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Cell kinase activity assay based on surface enhanced Raman spectroscopy

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ABSTRACT

Kinases control many important aspects of cell behavior, such as signal transduction, growth/differentiation, and tumorogenesis. Current methods for assessing kinase activity often require specific antibodies, and/or radioactive labeling. Here we demonstrated a novel detection method to assess kinase activity based on surface enhanced Raman spectroscopy (SERS). Raman signal was obtained after amplification by silver nanoparticles. The sensitivity of this method was comparable to fluorescence measurement of peptide concentration. When purified kinase enzyme was used, the detection limit was comparable to conventional radio-labeling method. We further demonstrated the feasibility to measure kinase activity in crude cell lysate. We suggested this SERS-based kinase activity assay could be a new tool for biomedical research and application.

1. Introduction

Protein kinases are key players in many aspects of cell activities such as growth, differentiation, migration, and in response to external stimuli [1–3]. Protein kinases also play critical roles in tumorogenesis, thus they have become important targets for drug development [4,5]. Kinase activity involves structural modification of substrate molecules by covalently conjugating a phosphate group. This structural change can be detected by radioactive labeling, or by antibody recognition. Current practice to monitor cell kinase activity are often time consuming and labor intensive. Methods involving radioactive material labeling and/or antibodies usually take 1–2 days to accomplish. Therefore, a simple, quick method to detect kinase activity is highly desired.

Raman spectroscopy allows direct monitoring of structural changes in macromolecules without further labeling/modification or denaturing of the target. However, this method is usually not sensitive enough for biological systems. Various methods of enhancement were developed to expand the detection limit. Among these, enhancement with heavy metal colloid nanoparticles (termed surface enhanced Raman scattering, SERS) was particularly interesting. With this method, probing single molecules absorbed onto a single silver nanoparticle or a single carbon nanotube was achieved [6–8]. The sensitivity of this method has been shown to be similar to that of the fluorescent detection method for the R6G molecule [6]. In this previous work, the amplification magnitude of SERS was estimated to be as high as 10^{14}.

Through different enhancement methods, the possibility of differentiating phosphorylated and non-phosphorylated peptides was explored recently [9–12]. Using synthetic, pure peptides at high concentration, characteristic Raman spectra on phospho-serine and phospho-tyrosine were reported [9,10]. However, a real test on kinase activity has not been reported. One possible hurdle could be that it is technically difficult to reach an ideal platform that combines signal amplification and the biological nature of kinase reaction.

Here we extended this line of research by directly measuring kinase activity via SERS. We wish to combine the highest sensitivity reported on SERS (i.e. single molecule detection), with a flexible platform that is suitable for biological molecular reaction. To this end, we employed a silver nanoparticle approach with enzyme substrate attached to the surface, and enable enzyme reaction in the liquid/solid interface. We first demonstrated the feasibility of this method using purified kinase in an in vitro reaction system. We then showed that it is also possible to measure kinase activity in crude cell lysate. Thus a SERS-based detection method can be readily applied to measure kinase activity in real biological system.

2. Materials and methods

2.1. Silver nanoparticle (NP) preparation

Silver NPs were prepared as described previously [13,14]. In brief, silver nitrate was precipitated by potassium hydroxide, and

