



Research article

Ultrasensitive surface-enhanced Raman scattering detection of biological pollutants by controlled evaporation on omniphobic substrates

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ABSTRACT

A simple and highly sensitive method, combining slippery liquid-infused porous substrates and surface-enhanced Raman spectroscopy (SLIPSERS) was used to detect biological pollutants at very low concentrations. Two commonly used rodenticides (brodifacoum and sodium monofluoroacetate) with long biological half-lives were selected as analytes. The SLIPSERS platform gives reproducible SERS enhancement and this allows “label-free” SERS detection of these environmental pollutants.

Analyte ions were detected down to a concentration of 10^{-14} M for brodifacoum and 10^{-9} M for sodium monofluoroacetate. The limit of detection, limit of quantification and limit of linearity for brodifacoum are 10^{-12} M, 10^{-10} M and 10^{-6} M respectively. The SLIPSERS method uses a physical process to significantly increase analyte concentration, and SERS enhancement and therefore can be generally applied to a range of environmental pollutants. The method can be successfully used for ultra-sensitive detection of several chemical and biological contaminants and meet the emerging needs of environmental monitoring and food safety analysis.

1. Introduction

Rodenticides find wide-spread use for the control of common pests such as rats and mice. Brodifacoum, a warfarin-like anti-coagulant is one of the world's most widely used pesticides. It is a lyophilic anticoagulant with a relatively long biological half-life. Sodium monofluoroacetate (also known as 1080) is a highly toxic pesticide that is used in the United States for predator control and control of introduced mammal pest species in New Zealand (Cooney et al., 2016). The residuals of these rodenticides in the environment can be accumulate to sufficiently high levels to cause fatality among non-target animals (Eason et al., 2002).

There are several methods to analyse warfarin and related compounds, but the challenge is to obtain high sensitivity, reproducibility and specificity (Duffield et al., 1979; Ray et al., 1989; Jin et al., 2007). Liquid chromatography-mass spectrometry (LC-MS) (Jin and Chen, 2006; Vindenes et al., 2008) and high-performance liquid chromatography (HPLC) (Kieboom and Rammell, 1981; Hunter et al., 1988; Kelly et al., 1993) have been applied but all these methods are relatively time-consuming and require significant sample extraction and preparation and the use of internal standards.

Surface-enhanced Raman spectroscopy (SERS) combines the specificity of Raman spectroscopy with the sensitivity of surface plasmon resonance methods (Srivatsan, 2014; Schlücker, 2014). SERS utilises nanostructured (colloidal) silver or gold as a substrate due to the ability of the optical response of the metal nanostructures to enhance the Raman scattering efficiency by many orders of magnitude. Analytical and environmental applications of SERS have been limited by the highly variable level of SERS enhancement and typically require either external or internal standards, with internal standards often implemented using sophisticated multi-component nanostructures, utilizing so-called Raman reporter molecules (Schlücker, 2014).

A necessary condition for efficient SERS is close contact between the analyte and the substrate and the SERS effect is strongest at the junctions between individual nanoparticles (so-called hotspots). In the aqueous phase, surface chemistry controls the analyte/substrate interaction, and this often limits SERS applications to a limited set of analytes that adsorb strongly to colloidal metal substrates.

The generation of a high density of hotspots and high surface coverage of the nanoparticle surface by the analyte can also be achieved by careful evaporation/drying of the solvent from a small droplet

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containing the analyte and silver nanoparticles. So called slippery liquid-infused porous surfaces (SLIPS) have been used to control droplet drying in SERS applications to provide a method (SLIPSERS) (Yang et al., 2016; Zhang et al., 2019) that can detect analytes at femtomolar concentrations with minimal sample preparation. Physically removing the solvent through drying overcomes limitations through chemical binding as the analyte is forced into contact with the metal nanoparticle, the meta-stable metal colloids are also forced into contact thereby generating a high density of hotspots (and therefore strong SERS intensities) with excellent reproducibility, and the analyte is highly concentrated into a small volume further increasing sensitivity and reproducibility.

