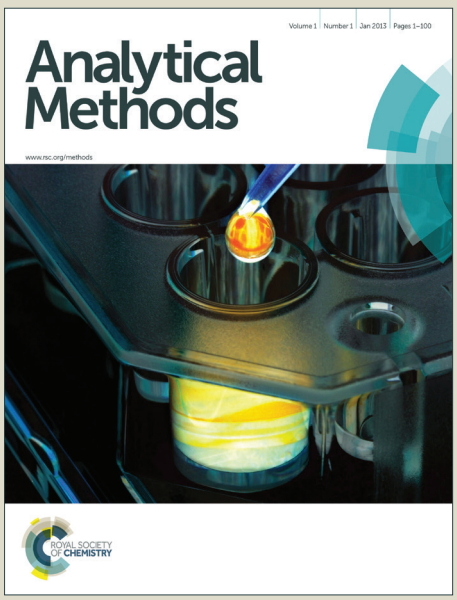
# Analytical Methods

Accepted Manuscript





ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

# Brushing, a simple way to fabricate SERS active paper substrates

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***Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX***
**DOI: 10.1039/b000000x**

A simple and facile method had been demonstrated to fabricate low-cost surface enhanced Raman scattering (SERS) active microfluidic paper chips using a painting brush. This strategy solved the problem of mass production of high reproducible SERS substrates without complicated or bulky micro- or nanofabrication instrument. Rhodamine 6G (R6G) was chosen as a probe molecule to evaluate the performance of SERS active chip. To further demonstrate the possibility of this method's potential application in environmental monitoring, trace malachite green (MG) was successfully analyzed on this chip. The performance of our chips was desirable. The paper substrate deposited with silver nanoparticles by brush was found to be cost-efficient, highly sensitive (LOD for R6G and MG are 1 nM and 10 nM, respectively) and good reproducibility (~15% relative standard deviation).

## Keywords

Surface-enhanced Raman scattering / Silver nanoparticles / Paper substrates/ Microfluidic

## Introduction

SERS is an excellent spectroscopy technique that can provide non-invasive detection, trace analysis and ultrasensitive determination[1-4]. Since it was firstly observed by Fleishcman et al in 1974[5], SERS has attracted much attention and been applied to chemical, biology analysis, environmental monitoring and food safety[1]. It is well-known that the two main mechanisms accounting for the origin of SERS are electronic mechanism and chemical mechanism[6,7]. The enhancement factor (EF) is commonly up to 6~8 orders, but in some reports, the EF can be 14~15 orders[3,8,9] that makes SERS detection limit down to single molecular level. SERS active platforms are generally based on various metallic substrates, such as metal films, nanowires, nanoflowers and nanoshells etc[10-14]. To date, most SERS active substrates usually require complex microfabrication or nanofabrication[15,16] by lithographic techniques such as electron beam lithography, focused ion beam (FIB) and nanosphere lithography. However, the bulky and expensive equipment with long time fabrication process is still a challenge. Herein, the key to expand the application of SERS is to find a simple way to implement low-cost fabrication of reproducible SERS substrates.

The rapid development of microfluidics has gained a lot of attention[17-20]. Moreover, increasing efforts have been invested into the paper-based microfluidic investigation because it represented a cheap and user-friendly way to realize simple operation, lightweight to transport and easy-to-use. In 2007, Whitesides's group proposed the concept of microfluidic paper analytical devices (μPADs) [21-23] and the origin of this technique could be traced back to 1949, that Müller used paraffin impregnation method to design channels[24]. Due to the great advantages of the inexpensive price, prominent device portability and low sample consumption, a large number of techniques including colorimetric, electrochemical, chemiluminescence and biochemistry have been reported on this versatile paper-based microfluidics platform and mainly focused on point-of-care (POC) [2,21,25], biochemical tests[26] and environmental monitoring[21], etc. As a non-invasive, high specificity and sensitivity technique, SERS has been introduced in the molecular analysis of μPADs[27-29]. Because of irregular stacking cellulose microfibers of paper that has abundant fibrils and wrinkles, noble metal layer could be deposited into a flexible plasmonic nanostructure containing a large-area SERS "hot spots" [11]. Furthermore, the capacity of fiber absorption of nanoparticles was also contributed to the formation of "hot spots" on the paper surface. Herein, the combination of SERS and paper-based microfluidic not only provides a facile method to fabricate highly reproducible and disposable SERS substrates but also reduces the detection limit of μPADs. White and coworkers[15,30,31] achieved highly sensitive SERS substrate by inkjet printing. Garnier and coworkers[32] used gold nanoparticle-paper as a three-dimension (3D) SERS substrate evaluated with 4-aminothiophenol, a common Raman molecule. Sun et al fabricated SERS strips by physical vapor deposition coating[11].

