Determination of aqueous antibiotic solutions using SERS nanogratings

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HIGHLIGHTS

\begin{itemize}
  \item Laser interference lithography was used to produce 1D periodic plasmonic structures (nanogratings).
  \item Surface-enhanced Raman scattering (SERS) was obtained from solutions of antibiotics with different concentrations.
  \item Advanced chemometrics was used to identify the spectral features and quantify each antibiotic in the mixture.
\end{itemize}

ABSTRACT

The emergence of antibiotics and their active metabolites in aquatic ecosystem has motivated the development of sensitive and reliable sensors to monitor traces of antibiotics and metabolites in drinking water sources (i.e. surface water). The surface enhanced Raman scattering (SERS) technique, which is widely recognized as a high sensitivity method for molecular vibrational detection, is potentially a powerful tool for trace environmental contamination analysis. The main goal of this work is to demonstrate pharmaceutical and metabolite multiplexing detection using the SERS approach. Periodic metallic nanostructures were fabricated using laser interference lithography (LIL) and used as SERS substrates (platform that supports the SERS effect). The LIL method allowed excellent substrate-to-substrate geometric parameters variations; for instance, the variations in periodicity were determined to be less than 1%. A common fluoroquinolone (FQ) parent-and-metabolite pair, enrofloxacin (ENRO) and ciprofloxacin (CIPRO), was targeted for multiplexing detection on the relative uniform substrates fabricated by LIL. The quantifications of the analytes mixtures were achieved by chemometric analysis (i.e. non-negative matrix factorization with alternating least square algorithm (NMF-ALS)). The limit of the quantification (LOQ) of the present method is in the ppm-level with less than 10\% spatial variation in the SERS signal.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Graphical abstract.}
\end{figure}

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1. Introduction

A variety of artificial organic compounds have been synthesized and developed to satisfy different societal needs (e.g. medical,
where in the environment, even in very remote places frequently, anthropogenic organic contaminants can be found every-agnostic and household applications) over the years. Consequence, anthropogenic organic contaminants include pharmaceuticals and personal care products. One class of anthropogenic contaminants are antibiotics, which have been used for many decades as antibacterials to treat both humans and animals. They are widely applied in the agriculture industry to prevent infectious diseases and promote the growth of poultry and livestock. They are increasingly used in animals, which can be pathogenic and dangerous for both humans and animals. The widespread application of quinolones led to their accumulation in the environment. These compounds are never absorbed entirely by humans or animals and most of the antibiotics are excreted either in their intact form or as their metabolites.

There are many possible pathways for quinolones (and other antibiotics) to enter into the environment: runoff or infiltration of animal sewage (i.e. urines or feces) [12,13]; wastewater from treatment plants [14,15]; leakage from landfills [16,17]; and many others. Farming sewage generally does not go through wastewater treatment systems before discharging into the environment. This increases the likelihood of surface water contamination due to the residues of antibiotics, including quinolones and their metabolites, from the agricultural waste.

The environmental fate of quinolone antibiotics is not completely understood. Several studies [19,20] have proposed that the residues of antibiotics and their metabolites in the aquatic environment, even at a very low concentration (parts per billion (ppb) level [21]), have a potential to become a threat to the ecosystem. They promote the breeding of drug-resistant microorganisms, which can be pathogenic and dangerous for both humans and animals. The presence of these emerging pollutants might also contaminate plants and animals as they uptake water [20]. As the demands for potable water increases, the reuse of water will be necessary [22,23]. The issue of surface water contamination has then motivated analytical chemists towards the development of sensitive and reliable sensors to monitor traces of antibiotics and metabolites in drinking water sources.

Enrofloxacin (ENRO), a fluoroquinolone (FQ) approved as a veterinary antibiotic in poultry and bovine industries, is proven to be biologically effective for a broad-spectrum of microbial and bacterial prevention [10,11]. ENRO, as other antibiotics, is never taken in fully by animals but excreted unchanged or in its primary active metabolite, ciprofloxacin (CIPRO). The states-of-the-art for FQ analysis from water samples is associated with chromatographic techniques in tandem with mass spectrometry (MS) for compound identification. The current detection method presents a reasonable low limit of detection (1–10 ppb) [27,28]; however, the method requires sophisticated instruments and well-trained personnel. In addition to that, the analysis requires extensive sample pre-treatment/pre-concentration, which is a time-consuming step in the process.

