Supporting Information

Multiplexed digital colloid-enhanced Raman spectroscopy for metabolite detection via selective molecular affinity

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METHODS

Materials and instrumentation.

Trisodium citrate dihydrate (TSC, AR, 99.8%), polyethyleneimine (PEI, 99%), tryptamine (Try, 98%), octopamine (Oct), O-aminophenol (OAP, 99%), and thymine (Thy, 99%) were obtained from Aladdin (Shanghai, China). Silver nitrate ($AgNO_3$, AR, 99.8%), itaconic acid (Ita, AR), and sodium borohydride ($NaBH_4$, AR) were purchased from Sinopharm (Shanghai, China). Spermine tetrahydrochloride (95%) was purchased from Macklin (Shanghai, China). Mucic acid (Muc, 98%) and dithiothreitol (DTT, 99%) were purchased from Meryer (Shanghai, China). Cytidine (Cyt, 99%), malic acid (Mal, 99%), and d-fructose 1,6-bisphosphate trisodium salt (FDP, 98%) were bought from Yuanye Bio-Technology (Shanghai, China) and ACMEC (Shanghai, China), respectively. All materials were used as received without further purification. Ultrapure water (18.0 MΩ) was used for all experiments.

Raman spectra were measured with a confocal Raman spectrophotometer (LabRAM XploRA INV, Horiba). The morphology of the colloids was characterized using a JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan). Extinction spectra were measured with a UV1900 UV–vis spectrophotometer (Aucybest, Shanghai, China). The hydrodynamic diameter and zeta potential of the colloids were measured using a Zetasizer Nano ZSP (Malvern, UK).

Synthesis of Cit-AgNPs.

Cit-AgNPs were synthesized according to the method reported by Lee and Meisel with slight modifications.[1] Briefly, 18 mg of $AgNO_3$ was dissolved in 100 mL of ultrapure water and heated to boiling. Then, 2 mL of a 10 mg/mL TSC solution was added to the boiling solution

under stirring. The boiling mixture was continuously stirred for 1 hour in the dark. Afterward, the mixture was cooled to room temperature, and the resulting Cit-AgNPs were stored at 4 °C.

Synthesis of Sp-AgNPs.

Sp-AgNPs were synthesized following a previously reported procedure with slight modifications.[2] Briefly, 100 μL of a 0.5 M $AgNO_3$ solution was added to 9.9 mL of ultrapure water, followed by the addition of 7 μL of 0.5 M spermine tetrahydrochloride under vigorous stirring. Subsequently, 25 μL of a freshly prepared 0.5 M $NaBH_4$ solution was added dropwise to the mixture under continuous stirring. The reaction mixture was then gently stirred for 20 minutes. Prior to synthesis, the glass vials were coated with a 0.2% (w/w) polyethyleneimine (PEI) aqueous solution for 2 hours and then thoroughly rinsed with ultrapure water.

Raman measurements.

Before measurement, Cit-AgNPs were centrifuged at 5,000 × g for 10 min to achieve a 5-fold concentration. Similarly, Sp-AgNPs were concentrated 4-fold by centrifugation at 5,000 × g for 15 min. The analytes were mixed with the respective silver nanoparticle colloids (Cit-AgNPs/Sp-AgNPs) at a 1:1 volume ratio and incubated at room temperature. The mixture was then subjected to brief gentle ultrasonication for 5 s to prevent agglomeration and precipitation. Subsequently, 10 μL of the sample-colloid mixture was injected into a quartz capillary (internal diameter: 1 mm; external diameter: 2 mm) for measurement using a confocal Raman spectrophotometer. All spectra were acquired in pointwise scanning mode with a 10× objective, using a 638 nm laser (power: 10.36 mW) at an acquisition time of 1 s and a 600 g/mm grating.

Data analysis.

All spectra were processed using Python 3.11. Specifically, spectral smoothing was performed via the Savitzky-Golay filter method, followed by baseline correction using the adaptive iteratively reweighted penalized least squares (airPLS) algorithm. The digitization criteria are as follows: if the maximum value in the characteristic peak window of the target metabolite (I_{peak}) is higher than the preset threshold, the voxel is assigned as positive ("1"); otherwise as negative ("0"). The threshold is determined by calculating the signal fluctuation in the noise window. To ensure quantitative specificity, exclusive characteristic peak windows were set for the four metabolites respectively. The signals of each window had no overlapping interference (515–590 cm^{-1} for FDP, 1345–1420 cm^{-1} for itaconic acid, 1190–1240 cm^{-1} for thymine, and 1265–1320 cm^{-1} for cytidine). The noise window was set to 1800–1860 cm^{-1} (no signals of target metabolites in this region). Thresholds were calculated as follows: Threshold = average signal of noise window (μ_{noise}) + N × standard deviation (σ_{noise}).

