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Quantification of an Exogenous Cancer Biomarker in Urinalysis by Raman Spectroscopy[†]

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We quantified an exogenous cancer biomarker, Acetyl Amantadine (AcAm), directly from urine solution using surface enhanced Raman spectroscopy (SERS). SERS was used for the detection of AcAm using a commercial Raman substrate after beta-cyclodextrin encapsulation for capture of the analyte. We achieved a detection limit of 1 ng/mL of AcAm in the mock urine in the absence of steroids without extraction or other pre-treatment methods required. With levels of corticosterone typical of urine, the limit of detection was 30 times higher. Since the approach works directly from samples containing the high concentrations of salts and organic co-solutes normal to urine, it has the potential to reduce cost and speed up processing with respect to methods that require pre-purification. Therefore, this is promising for clinical adoption for early cancer detection, particularly for lung cancer.

1 Introduction

Amantadine is acetylated by the enzyme spermidine/spermine N1 Acetyltransferase (SSAT) in the body; a process which is significantly up-regulated in cancer cells. Therefore, the acetyl amantadine (AcAm) can serve as an exogenous biomarker for the detection of cancers, specifically lung cancer which is difficult to detect at early stages by other methods¹⁻³. Previous work has used solid phase extraction (SPE) and tandem liquid chromatography with mass spectrometry (LCMS) to quantify AcAm from urine samples at approximately 10 ng/ml levels⁴. The use of LCMS, while highly effective in quantification, makes the process slow and costly. Further costs and delays result from the requirement for SPE. Therefore, it is highly desired to lower the costs and delays in processing by exploring different quantification approaches, ideally without the need for SPE processing.

Recent reports show an increasing trend in applying Raman spectroscopy in clinical applications, such as medical diagnosis^{5,6} and chemical sensing⁷⁻⁹. Raman scattering provides a "fingerprint" enabling characterisation and identification of the molecules, so it has inherent specificity. Despite the success of Raman spectroscopy, the technique still suffers from extremely low intensity with typical cross section from

10^{-28} to 10^{-24} cm² per molecule^{10,11}. Surface enhanced Raman scattering can be used to improve sensitivity in quantification.¹²⁻¹⁶

Due to the distance dependence of SERS, an important criterion is that the analyte of interest must be within a few nanometers of the nano structured surface. This can be achieved by using various surface functionalization techniques to bring analyte closer to the metal surface. A supramolecular host, like cyclodextrin (CD), can be incorporated on the metal surface to capture the analyte. CDs are a family of supramolecular hosts, which consist of six or more α -D-glycopyranose units. Due to its internal hydrophobicity, CDs can capture a variety of poorly water-soluble organic compounds^{17,18}. β -CD provides a hydrophobic small molecule binding pocket that has been used previously in Raman studies on polychlorinated biphenyls¹⁹ and canbendazim²⁰.

In this paper we investigate the use of surface enhanced Raman spectroscopy (SERS) to quantify AcAm in urinalysis. We prepare Raman substrates with hydrophobic pocket surface capture agents β -CD that work to extract the AcAm directly from the urine, thereby removing the need for SPE. We also investigate the influence of hydrophobic interferences on this detection approach.

2 Quantification approach

2.1 Materials

Sodium chloride, potassium chloride, potassium sulfate, urea, creatinine and corticosterone were purchased from Sigma Aldrich. Amantadine was purchased from Tokyo Chemical

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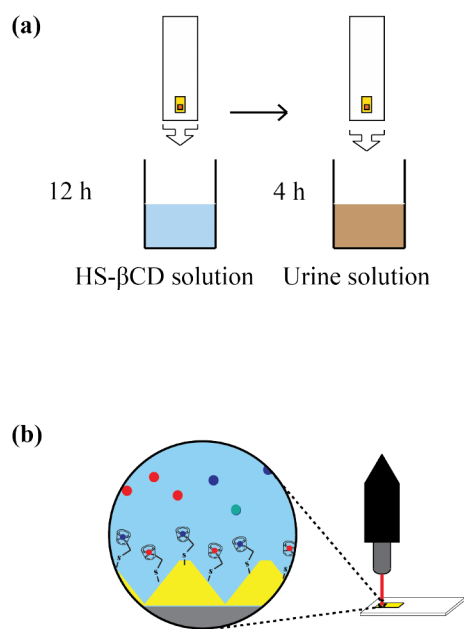


Fig. 1 Schematic of our approach to SERS quantification of AcAm. a) Substrate preparation; b) SERS measurement is taken using fiber probe on the β -CD functionalized Klarite.