Surface enhanced Raman scattering (SERS) is a large increase in the Raman cross-section (enhancement factor as high as $10^9$ – fold [29,30] as compared to the normal Raman) observed from molecules adsorbed in certain metal nanostructures (SERS substrates). SERS offers high sensitive allied to vibrational fingerprint information of the adsorbed molecules. SERS has been widely used for the detection of pharmaceuticals (or their metabolites), including anorexic (appetite suppressant) drugs and analogues [31], nicotine [32], and FQs in spiked samples (chicken or fish [33,34], urine [35,36], blood [37]. Most of the SERS work in the area has utilized random metal nanostructures, such as colloidal metal nanoparticles [31–38], dendritic metal nanostructures [39] and electrochemically roughened metal film [40]. Those random SERS substrates are known for their simplicity in terms of fabrication and by their high SERS enhancement efficiency. On the other hand, those random SERS platforms suffer from issues related to lack of spatial uniformity and SERS intensity reproducibility. These limitations can be addressed by using fabricated nanometric metallic patterns in solid supports as SERS substrates. Laser interference lithography (LIL) [41–43] is a fabrication method that is quick, cost-effective, and yields a relative uniform periodic pattern that supports SERS. Compared to other advanced nanofabrication approaches, such as electron beam lithography (EBL) [44,45] and focused-ion beam milling (FIB) [46–48], LIL allows the patterning of a large area (2″ x 2″, for instance) at a much more reasonable cost. SERS substrates generated through LIL have the potential to be miniaturized as portable or handheld devices [49,50] for remote environmental detection [51].

The main goal of this work is then to explore the possibility of pharmaceutical and metabolite multiplexing detection using an uniform SERS platform fabricated using LIL. As a proof of concept, the common FQ parent-and-metabolite pair, ENRO and CIPRO, was targeted for multiplexing detection. The quantifications of analytes mixtures were enabled by non-negative matrix factorization with alternating least square algorithm (NMF-ALS) to resolve the complicated spectral information of the mixtures into the concentration profiles for calibration purposes.

2. Materials and methods

2.1. Materials

Fisher finest premium plain microscope slides (3″ x 1″ x 1 mm, supplied by Fisher Scientific), Microposit SC 1827 positive photo-resist, Microposit thinner type P, Microposit 351 developer, deionized water (produced through Barnstead Millipore system with resistivity of 18.2 MΩ cm), hydrochloric acid (supplied by VWR International), enrofloxacin (ENRO) and ciprofloxacin (CIPRO) solid (both supplied by Sigma-Aldrich).

2.2. Template preparation via LIL

Microscope glass slides were cut from 3″ x 1″ standard size to 1 inch² and were used as the support. The glass slides were first sonicated in a water bath and then cleaned with copious amounts of organic solvents (acetone, methanol, ethanol and isopropanol). After oven-drying (T ~ 120 °C) and cooling to room temperature, the glass slides were ready to be spin-coated with 1:1 positive photo-resist diluted with thinner at 2000 rpsms for 30 s, as illustrated in...
The substrates were then pre-baked in an oven at 120 °C for 10 min to remove the solvent from the photoresist layer. Next, the glass substrate coated with the photoresist was then mounted on the interference lithography setup and exposed to a 458 nm laser once for 45 s (the laser power was 250 mW) [50,51]. After the laser exposure, the photoresist film was developed in 1:3 Microposit 351 developer diluted with deionized water for 45 s under gentle motion parallel to the grating axis to generate a uniform 1-dimensional photoresist template. The fabrication steps involving the photoresist were all realized under yellow light, and aluminum foil was used to protect the samples when room transfer was necessary. The photoresist templates were then coated with a 250 nm silver film (Angstrom Engineering glove-box evaporator, deposition rate of 1 Å s⁻¹).

