

SERS Detection of Breast Cancer-Derived Exosomes Using a Nanostructured Pt-Black Template

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At present, there are no cancer treatments that are both non-invasive and highly accurate. New tests that can diagnose cancer at an early stage would help to facilitate such improved therapies, and many recent studies have focused on the development of liquid biopsy tests for this purpose. Exosomes are extracellular vesicles secreted by cells as a means of communication that can be simply collected from blood samples. Current studies have shown the potential of surface-enhanced Raman spectroscopy (SERS) in differentiating cancerous cells from healthy cells. Herein, a bespoke platinum-black (Pt-black) SERS template is developed—via a cost-effective fabrication method of electroplating—to detect malfunctioned (cancerous) exosomes. The results demonstrate that the Pt-black SERS substrate exhibits stable and consistent spectra, which produces the high reproducibility required for a reliable diagnostic template. More importantly, using the Pt-black SERS template allows for the differentiation of cancer-derived exosomes (extracted from 4T1 cells—a triple-negative breast cancer cell line) and exosomes from healthy fibroblast cells with an 83.3% sensitivity and a 95.8% specificity. This study establishes the potential of the Pt-black template in detecting cancerous exosomes and lays a solid foundation for future studies in the clinical application of SERS in cancer diagnosis.

1. Introduction

It is well known that early diagnosis of cancer can significantly increase survival rates.^[1] Thus, advancements in diagnostic methods that allow early detection of cancer have great potential in reducing cancer mortality. Amongst currently available cancer diagnostics methods, tissue biopsy and liquid biopsy are the most common and definitive diagnostic approaches. Tissue biopsy is more prevalent, offers authoritative test results, and is regarded as the gold standard in cancer diagnosis.^[2] However, the test can be performed only when the tumor has grown to a size that can be detected via an imaging technique, such as computed tomography (CT), which limits its potential in early diagnosis.^[2] Another drawback of tissue biopsy is its failure to account for the highly heterogeneous nature within the primary tumor, as well as between metastases, which hinders the development of personalized treatment and contributes to subsequent therapeutic failure.^[3,4] Moreover, tissue biopsies

are invasive and carry the risk of spreading tumor cells to adjacent healthy tissues which result in tumor seeding.^[2,5] Finally, often biopsies are blindly obtained, and even if not, they can easily sample healthy tissue, and thus provide a false negative.

Liquid biopsy, on the other hand, is much less invasive since it only requires the collection of body fluids such as blood.^[6] By examining biomolecules secreted into the circulation by tumor cells, liquid biopsy takes the heterogeneity of tumors into account.^[6,7] Therefore, liquid biopsy has enormous potential in the early diagnosis of cancer and can provide more reliable decision support on the subsequent treatment methods. Currently available liquid biopsy tests include the cancer antigen 125 (CA-125) test for ovarian cancer, prostate serum antigen (PSA) test for prostate cancer, and mutation screening within circulating tumor DNA (ctDNA).^[8,9,10] However, the main problem with liquid biopsy tests is the high rate of false positives, which results in overdiagnosis and overtreatment, and false negatives that lead to misdiagnosis and postponed treatment.^[2,11]

Recently, numerous studies have examined the possibility of developing new liquid biopsy methods for the detection of circulating cancer cells (CTC), circulating tumor RNAs,

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tumor-educated platelets (TEPs), and extracellular vesicles (EVs) such as exosomes.^[8,12,13] Since exosomes carry more biological information and are more abundant compared to other circulating biomarkers,^[14,15] this study focused on the detection of exosomes in developing a novel liquid biopsy for early cancer diagnosis.

Exosomes are membrane-enveloped vesicles produced in most eukaryotic cells and are secreted by cells via exocytosis to achieve local and distant communication.^[16] They carry unique cargos, including nucleic acids, proteins, and lipids, that can be specifically taken up by their target cells at distant sites.^[16–19] It has been shown that exosomes play a significant role in cancer progression by facilitating angiogenesis, drug resistance, metastasis, and evasion of immune surveillance.^[20–23] Common methods used to analyze exosome composition mainly include proteomic and genomic techniques that identify the presence of oncogenic proteins and oncogenes.^[23–25] However, those techniques are usually time-consuming, costly, and require highly concentrated samples for detection, which make them less suitable for the development of a rapid, point-of-care liquid biopsy test for cancer diagnosis.

Raman spectroscopy is a non-destructive optical technique that can quantitatively analyze the biochemical composition of samples.^[26] Although many other optical techniques, such as fluorescent microscopy (FM), stimulated emission depletion (STED) microscopy, and dynamic light scattering (DLS), have been used for exosome analysis,^[27] those techniques can only be used to detect tagged molecules or provide morphological information.^[28,29] In contrast, Raman spectroscopy can provide the complete biochemical fingerprint of exosomes by recording the inelastic scattering of photons where there is a net exchange of energy between the incident laser and the sample molecules.^[26] It has been shown that Raman spectroscopy is able to differentiate cancerous cells from healthy cells using tissue samples of various cancer types.^[30–32] However, signals generated by standard Raman spectroscopy are relatively weak and are often accompanied by high fluorescent background signals.^[26] Therefore, to fully exploit the potential of Raman spectroscopy in the development of a novel non-invasive cancer diagnostic, surface-enhanced Raman spectroscopy (SERS) has been developed, which acts to enhance the observed Raman signals.

SERS is based on the interaction of incident laser light with a nano-structured surface, which results in a localized surface plasmon resonance (LSPR) effect.^[33] This LSPR induces enhancement of the Raman signal, and enhancement factors of 10^6 and above have been routinely reported. As a result, SERS has been proven capable of generating ultra-sensitive molecular fingerprints of biological samples^[34,35] and has considerable potential in the diagnosis and monitoring of diseases including cancer.^[36,37] Recent studies have used SERS to detect cancerous subcellular compartments and tumor-derived exosomes using a range of substrates, most commonly silver and gold (Au).^[38,39] Electrodeposited metals are often exploited for SERS-based sensing and other applications.^[40–43] However, there is currently no established technique for the detection of exosomes using SERS that can be clinically applied to the diagnosis of all cancer types. Therefore, this research investigated exosome detection using SERS combined with a novel electrodeposited platinum-black (Pt-black) substrate with the aim of demonstrating the future potential for cancer diagnostics. Pt-

black has been extensively used in electronic devices including in electrodes for implantable, wearable, and point-of-care devices for electrical stimulation;^[44–47] for electrophysiological signal recordings;^[44,48–51] in electrochemical sensors;^[52,53] and in bioimpedance sensors.^[54,55] The primary reason for the use of electrochemically deposited Pt-black in these applications is the resulting high level of nanostructuring of μm -scale electrodes and the consequent increase of electrode surface area, leading to a significantly reduced electrode interfacial impedance (which is beneficial for the electronic circuitry used in conjunction with these electrodes). However, the utility of Pt-black for SERS is yet to be reported.

