Quantitative analysis of creatinine in urine by metalized nanostructured parylene

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Abstract. A highly accurate, real-time multisensor agent monitor for biomarker detection is required for early detection of kidney diseases. Urine creatinine level can provide useful information on the status of the kidney. We prepare nanostructured surface-enhanced Raman spectroscopy (SERS) substrates without template or lithography, which provides controllable, well-organized nanostructures on the surface, for the quantitative analysis of creatinine concentration in urine. We present our work on sensitivity of the SERS substrate to urine samples collected from diabetic patients and healthy persons. We report the preparation of a new type of SERS substrate, which provides fast (<10 s), highly sensitive (creatinine concentration <0.5 μg/mL) and reproducible ($<5\%$ variation) detection of urine. Our method to analyze the creatinine level in urine is in good agreement with the enzymatic method. [DOI: 10.1117/1.3369002]

Keywords: urine; Raman; surface-enhanced Raman spectroscopy; creatinine; nanostructured poly(chloro-p-xylylene).

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1 Introduction

Acute renal failure (ARF) occurs in 5 to 7% of hospitalized patients[1] and results in a mortality rate of about 50%.[2] A particularly disturbing feature has been the inability since the mid-1960s to substantially lower mortality rates in acute renal failure.[3] The financial costs of acute renal failure are estimated to be 8 billion dollars per year, or about $130,000 per life-year saved.[3] It is unlikely that this high mortality and associated cost will be reduced until we have better tools for the early diagnosis of renal injury. Accordingly, a critical need exists to develop clinically useful markers of early kidney injury and clinically efficient means for their detection. Several authors have pointed out the deficiencies of standard measures of kidney function, e.g., serum creatinine, for the early detection of acute renal failure.[4,5] Early detection of ARF is essential to reduce the incidence, progression, and associated morbidity and mortality of these common problems. Currently, point-of-care testing for ARF is limited due to the lack of sensitivity of common markers, such as serum creatinine, and the complexity and expense of measuring more sensitive and specific biomarkers. The lack of real-time detection capabilities for ARF biomarkers also severely impedes progress in clinical research, which relies on the early identification of affected individuals. The use of detection technologies in doctor offices or at the bedside will require a sensitive, selective, and portable sensor. Recent advances in the fields of nanotechnology have enabled the development of a new class of detection based on surface-enhanced Raman spectroscopy (SERS), which provides highly accurate, portable, real-time detection technology for urine biomarkers. Here, we present a highly sensitive and robust SERS substrate for urine analysis.

Urine analysis is one of the most commonly used approaches to receive information of renal function and metabolic condition of the body. The feasibility of Raman spectroscopy for urine analysis has been investigated by several researchers. Dou et al.[4] and McMurdy and Berger[5] studied the quantitative Raman analysis of creatinine in human urine. Premasiri et al.[6] studied the potential of the SERS method for analyzing creatinine in urine using gold colloids as the SERS active substrate. Wang et al. reported the semiquantitative measurement of creatinine concentration in human urine samples also using gold colloids as the SERS active substrate.[7] Recently, urine samples have been analyzed on a SERS-based microfluidics device[8] using an array of polystyrene nanospheres as the SERS substrate. Furthermore, Qi and Berger[9] described the quantitative measurement of creatinine by Raman spectroscopy and optical fiber technique. Most of the SERS analyses of urine have been done on a SERS active metal colloid-based system. SERS has remarkable analytical sensitivity, but the difficulty in preparing robust and uniform SERS substrates with surface morphologies that can deliver maximum SERS enhancement with high reproducibility is a major problem for detection.[10]

We demonstrate the use of a nanostructured SERS substrate in urine analysis of healthy and diabetic patients. We showed that the SERS spectra of urine samples are highly reproducible, resistant to differences in ionic strength, and the concentration of chemical components can be measured quan-

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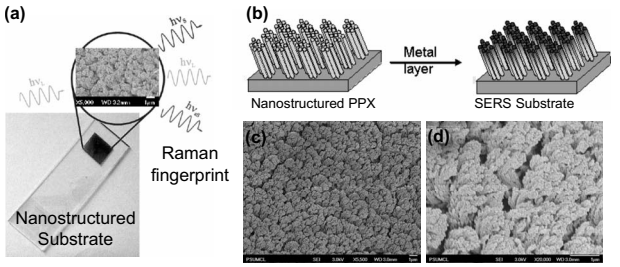

Fig. 1 (a) Surface-enhanced Raman substrate and (b) schematic of SERS substrate preparation are shown. The surface area of the SERS-active region is approximately 1 cm^2. SEM image of the Ag-PPX-Cl SERS substrate at (c) low magnification (5500×) and (d) high magnification (20,000×). Scale bar for SEM images is 1 μm.

titatively with high sensitivity (i.e., creatinine concentration as low as 0.5 μg/mL). Preparation of these SERS active substrates are inexpensive and easy to prepare.[11–13] The creatinine levels of urine samples from diabetic patients and healthy persons were measured quantitatively using metalized nanostructured parylene substrates. As a result, we showed the suitability of using our SERS substrates in urine analysis.

