Sensitive and label-free quantification of cellular biothiols by competitive surface-enhanced Raman spectroscopy

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Article info

Article history:
Received 4 January 2016
Received in revised form 27 January 2016
Accepted 1 February 2016
Available online 2 February 2016

Keywords:
Competitive adsorption
Plasmonic switching
SERS
Biological thiols
Optical detection

A B S T R A C T

A label-free surface-enhanced Raman spectroscopy (SERS)-based method for the rapid quantification detection of cellular biothiols at picomolar levels was developed by using a mechanism of binary competitive adsorption to regulate the plasmon coupling behavior of gold nanoparticles (Au NPs). 4,4'-Di- pyridyl (Dpy), a small organic ligand with two symmetrically located pyridine rings, was used to shorten the inter-particle space and generate a multitude of “hot spots”, which in turn amplified the fingerprint signals of Dpy molecules. When biothiols were introduced into the Dpy-containing solution of Au NPs, they competitively adsorbed to the metal surface through the much stronger S–Au linkage, leading to the disaggregation of Au NPs and SERS quenching of Dpy molecules. The change of SERS responses was quantitatively related to biothiols added to the solution, and the detection limit down to 0.14 pM for GSH was facilely achieved without any pre-concentration. The total assay time, including data analysis was within 8 min. Finally, the SERS-based method was successfully applied to measure cellular biothiols, indicating its potential applicability in biological and biomedical research.

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1. Introduction

Biological thiols, such as glutathione (GSH) and cysteine (Cys), play crucial roles in many physiological and pathological processes. GSH is the most abundant non-protein biothiol in mammalian and eukaryotic cells, which is synthesized endogenously from the precursor amino acids l-cysteine, l-glutamic acid, and glycine. It serves a key function in combating oxidative stress and maintaining redox homeostasis that is pivotal for cell growth and function [1]. Increasing studies have demonstrated that changes in the levels of GSH are closely associated with multiple disease states, including liver damage, cancer, osteoporosis, Alzheimer's disease and HIV infection [2–6]. In addition, GSH can transform to oxidized form (GSSG) after oxidation. The concentrations of GSH and GSSG and their molar ratio are related to cell functionality and oxidative stress. Cys is an essential amino acid in native proteins and plays a central role in biological systems, especially in folding and refolding mechanisms [7]. Elevated levels of Cys have been proved to be linked with neurotoxicity; while Cys deficiency has been correlated with several clinical symptoms, including slow growth, hair depigmentation, edema, lethargy, muscle and fat loss, skin lesions, and weakness [8–10]. Therefore, the development of novel means of biothiol detection is highly necessary and has attracted continuing interest in the fields of chemical and biological sciences [11,12].

Owing to their apparent advantages of high sensitivity, cost efficiency and simplicity, optical spectroscopies, such as fluorescent spectrometry [13–17], colorimetry [18,19] and surface-enhanced Raman scattering (SERS) [20–23], are the most available analytical techniques for the detection and quantification of thiols in biological samples. However, the conventional assays based on colorimetry and fluorescence usually suffer from some drawbacks to restrict their wide applications. Colorimetric assays usually only exhibit moderate sensitivity at micromolar or nanomolar levels [18,19]. For fluorescent spectrometry, most fluorescent probes relying on organic fluorophores are vulnerable to photobleaching and photolysis. Although the recently developed inorganic phosphors, such as quantum dots (QDs) [24], upconversion nanoparticles (UCPs) [14], silver nanoclusters [25] and carbon-based nanomaterials [6,19], hold promise for overcoming the above problems, such probes, however, either involve the employment of toxic heavy-metal elements (e.g., Hg²⁺ ions and Cd²⁺ ions), or are limited by their optical properties of low quantum yields or short-wavelength emission.