To investigate the potential of Pt-black in SERS-based biosensing, we have developed a novel, custom-made, nanostructured Pt-black sensing substrate as a SERS template using electrochemical deposition (a relatively simple and low-cost approach to realize micro/nanostructured surfaces). To demonstrate the potential for cancer diagnostics, we used the novel Pt-black SERS template to differentiate exosomes derived from 4T1 triple negative mouse breast cancer cells from human fibroblast exosomes and compared our results against data collected using a commercially available gold SERS substrate (RAM-SERS-AU, Ocean Insight; hereafter referred to as SERS-Au substrates). In differentiating between the two types of exosomes, the proposed Pt-black SERS template exhibited stable and repeatable SERS signals, as well as strong signal enhancement. Furthermore, multivariate analysis of the collected spectra demonstrated the capability to correctly identify cell type with 83.3% sensitivity and 95.8% specificity. Hence, overall, this study demonstrates the potential of SERS for early cancer diagnostics and proposes a low-cost and simple-to-fabricate Pt-black template as a substrate for this purpose.

2. Results and Discussions

2.1. Fabrication and Characterization of Electroplated Pt-Black Templates

To fabricate Pt-black templates, Au-coated silicon (Si) wafers were diced, cleaned, and insulated using laser patterned sealing films to allow the exposure of only five 4 mm diameter circular regions of the Au-coated side of the silicon wafers. This allowed the creation of 32 samples. Pt-black deposition leads to a visible change in the color of the electrode, from the original yellow of Au to a dark black. Figure S2, Supporting Information, shows the different steps in the realization of the Pt-black templates. Electrochemical impedance spectroscopy (EIS) measurements were used to further demonstrate the formation of the Pt-black layer by probing the properties of the electrochemical double layer formed at the interface between the metal electrodes and a surrounding phosphate-buffered saline (PBS) solution.

As discussed in more detail elsewhere, the difference in charge carriers (electrons on the electrode side and ions on the solution side of the interface) leads to the formation of double-layer impedance, which can be modeled as a capacitor (capacitance of the double layer = C_{DL}) in parallel with a resistor (resistance to charge transfer = R_{CT}). The value of this impedance, along with other parameters (e.g., temperature, number, and type of ions involved), depends on the surface area of the electrode and the electrode material. Au and Pt are noble metals, which are challeng-

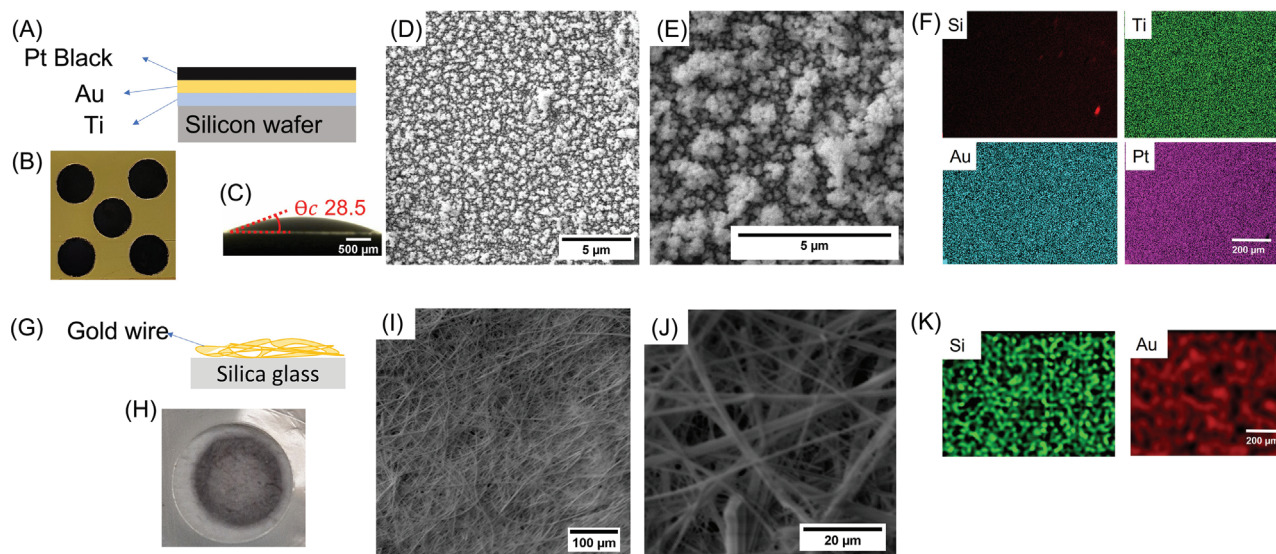


Figure 1. SEM and EDX characterization of the Pt-black template. A) Schematic illustrates the structure of the Pt-black template. B) Photograph of Pt-black template. C) Contact angle measurement of an exosome suspension. Contact angle (θ_c) was measured for both 4T1 and fibroblast exosomes as less than 28.5° demonstrating a high wettability (the same result was obtained using DI water). D,E) SEM micrographs of nanostructured Pt-black surface at two magnifications. F) Elemental mapping showing the distribution of the elemental components (Si, Ti, Au, and Pt) in the fabricated Pt-black templates. G) Schematic demonstrates the structure of the SERS-Au substrate. H) Photograph of SERS-Au substrate. I,J) SEM micrographs of nanostructured SERS-Au substrate at two magnifications. K) Elemental mapping showing the distribution of the elemental components (Au and Si) of the SERS-Au substrate.

ing to oxidize and reduce and thus demonstrate a high R_{CT} and a predominantly capacitive behavior. Deposition of Pt-black on Au will thus directly affect this interface impedance. Electrochemical measurements of the interface will also include measurement of the solution resistance (R_s).

EIS measurements were therefore taken in PBS to compare the interface impedance of the electrodes before and after Pt-black deposition. The recorded EIS spectra (impedance magnitude and phase) prior to and after deposition for all 32 fabricated samples are shown in Figure S1, Supporting Information. As expected, the behavior of the pure and highly flat Au electrode demonstrated a high interfacial impedance (Figure S1A,B, Supporting Information), with an impedance magnitude of $750 \pm 240 \text{ k}\Omega$ at 10 MHz decreasing toward $22 \pm 1 \text{ }\Omega$ at 1 MHz. Similarly, the phase exhibits large negative values at low frequencies, indicating a strong contribution to the measured impedance from the interfacial capacitance. The interface can, in this case, be roughly modeled with a parallel combination of a $14.5 \text{ }\mu\text{F}$ capacitor in parallel with a $1 \text{ M}\Omega$ resistor, as shown with the green trace in Figure S1A,B, Supporting Information. For simplicity, modeling of the interface has been performed with ideal R_s and C_s . More accurate modeling, which is beyond the scope of this paper, requires the use of a constant phase element (CPE) and other models used in electrochemistry to model electrode phenomena.

