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Biosensors and Bioelectronics 23 (2008) 980–986
BIOSENSORS & BIOELECTRONICS
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Protein kinase assay on peptide-conjugated gold nanoparticles

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Received 1 August 2007; accepted 2 October 2007
Available online 12 October 2007

Abstract

We demonstrate that protein kinase can be assayed with high sensitivity on peptide-conjugated gold nanoparticles (AuNPs). Phosphorylation of peptides on the AuNP-monolayers was detected by using an anti-phosphotyrosine antibody (α-pY) and Cy3-labeled secondary antibody (Cy3-α-mIgG) as a probing molecule. When compared to conventional self-assembled monolayers (SAMs), spherical and three-dimensional geometry of AuNPs led to high surface density of peptide substrate and easy accessibility to enzyme, and consequently the resulting AuNP monolayers gave rise to improved detection sensitivity. Blocking of peptide-conjugated AuNPs with a poly(ethylene glycol) (PEG) also contributed to a higher signal-to-background ratio in kinase and its inhibition assays. The use of AuNPs as the platform surface will enable highly sensitive detection of protein kinases in a high-throughput manner.

Keywords: Protein kinase; Phosphorylation; Gold nanoparticles; Peptide; Fluorescent assay

1. Introduction

Protein kinases play a key role in various cellular functions (Zolnierowicz and Bollen, 2000), and have been the targets for investigation of specific signaling pathways as well as for development of therapeutic drugs (Daucey and Sausville, 2003; Shults et al., 2005). Much efforts have been made to develop a method to assay these enzymes (Olive, 2004), but more sensitive and simple detection of kinase activity is still needed to facilitate the kinase-related studies. In particular, chip-based assay of protein kinase using peptide substrate has been of great interest due to the possibility of high throughput analysis as well as functional stability and facile synthesis of the peptide substrates (Reimer et al., 2002). To implement this assay format to a practical purpose, improved immobilization methods of peptides with more controlled density on a chip surface are still required. Over the past decades, self-assembled monolayers (SAMs) have been widely employed as the platform surface (Houseman et al., 2002; MacBeath et al., 1999). However, SAMs-based approaches still face several challenges, such as intricate derivatization steps, low surface density of substrate, and decreased enzyme accessibility caused by surface-confined immobilization of the substrate, which results in a low sensitivity in kinase assay. To enhance the sensitivity of kinase assay, high spatial density of peptide substrates and minimization of lateral steric hindrance on a solid surface are crucial.

In recent, gold nanoparticles (AuNPs) have attracted much attention as a platform surface to accomplish the sensitive detection in various biochip formats (Daniel and Astruc, 2004). However, despite the diverse roles of AuNPs including the electronic and surface plasmonic sensing systems (Shipway et al., 2000), there have been few studies about the use of AuNPs as an interfacial layer in a conventional fluorescence-based detection. Similar to dendritic or entangled polymers (Frey et al., 1995; Hong et al., 2005), we previously reported that AuNPs have a greater potential as the platform surface for immobilization of biomolecules owing to their spherical and biocompatible properties (Kim et al., 2006).

Here we demonstrate protein kinase assay on monolayers of peptide-conjugated AuNPs. Phosphorylated peptides on the AuNP-monolayers were readily detected by using an anti-phosphotyrosine antibody (α-pY) and Cy3-labeled secondary antibody (Cy3-α-mIgG) as a probing molecule. The use of AuNPs, as a platform interface, resulted in enhanced sensitivity in kinase and its inhibition assays compared to conventional SAMs, and this seemed to be due to high surface density and alleviated steric hindrance of the peptide substrate. With the extended average distance between a dye and AuNPs by

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doi:10.1016/j.bios.2007.10.001

using two antibodies, AuNPs-induced quenching effect was minimized in the fluorescence-based assay format. Details are reported herein.