To illustrate the versatility of the SLIPSERS methodology in this work we demonstrate that SLIPSERS can quantify persistent organic pollutants such as brodifacoum and aqueous anions such as monofluoroacetate. SLIPSERS can be implemented with readily available, low-cost chemicals and materials, and with the relatively wide-spread availability and ease-of-use of modern Raman spectrometers and Raman microscopes results can be obtained quickly and reliably.

2. Materials and methods

2.1. Chemicals and reagents

Sodium monofluoroacetate was purchased from MPbiomedicals Limited (Solon, OH, USA). Brodifacoum was purchased from Sigma-Aldrich (New Zealand). A stock solution of 0.01M of sodium monofluoroacetate was prepared by dissolving in MilliQ water (resistivity >18 M Ω). Similarly, a stock solution of 0.01M of brodifacoum was prepared by dissolving in acetone: water (1:1) mix. Silver nitrate (99.8%), trisodium citrate (99.0%), hydrogen peroxide (30% w/w), potassium bromide (99.0%) and sodium borohydride (NaBH₄) (98.0%) used for nanoparticle preparation were of high analytical grade. 0.01 M MgSO₄ was prepared with MilliQ water (resistivity>18M Ω).

2.2. Apparatus

All the Raman spectra were taken on a custom-built Raman spectrometer, based around an Olympus IX70 inverted fluorescence microscope and an Acton Spectrapro® 2500i spectrograph with a liquid nitrogen cooled Roper Scientific Spec-10 CCD detector operating at -110 °C. A 1200 g/mm holographic grating and slit width of 150 μ m was used. The spectrometer was calibrated using standard Raman bands of polymethylmethacrylate (PMMA), which was initially calibrated against Argon and Neon atomic emission lines. Winspec software was used for the collection and analysis of Raman spectra. Spectra were obtained using 532 nm excitation wavelength (with 12–14 mW laser power) focused onto the sample using a 40 \times , 0.65 NA objective. Rayleigh and Raman scattered light was collected in a back-scattered geometry using the same objective. Rayleigh scattered light was rejected at the spectrograph entrance slit using a Raman edge-filter from Iridian Spectral Technologies. Spectra were acquired with a 15 s exposure time and 4 accumulations.

Ultra-violet visible spectra were acquired with a Shimadzu 1800 spectrophotometer and zeta-potential and nanoparticle size was measured using Malvern Zetasizer Nano ZS.

Spectra were processed using Origin 8.5.1 and Python 3.6 software.

2.3. Preparation of silver nanoparticles

Silver nanoparticle preparation was performed in accordance with Frank et al., 2010. To describe it briefly, 4 mL of 1.25×10^{-2} mol L⁻¹ trisodium citrate, 1 mL of 3.75×10^{-3} mol L⁻¹ silver nitrate, 9 mL of Milli-Q water and 10 mL of 5.0×10^{-2} mol L⁻¹ hydrogen peroxide were mixed in a borosilicate vial. Then 0.80 mL of 1.0×10^{-4} mol L⁻¹ potassium bromide was added. Lastly, 5 mL of 5.0×10^{-3} mol L⁻¹ sodium

borohydride was added and stirred continuously for two minutes. A permanent yellow colour solution was formed immediately.

2.4. Preparation of SLIPSERS samples

For preparing the SLIPS substrate (Yang et al., 2016), Krytox™ GPL105 which is a polyfluoropolyether oil and Sterlitech Polytetrafluoroethylene (PTFE) unlaminated Teflon membrane filters of 0.2-micron pore size and diameter of 13mm was used. The white Teflon sheets were first placed at the top of the concave well of an indented glass slide. 15 μ L lubricant was dropped onto the centre of each Teflon sheet using a pipette. The glass slides were then tilted in every direction to ensure the lubricant completely covers the Teflon sheet before being placed on the spin coater. The spin coater was spun at 1000 rpm for 1 min to ensure excess lubricant was removed.