2 Results and Discussion

We recently demonstrated that nanostructured poly(chloro-p-xylylene) (PPX-Cl, also known as parylene) films can be fabricated by the oblique angle polymerization (OAP) method.[11–13] The nanostructured PPX films are deposited on a substrate from a directional vapor source. Vapor deposition and polymerization at an oblique angle relative to the substrate surface permits the fabrication of films possessing nanostructured morphology. The nanostructured PPX film comprises free-standing, slanted, parallel columns containing nanowires. Subsequently, we deposited a thin layer (i.e., ~60 nm) of Ag metal film onto the nanostructured PPX template. Figure 1(a) shows the metalized nanostructured PPX film coated on a glass slide. Figure 1(b) shows the schematics of nanostructured PPX film metallization. Scanning electron microscope (SEM) images show the nanostructured morphology of the metalized PPX film at two different magnifications [Figs. 1(c) and 1(d)]. Ag nanoparticles (~60 nm) cover the nanostructured PPX films uniformly. The SERS substrates have high uniformity (~5% signal variation in a 1 mm^2 area) and sample-to-sample reproducibility.[14] The enhancement factor (EF) was calculated as 10^5 for the Ag metalized substrates.[14,15]

Raman spectra of pure creatinine, artificial urine, and urine from a diabetic patient were measured on the nanostructured PPX-Cl substrate. Significant peaks at 700, 840, 900, and 1420 cm^{-1} were observed in the Raman spectra of creatinine [Fig. 2(a)]. However, in urine measurements, we found that 840- and 900-cm^{-1} peaks are stronger than the 700-cm^{-1} peak.

A significant advantage of our nanostructured substrate over traditional metal colloids is the enhanced stability of Raman signals when detecting samples with different ionic strengths. The variation of the ionic strength of the analyte can affect the aggregation process of metal colloids. It has been reported that the reproducibility of Raman signal drops drastically if the salt content of the urine varies.[8] In contrast, our nanostructured SERS substrates show superior stability due to the immobilized metal nanoparticles on the surface of the SERS surface. Figure 2(b) shows robustness of SERS signal with various potassium chloride (KCl) concentrations. The urea peak at 1000 cm^{-1} shows an intensity variation of less than 5%. Each spectrum is shifted for clarity. A concentration profile for varying creatinine concentration in artificial urine is measured.

The reproducibility of our SERS substrate is demonstrated using 1 to 3 μl of urine sample from a diabetic patient. 12 spots were chosen randomly on the surface of the nanostructured substrate to collect Raman spectra [Figure 3(a)]. From spot to spot, the urea signature peak at 1000 cm^{-1} (symmetrical C-N stretch) shows an intensity variation of less than 5%. This major peak can be used as an internal reference for urine

