Functional magnetic nanoparticle-based label free fluorescence detection of phosphorylated species†

Cheng-Tai Chen and Yu-Chie Chen*

Received 29th March 2010, Accepted 15th June 2010
DOI: 10.1039/c0cc00637h

A label free fluorescence method for rapid detection of the presence of phosphorylated peptides/proteins in sample solutions using functional magnetic nanoparticles (MNPs) as the detection probes is demonstrated.

Protein phosphorylation is one of the most studied post-translational modifications (PTMs) that can regulate cellular processes. Thus, developing appropriate quantitative analysis methods for monitoring the levels of phosphorylated species in a biological system is significant. Isotope labeling quantitative phosphoproteomics[1] has been developed for this purpose. However, isotope labeling reagents are expensive. Generally, a sample pretreatment must be performed by either immobilized metal ion affinity chromatography (IMAC)[2] or metal oxide affinity chromatography (MOAC)[3–5] to concentrate traces of phosphorylated proteins/peptides from complex samples prior to quantitative/qualitative analyses. Phosphorylated species concentrated by these affinity chromatography approaches are generally characterized using biological mass spectrometry. When aiming to monitor the levels of major phosphorylated species in a biological system, quantitative methods possessing the features of simplicity and rapidity are desirable.

Label-free fluorescence detection offers a convenient and sensitive means for rapidly sensing target species.[6] Since alumina nanoparticles have been demonstrated to be good affinity probes for phosphorylated species such as phosphopeptides and phosphoproteins,[4,5] we herein propose a method by immobilizing fluorescence molecules, namely, riboflavin-5′-monophosphate (RFMP), onto the surface of alumina-coated magnetic nanoparticles (RFMP-Fe_3O_4@Al_2O_3 MNPs) *via* Al-phosphate chelating as sensing probes for phosphorylated species. The magnetic feature of the MNP leads to fast isolation of the MNP-target species from the solution. The fluorescence of RFMP molecules is quenched while the molecules anchor on the surface of the MNPs. However, upon the addition of the RFMP-Fe_3O_4@Al_2O_3 MNPs into sample solutions containing phosphorylated species, the phosphorylated species spontaneously exchange with the RFMP molecules from the surfaces of the MNPs, resulting in the appearance of visible fluorescence in the solution. Thus, using RFMP-Fe_3O_4@Al_2O_3 MNPs as sensing probes for phosphorylated species in order to conduct quantitative analysis is possible. Furthermore, the attachment of phosphorylated species on the MNPs can be examined by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) for further confirmation. Fig. 1 presents the design of this label free approach for phosphorylated species by using fluorescence spectroscopy (FL) and mass spectrometry (MS) for quantitative and qualitative analysis, respectively. Blood coagulation abnormalities have been found in patients with cancers.[7] Fibrinopeptide A (FPA), one of the products of fibrinogen after digestion by thrombin, has been known to elevate abnormally in the blood of patients with gastric[8] and ovarian cancers.[9] Thus, rapidly determining the FPA level in serum samples can assist medical diagnostics. Furthermore, it has been found that 20–30% of FPA is phosphorylated in human blood.[10] Thus, phosphorylated FPA (PFPA) may be used as a potential risk factor for early detection and screening of high-risk-individuals. We have previously demonstrated that only PFPA-derived ions appear in the MALDI mass spectra after human serum samples were enriched by Fe_3O_4@Al_2O_3 MNPs.[4] That is, because PFPA is the major phosphorylated species in serum samples therefore the contribution from traces of other phosphorylated molecules is suppressed and may be neglected. In this paper, we examined the feasibility of employing the RFMP-Fe_3O_4@Al_2O_3 MNPs as sensing probes for rapid screening of the presence of PFPA in human serum based on the detection of fluorescence derived from RFMP molecules.

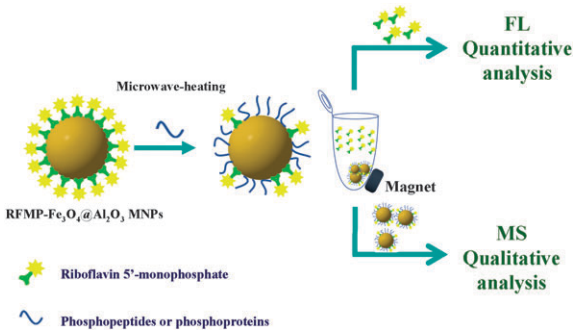

Fig. 1 Cartoon representation of the quantitative and qualitative detection of phosphorylated peptides/proteins by fluorescence spectroscopy (FL) and mass spectrometry (MS), respectively, using RFMP-Fe_3O_4@Al_2O_3 MNPs as affinity probes and reporters.