2. Materials and methods

2.1. Chemical reagents

All the related reagents were purchased from commercial sources: Abl tyrosine kinase (New England Biolab), monoclonal anti-phosphotyrosine antibody (α-pY, mouse IgG2b isotype from PY20, Sigma), Cy3-labeled anti-mouse immunoglobulin G antibody (Cy3-α-mIgG, sheep source, Sigma), mouse immunoglobulin G (mIgG, Sigma), staurosporine (Sigma), sulfosuccinimidyl 4-(*N*-maleimidometyl) chclohexane-1-carboxylate (SSMCC, Pierce), 11-amino-1-undecanethiol (AUT, Dojindo), methoxy-poly(ethylene glycol)-succinimidyl α-methylbutanoate (*m*PEG-SMB, MW 2000, Nektar Inc.), hydrogenetrachloroaurate(III) trihydrate (99.9% $HAuCl_4{\cdot}3H_2O$, Sigma-Aldrich), sodium citrate dihydrate (99.9%, 2-hydroxy-1,2,3-propanetricarboxylic acid trisodium salt, $C_6H_5Na_3O_7{\cdot}2H_2O$, Sigma-Aldrich), and sodium borohydride (99%, $NaBH_4$, Sigma-Aldrich). Amine-functionalized glass slide was purchased from Schott Nexterion (Slide A+, Germany). Peptide substrate for Abl kinase (Pep; Ac-IYAAPKKGGGGC) and its phosphorylated form (P-Pep; Ac-IYpAAPKKGGGGC) were synthesized by Peptron Inc. (Korea). A chambered silicon coverslip (50 wells, 3 mm × 1 mm, sterile) was from Sigma-Aldrich.

2.2. Synthesis of AuNPs

AuNPs were synthesized by a citrate-stabilization method as described elsewhere (Grabar et al., 1996). Briefly, 100 μL of a stock solution containing 300 mM $HAuCl_4{\cdot}3H_2O$ was added to 100 mL distilled water to give a final concentration of 300 μM followed by vigorous stirring for 1 min. To this solution, 1.5 mL of 30 mM sodium citrate dihydrate was added at a final concentration of 450 μM (the molar ratio of tetrachloroaurate to sodium citrate = 1:1.5), and stirred for 2 min. For fast reduction and formation of gold colloids, 100 μL of a stock solution containing 300 mM $NaBH_4$ was quickly added to the reaction solution followed by stirring for 5 min. The completely reduced solution was then stored at 4 °C. The clustering of AuNPs was checked by UV–vis spectroscopy (UV-2550, Shimadzu), and the average size of AuNPs was estimated to be 10.7 ± 2.7 nm according to an field emission scanning electron microscope (FE-SEM, Sirion, FEI, Netherlands).

2.3. Construction of AuNP-monolayers

AuNP-monolayers were prepared in wells formed by overlaying a chambered silicon coverslip (50 wells, 3 mm in diameter and 1 mm in height) on an amine-functionalized glass slide. A 10 μL solution of AuNPs was added to the wells, followed by incubation for 30 min on the glass surface. The wells were then washed with distilled water three times, and then dried with nitrogen gas. As a control surface without AuNPs, a 10 μL of 2 mM SSMCC dissolved in 50 mM sodium bicarbonate buffer (pH 8.5) was added to the wells on an amine-glass surface, followed by incubation for 30 min, and then dried with nitrogen gas. Cysteine-terminated peptides were employed for conjugation to the AuNP layers or the SSMCC-modified SAMs. Peptides dissolved in 10 mM phosphate buffer saline (PBS pH 7.4) were directly attached onto AuNPs in wells by incubation for 60 min, followed by thoroughly washing with distilled water and drying under a stream of N_2. The final concentration of single peptide or a mixture of two peptides (Pep and P-Pep) was 50 μg mL^{-1}. Afterwards, *m*PEG-SMB dissolved in a 50 mM bicarbonate buffer (pH 8.5) was added to the wells at a final concentration of 2 mM in order to block the remaining amine groups, and rinsed with water and dried.

2.4. Kinase reaction

Typically, the kinase reaction was initiated by adding 10 μL of a reaction mixture containing 10 nM Abl, 150 μM ATP and 30 mM $MgCl_2$ to the wells of a chambered silicon coverslip on a glass slide. Kinases and ATP were dissolved in 50 mM Tris buffer (pH 7.5, containing 0.05 mM EDTA, 0.015% Tween-20, and 0.1 mg mL^{-1} BSA). For a quantitative assay of kinase, the varied concentrations of Abl kinase were tested with 150 μM ATP. For an inhibition assay, inhibitor (staurosporine) dissolved in DMSO was diluted to appropriate concentration in distilled water, and added to the reaction mixture. Kinase reaction was carried out at 30 °C for 60 min in a reaction chamber with a tight sealing to prevent evaporation of water. After reaction, the wells were rinsed with PBS buffer and distilled water subsequently followed by drying under a stream of N_2. Primary and secondary antibodies were dissolved in 10 mM PBS (pH 7.4) at a final concentration of 10 μg mL^{-1}, and consecutively added to wells and incubated for 60 min with the washing and drying steps. Following a removal of a silicon coverslip, the resulting surfaces were subjected to a fluorescence scanning.