SERS spectroscopy has attracted considerable attentions in a variety of fields owing to the integration of high sensitivity, good specificity, and the capability to provide abundant molecular...
structural information. Raman signals can be enhanced by several orders of magnitude by the localized electromagnetic fields of metal nanostructures [26–28]. In particular, the electromagnetic field in the junction between adjacent nanoparticles, occurring for pairs, larger clusters, or even aggregate films of nanoparticles, can be drastically amplified, resulting in an extraordinary enhanced Raman signal. Therefore, the interjunction regions were nicknamed “hot spots” [29,30]. In comparison to the broad fluorescence spectra of organic molecules, the narrow line width feature of their SERS bands guarantees the specificity and permits the multiplex analysis in a single sample. Many researches have shown the great potential of using SERS as a robust tool for the sensitive detection of various biological species, including carbohydrates [31–33], DNA and RNA [34–36], protein [37], bacteria [38–40] and virus [41]. Although a few SERS-based thiols (GSH) detections are found in the literature, the low sensitivity still represents one of the main bottlenecks that need to be overcome. For example, the limits of detection (LODs) obtained through a reversed reported agent-based method [21] or a heat-induced SERS sensing method [20] were 1 μM and 50 nM, respectively. The sensitivity can be improved by using a magnetically-assisted strategy, with a sacrifice of rapidness and simplicity [22]. Considering the ultra-low amount of biothiols in living cells, the development of sensitive and rapid means of detecting and quantifying intracellular thiols is still highly necessary and challenging.

In the present work, we report a novel competitive adsorption-based SERS assay for label-free detection of cellular biothiols with high sensitivity and selectivity, in which 4,4′-dipyridyl (Dpy) molecules were used to shorten the inter-spaces between Au NPs and simultaneously enhance the Raman signature of themselves. Biothiols could be competitively bound to the surface of Au NPs in the presence of Dpy, which led to the disaggregation of Au NPs and SERS quenching of Dpy molecules. Compared with most existing SERS-based approaches, such protocol not only possesses the advantages of operational simplicity and rapidity, but also shows high sensitivity with the picomolar detection limits involving neither pre-concentration nor label operation. The whole process can be accomplished within ~8 min including the spectrum analysis. The method was applied to reliably measure the expression of biothiols from tumor cells. To the best of our knowledge, this is the first example of using a simple binary, competitive adsorption system for sensitive and rapid quantification of biothiols by SERS.

2. Experimental section

2.1. Chemicals

Chloroauric acid (HAuCl₄·4H₂O), sodium citrate dihydrate (Na₃C₆H₅O₇·2H₂O), glutamate (Glu), glycine (Gly) and arginine (Arg) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). 4,4′-Dipyridyl (Dpy), N-ethyl maleimide (NEM) and oxidized form glutathione (GSSG) were purchased from Aladdin Reagent (Shanghai, China), Cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and glucose (Glc) were obtained from Sigma-Aldrich. Ascorbic acid (VC) was purchased from Sangon-Biotech (Shanghai, China). All chemicals were of analytical grade or better and were used as received without purification. Ultra-pure Milli-Q water (18.2 MΩ cm) was used to prepare all the aqueous solutions.

2.2. Apparatus

UV–vis spectra were recorded with an Aglient HP8453 spectrophotometer. Transmission electron microscopy (TEM) images were taken with a JEOL JEM-2011 electron microscope at an acceleration voltage of 200 kV. For TEM imaging, the samples were deposited onto Cu grids, followed by drying in air. SERS measurements were performed on a Horiba XploRA confocal Raman microspectrometer with a 638 nm laser. The samples were excited with the laser power of 2.4 mW and an acquisition time of 10 s. In addition, the samples were added into a 96-well plate for SERS measurement.

2.3. Synthesis and characterization of Au NPs

Citrated stabilized Au NPs were prepared according to Frens’ method [42]. Briefly, 0.4 mL of 1% citrate solution was added into 50 mL of 0.01% AuCl₄·3H₂O boiling solution under stirring. The mixture was then refluxed for 15 min to complete the reaction, during which the initially faint-yellow solution turned dark blue in about 40 s followed by dark red after approximately 90 s. The size of the as-prepared Au NPs was 72.6 ± 6.3 nm, as verified from TEM measurements.

