Reagent- and separation-free measurements of urine creatinine concentration using stamping surface enhanced Raman scattering (S-SERS)

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Abstract: We report a novel reagent- and separation-free method for urine creatinine concentration measurement using stamping surface enhanced Raman scattering (S-SERS) technique with nanoporous gold disk (NPGD) plasmonic substrates, a label-free, multiplexed molecular sensing and imaging technique recently developed by us. The performance of this new technology is evaluated by the detection and quantification of creatinine spiked in three different liquids: creatinine in water, mixture of creatinine and urea in water, and creatinine in artificial urine within physiologically relevant concentration ranges. Moreover, the potential application of our method is demonstrated by creatinine concentration measurements in urine samples collected from a mouse model of nephritis. The limit of detection of creatinine was 13.2 nM (0.15 µg/dl) and 0.68 mg/dl in water and urine, respectively. Our method would provide an alternative tool for rapid, cost-effective, and reliable urine analysis for non-invasive diagnosis and monitoring of renal function.

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References and links


1. Introduction

Urine is an easily-accessible bodily fluid that provides metabolic information, including the renal status [1, 2]. Creatinine, a protein metabolite, is one of the major components of human urine besides urea. Since the content of creatinine excreted into the urine is relatively constant in the absence of renal disease, it is used as an internal standard to normalize variations in other urine analytes. Moreover, the detection of creatinine concentration in the urine is important for renal clearance tests, which monitor the filtration function of the kidney [3]. Serum creatinine concentrations are routinely monitored as an indicator of clinical renal function. In clinical diagnostics, serum and urine creatinine concentrations are measured, and generally a high creatinine level indicates kidney problems. For example, normal levels of blood creatinine are approximately 0.6 to 1.2 mg/dl in adult males and 0.5 to 1.1 mg/dl in adult females. In urine, however, creatinine is found to be 500-2000 mg over a 24-hour period. By simply dividing 500-2000 mg to the average urine volume of 1-2 L in an adult male, the lower limit of urine creatinine concentration is estimated to be ~25 mg/dL.

Due to the importance of creatinine in clinical research, a variety of analytical methods have been developed for detecting creatinine in urine, including Jaffe reaction spectrophotometric method [4], enzymatic method [5], capillary zone electrophoresis [6], high performance liquid chromatography (HPLC) [7], high performance thin-layer chromatography (HPTLC) [8], liquid chromatography tandem mass spectrometry (LC-MS) [9], gas chromatography mass spectrometry (GC-MS) [10], isotope dilution extractive electrospray ionization tandem mass spectrometry (ESI-MS) [11], Raman spectroscopy [12] and surface-enhanced Raman scattering (SERS) [13]. Compared to traditional analytical methods, Raman and SERS methods offer several advantages. They require no reagents or separation, are non-invasive, are capable of qualitative and quantitative measurements, and provide molecular structure information [14–21]. In particular, SERS is a highly-sensitive Raman spectroscopic technique where Raman scattering is enhanced primarily by near-field electromagnetic enhancement due to localized surface plasmon resonance (LSPR) [22]. Recent advances in the field of nanotechnology have paved the way for the development of SERS based detection [23–28].

Most creatinine SERS analyses have thus far been performed on metallic (e.g., Ag and Au) colloidal nanoparticles. In general, Au-based SERS substrates are more stable, nontoxic and biocompatible compared to Ag-based ones, although they have inherently lower (i.e. 10^2-10^3 fold) SERS effects than Ag-based substrates. By using gold colloids, the potential of SERS for qualitative and quantitative creatinine measurements was illustrated by W. R. Premasiri et al. [3], and the measurement of creatinine in human urine at concentrations ranging from 2.56 to 115.2 mg/dl was reported by T. L. Wang et al [29]. Y. Wang et al. [30] performed the detection of creatinine water solution with concentrations ranging from 10 to 280 mg/dl by mixing with silver colloids. R. Stosch et al. [31] described the determination of creatinine in human serum at physiologically relevant levels using silver colloids as SERS substrates. In addition, nanostructured metal surfaces have been employed for SERS measurements. Compared to metallic colloids, a significant advantage of nanostructure based approach is that SERS signals are more stable against sample ionic strength [32]. This is because the ionlc strength can affect the aggregation of metallic colloids and adversely influence reproducibility. H. Wang et al. [13] conducted quantitative analysis of creatinine in the urine of healthy and diabetic patients using Ag-coated parylene nanostructures as the SERS substrate, and successfully detected as low as 6.1 mg/dl urine creatinine. K. W. Kho et al. [32] analyzed urine samples in a microfluidic device embedded with Au-coated...
polystyrene nanosphere arrays as the SERS substrate. Among existing reports, the lowest detectable concentration was 0.1 μg/ml (~0.88 μM) in water [31], and 2.56 mg/dl in real urine samples [29].