2.5. Quartz crystal microbalance (QCM) analysis

A QCM measurement was performed by using a Q-Sense D300 system (Q-Sense AB, Sweden). A gold-coated AT-cut quartz crystal (Sensor Crystal QSX 301) was cleaned and modified with AUT SAMs according to the procedures of SPR gold chip. The gold chip was then modified either with AuNPs or with SSMCC to compare the adsorption of peptide. For measurements, the crystal was mounted in a thermal static liquid chamber, and the stable base line on the surface was then confirmed during the pre-washing step with 10 mM phosphate buffer saline (PBS pH 7.4). Adsorption of peptide (Ac-IYAAPKKGGGGC) was conducted by passing a 1 mL solution (50 μg mL^{-1} in PBS) of the peptide over the surface at room temperature, and then sequentially returned to PBS buffer for 5 min. Time-course changes in the resonant frequency (Δf) were traced at the fundamental resonant frequency (5 MHz).

2.6. Surface plasmon resonance (SPR) spectroscopy

SPR analysis was carried out using a BIAcore-X instrument and gold sensor chips (BIAcore). The chip surface was first cleaned with 0.1 N NaOH containing 0.1% Triton-X for 5 min. For the formation of SAMs, the sensor chips were immersed in a 2 mM ethanolic solution of AUT for 2 h, followed by a thorough rinsing with absolute grade ethanol and distilled water. The modification of a gold chip followed the procedures in order of AuNPs adsorption, peptide conjugation, and PEG coating as described above. As another control, a gold sensor chip was prepared without adsorption of AuNPs by the modification of SSMCC, peptide conjugation, and PEG blocking. Subsequently in both surfaces, Abl kinase reaction was carried out for 35 min at 30 °C by flowing a reaction mixture over the two channels at a flow rate of 1 μL min^{-1}. Afterward, 10 mM PBST containing 0.05% Tween-20 (pH 7.4) was flowed for 10 min at a flow rate of 2 μL min^{-1} at 25 °C. Of two channels, one channel was used to check a specific binding capacity by injecting the solution of α-pY (10 μg mL^{-1} in PBST) over the phosphorylated surfaces for 30 min. Mouse IgG (10 μg mL^{-1} in PBST) was also injected over the second channel as a control. Following a final rinsing with PBST, changes in the refractive index unit (RU) were traced.

2.7. Fluorescence readout

Cy3-specific fluorescence intensities were read out by using a fluorescence scanner (GenePix Personal 4100 A; Axon instruments, Union City, CA, USA), and analyzed by using a GenePix Pro 4.1 software provided by the manufacturer. Intensities of Cy3 taken from all spots were corrected for background intensity, and their mean values were collected and normalized to the intensity obtained when only the phosphorylated peptides had been immobilized.

3. Results and discussion

The principle of protein kinase assay is illustrated in Fig. 1. Monolayers of gold nanoparticles (AuNPs) were constructed on a glass slide as a platform surface (Fig. 1a). Amino groups of an APTES-coated glass surface were strongly associated with the negatively charged AuNPs that had been synthesized by a citrate-stabilized method, resulting in the formation of AuNP-monolayers. As a control, conventional self-assembled monolayers without AuNPs were compared under the similar conditions (Fig. 1b). As a substrate for tyrosine Abl kinase, cysteine-terminated peptides (Ac-IYAAPKKGGGGC) were preferentially oriented on surfaces by conjugation onto the AuNPs via a thiol bond (Fig. 1a), or by covalently binding to the amino group of the glass surface using a heterofunctional linker of SSMCC (Fig. 1b). To endow the effective accessibility of protein kinase, a glycine linker was also introduced to the C-terminal of the peptide sequence. In both cases, amine-reactive poly(ethylene glycol) (PEG, specifically *m*PEG-SMB, Nektar) was used to block the remaining amino groups on the glass surface. Unlike large blocking agents such as bovine serum albumin or casein, we reasoned that PEG (ca. 1–2 nm in diameter) would have a more efficient non-fouling effect in our system as reported elsewhere (Wegner et al., 2004). Since the exposed area of surface-attached AuNPs was preoccupied by peptide substrate, there seems to be little interactions between the PEG and AuNPs. Furthermore, the Fourier transform-infrared (FT-IR) spectroscopy revealed that the PEG was more preferentially

