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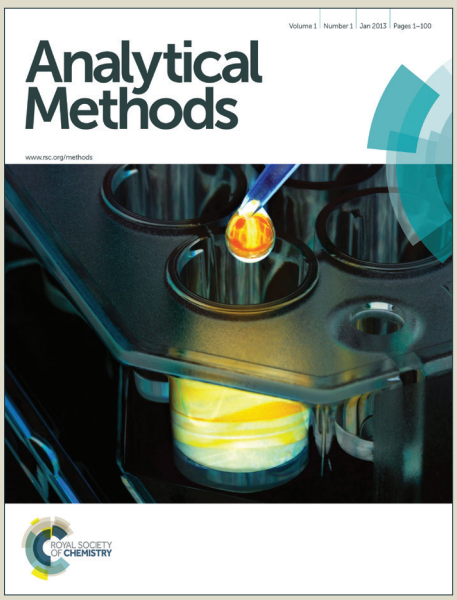

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Improving the Quantitative Accuracy of Surface-Enhanced Raman Spectroscopy by the Combination of Microfluidics with Multiplicative Effects Model

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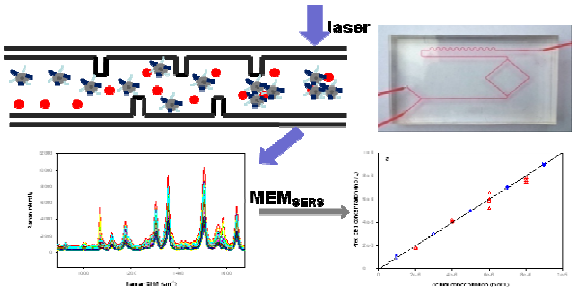
Graphical and text abstract



Accurate quantitative SERS analysis was achieved through the combination of microfluidics with Multiplicative Effects Model.

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ABSTRACT

In this contribution, the combination of polydimethylsiloxane microfluidics with a recently developed multiplicative effects model for surface-enhanced Raman spectroscopy (MEM_{SERS}) has been proposed to improve the accuracy and precision of quantitative SERS assays based on silver nanocolloids. The performance of the proposed method has been tested on two proof-of-concept systems and another real system (i.e., quantification of Rhodamine 6G by both internal standard addition and internal standard tagging detection modes, quantification of malachite green in fishpond water by internal standard addition detection mode). The average relative prediction error values of the proposed method for the test samples of the above three systems were 6.0%, 8.6% and 8.4% respectively. Conservatively speaking, these results demonstrated that accurate quantitative SERS analysis with an average relative prediction error less than 10% can be expected through the combination of microfluidics with MEM_{SERS} calibration model.

Key words: Quantitative Surface-Enhanced Raman Spectroscopy, Multiplicative Effects Model, Microfluidics

1. INTRODUCTION

Due to its unique advantages, such as narrow spectroscopic bands with excellent molecular specificity, reduced photo-bleaching, simple pretreatment, and ultra high sensitivity, surface-enhanced Raman spectroscopy (SERS) has attracted substantial research interests, since it was first observed in 1974 by Fleischman et al.[1] Though the full consensus on SERS enhancement mechanism has not yet been reached, SERS technique has been successful applied to many areas such as biological detection of cell and biomacromolecules,[2-4] monitoring of interactions between biological molecules,[5] characterization of biological tissue in vitro and in vivo[6, 7].

Although SERS has above-mentioned significant advantages, its limitations are also very straightforward. SERS effect relies heavily on the preparation of nano-rough metal enhancing substrates (such as nano-silver or gold colloids). The absolute intensities of SERS signals depend on not only the concentrations of the analytes of interest, but also the degree of aggregation, the particle size and shape of the metal colloids, and laser focusing position as well. Therefore, the heterogeneity of the enhancing substrates can cause significant variations in the absolute SERS intensities and hence significantly deteriorate the precision and accuracy of quantitative SERS analysis.

