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Simple Preparation of Positively Charged Silver Nanoparticles for Detection of Anions by Surface-Enhanced Raman Spectroscopy

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Modification of citrate and hydroxylamine reduced Ag colloids with thiocholine bromide, a thiol functionalized quaternary ammonium salt, creates particles where the zeta potential is switched from the normal values of ca. -50 mV to ca. +50 mV. These colloids are stable but can be aggregated with metal salts in much the same way as the parent colloids. They are excellent SERS substrates for detection of anionic targets since their positive zeta potentials promote adsorption of negatively charged ions. This is important because the vast majority of published SERS studies involve cationic or neutral targets Moreover, the fact that the modifier is a quaternary ammonium ion means that the negative surface charge is maintained even at alkaline pH. The modified colloids can be used to detect compounds which cannot be detected using conventional negatively-charged citrate or hydroxylamine reduced metal nanoparticles, for example the detection limit was 5.0 x 10^{-5} M for perchlorate and < 8.7 x 10^{-7} M for tetraphenylporphine tetrasulfonic acid (TPPS). More importantly, picric acid (an explosive) and diclofenac (a non-steroidal anti-inflammatory) could also be analysed quantitatively at low concentrations, 2.5 x 10^{-5} M and 1.9 x 10^{-5} M, respectively. Interestingly, the correct choice of aggregating agent is important for achieving high sensitivity since the anion in the aggregating salt may compete with anionic targets for surface binding sites. Finally, since the modification procedure simply involves reaction of nanoparticles with a small alkyl thiol derivative, it can easily be adapted to other particle morphologies or metals.

Introduction

Despite the advances made in the fabrication of solid plasmonic substrates, Ag and Au colloids continue to be very widely exploited as SERS enhancing materials.[1] This is due a combination of the high enhancement factors that aggregated nanoparticles provide and the simplicity of colloid preparation, which leads to low analysis costs and allows substrates to be discarded after a single use. The range of target compounds which have now been detected by SERS measurements carried out on metal colloid substrates is vast but the requirement that the analytes must adsorb on or near the enhancing surface still limits the types of targets that can be detected. In particular, the most popular enhancing substrates are prepared by chemical reduction of an appropriate soluble metal salt. Citrate-reduced silver[2] and gold[3] colloids and hydroxylamine-reduced silver colloids[4] are the most popular but borohydride reduction is also extensively utilized.[5] In all these cases the colloidal nanoparticles are charge-stabilized by a monolayer of anions adsorbed on the surface, which gives them a strongly negative zeta potential. This surface charge limits spontaneous aggregation of the particles, so they may be stored for weeks or months before use and it also promotes adsorption of cationic molecules, *e.g.* rhodamine 6G and crystal violet. However, it is important to recognize that the negative surfaces will also repel negatively charged analytes, and this can prevent their adsorption and subsequent SERS detection.[6] Here we report a method for modifying Ag nanoparticles so that they carry a strongly positive zeta potential and therefore allow detection of negatively charged target molecules, which are a significant proportion of all potential analytes but are very poorly represented in the SERS literature. .

The details of analyte adsorption onto colloidal metal nanoparticles do vary with different analytes and surfaces but consideration of a few simple factors can be extremely helpful in predicting whether a molecule will be adsorbed, and therefore detected, by a given metal colloid. In particular, it is useful to distinguish between co-adsorption and displacement mechanisms. In the former the analytes are co-adsorbed with an existing surface layer. The most obvious mechanism is through electrostatic attraction *e.g.* of a positive analyte for a particle with negative surface ions (Rhodamine 6G adsorbing to Ag colloid carrying surface Cl^- is the classic example) but hydrogen bonding interactions and π-stacking *etc* may also facilitate adsorption. Indeed, we and others have used chemical modification of the surface to provide favourable binding interactions with specific poorly-adsorbing targets.[7,8] In contrast, in the displacement mechanism the analytes bind strongly to the metal surface itself, rather than the adsorbed surface layer, so the analyte displaces the existing surface ions on adsorption. Well known examples of this mechanism would be the adsorption of akyl and aryl thiols onto Ag and Au nanoparticles through the formation of strong metal-sulfur bonds.[9]