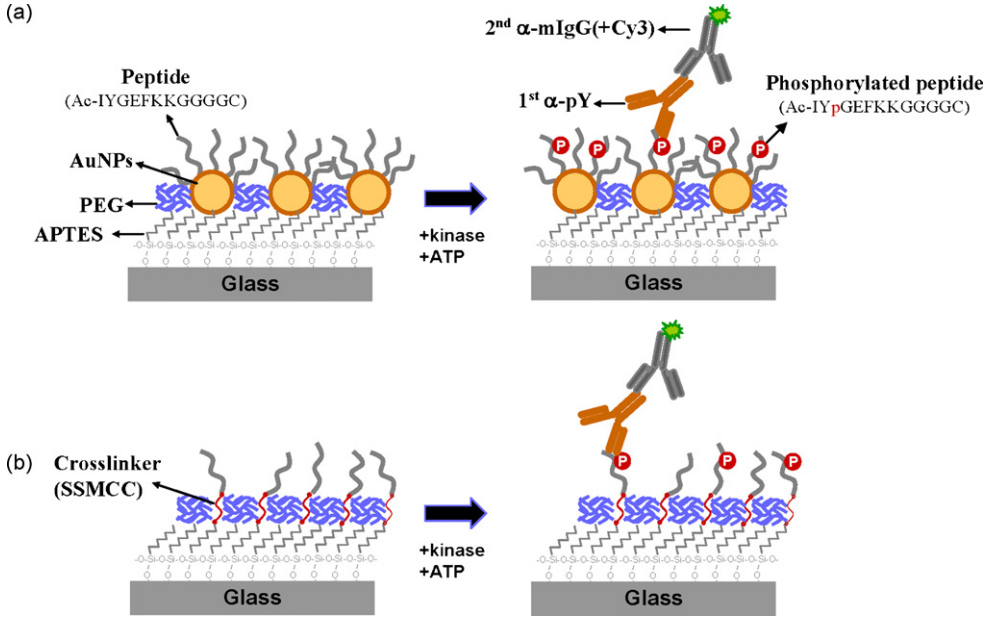

Fig. 1. Assay principle of protein kinase using an anti-phosphotyrosine antibody (α-pY) and a subsequent secondary antibody (Cy3-α-mIgG) on different peptide-conjugated monolayers: (a) monolayers of gold nanoparticles (AuNPs), and (b) SSMCC-modified self-assembled monolayers (SAMs) on glass. SAMs was used as a control surface against AuNP-monolayers. Cysteine-tethered peptide at its C-terminal (Ac-IY-EFGKKGGGGC) was oriented on both surfaces, and an amine-reactive poly(ethylene glycol) (PEG) was used to block the remaining amine groups on a glass surface.

