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A "turn-off" SERS assay for kinase detection based on arginine *N*-phosphorylation process

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Abstract

A sequential "turn-off" surface enhance Raman scattering (SERS) assay platform for the detection of protein arginine kinase McsB is constructed based on arginine *N*-phosphorylation process. The positive charged peptide initiates the aggregation of labelled Au nanoparticles (AuNPs) to form ''Hot Spots'', resulting to a higher SERS intensity. However, the aggregation of AuNPs could also be disbanded by the addition of McsB in which the peptides are phosphorylated on arginine residues, leading to the sharp decline of SERS signal, on account of two negative charges on the phosphate group. By this strategy, a novel ''turn-off'' SERS biosensor for McsB detection based on arginine *N*-phosphorylation was established with high sensitivity, selectivity and simplicity. Compared with other non-enzymatic amplification methods, the sensitivity of this newly demonstrated method was improved effectively. The detection limit for McsB is 46 pM. Besides, some controlled experiments show that the assay possesses good selectivity, which is mainly decided by the specificity of kinase. Since some kinase are important biomarkers, this assay could make great contribution in biomarkers detection in complex matrices which is based on signal amplification.

Key words: arginine *N*-phosphorylating; kinase detection; SERS; gold nanoparticle

1. Introduction

Protein phosphorylation is the process that protein kinase transfers the phosphoryl group from phosphor-donor to the phosphor-acceptor. As one of the key post-translational modifications (PTMs), it is involved in the regulation of protein structure, enzyme activity and signal transduction. For example, protein tyrosine kinase regulates the intracellular signal transduction pathways and mediates the multicellular communication [1]. In the past decades, great achievements have been made in *O*-phosphorylation, mainly focusing on serine, threonine and tyrosine residues [2, 3]. However, relatively slow progress has been achieved in *N*-Phosphorylation (arginine, histidine and lysine), even though this process plays an important role in the intracellular signal transduction in prokaryotes as well as eukaryotes [4]. Until now, there is only one arginine kinase related to *N*-phosphorylation which has been identified, that is McsB, the protein arginine kinase, in prokaryotes [5]. One of the reasons for less studies is that P-N bond in *N*-phosphorylation is very labile under acidic conditions [6]. Besides, the extremely low amount of phosphorylated substrates and kinases in biological samples also hamper its analysis [7]. Some research have been reported that kinase is a very important biomarker, such as senile dementia of the Alzheimer type [8]. Therefore, it is essential to establish reliable and sensitive analysis method for the detection of protein phosphorylation kinase and its substrates.

Some methods have been developed to detect the protein kinase, such as mass spectrometry (MS) [9], fluorescence [10], immunoassay [11], electrochemistry [12] and other methods [13]. However, there are some limitations of these strategies. The MS based quantification method always need some complicated enrichment processes and some stable isotope labeling like ^{13}C, which are very expensive [14]. Fluorescence requires the modification of proteins with organic group to reach a better performance. Meanwhile, the luminophore molecules are prone to photo bleach leading to unstable

signal. Moreover, the above mentioned methods are both used to detect the kinase for *O*-phosphorylation. The immunoassay has been reported to recognize and detect protein histidine *N*-phosphorylation. Tony Hunter's group made a breakthrough in histidine phosphorylation by designing and synthesizing the histidine phosphorylation analogues to obtain mono- and poly-antibodies [11]. Thompson's group and our laboratory have also developed the arginine phosphorylation analogues to obtain the high-affinity and specific phosphoarginine poly-antibodies [15, 16]. Those antibodies provide a powerful tool to identify and detect arginine phosphorylated protein. Nevertheless, few sensors have been reported to detect the protein arginine kinase, there are still enormous challenges to establish a sensitive, fast and simple strategy to detect *N*-phosphorylation kinase as well as substrates.