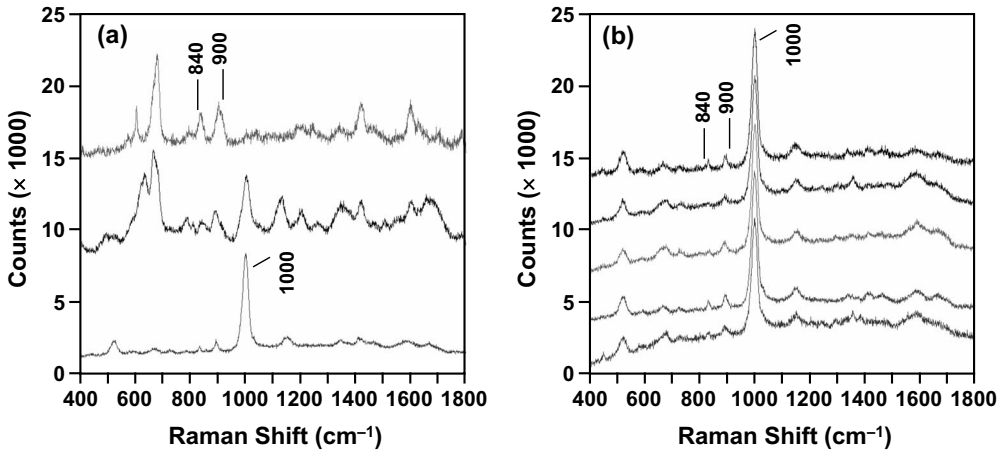

Fig. 2 (a) SERS spectra of: a creatinine solution (0.5 mM) (top), artificial urine solution composed of urea, creatinine, and uric acid (middle), and a urine sample (bottom). Artificial urine concentrations are 1639, 104, and 34 mg/dl, respectively, which is approximately the same level of healthy human urine.[18] All SERS measurements are colleted at 10-s acquisition time with four scans. (b) SERS spectra taken with different amounts of KCl added to a urine sample. Concentrations of KCl (from top to bottom) are 0 M, 0.5 mM, 5 mM, 50 mM, and 0.5 M, respectively (10-s acquisition time and four scans).

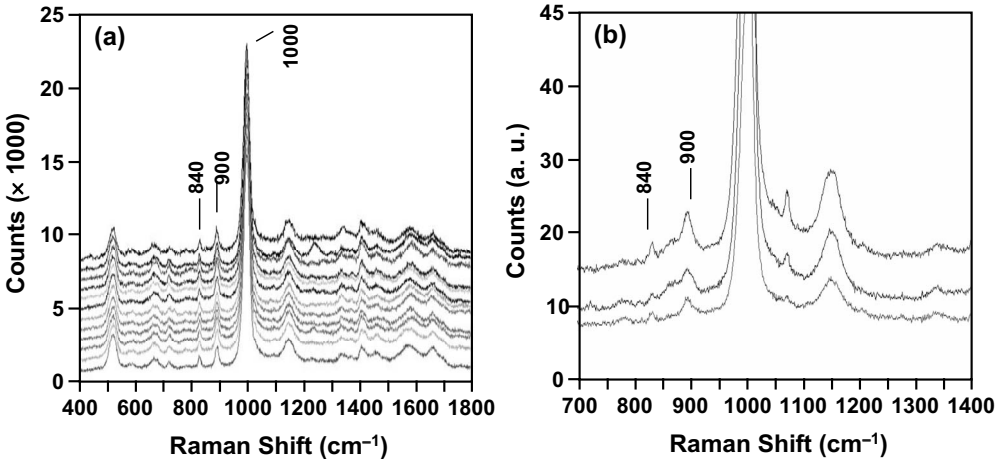

Fig. 3 (a) Reproducibility of SERS spectra of a urine sample. (b) SERS spectra from artificial urine samples. Concentrations of creatinine (from bottom to top) are 0.5, 5.1, and 10.2 μg/mL respectively.

analysis, considering the fact that urea is the dominant organic component of human urine, and the excretion rate of urea in urine is relatively stable. Figure 3(b) shows SERS spectra of creatinine at 0.5-, 5.1-, and 10.2-μg/mL concentrations, respectively. SERS intensity at 840 and 900 cm^{-1} are measured from artificial urine samples. These results also show that creatinine concentrations as low as 0.5 μg/mL can be detected easily by SERS method using our nanostructured substrate. Mass spectroscopy technique, commonly used in clinical chemistry, can detect creatinine concentrations of 0.5 μg/mL, with an uncertainty of approximately 3%.[16] Our substrate provides a similar sensitivity and reproducibility; hence it may be used as a quantitative technique in clinical measurements in the future.

Table 1 provides details of Raman measurement parameters and creatinine concentrations measured by enzymatic assays. The creatinine concentrations of clinical urine samples are measured by Diazyme's enzymatic creatinine reagent kit. This assay is known to have no interference from ascorbic acid, bilirubin, hemoglobin, or triglycerol. In this assay, creatinine is converted into creatine, and then transferred into sarcosine. The sarcosine is oxidized to hydrogen peroxide, which is measured by a Trinder reaction.

Figure 4(a) shows the quantitative measurement of creatinine from diabetic and healthy patients. Two peaks at 840 and 900 cm^{-1} are chosen in the SERS spectra for the quantitative analysis of creatinine. Creatinine has two major peaks at 840 and 900 cm^{-1}, but there is spectral overlap from other components of urine in the same region (e.g., urea and creatinine at 840 and 900 cm^{-1}). Therefore, the SERS data are analyzed by an ordinary least-squares method to generate linear predictive models of creatinine concentration of urine samples. There is a linear relationship between creatinine concentrations and SERS peak areas at 840 and 900 cm^{-1}. Percentage of variance R^2 equals 0.907 and 0.967, for 840 and 900 cm^{-1} data, respectively. The linear model is also analyzed with residual plots,[17] and the p-values were calculated by the Anderson-Darling test. We conclude that the residuals are normally distributed, since the p-values (0.514 and 0.245 for SERS data at 840 and 900 cm^{-1}, respectively) are greater than 0.05 (level of significance). Figure 4(b) shows the creatinine concentrations measured by SERS and the enzymatic method. The SERS data are in good agreement with the enzymatic data. The correlation coefficients are 0.907 (based on the SERS peak at 840 cm^{-1}) and 0.968 (based on the SERS peak at 900 cm^{-1}), respectively.