We first examined the binding constant of RFMP toward Fe_3O_4@Al_2O_3 MNPs according to the Langmuir adsorption equation. The dissociation constant (K_d) is *ca.* 6.82×10^{-7} M (see Fig. S1†). The binding process can be accelerated by placing the reaction under microwave-heating. The RFMP-Fe_3O_4@Al_2O_3 MNPs were generated by mixing RFMP with Fe_3O_4@Al_2O_3 MNPs under microwave-irradiation (power: 450 W) for 60 s. The maximum binding amount (Q_{max}) of RFMP molecules on the MNPs was *ca.* 76 nmol mg^{-1}, similar to that obtained from vortex-mixing for 2 h at room temperature. Additionally, microwave-assisted exchanges of

Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan. E-mail: yuchie@mail.nctu.edu.tw; Fax: +886-3-5723764; Tel: +886-3-5131527

† Electronic supplementary information (ESI) available: Experimental details and supplementary results. See DOI: 10.1039/c0cc00637h

monophosphopeptides from the surface of RFMP-Fe_3O_4@Al_2O_3 MNPs were examined. Fig. S2† presents the maximum emission intensity at 530 nm ($\lambda_{excitation}$ = 450 nm) obtained after incubating the monophosphopeptide (FQsEEQQQTEDELQDK) (10^{-6} M, 50 μL) with the RFMP-Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg mL^{-1}) under microwave-heating (power: 900 W) and vortex-mixing at room temperature for 10–60 s. Apparently, the emission intensity at 530 nm obtained after the sample was incubated with the RFMP-Fe_3O_4@Al_2O_3 MNPs under microwave-heating (plotted with circles) is higher than that obtained under vortex-mixing (plotted with squares) at room temperature. That is, microwave-heating can speed up the exchange of phosphopeptides with RFMP molecules from the MNPs.

We then employed RFMP-Fe_3O_4@Al_2O_3 MNPs as sensing probes and reporters for the samples of the monophosphopeptide (FQsEEQQQTEDELQDK) at different concentrations. Fig. S3a† presents the fluorescence spectra obtained by incubating the samples (50 μL) containing monophosphopeptides at different concentrations with the MNPs (3 μL, 1 mg mL^{-1}) under microwave-heating (power: 900 W) for 60 s. The maximum emission intensity at 530 nm increases as the concentration of monophosphopeptides in the samples increases. As the concentration of the monophosphopeptide reaches >2.5 μM, the fluorescence intensity of the sample solution does not change much. It indicates that the maximum amount of the target species can bind onto the surface of the MNPs. Fig. S3b† displays the corresponding plot to Fig. S3a† by plotting the emission intensity at 530 nm as a function of the concentration of the monophosphopeptides. There is a linear dynamic range between 10^{-8} M–5 × 10^{-7} M (R = 0.999), which indicates the possibility of using this approach to estimate the concentration of the phosphopeptides in sample solutions. Fig. 2 displays photographs of the solutions isolated from the same monophosphopeptide samples as those used for obtaining Fig. S3†. The concentrations of monophosphopeptide samples from left to right were 0, 10^{-5} M, 10^{-6} M, and 10^{-7} M, respectively. Apparently, the intensity of the fluorescence obtained from the sample at 10^{-7} M can still be distinguished from the sample that does not contain monophosphopeptides simply by the naked eye. The results indicate that this approach can be employed for quick sensing of the presence of monophosphopeptides at a concentration ≧10^{-7} M in a short period of analysis time by the naked eye.

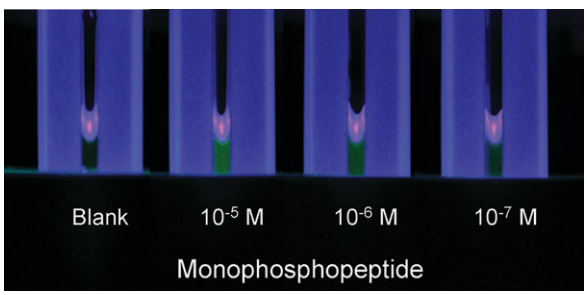

Fig. 2 Photographs of the resulting solutions obtained after the samples (50 μL) containing monophosphopeptides at 0, 10^{-5} M, 10^{-6} M, and 10^{-7} M (from left to right) were incubated with the RFMP-Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg mL^{-1}) under microwave-heating (power: 900 W) for 60 s followed by magnetic isolation. The photographs were taken under UV light irradiation ($\lambda_{maximum}$ = 365 nm).