Surface enhance Raman scattering (SERS) has aroused wide attention due to its applications in catalysis, cell imaging, sensor and identification of gems [17-21]. SERS demonstrates its power by linking analytes to the surfaces of nano-materials such as gold nanoparticles (NPs), silver NPs and copper NPs to produce enhanced signal. One of the advantages for SERS is that it doesn't necessitate tedious sample pretreatments, saving precious time and labor. There are many works showing that SERS is a powerful analytic technique with highly sensitivity and selectivity for the detection of biologic samples. Hu's group fabricated a SERS sensing platform to detect heparin by taking advantages of the electrostatic interaction using a chain of peptide and thrombin, indicating the feasibility of SERS in the application of biomolecule analysis [22]. In addition, Garry P. Nol's group developed a SERS strategy to monitor phosphorylation in living cells, further broaden the application of SERS in the biological field [23]. To date, SERS-based assays for detecting both of phosphorylated protein and protein kinase have been developed very well [24, 25]. However, none of them concentrated on the *N*-phosphorylated protein kinase. Therefore, it is essential to fabricate a new fast and simple assay to detect the kinase for *N*-phosphorylation.

Recently, Au nanoparticles-based signal amplification assays have widely been established to detect biomolecules such as protein, DNA and RNA. One of the most popular method is to fabricate multiple-cycle amplification by utilizing different DNA chain. For instance, Qiu's group described an ultrasensitive surface plasma resonance (SPR) approach using the aptamer-based cycle amplification, realizing the enhancement of the SPR signal [26]. In fact, compared with DNA-based amplification system, enzymatic amplification strategy has also been widely developed in the field of biological analysis. Hu's group developed another SERS biosensor based on enzyme assays by thrombin catalysis using AuNPs as SERS substrates [22]. However, even though specific catalytic cleavage of designed substrates has been reported based on enzymatic enhancement, few works concentrated on the kinase reaction such as protein and substrate peptide phosphorylation to amplify the signal. In fact, it is of great potential to construct a biosensor by combining the phosphorylated and non-phosphorylated state of substrate peptide, since the addition of a phosphate group can change the structure and the charge states of peptides or proteins to adjust the electrostatic interaction between the AuNPs and analytes. While the aggregation of negatively-charged AuNPs can be reflected by SERS signal. Some works employing the electrostatic interaction between AuNPs and substrates to realize the detection of bio-samples have been reported, indicating the feasibility of this method [22, 27].

In this study, we fabricated a "turn off" SERS assay for arginine kinase detection based on the decentralization of the aggregated AuNPs induced by arginine phosphorylation on substrate peptide. The positively-charged substrate peptide (KRGGGGYIKIIKV, KRV) of McsB, the protein arginine kinase in *Bacillus subtilis*, was added into AuNPs solution leading to the aggregation of AuNPs. McsB

can quantitatively phosphorylate arginine residues of substrate peptide, which neutralize part of the positive charge on the substrate peptide. The phosphorylated peptide has little contribution on the aggregation of AuNPs due to the electrostatic repulsion interaction of the negatively-charged hydroxyl of phosphate group. Therefore, ultrasensitive detection of arginine kinase can be realized in this platform. It is a novel method to detect protein arginine kinase McsB based on AuNPs aggregation in *N*-phosphorylation process. Since the kinase is sequence-dependent, the proposed strategy show high selectivity and sensitivity combined with SERS for arginine kinase. It also provides a new insight to detect the phosphorylation of other alkaline amino acid in biosensor.

2. Experimental

2.1 Materials

Chloroauric acid ($HAuCl_4 \cdot 4H_2O$), trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 4-mercaptobenzoic acid ($C_7H_6O_2S$, 4-MBA), adenosine triphosphate (ATP), magnesium chloride ($MgCl_2$), KRGGGGYIKIIKV (KRV), Tris base, Acrylamide/Bis-acrylamide (29:1, 30% solution), and Unstained Protein Marker IV, Mid Range (cat. No.: C600525) and *N, N, N', N'*-Tetramethylethylenediamine (TEMED) were purchased from Sangon Biotech (Shanghai, China). The protease inhibitor cocktail tablet as well as phosphatase inhibitor cocktail tablet were bought from Roche Diagnostics (Mannheim, Germany). Cyclin-dependent kinase 9 (CDK9) was purchased from Thermo Fisher (Thermo Fisher Scientific (China) Co. Ltd). Other proteins used in this work were expressed by ourselves. And other reagents used here were analytical grade. Ultrapure water was produced by Milli-Q system in our lab.