Although SERS has the potential for creatinine sensing, both the limit of detection and reproducibility need further improvement for practical application. As mentioned previously, colloidal SERS substrates suffer from sample ionic strength dependent aggregation. Planar nanostructures, on the other hand, may not provide low enough detection limit, because the surface area within the source laser footprint is small and the light-matter interaction is limited [33]. This calls for the development of robust, uniform, and reproducible SERS substrates and reliable measurement techniques. Recently, our group has developed nanoporous gold disk (NPGD), which is hierarchical with the external disk shape and the internal three-dimensional (3D) porous network. NPGD features large specific surface area, high-density plasmonic hot-spots, and tunable plasmonics [34–36]. We have demonstrated high-performance SERS substrates [24], efficient photothermal conversion and light-gated molecular delivery [37], and biomolecular detection in microfluidics [25, 26]. Based on NPGD SERS substrates, we have reported a technique called stamping SERS (S-SERS) for label-free, multiplexed molecular sensing, and large-area, high-resolution molecular imaging [38]. This technique provides several benefits such as reagent- and separation-free, low cost, high sensitivity and reproducibility.

In this work, we employ S-SERS to measure creatinine concentrations in samples of different levels of complexity. We first study the sensing reproducibility of our method using creatinine dissolved in water. Next, we demonstrate the detection limit, robustness and multiplexing capability using pure creatinine in water, mixtures of creatinine and urea in water, and artificial urine spiked with creatinine. Further, we perform creatinine concentration measurements in urines samples collected from a mouse model of nephritis.

2. Experimental

2.1 Sample preparation

Creatinine (C4255, anhydrous, ≥98%) and urea (U5378, powder) were purchased from Sigma-Aldrich. Sample solutions of individual molecules were prepared by dissolving the powders in deionized (DI) water, and then diluted to various concentrations. Sodium chloride, potassium, and sodium phosphate (monobasic, monohydrate) were purchased from Macron Chemicals. Artificial urine was prepared using 24.2 g urea, 10 g sodium chloride, 6.0 g potassium, and 6.4 g sodium phosphate (monobasic, monohydrate) dissolved in 1 L DI water. Polydimethylsiloxane (PDMS, Sylgard 184, Dow corning) was prepared by thoroughly mixing base and curing agent at a weight ratio of 10:1, and then poured over the bottom surface of a petri dish, degassed, and finally cured on a hot plate at 70 °C for 1 h.

2.2 NPGD substrate preparation

In our experiments, large-area, uniform, and reproducible NPGD arrays patterned on Au-coated silicon substrate were utilized as SERS substrates. NPGDs were fabricated with Ag-Au alloy (Ag₇₀Au₃₀, atomic ratio 70:30) using a process similar to nanosphere lithography (NSL). The detailed procedure was reported in our previous work [38]. Briefly, the fabrication process started with depositing layers (20 nm Cr/ 200 nm Au) by evaporation on a clean silicon wafer, and then a 120 nm-thick Ag/Au alloy layer by sputtering. After that, a monolayer of 460 nm-diameter polystyrene (PS) beads serving as masks was deposited onto the surface. A two-step plasma treatment was performed subsequently: oxygen and argon treatment to shrink PS beads and etch away the unprotected portion of the alloy film, respectively. Finally, NPGDs were formed by removing PS residues by sonication in chloroform, and dealloying in concentrated nitric acid.
2.3 SERS measurements

SERS measurements were performed using a home-built line-scan system with a 785-nm continuous-wave (CW) Titanium:Sapphire laser (3900S, Spectra-Physics) [39]. The excitation laser was shaped into a 1 X 133 $\mu$m$^2$ line at the sample plane. The SERS spectra were recorded by a spectrograph-charge coupled device (CCD) system (LS-785, PIActon) controlled by Winspec software (PIActon), and post-processed using Matlab (Mathworks). Spectroscopic backgrounds were approximated by a 5th order polynomial and removed [40]. Image curvature due to off-axis light incidence and the large-area CCD detector was corrected by a software technique [12, 41].