Pt-black deposition changes the interfacial impedance properties of the electrode (see Figure S1C,D, Supporting Information). The electrochemically deposited Pt-black nanoparticles create a nanostructured interface that greatly increases the surface area of the electrode. Interface impedance is inversely proportional to the electrode surface area and thus, as expected, the interfacial impedance is greatly reduced at 10 MHz from $\approx 750 \text{ k}\Omega$ to

$3.5 \pm 2.1 \text{ k}\Omega$. At the same time, with increasing frequency, the phase more rapidly approaches 0° , further supporting a diminished capacitive contribution to the measured impedance. In this case, the interface can be roughly modeled with a 4.5 mF capacitor in parallel with a $10 \text{ k}\Omega$ resistor, as shown with the green trace in Figure S1C,D, Supporting Information. This substantial decrease in interfacial impedance is achieved through the significant increase of electrode surface area generated by the electrodeposition of the nanostructured Pt-black layer.

The nanoscale structure and composition of the electroplated Pt-black templates were further investigated (and compared against the commercially available SERS-Au substrates) using scanning electron microscopy (SEM) and energy dispersive X-Ray (EDX). Schematic illustrations (depicting the stack-up of the Pt-black template and SERS-Au substrate) and photographs of the two substrates are shown in Figures 1A, 1B, 1G, and 1H, respectively. Five electroplated regions are clearly visible in Figure 1B. SEM micrographs of the Pt-black template (Figure 1D,E) show a uniform nanostructure of electroplated platinum as opposed to submicron fibers on the SERS-Au substrate (Figure 1I,J). Additionally, the wettability of the fabricated Pt-black templates was examined using contact angle measurements (using both DI water and exosome suspensions). High wettability with both DI water and exosome suspensions was evidenced by a contact angle not greater than 28.5° (Figure 1C) in both cases. Finally, the elemental composition of the fabricated Pt-black templates (Figure 1F) and SERS-Au substrates (Figure 1K) were ascertained using EDX and elemental mapping of the constituent substances (i.e., Si, Ti, Au, and Pt for Pt-black template; Si and Au for SERS-Au substrate). We note that the elemental components of the polymeric fibers in the SERS-Au substrates are not detected via EDX, which is mainly sensitive

Table 1. Acquisition parameters for Raman characterization measurements performed on crystal violet using Pt-black, SERS-Au, and silicon wafer substrates.

Substrate	Excitation wavelength [nm]	Laser power [mW]	Acquisition time [s]	Scans to average
Pt-black	532	1.0	1.0	10
SERS-Au	785	0.8	0.1	10
Silicon wafer	532	1.0 (at 532 nm)	1.0 (at 532 nm)	10
	785	0.8 (at 785 nm)	0.1 (at 785 nm)	

to inorganic substances. Together, these results further demonstrate the successful electrodeposition of a nanostructured Pt-black layer on the Au-coated silicon wafers and highlight the structural/compositional differences between the Pt-black and SERS-Au substrates.

2.2. SERS Properties of Pt-Black Templates

To characterize the SERS performance of the Pt-black templates, Crystal violet (CV) spectra were collected to determine the enhancement factor and the detection limit. This was performed both on the Pt-black templates and the commercially available SERS-Au substrates for comparison. For SERS-Au substrates, 15 μ L samples of 2.5–125 nM CV were each pipetted to the center of a substrate and air-dried. For Pt-black templates, 10 μ L samples of 5 nM–1 mM CV were each pipetted to the center of a substrate and air-dried. Spectra were collected as described in the Experimental Section (see Table 1). The average intensity at the 1620 cm^{-1} CV characteristic peak (see Figure 2) was plotted against the CV concentration to generate standard intensity versus concentration curves for both substrates (Figure 2B,D). A linear regression line was then used to fit a linear equation to the intensity versus concentration data, and hence, to calculate the limit of detection.

The enhancement factor, as defined in Equation (1), was used to determine the Raman signal enhancement capabilities of the SERS-Au and Pt-black templates when compared against the standard Raman signal acquired from pure Si substrates. For comparison with the SERS-Au, 10 μ L of 20 mM CV was pipetted to the silicon substrate and air-dried to be measured using the same acquisition parameters as used for the SERS-Au substrates (785 nm excitation, 0.5 mW laser power, 0.1 s acquisition time, and 10 scans). To compare with the Pt-black template, 10 μ L of 1 mM CV was pipetted to the silicon substrate and air-dried to be measured using the same acquisition parameters as used for the Pt-black templates (532 nm excitation, 1 mW laser power, 1 s acquisition time, and 10 scans). A CV concentration of 1 mM was chosen for excitation at 532 nm (as opposed to 20 mM at 785 nm) as the 532 nm laser was found to provide more efficient excitation of CV on Si substrates. The enhancement factor was calculated according to the equation below

$$\text{Enhancement Factor} = \frac{I_{\text{SERS}}/C_{\text{SERS}}}{I_{\text{Raman}}/C_{\text{Raman}}} \quad (1)$$

In this equation, I_{SERS} and I_{Raman} represent the average intensities at the 1620 cm^{-1} characteristic peak measured using the SERS substrates and silicon substrates, respectively. C_{SERS} and

C_{Raman} represent the CV concentrations used on SERS substrates and silicon substrates, respectively. To our knowledge, electrodeposited nanostructured Pt-black has not yet been evaluated as a SERS template, and thus one of the aims of this study involved establishing the stability and reproducibility of fabricated Pt-black SERS templates in comparison to commercially available SERS substrates. The SERS-Au substrates enhanced the CV signal by a factor of 207 (relative to Raman measurements on silicon substrates, see Figure 2A). The SERS-Au detection limit was calculated as 1.1 nM based on the CV standard curve (Figure 2B). The Pt-black template enhanced the standard Raman signal by a factor of 7.1 (Figure 2C) and the detection limit was calculated as 15 nM based on the standard curve (Figure 2D). Thus, the Pt-black template provided Raman signal enhancement, albeit with weaker enhancement and an inferior detection limit than the commercially available SERS-Au substrates. The characterization result was reliable since the peak at 1620 cm^{-1} , which was selected for calculations, was sharp and clear with both materials examined (Figure 2A,C). Moreover, the CV standard curves for both substrates had an R^2 value above 0.99, indicating their reliability in predicting the detection limit (Figures 2B and 3D).

While the SERS-Au substrates provided strong signal enhancement, they also demonstrated inconsistent background spectra with strong background signals that varied with respect to both wavelength and substrate (Figure S3A, Supporting Information). On the other hand, the Pt-black templates exhibited near-zero background signal, which was consistent across different substrates and across all wavelengths (Figure S3B, Supporting Information). This represents a significant advantage for the Pt-black templates, particularly for measurements of samples with low signal levels.