We further employed more biomolecules as the samples to examine the selectivity of this approach for phosphorylated species. Fig. S4a† presents the bar graph obtained from the fluorescence intensity contributed by RFMP in the solutions obtained after incubating the samples (10^{-6} M, 50 μL), including adenosine (1), L-serine (2), L-threonine (3), L-tyrosine (4), D-glucose (5), bradykinin (6), angiotensin I (7), the tryptic digest of cytochrome C (8), *O*-phospho-L-serine (9), *O*-phospho-L-threonine (10), *O*-phospho-L-tyrosine (11), D-glucose-6-phosphate (12), adenosine 5′-triphosphate (13), monophosphopeptide (14), and tetraphosphopeptide (15) with the RFMP-Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg mL^{-1}) under microwave-heating (power: 900 W) for 60 s. The results indicate that phosphorylated species have relatively high capability to replace RFMP molecules from RFMP-Fe_3O_4@Al_2O_3 MNPs, resulting in the noticeable increase of fluorescence intensity at the wavelength of 530 nm. We further employed proteins as the samples to examine the selectivity of this approach for phosphorylated proteins. Fig. S4b† displays the bar graph of the fluorescence intensity obtained after incubating the samples (10^{-6} M, 50 μL) including ubiquitin (1), cytochrome C (2), trypsinogen (3), carbonic anhydrase (4), BSA (5), IgG (6), α-casein (7), and β-casein (8) with the RFMP-Fe_3O_4@Al_2O_3 MNPs (3 μL, 1 mg mL^{-1}) under microwave-heating (power: 900 W) for 60 s. Among them, only α- and β-caseins are phosphoproteins. It seems that the I_f values are much higher than 0.7 when α- and β-caseins are used as the samples for examination. Apparently, the I_f value was >0.5 when phosphorylated species were used as the samples. The I_f value of IgG is ~0.5, which is slightly higher than those of the rest of the non-phosphorylated proteins. It may result from the high affinity between riboflavin and IgG[11] leading to more RFMP molecules being released from the MNPs to the solution. Furthermore, the I_f values obtained from acidic proteins such as BSA (pI = 4.7) and acidic peptides such as those derived from the tryptic digest of cytochrome C are much lower than 0.5, implying that the presence of these acidic species in samples can be ignored. The results indicate that this label-free fluorescence detection method can be used to distinguish the presence of phosphorylated species in the sample based on the relative level of RFMP released in the solution after incubating the samples with the RFMP-Fe_3O_4@Al_2O_3 MNPs for a short period of time.

To demonstrate the feasibility of using this approach in a very complex sample, we applied it to the examination of PFPA in serum samples since it has been demonstrated that PFPA-derived peptides dominate the MALDI mass spectra after being enriched by Fe_3O_4@Al_2O_3 MNPs from serum samples.[4] Fig. 3 displays the fluorescence spectra obtained after incubating a serum sample with the RFMP-Fe_3O_4@Al_2O_3 MNPs under microwave-heating for 60 s. The spectrum plotted by the solid line was obtained from the background of the RFMP-Fe_3O_4@Al_2O_3 MNP solution, while the spectrum plotted by the dotted line was obtained from the solution separated after incubating the diluted serum sample, which was stored at −80 °C, with the RFMP-Fe_3O_4@Al_2O_3 MNPs under microwave-heating (power: 900 W) for 60 s. The fluorescence intensity obtained from the solution after incubating the serum sample with the MNPs is dramatically

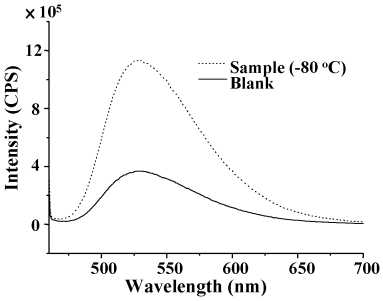

Fig. 3 Fluorescence spectrum ($\lambda_{excitation}$ = 450 nm) of the solution separated from the serum sample collected from a healthy individual, stored at −80 °C, incubated with the RFMP-Fe_3O_4@Al_2O_3 MNPs under microwave-heating (power: 900 W) for 60 s. The spectrum plotted by the solid line was obtained from the background of the RFMP-Fe_3O_4@Al_2O_3 MNP solution, while the spectrum plotted by the dotted line was obtained from the serum sample.