3. Results and discussion

Figure 1(a) shows the sample configuration used in the experiment. First, a 1 $\mu$L droplet of the prepared solution containing the target molecules (i.e. creatinine) was first pipetted onto a PDMS thin film (~1 X 1 cm$^2$, ~100 $\mu$m thick) laid flat on a glass coverslip. The droplet was then dried on the PDMS substrate, forming a film of target molecules after solvent evaporation. After that, a NPGD substrate (~0.5 X 0.5 cm$^2$) was stamped onto the PDMS surface bearing dried target molecules. Finally, the laser was focused at the PDMS surface to detect SERS signals from the sandwiched target molecules. Compared to directly drying the droplet onto the NPGD substrate, where target molecules are permanently chemically bound to the SERS substrate, the related issues like competitive adsorption among different molecules, surface and molecule affinity variability and uncertainty are reduced to some extent by the proposed S-SERS technique. Figure 1(b) shows the schematic of the NPGD arrays serving as Raman signal enhancers. Scanning electron microscope (SEM) images recorded are shown in Figs. 1(c) and 1(d) to illustrate the structure and morphology of the NPGD arrays. The fabricated NPGDs have average diameter, thickness, and pore size of ~300, 75, and 14 nm, respectively.

![Fig. 1. S-SERS configuration and NPGD substrate: (a) Photograph of the sandwich scheme used in the experiment for creatinine detection. (b) Schematic of NPGD based SERS substrate. SEM images of NPGD arrays at the magnification of 100,000 X (c) and 250,000 X (d). The scale bars in SEM images are 200 nm.](image)

3.1 Reproducibility of S-SERS for creatinine analysis

A 1 $\mu$L droplet of 100 $\mu$M creatinine was pipetted and dried on the PDMS surface, resulting in a spot with a diameter of ~0.5 mm. NPGD substrate was then gently stamped against the PDMS film with creatinine until intimate contact was formed, after which the sample was measured from the coverslip side. SERS spectra were acquired with 30 mW laser power and 10 sec acquisition time each. The laser power density was estimated to be 0.25 mW per $\mu$m$^2$. Figure 2 shows highly-reproducible SERS spectra from ten randomly chosen locations 100...
μm away from the circumference of the drying mark of creatinine droplet, where dominant SERS peaks corresponding to creatinine, i.e. 573, 602, 671, 836, 900, 1245, 1417, 1645, and 1707 cm$^{-1}$ [42] were identified. As shown in Fig. 2(b), the relative intensity variations of the major creatinine peaks were calculated to be within $\pm 12\%$ of the average intensity, when repeatedly assayed from different locations. Similar reproducibility has been obtained from previous NPGD substrates.

![Fig. 2. Reproducibility of S-SERS: (a) SERS spectra of 100 μM creatinine detected by S-SERS at ten different locations, and (b) relative intensity variations of major peaks for the ten locations.](image)

### 3.2 SERS detection of creatinine water solutions with different concentrations

In order to further assess the sensing capability of S-SERS for creatinine analysis, measurements were performed for creatinine water solutions of different concentrations ranging from 100 nM to 100 μM. The measurements for each creatinine concentration were performed five times with 30 mW laser power and 20 sec CCD integration time. The resulting averaged SERS spectra are shown in Fig. 3, where major creatinine Raman peaks were identified in all spectra, and the peak intensity increased with increasing creatinine concentration. The Raman spectrum of 10 μM creatinine before stamping is also presented at the bottom for comparison (black line), where creatinine peaks can hardly be identified. In contrast, S-SERS provides excellent creatinine spectrum at concentrations as low as 100 nM. The limit of detection of creatinine in water is estimated to be 13.2 nM based on the signal to noise ratio (22.7) at 100 nM. The inset shows a good linear relationship of intensity variations of the 836 cm$^{-1}$ peak as a function of creatinine concentration within the range of 100 nM to 100 μM.
3.3 Multiplexed SERS detection of creatinine and urea water mixture

Since urea is a dominant chemical constituent in urine samples, a reagent- and separation-free technique needs to provide selectivity based on intrinsic molecular fingerprints. To demonstrate the multiplexed sensing capabilities of S-SERS, we have performed measurements on samples by mixing 100 μM creatinine and 100 mM urea solutions at five different volume ratios, namely 10:1, 10:2, 10:3, 10:4, and 10:5. The resultant concentration of creatinine ranges from 66.7 to 90.9 μM, and urea ranges from 9 to 33 mM. Both concentration ranges correspond to physiological concentrations in urine, and are relevant for practical urine analysis. Five measurements were conducted for each volume ratio to obtain the averaged spectra and standard deviation. The averaged SERS spectra of creatinine and urea mixture at five different ratios are shown in Fig. 4. We can identify correlation between peak intensity and concentration for both creatinine (i.e. at 602, 671, 836 and 900 cm\(^{-1}\)) and urea (i.e. at 1003 cm\(^{-1}\)). The results show that concentration-dependent creatinine SERS can be obtained even in the co-presence of ~100X higher concentration of urea. In addition, we have compared the creatinine peak intensity from pure creatinine and mixture of creatinine and urea (i.e., Fig. 3 versus Fig. 4). The intensity at ~836 cm\(^{-1}\) from the mixture (Fig. 4) was about 17% lower than that from the pure sample (Fig. 3). This could be attributed to preferential surface crowding: urea, besides being present at higher concentration, may occupy more surface area on the PDMS film than creatinine. In addition, high concentration urea would change the refractive index, thus affecting laser spot size and collection efficiency.
3.4 SERS detection of creatinine in artificial urine

