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ORIGINAL PAPER

Quantitative analysis of methyl green using surface-enhanced resonance Raman scattering

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Abstract Surface-enhanced resonance Raman scattering (SERRS) spectra of aqueous solutions of the triphenylmethane dye methyl green have been obtained for the first time by use of citrate-reduced silver colloids and a laser excitation wavelength of 632.8 nm. Given the highly fluorescent nature of the analyte, which precluded collection of normal Raman spectra of the dye in solution and powdered state, it was highly encouraging that SERRS spectra showed no fluorescence due to quenching by the silver sol. The pH conditions for SERRS were optimised over the pH range 0.5–10 and the biggest enhancement for SERRS of this charged dye was found to be at pH 2.02, thus this condition was used for quantitative analysis. SERRS was found to be highly sensitive and enabled quantitative determination of methyl green over the range 10^{-9} to 10^{-7} mol dm^{-3}. Good fits to correlation coefficients were obtained over this range using the areas under the vibrational bands at 1615 and 737 cm^{-1}. Finally, a limit of detection of 83 ppb was calculated, demonstrating the sensitivity of the technique.

Keywords pH · Methyl green · Silver sol · Surface-enhanced resonance Raman spectroscopy (SERRS)

Introduction

Methyl green is a triphenylmethane dye used for identification of DNA, RNA, and other cell components; in histology it is a particularly useful counterstain that stains nuclei light green [1–7]. This dye has also been used for routine staining of biopsies from lymphoid glands to enable clinical pathologists to identify pyroninophilic cells [8] and has also been used to reveal sites of developing cartilage in human embryos [9].

Surface-enhanced resonance Raman scattering (SERRS) spectra of aqueous solutions of methyl green have been obtained in order to investigate the sensitivity of the technique as an alternative probe for the dye, and the concentration dependence of the signal intensities for quantitative analysis.

The theory of the SERRS effect has been outlined by Mullen et al. [10]. Briefly, SERRS signal enhancement arises from a combination of signal intensification via resonance Raman scattering (RRS) [11] and surface-enhanced Raman scattering (SERS) mechanisms [12–14] which in combination can increase the efficiency of the Raman scattering process by 10^{10}-fold or greater [15, 16]. SERS and SERRS have found wide utility as sensitive analytical and bioanalytical tools [17–20]. For maximum sensitivity, SERRS requires controlled aggregation of the colloidal sol used [21]. Surface enhancement of the Raman signals depend on the size of the colloidal particles [22], pH [23, 24], and the excitation wavelength employed. The surface plasmon absorption bands of metals such as silver and gold show wavelength-dependent shifts with metal particle size and a surface enhancement effect is achieved by choosing the Raman excitation wavelength to lie within the contour of the plasmon band [25].

The inability to interrogate intrinsically highly fluorescent molecules (for example methyl green) by Raman spectroscopy in the visible to near infrared (here defined as 488–830 nm because of the ready availability of lasers) can be overcome by using several alternative

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Raman analytical techniques. These include, but are not restricted to, the use of Fourier-transform (FT) Raman at 1064 nm excitation or deep UV resonance Raman below 260 nm [26, 27], anti-Stokes vibrational bands, shifted subtractive Raman spectroscopy [28], and the use of the Kerr gate effect in time-resolved Raman spectroscopy [29]. In addition, surface-enhanced methods (*viz*. SERS and SERRS) can also be used to overcome both fluorescence and the analytically (relative) low sensitivity generally associated with Raman scattering of molecules in solution. Different kinds of media/substrates can be used to obtain SERS and/or SERRS signals of analytes. These include, for example, roughened electrodes [30], silver and gold colloids [31], nanoshell colloids [32], and so-called "gel-colls" which employ a hydrophilic swelling polymer (such as polyacrylic acids) in combination with a metal colloid [33].

Although the average SERS signal enhancement of molecules adsorbed on SERS-active silver surfaces has normally been measured to lie in the range 10^5–10^6, it has been known for some time that the SERS enhancement can be 10^{10} (or greater) in specific "hot spots" [16]. The observed SERS signal enhancement is currently hypothesized to be caused by a combination of effects: "classical electromagnetic field (EM) enhancement" and "the chemical effect". The former refers to surface-plasmon polariton resonances in colloidal systems such as silver colloidal particles (and nanoparticles of a few other metals) used as SERS substrates. The chemical effect, actually due to a combination of effects, is primarily a "first-layer effect" caused by dynamic (i.e. optically excited) electron (or hole) transfer from the metal to the LUMO (or HOMO) of the adsorbed analyte(s) in the first monolayer of the metal (e.g. silver) sol [34, 35].