Industry. Triethylamine and acetic anhydride are both from Anachemia. Mono thiolated beta-cyclodextrin was obtained from Shandong Zhiyuan Bio-Technology. Milli-Q deionized water (resistivity: 18.2 M Ω /cm) was used for all preparations. All materials were used as received without further purification.

2.2 Synthesis of AcAm

Acetyl Amantadine were prepared using a previously developed protocol²¹. Amantadine (2.00 g, 10.7 mM) was dissolved in 30 ml dichloromethane under nitrogen atmosphere. Triethylamine ((3.77 g, 37.5 mM) was added to the mixture drop-wise. The resulting solution was stirred for 5 minutes at room temperature. After the addition of acetic anhydride (2.17 g, 21.3 mM), the solution was stirred for one hour at room temperature. After an addition of water, the aqueous layer was extracted. The organic extract was dried over sodium sulfate, and filtered. A rotavap was used to evaporate the solvent. High vacuum was applied to obtain AcAm as white solid form.

2.3 Methods

Figure 1 shows a schematic SERS quantification of AcAm in urine samples. First, a commercially available Raman substrate Klarite (Renishaw Diagnostics Ltd. Glasgow, UK)

was functionalized with thiol-terminated β -CD, by immersing overnight 10 mM β -CD in dimethyl sulfoxide (DMSO) solvent for 12 hours before being removed and washed thoroughly with DMSO to remove any excess thiols and residues. We observed the surface of Klarite substrate before and after β -CD functionalized using scanning electron microscope (SEM), atomic force microscope (AFM) and SERS. The SEM and AFM images are shown in Fig. S1 and Fig. S2 (See ESI†).

The functionalized substrate was immersed for four hours in artificial urine solution containing various quantities of AcAm. We found that 4 hours incubation time was enough to saturate the surface (See ESI†). Artificial urine (AU) contains sodium chloride (8.0 g/L), potassium chloride (1.64 g/L), potassium sulfate (2.63 g/L), urea (13.40 g/L) and creatinine (1.5 g/L). Am stock was made from dissolving Amantadine (18.8 g) in 5 mL MeOH and diluted to final volume of 10 mL with AU. AcAm stock was made from varying amounts of AcAm in 2 mL and diluted to a final volume of 10 mL with AU to give a final concentration in urine sample. Then 1 mL AU was combined with 1 mL Am stock and 1 mL AcAm stock to prepare urine samples containing salts, urea, creatinine, Am (3.3 μ M) and AcAm at varying concentrations. The Am was added since it is present as an interfering analyte in clinical urinalysis. After four hours immersion, we dried the Klarite substrate, rinsed with DI-water to remove the hydrophilic molecules including urea, creatinine, salts and Am.

Finally, Raman analysis was performed using a low-cost Raman system: A 785 nm fiber-coupled laser diode (Innovative Photonic Solution, 23 mW) was used for excitation, a QE 65 pro (Ocean Optics) portable spectrometer was used for detection, and a fiber optic probe (InPhotonics) was used for excitation, collection and filtering. Twenty spectra were acquired for each sample with 30 s integration time for each spectrum at different random locations. The QE 65 pro spectrometer allows for up to 15 minutes exposure.

In contrast to previous reports in the literature on the SERS detection of Amantadine²², we located multiple peaks of AcAm and chose 5 regions of interest (729-749 cm^{-1} , 769-789 cm^{-1} , 1179-1199 cm^{-1} , 1244-1264 cm^{-1} and 1428-1448 cm^{-1}) which are characteristic for AcAm to maximise the quantification signal. We calculated the intensity of AcAm signal by using the data points in the regions of interest, subtracting equal numbers of data points on either side of the regions to remove the background. The summed intensity is used to obtain a higher count rate and thereby increase the signal to noise ratio, which scales as the square root of the count rate. It is also used to add spectral diversity so that the response is less susceptible to spurious spectral artifacts occurring in a limited wavenumber range. Integration and standard deviation of the spectra were determined for each sample using Matlab. There is a previous report in the literature on the SERS detection of Amantadine.

We further studied the influence of hydrophobic steroids on our Raman approach. Corticosterone is the most prominent steroid found in urine, so we focussed on that as a representative steroid. To make corticosterone (50 mM) stock, 17.4 mg dissolved in DMSO (2 mL) and diluted to a volume of 10 mL with AU. AU with corticosterone was made by combining 1 mL corticosterone stock with 1 mL AcAm stock and 1 mL Am stock to prepare urine samples. For this experiment, five spectra were taken for each sample with 30 s integration time instead of 20 spectra to save time.

The results showed good reproducibility when repeating over a period of 4 months. Furthermore, the Klarite substrates could be reused more than 10 times by cleaning with plasma etching for 1 hour.