2.4. Sensitivity and selectivity of biothiols detection by SERS

In a typical procedure for GSH, GSSG or Cys detection, 35 μL of different concentrations of GSH, GSSG or Cys aqueous solution was firstly incubated with 200 μL of Au NPs in a 1.5 mL centrifuge tube for 5 min, followed by adding 15 μL of 10⁻⁵ M Dpy into the mixture and reacted for 1 min. Finally, the mixture was added into a 96-well plate for SERS measurement. SERS spectra were recorded using a 10× objective in the wavenumber range from 500 to 2000 cm⁻¹ with an acquisition time of 10 s. Other biologically relevant species such as Glc, VC, Glu, Gly, Arg and Hcy were also detected in the same process to evaluate the selectivity of this approach.

2.5. Measurement of cellular biothiols

HeLa cells were routinely grown on a culture flask in DMEM media at 37 °C in a 5% CO₂ incubator. Cell extract was prepared following the reported literature [22]. When cells reached 80–90% confluency, they were lifted with trypsin-EDTA. The trypsinized cells were suspended in PBS buffer solution and counted by a hemocytometer. About 1.6 × 10⁶ cells were collected after centrifugation and precipitated pellets were mixed with 10% trichloroacetic acid and shaken mildly for 6 h. Next, the sample was centrifuged and the supernatant containing biothiols was collected. The pellet of proteins was washed twice with 10% trichloroacetic acid and the supernatants were mixed together and taken for quantification of biothiols in cell.

The concentrations of cellular biothiols were determined by the standard addition method using GSH as the standard [43,44]. Before measurement, cell extract was diluted 100-fold in order to be consistent with the linear range of our method. Briefly, aliquots (0, 5, 10, and 15 μL) of GSH standard solution (5 nM) and diluted cell extract (20 μL) were added into Au colloid (200 μL) and incubated for 5 min, followed by adding 10 μL of Dpy (10⁻⁴ M) and reacted for 1 min. Then, aliquots of water were added into the solution to give final volumes of 300 μL. In a control experiment, cell extract was pretreated by using NEM (0.1 mM) as a thiol-blocking reagent. Then, cell extract was diluted and reacted with Au NPs and Dpy.

3. Results and discussion

3.1. Label-free SERS detection of biothiols based on the competitive
Scheme 1 illustrates the sensing mechanism for SERS detection of biothiols by using colloidal Au NPs as the SERS enhancing substrate and organic ligand Dpy as the signal reporter. Previous studies have shown that pyridine substituted with an electron-donating group in the para position of the nitrogen heterocycle can bind to gold surfaces in a vertical orientation through the endocyclic nitrogen [45–47]. The Dpy molecule consists of two symmetrically located pyridine rings, each of which can be considered as the para substituent group for its partner ring. Therefore, each Dpy molecule acts as a molecular bridge to bring two Au NPs into close proximity through the formation of two nitrogen–gold bonds, which efficiently decreases the inter-space between neighboring Au NPs, generating large amounts of “hot spots” for a strong electromagnetic enhancement of Raman signals (Scheme 1a). This Dpy triggered enhancement, however, is effectively or even completely inhibited in the presence of biothiols such as GSH/Cys because of the much stronger sulfur–gold bonding energy compared to the nitrogen–gold bond. That is to say, Au colloids will maintain the original monodisperse state owing to the competitive adsorption at the metallic surfaces between biothiols and Dpy molecules, which impedes the formation of plasmon-enhanced optical fields and the observation of the strong Raman fingerprint of Dpy molecules (Scheme 1b). Thus, the stronger sulfur–gold affinity significantly quenches the Raman signals of Dpy in Au NPs aqueous, which provides a highly sensitive and facile means of detecting biothiols.