Detection of negatively charged analytes by co-adsorption onto conventional Ag and Au colloids which have a negative zeta potential

will clearly be difficult because of the electrostatic repulsion between analyte and surface ions. A partial solution to this problem is to use the displacement approach i.e. to prepare colloids with surface ions that bind more weakly to the particles than the target molecules, so that when the target molecules are added they can displace the existing layer and be detected. Of course, in this approach it is also important that competing ions are not introduced during aggregation when excess metal salts are added.[10] We have used this is a strategy for detection of mononucleotides, which carry a negative charge but can still be detected by SERS because they can adsorb onto citrate-reduced Ag colloid by displacing the surface citrate ions.[11] Critically, this is only possible if the colloid is aggregated by an excess of salt which contains weakly binding anions, such as sulfate, rather than strongly binding Cl^- because the mononucelotides cannot compete with high concentration chloride for surface sites. A similar effect is also observed for DNA.[12] Unfortunately, this displacement method is limited to those targets which bind more strongly than existing surface ions, so a large subset of all possible analytes is still excluded. An alternative, which has been more widely adopted, is to overcome the charge repulsion of anionic targets by using positively charged polymers, such as poly(L)lysine, as the aggregating agents or as coatings[13]. Alternatively, positively charged particles have been prepared by including a suitable capping ligand such as PEI,[14] thiocholine[15] or cetyltrimethylammonium bromide[16] in the reduction step. However, it is unclear how much control over the size and shape of the resulting particles is afforded using these syntheses, and, considering that many different shapes and morphologies of nanoparticles, including prisms, rods, cubes, nanostars, and nanoshells,[1,17] have been developed in recent years, it would clearly be better these engineered particles could first be prepared and then have their surfaces modified to carry a positive charge. A partial solution is to functionalize the surface of pre-formed particles with thiol modifiers which have primary or secondary amine functional groups but these have the disadvantage that they need to be protonated before they atttract anions., so they must be used in a strongly acidic environment.[18] An alternative technique is the use of a core-shell approach, where each particle is coated in a thin layer of silica, before grafting positive modifiers to the shell.[19] However, this method requires reaction with silane compounds, which is inefficient in aqueous solution, and the silica shell can diminish the SERS activity of the nanoparticles. In this paper, we show a more direct approach of modifying silver colloids with a short chain quaternary ammonium-terminated thiol, thiocholine, which is easily synthesized by acid catalysed hydrolysis of acetylthiocholine.[20] The thiol functional group has a strong affinity for the surface of the silver particles, and displaces the anionic surface layers of both citrate- and hydroxylamine- reduced colloids to yield stable, positively charged colloidal particles. These retain their positive zeta potential at neutral or alkaline pH and can therefore promote adsorption of negatively charged analytes which do not spontaneously adsorb to normal colloids. Here these modified nanoparticles have been tested with a range of analytes including examples of therapeutic drugs (diclofenac, a common non-steroidal anti-inflammatory) and ions which are of interest for environmental monitoring (nitrate and perchlorate) or anti-terrorist (picric acid) applications.

Experimental

Materials. All chemicals used, except sodium hydroxide, were purchased from Sigma Aldrich. Sodium hydroxide was purchased from Riedel-de Haën. All chemicals were used as received without further purification. All solutions were prepared from distilled, deionized (DDI) water resistivity > 18.2MΩ, from a Branstead Nanopure Diamond system. Thiocholine bromide was synthesized from acetylthiocholine bromide using a previously published procedure (supporting information).[20]

Silver Colloid Synthesis. Hydroxylamine-reduced silver colloid (HRSC) was synthesized following the method published by Leopold and Lendl.[4] 5 mL of NaOH (0.1 M) was added to 5 mL of aqueous hydroxylamine hydrochloride (6 mM), then the whole mixture added to 90 mL of aqueous $AgNO_3$ (0.1 mM) with stirring. The colloid formed spontaneously and was left stirring for ~20 minutes before use. The nanoparticles had a mean diameter of 74 nm, measured by dynamic light scattering, and a zeta potential of -49.5 mV, recorded by laser Doppler electrophoresis, both using a Malvern Zetasizer Nano ZS.

Citrate reduced silver colloid (CRSC) was synthesized according to Lee and Meisel's method.[2] $AgNO_3$ (45 mg) was dissolved in 250 mL and heated until boiling. When boiling temperature had been reached, 5 mL of 1% sodium citrate solution was added dropwise with stirring. The mixture was stirred under reflux for 90 minutes and then was allowed to cool to room temperature. These nanoparticles had a mean diameter of 71 nm as measured by dynamic light scattering, and a zeta potential of -48.6 mV.

