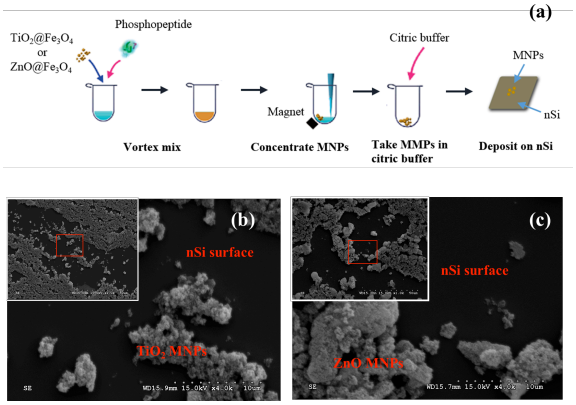

Figure 3: (a) MNPs/nSi-MS analysis procedure. SEM image of (b) TiO_2 and (c) ZnO coated MNPs deposited on the nSi surface

The nSi surface morphology play an import role in nSi-MS ionization. In our previous research report, we detail investigate the nSi surface morphology effects to the MS analysis efficiency[7]. In this work, in addition to the nSi surface, the critic buffer may also contribute to the nSi-MS analysis in the MNPs-nSi coupling procedures. Therefore, the citric buffer effect were first evaluate. Citric buffer composition (DAHC/CA) to the nSi-MS ionization efficiency were tested by Bk (5×10^{-9} g/ml), PC (5×10^{-8} g/ml), Erk (10^{-7} g/ml) and Erk_pY (10^{-7} g/ml) peptide mixture. As shown in Figure 4, Bk, Erk and Erk_pY shows increased MS signal intensity with presence of citric buffer. Compare with native nSi (control) surface, Bk enhanced 2.5 times at 3:1 DAHC/CA ratio. Erk and Erk_pY shows maximum MS signal intensity enhancement of 4 and 9 fold at 4.5:1 DAHC/CA ratio. Therefore, we use 4.5:1 critic

butter (pH range: 4.3~4.5) composition in the following MNPs/nSi-MS for phosphopeptide analysis.

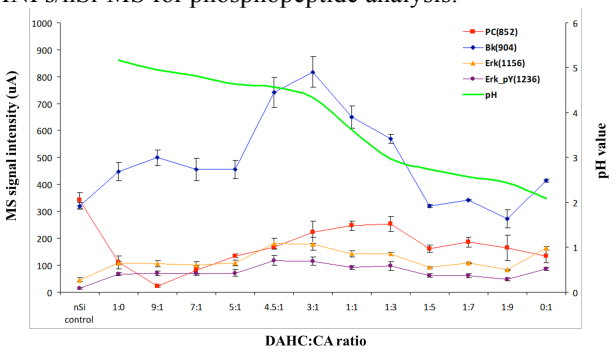

Figure 4: Citric buffer effects to the nSi-MS analysis. Error bars were obtain from three individual MS measurements

3.2 MNPs/nSi-MS Phosphopeptide Analysis

The MNPs/nSi-MS phosphopeptide analysis were evaluated by a standard peptide mixture which consists with 5 peptides (PC= 10^{-7} g/ml, BK= 10^{-8} g/ml, His= 10^{-8} g/ml, Erk= 10^{-6} g/ml and Erk_pY= 10^{-6} g/ml). Figure 5 shows MS spectrum of standard nSi-MS analysis without MNPs. All of the peptides were found in the native nSi-MS analysis. This native nSi-MS show comprehesive analyis and didn't selectively capture the phosphorylated peptide, Erk_pY, with MS signal intensity around 150 µA range. To future enhance the MS signal selectivity and sensitivity for phosphopeptide analysis, we use the MNPs/nSi-MS approach to selective concentrate phosphorylated peptide (Erk_pY) and perfroming nSi-MS on a chip chip. Both TiO_2 and ZnO coated MNPs surface are evaluated.

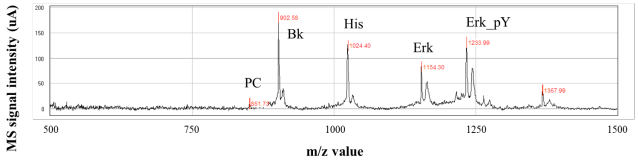

Figure 5: nSi-MS spectrum of standard peptide mixture

Figure 6 shows the MS spectrum of conventional MALDI-MS (matrix: DHB) and the TiO_2@MNPs/nSi-MS analysis the peptide mixture with series concentrations. For MALDI-MS analysis, Erk_pY can been identified in the 100% sample concentration (Figure 6a). However, due to the presence of matrix, numerous noise signals were also observed in the 500~1000 mass range. With the sample concentration dilute to 10% (Figure 6b) the Erk_pY MS intensity in MALDI-MS become low (signal intensity: 181.31 µA, S/N: 3.15). In the lowest 1% sample concentration (Figure 6c), Erk_pY can't be identified in the MALDI-MS.