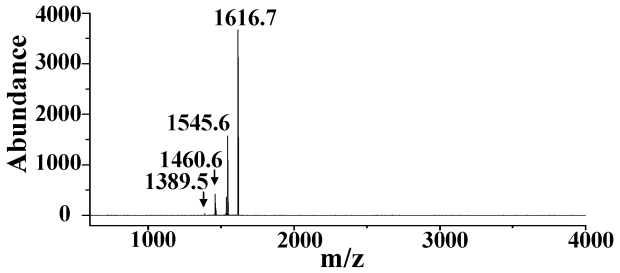

Fig. 4 MALDI mass spectrum of the sample obtained after using the RFMP-Fe_3O_4@Al_2O_3 MNP to trap target species from the serum sample under microwave-heating (power: 900 W) for 60 s. A mixture containing 2,5-DHB (15 mg mL^{-1}) and CHCA (15 mg mL^{-1}) prepared in acetonitrile/deionized water (2/1, v/v) containing 1% phosphoric acid was used as the MALDI matrix.

enhanced. We suspected that it was mainly contributed by PFPA replacements of RFMP from the MNPs. Thus, MALDI MS was employed to confirm the result. Fig. 4 displays the MALDI mass spectrum obtained from the MNPs that had been incubated with the serum sample. The ions at *m*/*z* 1616.7, 1545.6, 1460.6, and 1389.5 correspond to the sequences of ADsGEGD-FLAEGGGVR (#1–16), DsGEGDFLAEGGGVR (#2–16), ADsGEGDFLAEGGGV (#1–15), and DsGEGDFLAEGGGV (#2–15), derived from PFPA, which had been observed in a previous study.[4] Since all the peaks are derived from PFPA, the results indicate this approach can be potentially used to rapidly estimate the presence of the relative level of PFPA from very complex serum samples.

In conclusion, we have demonstrated the feasibility of using the RFMP-Fe_3O_4@Al_2O_3 MNPs as affinity probes and reporters for phosphorylated species in terms of quantitative and qualitative analyses. The exchange of phosphorylated species and the RFMP molecules on RFMP-Fe_3O_4@Al_2O_3 MNPs is speeded up with the assistance of microwave-heating. The RFMP molecules released from the MNPs can be used as reporters for the presence of phosphorylated species, while the phosphorylated species retained on the MNPs can be directly characterized by MALDI MS. Within a minute, the level of the RFMP resulting from the exchange on the MNPs with monophosphopeptides in the sample at a concentration $\geqq 10^{-7}$ M can be observed simply by the naked eye. This approach provides a rapid means for quantitative and qualitative analysis simultaneously. We believe that this method should be potentially useful for rapid screening of phosphorylated molecules from complex samples. Additionally, one of the potential applications of this approach should be in the rapid screening of the presence of the relative level of PFPA in serum samples obtained from cancer patients. Therefore, the possibility of using this approach for evaluating PFPA as a potential biomarker can be explored. Additionally, the presence of phosphates or phosphoric acid may affect sensing results. The use of phosphate buffer solution should be avoided when preparing samples. Small phosphate species may be present in biological fluids. Thus, it is required to remove small phosphates by desalting to avoid possible influence derived from these undesirable species.

We thank the National Science Council (NSC) of Taiwan for financial support of this work.

Notes and references

1 (*a*) E. B. Erba, R. Matthiesen, J. Bunkenborg, W. X. Schulze, P. Di Stefano, S. Cabodi, G. Tarone, P. Defilippi and O. N. Jensen, *J. Proteome Res.*, 2007, **6**, 2768–2785; (*b*) G. A. Zhang, D. S. Spellman, E. Y. Skolnik and T. A. Neubert, *J. Proteome Res.*, 2006, **5**, 581–588; (*c*) E. Salih, *Mass Spectrom. Rev.*, 2005, **24**, 828–846; (*d*) R. Amanchy, D. E. Kalume, A. Iwahori, J. Zhong and A. Pandey, *J. Proteome Res.*, 2005, **4**, 1661–1671.