The competitive interaction-based detection mechanism was firstly confirmed by the UV–vis extinction measurement. The as-prepared Au NPs were dark red when viewed in transmission (no picture given) and had a characteristic plasmon band centered at approx. 532 nm (black solid line in Fig. 1A). Introducing GHS into colloidal solution caused almost no variation in the spectral feature of Au NPs (green dotted line in Fig. 1A), indicating the negligible influence of the adsorption of GSH on the dispersion state of Au NPs. In contrast, when Dpy was added to the suspension of metal colloids, the peak intensity at 532 nm was decreased dramatically, accompanying the appearance of an intense broad band at approx. 835 nm (red dotted line in Fig. 1A), a characteristic band of plasmon coupling between neighboring particles. This suggested that Dpy could significantly shorten the distances of adjacent Au NPs by adsorbing on gold surfaces. However, when GSH was added in the solution, the broad band at 835 nm was not emerged (orange dotted line in Fig. 1A). This indicated that the aggregation of Au NPs induced by Dpy molecules was impeded by GSH, owing to the competitive adsorption on the metal surfaces between GSH and Dpy molecules. In addition, the change of SERS signal of Dpy in the absence and presence of GSH was shown in Fig. 1B. According to the literature, several SERS characteristic bands of GSH are located at 660, 795, 905, and 1414 cm\(^{-1}\) and they are assigned respectively to C–S stretching, –COO\(^-\) bending, C\(-\)COO\(^-\) stretching and –COO\(^-\) symmetric stretching [20]. These are totally different from that of Dpy, indicating that the signatures in Fig. 1B are not contributed by GSH molecules. Therefore, the obviously reduced SERS signal of Dpy in the presence of GSH (Fig. 1B) indicated that the aggregation of Au NPs triggered by Dpy was prevented by GSH. To further validate the detection scheme, we next varied the order of the reagent addition, which was Au-Dpy-GSH or Au-GSH-Dpy. As shown in Fig. S1 in the supporting information, the SERS intensity was decreased in both cases, indicating the rationality of the detection mechanism. However, when GSH was firstly introduced in the solution, the change of signal was more obvious. To improve the sensitivity of this method, the order of Au-GSH-Dpy was chosen in the following test. The above mechanism was also demonstrated by TEM. As shown in Fig. 2A, the as-prepared colloidal Au NPs are monodisperse with the diameter of 72.6 ± 6.3 nm. The aggregation of Au NPs was indeed triggered by the introduction of Dpy molecules (Fig. 2B), which was almost completely inhibited in the presence of Cys even at a very low concentration (14 nM) (Fig. 2C). In order to confirm that the observed aggregation (Fig. 2B) was not caused by self-assembly of NPs during the sample preparation in TEM.
analysis, in situ dynamic light scattering (DLS) was further employed to verify our detection mechanism (Fig. S2). Unsurprisingly, the variation tendency of the Au NPs diameter distribution was in good agreement with that observed in the TEM measurement. It was reported that Cys at high concentrations could induce the aggregation of Au NPs [48,49], however, no evidence of apparent aggregation was observed in the present work mainly because of the low concentration (100 nM) of Cys we employed [50,51]. All these results manifested that the competitive interaction-based method was feasible and sensitive for the detection of biothiols.

3.2. Sensitive detection of biothiols

The influence of Dpy concentrations on the aggregation state of Au NPs was investigated through SERS and UV–vis dual modes. The SERS spectrum of Dpy on Au NPs with several characteristic bands at 1023, 1072, 1229, 1294, 1509 and 1610 cm\(^{-1}\) was shown in Fig. 1B and the assignments of these characteristic bands were listed in Table S1. All these results manifested that the competitive interaction-based method was feasible and sensitive for the detection of biothiols.

