Towards quantitatively reproducible substrates for SERS†

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There is a need for a method to facilitate the development of novel, reproducible colloidal surface-enhanced Raman scattering (SERS) substrates to encourage the use of SERS in applied studies. In this study we show for the first time that by using suitably designed SERS experiments in conjunction with multivariate analysis of variance (MANOVA), an objective assessment of colloidal SERS reproducibility can be made. This is demonstrated with the analyte cresyl violet, but could be extended to any analyte of interest for which reproducible SERS data are needed.

Introduction

Surface-enhanced Raman and resonance Raman scattering (SERS and SERRS) are powerful techniques that can be used to increase the sensitivity of a conventional Raman experiment.[1] Although the SERS effect was discovered over 30 years ago,[2] its exploitation in applied studies has been hampered by relatively poor reproducibility, despite extensive work on quantitative SERRS for DNA multiplexing.[3] In colloidal SER(R)S it is understood that experimental error can result from variability in particle size and morphology.[4] In practice these parameters can differ greatly between, and indeed within, independently prepared colloidal batches and this depends largely on the preparation method used.

As a consequence, whilst much effort has been focused on developing morphologically reproducible substrates for SERS, there has been no clear strategy for determining comparative substrate reproducibility in an objective fashion.[5] Typically, for the characterisation of substrates, despite no clear correlation with signal enhancement,[6] researchers are reliant upon UV-visible absorbance measurements, electron microscopy imaging and other physical measurements (XRD and SPR) and, of course, exploratory SERS measurements on dye standards such as rhodamine 6G or cresyl violet. Assessment of the reproducibility of SERS measurements is often highly subjective because a 'stare and compare' approach is adopted, which is non-mathematical and fails to take in to account quantitative differences throughout the whole spectrum accurately. Herein, we report the characterisation of a number of novel and conventional SERS substrates, and demonstrate how multivariate analysis of variance (MANOVA[7]) can be applied to assess, in an objective and statistical manner, the reproducibility of SERS measurements acquired using these colloids on multiple batches of the same colloidal preparations.

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† Electronic supplementary information (ESI) available: experimental details; Fig. S1, absorbance measurements taken of 8 sols; Table S1, *S/N* ratios for the median spectra of the SERRS bands used in MANOVA; Tables S2(a) and S2(b), a comparison between ANOVA and MANOVA; Fig. S2, log–log calibration model for cresyl violet; Table S3, *R* values and linear coefficients for calibration models calculated from all bands in the SERRS spectrum of cresyl violet; Fig. S3. histogram for a bootstrap analysis of the correlation coefficient for the log–log relationship for area under the cresyl violet SERRS band at 930 cm^{-1}. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/b800340h

Materials and methods

Preparation of colloids

Eight sols were prepared according to the following published protocols. For the citrate reduced colloidal silver and gold sols the Lee & Meisel method[8] was used. EDTA reduced colloidal silver was prepared as described by Fabrikanos *et al.*[9] Fructose and glucose reduced colloidal silver solutions were prepared according to Pal *et al.*[10] Oleylamine reduced colloidal silver was prepared by the Hiramatsu and Osterloh method.[11] PVP capped colloidal silver sols were prepared using the method developed by Sun and Xia.[12] Finally, thiol capped colloidal silver was prepared according to Korgel and Fitzmaurice.[13]

To our knowledge, of these eight colloids, only the citrate reduced silver, gold sols, and the EDTA reduced silver sol have been adopted previously for use in SERS; for the other four sols no Raman measurements have been made. For each colloidal substrate, three independent batches were prepared, and in line with accepted practice UV-visible absorbance data were collected in an effort to gain an understanding of the properties of these colloids. Absorption spectra were acquired with a Spectronic UV1 scanning spectrophotometer (Thermo Electron Corp.).

Surface-enhanced Raman scattering

SERS active samples were prepared by mixing together 298 μL of colloid, 100 μL of 10^{-2} M KCl (Sigma-Aldrich, UK) and 2 μL of cresyl violet perchlorate (Sigma-Aldrich, UK) at 10^{-3} M,

resulting in a final concentration of 5×10^{-6} M. The samples were then presented in a 96 well microtitre plate (Greiner Bio-One Ltd.). SERS spectra were recorded on a Renishaw System 2000 microscope, with a resolution of ~6.5 cm^{-1} employing a high power (300 mW) 830 nm NIR diode laser source. Power at the sampling point was approximately 80 mW and static SERS spectra centred at 1050 cm^{-1} were each integrated for 1 s. This resulted in spectra from 754.8 cm^{-1} to 1333.5 cm^{-1} and each spectrum was represented by 574 bins.