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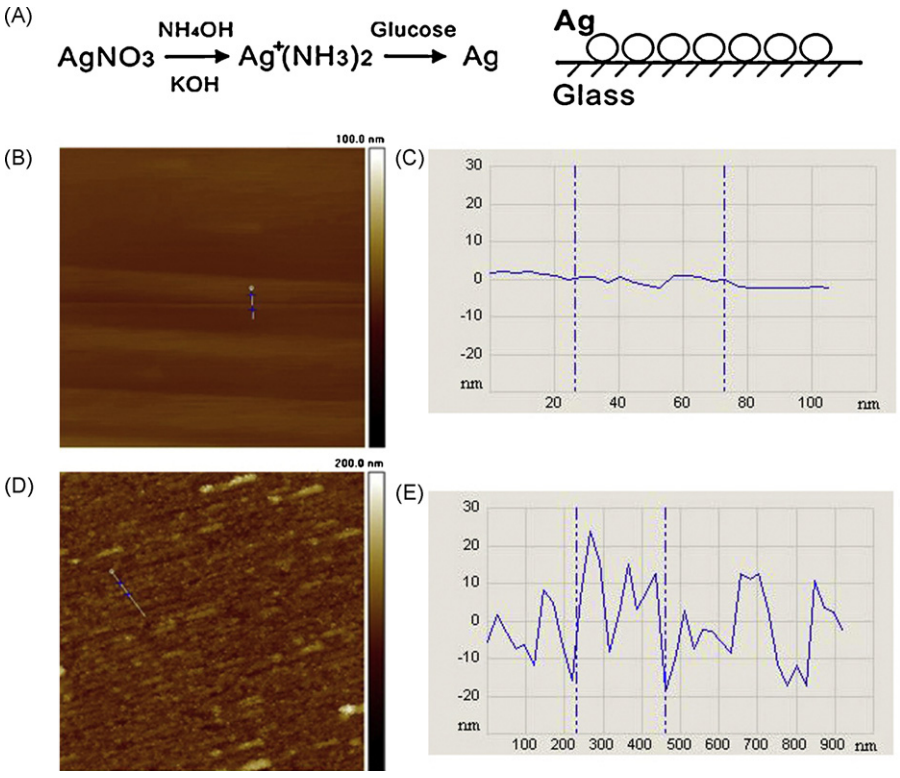

Fig. 1. AFM analysis of silver nanoparticles (NPs) deposited on glass surface. (A) Diagram showing the chemical reaction for silver colloid production and deposition on glass surface. (B–E) AFM characterization of silver NPs on glass surface. B and C, glass surface; D and E, silver NPs on glass. Width is about 100 nm, and height is about 30 nm.

dissolved by ammonium hydroxide. This "active" silver solution was then reduced by glucose to produce a silver colloid solution. Silver NPs were deposited onto a clean glass surface. We found that a short deposition time of about 15–25 s was sufficient to produce a thin layer of silver NPs.

2.2. AFM characterization

Atomic force microscopy (AFM) was used to characterize the morphology of the silver NP layer. Measurements were taken in air under tapping mode with a VEECO Dimension 3100 Atomic Force Microscope (Veeco Instruments Inc., USA), using RTESP silicon cantilever probes (Veeco Probes, CA, USA) (nominal spring constant 40 kN/m, resonance frequency 300 kHz) at a scan rate of 1 Hz on an area of 5 μm × 5 μm with a resolution 256 × 256 pixels. Images were captured with Nanoscope 6.12r2 software (Veeco Instruments Inc., USA) without further processing.

2.3. Raman detection and data processing

Raman spectra were acquired using a Renishaw in Via Raman microscope. Spectral data were acquired with excitation at 633 nm (for FITC-peptide) or 532 nm (for other peptides) and 200 mW, using Renishaw v.1.2 WiRE software coupled with Grams/AI (Thermo Galactic, USA). Spectral data were further processed with 5-point averaging in Origin software to reduce noise without changing the peak positions.

2.4. In vitro kinase reaction

Purified Akt and Jak3 kinases were purchased from Upstate Biotechnology Inc. The substrate peptides, Akt-tide (CGGGRPRTSSFAEGKK) and Jak3-tide (CGGGGEEEEYFELVKKKK) were synthesized by GeneScript Inc. One extra cysteine residue together with two glycine residues was added to the N-terminus for anchoring purposes. The FITC conjugated peptide EMP17 FITC-LC labeled (FITC-LC-TYSCHFGPLTWVCKPQGG) was purchased from Anaspec Inc. In vitro kinase reactions were setup as instructed by the manufacture (Upstate Biotech). Briefly, in a 25 μl reaction, 5 μl 5× reaction buffer (40 mM MOPS/NaOH pH 7.0, 1 mM EDTA), 10 μl 2.5× ATP cocktail (25 mM MgAc and 0.25 mM ATP), 5 μl water, and 5 μl enzyme solution or cell lysate were added. The enzyme or cell lysate was diluted with TE buffer (10 mM Tris pH 8.0). Kinase reactions were incubated for 10 min at 30 °C. Reactions were stopped by wash with DI water for three times.