An ideal SERS substrate should be reproducible when exhibit high SERS enhancement performance with a low cost. In this approach, we proposed a cheap and user-friendly way as an alternative method to prototype SERS active paper-microfluidic arrays on filter paper by a painting brush in some situations. By the aid of brush, silver nanoparticles (AgNPs) were deposited into designated areas onto the paper to form SERS active region. Our work demonstrated the ability to identify and semi-quantify trace Rhodamine 6G (R6G) and Malachite green (MG). Furthermore, the performance, fabrication and physical characterization of our

SERS active μPADs were also discussed.

## Experimental Section

### Chemicals and Materials.

All chemicals used were of analytical grade. Silver nitrate ($AgNO_3$, 99.8%) and sodium hydroxide (NaOH, 96%) were obtained from Sinopharm Chemical Reagent Co. Ltd, hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$, 98.5%) was purchased from Guangcheng Chemical Reagent Co. Ltd. R6G was obtained from Aladdin. All glassware was cleaned with aqua regia and doubly distilled water (Mill-Q, 18Ω·cm$^{-1}$ resistance) prior to use.

### Instrumentation.

The morphology of the AgNPs in the paper chip were characterized by scanning electron microscopy (SEM) with a Hitachi-4800 scanning electron microscope operated at an accelerating voltage of 5 kV and a Malvern particle size analyzer (Zeta Sizer nano-zs90). All SERS spectra were recorded by a DXR Raman Microscope (Thermo Fisher, USA). A 632.8 nm He:Ne gas laser was focused by a 10× microscope objective with a power of 3.8 mW. The estimated spectrum resolution of the DXR Raman Microscope is 5 cm$^{-1}$ (FWHM), with 1 μm x, y spatial resolution and 2 μm depth resolution. The exposure time was 4s and each of the signals was collected twice.

### Synthesis of AgNPs.

AgNPs were synthesized by reducing silver nitrate with hydroxylamine hydrochloride according to Leopold's method with slight modifications[33]. Briefly, 1 mL of NaOH solution (0.3 M) was added to 89 mL hydroxylamine hydrochloride solution under stirring, and then 10 mL of 0.01 M silver nitrate was slowly added to the above solution drop by drop and the solution turned to pale yellow. Subsequently, the solution should be continuously stirred for another one hour. The obtained colloids were stored at room temperature. SEM and UV/vis spectroscopy were used to characterize the dispersity and particle size of the colloid. The particle size was measured by Malvern particle size analyzer and its statistic hydration particle size was estimated about 61 nm.

### Depositing AgNPs Operational Procedure by Brush.

To get higher SERS signals, AgNPs were centrifuged at 7000 rpm to concentrate the colloids; 95% of the supernatant was removed. The final concentration of AgNPs was about 20 mM. The No.8 gouache brush which was supplied by Qingdao Meteor Stationery Co. Ltd was used as painting brush. First, the bristle of the gouache brush was immersed into the colloids solution for 5 s which was under magnetic stirring. Then we used it to brush AgNPs slightly on the designed region (the rectangle in Fig. 1C) of microfluidic paper. The brush direction was forwardly vertical to the channel and the brush region was in the middle of the rectangle covering four detection reservoirs. When the paper chip was dried, it was then brushed for a second time with a counter direction of the first time in order to obtain a uniformity of nanoparticles deposition. The brush action should be very softly and the reasons were mainly due to: (1) It can effectively deposited the AgNPs and avoid the damage of chip surface (2) Because the brushing force was relatively slightly and the hydrophilic bristle was also soft, the different operators could easily obtained similar depositing effect without obvious differences (As detailed in the subsequent discussion).