3 Results

3.1 Quantification of AcAm in urine

Figure 2(a) shows a representative spectrum of AcAm powder. The regions of interest are shown in grey where the data is summed, and the background around these peaks is subtracted.

Figure 2(b) shows example spectra for 100 ng/mL AcAm in artificial urine, following the procedure above. Also shown is 100 ng/mL of AcAm without the use of the capture agent, β -CD. This shows clearly that β -CD acts to effectively capture the AcAm at the surface of the Raman substrate. Compared to the spectrum of powdered AcAm, there are some other peaks appearing in Fig. 2b: the peak at 1000 cm^{-1} is from urea, the peak at 840 cm^{-1} is from creatinine²³ and the peaks at 534, 674, 948 cm^{-1} are from β -cyclodextrin (See ESI†).

Figure 2(c) shows the summed intensity increases with the concentration of AcAm. The errors are the standard error on the mean, taken from repeating the measurement 20 times at different locations on the Klarite. It is clear from this figure that the limit of detection is 1 ng/mL. The limit of detection (LOD) was calculated by three times standard error on mean of the blank samples plus the signal of black sample (background level) and by the slope of the linear fit at low concentrations. The curve also shows saturation for higher concentrations > 300 ng/mL, where all the binding sites are filled.

3.2 Influence of corticosterone

Figure 3(a) shows the Raman spectrum of corticosterone powder. From the spectrum, we could observe that a prominent peak of corticosterone is at 1650 cm^{-1} .

Figure 3(b) shows the example spectra for 100 ng/mL AcAm with (red) and without corticosterone (black). Compared with the spectrum which did not have corticosterone, it

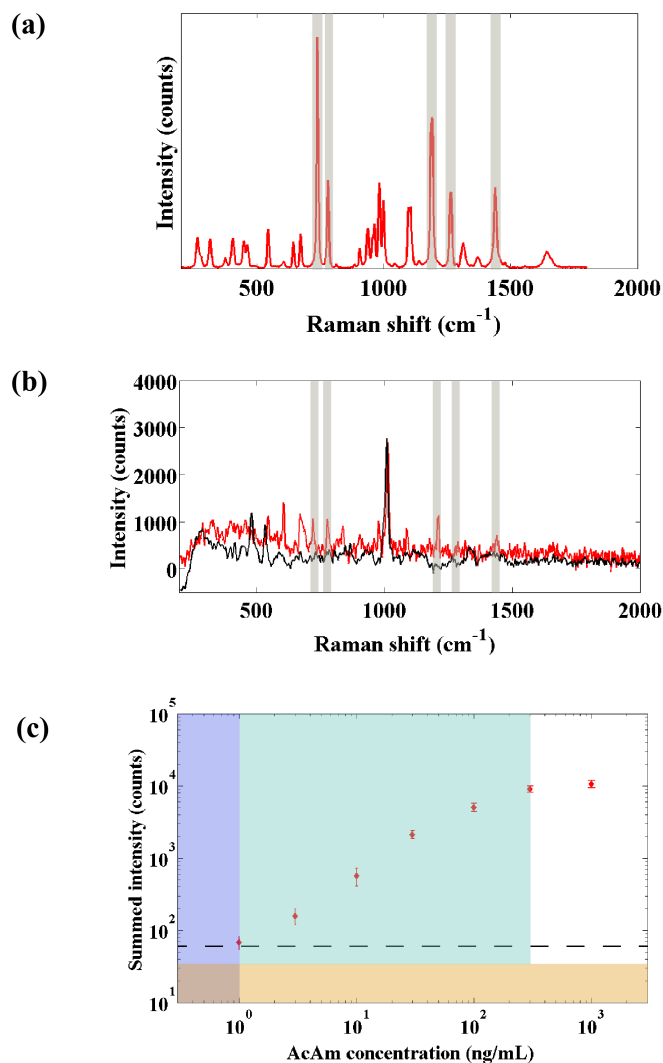


Fig. 2 a) Powdered AcAm of Raman spectrum and the regions of interest shown in grey. b) Spectra for 100 ng/mL of AcAm (red) with β -CD and the 100 ng/mL of AcAm without using β -CD (black). The regions of interests are shown in grey. c) Summed intensity versus AcAm concentration. The background level is shown in orange and limit of detection is shown in light blue, which corresponds to 1 ng/mL. The dynamic range is shown in light green, which is from 1 ng/mL to 300 ng/mL. The dash line shows the calculated signal of AcAm, which equals background level plus three times standard error of blank sample.