The lubricated membrane was placed in an oven set at temperature 64 °C and a 50 μ L drop of analyte solution was placed on the Teflon membrane. 10 μ L of Ag nanoparticles with 1 μ L 0.01 M MgSO₄ was injected onto the droplet. MgSO₄ is used to promote colloid aggregation. The slide was removed once the aggregate was dried. It took approximately an hour to dry the sample. To accelerate the evaporation process, the optimum temperature for drying is required. Overheating may degrade the sample. The optimum temperature for drying of the SLIPS surface was found to be 64 °C (in accordance with Yang et al.). After evaporation, the aggregate formed as a single black dot, characteristic of aggregated metal colloids, visible to the naked eye. Finally, the aggregate was transferred to a coverslip for SERS analysis.

For SERS testing, the laser power was kept as low as possible, ~1 mW, to avoid sample damage and an exposure time of 15 s with 4 accumulations.

3. Results and discussion

3.1. Characterisation of silver nanoparticles

Silver colloidal particles were prepared using a modified borohydride reduction method according to Kitaev et al. Figure 1 presents the surface

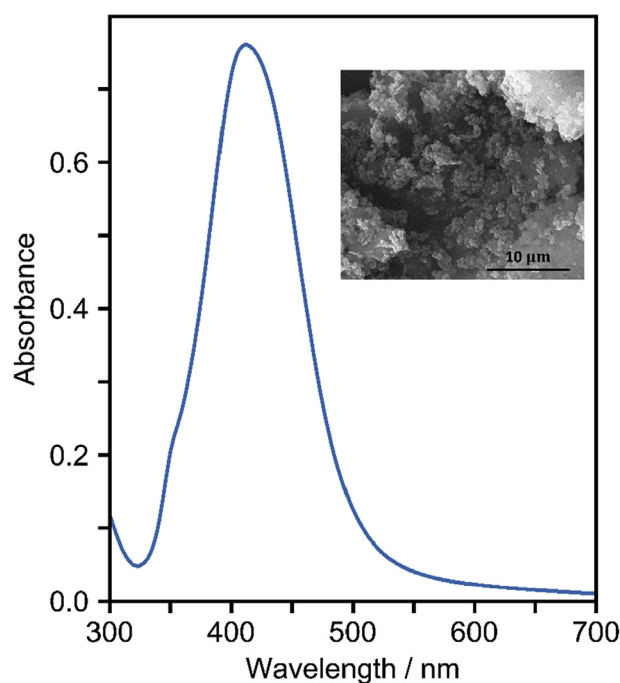


Figure 1. UV-Vis spectrum of colloidal silver nanoparticles in aqueous solution. Inset shows SEM image of aggregated silver nanoparticles after drying. Scale bar is 10 μ m.

plasmon resonance peak of the silver nanoparticles. The peak maximum occurred at 410 nm which is dominated by absorption (with negligible scattering). The inset shows an SEM image of a dried silver nanoparticles aggregated with magnesium sulphate. These nanoparticles had a mean diameter of 45.78 nm and a zeta potential of -55 mV as which is in close agreement with literature (Stewart et al., 2015).

3.2. SLIPSERS analysis of brodifacoum

Brodifacoum (structure shown in Figure S1) has several aromatic rings and extensive conjugation and thus should be an efficient Raman scatterer. The normal Raman spectrum of 1 mM brodifacoum is shown in Figure S2.

Figure 2 shows the SLIPSERS spectra of brodifacoum with concentration down to 10^{-10} M. Bands at 766, 1121, 1176, 1278, 1302, 1356, 1504, 1564, and 1644 cm^{-1} were clearly observed. A density functional theory calculation (B3LYP/6-31G(d)) was used to provide mode descriptions. Figure S3 shows the calculated spectrum which shows excellent agreement with the normal Raman spectrum. Table S1 presents the calculated frequencies and intensities, the assignments of the main peaks in the SLIPSERS Raman spectrum and mode descriptions. The strong bands at 1356, 1504, and 1644 cm^{-1} were selected for the quantitative analysis presented below. In the concentration range shown in Figure 2 i.e. 10^{-6} – 10^{-10} M, the intensities of all bands decrease monotonically, and the intensity ratios of most bands are constant. Considering the concentration range and the focal volume of the microscope object, simple calculations (Calc. S1, Figure S3) show there are many brodifacoum molecules in the focal volume. The observed spectrum in each case is the average spectrum over all the brodifacoum molecules adsorbed on the metal nanoparticles. The constant peak ratios observed across the concentration range in Figure 2 further suggests that the average spectrum is very similar in all cases and allows a quantitative relationship to

be established between the measured Raman intensity and the brodifacoum concentration.