## Results and Discussion

We firstly provided a very simple and robust way to deposit AgNPs on the paper surface only using a common painting brush. The schematic diagram of fabricating SERS active microfluidic paper is illustrated in Fig. 1. The chip design was implemented with drawing software (Adobe Illustrator) and then directly printed onto the filter paper (Whatman chromatography No.1 paper, GE) by the wax printer (XEROX Phaser 8560DN). After 20 seconds of wax melting at 150°C, the wax penetrated into the paper and it could block the flow of analytes. The diameter of detection area was 4.5 mm, while the analyte introducing reservoir was 2.5 mm (displayed in Fig. 1C). The painting brush, No.8 gouache brush that could be obtained in usual shop, was about 21 cm long. And the painting brush had a width of the bristle of 10 mm, which fit over two detection areas with the 1 mm gap between them. The reason why we chose gouache brush was that it had a preferably soft and hydrophilic bristle which could absorb AgNPs better. Four detection pools were composed of two parallel groups that could be evenly brushed according to the one forward direction and one backward direction during two cycles. Therefore, the AgNPs were uniformly deposited onto the paper. As illustrated in Fig. 1C, four circles in the white rectangle were detection areas, and the other four out of the white rectangle were analyte reservoirs that connect to the detection pool through the flowing channel.

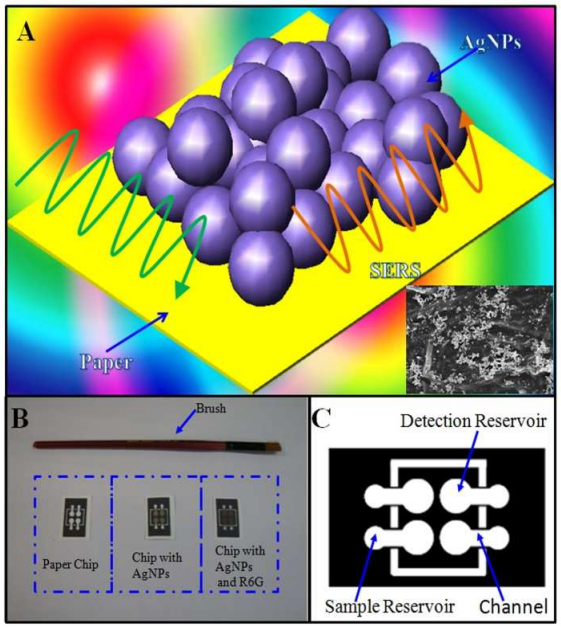


**Fig. 1** (A) Schematic diagram of using painting brush to fabricate low-cost SERS active microfluidic paper chips. (B) The pictures of raw chip, the chip deposited with AgNPs, and R6G was introduced to the chip deposited with AgNPs, respectively (from left to right). (C) The designed chip's diameter of detection area was 4.5 mm, while the sample introducing reservoir was 2.5 mm. The dimension of channel was 1.8 mm wide, 3 mm long.

The filter paper used in this study was almost completely composed of alpha-cellulose according to previous studies[2, 32], which ensured minimal interference between process components such as polymers and coatings. It was also well-known that cellulose fibers of filter paper were consisted of wood cells which were several millimeters long, and one to tens of micrometers wide[11]. All of these structures contributed to the fact that AgNPs could be absorbed on the surface of filter paper within a short time and then aggregated to form "hot spots" during the detection process. From Fig. 2A and 2B, it was also easy to find that there were many nanoclusters distributed on the surface of filter paper, which were beneficial to forming "hot spots" effect. Hence, paper has been applied in SERS substrates with a bright prospect.