In recent decades, there have been many technological developments to improve the accuracy and precision of quantitative SERS assays, which can be roughly classified into the following three categories: (1) Designing and fabricating highly sensitive and reproducible SERS enhancing substrates. It has been a major preoccupation of researchers interested in developing practical SERS assays.[8-12] Due to their ease of preparation, excellent enhanced effect and especially the ability to be dispersed in macrostructure such as cells and tissue samples, Ag and Au nanoparticle colloids are the most commonly used SERS enhancing substrates. Nevertheless, noble metal nanoparticle colloids especially silver colloids are notoriously difficult to produce with high reproducibility, which presents a major challenge for quantitative

SERS assays; (2) Utilizing internal standard method or microfluidic technology to mitigate the confounding effects on SERS signals caused by variations in the physical properties of enhancing substrates, the intensity and alignment/focusing of laser excitation source.[13, 14] But the application of conventional internal standard method requires that the internal standard used must have one or more SERS peaks in spectrally silent regions of the analyte of interest, other coexisting SERS-active compounds and possible background fluorescence interference. Such a stringent requirement renders the conventional internal standard method hardly applicable in practice; (3) Adopting multivariate calibration methods such as partial least squares (PLS) to improve the quantitative accuracy and precision of SERS assays.[15, 16] Compared with univariate calibration approaches, the application of multivariate calibration methods can indeed result in somewhat improvement in the precision and accuracy of quantitative SERS analysis. However, the improvement potential is rather limited considering that existing multivariate calibration methods do not explicitly model the relationship between the physical properties of SERS substrates and the SERS intensities of analytes. Methods which can effectively eliminate the confounding effects caused by variations in the physical properties of enhancing substrates, the intensity and alignment/focusing of laser excitation source are therefore highly desirable to upgrade SERS technique to routine quantitative tool.

In this contribution, we combined microfluidic technology[17, 18] with a multiplicative effects model for surface-enhanced Raman spectroscopy[19] to improve the quality of results in quantitative SERS assays. The utilization of microfluidics in SERS detection aims to solve the problems associated with the static SERS detection mode, such as varying mixing time, varying scattering geometry, localized heating, and photo-dissociation. Compared with the static detection mode, SERS detection in a fluidic channel might provide more reproducible results, which is due to more homogeneous mixing, more consistent geometries and more efficient heat dissipation in the flow detection mode. The multiplicative effects model is then adopted to further

eliminate the confounding effects on SERS signals caused by possible variations in the physical properties of nanocolloids as well as the intensity and alignment/focusing of laser excitation source, and finally realize accurate quantitative SERS assays.

2. EXPERIMENTAL

Reagents and Chemicals. $AgNO_3$, sodium citrate and KCl were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Rhodamine 6G (R6G), butyl rhodamine B, *p*-thiocresol, malachite green (MG) and concentrated nitric acid were obtained from Aladdin Chemistry Co., Ltd. (Shanghai, China). All chemicals were of analytical grade, and were used as received without any further purification. Ultrapure water (18.25MΩ.cm) produced by Direct-pure plus water system (Aquapro, Chongqing, China) was used throughout this study**.**

Preparation of Silver Nanocolloids. Silver nanocolloids were prepared according to the Lee-Meisel method.[20] Briefly, 100 ml of silver nitrate solution was prepared by adding 18 mg silver nitrate to appropriate volume of ultrapure water and heated to boil. And then 2 ml of sodium citrate aqueous solution was added to the boiling silver nitrate solution under vigorous stirring. The mixture was kept at a gentle boil for 1 h. It was then cooled naturally to room temperature and stored in the fridge at 4 ^{o}C. Before using, the silver nanocolloids were redispersed by ultrasound for five minutes.

Microfluidic Chips. PDMS microfluidic chips with upper and lower zig-zag type blocks ordered from WenJing chip company (Suzhou, China) were used to ensure relatively more homogeneous mixing, more consistent geometries and more efficient heat dissipation during SRES assays (Fig.1). The PDMS microfluidic chips can be recycled by nitric acid washing (Supporting Information). During SERS assays, solution A (a mixture of the analyte of interest, silver nanocolloids and the internal standard) and solution B (0.5 M KCl) were injected into the microfluidic channel

through syringe pump with the same rate of 10 ul per minute. The confluent streams traveled along the microfluidic channel. Laminar flow was devastated by the upper and lower blocks and mixed efficiently. As the fluid was flowing through the channel, SERS spectra were measured at the detection point.