2 (*a*) L. M. Nuwaysir and J. T. Stults, *J. Am. Soc. Mass Spectrom.*, 1993, **4**, 662–669; (*b*) S. H. Li and C. Dass, *Anal. Biochem.*, 1999, **270**, 9–14; G. K. Bonn, K. Kalghatgi, W. C. Horne and C. Horvath, *Chromatographia*, 1990, **30**, 484–488; (*c*) M. C. Posewitz and P. Tempst, *Anal. Chem.*, 1999, **71**, 2883–2892; (*d*) P. Cao and J. T. Stults, *Rapid Commun. Mass Spectrom.*, 2000, **14**, 1600–1606; A. Stensballe, S. Andersen and O. N. Jensen, *Proteomics*, 2001, **1**, 207–222; (*e*) C. S. Raska, C. E. Parker, Z. Dominski, W. F. Marzluff, G. L. Glish, R. M. Pope and C. H. Borchers, *Anal. Chem.*, 2002, **74**, 3429–3433; (*f*) Y.-C. Li, Y.-S. Lin, P.-J. Tsai, C.-T. Chen, W.-Y. Chen and Y.-C. Chen, *Anal. Chem.*, 2007, **79**, 7519–7525.

3 (*a*) F. Wolschin, S. Wienkoop and W. Weckwerth, *Proteomics*, 2005, **5**, 4389–4397; (*b*) H. K. Kweon and K. Hakansson, *Anal. Chem.*, 2006, **78**, 1743–1749; (*c*) C.-Y. Lo, W.-Y. Chen, C.-T. Chen and Y.-C. Chen, *J. Proteome Res.*, 2007, **6**, 887–893; (*d*) C.-T. Chen and Y.-C. Chen, *Anal. Chem.*, 2005, **77**, 5912–5919.

4 C.-T. Chen and Y.-C. Chen, *J. Biomed. Nanotechnol.*, 2008, **4**, 73–79.

5 C.-T. Chen, W.-Y. Chen, P.-J. Tsai, K.-Y. Chien, J.-S. Yu and Y.-C. Chen, *J. Proteome Res.*, 2007, **6**, 316–325.

6 (*a*) S.-J. Chen and H.-T. Chang, *Anal. Chem.*, 2004, **76**, 3727–3734; (*b*) Y. S. Wang and B. Liu, *Anal. Chem.*, 2007, **79**, 7214–7220; (*c*) M. Schuttpelz, C. Muller, H. Neuweiler and M. Sauer, *Anal. Chem.*, 2006, **78**, 663–669; (*d*) A. Ojida, M. Inoue, H. Tsutsumi, K. Sada and I. Hamachi, *J. Am. Chem. Soc.*, 2006, **128**, 2052–2058; (*e*) T. Anai, E. Nakata, Y. Koshi, A. Ojida and I. Hamachi, *J. Am. Chem. Soc.*, 2007, **129**, 6232–6239.

7 F. R. Rickles, R. L. Edwards, C. Barb and M. Cronlund, *Cancer*, 1983, **51**, 301–307.

8 M. P. A. Ebert, D. Niemeyer, S. O. Deininger, T. Wex, C. Knippig, J. Hoffmann, J. Sauer, W. Albrecht, P. Malfertheiner and C. Rolcken, *J. Proteome Res.*, 2006, **5**, 2152–2158.

9 (*a*) A. Gadducci, U. Baicchi, R. Marrai, B. D. Bravo, F. P. Vittorio and V. Facchini, *Gynecol. Oncol.*, 1994, **53**, 352–356; (*b*) Y. Ogata, C. J. Hepplmann, M. C. Charlesworth, B. J. Madden, M. N. Miller, K. R. Kalli, W. A. Cilby, H. R. Bergen III, D. A. Saggese and D. C. Muddiman, *J. Proteome Res.*, 2006, **5**, 3318–3325.

10 (*a*) C. G. Binnie, J. M. Hettasch, E. Strickland and S. T. Lord, *Biochemistry*, 1993, **32**, 107–113; (*b*) M. C. Maurer, J.-L. Peng, S. S. An, J.-Y. Trosset, A. Henschen-Edman and H. A. Scheraga, *Biochemistry*, 1998, **37**, 5888–5902.

11 A. H. Merrill Jr, J. A. Froehlich and D. B. McCormick, *Biochem. Med.*, 1981, **25**, 198–206.

