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Detection of low concentrations of Ampicillin in Milk

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Ampicillin, a common antibiotic, is detected at trace concentrations in milk using surface enhanced Raman spectroscopy in a microfluidic device, using less than 20 µL of sample, in 10 minutes, with minimal off-chip preparation. The device is configured so as to favor the interaction of the analyte with colloidal silver, and the optimization of the aggregation of the silver nanoparticles so as to increase the SERS intensity and the consequential sensitivity of analyte detection.

In addition to being vital in modern healthcare, antibiotics also play a major role in the food industry. However, overuse of antibiotics in raising of farm animals has led to the development of strains of antibiotic resistant bacteria, adding significant risk to the consumer and cost to patient treatment[1,2]. Residual antibiotics used to treat animals have been reported in meat[3], poultry[4], fish[5], and milk[6]. Additionally, the excessive use of antibiotics creates a baseline presence of antibiotics in the environment and the water supply[7,8], exposing the general population to antibiotics, even when not treated for a bacterial infection. The significant growth in production of organic meat and dairy over the last two decades is in part due to increased demand for antibiotic-free food[9]. Additionally, certain types of veterinary antibiotics are prohibited due to side effects in humans, such as carcinogenicity, and need to be monitored[10]. Assays currently used for antibiotics testing rely mainly on bacterial cultures, and require hours to complete[11]. Methods for quickly and cheaply screening food samples for traces of antibiotics are therefore of interest to healthcare, as well as for financial and regulatory compliance reasons. Here we report a microfluidic system for the detection of antibiotics in milk samples at parts per billion concentrations, using a vacuum-driven microfluidic device together with surface enhanced Raman spectroscopy (SERS) detection. The microfluidic device is engineered to control the interactions between the analyte and a silver nanoparticle (AgNP) suspension for optimal signal intensity and detection. Ampicillin is used to demonstrate this methodology in raw milk samples.

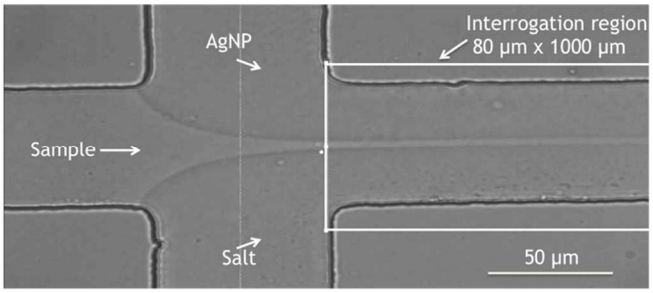

Figure 1 Flow focusing of the sample at the microfluidic junction. Under laminar flow conditions, the incoming sample stream is hydrodynamically focused between two side-streams, carrying AgNPs and a salt solution respectively, where the species mix by diffusion. As the AgNPs aggregate they become SERS-active, enabling rapid and specific detection of the antibiotic.

SERS has been previously shown to be applicable for the detection of and discrimination between multiple penicillin-type antibiotics, including ampicillin[12]. These studies employed concentrations of antibiotics at levels that, while useful in clinical settings, are not low enough for food safety applications. FDA required maximum levels for ampicillin in food is 10 ppb, corresponding approximately to 30 picomolar concentration. What we show here is that by combining microfluidics with SERS, it is possible to quickly detect antibiotics at appropriately low concentrations. Other groups have shown that it is possible to reach even lower limits of

detection, by using engineered SERS substrates such as silver dendrites[13] or silver films[14], although in pure analyte solutions and not in commercially relevant samples.

The central features of the analysis we used, as well as the specifics of the microfluidic device, were previously described by us in the context of detecting recreational drugs in saliva[15]. Briefly, a small aliquot of aqueous sample containing the analyte is introduced into a microfluidic device where it is hydrodynamically focused between two enveloping flows. One of the side-flows is an aqueous sol of silver nanoparticles, while the other is an aqueous solution of LiCl. The streams meet at the flow-focusing junction, as shown in Figure 1. The species resident in each of the flows convect downstream, while mixing with the other flows by diffusion. Small molecules, such as ampicillin, diffuse faster than larger molecules present in the sample, populating the side-channels. Importantly for the detection scheme, the antibiotic interacts with the AgNPs adsorbing on their surface. The salt ions also diffuse quickly, diluting as they migrate across the central channel to the opposite side channel containing the silver where they induce aggregation of the AgNPs by ionic screening. The analyte-covered AgNP aggregates becoming a strongly SERS-active species, which upon interrogation with the laser produce the SERS spectra identifying molecules adsorbed on their surface. An optical micrograph of the flow-focusing junction under operating conditions is shown in Figure 1, in which the focused stream is made visible by its slightly different refractive index from those of the enveloping flows, as well as any particulate matter therein.