The SERS-Au substrates comprise of polymer fibers with integrated aggregates of gold nanoparticles (AuNPs) (as shown in Figure 1I–K). The background peaks observed from these slides have previously been attributed to signals from the polymer fibers and/or from chemicals used in the fabrication process.^[56] The Pt-black templates, on the other hand, are fabricated via direct electro-deposition of Pt-black onto Au-coated substrates. Thus, the reduced background signals in the Pt-black substrates (relative to the SERS-Au substrates) are tentatively attributed to the more homogeneous structure and simpler fabrication protocol (i.e., involving direct deposition of Pt-black rather than chemical fabrication of AuNPs).

2.3. Exosome Classification Using SERS-Au Substrates

Exosomes from 4T1 cells and fibroblasts were measured using the commercially available SERS-Au substrates. Prior to the

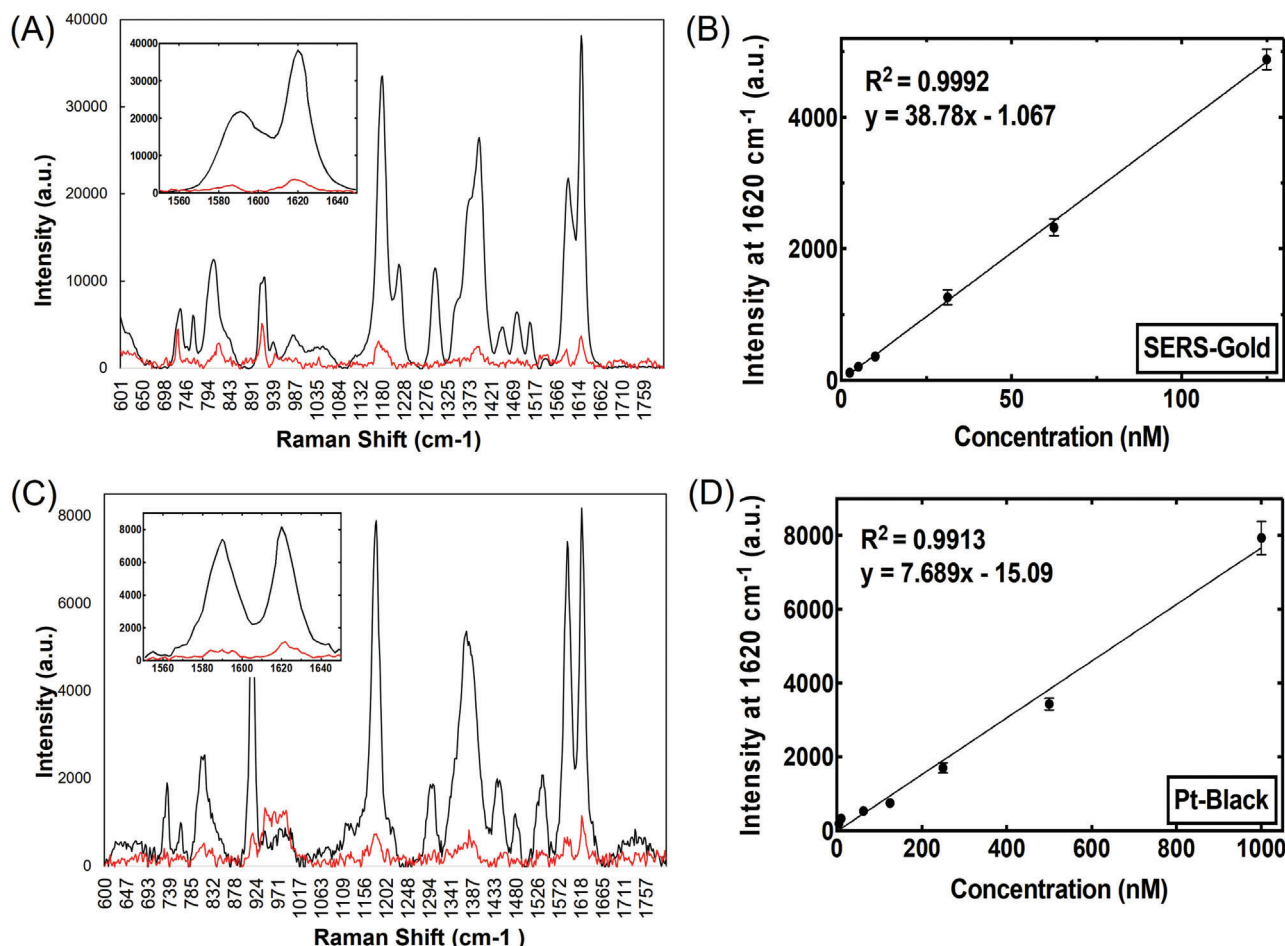


Figure 2. Enhancement factor and detection limit of SERS-Au and Pt-black templates. A) Average spectrum of 1 mM CV on SERS-Au substrate (black line) and 20 mM CV on silicon substrate (red line). B) The intensity at a Raman shift of 1620 cm⁻¹ at different CV concentrations measured using the SERS-Au substrate. C) Average spectrum of 1 mM CV on Pt-black (black line) and silicon substrates (red line). D) The intensity at a Raman shift of 1620 cm⁻¹ at different CV concentrations measured using the Pt-black template. Spectra in (A) were collected using 785 nm excitation, 0.5 mW laser power, 0.1 s acquisition time, and 10 scans. Spectra in (C) were collected using 532 nm excitation, 1 mW laser power, 1 s acquisition time, and 10 scans. Data are presented as means $\pm$ SD calculated from five technical replicates. Inset images in panels (A) and (C) show a zoomed-in region of the full spectra.

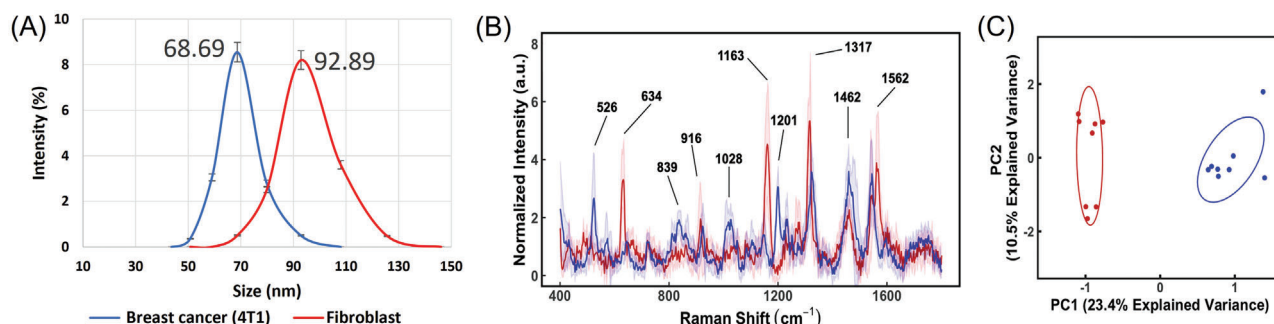


Figure 3. Discrimination of 4T1 breast cancer exosomes from fibroblast exosomes using the SERS-Au substrate. A) DLS shows an average size distribution of 68.7 and 92.9 nm for exosomes extracted from 4T1 and fibroblast cells, respectively. B) Average SERS spectra of 4T1 (blue) and fibroblast (red) exosomes. Peak positions in Raman shift (cm⁻¹) are labeled. Spectra are presented as means $\pm$ SD. Measurement points per sample = 8. C) PCA plot showing clear separation of exosomes derived from different cell lines. The axes represent the two PCs that account for the greatest percentage of variance between groups. The ellipses represent 95% confidence intervals.