Table 1 Creatinine concentration of urine samples from diabetic patients and healthy samples, and Raman data collection parameters are shown.

Patient ID	Standard creatinine concentration (mg/dL)	Scan times (10 s/scan)	Laser power (%)
CR24 (M, diabetes type 1)	126.3	8	5
HD46 (F, diabetes type 2)	100.1	8	5
HK17 (F, diabetes type 2)	65.5	1	10
MC42 (F, diabetes type 2)	52.0	16	5
SR45 (M, diabetes type 2)	114.2	4	10
SV29 (F, diabetes type 1)	114.2	1	10
TJ008 (M, diabetes type 2)	6.1	1	5
RN37 (F, diabetes type 1)	54.7	1	10
GC006 (F, diabetes type 2)	73.0	1	10
ML36 (M, diabetes type 2)	158.1	1	10
MB38 (F, diabetes type 2)	41.8	16	5
MR53 (healthy control)	173.0	1	10
AS19 (healthy control)	99.3	1	5

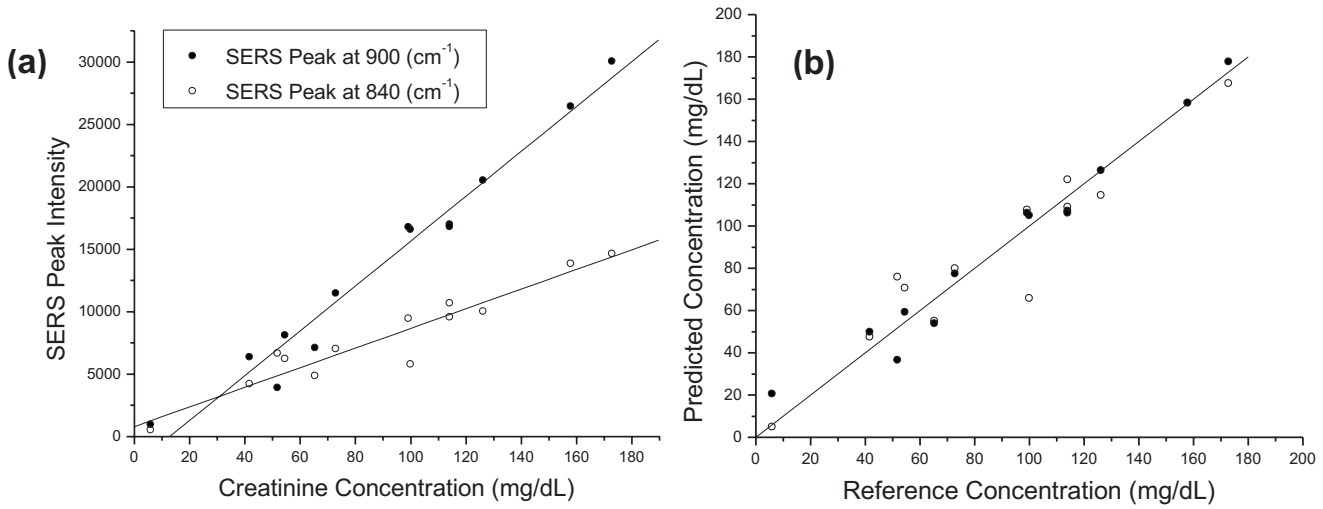

Fig. 4 (a) Creatinine concentration of 13 clinical urine samples and corresponding SERS peak area at 900 and at 840 cm^{-1} are plotted. (b) Creatinine concentration of 13 clinical urine samples from SERS and as enzyme-based method are plotted. Open and closed circles denote SERS peak at 840 cm^{-1} (correlation coefficient equals 0.907) and at 900 cm^{-1} (correlation coefficient equals to 0.968), respectively.

3 Experimental

3.1 *Materials*

All chemicals were of ACS reagent grade and were used as-received. Deionized water of 18.1 MΩ from a Barnstead Nanopure Diamond dispenser was used for all experiments. Poly(chloro-p-xylylene) (PPX-Cl) films were prepared from dichloro-[2.2]paracyclophane (DCPC), purchased from Parylene Distribution Services (Katy, Texas) and deposited on *p*-type Si (100) wafers (Wafernet, Incorporated, San Jose, California). Diazyme's enzymatic creatinine assay kits were purchased from Diazyme Laboratory (Poway, California). Urine samples were collected from 11 diabetic patients and two healthy persons under a protocol approved by the Institutional Review Board of the Pennsylvania State Hershey Medical Center, and stored in −80 °C before testing.