![Fig. 2. TEM images of (A) citrate stabilized-Au NPs, (B) of Au NPs in the presence of Dpy, and (C) in the presence of both Dpy and Cys. Scale bars represent 100 nm.](image)

![Fig. 3. (A) Spectral changes of Dpy upon addition of different concentrations of GSH. (B) Intensity of the 1610 cm\(^{-1}\) band as a function of the GSH concentration. Inset: Linear plot of the 1610 cm\(^{-1}\) peak intensity against Dpy concentrations from 0.01 to 0.2 μM. (C) UV–vis spectra of Au NPs at different concentrations of Dpy. (D) Relative absorbance of \(A_{870}/A_{532}\) as a function of Dpy concentrations from 0 to 1000 nM. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)](image)
increased with the addition of Dpy until the concentration of Dpy up to 0.3 μM (Fig. 3A and B), indicating that the aggregation state of Au NPs was intensified and concomitantly formed more and more “hot spots” with the addition of Dpy. Au NPs were sensitive to Dpy because even 10 nM of Dpy could induce the distinct increase in SERS signal at 1610 cm⁻¹ (red line in Fig. 3A), giving a linear dependence ($R^2$ of 0.9754) of the single intensity against the concentrations of Dpy from 10 nM to 200 nM (See insert of Fig. 3B). The UV–vis spectra in Fig. 3C and Fig. 3D depicted the similar results. In Fig. 3C, the absorbance at 532 nm gradually declined with the increase of the concentration of Dpy until 0.3 μM. At the same time, a new band at 835 nm emerged and slowly red shifted to 870 nm. The intensity of this band was also increased until the concentration of Dpy up to 0.3 μM. The new band at the near infrared region strongly indicated that the plasmon couplings were formed between neighboring particles due to the shortage of the inter-particle gaps and the formation of Au clusters by Dpy adsorbed on the gold surfaces. Fig. 3D clearly indicated that the absorbance ratio of 870 nm and 532 nm was increased with the addition of Dpy from 0 to 0.3 μM. The change of the absorbance ratio was small when the concentration of Dpy was higher than 0.3 μM. Based on the above results, 0.3 μM of Dpy was the optimal condition to achieve the highest sensitivity.

In addition, the reaction kinetics between Au NPs and Dpy was further discussed with SERS and UV–vis spectroscopies. As shown in Fig. 4A, upon addition of Dpy, the SERS intensity at 1610 cm⁻¹ increased remarkably and reached a plateau in 2 min, indicating a fast interaction between Au and the pyridine nitrogen. The same result was also observed in the UV/vis measurement, where the relative absorbance ($A_{\text{max}}/A_{532}$) increased as a function of time and reached its maximum at about 2 min (Fig. 4B). Moreover, the reproducibility of the signal intensity is an important parameter for SERS-based detection. We examined the signal fluctuation by estimating the relative standard deviation (RSD) from ten independent measurements, which was found to be 7.8% (Fig. S3), suggesting the good reproducibility of our strategy.

Fig. 5A shows the SERS spectral changes of Dpy molecules after introducing different concentrations of GSH. The SERS intensities decreased gradually with the incremental concentrations of GSH ranging from 0.14 pM to 1400 nM. And a linear relationship between the SERS response and the GSH concentration was observed from 0.7 pM to 7 nM with a detection limit of 0.14 pM (Fig. 5B), which was lower than most SERS-based sensors for biothiols detection (Table S2). Compared with the UV/vis detection (Fig. S4), such SERS-based assay exhibited apparent superiority in terms of its higher sensitivity and wider dynamic range. The good performance could also be achieved for Cys and GSSG and the linear ranges were from 70 pM to 140 nM for Cys and from 0.35 pM to 70 nM for GSSG with good linear correlation coefficients of 0.9947 and 0.9794, respectively. The detection limits of Cys and GSSG were 70 pM and 0.35 pM, respectively (Fig. 5), which were much superior to the results of UV/vis detection (Figs. S5 and S6).