Modification of Colloids with Thiocholine. Both HRSC and CRSC were modeled as spheres of uniform diameter and the total surface area of the colloid was calculated based on the mass of silver per sample and mean nanoparticle diameters found above. The amount of thiocholine required was estimated by dividing the total surface area of the colloidal sample by the surface area of one mercaptopropionic acid molecule, calculated by Spégel *et al* to be 20.2 $Å^2$.[21]

HRSC was modified directly without further treatment; 6 mL of $1x10^{-4}$ M thiocholine solution (approximately a two-fold excess) was stirred vigorously at room temperature in a conical flask, while adding 50 mL of HRSC dropwise over several minutes to produce the positive colloid, HRSC+. During the modification procedures it was common for some particles to adhere to the glassware, and metal-like films to form at the water/air interface. These metal films, as well as excess thiocholine and other ionic species could be removed by centrifugation (1500 *g* for 30 minutes) and resuspension in DDI water. The final zeta potential varied with each batch, but was always strongly positive, with a mean value of $\zeta = 47.9 \pm 11.6$ mV across 4 batches.

CRSC could not be modified directly (see Results and Discussion), but it could be modified after displacement of surface citrate by Cl^- by adding 2% (v/v) 0.01 M NaCl then centrifuging at 3000 rpm for 30 minutes. The pale yellow supernatant was removed and the nanoparticles were resuspended in DDI water up to the original volume. This process was repeated once more, at which point no citrate peaks were visible on in the SERS spectrum of the colloid. 30 mL of this modified colloid was added to 3 mL of $1x10^{-4}$ M thiocholine solution dropwise with vigorous stirring to produce the positive colloids CRSC+. After cleaning by centrifugation at 1500 *g* for 30 minutes and resuspension in DDI H_2O, the colloids were strongly positive with $\zeta = 45.9 \pm 16.8$ mV across 4 batches.

Nanoparticle Analysis. Colloids were prepared for SERS analysis by transferring 200 □L aliquots of each into a 96-well plate and aggregating through addition of 20 □L of a salt, such as 1 M NaCl, and stirring vigorously for 5 seconds. Raman spectra were acquired using an Avalon R2 Ramanstation, which has a 785 nm diode laser delivering 100 mW at the sample, an echelle spectrograph and a thermoelectrically cooled CCD. The SERS analysis of anionic targets was carried out by adding 20 □L of the analyte solution to the colloid and mixing vigorously for a few seconds before addition of the aggregating agent.

Nanoparticle size and zeta-potential were measured by dynamic light scattering and laser Doppler electrophoresis, respectively, using

a Malvern Zetasizer Nano ZS equipped with a 633 nm laser. UV-vis measurements were performed using an Agilent Agilent 8453 UV-Vis spectrometer.

Results and Discussion

In this work both citrate- and hydroxylamine-reduced silver colloids were modified with thiocholine. This would be expected to be a reasonably straightforward procedure since it is well known that alkyl thiols bind rapidly to the surface of Ag colloids through the formation of strong Ag-S bonds and simple addition of an appropriate concentration of thiols normally results in displacement of the surface ions by thiol modifiers.[22] However, in this case it was found that when thiocholine (0.1 mM) was added dropwise with vigorous stirring to HRSC the colloid aggregated during the modification process, and the nanoparticles irreversibly sedimented out of solution within a few minutes. Addition of thiocholine solution to silver colloids at different rates, with heating and/or using sonication still failed to yield stable, positive colloids In contrast, reversing the addition procedure dramtically improved the result. Adding HRSC slowly to a dilute thiocholine solution with stirring did not result in gross aggregation; the colloid became slightly darker but UV-Vis and DLS measurements confirmed that the colloid remained unaggregated.

It has been suggested that for amine-substitued thiols the positive ammonium head group and the thiol will compete for sites on the silver surface.[19] This model could explain how gradual addition of thiocholine aggregates a negatively charged colloid, as the positive head group would be attracted to the negative surface and reduce the charge on the nanoparticle, causing the colloid to aggregate even without direct reaction between the thiol and the Ag surface.