SERS classification of the isolated exosomes from cell culture media, their size distributions were characterized using DLS. As shown in Figure 3A, the average size distribution of 4T1 and fibroblast exosomes was determined to be around 68.7 and 92.9 nm for 4T1 and fibroblast cells, respectively. Despite the average size differences in the isolated exosomes from the aforementioned cell lines, an average intensity of $\approx 8.5\%$ was observed in both cases indicating a similarity in the exosome concentration.

Figure 3B shows the average recorded SERS spectra (mean $\pm$ SD) obtained from two SERS-Au substrates, one with 4T1 exosomes and one with fibroblast exosomes. There were visible spectral differences between 4T1 exosomes and fibroblast exosomes (Figure 3B). The exosomes extracted from 4T1 cells exhibited characteristic peaks at 526, 655, 1021, 1024–1028, and 1224 cm^{-1} that were not observed in the Raman spectra of fibroblast exosomes (Figure 3B). For fibroblast exosomes, unique peaks were observed at 634, 1163, 1280, 1317, and 1562 cm^{-1} (Figure 3B). Peak assignments for the observed spectral peaks are tabulated in Table S1, Supporting Information.

Often visual analysis is insufficient to identify differences between the spectra of different exosomes.^[39,56,57] This is possibly due to the presence of proteins that are common in all types of the exosome,^[58] which could also explain the overlap of fibroblast and 4T1 exosome spectral peaks. Nonetheless, there should still be differences between spectra, since there are tumor-specific proteins and genetic materials in cancerous exosomes, that are, however, much less abundant than the commonly shared proteins.^[59] SERS has been widely used to differentiate the biomarkers of cancerous from healthy cells with the help of techniques such as principal component analysis (PCA) and deep learning methods.^[39,60]

The variation in the SERS spectra obtained from breast cancer (4T1) and fibroblast exosomes was thus further investigated using PCA (Figure 3C). PCA demonstrated clear separation of the exosome spectra according to cell type (Figure 3C), with the 1st principal component (PC1) accounting for 23.4% of the total explained variance and PC2 accounting for 10.5%. This indicated the potential to identify exosomes according to their Raman spectra using the SERS-Au substrates.

To investigate the repeatability of these findings, the experiments were repeated using two sample replicates. The mean spectra recorded from these replicates are shown in Figure 4A,B. As for the results presented in Figure 3, spectral differences were observed between the 4T1 and fibroblast exosomes for all replicates (Figure 4A,B). Furthermore, PCA plots showed similar separation of 4T1 and fibroblast exosomes (Figure S4, Supporting Information). However, when comparing the spectra shown in Figures 3B and 4A,B, it is clear that the exosome SERS spectra acquired from each cell line using SERS-Au substrates were not consistent across replicates. This issue was further exemplified by the mean spectra calculated across all replicates (Figure 4C,D), which exhibited large standard error ribbons for both cell lines. Thus, while we observed some unique peaks for each exosome type, we did not see consistency across different samples.

Moreover, PCA was unable to differentiate exosomes derived from the two cell lines when data were combined (Figure 4E). According to the combined PCA plot, 4T1 exosomes were separated into three clear clusters corresponding to each replicate, further confirming the inter-replicate variation (Figure 4E). Therefore,

while exosome classification was achieved for individual replicates, this was not possible when combining replicates recorded using different SERS-Au substrates. Taken together, these results suggested that there were other sources of variation within the data. We, therefore, identified and investigated three possible sources of variation: the reagents, substrates, and exosome samples.

Reagent controls were prepared by mixing the tissue culture media specific for each cell line, the exosome isolation reagent, and PBS at a ratio that best mimicked the real experimental settings. The result showed that there was hardly any signal from the reagent that can be detected using the SERS-Au substrate (Figure S5, Supporting Information), excluding reagents as the source of variation.

The SERS-Au substrates were confirmed to have variable background signals with high peak intensities (comparable to exosome peak intensities) in some cases (Figure S3A, Supporting Information). Although substrate signals were subtracted from the sample signal, the high peak intensities and the variability across substrates meant that this led to significant variation in the exosome spectra. Therefore, the inter-replicate variation in the exosome spectra can be attributed (at least to some extent) to the strong background signals observed from the SERS-Au substrates, which varied considerably from one substrate to the next (Figure S3A, Supporting Information).

There may also be variations in the composition of the exosomes derived from the same cell line but prepared on different experimental days. This was partially supported by the three separated clusters of 4T1 exosomes in the combined PCA plot of Figure 4E (although this may also be explained by differences in the substrate background signals for the three substrates used). Variation in exosome spectra may be due to different states of cells (e.g., depending on the passage number and the time after recovery), which could affect the intercellular communication guided by exosomes.^[61] Overall, however, our results showed that SERS-Au substrates were unable to generate reproducible spectra of exosomes (leading to an inability to classify exosomes using PCA) and that this was most likely due to high and variable background signals from the SERS-Au substrates themselves.

2.4. Exosome Classification Using Pt-Black Template

To address the strong background signals and high inter-replicate variability observed with the SERS-Au substrates, we examined the exosome classification capability of the custom-made electrodeposited nanostructured Pt-black SERS templates. Spectra collected for a single replicate (using a pair of Pt-black templates) demonstrated that 4T1 cancerous and fibroblast exosomes were similar and shared most of the same signal peaks (which were observed at Raman shifts of 81, 280, 363, 843, 1063, 1142, 1238, 1279, and 1487 cm^{-1}) with small variations observed in peak intensities between the two cell lines (e.g., at 363, 843, 1142, 1279, and 1487 cm^{-1} ; see Figure 5A). This observation is supported by several previous SERS studies.^[39,57,58] The peaks corresponded to specific chemical bonds present in proteins, lipids, and nucleic acids and their assignment is tabulated in Table S2, Supporting Information. PCA of the spectra recorded