By contrast the SLIPSERS spectra shown in Figure 3 show considerable variation. This behaviour is well-known in SERS spectra obtained from dried colloid solutions (Costa et al., 2010). The spectra recorded at 10^{-11} M and 10^{-12} M show good agreement with each other (and with the spectra in Figure 3), but the 10^{-13} M and 10^{-14} M spectra have a very different set of enhanced bands. This is because the concentration of the brodifacoum molecules has reached the single molecule regime.

The single molecule regime is reached when the number of analyte molecules, on average, in the focal volume is very small, often with one or no molecules present in the focal volume. In this case there will be very limited or no averaging of the spectra. As the 10^{-13} M and 10^{-14} M spectra show, there can be substantial variation in the intensity pattern which arises from variation in the plasmonic enhancement by the nanostructure metal substrate and variation due to the orientation of the molecules at the surface relative to the polarization direction of the incident laser beam (Marshall et al., 2017). The single molecule regime places a lower bound on the limit of quantification for surface-enhanced Raman methods.

Raman scattering is highly anisotropic but for molecules in solution or for scattering obtained from many molecules on a solid SERS substrate orientational averaging occurs and a relatively constant “average spectrum” is obtained. In the single molecule regime molecular orientation relative to the electric field of the radiation plays an important role in determining the mode enhancement patterns (Marshall et al., 2017; Wilson and Willets, 2016). For the spectra shown in Figure 3 it is likely that the orientations of the molecules in the 10^{-13} M and 10^{-14} M are different from each other. In this case the polarised electric field from the incident laser selectively enhances the modes where the polarizability ellipsoid aligns closely with the laser polarisation.

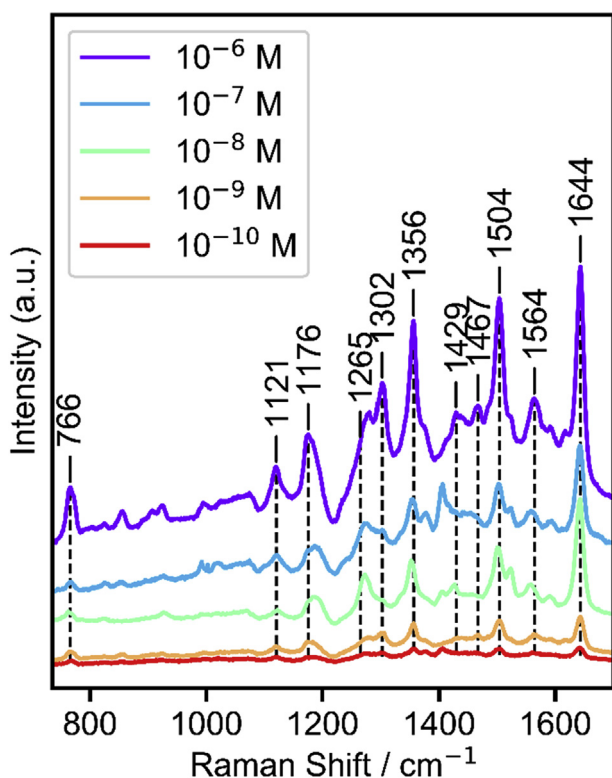


Figure 2. SLIPSERS detection of brodifacoum molecules in aqueous solutions at different concentration (10^{-6} to 10^{-10} M) (top to bottom) acquired with 532 nm laser excitation with 40 \times objective, laser power of 12 mW and 15 s exposure time with average spectra from four accumulations.