2.2 Apparatus

UV-vis spectra of AuNPs and the mixture of AuNPs and peptide were both recorded by a Shimadzu UV-2550 at room temperature using a 300 μL quartz cuvette with 1 cm path length. Raman measurements were performed with a confocal microscope (Xplora, Horiba). He-Ne laser with 632.8 nm radiations was used as excitation for all the SERS measurements. The signals were integrated for 5 s and twice accumulations. Spectra were collected with 50× long working distance lens and smoothed once at degree of 3. Transmission electron microscopy (TEM) for AuNPs was performed on a JEOL JEM-1400 microscope. Zeta potentials of sample were measured on a Nano-ZS ZEP3600 particle sizer (Malvern Instruments) to record the size of AuNPs.

2.3 Preparation of AuNPs

AuNPs were synthesized according to the reported work using sodium citrate as reductant [28]. Before the reduction of H_2AuCl_4, the two-necked flask and the condenser-Allihn type were both soaked by fresh aqua regia for two hours. Then, rinsed well under running water several minutes. Finally, washed them using ultrapure water three times. In brief, 50 mL of 0.01% (w/w) $HAuCl_4$ was added to a flask and heated to boiling in oil bath with vigorous stirring, followed adding 0.75 mL of 33.8 mM sodium citrate into the boiling solution. The mixture was kept heating and stirring for 30 min. Finally, the prepared AuNPs cooled to room temperature naturally. AuNPs were centrifuged and washed by ultrapure water several times before characterization.

2.4 Expression and purification of McsB

McsB was obtained according to the previous report in our work [15]. Firstly, the plasmid was transformed to *E. coli* BL21 (DE3)-pLys strain. Then, they were grown in Luria–Bertani (LB) media with the addition of ampicillin, placing in the table concentrator at 37℃ and setting speed to 220 rpm. 100 μL of overnight cultures was added to 100 mL of media with shaking for more than 2 h at 37 ℃. After adding IPTG (500 μM), the bacteria were cultured for another 12 h. Finally, the cell pellet was collected from LB media by centrifugation at 6000 rpm and resuspended in Tris buffer (300 mM NaCl,

50 mM Tris/HCl pH 8.0). The lysate was obtained by sonication. Then the resulting lysate was purified by Ni^{2+}-nitrilotriacetate (Ni-NAT) column and size exclusion chromatography (SEC, Superdex 75). The pure protein was stored in -20℃ before to use.

2.5 Detection of McsB

In order to accurately detect McsB, a series of standard concentration of McsB solution were prepared ranging from 0 pM to 1 mM. Then it was added to the solution containing $MgCl_2$ (5 mM), ATP (10 mM) and peptide (0.5 mg/mL), incubating for 3 h at 40℃. AuNPs were mixed with the mixture containing different concentration of McsB and should be incubated some time before SERS test.

3. Results and discussion

3.1 The characterization of AuNPs and McsB

The AuNPs with about 25 nm diameter was prepared by the reducing method. The UV-vis was firstly employed to characterize the size of AuNPs. It can be observed that the absorption peak of AuNPs is at 522 nm (Fig. S1), indicating the size of AuNPs was 25 nm [29, 30]. Dynamic Light Scattering (DLS) was also used to further confirm the size of AuNPs, which showed that the diameter approximately distributed from 10 nm to 80 nm with the average diameter of 25 nm (Fig. S2). McsB was obtained by transforming the plasmid into *E. coli* and grown in LB media. After purified by SEC chromatograph, McsB was characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. S3). And the Bradford method was used to calculate the protein concentration.