In TiO_2@MNPs/nSi-MS, it shows selecvtive detecion of Erk_pY with MS signal intensity of 1686.1 µA and S/N of 53.26 where unphosphorylated peptides peaks were vanish comparing with the native nSi-MS analysis shown in Figure 5. At lower sample concentration, Erk_pY can further analyzed with signal intensity: 411.0 µA, S/N: 15.5 and signal intensity: 146.3 µA, S/N: 4.7 for 10% (Figure 6b) and 1% (Figure 6c) concentration respectively while MALDI-MS can't effectively detect Erk_pY these low sample concentrations.

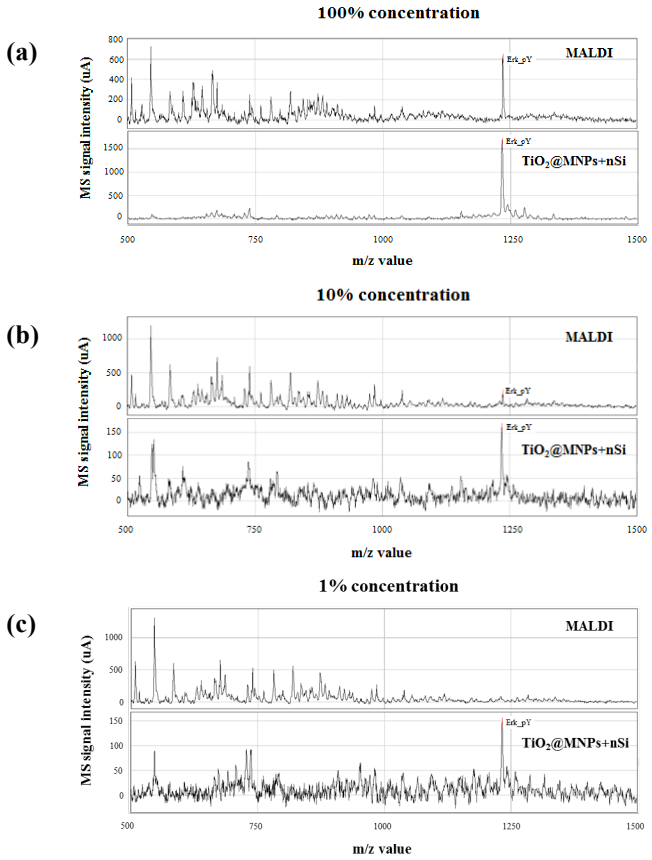

Figure 6: MS spectrum of TiO_2@MNPs/nSi-MS and MALDI-MS analyzing peptide mixture with (a) 100%, (b) 10% and (c) 1% sample concentration

ZnO@MNPs/nSi-MS analysis also exhibit similar selective detection performance of Erk_pY with TiO_2@MNPs/nSi-MS. Erk_pY peak were selectively detected with 209.6 µA, 8.8 S/N detection sensitivity in the 100% (Figure 7a), 160.0 µA, 6.3 S/N detection sensitivity in the 10% (Figure 7b) and 81.6 µA, 3.3 S/N detection sensitivity in the 1% (Figure 7c) sample concentrations. In MALDI-MS analysis, Erk_pY can only been detected in 100% (Figure 7a) and 10% (Figure 7b) sample concentration conditions with low S/N and numerous of noise backgrounds.

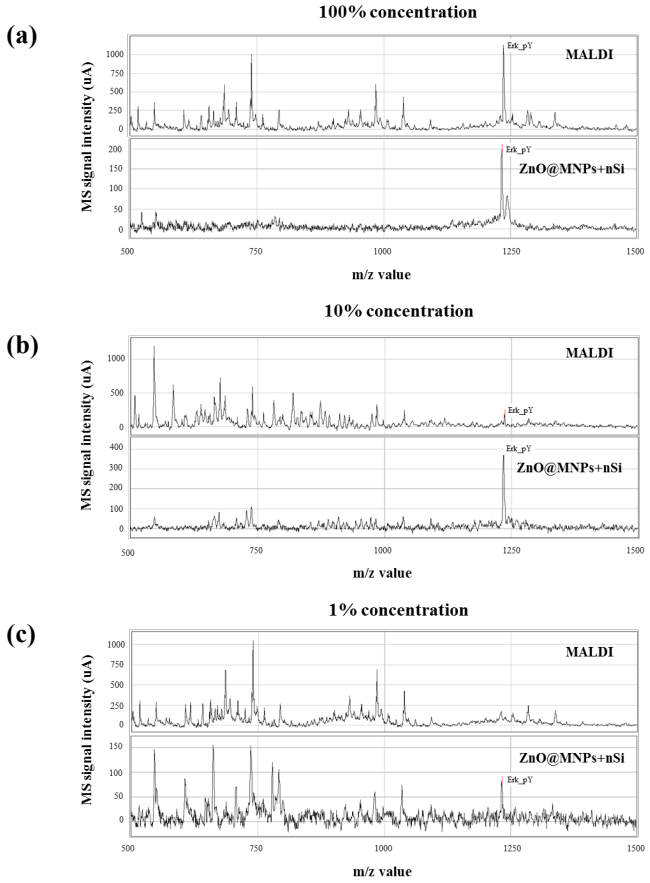

Figure 7: MS spectrum of ZnO@MNPs/nSi-MS and MALDI-MS analyzing peptide mixture with (a) 100%, (b) 10% and (c) 1% sample concentration