SERS spectra are obtained by interrogating the area immediately downstream of the junction with a micro Raman system in a rectangular array, producing a "chemical map" of the channel. Initially we interrogated a larger area (80 × 1000 μm^2) as shown in Figure 1, and observed that most of the useful signal comes from the first 500 μm after the junction. Subsequent scans were performed on a grid of 7 × 50 equally spaced points spanning 70 μm across the channel, and 500 μm along the streamwise direction of the channel. Typical spectra are shown in Figure 2A recorded from the interrogation region demarcated in Figure 1. A typical chemical map is shown in Figure 2B for a 100 ppm ampicillin solution in DI water. By mapping the intensities of specific Raman bands, we can obtain a measure of the nanoparticle aggregation process, in addition to determining the presence of the analyte. The blue contours map the intensity of the 490 cm^{-1} Raman band, corresponding to PDMS, which, predictably, is most intense outside the channel. The green contours indicate the intensity of the 230-240 cm^{-1} band, corresponding to AgCl, which we use to locate the areas with optimal AgNP aggregation, as previously discussed by Andreou *et al.*[15] Finally, the red contours (mapping the 1115 cm^{-1}) indicate the areas of high ampicillin signal, which, as expected, largely lie within the regions of maximal AgNP aggregation (green contours).

Milk is a complex fluid, an emulsion of lipid droplets suspended in water, which also contains a variety of sugars and proteins[16]. When homogenized milk was introduced into

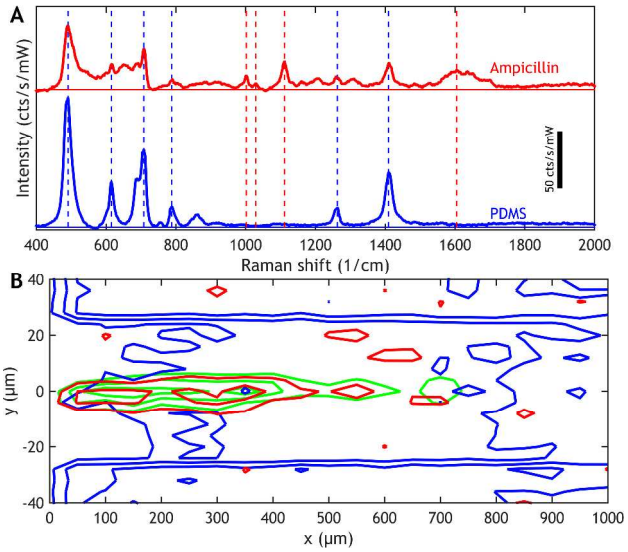

Figure 2 A) Reference spectra for PDMS and ampicillin (100 ppm in DI water) collected from the channel. B) Contour map of intensities of bands corresponding to the significant chemicals present in the device: blue – PDMS (490 cm^{-1}), red – ampicillin (1115 cm^{-1}), and green – AgCl (230-240 cm^{-1}).

the device, phase separation of the lipids occurred near the flow-focusing junction, resulting in an accumulation of lipid aggregates, quickly blocking the junction and impeding the fluid flow. This phase separation, which was caused by the low pH of the citrate buffer carrying the AgNP suspension, was not observed in a control experiment using salt solution alone. To remedy this, we carried out a lipid-removal procedure prior to introducing the sample into the device. We pre-aggregated the lipids in the milk by adding a strong acid to the milk drop-wise at room temperature until the pH was lowered to 2 (typically 5 μl sulfuric acid per 1 ml milk), at which point the lipids aggregated (curdled) allowing the solids to be easily removed using a 0.2 μm syringe filter, yielding a clear filtrate, which did not clog the channel.

For preparation of the samples used in the analysis, whole organic raw milk was purchased from Organic Pastures (Fresno, CA). 1 ml aliquots were spiked with ampicillin to the desired concentrations, and then treated for the removal of lipids, as described above. Samples of ampicillin in DI water were also prepared as control samples. No effect to the aggregation profile or SERS intensity was observed by mock treating the DI samples in the same way as the milk samples (lowering pH and filtration). The AgNPs used were citrate-capped BioPure particles of 20 nm diameter (nanoComposix, Inc., San Diego, CA) at a concentration of approximately 0.4 nM in 20 μM citrate solution (100-fold dilution with DI from stock). A 0.2 M aqueous LiCl solution was used as the aggregation-inducing agent.