3.2 Principle of the proposed biosensor

The principle of demonstrated kinase assay was shown in Scheme 1. First, AuNPs with the diameter about 25 nm were functionalized with 4-MBA as the SERS output signal. There are two vibration modes of 4-MBA. One is an aromatic ring-breathing vibration with the Raman shift in 1077 cm^{-1}, while the other one is C=C stretching vibration mode located in 1580 cm^{-1}. In this study, the Raman band at 1077 cm^{-1} was selected as the quantitative standard. Since AuNPs were synthesized by the chemical reduction method using sodium citrate as the reducing agent, they were usually negatively charged, confirmed by DLS and the average value is -42 mV. The KRV peptide was chosen as the model substrate which can be specifically recognized by McsB in vitro [5]. There are four positively charged amino acids including three lysine and one arginine residue in the model peptide. When KRV was added to AuNPs solution, the color of AuNPs colloid changed from red to blue immediately, demonstrating the aggregation of AuNPs. This was induced by electrostatic interaction among the lysine, the arginine residues in peptide chain and the negatively charged AuNPs. The aggregated AuNPs enables enhance electromagnetic field, thus the Raman signal of 4-MBA could be remarkably enhanced. However, once KRV was phosphorylated by McsB in the present of ATP and Mg^{2+}, parts of the positive charge in the peptide chain were neutralized by attaching the anionic phosphate group to the guanidine of arginine. The structure of phosphorylated KRV (pKRV) was shown in Fig.S4. Apparently, pKRV can reduce the electrostatic interaction and result in declined SERS intensity. Thus, the addition of peptide can enhance the SERS intensity, while *N*-phosphorylation of substrate peptide by kinase McsB could weak the SERS intensity. In addition, there was a linear relationship in certain concentrations which can realize the detection of the arginine kinase McsB.

To further test the feasibility of the demonstrated method, some control experiments have been carried out. By Raman spectra which were shown in the Fig. 1A, a weak Raman intensity could be found in the AuNPs labelled with 4-MBA in aqueous solution (curve a). When it was dispersed in Tris buffer, the intensity slightly rose (curve b), caused by salt effect. There was a drastically increase of

SERS intensity when the KRV was put into the AuNPs dispersed in Tris buffer (curve c). While other contrastive tests with the functioned 4-MBA AuNPs added into the mixture of KRV, ATP, $MgCl_2$ and McsB (curve d), the solution only containing of ATP, $MgCl_2$ and McsB without KRV (curve e) were also studied respectively. Compared with the absorption band of AuNPs modified 4-MBA containing KRV (curve c), the intensity of SERS intensity (curve a, b, d and e) declined remarkably, which illustrated that the sharp change of SERS intensity was only related to the KRV and pKRV.

Further, the 4-MBA labelled AuNPs was also characterized by UV-vis spectroscopy, the result of which was coincided well with Raman spectroscopy. As shown in Fig. S5, there was an obvious absorption band at λ=522 nm corresponding to the spherical AuNPs (curve a) [31]. When the KRV with $MgCl_2$ and ATP were added to AuNPs colloid, a red shift of absorb band (curve b) can be observed, indicating the aggregation of AuNPs. Once the above KRV solution was incubated with the McsB, the absorption peak of AuNPs has no change (curve c), which illustrated the electrostatic interaction between the substrate peptide and the AuNPs was changed. Hence, through the results of SERS measurements and UV-vis spectra, it could be concluded that the aggregation of AuNPs was mainly due to the addition of KRV. TEM images also confirmed that AuNPs aggregated after adding the KRV (Fig. 1B, Fig. S6). Once the peptide was *N*-phosphorylated by McsB, it reduce the aggregation of AuNPs due to the electrostatic repulsion.

3.3 Optimization of assay parameters

With the purpose of obtaining more sensitive and stable SERS signal, some experimental parameters should be optimized. In this work, the factors influencing the assay sensitivity mainly included the concentration of KRV, pH and incubation time after adding the enzymatic reaction solution into the AuNPs labelled with 4-MBA.