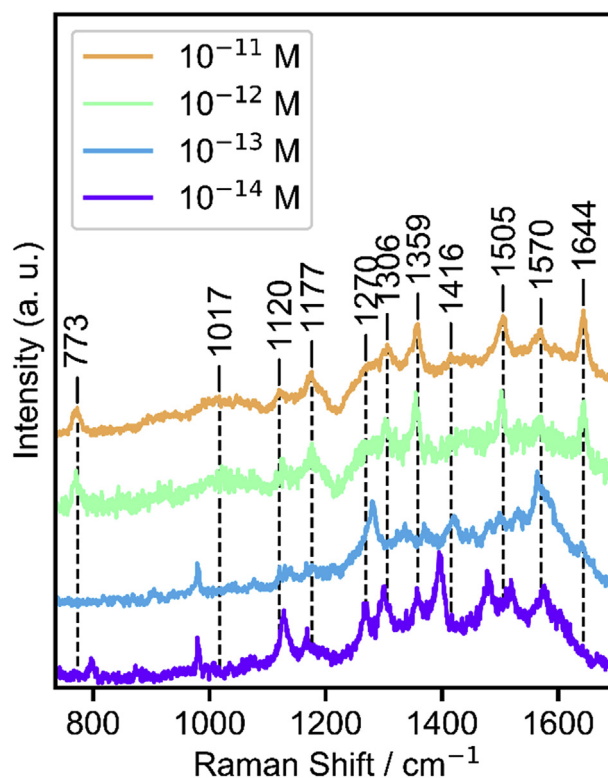


Figure 3. SLIPSERS detection of brodifacoum molecules in aqueous solutions at different concentration (10^{-11} M - 10^{-14} M, top to bottom) acquired with 532 nm laser excitation with 40 \times objective, laser power of 12 mW and 15 s exposure time with average spectra from four accumulations.

3.3. Quantitative analysis of brodifacoum with SLIPSERS

a) Linearity

The linearity of the method for brodifacoum was evaluated by taking four calibration standard points against the intensity of the 1644 cm^{-1} brodifacoum peak for concentrations from 10^{-6} to 10^{-12} M (Figure 2). The band at 1644 cm^{-1} which is assigned to the ring stretching modes of the biphenyl rings was selected due to its strong intensity and clear separation from other bands in the spectrum.

Logarithmic transformation of data is quite useful in handling the wide concentration range and is frequently used for SERS calibration curves (Sarfo et al., 2017; Jiang et al., 2016). The log concentration vs intensity spreads out the data so that the shape and quality of the fit are clearly visible when the concentrations cover a wide range. When the data is exhibited in log concentration, the data yields a linear response for concentrations between 10^{-6} – 10^{-10} M. All spectra were averaged using three random points on each sample substrate to get reliable data.

The calibration curve for brodifacoum in aqueous solution is shown in Figure 4, where the error bars indicate the standard error of Raman intensity for each concentration. Excellent linearity is observed with the linear equation of $y = 16271.4x + 17164$ and R^2 value of 0.99.

b) Limit of Detection (LOD), Limit of Quantification (LOQ) and Limit of Linearity (LOL)

An acceptable parameter for determining LOD is a signal-to-noise ratio (SNR) equal to three whereas the limit of quantification (LOQ) requires $\text{SNR} = 10$.

The limit of detection (LOD) is estimated as 10^{-12} M by comparison with the blank (shown in Figure S6). The lowest amount of brodifacoum in aqueous solution which can be determined quantitatively with a suitable precision and accuracy (LOQ) was 10^{-10} M (Shrivastava and Gupta, 2011).