Multivariate analysis of variance

Multivariate analysis of variance (MANOVA[7,14]) uses exactly the same principle as univariate analysis of variance (ANOVA), except the data comprise p variates instead of a single variate. Hence, in MANOVA the sums of squares (SS) are replaced by sums of squares and products matrices (SSPM) for the p variates. A comparison between ANOVA and MANOVA can be seen in Tables 1 and 2. In these tables the generalised forms for one-way ANOVA and one-way MANOVA are shown for testing differences among two or more independent groups.

There are no quantities which correspond to the mean squares (M) of ANOVA, instead the test statistic, termed Wilks' Lambda (Λ) is calculated as the ratio of the determinant of matrix $\boldsymbol{R}$ divided by the determinant of the matrix which is the sum of both $\boldsymbol{H}$ and $\boldsymbol{R}$. The F-statistic approximation and its degrees of freedom (d.f.) are then calculated as described in eqn (1).

$$\frac{1-\Lambda^{1/b}}{\Lambda^{1/b}}\frac{(ab-c)}{ph} \approx F_{[ph,ab-c]} \quad (1)$$

Where

$$a=\left(r-\frac{p-h+1}{2}\right), \quad b=\sqrt{\frac{p^2h^2-4}{p^2+h^2-5}}, \quad c=\frac{ph-2}{2},$$

Table 1 An ANOVA table for a one-way situation, where there are t treatments each replicated n times. The other symbols (M and s^2) are as defined in the table, F is the test statistic. Table adapted from ref. 15

	Sum of squares	Degrees of freedom (d.f.)	Mean square	F
Treatment	$(t-1)M$	$(t-1)$	M	$\frac{M}{s^2}$
Residual	$t(n-1)s^2$	$t(n-1)$	s^2	—
Total	Sum of above	$tn-1$	—	—

and h, r and t are defined in Table 1 and p is the number of variates.

It is important to note that MANOVA cannot handle collinear matrices, which give rise to matrix singularities, and therefore rather than work directly on the SERS spectra (which contained 574 variates) we first of all need to reduce the number of independent variables. We chose to take 4 normalised intensity measurements for clearly defined (non overlapping) SERS bands arising from cresyl violet using each colloidal preparation; further details of the bands used are provided below. The normalised intensity calculation used the ratio of maximum peak intensity to the intensity of the bin at 1324 cm^{-1}, which represented a position on the spectrum where no Raman signal was observed across all spectra acquired for this experiment.

All data analyses were performed using MATLAB v. 6.5 (The Mathworks, USA) with the statistical toolbox.

Results and discussion

UV-visible absorbance spectroscopy of colloids

In Fig. 1 the absorbance data are summarised by averages and standard deviation errors bars for the absorption (λ) maximum (for which a larger value equates to a larger particle size); the

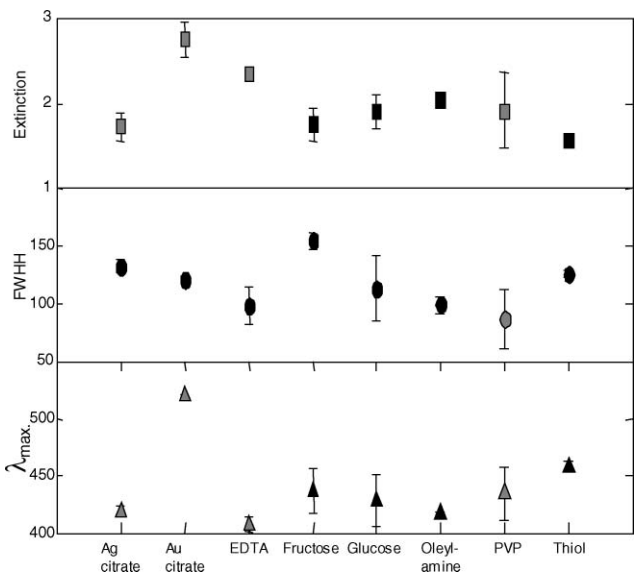

Fig. 1 Mean and standard deviation error bars for absorbance measurements taken from three independent batch preparations of the eight sols that were subsequently assessed for SERS activity. λ = absorption maximum; FWHH = full width at half height.