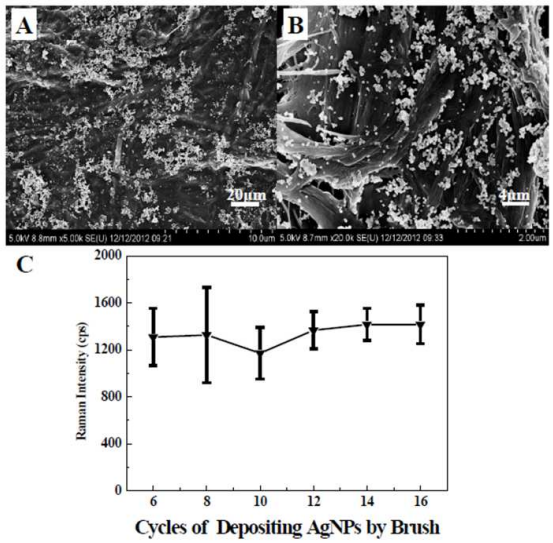


**Fig. 2** (A, B) SEM images of microfluidic paper chip and its enlarged portion. The scale bars of the original SEM image and its enlarged one were 20 and 4 μm, respectively. (C) SERS effect by different depositing cycles through brush for the investigations of the intensity of SERS spectra were obtained from $1.0\times10^{-6}$ M R6G (n=4).

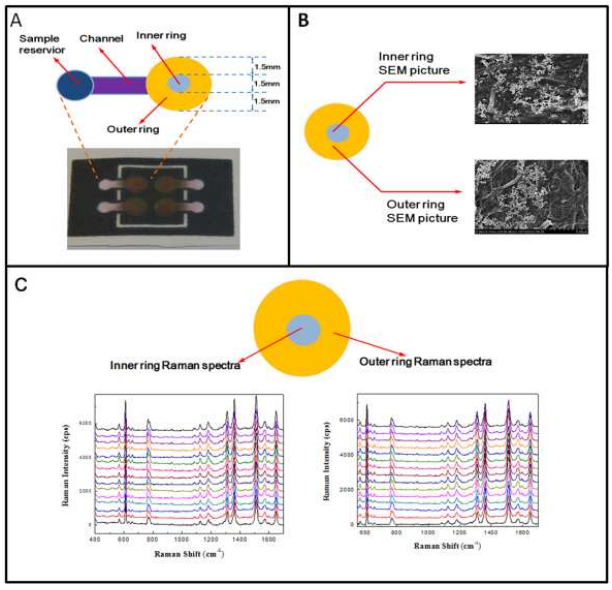


**Fig. 3** (A) The illustrated picture of inner and outer ring of detection area (D=4.5 mm) to further study the uniformity of AgNPs deposition by brushing. The diameter of inner ring was 1.5 mm. (B) The SEM pictures for the AgNPs depositing on the inner and outer ring area. (C) Raman spectra of 15 points randomly selecting from inner (gray) and outer (yellow) ring by analyzing $1.0\times10^{-6}$ M R6G. The RSDs for inner ring and outer ring were 13.7% and 14.4% respectively.

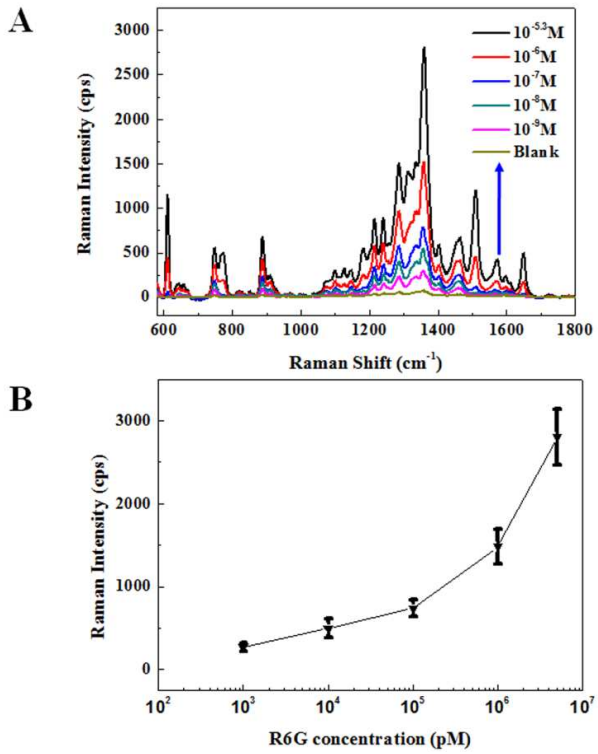


**Fig. 4** (A) Raman spectra of different concentrations of R6G. The R6G concentrations were $1.0\times10^{-9}$, $1.0\times10^{-8}$, $1.0\times10^{-7}$, $1.0\times10^{-6}$, $5.0\times10^{-6}$ M. (B) The plot of the concentration vs. 1360 $cm^{-1}$ peak intensity (n=4).