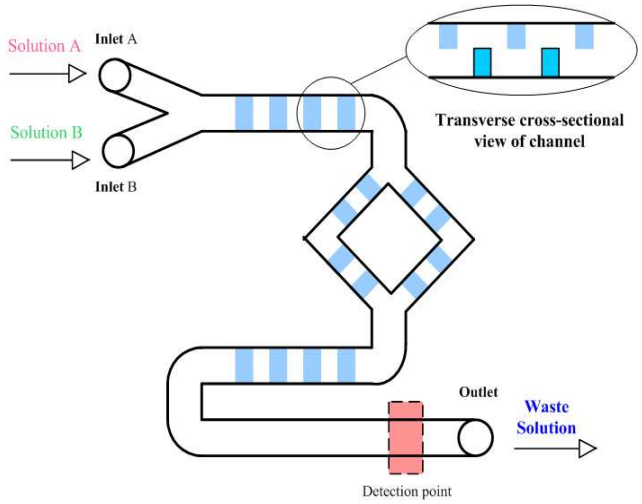


Fig.1. The schematic illustration of the PDMS microfluidic chip.

Quantification of R6G by Internal Standard Addition Detection Mode. A total of nine samples of R6G were prepared by diluting appropriate volumes of R6G stock solution (1.00×10^{-5} M) with ultrapure water. The concentrations of R6G in the nine samples were 1.00×10^{-6} M, 2.00×10^{-6} M, 3.00×10^{-6} M, 4.00×10^{-6} M, 5.00×10^{-6} M, 6.00×10^{-6} M, 7.00×10^{-6} M, 8.00×10^{-6} M and 9.00×10^{-6} M, respectively. The five samples with concentrations of R6G equaling to 1.00×10^{-6} M, 3.00×10^{-6} M, 5.00×10^{-6} M, 7.00×10^{-6} M, and 9.00×10^{-6} M formed the calibration set. The test set consisted of the remaining four samples. Solution A consisted of 20 ul R6G sample solution, 20 ul 3.50×10^{-6} M butyl rhodamine B solution (as internal standard) and 80 ul silver nanocolloids solution. Each sample was measured five times at the same focusing position.

Quantification of MG in Fishpond Water by Internal Standard Addition Detection Mode. MG had been widely used as a fungicide and antiseptic in the aquaculture industry.[21] Nowadays, as being suspected of having genotoxic and

carcinogenic potential[22, 23], MG has been banned for using in aquaculture. However, MG is still used illegally in fish farming industry in some areas due to its low cost, easy availability and efficacy. Therefore, methods for its rapid quantification are desirable. In this contribution, a total of eleven samples with different concentrations of MG equaling to 0.50×10^{-6} M, 1.00×10^{-6} M, 2.00×10^{-6} M, 3.00×10^{-6} M, 4.00×10^{-6} M, 5.00×10^{-6} M, 6.00×10^{-6} M, 7.00×10^{-6} M, 8.00×10^{-6} M, and 9.00×10^{-6} M, 1.00×10^{-5} M, respectively, was prepared by mixing the water from a fishpond (it is absolutely MG free) with standard stock solution of MG (1.00×10^{-3} M) in appropriate volume ratios. The six samples with concentrations of MG equaling to 0.50×10^{-6} M, 2.00×10^{-6} M, 4.00×10^{-6} M, 6.00×10^{-6} M, 8.00×10^{-6} M and 1.00×10^{-5} M formed the calibration set. The remaining five samples formed the test set. Solution A was made by mixing 20 ul MG, 20 ul butyl rhodamine B (8.00×10^{-6} M) as internal standard, and 80 ul silver colloids solution. Each sample was measured four times at the same focusing position.