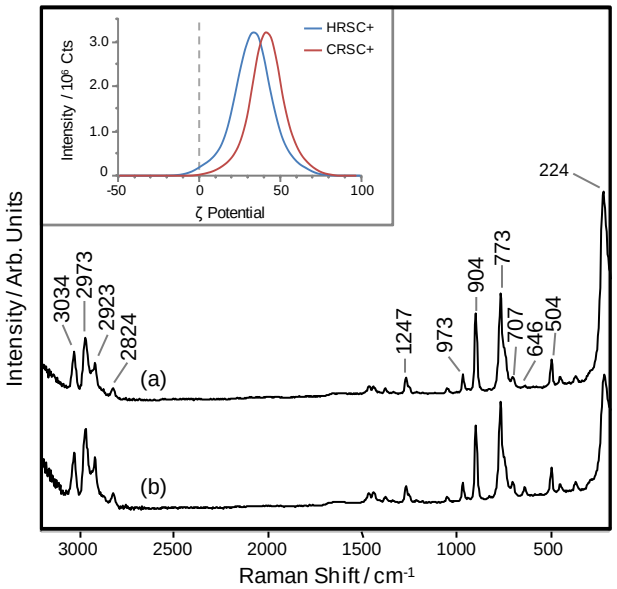
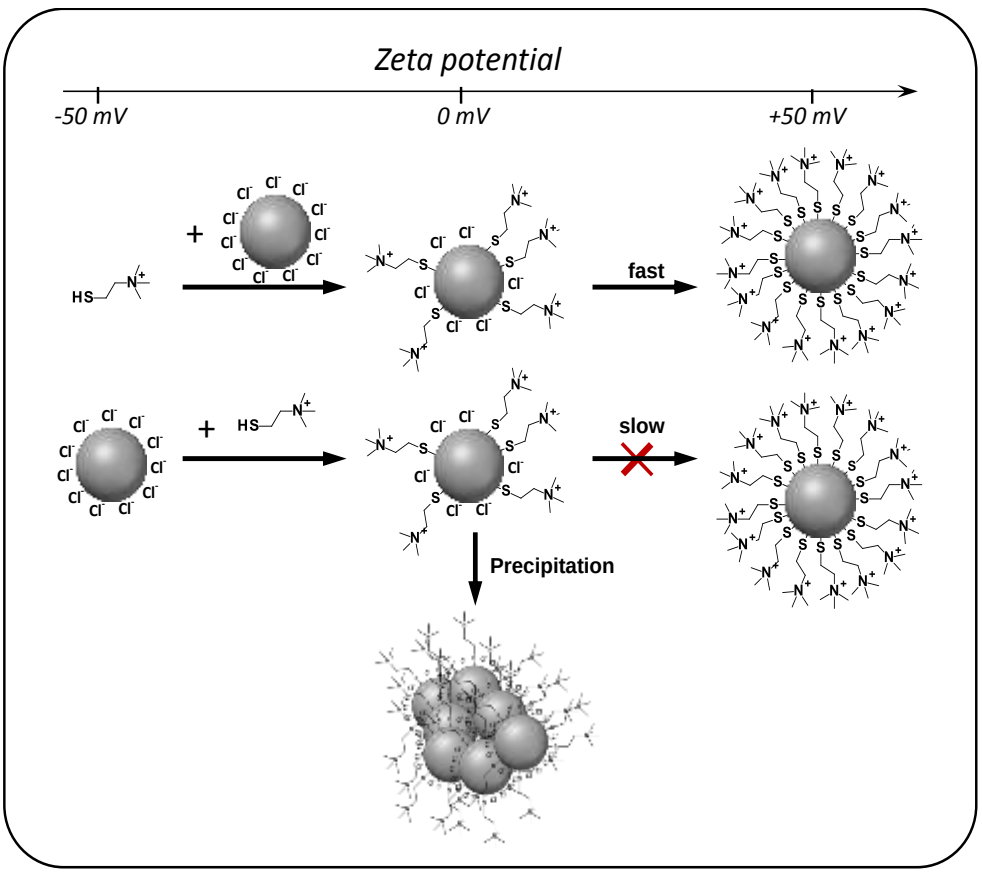


Figure 1. SERS spectra of (a) HRSC+ and (b) CRSC+. Inset shows the ζ potential distribution for each colloid.

However, the thiol group's high affinity for silver, makes it much more likely that the thiocholine would displace the negatively charged ions at the surface than to co-adsorb with the negative chloride ions.