To investigate the SERS detection capability of the chips, R6G was chosen as the probe molecule. 10 μL analytes were firstly introduced to the sample reservoir by micropipette. Then the sample solution flowed into the detection area along with the channel quickly by means of capillary force. After the paper was dried completely, SERS measurements were performed. The conclusions section should come at the end of article. When we brushed the AgNPs onto the paper, whether there was the deposition variability between the center and edge of the sensing area during the brush stroking that was worth considering. In order to prove it, the detection area (D= 4.5 mm) was divided into two parts, the inner and outer rings (as shown in Fig. 3A). The inner ring diameter was 1.5 mm. 15 points were randomly selected to collect the Raman spectra in the inner and outer ring area respectively. As displayed in Fig. 3C, The RSD of $1.0\times10^{-6}$ M R6G Raman intensity for inner ring and outer ring were 13.7% and 14.4% (n=15) after calculation, respectively. The results implied that the AgNPs deposition had no obvious difference between the outer and inner ring area when we brushed the AgNPs onto it. The SEM images (Fig. 3B) were agreed with the results.

Each detection region was determined four times repeatedly, and the average value was the Raman intensity of detection region. Figure 4A shows SERS spectra of different

concentrations of R6G varied from $1.0\times10^{-9}$ to $5.0\times10^{-6}$ M. From the spectra, the characteristic peaks of R6G at 610 $cm^{-1}$, 1360 $cm^{-1}$, 1510 $cm^{-1}$ and 1650 $cm^{-1}$ can be easily found, that correspond to one C-C-C ring bending vibration (610 $cm^{-1}$) and three aromatic C-C stretching mode (1360 $cm^{-1}$, 1510 $cm^{-1}$, 1650 $cm^{-1}$), respectively[34]. Apparently, the Raman band at 1360 $cm^{-1}$ was the most prominent one and was selected as a typical peak for the analysis of R6G. As illustrated, even R6G concentration down to 1nM, SERS signals could be identified clearly, which was indicative of the excellent enhancement of our chips. Furthermore, in order to obtain an ideal performance of the chips, the optimal number of depositing cycles was investigated. As seen from Figure 2C, the SERS intensity and the chip homogeneity were enhanced with the increase of brushing cycles because of the improvement of the amount and uniformity of AgNPs deposited on the chip. When the brushing cycles were increased to 14, the signal strength reached a plateau. Moreover, compared with other depositing cycles, it showed the lowest relative standard deviation (RSD, 14.2%). Hence, from the view of efficiency and chip homogeneity, the optimal depositing cycle was selected as 14. In addition, though there were some inherent defects of the filter paper mainly coming from its irregular structure, the lower RSD had also proved that brushing SERS active microfluidic paper chips was noteworthy because of its simple fabrication and good reproducibility. Because paper has some background interference for the R6G, Figure 4B depicted the plot of the intensity of 1360 $cm^{-1}$ band with different R6G concentrations after removing the paper's background. And it showed a monotonic increase of the Raman intensity with R6G concentration.