2.5. Cell transfection and lysis

Cell transfection and lysis were described previously [24]. C2C12 myoblasts were cultured and transfected with a constitutively active Akt together with EGFP plasmid (3:1). After 2 days, 30–40% cells were GFP positive. Cells were then lysed and Akt expression level was monitored by western blot as described.

3. Results and discussion

3.1. Silver nanoparticle preparation and characterization

In order to construct a versatile platform for the purpose of detecting kinase activity, we used silver nanoparticles as a carrier for kinase substrates. This offers several advantages. First, silver NPs can readily be made and deposited on glass slides [13,14]. The size of NPs can also be controlled. Second, to measure different kinases, the different substrates can be selectively conjugated to the silver NPs. Moreover, by depositing different substrates on one slide, we can also achieve a "kinase substrate array" for high throughput measurements. Third, the reaction system is on a glass slide, which makes it highly flexible, easy to operate and minimizes reagent consumption.

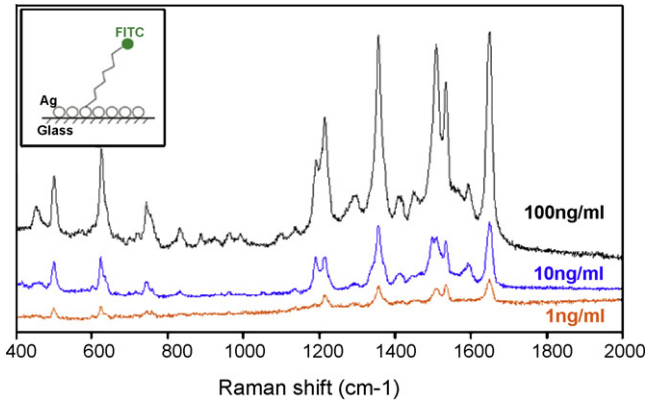

Fig. 2. FITC-peptide coupled to silver NPs. The peptide, EMP17 FITC-LC labeled (FITC-LC-TYSCHFGPLTWVCKPQGG) was linked to the silver NPs through a thiol group in the cysteine residues. The concentration of peptide range from 100 to 1 ng/ml. Due to the strong absorption pattern of the FITC molecule, vibrational patterns from the peptide residues were obscured. The integration time is 15 s.