The range of SLIPSERS intensities gave a linear response against log concentration. The lower limit of the linear range is due to subsampling issues (i.e. as the single molecule regime is approached). We note that this linear response is obtained from measuring peak intensity and not peak intensity ratio with an internal standard. Peak intensity variations occur due to incident laser power fluctuation, and variations in the number of hot spots and analyte molecules in the focal volume of the Raman microscope. The controlled drying/aggregation of the nanoparticles

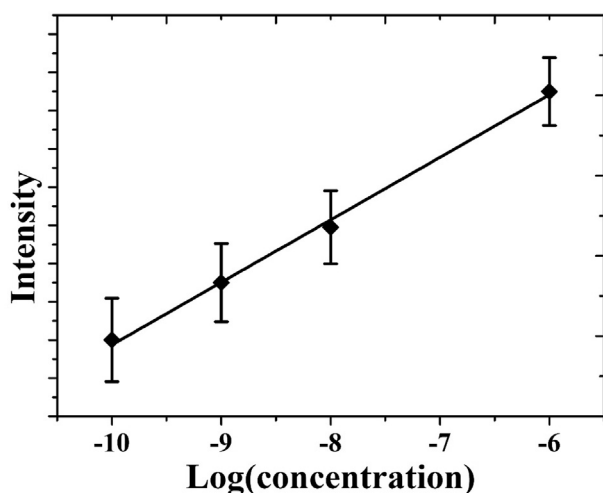


Figure 4. Calibration curve for SLIPSERS detection of different concentration of brodifacoum to intensity at 1644 cm^{-1} with error bars representing the standard error.

reduces these variations by producing a reproducible hot spot and analyte density in the focal volume.

At high concentration, the limit of linearity (10^{-6} M) is reached due to a loss of intensity enhancement. At concentrations above 10^{-6} M, there are sufficient molecules to form multiple layers of analyte over the available silver nanoparticle surface. Molecules that are subsequently added to the outer layer of adsorbed analyte receive increasingly less intensity enhancement from the plasmonic nanoparticle so the SLIPSERS intensity decreases. In addition, the adsorbed molecules scatter the incident laser before it reaches the nanoparticle surface preventing enhancement via plasmon resonance (Figure 5). shows the linear response and single molecule regions.

The linear and single molecule regions were also observed for the SERS of 2,4-dichlorophenoxyacetic acid (Costa et al., 2010). Costa et al. employed a bi-layer gold substrate (gold nanoparticles with a second layer gold deposited from tetrachloroauric acid), in their work the cross-over from linear to single molecule region was observed at 10^{-8} M, thus it appears that the controlled drying employed by the SLIPS substrate might be capable of extending the linear SERS response to lower concentrations.

c) Precision & Accuracy

Triplicate analysis was done using two concentrations: 10^{-6} M and 10^{-10} M at three different sample positions using the 1644 cm^{-1} band. To evaluate precision and accuracy, the coefficient of variance (CV) and relative standard deviation (RSD) from the predicted values (Lu et al., 2018) were measured from the regression equation.

Coefficient of variation, also known as relative standard deviation is used to measure precision and repeatability of the results. RSD (%) was calculated after dividing the standard deviation by the average intensity of three replicated for a specific concentration.

The accuracy was determined by comparing the mean calculated concentration of the samples with the true value obtained from the calibration curve. Accuracy can also term as recovery, that is, the ability to detect the amount of analyte present in a sample. So, the recovery rate was calculated by comparing the known analytical concentration of brodifacoum in the solution with the predicted concentration obtained from the regression equation and this can determine the accuracy of the chosen method (Vicario et al., 2015).

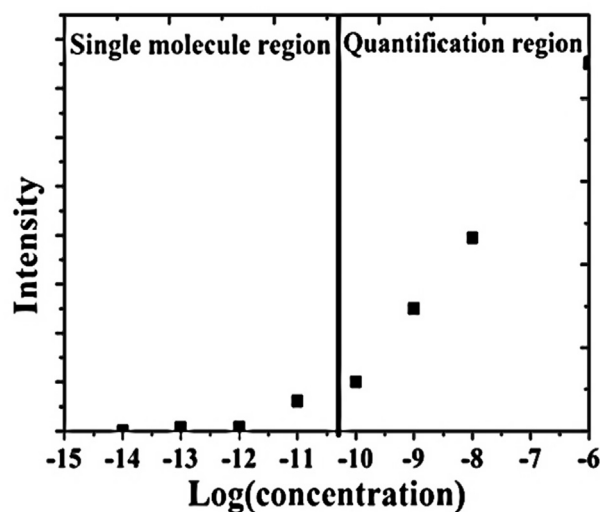


Figure 5. SLIPSERS intensity at 1644 cm^{-1} as a function of different concentrations of brodifacoum.

$$\text{Accuracy (\%)} = \frac{[A]_{\text{predicted}}}{[A]_{\text{known}}}$$

where $[A]$ is the concentration of analyte. Table 2 (SI) demonstrates the values obtained that provide a satisfactory level of quantification with relative deviation of both higher and lower concentration of less than 3%. Therefore, the accuracy and precision values are excellent and were well within the acceptable limits of RSD <20% (Doering and Nie, 2002).