In order to test the capability of S-SERS for quantitative analysis of creatinine in a more complex mixture, we spiked creatinine in artificial urine with resultant concentrations of 10, 25, 50 and 200 μM. Each sample was measured five times, and the averaged SERS spectra are shown in Fig. 5. From spot to spot, the urea signature peak at 1003 cm\(^{-1}\) shows an intensity variation of less than 5%, which further indicates the high reproducibility of S-SERS. Moreover, we find that with the increase of creatinine concentration, the major peaks for creatinine (i.e. 602, 671, 836 and 900 cm\(^{-1}\)) increased in intensity. The inset indicates the variations of creatinine peak intensity at 836 cm\(^{-1}\) as a function of creatinine concentration, and a good linearly relationship is observed. We have also compared the creatinine peak intensity at the same concentration (i.e. 10 μM) from pure creatinine versus creatinine in artificial urine (i.e., Fig. 3 versus Fig. 5). The intensity at ~836 cm\(^{-1}\) from the doped artificial urine sample (Fig. 5) was about 35% lower than that from the pure creatinine sample (Fig. 3). We believe this was primarily due to preferential surface crowding by other molecules in the mixture.
3.5 SERS detection of creatinine in nephritic mouse nephritic mouse urine samples

We further employed S-SERS to measure creatinine in urine samples collected from nephritic mice with anti-GBM disease induced as described before [43, 44]. The “ground truth” creatinine concentrations in these samples from different mice were determined using a commercial colorimetric assay kit based on a coupled enzyme reactions (#80350, Crystal Chem, Inc.) with a detection limit ~0.15 mg/dl according to product specifications. In this assay, the creatinine is broken down to creatine, and then converted to sarcosine. The sarcosine is oxidized to hydrogen peroxides, which react with peroxidase, and can be quantified by measuring light absorption at 550 nm. The entire assay from warming up reagents to room temperature to calculating creatinine concentration would take ~1 hour at the very least. Figure 6(a) shows averaged SERS spectra of the different mouse urine samples with creatinine concentrations at 2.08, 6.16, 12.55, 22.55, and 30.30 mg/dl. We note that 1 mg/dL corresponds to 88.4 μM for creatinine in water. The major SERS peaks of creatinine at 602, 671, and 836 cm\(^{-1}\) can once again be identified in the mouse urine samples. A good linear relationship is observed by plotting the peak intensity at 836 cm\(^{-1}\) versus creatinine concentrations as shown in Fig. 6(b). Although the lowest concentration in this experiment was 2.08 mg/dl, the limit of detection is estimated to be 0.68 mg/dl based on the signal to noise ratio (9.14) at 2.08 mg/dl. When one compares the creatinine peak intensity at ~836 cm\(^{-1}\) from real urine to that of creatinine water solution at the same concentration (i.e. Fig. 3), we find that the intensity from real urine is about 6-fold lower than that from the creatinine water solution. Besides preferential surface crowding from other urine analytes and proteins, this difference may relate to the non-transparent, dark yellow color of the urine, which might have caused light attenuation and local refractive index changes. The results show that this new approach provides a reagent- and separation-free method for creatinine concentration measurements in real urine samples. It is remarkable that S-SERS is still able to capture the creatinine-specific signature despite the co-existence of >70 proteins in these nephritic urine samples [44]. This is attributed to the intrinsic molecular fingerprinting capability of Raman and SERS spectroscopy.
4. Conclusions

In this work, we have developed a reagent- and separation-free method to determine creatinine concentrations in urine based on stamping SERS and nanoporous gold disk SERS substrates. The reproducibility of our method has been demonstrated by detecting 100 μM creatinine with relative intensity variations ranging from −12% to 12% across multiple samplings. The detection of creatinine water solutions in the 100 nM to 100 μM range has been demonstrated with good linearity, representing the first demonstration of sub-μM creatinine sensing by any reagent- and separation-free technique. Moreover, excellent concentration-dependent creatinine sensing was successfully demonstrated in samples of increasing complexity: urea water mixture, artificial urine, and urine from nephritic mice. In particular, an unprecedented detection limit of ~0.68 mg/dl has been achieved in urine samples from nephritis mice. This is a significant milestone because it approaches the performance of commercial creatinine kits, however, without the need for reagent and time-consuming assays. We anticipate that this method will provide a viable alternative for urine creatinine quantification without the need for reagent or separation. Such as technology would provide invaluable information and utility in renal function monitoring and disease diagnosis at both hospitals and point-of-care scenarios.

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