In previous investigations we carried out SERS and SERRS studies on a wide range of dyes belonging to different structural classes and illustrated the power of Raman spectroscopy, when coupled with a surface enhancement effect, for characterisation of these dyes [23, 36–40]. In the current investigation reported herein we investigated SERRS spectroscopy, with laser excitation at 632.8 nm, to generate information-rich spectra from the triphenylmethane dye methyl green (Fig. 1). We investigate the pH dependence of the dye solutions for generating good SERRS. Following this optimisation we used these conditions for quantitative analysis of the dye.

Experimental

Reagents

Methyl green (Aldrich), poly(l-lysine) hydrobromide, M_r 4,000–15,000 (Sigma), silver nitrate (BDH), trisodium citrate, sodium hydroxide, and hydrochloric acid (Fisher) were of analytical grade. The dye was used without further purification. Double-deionized water was used for all experiments.

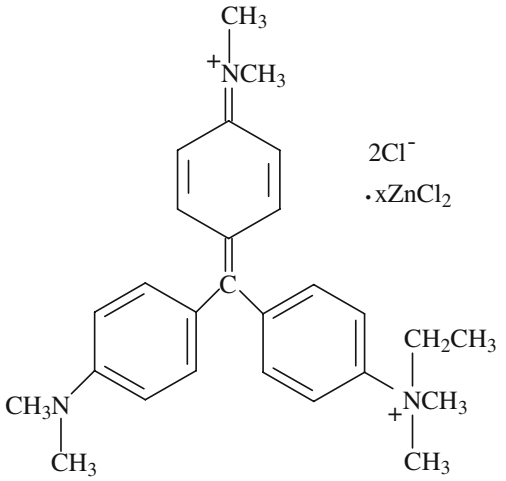

Fig. 1 Schematic diagram of the structure of methyl green

Methyl green solutions

Aqueous solutions of the dye in the concentration range 10^{-9}–10^{-3} mol dm^{-3} were prepared in double-deionised water. Samples were always made up fresh, immediately before analysis was carried out.

Colloid preparation

A silver colloid was prepared according to a modified Lee-Meisel procedure [25, 41]. All glassware was acid-washed with aqua regia (HNO_3-HCl, 1:3*v*/*v*) followed by gentle scrubbing with a soap solution. Silver nitrate (90 mg) was suspended in 500 mL deionised water at 45 °C and rapidly heated to boiling before addition of a 1% solution of trisodium citrate (10 mL) under vigorous stirring. The solution was held at boiling for 90 min with continuous stirring. The quality of the resulting colloid was checked by determining the wavelength of the absorption maximum in the visible region on a Perkin-Elmer Lambda-2 UV-visible spectrometer. Good quality silver colloids for SERS apparently have an absorption maximum at approximately 404 nm and this peak has a full width half height (FWHH) of ~60 nm [41] which demonstrates a narrow distribution of the particles. In addition, a Jeol JEM-200CX transmission electron microscope was used to inspect the colloids and revealed a mean of diameter of 50 nm (±1 nm standard deviation) for citrate-reduced sols (see Fig. 2 for an example TEM image). The nature of the Lee-Meisel colloid [15, 42], has been examined using visible absorption, photon

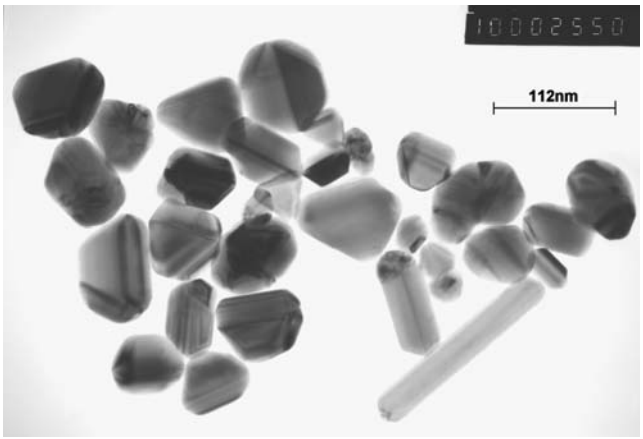

Fig. 2 Representative TEM image of citrate-reduced sols (magnification × 50,000)

correlation, and NMR spectroscopic techniques (data not shown) which confirm that the surface of the silver particles are covered with a layer of citrate with pendent negatively charged groups. As the methyl green dye itself is negatively charged (Fig. 1) and would repel the colloid, the subsequent addition of poly(l-lysine) coats the surface with pendent positively charged groups on the colloidal surface [43] thus allowing colloid and dye to aggregate. We thus adopted this approach to obtain good SERRS signals from the methyl green dye.