For each target analyte, the multiplier N was determined using a statistics-based maximum-value method ensuring strict control of false positives.[3, 4] Noise intensity follows a normal distribution $X \sim N(\mu_{noise}, \sigma_{noise}^2)$. For a given analyte, the characteristic Raman peak window contains n independent spectral data points, and I_{peak} is the maximum value of the window. We set the false-positive probability to be less than $\frac{1}{k}$ (where k is the number of acquired spectra). Based on the cumulative distribution function of the maximum, we obtain:

$$F(X_n > x) = 1 - F(X_n \leq x) = 1 - \prod_{i=1}^{n} F(X_i \leq x) = 1 - [F(x)]^n < \frac{1}{k}$$

The value of n (the number of points in its peak window) depends on the spectral resolution and the chosen wavenumber range. With a fixed $k = 500$ for all measurements, the corresponding x is calculated individually.

Taking Cytidine as an example, the window of the characteristic peak was set from 1265 to 1320 cm^{-1}, with an intensity number (n) of 23. A total of 500 spectra were collected for each test (k = 500). Then,

$$F(X_n > x) = 1 - [F(x)]^{23} < \frac{1}{500}$$

$$F(x) \approx 0.9999129602 \approx F(x = 3.7539)$$

Since $\sigma_{\text{peak1265 - 1320}} \approx 1.51 * \sigma_{\text{noise}}$, thereby the N was determined to be 6 $(> 1.51 * 3.7539)$for Cytidine, the threshold was determined to be ($\mu_{noise} + 6\sigma_{noise}$). The calculation process for other analytes is similar. Please see Table S2 for details. The ratio of positive voxels (RPV) was calculated as the percentage of positive spectra relative to all acquired spectra. Then, calibration curves for RPV (or positive counts) versus concentration were established. The LOD concentration was calculated based on the calibration curve using the false positive level, defined as the mean RPV of blank controls plus two times the standard deviation.

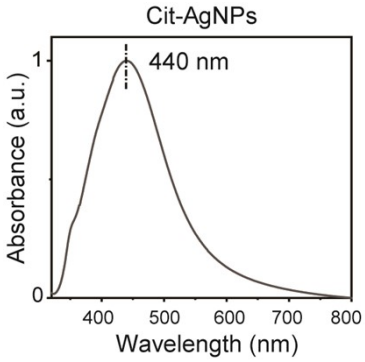

Figure S1. The UV-Vis spectrum of Cit-AgNPs showed an absorption peak at 440 nm.

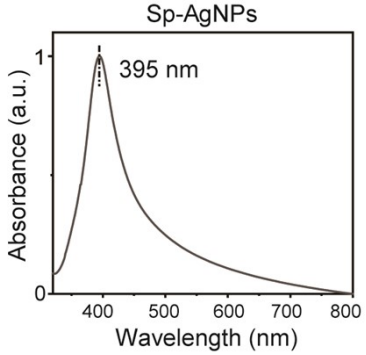

Figure S2. The UV-Vis spectrum of Sp-AgNPs showed an absorption peak at 395 nm.

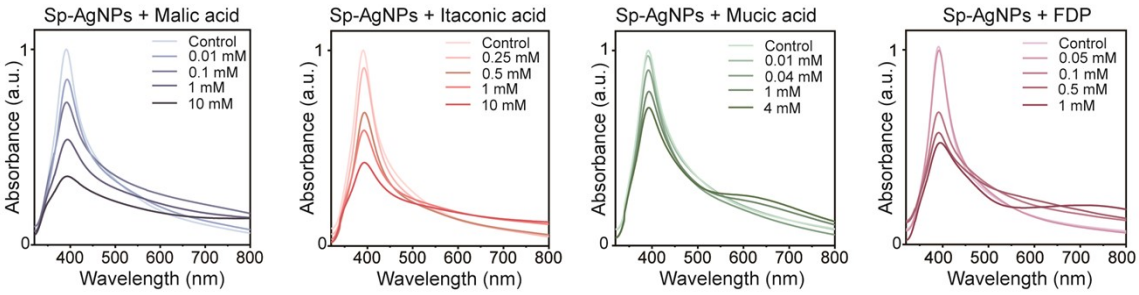

Figure S3. The UV-Vis spectra of Sp-AgNPs with metabolites (malic acid, itaconic acid, mucic acid, and FDP) at different concentrations. The line color varies from light to dark, indicating an increase in metabolite concentration.