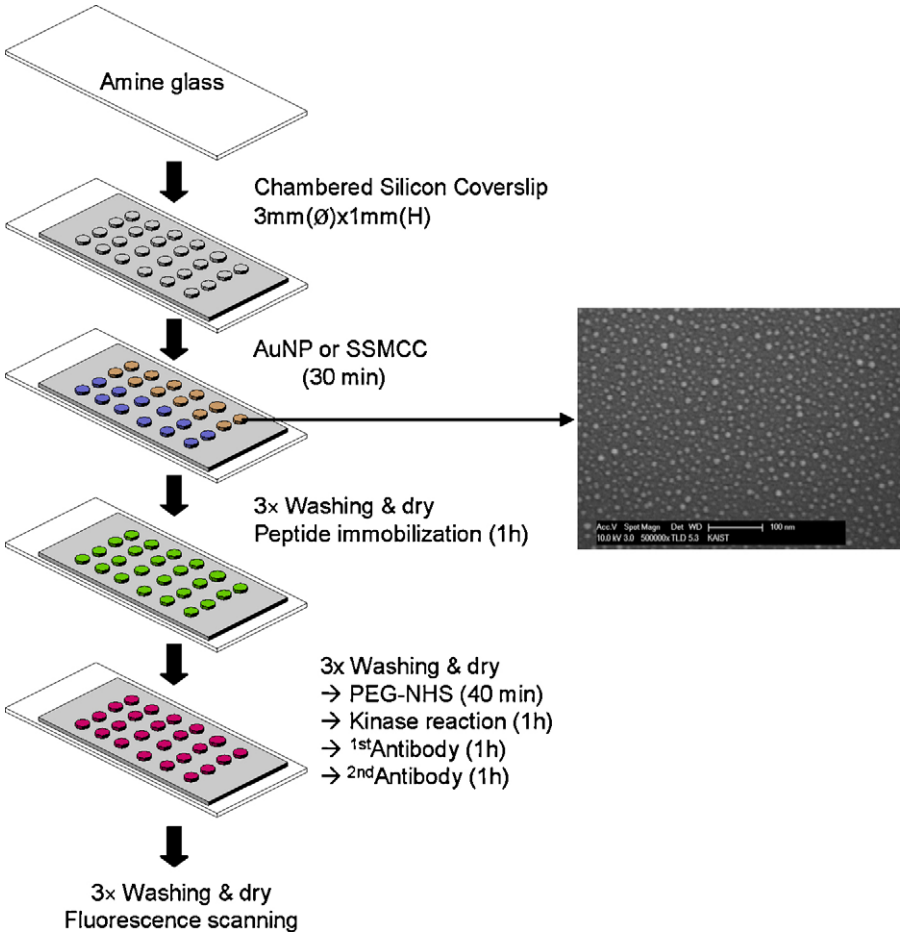

Fig. 2. Schematic procedure of chip-based fluorescence detection for phosphorylation. AuNP-monolayers and SSMCC-modified SAMs were constructed on a glass slide. The homogeneous distribution of AuNPs with a size of 10 nm on an amine-functionalized glass surface was imaged by using FE-SEM.

conjugated with the amino groups on SAMs, rather than with the lysine residues of peptides (data not shown). Following a Abl kinase reaction, phosphorylation of surface-attached peptides was probed by using an indirect antibody method; that is, phosphorylation of tyrosine residue was recognized by anti-phosphotyrosine antibody (α-pY) and subsequent secondary Cy3-labeled antibody (Cy3-α-mIgG). According to the above procedure, chip-based kinase assay was carried out by overlaying a chambered silicon coverslip onto the glass surface, as depicted in Fig. 2. In the case of AuNP monolayers, homogeneous distribution of AuNPs was confirmed from the FE-SEM images in Fig. 2.

To test the specificity of antibody-based fluorescence detection for phosphorylated peptides, various ratios of Abl peptide substrate (Pep; Ac-IYAAPKKGGGGC) and its synthetic phosphorylated form (P-Pep; Ac-IYpAAPKKGGGGC) were quantified without kinase reaction by capturing a phosphotyrosine-specific antibody on different surfaces. Phosphorylation efficiency was calculated from the normalization to the signal that had been obtained with 100% phosphorylated peptide. In the case of AuNP-monolayers, fluorescence intensities by primary α-pY and secondary Cy3-α-mIgG significantly increased with the increase of the relative amount of P-Pep, resulting in a linear correlation between the amount of phosphorylated peptides and phosphorylation efficiency (Fig. 3a and c). In contrast, direct conjugation of peptides onto SSMCC-modified SAMs displayed a much less increase in phosphorylation efficiency even with the increase of P-Pep. The extent of non-specific protein binding was significantly reduced on AuNPs by the treatment with PEG, compared to those on SAMs and AuNPs with no PEG treatment (Fig. 3b). This result indicates that AuNP-monolayers with PEG treatment enabled a more sensitive assay of protein kinase than conventional SAMs-based format. Feasible reason for this superior property might stem from high surface density of peptides and less steric hindrance of antibody on AuNP-monolayers. We previously reported that surface-attached AuNPs induce a high loading capacity of peptides and easy accessibility of enzyme due to their globular structure, when compared to a flat bare gold surface (Kim et al., 2006). In order to get some insight into the surface density of peptides on AuNPs, we determined the amount of peptide substrates bound to the AuNP-modified crystal by using a quartz crystal microbalance (QCM). The resulting frequency shift (Δf) of peptides represented −3.6 Hz (solid line, Fig. 4), which can be easily converted to a gain in mass of ∼64 ng cm^{-2} (equal to be ∼59 pmol cm^{-2}) by taking into account a mass change of 17.7 ng cm^{-2} per −1 Hz. In contrast, the surface density of peptides on SSMCC-modified SAMs (−1.7 Hz, equal