Scheme 1. Schematic showing how adding thiocholine (TC) to a silver colloid results in a low effective concentration and charge neutrality on the surface (a). Slowly adding colloid to thiocholine gives a high effective concentration of modifier and overcomes charge neutrality (b).

This would also account for the observed aggregation since gradually increasing thiocholine concentration in the solution (and therefore also the surface) would give a corresponding decrease in ζ-potential, until somewhere around the point of charge neutrality, aggregation would occur. A similar effect has been observed with Au nanoparticles modified by mixed thiol monolayers.[23]
In contrast, reversing the procedure and adding colloid slowly to a solution of thiocholine (Scheme 1) gives an instantaneous jump in the effective concentration of the modifier surrounding each nanoparticle, which causes rapid exchange of thiol for chloride. This means that the point of charge neutrality is passed rapidly, before aggregation can occur, so direct conversion of a negatively- to positively-charged colloid (HRSC+) is possible.

Surprisingly, attempts to directly modify CRSC in the same way as was successful for HRSC still resulted in aggregation of the colloid. In this case the aggregation was found to be due to a direct interaction between the citrate initially present in the colloid and the thiocholine-modified CRSC+ colloids which was formed, since the same unexpected aggregation could also be induced in pre-prepared HRSC+ by adding a low concentration of trisodium citrate. Even citrate at a concentration of 6.8 x 10^{-5} M (in the colloid solution), which is much too low to aggregate normal CRSC and HRSC, caused the colloid to aggregate rapidly. This suggests that citrate directly adsorbs onto thiocholine modified colloids (HRSC+ or CRSC+) and reduces the surface charge to the point where aggregation occurs. It was found that the problem could be circumvented by replacing the surface citrate ions in CRSC with chloride ions before attempting modification with thiocholine. This was achieved by treating the nanoparticles with aqueous NaCl and centrifuging/resuspending them in DDI water. This process was repeated until citrate anions could not be detected in the SERS spectrum of the colloid. The nanoparticles could then be modified using thiocholine in the same way as HRSC to yield CRSC+.

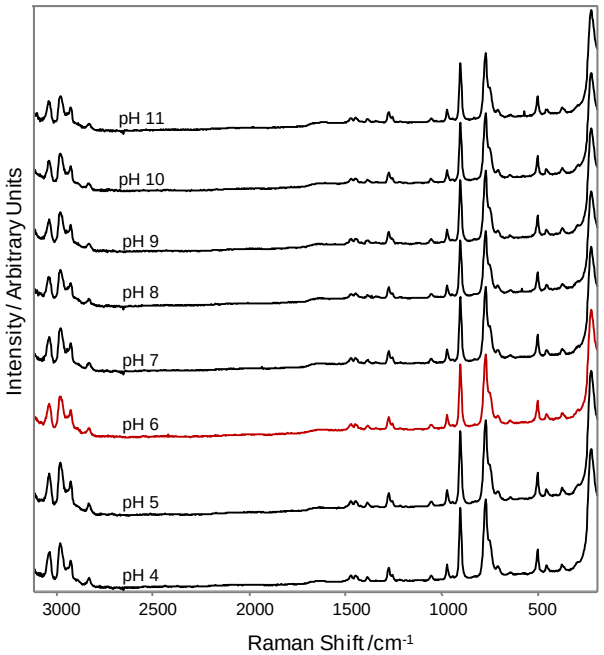


Figure 2. Raw SERS spectra of HRSC+ at varying pH values. The pH was adjusted using HCl or NaOH, before drawing 200 uL aliquots and aggregating with 20 uL 1 M NaCl. Spectra have been vertically offset for clarity, but are displayed on the same scale and are completely unprocessed. The pH 6 spectrum is highlighted as the typical pH of HRSC+ without adjustment.

The zeta potentials of both positive colloids were very similar (see insert in Figure 1), as were their SERS spectra, shown in Figures 1(a) and (b), which were dominated by bands due to the thiocholine surface modifier. The vibrational modes of the alcohol analogue, choline, have been assigned by Akutsu [24] and these have been used as the basis for our assignments of the main bands of thiocholine, which are shown in the Supporting Information.