Sample preparation

Aggregation of the silver colloid particles was induced by poly(l-lysine). An aqueous solution of poly(l-lysine) (0.01%, 150 μL) was added to 1 mL silver colloid which had been diluted with 1 mL deionised water, followed by 150 μL methyl green solution and 35 μL of a 1 mol dm^{-3} aqueous solution of either HCl or NaOH. For quantification (*vide infra*) all the SERRS spectra were collected three times using the same analyte, colloid, and aggregating agent prepared three times; all measurements were made on the same day.

Instrumentation

Raman spectra were obtained using a Renishaw (Old Town, Wotton-under-Edge, Gloucestershire, UK) System 2000 Raman microscope, with a resolution of ~6.5 cm^{-1}. A 20-mW 632.8 nm HeNe laser source was used for all measurements and there was ~2 mW power at the sample. All SERRS spectra were collected by using 180° back-scattering geometry. An Olympus microscope objective of magnification 50× was used both to focus the incident laser light and to collect the back-scattered Raman light.

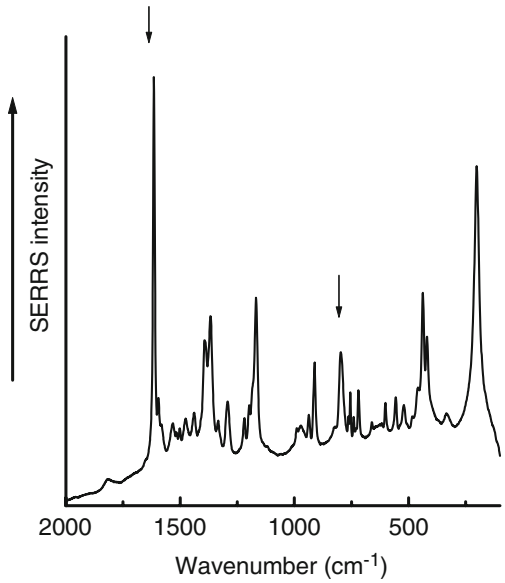

Fig. 3 A typical unprocessed (raw data) SERRS spectrum of 8.1×10^{-6} mol dm^{-3} methyl green using an excitation wavelength of 632.8 nm and a laser power at the sample of 2 mW. Vibrational bands at 797 cm^{-1} and 1615 cm^{-1} used for quantitative analysis are indicated by *arrows*

SERRS pH dependence

A series of SERRS spectra from 10^{-7} mol dm^{-3} methyl green dye were collected over the pH range 0.5 to 10.0. The optimum pH conditions were determined by plotting the logarithm (log_{10}) of peak area of the vibrational band at 1615 cm^{-1} from methyl green *vs*. pH.

Reproducibility/time dependence

Following mixing of the dye solution with the silver sol and aggregation of the colloidal particles, SERRS signal intensities of methyl green grew with time until after ~5 min when

Table 1 Tentative SERRS[a] vibrational band assignments for methyl green

Wavenumber (cm^{-1})	Tentative band assignments
797	CH_3 stretching vibration
1369	CH_3 sym deformation
1400	CH_3 antisym deformation
1445	CH_3 antisym deformation
1479	CH_2 scissoring
1603	Ring stretch
1615	Ring stretch
1620	C=C stretch