2.3. Single and bi-analyte SERS detection

The silver-coated SERS nano-grating substrates were drop-coated with 20 μL of one of the following solutions: (i) aqueous solution containing only enrofloxacin (ENRO), (ii) aqueous solution containing only ciprofloxacin (CIPRO), or (iii) aqueous solution containing a mixture of ENRO and CIPRO. The concentration range explored was from 0 to 150 ppm. The droplets were gently dried under nitrogen. In order to account for the effect of spatial variation due to drying, SERS spatial mappings were obtained for each droplet. Three areas of ~400 μm² were mapped for each droplet. The SERS maps were obtained using a Raman microscope (Renishaw inVia system) equipped with 785 nm excitation (laser power 31.6 mW, 2 s acquisition, single accumulation). The spectra were obtained under 100× objective (NA = 0.90).

2.4. Data analysis

Chemometric methods, including principal component analysis (PCA) [52,53] and non-negative matrix factorization with alternating least square algorithm (NMF-ALS) [54,55], were applied to process the SERS mapping datasets. Routines developed in house were run in Matlab (version 7.12.0) to implement the analysis [56]. The dataset was pre-processed for baseline correction (asymmetric least squares (AsLS) algorithm [57]; λ = 10⁴ and p = 0.01) and smoothing (Savitzky-Golay algorithm [58]; window of 15 and polynomial degree of 1). Prior to NMF-ALS, PCA was employed to determine the number of factors (i.e. number of chemical species in the sample) using the mean-centered datasets. It followed by NMF-ALS data processing, where a data matrix is formed from each SERS mapping. Each spectrum in the SERS mapping is allocated on the sample) and the columns of the data matrix are the SERS intensity values in each wavenumber (i.e. score matrix that related to the concentration of the species). A ‘pseudo calibration curve’ was constructed by plotting the scores from NMF-ALS (from a series of the mapping datasets) against to the actual concentrations of analytes. More information about the data analysis is included as supporting material.

3. Results and discussion

3.1. SERS substrate characterization — surface morphology and reproducibility

The IL fabrication process [43,50,51] of large area nano-grating substrates is illustrated in Fig. 1. The final patterned area was ~1 inch² and silver film thickness was 250 nm. The surface morphology of the fabricated substrates was characterized by using scanning electron microscopy (SEM) (Fig. 2). The nano-gratings structure fabricated is morphologically reproducible with periodicity ~450 nm and grating width ~200 nm (periodicity variation ~ 0.3%, assessed through atomic force microscopy). The IL template fabrication has been found to be relatively simple, cost and time-effective. The entire process of fabrication, starting from photoresist spin-coating to template solvent development, took less than 15 min in total for one nanograting substrate. The fabrication time could be even shorter if mass-produced. The homogeneity of SERS performance within a substrate was estimated by the standard errors obtained from MCR analysis for a single analyte mapping dataset (~900 μm²). The spatial variation in SERS intensity within a single mapped area was found to be less than ~10%. In addition, the SERS reproducibility between different substrates was evaluated through the mapping measurements (~400 μm² each) on three substrates previously drop-coated with Nile Blue A (10 μM) solution. The variation of average SERS intensity was ~20% between the substrates.