3.2 *Nanostructured Poly(chloro-p-xylylene) Film Preparation*

Silicon wafers were first washed in deionized water and dried under nitrogen flow, and then added into a 1:1-v/v solution of HCl and methanol. After 30 min, silicon wafers were removed and sonicated in deionized water for 10 min and dried under nitrogen flow. The silicon wafers were then kept in concentrated sulfuric acid for another 30 min, after which they were sonicated again in deionized water for 10 min. After thoroughly dried under nitrogen flow, the silicon wafers were immersed into a toluene solution containing 1% allyltrimethoxysilane (Gelest, Pennsylvania) and 0.1% acetic acid at room temperature to form a self-assembled monolayer (SAM) on their surfaces. The silicon wafers were removed from the solution after 60 min, sonicated in anhydrous toluene for 10 min, and then dried under nitrogen flow. The silicon wafers were heated on a hot plate at 150 °C for 4 min to bind the SAM onto the silicon surface. Nanostructured PPX-Cl films were deposited onto the wafers using 0.3 g of DCPC. The vaporizer and the pyrolysis chamber temperatures were maintained at 175 and 690 °C, respectively. The angle between the substrate and the flux was held at 10 deg.

3.3 *Metal Deposition*

The preparation of substrate for SERS experiments was started from the structured PPX-Cl film templates. The silver was thermally deposited from resistively heated tungsten and tantalum boats onto the surface at about 1×10^{-8} Torr base pressure in a cryogenically pumped deposition chamber.

3.4 *Scanning Electron Microscope*

High-resolution SEM images of the metallized PPX films were obtained using a field emission scanning electron microscope (Fesem, JEOL 6700F, Japan) operated at 3-kV accelerating voltage.

3.5 *Surface Enhanced Raman Spectroscopy Measurements*

Renishaw inVia microRaman equipment (Renishaw, Gloucestershire, United Kingdom) was used for studying the SERS substrate. The instrument consisted of a 35-mW HeNe laser (632.8 nm) as the source, a motorized microscope stage sample holder, and a CCD detector. Raman spectra were collected at 5 to 10% laser power. The motorized microscope stage allowed SERS maps of the surface to be formed. The instrument parameters were 50× objective and 10-s acquisition time. 1 to 16 scans were used to obtain high signal-to-noise ratios (SNRs) if needed. The data are plotted without filtering or smoothing in all figures. The surfaces of SERS substrates were treated by a UV-Ozone cleaner for 2 min, then 5 μL of a urine sample was added onto the substrate and dried in air. No dilution or pretreatments were needed for the urine samples.

3.6 *Urine Collection*

Urine samples from patients were collected at the nephrology department of Penn State Medical School under a designated

Institutional Review Board (IRB). Artificial urine was prepared using 36.4 g of urea, 15.0 g of sodium chloride, 9.0 g of potassium, chloride and 9.6 g of sodium phosphate (monobasic, monohydrate) dissolved in 1.5 L of water.

4 Conclusion

We use SERS to quantitatively measure the creatinine level of urine samples from diabetic patients and healthy persons using our metalized nanostructure parylene (PPX-Cl) film as a SERS substrate. The reproducibility and robustness of our SERS substrate are demonstrated at various ion concentrations. The advantage of these new types of substrates is that no template or lithography is involved, thus providing a simple, inexpensive, and quick production method to achieve highly sensitive and spatially uniform signals. We demonstrate a highly sensitive (i.e., concentration as low as 0.5 μg/mL) and reproducible (i.e., signal variation less than 5%) substrate that can be used in clinical measurements. Rapid data analyses (i.e., 10-s integration time) at low laser power (i.e., 2.5-mW laser at 632 nm) can be performed to determine creatinine concentration in urine. These advantages may improve the feasibility of using SERS as a fast, effective, and inexpensive tool for urine analysis in hospitals. We should also note that these data were collected at 2.5-mW laser power and 10-s integration time, which can be preferable for integration of these substrates into a low-power handheld diagnostic device.

Our method is also applicable to blood creatinine level measurements. The normal creatinine level in blood is about 0.5 to 1.2 mg/dl,[16] which can be detected by our SERS substrate. However, a preseparation (e.g., centrifugation and filtration) may be required to remove the interfering constituents. We will focus on the feasibility of selective biomarkers and metabolites in blood in the future.

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