[a] λ_{exc} 632.8 nm

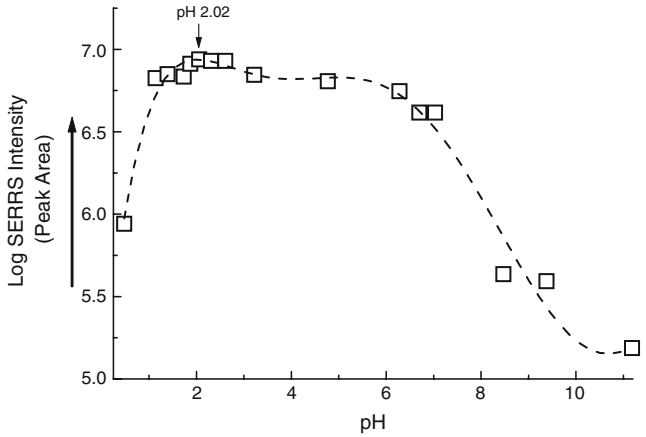

Fig. 4 SERRS pH profile for methyl green using a dye concentration of 5.5×10^{-7} mol dm^{-3}. Logarithm (Log_{10}) intensity (peak area) of the 632.8 nm-excited SERRS vibrational band at 1615 cm^{-1} of methyl green is plotted against the pH. The optimum SERRS response is indicated by an *arrow*. *Points* represent averages from three measurements

they remained constant. Therefore all spectra were collected 5 min post-aggregation.

Quantification of methyl green

The concentration-dependence of the SERRS signal from methyl green was determined by plotting the logarithm intensity (peak area) *vs*. the logarithm of methyl green concentration. For quantitative analysis the area under the vibrational bands at 1615 and 797 cm^{-1} were used; this was because all the other bands showed very similar trends and similar limits of detection (data not shown).

Results and discussion

All Raman and SERRS spectra were collected using exciting radiation of 632.8 nm on a Renishaw 2000 Raman microspectrometer. Because of the highly fluorescent nature of the methyl green dye normal Raman spectroscopy of the dye in the solution and solid state revealed strong fluorescence backgrounds (data not shown) and an absence of any Raman vibrational bands. By contrast, strong vibrational bands were observed at 201, 483, 755, 797,

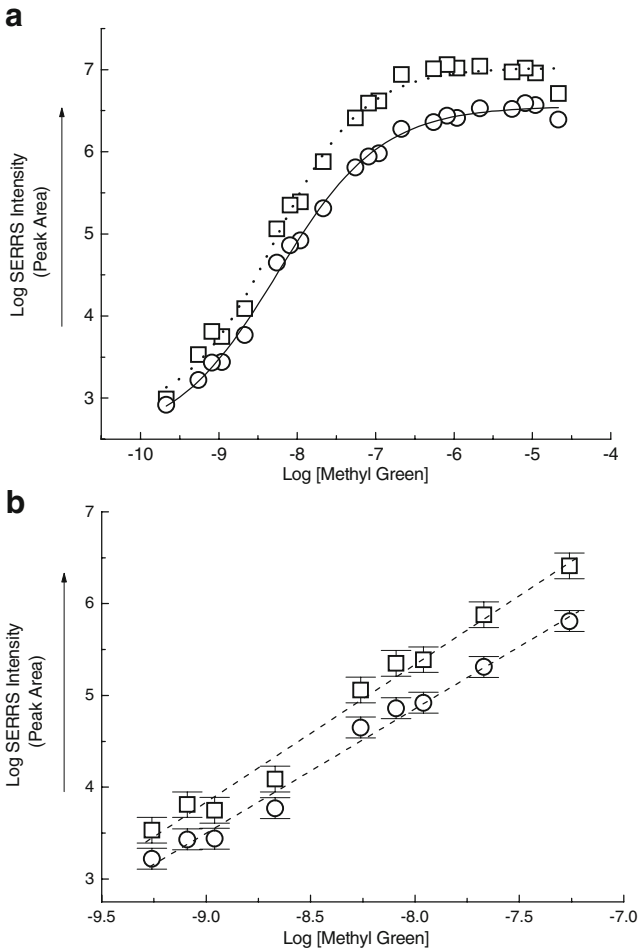

Fig. 5 (**a**) Methyl green in the concentration range 2.14×10^{-10} to 2.20×10^{-4} mol dm^{-3}. SERRS vibrational bands used for analysis are 1615 cm^{-1} (*squares*) and 797 cm^{-1} (*circles*). (**b**) Linear region used for semi-quantitative analysis in the concentration range 10^{-10} to 10^{-7} mol dm^{-3} (see Table 1). The excitation wavelength was 632.8 nm, and the laser power at the sample was 2 mW. *Points* represent averages from three measurements and *error bars* represent the standard deviation

1218, 1369, and 1615 cm^{-1} (Fig. 3) from the dye in solution. Tentative vibrational band assignments are given in Table 1. The excitation wavelength of 632.8 nm lies within the strong electronic absorption band centred on 635 nm, so these spectra are resonance-enhanced and surface enhanced from the silver colloid. In addition, it

