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

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ABSTRACT

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′-Dipyridyl (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.

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 defolding 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., Hg^{2+} ions and Cd^{2+} 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

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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 carbohydrate [31–33], DNA and RNA [34–36], protein [37], bacteria [38–40] and virus [41]. Although a few SERS-based thiol (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 simultaneous 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 ($HAuCl_4 \cdot 4H_2O$), sodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$), 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 (Hcys), 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. Ultrapure 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

Citrate 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% $AuCl_4^-$ 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 approximate 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 Hcys 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% CO_2 incubator. Cell extract was prepared following the reported literature [22]. When cells reached 80–90% confluence, they were lifted with trypsin-EDTA. The trypsinized cells were suspended in PBS buffer solution and counted by a hemocytometer. About 1.6×10^6 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^{-5} 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