First, the concentration of KRV leading to the controllable aggregation of AuNPs was studied. The aggregation level increased with the increasing amount of KRV. As shown in Fig. 2A, the SERS intensity enhanced sharply after adding more KRV to AuNPs functioned with 4-MBA colloid. When its concentration reached 0.5 μg /mL, the signal became weak. In order to get a stronger signal during the experiment, the optimal concentration of KRV was 0.5 μg/mL.

The pH value of phosphorylation of arginine in peptide with McsB was decided according to the literature [5]. The whole phosphorylation reaction was carried out in the 100 μL Tris buffer (20 mM, pH=8.0). AuNPs were synthesized by the reduction of sodium citrate which acted as both reducing agent and protective agent. Therefore, the residual sodium citrate endowed acidic property to the whole solution. However, the phosphoarginine was easily hydrolyzed through P-N bond cleavage under acidic condition, the effect of pH of mixture solution between AuNPs and kinase reaction should be investigated in detailed. As was shown in Fig. 2B, the pH was studied in the range of 6.0 to 9.5. Raman signals reached the maximum when the pH was 8.0. Therefore, 8.0 was adopted as the optimal pH value.

In order to acquire the stable Raman signal, we also took the incubation time into account. The result was shown in Fig. 2C. Obviously, the signal intensity was increasing with the longer incubation time of kinase reaction solution. After 10 min, the intensity of Raman signal reached the maximum value, while there was little decline between 10 min to 45 min. Then it reached a plateau after 45 min. Therefore, it was decided that the stable signal can be obtained after 45min.

3.4 Quantitative detection of McsB

To explore the application of this assay to quantitative detection of McsB, a series concentration of McsB were prepared to incubate with AuNPs labelled with 4-MBA under the optimal conditions.

Different concentrations of McsB were reflected by the intensity of 4-MBA at 1077 cm^{-1}. In order to reduce the background signal interference, the difference intensity between AuNPs modified 4-MBA solution within or without certain KRV was decided as vertical coordinates. Triplicate samples at the same concentration of McsB were tested at least. Besides, in order to assess the reproducibility, we tested seven times parallel samples to obtain Raman signal. The results was shown in Fig. S7, it indicated that there was a stable Raman intensity among different sample. The relative standard deviation (RSD) was 7.8 %, illustrating the stability of the aggregation. From Fig. 3A, it could be observed that the SERS intensity dramatically increased with the reduction of the McsB. It illustrated that more arginines in the KRV were phosphorylated when introduced the larger amount of McsB. Furthermore, the intensity of Raman signal produced by 4-MBA reached the maximum value with the concentration of McsB deduced to 250 pM (Fig. 3B). When the concentration of McsB in the sample was more than 500 nM, the intensity of Raman signal did not change too much, all of which were relatively low. Under this circumstances, the electrostatic repulsion between AuNPs and KRV increased because the arginine was phosphorylated by McsB.

In addition, Fig. 3B inset demonstrated the relationship between the concentration of McsB and the SERS intensity of AuNPs labeled by 4-MBA. A good linear correlation obtained with the concentration of McsB in the range from 1 nM to 100 nM. The regression equation was $y=-52.38X+7052$ (x represents the concentration of McsB in nM, while y was the intensity of Raman signal of 4-MBA) with a squared correlation coefficient of 0.9806. LOD was determined to be 46 pM based on three times the standard deviation of the blank signal. Compared with the reported methods based on flourescence [10], Raman spectra [23, 25], the suggested protocol was more sensitive and effective.