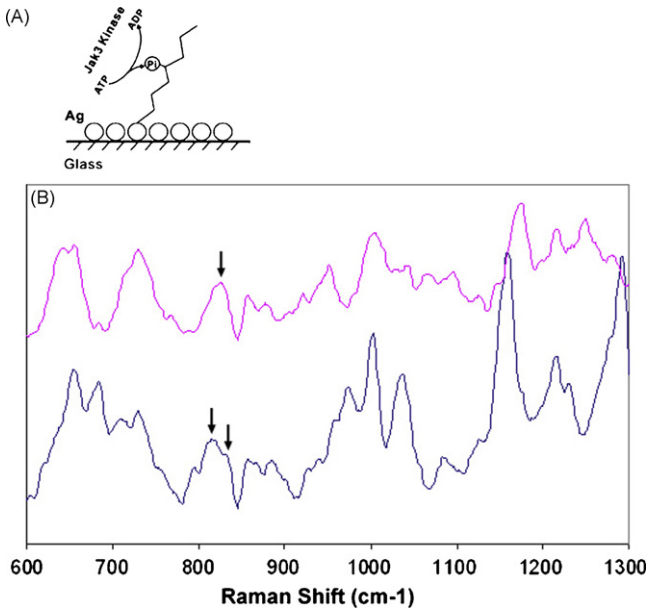

Fig. 3. Detection of purified Jak3 kinase in an in vitro kinase assay. Jak3 kinase is a tyrosine kinase. (A) Diagram showing the in vitro kinase reaction, where the kinase catalyzed the transfer of the phosphate group onto the peptide chain from ATP molecules. (B) The Raman shift of control (blue, no enzyme) and experiment (red, 5 ng/ml Jak3 kinase enzyme). Notice the characteristic collapse of 830 and 815 doublets after kinase reaction (arrows). This detection limit was similar to a radio-labeling method (1–5 ng/ml). The integration time is 5 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Previous work suggested that silver NPs sized 10–100 nm can be readily used to enhance Raman signal. A Raman "hot-spot" could be a single NP, a group of NPs, or rod-shaped [6]. In our preparation, silver colloid made from silver nitride was deposited on glass slides after a reduction by glucose. By controlling the deposition timing, we could reach a mostly homogenous coating on the glass surface. AFM analysis revealed the silver NPs were about 100 nm wide, and 50 nm high (Fig. 1). This coating was reasonably stable when incubated in phosphate buffer saline, washed with water and dried in air.

3.2. Measuring FITC-peptide

To determine if this silver NPs deposition on glass can be used to amplify Raman signal, we conjugated a FITC-peptide to the NPs through a thiol group in the peptide. After several washes, successful conjugation of the peptide can be easily monitored under a fluorescent microscope. After diluting an initial stock solution of 1 mg/ml by 10^6 (to 1 ng/ml), we approached the lower limit of detection using the fluorescence microscope (data not shown). When using the Raman spectroscopy method, we obtained significant signals within the dilution range between 100 and 1 ng/ml (Fig. 2). Using either 532 nm (data not shown) or 633 nm excitation wavelength, we got similar absorption patterns. Because FITC fluorescence excitation peak is at 488 nm and emission peak is at 522 nm, which are close to 532 nm, we choose to use 633 nm as the excitation wavelength. Due to the strong Raman signal from FITC, signals from peptides were obscured. Actually at the experiment condition (15 s integration time and peptide concentration range), non-labeled peptide only show base signal (data not shown). The Raman spectrum was comparable to previous reports of fluorescein molecule [15,16]. Therefore, this sensitivity is due to the FITC group in the molecule.

3.3. Measuring Jak3 kinase activity as a purified enzyme

Previous work on phosphorylation focused on the comparison between synthetic peptides with or without phosphorylation on the serine/tyrosine residues [9–12]. This was necessary for the purpose of establishing the characteristic Raman shift. Novel phenomena associated with SERS were also discovered, namely preferential amplification of specific bands due to molecular orientations. This lead to the suggestion that in SERS only the Raman shift position but not the absorption intensity was meaningful [12]. Due to the complexity of peptide structures, assigning each absorption band to a unique molecular structure is often difficult. Secondary structure may also contribute to the Raman shift and absorption intensity [10,12].

To explore if SERS can be readily used to detect kinase activity directly, we monitored Jak3 kinase activity using a purified, constitutively active enzyme and a synthetic peptide, Jak3-tide as a substrate. Jak3 kinase is a tyrosine kinase, which may play important roles in mediating Jak/Stat signal transduction [17]. Jak3-tide was synthesized with a cysteine at the N-terminus, which allowed its conjugation to the silver NPs on the glass slide. In an in vitro kinase reaction, a purified Jak3 kinase preparation was mixed with Jak3-tide substrates to catalyze the covalent attachment of a phosphate group to the tyrosine residue on the Jak3-tide substrate (Fig. 3A). The reaction was then washed and Raman spectra were taken for both modified and unmodified Jak3-tide substrates.