3.3. Selectivity of the competitive interaction-based boithiols assay

To evaluate the selectivity of such SERS sensor, Glc, VC and several common amino acids, namely Glu, Gly and Arg were investigated with the same method. Molecular structures of these substances were summarized in Fig. S7. As expected, the respective introduction of Glc, VC, Glu, Gly and Arg in the Au NPs solution had no remarkable SERS quenching of Dpy even the concentrations of them were ten times higher than that of GSH and Cys (Fig. 6). This can be explained by the lack of the mercapto group in molecular structures of these species, resulting in no competitive interaction with Dpy. We further evaluated the selectivity of this method toward other thiol-containing species, such as Hcys and the result was shown in Fig. 6. It is challengeable to recognize Cys in the presence of Hcys because the molecular structure of Hcys is similar to that of Cys [44,52]. Hcys has only one more methylene than Cys. However, the property of Hcys is different from that of Cys in Au NPs solution. Previous researches have shown that the aggregation capacity for Au NPs was in the order of HCys ≫ Cys > GSH [53,54]. That is to say, compared with the little influence of GSH and Cys on the dispersion of Au NPs, the presence of Hcys could facilitate the aggregation of the colloidal NPs and resulted in the formation of plasmonic “hot spots”. When Dpy molecules located within the enhanced electromagnetic field of “hot spots”, Raman signals of Dpy molecules would be amplified. As shown in Fig. 6, with the same concentration of GSH and Cys, Hcys indeed induced no obvious quenching of the Dpy Raman emission. These results indicated that the as-prepared competitive SERS-based strategy had impressive specificity to GSH, GSSG and Cys.

3.4. Analysis of cellular biothiols

In order to evaluate the biological application of the competitive SERS-based strategy, the detection of total biothiols content in HeLa cells was further investigated. Considering the possible interference from non-specific adsorption of intracellular proteins and other thiol-containing macromolecules on the Au NP surfaces, the trichloroacetic acid-based method was used to extract free thiols from the cells [22]. A control experiment was performed where NEM was respectively added into GSH standard solution.

![Fig. 4](image-url)
and cell extract before the detection for blocking of free thiol groups. Obviously, SERS intensity was decreased after the addition of cell extract, indicating that cellular biothiols competitively bond to the surface of Au NPs (Fig. 7A). In contrast, the pretreatment of the cell extract with NEM resulted in no distinct decrease of the SERS intensity (Fig. 7A). The detections of GSH standard solution with and without NEM had the similar results with cell extract. These observations show that SERS changes are caused by cellular biothiols. The concentrations of cellular biothiols were estimated by the standard addition method [43,44]. The decreased intensity at 1610 cm\(^{-1}\) was plotted against concentration of known amount of GSH added. Concentration of unknown biothiols in the cell extract was calculated from the slope of the straight line (Fig. 7B). The level of biothiols was \(\sim 3.79\) fmol/HeLa cell, which was in good agreement with the reported value [22].

4. Conclusion

We have developed a highly sensitive and rapid SERS sensor for label-free detection of biothiols based on a simple binary competitive adsorption mechanism. Dpy with two symmetric nitrogen heterocycles could dramatically shorten the interspaces between Au NPs, generating a large amount of plasmonic “hot spots” and simultaneously enhancing the SERS signal of itself. Due to the much stronger Au–S covalent bond, the presence of biothiols could weaken or even completely quench SERS emission of Dpy because of competitive adsorption of biothiols on the gold surfaces to protect the particles from direct contact with Dpy molecules. The lower detection limit down to pM level (0.14 pM for GSH) was facilely achieved within a few minutes (< 8 min). The sensor exhibited high selectivity for GSH and Cys that excluded the interference of other important biological substances, even including Hcys without any derivatization process. We had also demonstrated that the competitive SERS-based strategy was sensitive enough to monitor the expression level of cellular biothiols. This SERS-based biosensor with the advantages of operational simplicity, rapidity and high sensitivity could open a perspective protocol for the detection of trace biological thiols.
Fig. 7. (A) Intensity changes at 1610 cm⁻¹ of the assay system for GSH standard solution and cell extract with and without the thiol blocking agent (NEM). (B) Standard addition plot for the determination of biothiols in the cell extract. The error bar represents the standard deviation of three measurements.

Acknowledgment

This work was supported by the National Natural Science Foundation of China (Nos. 21375022, 21170508 and 21105014) and China Postdoctoral Science Foundation (No. 2015M580287).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2016.02.008.

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