3.5 Selectivity of the demonstrated sensor

Since this method exhibited the great performance for the detection of arginine kinase, it was essential to monitor whether the obtained signal can be disturbed by other interferents. However, the arginine kinase has not been reported in the eukaryotes so far. And there was only one arginine kinase, McsB, to be identified. Thus, some general protein including lysozyme, β-casein from bovine milk, albumin from chicken egg, proteinase K and catalase, were selected as interfering agents. Besides, other serine kinase/threonine kinase (CDK7) and tyrosine kinase (*c*-Src) were also chosen as interferents. The experiments were carried out by keeping the concentration of interferents at 1 μg/mL respectively. After incubated with KRV, the reaction mixture was put into AuNPs labelled with 4-MBA. As was shown in Fig. 4A, there was no significant change of the signal intensity at 1077 cm^{-1} after adding each interferent into the mixture as above mentioned. Only when KRV was incubated with McsB, the sharp decrease of SERS intensity could be observed. This phenomenon was due to the phosphorylated arginine increasing the electrostatic repulsion between KRV and AuNPs. The results further illustrated that the suggested method showed excellent anti-interference performance.

3.6 Analysis of McsB in *Bacillus subtilis* lysate

To test the reliability of the suggested method in a complex sample, the McsB detection assay in normal *Bacillus subtilis* lysate was verified. Here, the *Bacillus subtilis* cell lysate was used as test medium to assess the ability of the assay in the complex matrices with various interferents. The cell lysate was obtained after ultrasonic decomposition and centrifugation. In order to reduce the interference of phosphatase in the lysate, some phosphatase inhibitors were used here. The standard McsB with the concentrations of 20 nM, 40 nM, 60 nM, 80 nM and 100 nM were added to the lysate respectively. After the SERS measurements, the measured concentrations were calculated by the calibration equation acquired by the test of the standard McsB without any interferents. From Fig. 4B,

there was a line decrease for the SERS intensity with the increasing of the McsB. The results were conformed to the experiment phenomena carried out in the Tris buffer. By calculation, the recovery of McsB in lysate ranged from 92% to 106% (Table. S1), indicating the accuracy of this assay. We also carried out some comparative experiments in other cell lysate, such as *c* and *Escherichia coli* (Fig. S8), further confirming the feasibility of proposed method.

4. Conclusions

In summary, we successfully designed and constructed a novel "turn-off" SERS assay platform for arginine kinase McsB detection. The design strategy is based on the interaction between the 4-MBA labelled AuNPs and KRV peptide with the phosphorylation regulation by McsB. The above method possesses good sensitivity, selectivity and convenience, and can perform the quantitative determination of McsB with the detection limit of 46 pM. It is constructive to use the process of arginine phosphorylation to adjust the amplified Raman intensity by adding a negative-charged phosphate group to the guanidine of arginine. Compared with the other methods of analysis to detect the kinase, this strategy provides a novel insight to detect the bio-samples by combining the *N*-phosphorylation process with the AuNPs.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version.

Conflict of interest

The authors declare that they have no conflict of interest.

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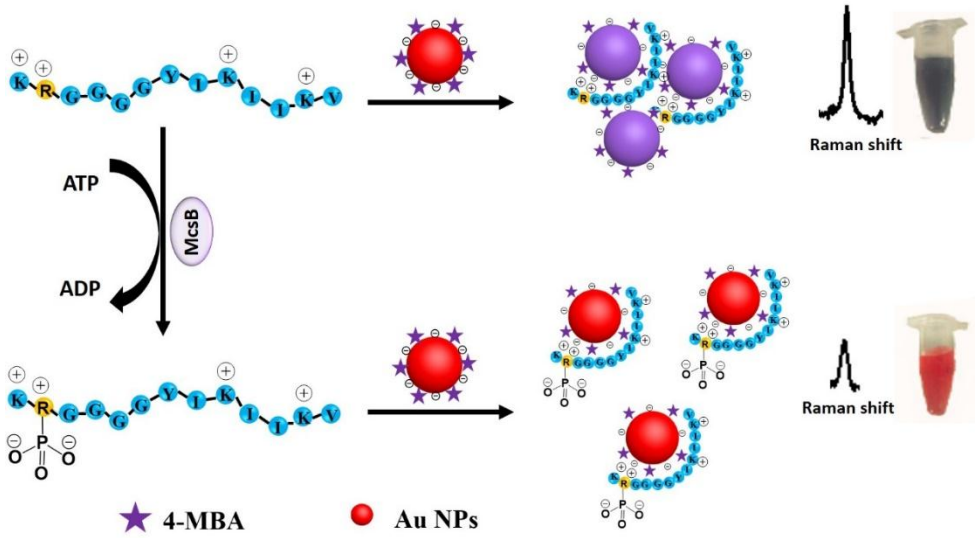

