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DNA cross-linked SERS probes†

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We propose an ultrasensitive SERS-based peptide biosensor platform to monitor phosphorylation catalyzed by kinase in a dynamic format. The developed SERS strategy has a short response time with potential to monitor phosphorylation in live cells.

Protein phosphorylation catalysed by kinase is known to be involved in various physiological processes and has been implicated in numerous diseases, such as Alzheimer's, Cancer, Immunodeficiency syndrome, among others.^{1–6} To enhance our understanding of phosphorylation dynamics and to assist in the development of therapeutic targets, analytical tools with a fast response time is critical. To record phosphorylation in a dynamic format the detection strategy should be specific, sensitive and rapid with probes suitable for monitoring the activity of multiple kinases. Until now, various methods are available to detect phosphorylation *in vitro*,^{7–11} and only a limited few based on fluorescence can detect phosphorylation in a dynamic format or in live cells. Although unlike the HPLC/GC-MS, Raman or electrochemistry based methods, fluorescent methods usually do not need a cell lysis or cell fixation step, however, they are limited due to cellular autofluorescence or photobleaching of the probes necessitating complex procedures and/or expensive instrumentation.

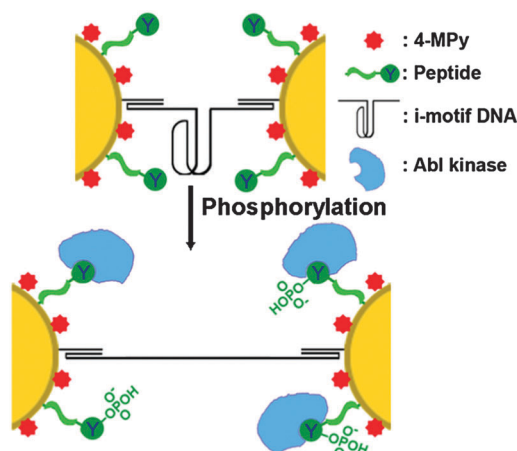
Past work has shown that SERS can be a powerful bioanalytical tool due to the strong enhancement and potential for multiplex detection, quantification, and in bioimaging.^{12–17} SERS has been used to a limited extent to detect phosphorylation by identifying the spectral signature of the kinase-substrate peptide fingerprint before and after phosphorylation.^{18–20} However, the complicated

detection procedures and poor sensitivity of the existing methods have limited further efforts in SERS-based sensors for biological process monitoring. Key limitations are the weak signal from the peptide substrate, noise from competing molecules, almost indiscernible change in spectral signature of the peptide after phosphorylation and the need for complicated data processing steps. In addition, the nanoparticle arrays commonly used as SERS substrates also limit the application of SERS for dynamic phosphorylation monitoring in live cells. Although attempts based on antibody targeting phosphorylated amino acid residue have been reported to monitor phosphorylation based on SERS of Raman tags modified on probes,^{21,22} the application is limited due to the complexity of the procedure. Meanwhile, based on the antibody modified SERS probes, phosphorylation detection was performed in fixed cells, but the required wash step limits its applicability in live cells.²³ Thus, a dynamic SERS-based approach to track phosphorylation has not been possible because of the lack of suitable biosensors with high sensitivity.

Herein we present the very first non-fluorescence technique based on SERS for monitoring phosphorylation in a dynamic format *in vitro* with a possible extension to live cell monitoring. Nanoscale biosensors were designed to record phosphorylation based on SERS intensity *in vitro* and in live cells. Upon Abl kinase catalysed phosphorylation, phosphate groups from adenosine triphosphate (ATP) are transferred onto the tyrosine residues in the substrate peptide by the action of the enzyme. The affinity between Abl kinase with the phosphorylated peptide is demonstrated in prior work.^{24–26} The transfer of phosphate groups accompanied by the interaction between the Abl kinase and the phosphorylated peptide results in a change in the local hydrogen ion concentration in the microenvironment near the GNP surface and can be used as an indicator of phosphorylation. Herein, as illustrated in Scheme 1, we propose a novel strategy with i-motif DNA cross-linked GNP probes (iDCL GNP probes, results of the probe characterization are shown in Fig. S3 and S5–S8, ESI†), which are networks of numerous modified GNPs to report on phosphorylation based on SERS. The designed probe consists of i-motif DNA cross-linked gold nanoparticles

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† Electronic supplementary information (ESI) available: Experimental details, simulation of SERS activity of the iDCL GNP probes before and after phosphorylation, negative control experiments, UV spectral characterizations of the preparation of probes and negative control, morphological image by TEM and SEM of the probes, and the representative SERS spectra from live PC3 cell treated with and without phosphorylation inhibitor. See DOI: 10.1039/c5cc06566f



Scheme 1 Phosphorylation tracking by SERS.