Quantification of R6G by Internal Standard Tagging Detection Mode. Internal standard tagging SERS enhancing substrate mixture for quantitative analysis of R6G was prepared by adding 650 μl of *p*-thiocresol solution (1.00×10^{-4} M) as an internal standard to 26 ml silver colloids solution under ultrasound treatment at a frequency of 28KHz for 10 min. Eleven samples were prepared by adding appropriate amount of water, R6G standard stock solution (1.00×10^{-5} M) into 1.6 mL of the above SERS enhancing substrate mixture to make a final volume of 2 ml. The ultimate concentrations of R6G in the eleven samples were 0.50×10^{-7} M, 0.80×10^{-7} M, 1.00×10^{-7} M, 2.00×10^{-7} M, 3.00×10^{-7} M, 4.00×10^{-7} M, 5.00×10^{-7} M, 6.00×10^{-7} M, 7.00×10^{-7} M, 8.00×10^{-7} M, and 9.00×10^{-7} M, respectively. The six samples with concentrations of R6G equaling to 0.50×10^{-7} M, 1.00×10^{-7} M, 3.00×10^{-7} M, 5.00×10^{-7} M, 7.00×10^{-7} M, and 9.00×10^{-7} M formed the calibration set. The test set was comprised of the remaining five samples. Each sample was measured four times at the same focusing position.

Instruments. SERS measurements were collected at room temperature by an invia-reflex laser confocal inverted microscopic Raman spectrometer (Renishaw, UK) equipped with a 633 nm laser for excitation, a near-infrared enhanced deep-depleted thermoelectrically Peltier cooled CCD array detector (576×384 pixels), and a 50×(NA 0.50) Leica DMLM objective microscope. SERS spectrum of each samples were acquired using 4 scans with a resolution of 1 cm^{-1} over the range 717 ~ 1828 cm^{-1}. It is worth pointing out that during the experiment, the laser was carefully focused on the middle region of the microfluidic channel to avoid the interference from PDMS.

Data Analysis. SERS measurements in the appropriate Raman shift ranges were selected for subsequent data analysis to ensure that the SERS peaks of both the analyte of interest and the internal standard can be readily observed in the selected regions. For quantification of R6G by internal standard addition detection mode, SERS signals in the range of 1010.9 to 1712.4 cm^{-1} were selected (Fig.2a); while for the quantification of MG, the spectral measurements ranging from 953.1 to 1693.1 cm^{-1} were used for quantitative analysis (Fig.2b); For the quantification of R6G by internal standard tagging detection mode, the spectral data between 894.7 and 1681.9 cm^{-1} were selected (Fig.2c).

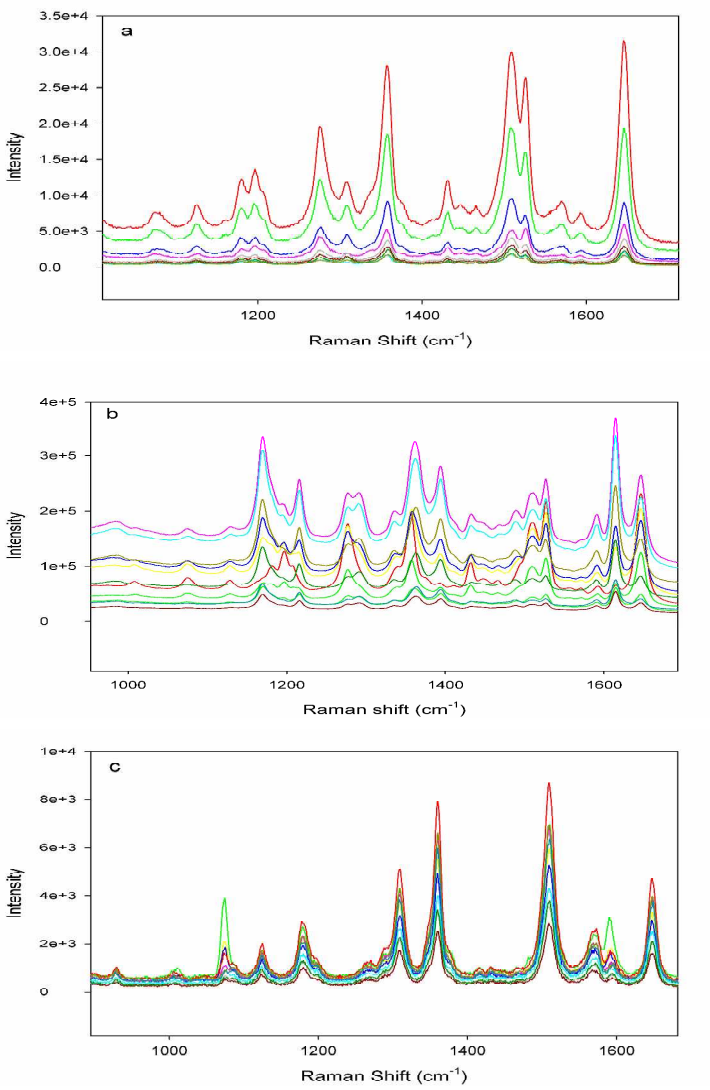


Fig.2. SERS measurements in the appropriate Raman shift ranges selected for quantification of R6G by internal standard addition detection mode (a), quantification of MG in fishpond water by internal standard addition detection mode (b), and quantification of R6G by internal standard tagging detection mode (c).