Scheme 1. Schematic diagram of SERS-based kinase assay for McsB

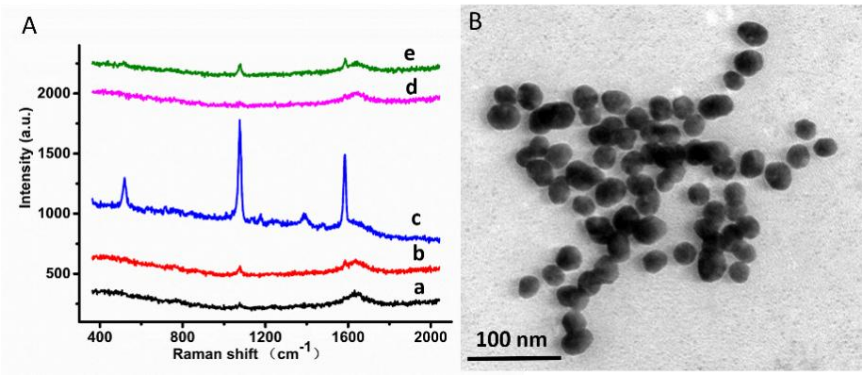

Fig. 1. (A) SERS spectra: functioned by 4-MBA (a) in dispersed form, (b) in Tris Buffer (pH=8.0), (c) with peptide KRV (5 μM), (d) with phosphorylated peptide pKRV (5 μM) (e) with McsB (1 μM), ATP and Mg^{2+} (B) the TEM image of AuNPs

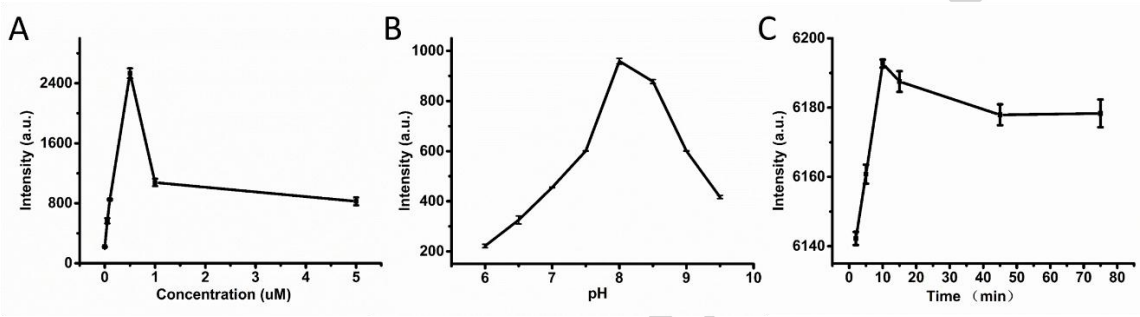

Fig. 2. Optimization of (A) the concentration of KRV, (B) pH, (C) incubation time