Twenty microliters of sample were loaded into the device and allowed to flow for 7 minutes prior to interrogation. Flow in the device was induced by a single vacuum pump, attached to the outlet. Spectra were recorded using a confocal micro-

Raman system (LabRam Aramis spectrometer (Horiba, Kyoto, JP), with 3.8 mW of 633 nm laser, 1 s acquisition time per point). Subsequently, baseline subtraction for each spectrum was performed using a Whittaker filter (λ=60 cm^{-1}). The regions in the microchannel where the SERS signal is most intense depend on the flow rate and the salt concentration, and occur in the same area of the channel for each run. To account for the small variability of the device alignment and positioning between runs, the high-intensity areas were identified by measuring the intensity of the SERS peak at around 230 cm^{-1}, which we have previously interpreted to be an enhanced AgCl vibration that can be taken as an indicator of the aggregation state of the silver even in the absence of analyte. For each experiment, the spectra within this high-intensity region were normalized based on the maximum intensity between 800 and 2000 cm^{-1} of the population average, and subsequently averaged to give a representative spectrum. This normalization method resulted in spectra of comparable intensities and was found to be more robust than other normalization factors, such as division by the maximum or the area.

Spectra collected in this fashion are shown in Figure 3 for water and milk samples with varying concentrations of the antibiotic. The characteristic bands of the phenyl ring in the 1007 and 1035 cm^{-1} appear in all ampicillin-positive samples down to a concentration of the antibiotic at least as low as 10 ppb. For concentrations tested below 10 ppm, the intensity of these bands was not indicative of the concentration. We believe this to be due to the fact that very few molecules in SERS-active clusters provide most of the signal. The band around 1600 cm^{-1}, also associated with an aromatic ring vibration, broadens as the AgNPs aggregate and is removed by our baseline subtraction. As a result we are doubtful that it can be used as an ampicillin specific band at the lowest concentrations. The band around 1115 cm^{-1}, likely stemming from the secondary amide, appears to scale approximately with the concentration of ampicillin in the sample. The bands that appear at 1415 cm^{-1} and 1267 cm^{-1} are Raman signals of the PDMS.

Conclusions

A rapid microfluidics-based SERS methodology is demonstrated for determining the presence of ampicillin in raw milk samples at concentrations relevant to commercial antibiotic screening, which might be useful to the food industry and regulating agencies, such as the FDA, and may have further applications in healthcare. Moreover, on-chip sample pre-treatment steps, such as lipid particle removal, are potentially achievable by incorporating additional microfluidic components. The microfluidic device is amenable to automation, and by combining with a miniaturized spectrometer and a cartridge-based system, could provide a cost-effective, rapid, and field-deployable analytical tool.

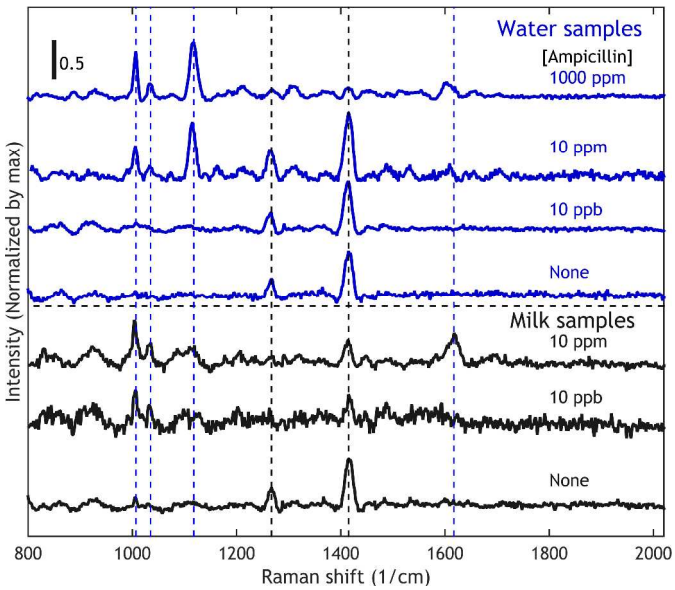

Figure 3 Average spectra collected. Peaks related with ampicillin were identified from the DI water samples and are marked with the dashed blue lines. These peaks appear in the milk samples spiked with ampicillin, but not in the negative controls. The dashed black lines denote bands at 1267 and 1415 cm^{-1} stemming from the Raman signal of PDMS.

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