Table 2 Analytical data obtained from linear regression for analysis of methyl green using SERRS at 632.8 nm

	Linear regions for calculations below							
Band	Slope	Intercept	Correlation coefficient (R)	Concentration range (mol dm^{-3})	RSD (±)	Orders of magnitude	X^2	LOD[a] (ppb)
1615 cm^{-1}	1.51	17.46	0.992	10^{-9}–10^{-7}	0.139	2	0.016	117
797 cm^{-1}	1.36	15.76	0.993	10^{-9}–10^{-7}	0.114	2	0.06	83

[a] Limits of detection (LOD) were determined by taking three times the standard deviation of the intercept (of non-log plots) and dividing by the slope, as adopted by Womack et al. [45]

was also encouraging to observe in these and all the SERRS spectra collected that the fluorescence was completely quenched.

As one can observe from the chemical structure of methyl green (Fig. 1) this dye is a charged molecule and its ionisation state depends on the pH of the surrounding medium. It has been recently demonstrated that the SER(R)S response is dependent on the pH of the solution of the analyte [23, 24], and therefore we decided to conduct a "pH profile" of the methyl green in order to optimise the signal enhancement. In this optimisation all conditions were kept constant except the pH which was adjusted using NaOH and HCl to lie between pH 0.5 and 10.0. The dye concentration was 5.5×10^{-7} mol dm^{-3} and the largest surface and resonance-enhanced peak at 1615 cm^{-1} was used to assess signal enhancement. Figure 4 shows the pH profile from this set of SERRS spectra and it was interesting to note that good SERRS spectra can be obtained for methyl green over the whole pH profile. Moreover, the optimum pH was found to be 2.02 and this was used for quantification of methyl green.

SERRS spectra of methyl green were collected over the concentration range 5×10^{-9} to 5×10^{-4} mol dm^{-3}. Plots of the area under two SERRS peaks at 1615 cm^{-1} and 797 cm^{-1} that gave good reproducibility are shown in Fig. 5a; these bands are chosen for illustrative purposes because very similar results were obtained for all other bands (data not shown). In this figure it can be seen that the signal enhancement reaches a plateaux after 10^{-7} mol dm^{-3} and this was because of the coverage of the colloidal silver particles being in excess of full monolayer of the dye, a phenomenon that we have observed previously [44]. The region from 10^{-9} to 10^{-7} mol dm^{-3} shows very good linearity (in this log-log plot) over two orders of magnitude (Fig. 5b) and this is confirmed using correlation coefficients and error measurements on the repeated acquisition (three in total) over this range (see Table 2 for the statistics). In Fig. 5b the mean peak areas of each vibrational band at 1615 cm^{-1} and 797 cm^{-1} are shown, with standard deviation error bars. The percentage difference for the three replicates was ~3% which is excellent for SERRS. We note that the slope of these two measurements was slightly different (Table 2); for the band at 1615 cm^{-1} this was 1.51 whereas for the 797 cm^{-1} band it was 1.36. This could indicate different mechanisms for the two lines (i.e., a mixture of electromagnetic *versus* chemical enhancement) but there was no direct evidence of this.

The limits of detection (LOD) were determined by taking three times the standard deviation of the intercept (of non-logged plots) divided by its slope, as adopted by Womack et al. [45] and used by us previously [23, 36–40]. These calculations are shown in Table 2 for the two bands and it is clear that the LOD for SERRS was 83 ppb (10^{-9} mol dm^{-3}) for the band at 797 cm^{-1}.

In conclusion, this is the first study to show SERRS from the triphenylmethane dye methyl green. SERRS has been shown to be a very sensitive technique for quantitative determination of methyl green with the advantage of fluorescence quenching by the silver colloids. The benefits of the SERRS technique are clearly demonstrated in that it was not possible to obtain normal Raman spectra of the dye in the solution and solid states. For quantitative analysis good linear fits over the 10^{-9} to 10^{-7} mol dm^{-3} range were observed. For this molecule SERRS spectra could also be obtained across a broad pH range, and the optimum pH was calculated to be 2.02. Thus we have clearly demonstrated excellent SERRS spectra of aqueous solutions of methyl green in order to investigate the sensitivity of the technique as an alternative probe for the dye. Future work will concentrate on investigating SERRS of this dye in histological sections.

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