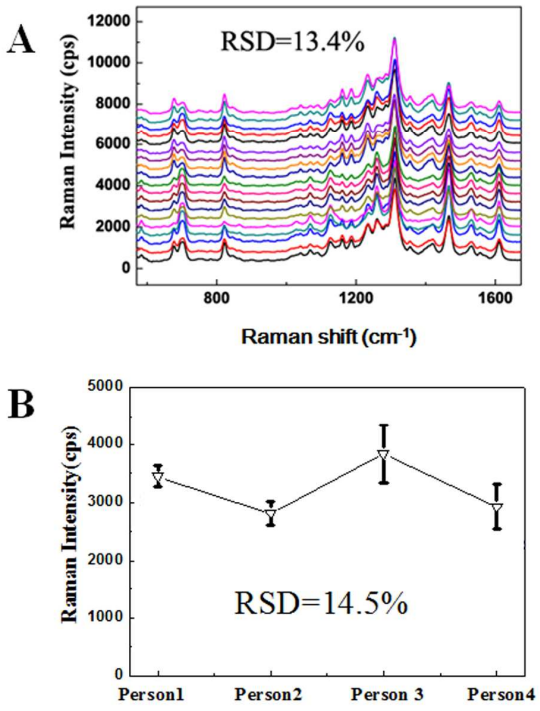


**Fig. 5** (A) Raman spectra of $5.0\times10^{-6}$ M R6G from 20 deposited SERS active chips (n=20). The RSD was 13.4% (B) Raman spectra of $5.0\times10^{-6}$ M R6G from every five pieces of SERS active chips made by four different people including two men and two women. The RSD for different people was 14.5%

The SERS enhancement factor of our chip was calculated by comparing the acquired signals from chip with and without AgNPs. For a 10 mM R6G solution without SERS enhancement effect, the obtained SERS signal (n = 4) at 1360 $cm^{-1}$ was approximately equal to the signal gained from $4.5\times10^{-10}$ M R6G on chip with AgNPs. Furthermore, the analytical SERS enhancement factor can be calculated using the following equation: $EF = (I_{sers}/I_s) \times (C_s/C_{sers})$, where $C_s$ is the concentration of the analyte solution which produces a spontaneous Raman signal, and $C_{sers}$ represents the concentration of analyte which was analyzed on a SERS active paper chip. The $I_s$ and $I_{sers}$ indicate their Raman signals under the above experimental conditions respectively. Therefore, the enhancement factor could be calculated as $2.2\times10^7$, which was in agreement with previous literatures[15,35].

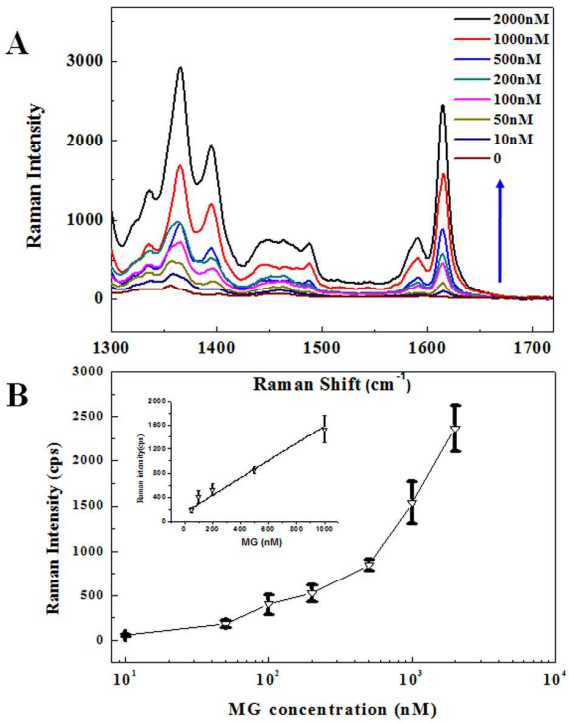


**Fig. 6** (A) Raman spectra of different concentrations of MG. The MG concentrations were $1.0\times10^{-8}$, $5.0\times10^{-8}$, $1.0\times10^{-7}$, $2.0\times10^{-7}$, $5.0\times10^{-7}$, $1.0\times10^{-6}$, $2.0\times10^{-6}$ M. (B) The plot of the concentration vs. 1616 $cm^{-1}$ peak intensity (n=4). The inset was the quantitative calibration curve of different MG concentration ($5.0\times10^{-8}$, $1.0\times10^{-7}$, $2.0\times10^{-7}$, $5.0\times10^{-7}$, $1.0\times10^{-6}$ M). The linear equation was $y = 1.442x + 139.4$ ($R^2 = 0.97$, n=4).