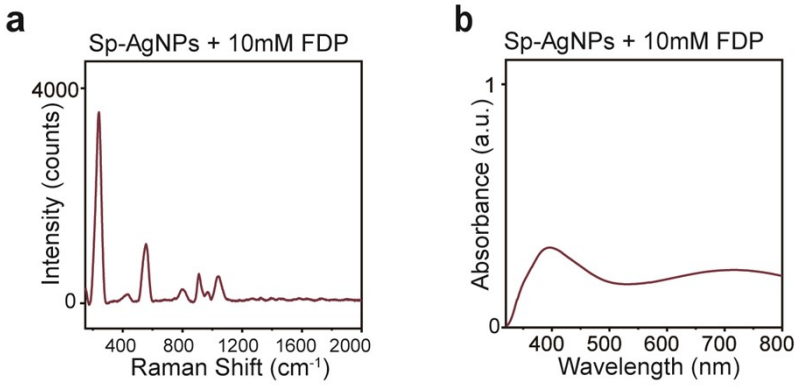

Figure S4. (a) The SERS spectrum of 10 mM FDP. (b) The UV-Vis spectrum of the Sp-AgNPs with 10 mM FDP.

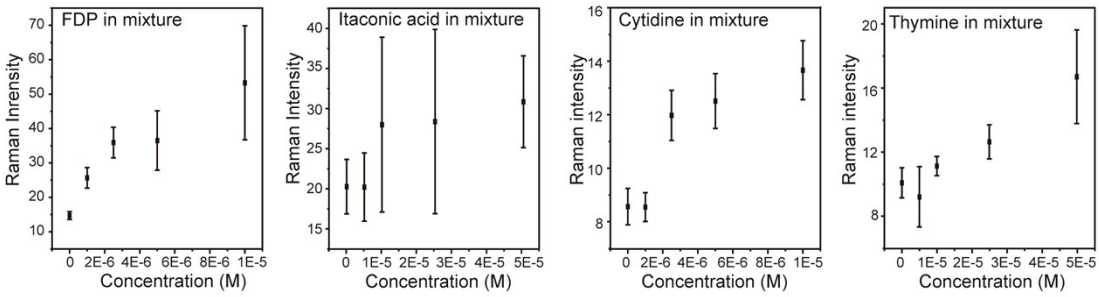

Figure S5. The peak intensity from the average spectra versus metabolite concentration, showing poor distinction from the blank control at low concentrations and overlapping error bars.

Table S1. Concentrations of metabolites in the mixture.

Target metabolite	FDP (μM)	Itaconic acid (μM)	Cytidine (μM)	Thymine (μM)
FDP	0, 1, 2.5, 5,10	10	5	5
Itaconic acid	1	0, 5, 10, 25, 50	5	5
Cytidine	1	10	0, 1, 2.5, 5, 10	5
Thymine	1	10	5	0, 5, 10, 25,50

Each target metabolite was measured at five concentration levels (as shown in the corresponding column), while the concentrations of the other three metabolites were kept constant.

Table S2. The detailed parameters for dCERS data processing.

Target metabolite	n	$\frac{\sigma_{peak}}{\sigma_{\text{noise}}}$	x	N
FDP	28	1.46	3.8029	6
Itaconic acid	31	1.17	3.8281	4.5
Cytidine	23	1.51	3.7539	6
Thymine	21	1.49	3.7311	6

Table S3. Limits of detection (LOD) for the four metabolites using dCERS method.

Target metabolite	LOD (μM)
FDP	0.39
Itaconic acid	3.61
Cytidine	0.631
Thymine	3.33

REFERENCES

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2. D. van Lierop, Ž. Krpetić, L. Guerrini, I. A. Larmour, J. A. Dougan, K. Faulds and D. Graham, Chemical Communications, 2012, 48, 8192-8194.
3. X. Bi, D. M. Czajkowsky, Z. Shao and J. Ye, Nature, 2024, 628, 771-775.
4. Z. Luo, X. Bi, Z. Yuan, X. Yu, M. Huang, Z. Shao and J. Ye, ACS Applied Materials & Interfaces, 2026, 18, 24159-24169.