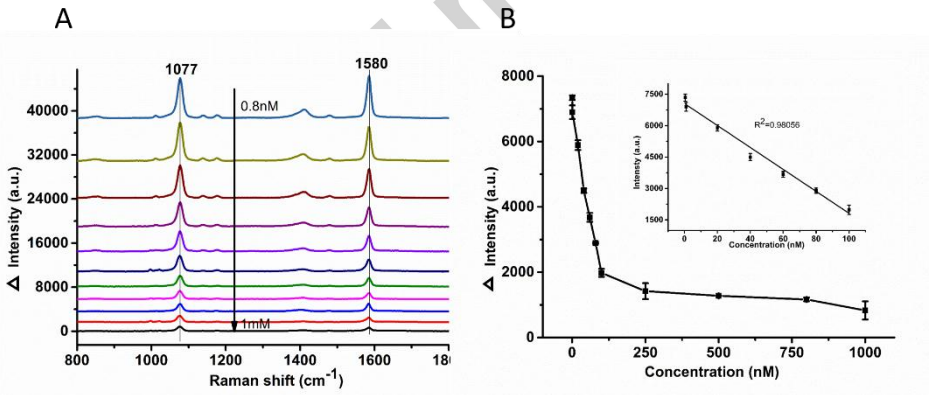

Fig. 3. (A) SERS spectra of AuNPs functioned by 4-MBA incubated with peptide incubated with different concentration of McsB for 3 h and (B) McsB concentration range from 250 pM to 1 mM dependent normalized Raman intensity of 1077 cm^{-1} peak. The inset shows the calibration curves at 1077 cm^{-1} against the concentration of McsB. Each error bar represents the standard deviation in normalized Raman intensity associated with n=3.

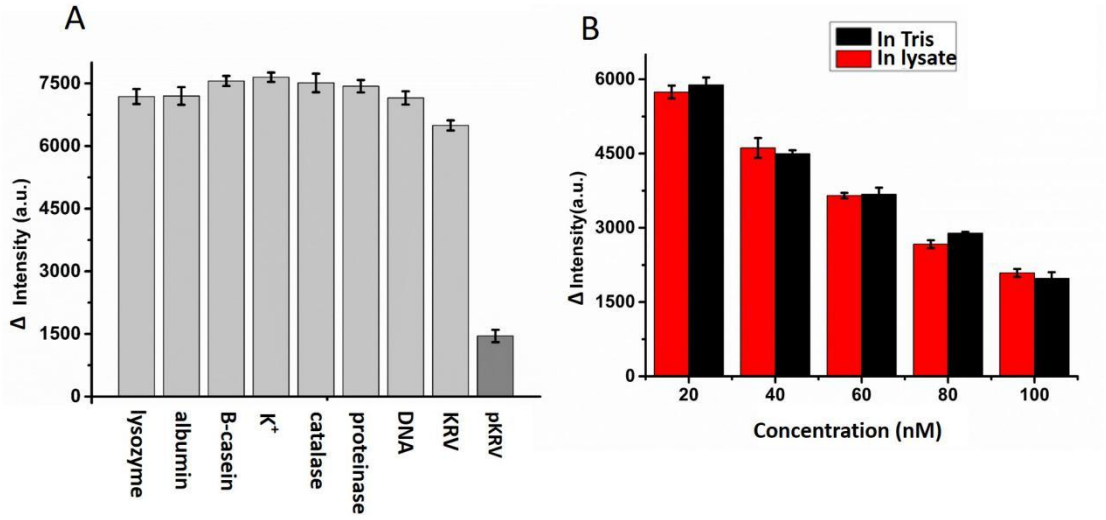

Fig. 4. (A) Evaluation of the specificity of the SERS assay. Variation of SERS intensity of 4-MBA modified AuNPs at 1077 cm $^{-1}$ after separate incubation of KRV (0.5 μM) with lysozyme (100 nM), β-casein from bovine milk (100 nM), albumin from chicken egg (100 nM), proteinase (100 nM) and catalase (100 nM), serine kinase (CDK9) (100 nM) and tyrosine kinase (*c*-Src with GST) (100 nM) and (B) Recovery test of McsB spiked in cell lysate. Each error bar represents the standard deviation in normalized Raman intensity associated with n=3.

Highlights

- A novel method was proposed to detect protein arginine kinase McsB based on AuNPs aggregation.
- A "turn off" SERS assay was designed based on arginine phosphorylation on substrate peptide.
- We demonstrated an extensible, facile and sensitive assay for arginine kinase detection.

Graphical abstract