The following multiplicative effects model for surface-enhanced Raman spectroscopy (MEM_{SERS})[19] was adopted for the subsequent quantitative analysis:

$$\mathbf{x}_k = \sum_{j=1}^{J} b_k \cdot c_{k,j} \cdot \mathbf{r}_{chem,j} + \mathbf{d}_k ; \quad (k = 1,2,\cdots,K) \tag{1}$$

Where, $\mathbf{x}_k$ is the SERS spectrum of the k-th sample; $c_{k,j}$ is the concentration of the j-th chemical component in the k-th calibration sample; $\mathbf{r}_{chem,j}$ represents the

molecular scattering properties of the *j*-th chemical component; The multiplicative parameter b_k explicitly accounts for the multiplicative confounding effects on SERS intensities caused by changes in variables other than the concentrations of analytes in the *k*-th calibration samples, such as physical properties of enhancing substrates, the intensity and alignment/focusing of laser excitation source; $\mathbf{d}_k$ is a composite term that represents background interference(s) and the non-multiplicative effects caused by variations in physical properties of the enhancing substrates on the *k*-th sample.

The multiplicative parameters b_k (k=1, 2, …, K) for K calibration samples in the above MEM_{SERS} model can be estimated from their SERS spectra by the modified optical length estimation and correction (OPLEC_{m}) method.[24, 25] Let's arbitrarily assume that the first chemical component in eq.1 is the analyte of interest. The following two calibration models can then be built by multivariate linear calibration methods such as partial least squares regression (PLS).

$$\mathbf{b} = \alpha_1 \mathbf{1} + \mathbf{X}_{cal}\boldsymbol{\beta}_1; \quad diag(\mathbf{b})\mathbf{c}_1 = \alpha_2 \mathbf{1} + \mathbf{X}_{cal}\boldsymbol{\beta}_2 \tag{2}$$

Here, $\mathbf{X}_{cal} = [\mathbf{x}_1; \mathbf{x}_2; \cdots; \mathbf{x}_K]$; $\mathbf{c}_1 = [c_{1,1}; c_{2,1}; \cdots; c_{K,1}]$; $diag(\mathbf{b})$ denotes the diagonal matrix in which the corresponding diagonal elements are the elements of $\mathbf{b}$; $\mathbf{1}$ is a column vector with its elements equal to unity. After the estimation of the model parameters α_1, α_2, $\boldsymbol{\beta}_1$, and $\boldsymbol{\beta}_2$, the concentration ($c_{test,1}$) of the target analyte in any test sample can be determined from its measured SERS spectrum $\mathbf{x}_{test}$ according to eq.3. The confounding multiplicative effects of physical properties of enhancing substrates, the intensity and alignment/focusing of laser excitation source on the quantitative results has been readily corrected.

$$c_{test,1} = \frac{\alpha_2 + \mathbf{x}_{test}\boldsymbol{\beta}_2}{\alpha_1 + \mathbf{x}_{test}\boldsymbol{\beta}_1} \tag{3}$$

The quantitative performance of MEM_{SERS} model was compared with that of PLS model in terms of root-mean-square error of prediction (RMSEP) and average relative