3.2. Raman and SERS spectra of ENRO and CIPRO

Fig. 3 presents the normal Raman and SERS spectra of ENRO and CIPRO. The molecular structure from these species are shown in Scheme 1. The normal Raman spectra are from solid ENRO and CIPRO samples deposited on a glass slide and probed with the 100× objective. The SERS spectra are from aqueous samples dried in the nanograting substrate, as described in the experimental section (section 2.3). As expected, the SERS spectra in Fig. 3(c) and (d) generally presents broader vibrational features when compared to the bands of the normal Raman spectra of the solids (Fig. 3(a) and (b)).
The interaction of adsorbates with metallic surfaces lead to shifts in the vibrational energy and the broadening of vibrational bands [59,60]. Moreover, the relative intensities of the adsorbed species also change (when compared to the solid) due to the surface selection rules [61–63]. Some characteristic SERS bands were found in both ENRO and CIPRO, including features at 747 cm\(^{-1}\) (methylene rocking), 1391 cm\(^{-1}\) (O–C–O stretching), 1461 cm\(^{-1}\) (benzene ring vibration), 1594 cm\(^{-1}\) (benzene ring stretching/breathing) and 1627 cm\(^{-1}\) (C=O stretching). The SERS bands at 1627 cm\(^{-1}\), assigned to the carboxyl stretching (in both species) was significantly stronger than observed in the previous reported SERS spectra for ENRO and/or CIPRO [33,38]. This might be related to the high degree of carboxyl group protonation [64,65] in both ENRO and CIPRO solutions, as they were prepared in acidic medium to promote dissolution of the solids. Although both CIPRO and ENRO present strong similarities in their molecular structure (see Scheme 1), it is still possible to find variations in their vibrational signatures presented in Fig. 3. For instance, the SERS spectra for ENRO (Fig. 3(d)) and CIPRO (Fig. 3(c)) present unique features in the spectral region below 900 cm\(^{-1}\), including the SERS band at 850 cm\(^{-1}\) which is only present in the SERS spectrum of ENRO (Fig. 3(d)). Although it would be possible to concentrate on that particular band (and others, in the 1400 cm\(^{-1}\) - region, for instance) to determine the relative presence of each species in a mixture, a more representative quantification was obtained using chemometrics (this will be discussed further in section 3.3). In that case, the entire SERS spectral window (from 350 to 1650 cm\(^{-1}\)) was taken into consideration to interpret the SERS spectra of CIPRO and ENRO mixtures.

### 3.3. FQs detection in mixtures

The goal of this work is to quantify a mixture of an antibiotic and its metabolite in aqueous solution using SERS from nanogratings. ENRO and CIPRO, the parent-and-metabolite FQ antibiotics pair used in our proof of concept measurements, are relatively similar in their molecular structures (see Scheme 1), resulting in several overlap in their SERS spectral features (Fig. 3). The structural similarity provides a challenge for the quantifications of these species from mixtures. As discussed in section 3.2, it is possible to find a few non-overlapping regions in their vibrational fingerprinting, but a restrict analysis of those regions might limit the possibility of quantification. Alternatively, chemometric methods were used to take advantage of the whole spectral range. All SERS mapped datasets were analyzed through non-negative matrix factorization with alternating least square algorithm (NMF-ALS); a class of algorithms under the multivariate curve resolution methods [66]. Basically, this unsupervised learning chemometric technique resolves the complicated spectral information of the mixture into loadings (simulated pure spectra for each individual analyte) and scores (simulated concentration profiles of the analytes) This technique was previously applied in SERS environmental contaminants detection (i.e. malathion in fruit peels [56], antibiotics detection in tertiary mixtures [67]) and was proven to be effective to recover both the spectral and concentration information of the species of interest, without being affected by sample matrix interferences.

Although the final goal was to detect FQ mixtures using our IL fabricated silver nano-gratings, preliminary experiments concentrated on the quantification of the individual components (CIPRO and ENRO) in aqueous sample. Fig. 4 shows representative SERS spectra of CIPRO adsorbed from different concentrations in the nanograting substrate. A series of CIPRO standard solutions within a concentration range between 1 ppm and 120 ppm were deposited (20 μL) on the nanogratings. SERS mappings were obtained for each concentrations and the results analyzed by NMF-ALS. The generated scores (pseudo-concentration profiles) were plotted against the actual CIPRO concentration and presented in Fig. 4(b). A linear
A relationship \( R^2 = 0.983 \) was found for the entire measured calibration range, from 1 to 120 ppm, of individual CIPRO. This reasonable linear fit was obtained within less than 10% of relative standard deviation (RSD) in spatial variation for each SERS map. This data spreading was reflected in the error bars of the calibration curve (Fig. 4(b)). The % RSD was assessed through the ratio of the NMF-ALS processed standard errors (pixels to pixel variation in the mapped datasets) and the average scores from the replicates of mapped measurements. It is also notable that each of the data point in the calibration curve in Fig. 4(b) was obtained from the average of three SERS mappings of 400 \( \mu \text{m}^2 \) for each concentration (~1200 spectra). The limit of quantification (LOQ), defined as the smallest concentration of analytes that produces reasonable characteristic SERS spectrum of the species, was found to be ~1 ppm. As the concentration decreased further, the spatial variation becomes stronger and the error in the determination was overwhelming. This is an interesting characteristics of SERS, since the enhancement occurs from very localized regions, strong spatial variations are expected for low surface coverages. The quantification in those conditions are challenging, but we are developing a method to deal with this issue that will be presented in future works.