The covalent attachment of the phosphate group to the tyrosine residue resulted in a prominent change in the Raman spectra, exhibiting a collapse of the 848/828 cm^{-1} doublet to a single peak around 830 cm^{-1} (Fig. 3B). This is a prominent spectral signature produced by phosphorylation as previously reported [9,10]. Moreover, a red-shift in the amide III band around 1200 cm^{-1} was also observed. The peaks around 1000 cm^{-1} were assigned to phenoalanine absorption. The modification of this area was presumably due to a change in the secondary structure of the peptide after tyrosine phosphorylation. The integration time for this Raman measurement is 5 min.

The reaction system contains the Jak3 kinase at a concentration of 5 ng/ml. A method based on ^{32}P labeling also measured Jak3 kinase at a concentration of about 5 ng/ml (data not shown). Therefore, our SERS-based method is comparable to the method based on ^{32}P labeling for this kinase.

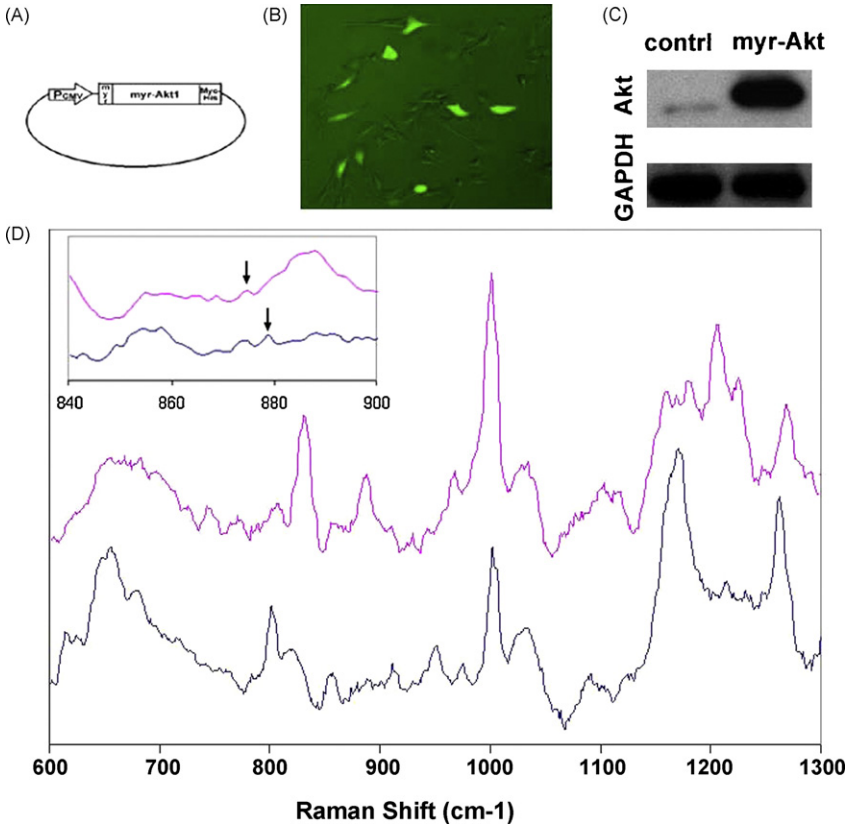

Fig. 4. Akt kinase analysis in cell lysate. Akt is a serine/threonine kinase. (A) A plasmid encoding a constitutive active form of Akt1 (Upstate Biotech) was used to transfect C2C12 cells. (B) A plasmid encoding GFP (green color) was co-transfected to show the efficiency of transfection. About 30–40% of the cells were GFP positive. (C) Cell lysate was measured for Akt expression by western blot. Equal loading was shown by GAPDH level. (D) Cell lysate from about 1000 cells in an in vitro kinase assay. Notice the characteristic peak shift of 880–875 cm^{-1} (insert: blue, control lysate; red, Akt-transfected cell lysate). The integration time is 5 min. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.4. Measuring Akt kinase activity in cell lysate

We further explored the possibility of using this method to directly measure cell kinase activity in crude cell lysate. This was particularly challenging because the lysate was a complex mixture of many proteins and small molecules. Although previous studies suggested that SERS measurements of peptide structures could be made in the presence of BSA and phosphates [12,18], a measurement in the presence of complex biological fluids that contain surfactant detergent (used for cell lysis), physiological buffer saline, and many unknown proteins/lipids from the cells has not yet been reported.