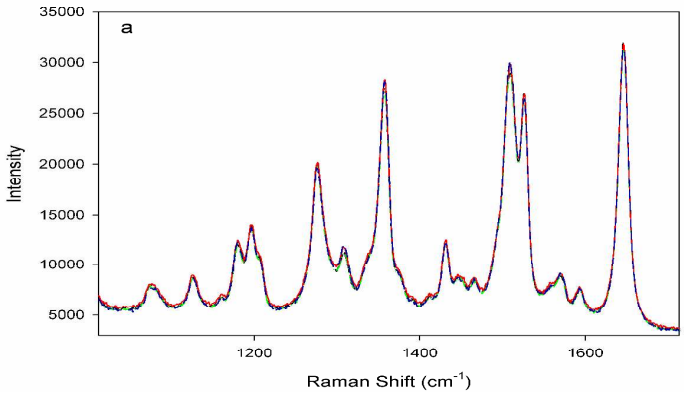
prediction error (ARPE). Leave-one-out cross validation was employed to determine the optimal MEM_{SERS} and PLS calibration models

$$RMSEP = \sqrt{\sum_{i=1}^{N}(c_{i,1} - \hat{c}_{i,1})^2 / N}\ ; \qquad ARPE = \frac{1}{N}\sum_{i=1}^{N}\left|(c_{i,1} - \hat{c}_{i,1})/c_{i,1}\right| \times 100\% \tag{4}$$

Where, $c_{i,1}$ and $\hat{c}_{i,1}$ are the actual and predicted concentrations of the analyte of interest in the *i*-th test sample, respectively; *N* is the number of test samples.

3. RESULTS AND DISCUSSION

In order to improve the reproducibility of SERS measurements, PDMS microfluidic channel was used to ensure relatively more homogeneous mixing and more consistent geometries during SRES assays. Fig.3 shows the SERS spectra of the same R6G sample measured continuously when the sample went through the PDMS microfluidic channel. It can be seen that the SERS spectra exhibit good reproducibility with relative deviation of about 10%.



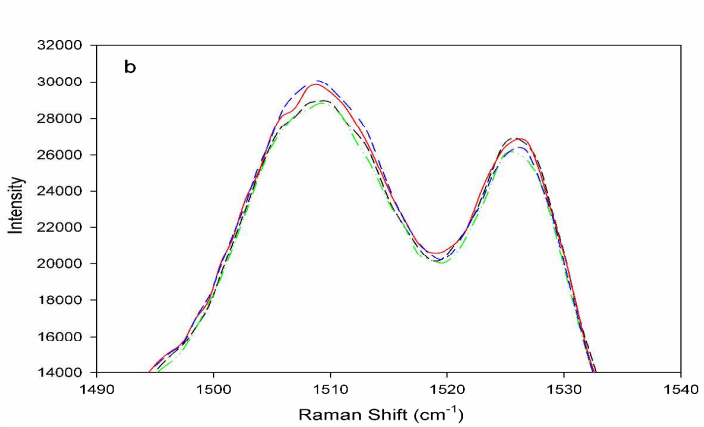


Fig.3. (a) SERS spectra of the same R6G sample measured continuously when the sample went through the PDMS microfluidic channel; (b) the zoomed part of the same set of spectra within the range of 1490 to 1540cm^{-1}.

However, as listed in table 1, the concentration predictions for R6G in both the calibration and test samples obtained by PLS calibration model significantly deviate from their actual values. The RMSEP values of PLS calibration model for the calibration and test samples were 1.75×10^{-6} M and 1.68×10^{-6} M, which are equivalent to ARPE values of 39.8 % and 42.0 %, respectively. These results suggested that though the application of PDMS microfluidic channel can render good reproducibility in SERS spectra of the same sample collected continuously when the sample went through the PDMS microfluidic channel, the application of microfluidics alone could not realize accurate quantitative SERS assays. The possible differences in the physical properties of the silver colloids across samples complicated the relationship between the concentrations of R6G in samples and their corresponding SERS spectra, which undermined the underlying linearity assumption of PLS calibration method. The influence of the physical properties of silver colloids on the SERS spectra of R6G samples has a multiplicative nature. To evaluate the significance of the multiplicative effects of the physical properties of silver colloids on SERS spectra, the multiplicative parameters b_k (k= 1, 2, ⋯, K) for the R6G calibration samples were estimated by OPLEC$_m$ from their SERS spectra measured under internal standard addition detection mode. As expected, b_k varied significantly, ranging from 1 to 31 (not

shown). Multivariate linear calibration methods such as PLS are incapable of dealing with so severe multiplicative effects. This is the reason that the concentration predictions of PLS calibration model have such large errors.