The LOQ achieved in this work (~1 ppm) is higher than those reported for SERS antibiotic detections [33–37,39], which ranges from 20 ppb [33,39] to a few hundreds ppb [34,36]. All the previous work on the quantification of antibiotics by SERS used random metallic nanostructures that are known to be less reproducible and reliable. By using a periodic SERS substrate that can be mass fabricated by a relative simple method, the giant SERS efficiency
(observed in random substrates) was traded off by spatial uniformity and better sample-to-sample reproducibility. Environmental methods for the analysis of water generally employ pre-concentrated steps using solid phase extraction [68]. Although the nanogratings described here could potentially substitute some of the advanced instrumentation employed in the state-of-the-art water analysis, the pre-concentration step could be required to achieve competitive sensitive levels. This comes from a compromise between a reasonable enhancement factor and the reproducibility of the surface achieved using these nanogratings as platforms for SERS.

SERS quantification of biamalyte mixtures of aqueous solution containing ENRO and CIPRO was demonstrated. A set of multi-analyte standard solutions with varying concentrations of ENRO but constant CIPRO concentration were employed. The SERS spectra of some of the mixtures are presented in Fig. 5(a). The SERS bands at 850 cm\(^{-1}\) noticeable increases in intensity it with the ENRO concentration, as illustrated in the enlarged view of that portion of the spectra in Fig. 5(b). The pseudo-concentration information (scores obtained using the whole spectrum, rather than selected bands) were retrieved a NMF-ALS data analysis of the spectra in Fig. 6 and the results are plotted in Fig. 6(a). The ENRO scores from the mixtures yielded a linear relationship with the concentration \((R^2 = 0.981,\) as shown in Fig. 6(a)). Fig. 6(b) and (c) compared the recovered pseudo spectra (loadings) from the NMF-ALS analysis to the experimental SERS data. The 'pseudo' spectra for both analytes were observed to match well to the actual SERS spectra of the individual analytes (Fig. 6(b) and (c)). This is a strong indication of the successful implementation of the NMF-ALS analysis for bi-analyte mixtures of FQs by SERS.

The standard errors for each data point in Fig. 6(a) (averaged from all mappings in a particular concentration, ~1200 spectra altogether) were slightly higher (ranging from 0.51 to 4.1% relative to the average scores) as compared to the individual CIPRO concentration.

![Fig. 6](image-url)
quantification shown in Fig. 5(b) (magnitude of 0.37–3.9% relative to the average scores). This suggests that the spatial reproducibility of the mappings was marginally poorer for the mixture than for the case of individual analytes. The higher error can be attributed to the processing of mixed spectra from two almost identical molecules is challenging and it is expected to carry more uncertainty in the concentration information (scores).

4. Conclusions
We have successfully employed the LIL fabricated nanoragins in SERS detection of an antibiotic and its metabolite (i.e. ENRO and CIPRO), as proof-of-concept of SERS applications in environmental detection. The quantification was achieved using NMF-ALS. This technique has proven to be successful in recovering the concentration and spectral profiles of the analytes, up to bi-mixtures. Ongoing effort is still required to optimize the ENRO and CIPRO quantifications in tertiary mixtures or other more complex mixtures (i.e. real environmental samples). Notwithstanding, the SERS substrates (i.e. silver nanoragins) fabricated through the LIL technology has high potential to apply into the environmental monitoring due to their uniformity. This type of substrate can potentially be integrated in portable handheld devices or compact bench-top readers. These would be useful for on-site monitoring and detection of emerging contaminants.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaca.2017.05.025.

References