3.4. SLIPSERS analysis of 1080

Sodium monofluoroacetate is a small compound with no aromatic rings or polarisable groups and so behaves as a poor Raman scatterer due to its inherently low Raman scattering cross-section. Solid 1080 and an aqueous solution of 1080 (0.01 M) did not show any observable Raman bands even at this relatively high concentration. Plasmon enhancement is therefore essential for the detection of 1080 by Raman methods.

SLIPSERS spectra for 1080 are shown in Figure 6. Below 10^{-10} M no prominent peaks were observed, only the bands from the silver nanoparticles (compare with the blank spectrum shown in Fig S6). The major peaks observed in the 1080 spectra were 1376, 1498 and 1642 cm^{-1} . In the absence of a “normal” Raman reference spectrum (Figure S4) the 1080 bands in the SLIPSERS spectra were identified by comparison to calculated frequencies and intensities shown in Table S2. The most intense peak corresponds to the O–C–O stretching band of the carboxylate group (note that this band appears as a shoulder on the citrate O–C–O band in low concentration solutions of 1080). The strong 1642 cm^{-1} carboxylate band is confounded by the presence of citrate ions at the surface of the silver nanoparticles (and indeed, the fluoroacetate anion must compete with binding to the silver surface with any citrate anions present). Therefore the 1642 cm^{-1} band cannot be used for quantification. Instead, the band at 1376 cm^{-1} (in-plane bending of C–H bond) was selected. This band appears as a shoulder on yet another citrate band, but comparison with the remaining bands reveals this band to be the best possible choice. Besides these vibrational modes for monofluoroacetate, there is also a weak C–F stretching band at 1196 cm^{-1} . Further development of SLIPSERS for monofluoroacetate detection will involve utilizing a citrate-free SERS substrate, and ideally, a substrate with a positive zeta potential (Stewart et al., 2015).

The calibration curve for 1080 in aqueous solution is shown in Figure 7. The linear relationship of SLIPSERS intensity and 1080 concentrations is $y = 8897.4x + 98384.29$ with $R^2 = 0.99$. The lowest amount of 1080 in a sample, which can be detected but not quantified, is estimated as 10^{-9} M (LOD), and the lowest amount of 1080 in a sample which can be determined quantitatively with a suitable precision and accuracy (LOQ) was 10^{-8} M (Table S3).

Brodifacoum and 1080 both are rodenticides; one a conjugated structure with aromatic rings while the other is a simple anion with no aromaticity. Brodifacoum showed high SERS activity with good enhancement factor that is a new addition to the list of analytes detected by SERS. 1080 is poor Raman scatterer due to its small cross section whereas SLIPSERS for 1080 is quite achievable. Therefore, it is evident that SLIPSERS can detect two analytes that have a similar biological and environmental function but very different physical and chemical properties.

Several studies have been done for the detection of anti-coagulant rodenticide residues in dog's liver, human blood and water using GC-MS, LC-MS or HPLC but no SERS detection has been attempted to date. Ray et al. have detected brodifacoum in dog liver using GC-MS with LOD as 1.1 mg/kg (Ray et al., 1989) and in whole blood as 10 ng/mL using LC-MS (Yan et al., 2016). In our current work, a simple system of rodenticide detection in aqueous solution was demonstrated and we have compared the LOD values from our work with different analytical methods from the in Table S4.