Table 1. Concentration predictions for R6G in the calibration and test samples obtained by PLS and MEM_{SERS} models, when internal standard addition detection mode was adopted

Sample Category	Replicates	Actual Conc. ($\times10^{-6}$ M)	Mean predicted Conc. ($\times10^{-6}$ M)	
			PLS	MEM_{SERS}
Cal.	5	1.00	2.04 (0.15[a])	0.99 (0.08)
Cal.	5	3.00	2.67 (0.45)	3.00 (0.01)
Cal.	5	5.00	7.23 (0.27)	4.99 (0.03)
Cal.	5	7.00	6.99 (0.46)	7.01 (0.06)
Cal.	5	9.00	6.07 (0.35)	9.00 (0.10)
		RMSEP	1.75	0.06
		ARPE	39.8 %	1.7 %
Test	5	2.00	-0.28 (0.70)	1.79 (0.04)
Test	5	4.00	4.47 (0.23)	4.07 (0.10)
Test	5	6.00	6.95 (0.22)	5.86 (0.49)
Test	5	8.00	5.90 (0.17)	7.63 (0.19)
		RMSEP	1.68	0.33
		ARPE	42.0 %	6.0 %

a. The numbers in the bracket denote standard deviation

The results listed in table 1 also showed that the combination of PDMS microfluidic channel with MEM_{SERS} could effectively mitigate the SERS spectral variations caused by possible changes in the physical properties of enhancing substrates, the intensity and alignment/focusing of laser excitation source, and hence achieved much more precise concentration predictions. The RMSEP values of MEM_{SERS} calibration model for the calibration and test samples were 0.06×10^{-6} M and 0.33×10^{-6} M, respectively. The ARPE value for the test samples was 6.0%, which was only one seventh of the corresponding value of PLS calibration model. Considering the notorious heterogeneity of silver colloids, such results were rather commendable.

The success of the combination of PDMS microfluidics with MEM_{SERS} on just one model compound R6G is not enough to justify its application potential in routine

quantitative SERS assays. MG, a chemical compound once used as fungicide and antiseptic in the aquaculture industry, was therefore selected to test the performance of the proposed method. Once again, PLS calibration model failed to provide acceptable concentration predictions for MG in the test samples (Fig.4). The corresponding RMSEP and APRE values were 1.47×10^{-6} M and 62.6%, respectively. Interestingly, MEM_{SERS} achieved rather similar accuracy for MG as it did for R6G (Fig.4). Its RMSEP and APRE values were 0.24×10^{-6} M and 8.4%, respectively. This consistency can be explained by the facts that the same internal standard and quite similar concentration ranges were used in these two systems. Nevertheless, on the other hand, it has also demonstrated the effectiveness and robustness of the proposed method in improving the quantitative accuracy and precision of SERS assays.

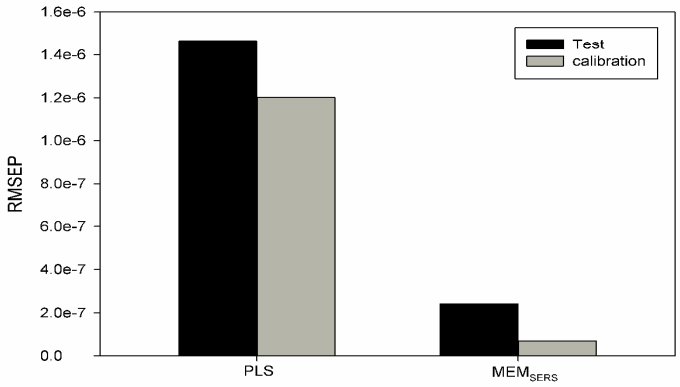


Fig.4. The RMSEP values for the concentrations of MG in both the calibration and test samples predicted by PLS and MEM_{SERS} calibration methods.

In the internal standard addition detection model, the internal standard butyl rhodamine B has both molecular structure and SERS activity similar to the target analytes R6G and MG. In practice, it is rather unrealistic to find such an internal standard for an arbitrary analyte of interest. Therefore, the performance of the proposed method in this contribution was further tested under internal standard tagging detection model where a generic internal standard, *p*-thiocresol, was adopted. Fig.5 showed the pure SERS spectra of R6G and p-thiocresol measured on silver

colloids. It can be seen that the SERS spectrum of *p*-thiocresol is quite different from that of R6G. Moreover, the main SERS peaks of the internal standard *p*-thiocresol are overlapped with those of R6G.