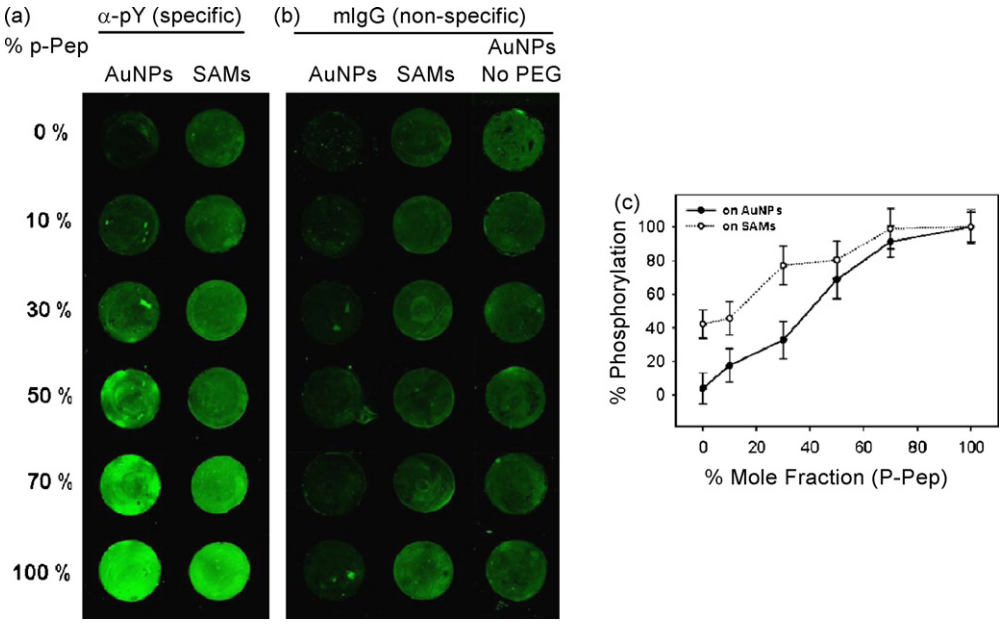

Fig. 3. Fluorescent images by (a) specific monoclonal antibody (α-pY) and (b) non-specific antibody (mIgG) at varied ratios of Abl peptide (Pep; Ac-IYAAPKKGGGGC) to its phosphorylated one (P-Pep; Ac-IY(p)AAPKKGGGGC) on the AuNP-monolayers and SAMs. The fluorescence intensity was obtained by using a secondary Cy3-labeled antibody. (c) Fluorescence intensity shown in (a) was converted to the phosporylation efficiency and represented with respect to a mole fraction of phosporylated peptide. Phosphorylation efficiency was calculated by normalizing the respective spot intensity to that obtained at the maximum phosphorylation efficiency.

to be ∼26 pmol cm^{-2}) was ∼2-fold lower (dotted line, Fig. 4) than that on AuNPs. Thus, this result strongly implies that three-dimensional structure of AuNPs provides more effective binding sites for peptides than SAMs.

For a quantitative analysis of phosphorylation by protein kinase on different surfaces, phosphorylation of peptides by kinase reaction was examined on the AuNP-modified and SAM-modified surfaces by using a surface plasmon resonance (SPR) spectroscopy. Specific and non-specific binding were monitored by flowing α-pY and mIgG over respective surface, respectively. Prior to flowing the antibodies, the surface-tethered peptide substrate had been phosphorylated by flowing a solution containing Abl kinase and ATP. Changes in RU by specific (α-pY) and non-specific antibodies (mIgG) on the AuNP-monolayers were estimated to be 3400 RU and 490 RU, respectively (Fig. 5a), whereas those on the SSMCC/SAM-modified chip were about 1630 RU and 530 RU, respectively (Fig. 5b). This result indicates that the AuNP-monolayers gave rise to more efficient phosphorylation with less steric hindrance compared to the flat SAM, resulting in a much higher binding of specific antibody. We further tested the AuNP-monolayers on a glass slide for assay of protein kinase and its inhibition. As shown in Fig. 5c, strong fluorescence intensity was observed from a spot by addition of protein kinase, and the phosphorylation efficiency reached about 80%, which is well coincident with the result by SPR. However, addition of kinase inhibitor (1 μM staurosporine) caused a remarkable reduction in fluorescence intensity. By blocking the remaining surface by PEG, the resulting surface displayed an improved signal-to-background ratio due to the minimized background noise.