Table S4 demonstrates the promising potential of SLIPSERS for detecting brodifacoum (as an example of a persistent organic pollutant)

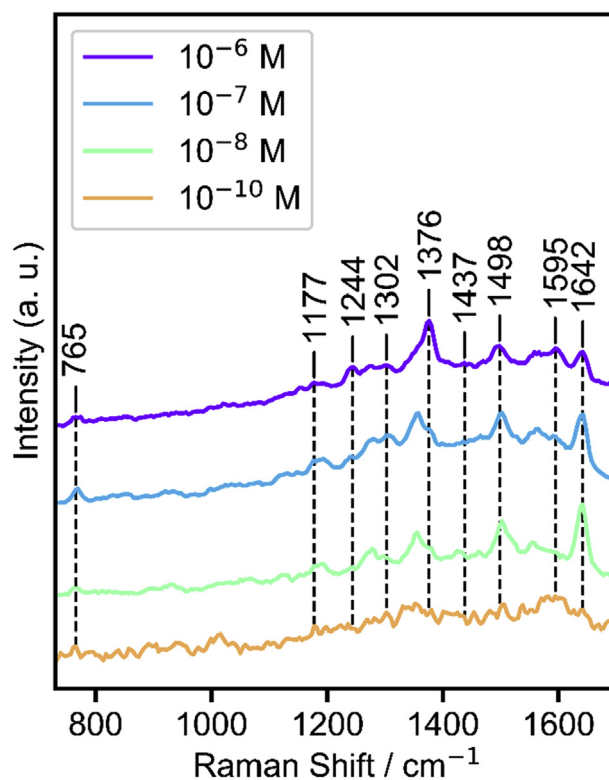


Figure 6. SLIPSERS detection of 1080 in aqueous solution acquired at 532 nm laser wavelength with 40× objective, laser power of 12 mW and 15 s exposure time with average spectra from four accumulations.

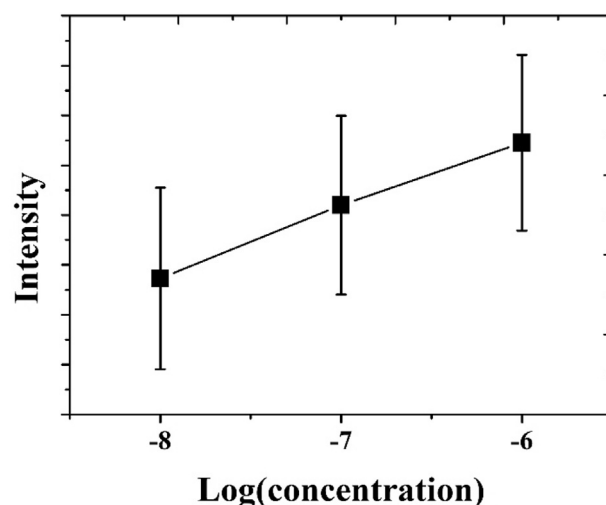


Figure 7. Calibration curve for SLIPSERS detection of different concentration of 1080 to intensity at 1373 cm^{-1} with error bars representing the standard error.

to a concentration 100-fold lower than those reported in literature whereas 1080 also has achieved similar lowest detection through SLIPSERS.

4. Conclusion

The results obtained in this study demonstrate the capability of SERS using an omniphobic substrate, SLIPS, that overcomes some of the drawbacks faced by SERS regarding the distribution of analytes molecule over SERS hot-spots. Both brodifacoum and 1080 are used extensively as rodenticides but have very different physical and chemical properties. The limit of detection for 1080 is 10^{-9} M and the limit of quantification is

10^{-8} M. For brodifacoum, the limit of detection is 10^{-12} M and the limit of quantification is 10^{-10} M. The limit of linearity for both analytes is 10^{-6} M.

SLIPSERS provides a versatile platform for the rapid detection of trace amounts of brodifacoum and 1080 that brings the lowest detected concentration to picomolar level. SLIPSERS is much faster, simpler than existing analytical techniques like HPLC, GC-MS and LC-MS and provides a lower detectable concentration than SERS (with greater reproducibility).

Declarations

Author contribution statement

Mark Waterland: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Megha Mehta: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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