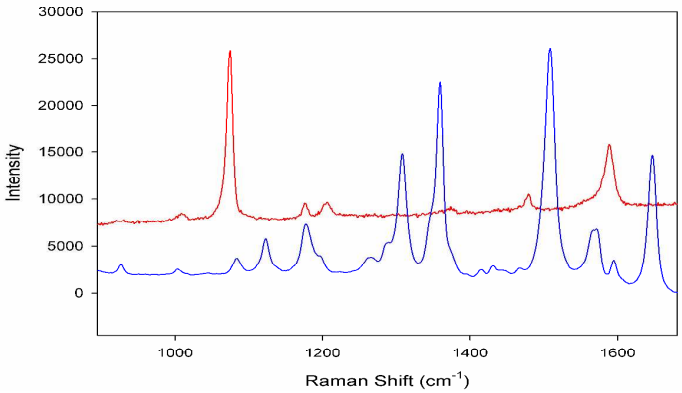


Fig.5. the SERS spectra of R6G (blue line) and p-thiocresol (red line) measured on silver colloids.

As displayed in Fig.6a, though the PLS calibration model with 7 underlying components fitted the calibration samples quite well, its concentration predictions for R6G in the test samples were very poor with a RMSEP value of 1.72×10^{-7} M, equivalent to an ARPE value of 49.6%. In contrast, the results of MEM_{SERS} were far more accurate (Fig.6b). Its RMSEP and APRE values for the test samples was 0.43×10^{-7} M and 8.6%, respectively. These results demonstrated the capability of the combination of PDMS microfluidics with MEM_{SERS} in realizing accurate quantitative SERS assays under internal standard tagging detection mode, even when a generic internal standard was utilized.

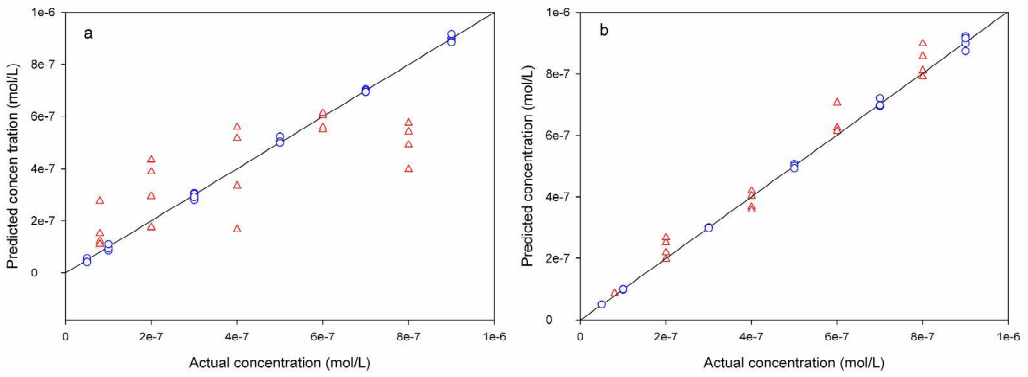


Fig.6. Concentration predictions for R6G in the calibration (blue circle) and test (red triangle) samples obtained by (a) PLS and (b) MEM_{SERS} when internal standard tagging detection mode was adopted

4. CONCLUSIONS

Variations in the physical properties of silver nanocolloids exert significant multiplicative influence on the SERS intensities of the analyte of interest and significantly distort the linear relationship between the concentration of the analyte of interest and SERS measurements. It is therefore very difficult to carry out quantitative SERS assays. Our experimental results revealed that the application of PDMS microfluidic channels can render good reproducibility in SERS spectra of the same sample collected continuously when the sample went through the PDMS microfluidic channel. However the application of microfluidics alone could not realize accurate quantitative SERS assays. It was also found that the combination of microfluidics with MEM_{SERS} calibration model could achieve very satisfactory results in quantitative SERS assays. Based on the results for two proof-of-concept systems and another real system, conservatively speaking, accurate quantitative SERS analysis with a mean relative prediction error of less than 10% can be expected through the combination of microfluidics with MEM_{SERS} calibration model.

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