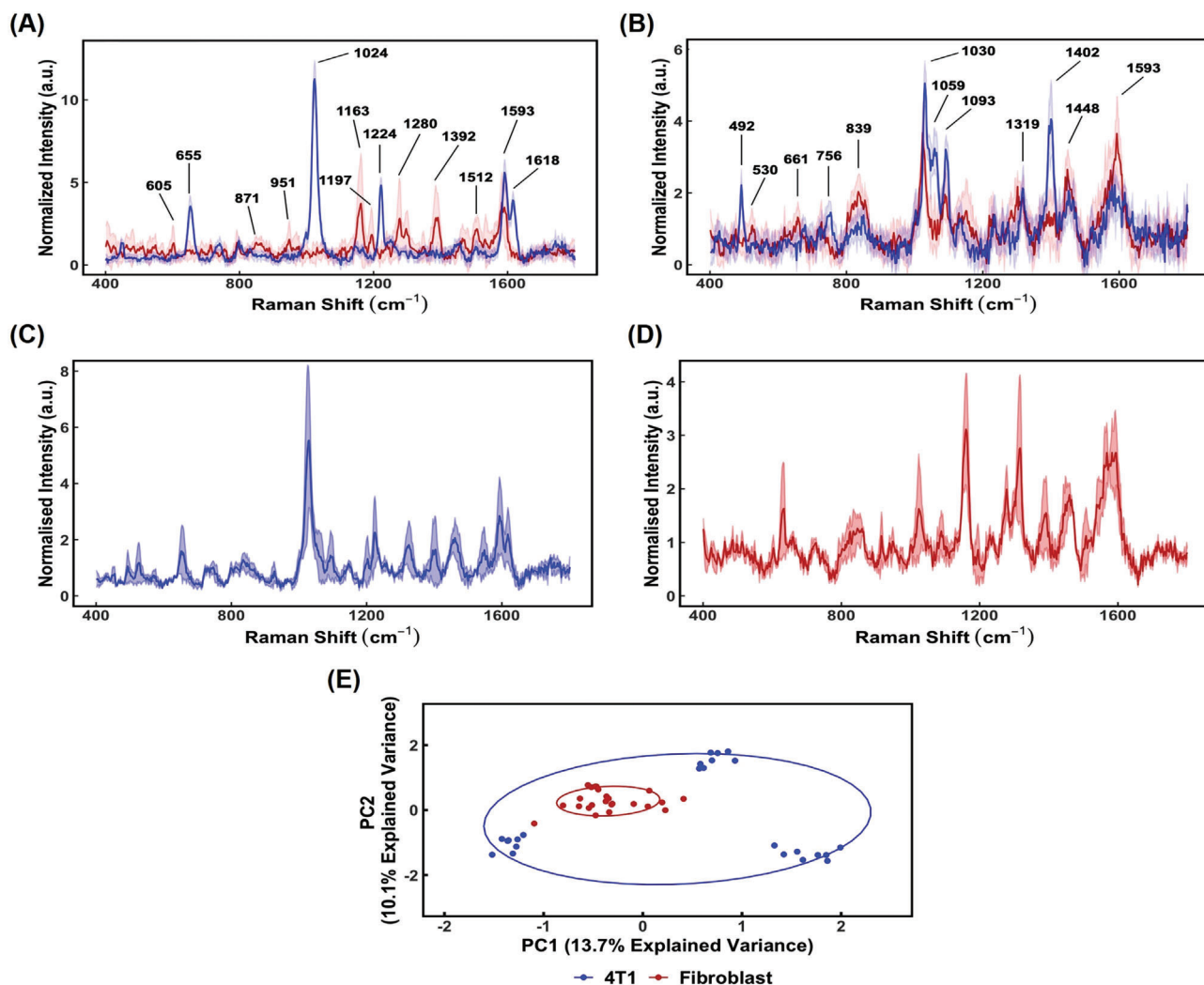


Figure 4. Repeatability assessment of exosome classification using SERS-Au substrates. A,B) SERS spectra from 4T1 (blue) and fibroblast (red) exosomes for two individual sample replicates. Peak positions in Raman shift (cm^{-1}) are labeled, and spectra are presented as means $\pm$ SD. Measurement points per sample = 8. C) Average SERS spectrum from 4T1 exosomes for all replicates combined (mean $\pm$ standard error [SE] calculated across all replicates). D) Average SERS spectrum from fibroblast exosomes for all replicates combined (mean $\pm$ SE calculated across all replicates). E) PCA of combined replicates showing no separation of exosomes derived from different cell lines. The axes represent the two PCs that account for the greatest percentage of variance between groups. The ellipses represent 95% confidence intervals. Data collected across three experimental replicates, $n = 3$.

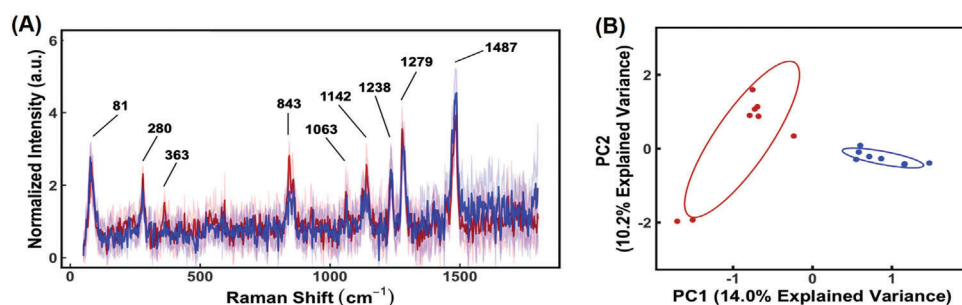


Figure 5. Differentiation of 4T1 breast cancer exosomes from fibroblast exosomes using Pt-black template. A) Average spectra of 4T1 and fibroblast exosomes from a single experimental replicate. Annotations show peak positions in Raman shift (cm^{-1}). Spectra are presented as means $\pm$ SD. Measurement points per sample = 8. B) PCA shows a clear separation of exosomes derived from different cell lines. The axes represent the two PCs that account for the greatest percentage of variance between groups. The ellipses represent 95% confidence intervals.