We first demonstrated that a detection method based on SERS could be used to detect the serine/threonine kinase Akt activity with a purified enzyme in an in vitro reaction system (data not shown). A constitutively active Akt kinase and a synthetic peptide substrate, Akt-tide was used. We then transfected C2C12 cells with a plasmid encoding a constitutively active Akt kinase together with an EGFP expression plasmid. After 48 h, cells were monitored for GFP expression as a measure of transfection efficiency. Approximately 30–40% of transfected cells were GFP positive when viewed under a fluorescence microscope. Cells were then lysed and the expression of the Akt kinase was confirmed by performing western blot analysis (Fig. 4A–C). For SERS detection, cell lysate from about 10^3 cells was used for each assay (Fig. 4D). The integration time we used was 5 min. The characteristic spectral shift from 880 to 875 cm^{-1} was readily observed (Fig. 4D insert). The phenoalanine absorption at 1000 cm^{-1} was unaltered. The amide III backbone vibration pattern was changed, suggesting an alternation in secondary structure after serine/threonine phosphorylation. This is close to the detection limit of the radio-labeling method, where about 100 cells could be used for an assay [19].

Altogether, these results indicated that the SERS method is very resistant to noise, even in a complex biological fluid such as the crude cell lysate. The sensitivity of this method is similar to current methods where radioactive labeling is used.

4. Discussion

In this work, we developed a novel method for the qualitative detection of protein kinase activity based on surface enhanced Raman spectroscopy. Current methods used to measure cell kinase activity often rely on phospho-specific antibody recognition, and/or radioactive labeling. This usually takes 1–2 days to complete. Using a method based on Raman spectroscopy, structural changes on the substrate molecules can be detected in much less time. This is based on the principle that the covalent conjugation of a phosphate group onto the substrate will cause changes in the molecular vibrational pattern, as shown by others [9–12] and by us here. There are several advantages associated with this method. First, it is fast and does not require further labeling or recognition of the substrate molecules. Collection of Raman spectra usually only requires a few seconds to a few minutes. No antibody or radioactive materials were needed. Second, this can potentially be a platform for high throughput analysis. Different kinases have preferred substrate molecules. By conjugating specific substrate molecules onto NP arrays, we can measure many kinases at the same time.

To our surprise, SERS measurements based on silver NPs reported here works well with crude cell lysate, where most cellular components are unknown. This may due to the fact that in

our system specific kinase-recognition peptides were covalently linked to the silver NPs through a thiol group in the amino terminus of the peptides. All other components were washed away after the kinase reaction and no significant absorption of miscellaneous cellular components onto the silver NP surface was observed. Previous work showed that SERS can withstand BSA and ATP/ADP in solution [12,18]. Surface enhancement of Raman spectroscopy only occurs when the molecules were "very close" to the silver NP. This means that other than direct absorption or covalent conjugation, molecules in solution will not interfere or amplify spectral signal from silver NPs. This special property may be the key to the success of this methodology.

Unlike fluorescence, Raman scattering provides a spectrum of signals reflecting the vibrational patterns of a molecule. Characteristic patterns can be assigned to specific molecular properties, although it is often difficult to interpret each of the individual peaks. Previous work showed that tyrosine and serine/threonine phosphorylation induces specific changes in the spectra, and the specific changes provided a more reliable indication of the molecular structure. However, it seems that these changes could be small and further development on signal amplification methodology will be helpful.

For future work, an array-like kinase assay platform based on this method is very attractive. Such a platform will be valuable in biomedical research and drug development. Simultaneous monitoring of multiple kinase activities in a biological system is very important [20,21]. Moreover, heavy metal NPs with special Raman properties can be readily fabricated [22,23]. This development may potentially lead to cell kinase activity detection inside the cell, a near real-time sensor for cell signaling events.

Acknowledgements

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