Although TiO_2@MNPs and ZnO@MNPs have shown good MS detection selectivity for phosphorylated peptides on the nSi surface with high detection sensitivity comparing with MALDI-MS and nSi-MS. The MS signal intensity of MNPs/nSi-MS is relatively low around the 100~400 μA range. One of the possible reason may due to MNPs deposited on the nSi surface were partially block by the MNPs resulting insufficient ionization. Organic matrix is an ideal biomolecule ion source for LDI-MS analysis. Therefore, we use DHB maxtix in the MNPs/nSi-MS analysis to enrich the ion source for efficient ionization. Figure 8 shows the MS spectrum of MNPs/nSi-MS analysis with DHB matrix addition. MS experiment results shows that MS intensity enhanced to 2148.9 μA (S/N = 65.7) for TiO_2@MNPs and 1173.7 μA (S/N = 65.7) for ZnO@MNPs. Besides, little MS noise background were observed at 500~1000 m/z range in this MNPs/nSi-MS approach even with matrix addition. These results suugest that matrix can also been used in the MNPs/nSi-MS appraoch to enhance MS signal intensitsy without adding excessive noise grounds.

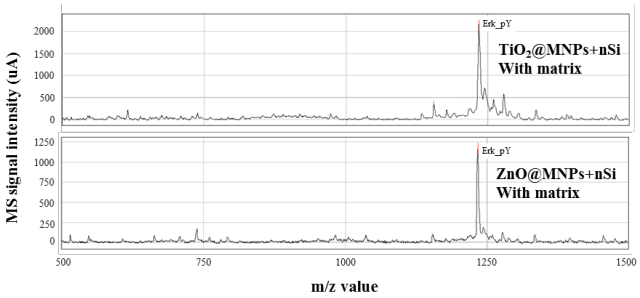

Figure 8: TiO_2@MNPs/nSi-MS and ZnO@MNPs/nSi-MS analyzing peptide mixture with matrix

4 CONCLUSION

MNPs coupled with nSi for DIOS-MS analysis was first successfully presented in this paper. TiO_2@MNPs/nSi-MS and ZnO@MNPs/nSi-MS have been demonstrated to selective capture phosphorylated peptide (Erk_pY) with good MS detection sensitivity and selectivity comparing to convectional MALDI-MS or nSi-MS methood. In addion, we aslo found that matrix addition in the MNPs/nSi-MS approch can futrther enhanced the MS signal intensity without creating excessive noise backgrounds during MNPs/nSi-MS analysis.

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REFERENCES

[1] Z. X. Shen, J. J. Thomas, G. Siuzdak, and R. D. Blackledge, "A case study on forensic polymer analysis by DIOS-MS: The suspect who gave us the SLIP (R)," *Journal of Forensic Sciences,* vol. 49, pp. 1028-1035, Sep 2004.

[2] Y. C. Cheng, K. H. Chen, J. S. Wang, W. L. Hsu, C. C. Chien, W. Y. Chen*, et al.*, "Rapid analysis of abused drugs using nanostructured silicon surface assisted laser desorption/ionization mass spectrometry," *Analyst,* vol. 137, pp. 654-61, Feb 7 2012.

[3] O. Yanes, H. K. Woo, T. R. Northen, S. R. Oppenheimer, L. Shriver, J. Apon*, et al.*, "Nanostructure Initiator Mass Spectrometry: Tissue Imaging and Direct Biofluid Analysis," *Analytical Chemistry,* vol. 81, pp. 2969-2975, Apr 15 2009.

[4] C. W. Tsao, C. H. Lin, Y. C. Cheng, C. C. Chien, C. C. Chang, and W. Y. Chen, "Nanostructured silicon surface modifications for as a selective matrix-free laser desorption/ionization mass spectrometry," *Analyst,* vol. 137, pp. 2643-2650, 2012.

[5] C. T. Chen and Y. C. Chen, "Fe3O4/TiO2 core/shell nanoparticles as affinity probes for the analysis of phosphopeptides using TiO2 surface-assisted laser

desorption/ionization mass spectrometry," *Analytical Chemistry,* vol. 77, pp. 5912-5919, Sep 15 2005.
[6] W. Y. Chen and Y. C. Chen, "Functional Fe3O4@ZnO magnetic nanoparticle-assisted enrichment and enzymatic digestion of phosphoproteins from saliva," *Analytical and Bioanalytical Chemistry,* vol. 398, pp. 2049-2057, Nov 2010.
[7] W. Y. Chen, J. T. Huang, Y. C. Cheng, C. C. Chien, and C. W. Tsao, "Fabrication of nanostructured silicon by metal-assisted etching and its effects on matrix-free laser desorption/ionization mass spectrometry," *Analytica Chimica Acta,* vol. 687, pp. 97-104, Feb 21 2011.