One of the main advantages of HRSC+ and CRSC+ is that, in contrast to surfaces modified with primary or secondary amines, they are expected carry a positive surface charge over a broad pH range because they do not rely on the solution being sufficiently acidic to protonate their basic nitrogen atom. This is confirmed by their SERS spectra, which are identical over the pH range 4 to 11, as shown for HRSC+ in Figure 2. This is important because it means that in addition to acidic pHs they can be used at neutral and alkaline pH, which allows for detection of acid-sensitive compounds and also for samples at physiological pH.

It is now very well established that colloids must be aggregated to obtain maximum SERS enhancement. The UV/visible extinction spectra of HRSC+ aggregated with NaCl are shown in Figure 3. The spectra are unremarkable, aggregation gives rise to a broad plasmon band at the red end of the visible spectrum in much the same way as was observed for their negatively-charged precursors. This means that SERS spectra can be obtained using these colloids with 785 nm excitation.

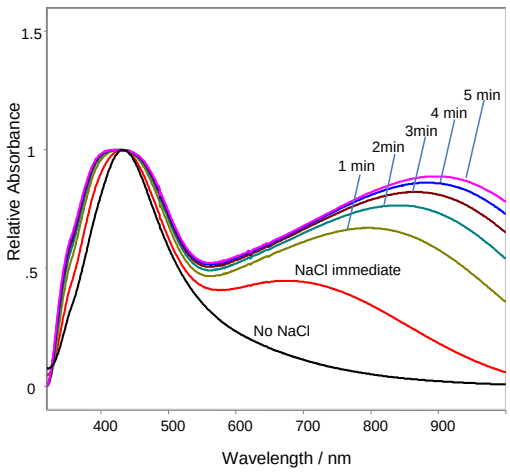


Figure 3. UV/vis spectra of HRSC+ over a period of 5 minutes when NaCl is added.

Although aggregating agents are essential for creating the appropriate plasmons for SERS enhancement, the aggregating agents are often not simply spectators but may compete with the analyte for surface sites. As discussed above, it well known that with negative colloids, analytes that can be detected using one aggregating agent may be completely undetectable using a different one. For example, aggregating normal CRSC with NaCl covers the surface in chloride anions that displace weakly binding analytes such as dipicolinic acid.[25] Alternatively, dipicolinic acid can be detected if more easily displaced anions, such as SO_4^{2-} or NO_3^-, are used for aggregation. Similar considerations apply to the positively charged colloids but in this case we have found that the anions of the aggregating salts interfere by coadsorbing to the modified surfaces of the positive colloids, blocking coadsorption by the target anions, rather than by directly displacing the surface layer on the silver. This is hardly surprising since the colloids are modified with thiocholine specifically

to promote absorption of anions but it means that it is important to select aggregating salts whose anions have a reasonably low affinity for the modified surface. One method of testing the extent to which this effect occurs is simply to aggregate the colloid with a series of different aggregating agents and to record their SERS spectra, looking for the characteristic bands of the aggregating agent in the spectra. Data for 12 different aggregating agents are shown in the Supporting Information. This is a reasonable guide to which aggregating agents will show a significant degree of interference but it is better to test the aggregating agents under the conditions where they will actually be used, *i.e.* in competition with the particular target anion of interest.

Here we show an example of this procedure using picric acid as the test analyte. Picric acid is of interest because it is an explosive that was once manufactured and used on a large scale but fell out of favor as more modern explosives were developed. However, it is currently freely available in most countries and therefore has potential to be reinstated for use in terrorist weapons. We have found that picric acid cannot be detected at low concentration using traditional metal colloids because it does not adsorb to their negative surfaces (data not shown) but it would be expected to adsorb to HRSC+, even at neutral pH, since its phenolic oxygen will be deprotonated and will therefore carry a negative charge. Figure 4(a) shows the SERS spectra of 3.6 x 10^{-4} M picric acid in HRSC+ after aggregation with 4 different salts. Aggregation with $MgSO_4$ or sodium acetate (NaOAc) gives signals which are dominated by strong picric acid bands, although they the spectra do show relatively weaker features due to the TC modifier, which remains bound. In contrast, with strongly competitive anions such as Br^- the intensities of the picric acid bands decrease relative to those of the TC modifier because the picric acid cannot compete with the Br^- for the adsorption sites. The spectra in Figure 4(a) were normalized to the thiocholine band at 774 cm^{-1} to emphasize this effect.