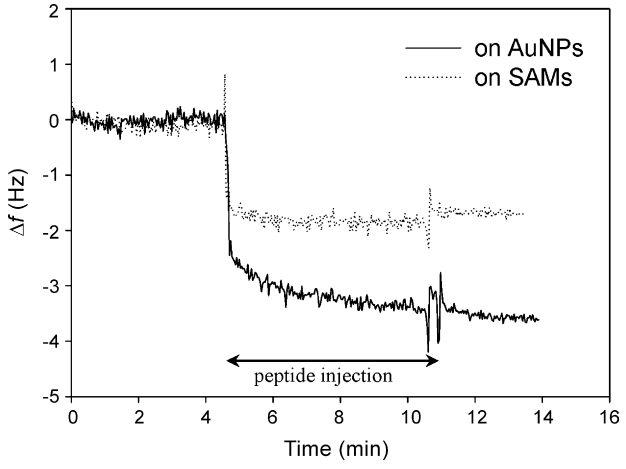

Fig. 4. QCM response of Pep (Ac-IYAAPKKGGGGC) attached on AuNPs (solid line) and SAMs (dotted line). Changes in frequency were traced in a 5 MHz crystal. Change in 1 Hz corresponds to a mass change of 17.7 ng cm^{-2} at a fundamental frequency ($f_0 \approx 5$ MHz).

Sensitivity of protein kinase assay on the AuNP-monolayers was checked by measuring the phosphorylation efficiency with respect to the kinase concentration in the absence of an inhibitor. As can be seen Fig. 6a, the phosphorylation efficiency increased with the increasing kinase concentration ranging from as low as 50 pg mL^{-1} to 10 ng mL^{-1}. Detection sensitivity of kinase assay was about 50 pg mL^{-1} (corresponding to 1 pM), which is much lower than the reported level of 1 ng mL^{-1} (Rodems et al., 2002). In the case of inhibitor assay, the phosporylation efficiency was determined as a function of the inhibitor concentration. A dose-dependent decrease in kinase activity was clearly observed (Fig. 6b), and IC_{50} value of staurosporine was

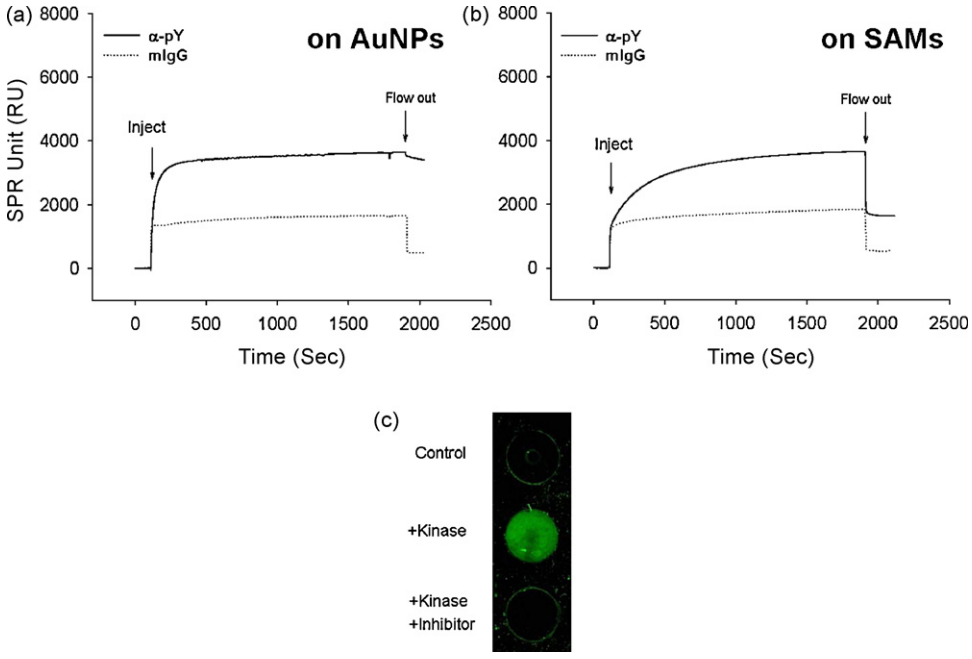

Fig. 5. Analyses of phosporylation by SPR and fluorescence measurement on different surfaces. (a) Changes in sensorgram by binding of specific antibody (α-pY) and non-specific antibody (mIgG) on AuNP-monolayers (b) changes in sensorgram on SAMs. Antibody binding was traced after kinase reaction on respective surfaces containing a peptide substrate. (c) Chip-based assay of protein kinase and its inhibition using the fluorescence measurement on AuNP-monolayers. Non-specific binding of proteins was minimized by PEG treatment, and the concentration of inhibitor was 1 μM.