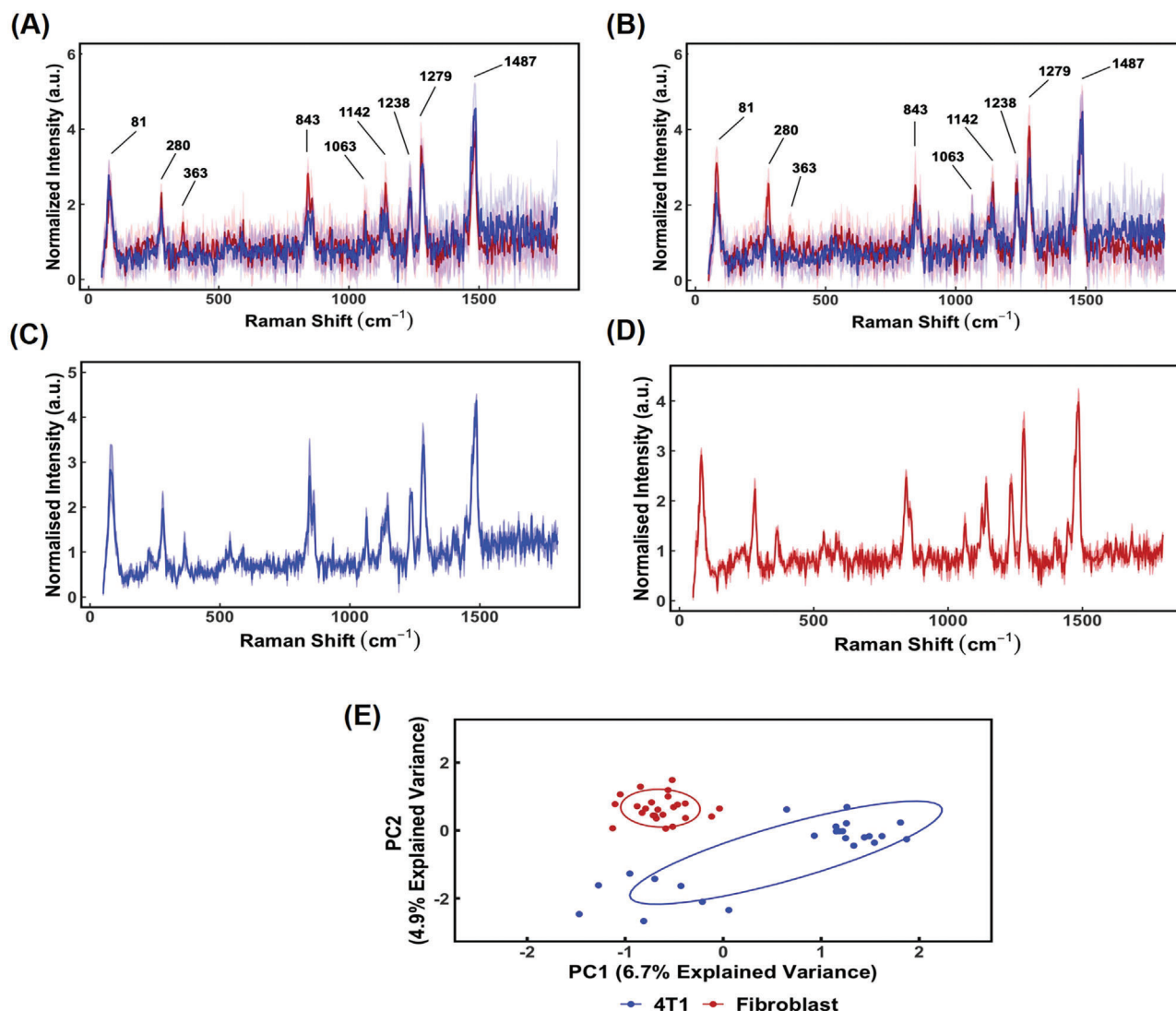


Figure 6. Successful discrimination of 4T1 breast cancer exosomes from fibroblast exosomes using Pt-black templates when replicates were combined. A,B) Average SERS spectra of 4T1 (blue) and fibroblast (red) exosomes for two individual sample replicates. Annotations show peak positions in Raman shift (cm^{-1}). Spectra are presented as means $\pm$ SD. Measurement points per sample = 8. C) Average spectrum of 4T1 exosomes for all replicates combined (mean $\pm$ SE calculated across all replicates). D) Average spectrum of fibroblast exosomes for all replicates combined (mean $\pm$ SE calculated across all replicates). E) Principal component analysis of combined replicates showing good separation of exosomes derived from the two different cell lines. The axes represent the two PCs that account for the greatest percentage of variance between groups. The ellipses represent 95% confidence intervals. Data collected across three experimental replicates, $n = 3$.

for this replicate demonstrated clear separation of the 4T1 and fibroblast exosomes (Figure 5B).

In contrast to the commercial SERS-Au substrates, the proposed Pt-Black templates had a lower background signal (Figure S3B, Supporting Information), which could be beneficial in reducing the possible variation caused by substrates. To examine the repeatability of the results with the Pt-black templates, two further experimental replicates were performed (Figure 6A,B), which revealed similar spectral profiles to those observed in the first replicate (Figure 5A). Mean spectra calculated across all three replicates (Figure 6C,D) exhibited comparable results (demonstrating small but visible spectral differences between 4T1 and fibroblast exosomes). The mean spectra also exhibited much thin-

ner standard error ribbons than those observed using the SERS-Au substrates, indicating a smaller degree of inter-replicate variability (i.e., compare Figure 6C,D against Figure 4C,D). PCA of the combined data revealed clear separation of the exosome spectra according to cell line (Figure 6E), indicating the potential to reliably classify exosomes based on their Raman spectra when using the Pt-black templates.

As opposed to the commercial SERS-Au substrate, there were only small inter-replicate variations using the Pt-black substrates, and the spectra collected were consistent across replicates (i.e., see Figures 5A and 6A,B). This was attributed to the low background signals exhibited by the Pt-black substrates (Figure S3B, Supporting Information). These low background levels also

Table 2. Random forest algorithm LOOCV using exosome spectral data.

(A)		
	LOOCV	
Sensitivity [%]	83.3	
Specificity [%]	95.8	
PPV [%]	95.2	
NPV [%]	85.1	
Accuracy [%]	89.5	
(B)		
	Actual 4T1	Actual fibroblast
Predicted 4T1	20	1
Predicted fibroblast	4	23

A) LOOCV results presented as sensitivity (%), specificity (%), positive predictive value (PPV) (%), negative predictive value (NPV) (%), and accuracy (%). Here, sensitivity represents the correct positive classification of 4T1 exosomes and specificity represents the correct positive classification of fibroblast exosomes. The normalized spectral intensities at 908 wavenumber locations from 50–1800 cm^{-1} were selected and used as features for learning; B) Confusion matrix of LOOCV results. Data shown were collected across three experimental replicates ($n = 3$) with eight measurements made per replicate, giving a total of 24 measurements per cell type.

meant that the Pt-black templates were able to detect signals from the reagents, which demonstrated similar spectral profiles to the exosomes (Figure S6A,B, Supporting Information). This similarity was most likely caused by reagent residues in the exosome samples (and vice versa). Despite this, PCA confirmed that there were differences between the reagent spectra and the exosome spectra (Figure S6C,D, Supporting Information). Nonetheless, these results demonstrate the potential to further optimize the exosome isolation protocol to reduce interference from reagents.

Importantly, as described above, the Raman measurements performed on both the SERS-Au and Pt-Black substrates were repeated multiple times. Eight measurements were made on each substrate (at different locations) and all experiments were repeated in triplicate (i.e., with exosomes isolated from different cell culture batches and with experiments performed on different SERS substrates and on different days). Thus, the experiments presented here demonstrate the repeatability of the technique over different days and across different substrates (for both the Pt-Black and SERS-Au substrates).