This competition is better illustrated by the plots shown in Figure 4(b), where the intensity of the strong picric acid band at 822 cm^{-1} (relative to the 904 cm^{-1} thiocholine band) is plotted against the concentration for colloids aggregated with three different salts: $MgSO_4$, NaCl and NaOAc. It is clear that, as expected from the data in Figure 4(a), aggregation with NaOAc gives strong picric acid signals even at low picric acid concentrations. Indeed, the data show a classic saturation curve where the signals plateau at approximately $1x10^{-4}$ mol dm^{-3}, presumably corresponding to monolayer coverage. For $MgSO_4$ there is clearly some competition between the SO_4^{2-} and picric acid because the signal only reaches saturation at higher picric acid concentrations. Finally, with NaCl aggregation the highest picric acid concentrations still gave much smaller signals than were obtained with either of the other aggregating agents, presumably because full monolayer coverage of picric acid was never achieved due to competition by coadsorbing Cl-.

Much of the previous work on SERS with positively charged modified colloids has focused on small inorganic anions, particularly those relevant for ground water analysis.[26] We have found that TC modified silver colloids are generally excellent for detection of such anions and data for ClO_4^- and NO_3^- are given in the Supporting Information but here we are more interested in detecting larger molecules which carry a negative charge. The first large molecule studied was tetrakis(4-sulphonato)phenyl porphyrin (TPPS) which is a interesting because the peripheral sulfonate groups on the porphyrin give it a high overall 4- charge but it does not adsorb to conventional unmodified colloids. Data for successful detection of TPPS with HRSC+ with a detection limit $<10^{-6}$ mol dm^{-3} is given in the Supplementary Information but here we have chosen to show the detailed data for a real potential target compound, diclofenac sodium, rather than the unusually highly charged TPPS test compound.

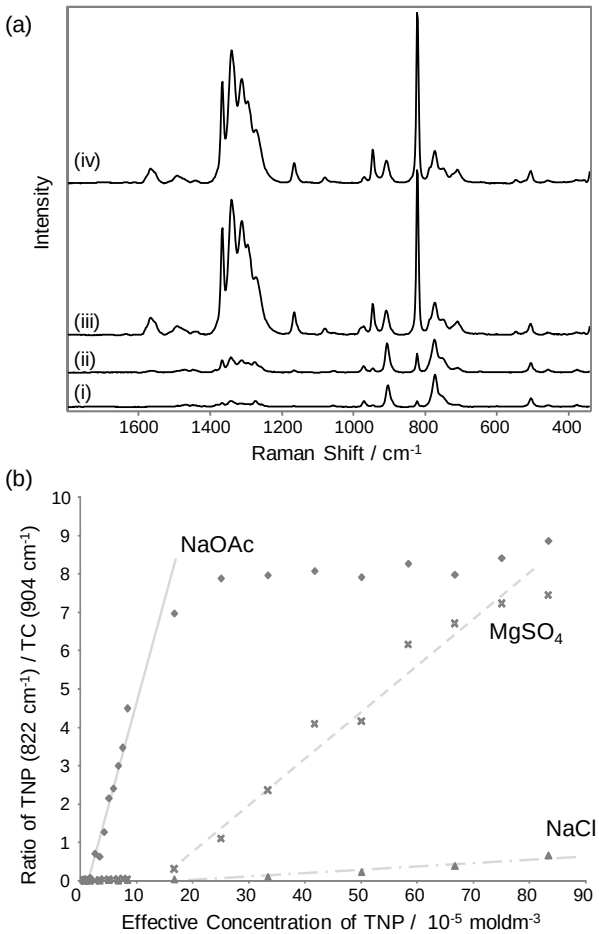


Figure 4. (a) Spectra obtained when $3.6x10^{-4}$ M picric acid was added to HRSC+ and aggregated with (i) 1 M KBr, (ii) 1 M NaCl, (iii) 0.01 M $MgSO_4$, and (iv) 1 M NaOAc. Spectra have been normalized to the 774 cm^{-1} band of thiocholine. (b) Change in picric acid signal with concentration when using different aggregating agents, 1 M NaCl, 0.01 M $MgSO_4$ and 1 M NaOAc.