Table 2 A MANOVA table for a one-way situation, where there are t treatments each replicated n times. The square matrices $\boldsymbol{H}$, $\boldsymbol{R}$ and $\boldsymbol{T}$ are $p \times p$ in dimension, where p is the number of variates involved. F is the test statistic approximation calculated from Wilks' Lambda (Λ)

	Sum of squares & products matrices	Degrees of freedom (d.f.)	Wilk's Lambda Λ	~ F
Treatment	$\boldsymbol{H}$	$h=(t-1)$	$\frac{\lvert R\rvert}{\lvert H+R\rvert}$	eqn (1)
Residual	$\boldsymbol{R}$	$r=t(n-1)$	—	—
Total	$\boldsymbol{T}$, sum of above	$t=tn-1$	—	—

full width at half height (FWHH, a larger FWHH indicates wider particle size distribution) and the extinction (a lower value for the extinction indicates greater aggregation).[16] Major differences between the single gold sol and the seven silver sols can be seen with respect to the extinction, which is greatest for gold, and the absorption maximum, which is also greatest. The larger standard deviation errors for the glucose reduced and PVP capped Ag sols would suggest poor batch-to-batch reproducibility when compared against the other preparations, although the MANOVA result contradicts this for the PVP case. It would not be unfair to comment that UV-visible absorbance data do not act as good predictors for the quality of repeat SERS measurements taken using different substrate batch preparations.

On testing the sols for SERS activity, only four of the substrates yielded a SERS response for cresyl violet under the conditions described above; these were the citrate reduced silver and gold sols, PVP capped silver and EDTA reduced silver. The data shown in Fig. 1 do not show any correlation with SERS response, as there is no characteristic trend which separates these colloids (gray markers) from those which did not give SERS (black markers). For those that did work, an experiment was designed that could be used to determine the level of reproducibility for each. The method simply required the acquisition of replicate SERS measurements of cresyl violet from each batch of colloid. To enable statistical analysis of these data, five spectra were obtained using each batch ($n = 3$), giving a total of 60 SERS spectra.

A previous SERRS study of cresyl violet used spectra acquired with green and red excitation facilitated by a silver island films.[17] In conjunction with density functional theory calculations, these researchers were able to derive a comprehensive list of band assignments for the cresyl violet SERS spectrum, and we have used this resource in our analysis. In Fig. 2 the average SERS spectrum of cresyl violet acquired using each of the substrates is plotted. Clear overlaps across each colloid exist for bands at *ca* 877 cm^{-1} (C–H wag & twist, reported as a very weak vibration previously), 985 cm^{-1} (NH_2 in phase rock + C–O stretch, weakly scattering with visible excitation), 1230 cm^{-1} (C–H bend & rock + NH_2 rock) and 1277 cm^{-1} (C–H vibrations). Although for citrate reduced colloidal gold, the bands at 846 cm^{-1} (possibly related to the C–O–C bend) and 985 cm^{-1} are striking by their absence. This suggests that the adsorption characteristics of cresyl violet onto gold as opposed to silver colloid is different, which gives rise to a significant chemical effect. For the gold substrate, there is a possibility that this could be explained by the bonding ligand being formed at the sole oxygen atom, which would explain an absence of oxygen related vibrational structure (the peaks at 985 cm^{-1} and 846 cm^{-1}) in these spectra. Furthermore, for citrate reduced colloidal gold the nanoparticles exhibit a net positive charge,[18] that would make this a more realistic scenario than bonding at the amino groups. Conversely, for the citrate reduced silver substrate, the colloids have a net negative charge,[16] which makes bonding at the amino groups more likely. However, at this stage we have no direct evidence to support these comments, and this shall be an area of future work.

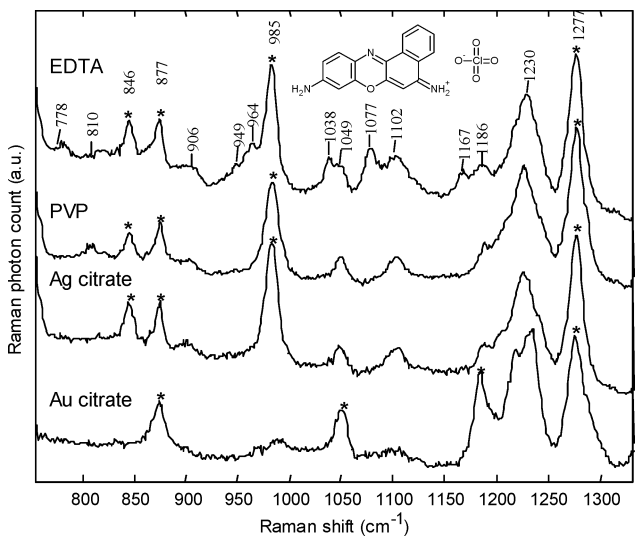

Fig. 2 Mean SERS spectra of cresyl violet acquired using the four colloidal substrates that were found to be SERS active. normalised intensity was calculated from the peaks marked by asterisk and then used in the MANOVA calculation. The structure of cresyl violet perchlorate is inlaid.