(GNPs) modified with short peptides containing a recognition motif for phosphorylation by Abl kinase (termed as Abl peptide, sequence showed in ESI†) and a Raman tag, 4-MPy. Unlike published methods, in our strategy, the extent of Abl kinase phosphorylation is characterized by a change in the SERS intensity of 4-MPy, instead of the change in the peptide SERS signature reported previously. 4-MPy is chosen as the Raman reporter because of its excellent SERS intensity and sensitivity to the change in microenvironment upon phosphorylation.^{27,28} i-Motif DNA, first used for phosphorylation detection in this work, is a cytosine-rich single stranded oligonucleotide which links the modified GNPs and elicits a change in its configuration with respect to the microenvironment: the conformation of the oligonucleotide changes from a linear configuration to a quadruplex-like folding pattern based on a change in pH of the microenvironment is known from prior reports.^{29,30} The increased interparticle distance of the probe upon phosphorylation is confirmed with the scattering spectra shown in Fig. S4 (ESI†). Our simulation results in Fig. S1 (ESI†) show that an increase in interparticle distance will affect the corresponding SERS activity of the iDCL GNP probes. Upon phosphorylation, a change in the microenvironment is expected due to the transfer of phosphate groups from ATP to the tyrosine residues in the Abl peptide biosensor accompanied by the interaction between the Abl kinase and the phosphorylated Abl peptide. This microenvironment change will result in a reduction in the SERS intensity of the iDCL GNP probes and used as an indicator to monitor phosphorylation in our scheme. As a simple and elegant method, our strategy exhibits high sensitivity and rapid response, making our technology a valuable analytical tool for real-time investigation of phosphorylation.

To demonstrate the reduction in SERS intensity of 4-MPy upon phosphorylation, positive control experiments were performed with iDCL GNP probes and i-motif DNA cross-linked GNPs modified with synthetic phosphorylated Abl peptides. The corresponding normalized SERS intensity of the peak at 1095 cm^{-1} is shown in Fig. S2 (ESI†). It can be seen that the SERS signal from the iDCL GNP probes modified with the unphosphorylated Abl peptides is distinct and much stronger than the signals

from i-motif DNA cross-linked GNPs modified with the synthetic phosphorylated Abl peptides (positive control). These results indicate that the phosphate group transferred to the tyrosine residue in the Abl peptide upon phosphorylation will reduce the SERS intensity of 4-MPy. To estimate the effect of interaction between the Abl kinase and the Abl peptide on the reduction in SERS intensity, we compared the samples of the iDCL GNP probes phosphorylated by Abl kinase and samples of i-motif DNA cross-linked GNPs modified with the synthetic phosphorylated Abl peptides. Our experiments revealed that the SERS intensity of the iDCL GNP probes phosphorylated by the Abl kinase is only about 20% of the signals from i-motif DNA cross-linked GNPs modified with the synthetic phosphorylated Abl peptides. These results show that the interaction of Abl kinase with the phosphorylated Abl peptide substrates on the iDCL GNP probes will also significantly reduce the SERS intensity. From the SERS intensity shown in Fig. S2 (ESI†), it can be established that due to phosphorylation, the SERS intensity of iDCL GNP probes decreased because of the effect of phosphate group transfer accompanied by the interaction between the Abl kinase and the phosphorylated Abl peptide. The reduction in SERS intensity of the probes is then utilized to monitor phosphorylation in our strategy.