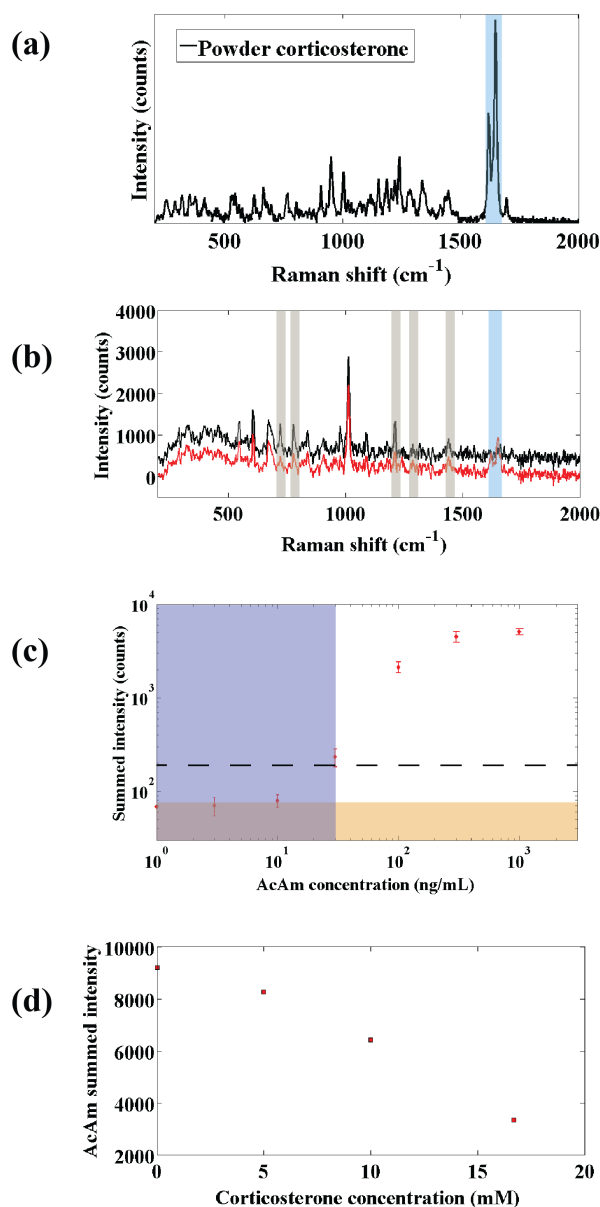


Fig. 3 a) Raman spectrum of corticosterone powder. b) Spectra for 100 ng/mL AcAm in artificial urine with and without corticosterone. The regions of interest are shown in grey (AcAm) and blue (corticosterone) c) Summed intensity versus AcAm concentration. The background level is shown in orange and LOD level is shown in light purple, which corresponds to 30 ng/mL. The dash line shows the calculated signal of AcAm. d) AcAm summed intensity with different concentrations of corticosterone. The concentration of AcAm was 300 ng/mL.

it is easy to see that corticosterone peak appeared and the AcAm signal decreased.

Figure 3(c) shows the summed intensity of the sample with corticosterone. From the figure, the LOD is 30 ng/mL. The curve also shows the saturation at 300 ng/mL.

Figure 3(d) shows AcAm summed intensity in AU with different concentrations of corticosterone. The AcAm concentration we used in this case was 300 ng/mL. From the figure, we see that the AcAm signal decreased as the corticosterone concentration increased, which shows corticosterone was competing with AcAm for β -CD binding.

4 Discussion

Past clinical studies have looked at samples containing typically around 10 ng/mL of AcAm in urine²⁴. Those studies used SPE and other extraction methods to purify the sample before mass spectrometry. In the absence of steroids, we have achieved a LOD below this value without any preprocessing of the urine. Furthermore, we achieved the desired dynamic range, which is from 1 ng/mL to 300 ng/mL²⁵.

Corticosterone was used as a representative steroid that is present at relatively high concentrations in human urine. Corticosterone reduces the LOD and changes the quantification levels, so that future work will look at different approaches to manage this interference. One approach is using other binding pockets such as cucurbiturils (CBs)^{26–29} that do not capture steroids. Another approach is to work on the quantification of the steroids from their Raman spectra as well, which will allow for characterizing the degree of competition and thereby allow for extraction of the AcAm concentration accurately.

We also note that urea is still present in the spectra due to its high concentration in urine, even after copious washing. That does not affect the analysis since the peak is well separated from AcAm peaks.

5 Conclusions

A method for quantifying a lung cancer biomarker AcAm was presented using a commercial SERS substrate with β -CD encapsulation and using multiple peaks for quantification. With this method, we achieved the LOD of 1 ng/mL AcAm directly from artificial urine without any additional purification. The LOD is reduced by the presence of steroids. The approach is low cost and rapid, which is both promising features for future clinical adoption. In the future, we plan to use Raman analysis to quantify the steroids as well, and/or use other capture agents, such as CBs to select AcAm against steroid.

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