As shown in Fig. 5A, in order to further prove the reproducibility, we obtained SERS signals from 20 different SERS active chips deposited with AgNPs and the analyzing concentration of R6G was $5.0\times10^{-6}$ M. No significant difference could be observed between 20 different fabricated chips and the standard deviations were 13.4%. Furthermore, to prove our method has the universal usability and accessible to end users, we also studied the brushing effects by different people. Four non-skilled people were selected to brush the SERS active chips individually according to our standard procedure. Every person fabricated 5 pieces of SERS active chips. Fig. 5B showed the Raman spectra of $5.0\times10^{-6}$ M R6G from the chips (n=5) fabricated by two men and two women. The standard deviation of 4 different people was only

14.5%, which was similar to the result of the same person (13.4%) and proved that this method was robust and user-friendly. And the standard deviation of the total collective data (4 persons, every person repeated 5 times) was 15.0%. Consequently, according to the depositing procedure mentioned above, the operators could feasibly use this technique and obtained the satisfied results without specific skill training.

MG is a kind of shiny metal green crystals belonging to cationic triphenylmethane dyes[36]. As a fungicide and preservative, MG has been widely used in the industry. However, because of genotoxic and carcinogenic effect, MG was classified under category C.III by FAO/WHO[37]. Nevertheless, some unscrupulous producers are still using MG in order to reduce the cost of aquaculture. It is urgently in need of a cheap, fast and sensitive analysis method for trace detecting MG. Considering these circumstances, we extended our chips in the application of MG determination. A series of different concentrations MG were investigated and their concentrations were $1.0\times10^{-8}$, $5.0\times10^{-8}$, $1.0\times10^{-7}$, $2.0\times10^{-7}$, $5.0\times10^{-7}$, $1.0\times10^{-6}$, $2.0\times10^{-6}$ M, respectively. The SERS signals from 4 different SERS active chips and the corresponding Raman curves, based on the variations in the peak area, were illustrated in Fig. 6A. According to previous reports[36], we chose 1616 $cm^{-1}$ as the characteristic Raman peak of MG because the background interference was mainly around 1360 $cm^{-1}$. The plot of the Raman intensity of the 1616 $cm^{-1}$ band corresponding to the concentration of MG was monotonic increased (Fig. 6B). And the spectrum of 10 nM MG could be easily detected. Furthermore, from the insert diagram in Fig. 6B, we could find that the Raman intensity of MG had a good linear relationship ($R^2$=0.97) with the concentration of MG from 50 nM to 1000 nM. And the SERS active chips were also tested by standard addition and the recovery of spiked MG ranges from 89.19 % to 108.58 % (Table 1). This indicated that our chips could be applicable to the quantification of MG in practical samples. The proposed method made it easily accessible to end-users, without performing other complicated fabrication steps.

**Table 1** The recovery of spiked MG in local lake water. The standard deviation of the samples was detected by three measurements.

| Sample | Added(nM) | Detected(nM) | RSD(%) | Recovery(%) |
|---|---|---|---|---|
| 1 | 800 | 868.63 | 9.15 | 108.58 |
| 2 | 300 | 267.57 | 14.40 | 89.19 |
| 3 | 100 | 91.60 | 12.17 | 91.60 |

## Conclusions

In this paper, a simple, robust and low-cost fabrication technique of SERS substrates had been proposed through depositing silver nanoparticles on the surface of filter paper by brushing. Compared with previous methods, the fabrication of our SERS active substrates didn't need expensive equipment and tedious procedure. The rapid mass production could be approximately 150 chips per hour. The RSDs were below 15% on different papers or different deposited locations that indicated good reproducibility for the presented approach. Furthermore, by taking advantage of the excellent Raman enhancement of our substrates, the trace Rhodamine 6G and malachite green were successfully analyzed on the paper chip. And the result of recovery experiments of spiked MG in local lake water was satisfied. Combing with paper's attractive characteristics of lightweight and simplicity, this robust and feasible method will be a potential candidate implemented in various chemical and environmental pollutant sensing applications.

## Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (grant no. 21205131, 21275158), the Program of Zhejiang Provincial Natural Science Foundation of China (grant No. R5110230), the Science and Technology Development Plan of Yantai (2011071) and the 100 Talents Program of the Chinese Academy of Sciences.

## Notes and References

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# Graphic Abstract

A cheap, robust and facile method was proposed to create paper-based SERS active substrates by using a painting brush to deposite Ag nanoparticles.