estimated to be 61 nM, which is comparable to that in solution (Rodems et al., 2002). As the distance between dye and AuNPs is closer, the fluorescence quenching of dye can occur due to the electromagnetic field of metal surface. However, the Cy3 was sufficiently away from the surface of metallic AuNPs, owing to a vertical orientation of two consecutive antibodies. Each antibody molecule is assumed to have a size of more than 12 nm (plus 3–4 nm for peptide sequences attached onto AuNPs) (Marquart et al., 1980). Therefore, quenching of fluorescent dye by energy-transfer between dye and AuNPs might be insignificant. It has been suggested that long separation distance (>20 nm) between dye and metal surface causes negligible quenching (Yu et al., 2003; Yun et al., 2005).

Based on the results, it is evident that the peptide-conjugated AuNP-monolayers enabled simple and sensitive assay of protein kinase and its inhibition on a chip surface without the necessity of intricate derivatization steps (MacBeath et al., 1999) or laborious radioactive labeling on the conventional SAMs (Houseman et al., 2002). When compared to the bio-interfaces such as dendritic polymers or hydrogels, the monolayer of AuNPs provides a stable and homogeneous binding site for peptides, consequently resulting in enhanced sensitivity in a chip-based kinase assay. Moreover, AuNP-based chip surface can be easily prepared and applied to various solid substrates (glass, Si, Au, etc.) and biomolecues (DNA, aptamer, peptide, antibody, and other proteins), offering a possibility of a high-throughput analysis. Especially by controlling the size and density of AuNPs, the detection sensitivity of phosphorylation on the peptide-tethered AuNP monolayers might be further improved.

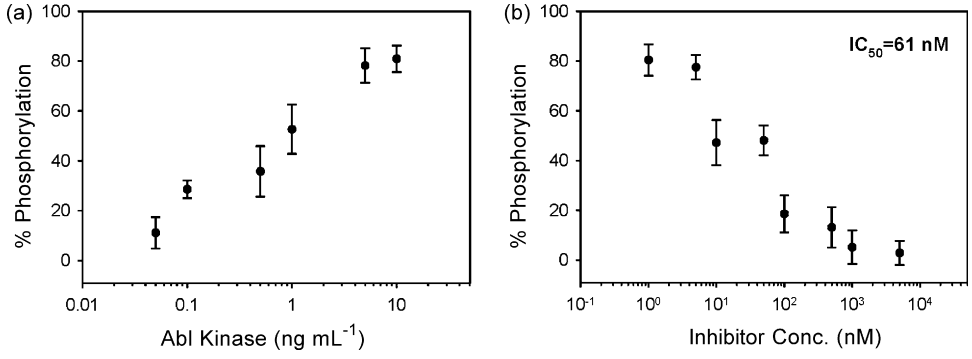

Fig. 6. (a) Change in phosphorylation efficiency as a function of Abl kinase concentration. (b) Inhibition assay of Abl kinase. Phosphorylation efficiency was determined at different concentrations of staurosporine. The concentration of Abl kinase was fixed at 10 ng mL^{-1}. The error bar indicates the standard deviation ~ in duplicate experiments.

4. Conclusion

We have demonstrated that protein kinase and its inhibition can be assayed with high sensitivity on peptide-conjugated gold nanoparticles (AuNPs). With construction of the AuNP-monolayers on an amine-functionalized glass surface and poly(ethylene glycol) (PEG) treatment, the enhanced signal-to-background ratio for phosphorylation was obtained. In contrast to conventional self-assembled monolayers, the AuNP-monolayers generated higher density of peptide substrates and easier accessibility of protein kinase, due to the spherical and three-dimensional geometry on surfaces, resulting in highly sensitive assay of protein kinase and its inhibition. Our approach will open a route to high quality assay of protein kinases as well as other enzymes in a high throughput manner.

Acknowledgments

This work was supported by the Nano/Bio Science & Technology Program (M10503000218-05M0300-21810) and the Nano Science & Technology Program (M10536090002-07N3609-00210) of MOST, the Korea Health 21C R&D Project (0405-MN01-0604-0007) of MHW, and the Next-Generation New-Technology Development Program for MOCIE.

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