As shown in Fig. 1, results from the SERS spectra and intensity change upon phosphorylation for different incubation time is recorded for different concentration of Abl kinase. The SERS spectra shown in Fig. 1 are assigned to 4-MPy.^{31,32} Compared to the reported SERS spectra of the peptides, the intense SERS signal from the iDCL GNP probes in our strategy provided a significant improvement in sensitivity. Fig. 1A shows that phosphorylation can be detected in 5 minutes after the addition of Abl kinase as observed from the reduction in SERS intensity, signifying the rapid response of the biosensor. The phosphorylation reaction rate represented by the SERS signal from the probes decreased in proportion to an increase in the Abl kinase concentration from 0.263 nM to 2.10 nM. It was also noted that samples with Abl kinase concentration greater than 1.05 nM exhibited similar SERS intensity, implying a saturated SERS response upon phosphorylation possibly due to the amount of participating probes. Similar reduction in SERS intensity can be noted for Abl kinase concentration at incubation times of 35 minutes and 65 minutes. The calibration curves of the normalized SERS intensity of the peak at 1095 cm^{-1} to Abl kinase concentration for different incubation times are also shown in Fig. 1D. It can be seen that for a particular incubation time, the phosphorylation reaction rate in proportion to the Abl kinase concentration can be assessed as a function of SERS intensity from the iDCL GNP probes, thus providing a rapid means to monitor Abl kinase concentration. Compared to the reduction of the signal from blank attributed to the aggregation of the probes triggered by the addition of MgCl_2 , a further reduction in SERS intensity in proportion to the concentration of Abl kinase in samples was noted (Fig. 1D) as an indicator of phosphorylation.

To demonstrate specificity of the detection strategy, two different i-motif DNA cross-linked GNPs were used as negative

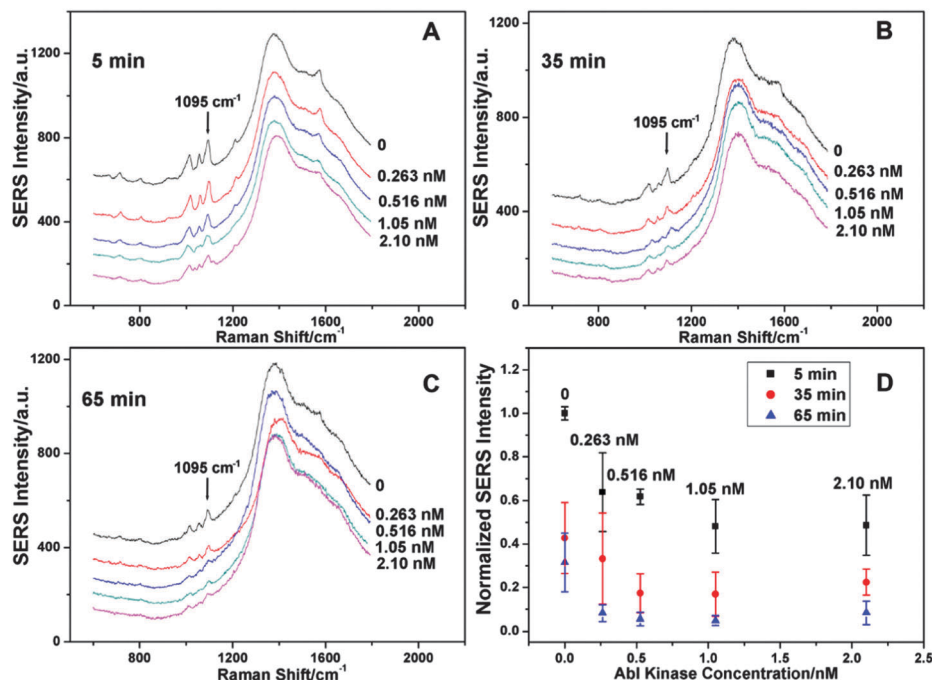


Fig. 1 SERS spectra of iDCL GNP probes phosphorylated at various concentration of Abl kinase for an incubation time of 5 minutes (A), 35 minutes (B), and 65 minutes (C). Calibration curves are also presented (D) [mean $\pm$ SD, $n = 3$].

control. One of the i-motif DNA cross-linked GNPs was not modified with any peptides and the other was modified with Hck peptides. As shown in Fig. 2, for the samples with iDCL GNP probes, a further reduction in the SERS intensity compared to the blank was observed for serial incubation times. However, for samples with negative control using the i-motif DNA cross-linked GNPs modified with no peptides, Fig. 2B shows a similar SERS signal in the presence of Abl kinase to the blank at different incubation times, indicating that without phosphorylation the SERS intensity will not decrease. Use of Hck peptide, which is not the substrate peptide for Abl kinase, should not result in an interaction between Abl kinase and Hck peptide; further, the Hck peptide cannot be phosphorylated by the Abl kinase due to a lack of sequence specificity. Therefore, the presence of Abl kinase will not reduce the SERS intensity of i-motif DNA cross-linked GNPs modified with Hck peptides. As illustrated in Fig. 2C, for the

negative control using the i-motif DNA cross-linked GNPs modified with Hck peptides, the SERS intensity of the samples with Abl kinase is also similar to that of the blank, indicating that without Abl peptide phosphorylation the SERS intensity will not decrease. Similar SERS intensity from negative control with increased kinase concentration can be noted in Fig. S9 (ESI[†]). Results from negative control experiments demonstrate a high level of specificity of the SERS strategy to monitor phosphorylation.