Diclofenac sodium (structure insert in Figure 5) is a non-steroidal anti-inflammatory drug which is negatively charged at neutral pH (the p*K*a of its carboxylic acid substituent is 4.9).[27] Some previous studies have reported the detection of this compound using standard CRSC but the response was poor, even at high concentrations.[27] In contrast, we have found that diclofenac can be detected at neutral pH using normal CRSC when it is aggregated with $MgSO_4$. However, although the lowest observable concentration under these conditions of 6.7 x 10^{-5} M is much better than that found in previous SERS studies, the assay is still very susceptible to interference by chloride ions. For example, unmodified CRSC aggregated with 1 M NaCl gives no signal from diclofenac, even at 8 x 10^{-4} M. The fact that strongly binding chloride ions interfere with the detection of diclofenac suggests that they prevent binding and conversely, in their absence diclofenac adsorbs to CRSC by displacing the surface citrate.

Chloride interference is a potential problem for physiological monitoring, for example, but it would be expected that HRSC+ would be less susceptible to interference by direct chloride adsorption on the surface, although some competition between the target anion and other anions in solution for binding at the positive surface might occur. Three different aggregating agents, NaCl, NaOAc and $MgSO_4$

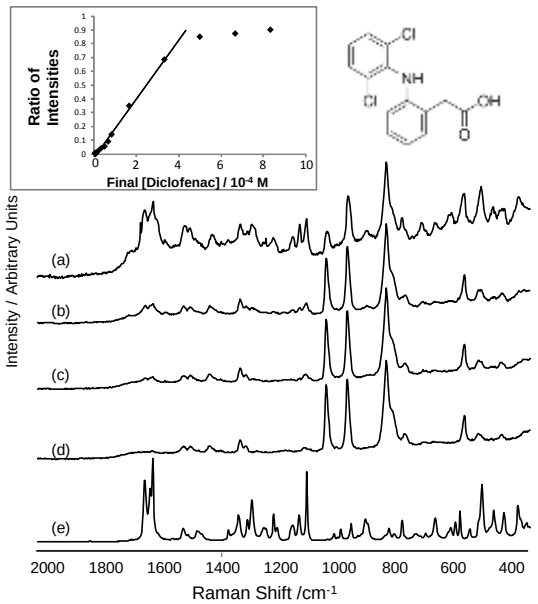


Figure 5. SERS spectra of different concentrations of diclofenac adsorbed onto HRSC+ and aggregated with 0.1 M $MgSO_4$. Concentration of diclofenac is (a) $8.3x10^{-4}$ M, (b) $8.3x10^{-5}$ M, (c) $5x10^{-5}$ M, (d) 0. Spectrum (e) is a normal Raman spectrum of diclofenac sodium powder for reference. Spectra have been scaled and vertically offset for clarity. Inset shows a plot of the intensity ratio of diclofenac/TC marker bands against [diclofenac].

were screened to find the most suitable for diclofenac detection. The detection limits (calculated from 3 × the noise on the spectra) were 1.1 x 10^{-4} M, 3.4 x 10^{-5} M and 1.9 x 10^{-5} M for NaCl, NaOAc, and $MgSO_4$, respectively. In contrast to the generally observed trend, $MgSO_4$ gave better spectra and a lower detection limit than NaOAc (see Figure 5), so that for diclofenac detection, $MgSO_4$ > NaOAc > NaCl. However, the main conclusion is that the positively charged colloids clearly give a significant improvement in sensitivity over negatively charged colloids. In particular, the dramatically improved response in the presence of high concentrations of NaCl mean that thiocholine modified colloids could even be used for samples in physiological fluids.

Conclusions

Colloidal silver nanoparticles modified with thiocholine are excellent SERS substrates for detection of anionic targets since their positive zeta potentials promote adsorption of negatively charged ions. Moreover, the fact that the modifier is a quaternary ammonium ion means that the negative surface charge is maintained even at alkaline pH, something which is not possible with modifiers based on primary or secondary amines. The modified colloids can be used to analyse compounds which cannot be detected using conventional negatively charged reduced metal nanoparticles, examples here included picric acid and diclofenac. Finally, since the modification procedure simply involves reaction of nanoparticles with a small alkyl thiol derivative it can easily be adapted to other particle morphologies such as nanoprisms and nanoboxes, other metals such as Au or Cu and other nanostructured enhancing substrates, such as island films.

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Notes and references

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Electronic Supplementary Information (ESI) available: Thiocholine preparation; characterization data for HRSC+ and CRSC+, including UV-vis, DLS size data, SERS and Zeta potential distributions; comparison of aggregating agents; spectra of other analytes on HRSC+. See DOI: 10.1039/b000000x/

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