Statistical comparison of SERS reproducibility

Table 3 lists the normalised intensities for the median spectra of the SERS bands used subsequently in MANOVA (those marked by asterisk in Fig. 2), since this metric is of interest to those attempting to optimise a SERS experiment. Whilst any differences in the intensity of SERS signal in the enhanced spectra for different substrates are fairly small, the normalised intensity for both citrate reduced and PVP capped colloidal silver are greater than those for both citrate reduced gold and EDTA reduced silver.

Many researchers use single bands (usually the most intense) for assessing reproducibility and typically quote relative standard deviations. This undervalues and ignores valuable information across the whole spectrum and we believe that a multivariate approach is more appropriate. In principle, MANOVA parallels the univariate ANOVA method, that is used widely; but MANOVA has the advantage that it enables the assessment of the statistical significance of the factors and their interactions within the experimental design. In MANOVA, the approximate F value is calculated from Wilks' Lambda (Λ[19]) and its significance determined by reference to the F distribution statistical table. Here, we are applying MANOVA to assess the reproducibility between batches of colloidal metal substrates

Table 3 Normalised intensities observed in the median SERS spectra of cresyl violet for the vibrational bands used in MANOVA

	877 cm^{-1}	1049 cm^{-1}	1186 cm^{-1}	1277 cm^{-1}	$\bar{x}$
Au citrate	1.24	1.20	1.58	1.88	1.47
	846 cm^{-1}	877 cm^{-1}	985 cm^{-1}	1277 cm^{-1}	
Ag citrate	1.23	1.28	1.81	2.10	1.60
EDTA	1.09	1.11	1.44	1.72	1.34
PVP	1.29	1.28	1.93	2.03	1.63

Table 4 MANOVA on the normalised intensities calculated from the SERS bands identified in spectra of cresyl violet, from four active substrates, where in each case d.f.1 = 8 and d.f.2 = 18 w.r.t. the *F* statistic[14]

	Ag citrate	Au citrate	EDTA	PVP
Raw SERS spectra				
Wilks' Λ^a	0.429	0.061	0.122	0.300
~ F^b	1.187	6.890	4.187	1.856
P^c	NS	0.000	0.006	NS
Row normalised SERS spectra				
Wilks' Λ^a	0.543	0.141	0.577	0.560
~ F^b	0.803	3.749	0.712	0.757
P^c	NS	0.009	NS	NS

[a] Wilks' lambda (Λ), a statistic for testing the null hypothesis that the group centroids do not differ. [b] *F* statistic calculated from Wilks' Λ. [c] The probability level at which *F* is significant (NS indicates that *F* is not significant).

for SERS using the null hypothesis that there is a significant difference between batch means, *i.e.* the *F* statistic is significant at a level of 5% or less.

MANOVA of the raw data showed a highly significant difference between batches for Au citrate and EDTA (Table 4), which allows us to conclude that in these cases the reproducibility of SERS measurements is poor. However, for the Ag citrate and PVP preparations no significant differences are observed which shows good reproducibility. To account for variability in overall signal intensity, because either more analyte/colloid was present in the collection volume, we row normalised (centred each spectrum about the mean and scaled to unit variance) the SERS spectra and repeated MANOVA (Table 4). In this case three out of the four colloids, the exception being the Au citrate preparation, show no significant differences between batches. The value for the *F* statistic is also substantially lower across each of the four MANOVA results, which indicates a reduction in variability between SERS measurements following this simple pre-processing step.

Conclusions

We have demonstrated a novel method for determining objectively the multivariate reproducibility of colloids used for SERS measurements across multiple batch preparations. If colloidal SERS is to become a viable analytical method in applied science, it is necessary to consider experimental designs that take into account batch-to-batch variability, and provide a suitable statistical treatment of the data in order to draw objective comparisons between different methods. In this study we have shown that MANOVA is a very powerful tool for conducting such analysis, which until now has not been applied in SERS studies.

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