As a final proof of concept validation, we apply the developed method to detect phosphorylation in live cells. Here we used imatinib, an Abl kinase inhibitor, to inhibit phosphorylation in live cells. PC3 cells were incubated with the iDCL GNP probes and the SERS spectra of the probes were recorded in live PC3 cells. SERS intensity from the cells treated with and without the inhibitor are shown in Fig. 3 (typical SERS spectra are shown in Fig. S11, ESI[†]). It can be observed that the SERS intensity from

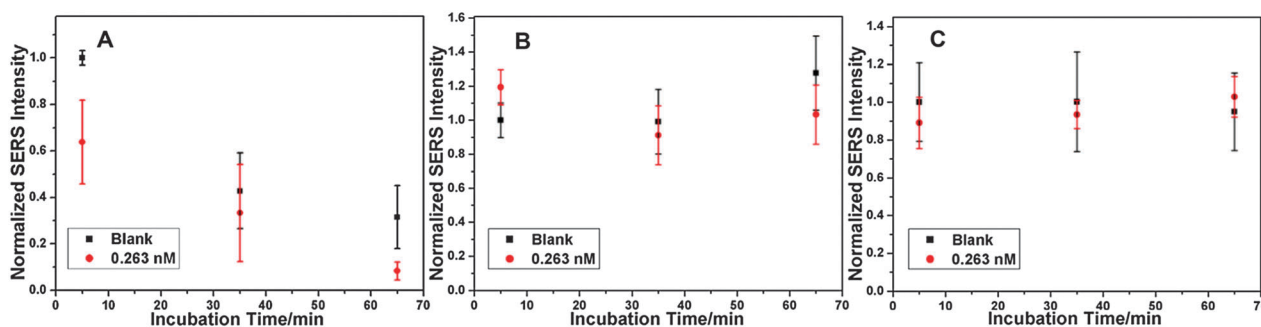


Fig. 2 Normalized SERS intensity from the iDCL GNP probes phosphorylated by Abl kinase (A), i-motif DNA cross-linked GNPs modified without peptide (B), and Hck peptides (C) mixed with Abl kinase [mean $\pm$ SD, $n = 3$].

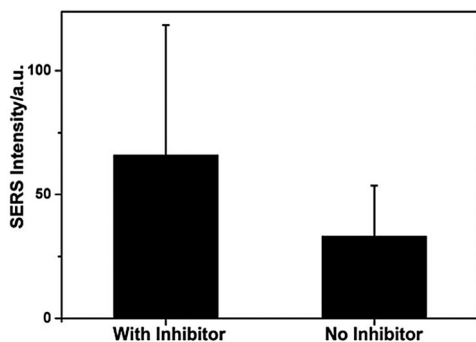


Fig. 3 SERS intensity of the iDCL GNP probes in live cells treated with and without inhibitor.

the cells treated with the inhibitor is obviously stronger than that from the cells without the inhibitor, while the deviation in SERS intensity could be attributed to the nonuniform distribution of probes. As established above, the phosphorylation of Abl peptides on the iDCL GNP probes would decrease the SERS intensity of the probes, accompanied by the increased interparticle distance of the probe demonstrated with the scattering spectra in Fig. S12 (ESI[†]). Since imatinib inhibits phosphorylation, compared to the sample without imatinib the iDCL GNP probes exhibited a stronger SERS signal in live PC3 cells treated with imatinib. The results obtained in live PC3 cells clearly show that, based on the SERS intensity of the iDCL GNP probes, the method developed can be used to monitor live cell phosphorylation.

In summary, we present a highly sensitive SERS strategy to monitor phosphorylation in a dynamic format. Our method is based on the enhanced signal with a short response time from the iDCL GNP probes. The strong SERS signal allows for the monitoring of phosphorylation for Abl kinase concentration as low as 0.263 nM within 5 minutes in a temporal manner. Our unique approach can be extended to record phosphorylation in live cells and to assess drug response. Compared to the past strategies based on SERS fingerprinting of substrate peptide, the developed approach is robust and pertinent for phosphorylation investigation, and will pave the way for real-time multiple signaling pathway monitoring in live cells. Other peptide sensors targeting multiple kinases could be designed and integrated with our SERS strategy with different Raman labels for multiplex kinase signal monitoring.

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