Finally, a leave-one-out cross-validation (LOOCV) analysis based on the random forest algorithm was performed to determine the sensitivity and specificity of Raman spectroscopy using the Pt-black SERS templates for the classification of 4T1 and fibroblast exosomes. The random forest algorithm was chosen for this purpose as it has been applied to Raman data in numerous prior studies (e.g., see ref.[63] and was thus well suited to the proof-of-concept analysis performed here. The results showed an 83.3% sensitivity (i.e., correct positive classification of 4T1 exosomes) and a 95.8% specificity (i.e., correct positive classification of fibroblast exosomes) with an overall accuracy of 89.5% (Table 2A). This corresponded to the correct identification of 20/24 4T1 exosome samples and 23/24 fibroblast exosome samples (Table 2B).

Together, this demonstrates the potential of SERS for exosome-based cancer diagnostics and highlights the importance of the

novel Pt-black templates for this purpose. The combination of low, consistent background signals, low variability across substrates, and modest Raman signal enhancement capabilities means that these substrates are ideally suited to applications in samples with low signal levels and facilitated accurate classification of exosomes in this study.

3. Conclusion

This study reported the development of a novel Pt-black SERS template and its application to exosome classification. Our results show that the low background signals, consistency across substrates, and modest signal enhancement provided by the Pt-black templates allow for accurate identification of exosomes derived from two different cell lines (4T1 breast cancer cells and fibroblast cells). Importantly, accurate and repeatable classification of exosomes was not possible using a commercially available SERS substrate (SERS-Au) due to strong and variable background signals, which effectively swamped the exosome signatures. Thus, this indicates the potential of the novel Pt-black SERS template for the future development of point-of-care cancer diagnostics based on exosome detection. Such a diagnostic tool would also be applicable to other medical and biological applications where the low substrate background signals may be beneficial in investigating samples with low Raman spectral intensities. Future work will involve the assessment of exosomes derived from different types of healthy and cancerous cells (including clinical samples such as urine and blood) to further investigate the diagnostic potential of the Pt-black templates for clinical applications. Further insight into the functionality of the studied materials could also be obtained through simulations, and this will also be investigated in the future. In addition, we plan to explore the use of the proposed template to analyze samples over long periods of time as opposed to a single-use disposable platform for point-of-care applications. Finally, the Pt-black-modified electrodes also have the potential for applications such as cell electroporation, electrical stimulation, and electrical (e.g., bioimpedance) sensing. Combined with Raman measurements, this presents a promising route toward multiparametric sensing and multimodal diagnostic systems and cell analysis platforms.

4. Experimental Section

SERS Substrate Fabrication: To prepare the templates for Pt-black deposition, high-quality polished single-crystal p-type B-doped silicon wafers with a crystallographic orientation of (100) and $625 \pm 25 \mu\text{m}$ thickness (W-SI WAFER 06/007-P) coated with 5 nm Titanium (Ti) and 100 nm gold (Au) were purchased from Inseto Ltd. (Hampshire, UK). After dicing the wafer into 2 cm^2 square-shaped samples, the samples were ultrasonically cleaned in an acetone and isopropyl alcohol (IPA) bath for 15 min followed by rinsing with deionized water (DI) and drying (see Figure S2A, Supporting Information). A CO_2 laser cutter (VLS 6.60, Universal Laser Systems, USA) was then used to pattern a standard well plate sealing film (clear polyester sealing film, E2796-0794, Starlab international GmbH, Germany) into a film that will electrically insulate the back and sides of the wafer and allow only five 4 mm diameter circular openings exposing the Au layer on the silicon substrate (see Figure S2B, Supporting Information).

After laser cutting, the patterned adhesive films were blown with pressurized air to remove any residual debris and were then cleaned with IPA. Prior to the attachment of the sealing film on the wafer pieces (see Figure

S2D, Supporting Information), a wire was attached to the Au using a silver paste (186-3600, RS-PRO, UK) and copper tape and further fixed and insulated with a cyanoacrylate-based adhesive (Loctite super glue, Henkel, UK). Wire attachment is shown in Figure S2C, Supporting Information. Kapton tape was then used around the edges of the samples to further insulate the samples and ensure no areas are exposed to the electrodeposition solution, as shown in Figure S2E, Supporting Information. The completion of the assembly was followed by cleaning with IPA. All the above steps took place in a Class 1000 ISO6 clean room and under a laminar flow hood.

The completed devices were sealed in individual containers and were ready for further processing. These then served as the working electrodes (WE) in a three-electrode electrochemical cell for electrochemical deposition. In brief, the solution used for electrodeposition was composed of 12.5 mL of chloroplatinic acid (8 wt% in H₂O, Sigma-Aldrich Prod. No.: 262587), 2.5 μ L hydrochloric acid (1 M, 1 N, Sigma Aldrich Prod. No.: 1.09057), and 10 mg lead acetate trihydrate (Sigma-Aldrich Prod. No.: 215902), filled with deionized water to reach a total volume of 100 mL. The three-electrode cell was completed with a glass-based Ag/AgCl reference electrode (RE-5B, MF-2052, BASi, USA) and a custom Pt counter electrode (PT199001, 0.25 mm thick, 25 mm $\times$ 25 mm sheet of 99.99% purity Pt, Advent Research Materials, UK) with a surface area much greater than 10 times that of the WE (see Figure S2F, Supporting Information). The WE was held in place during the deposition process with a custom 3D-printed fixture (Figure S2G, Supporting Information). Pt-black deposition on the working electrode was performed for 100 s with a current of 28 mA cm⁻²—that is, a current of 9.35 mA was applied to deposit Pt-black simultaneously on all five circular regions defined by the laser patterned sealing films. The electrochemical cell (shown in Figure S2H–J, Supporting Information) was subjected to ultrasonication during the electrodeposition process to ensure only well-attached nanoparticles remain on the electrode.^[46,62,63] The electrodeposition set-up showing the ultrasonic probe is presented in Figure S2H, Supporting Information.

It was found experimentally that depositing good quality, uniform, and reproducible Pt-black films over the whole 2 cm² wafer pieces was challenging. Reducing the deposition area using the sealing film addressed this issue. After the electrodeposition process was completed, the electrodes were rinsed with DI water and IPA and left to dry at room temperature. Before and after electrodeposition, EIS spectra were recorded, using the same three-electrode cell in PBS (P4417-100TAB PBS tablets dissolved in 200 mL DI water, Sigma-Aldrich, USA) as shown in Figure S2I, Supporting Information. Measurements were obtained using a 50 mV small signal perturbation with a frequency ranging between 10 and 1 MHz with 12 frequencies per decade. Electrodeposition and EIS were performed using a CHI760E Electrochemical Workstation (CH Instruments, Austin, TX, USA).

To assess the SERS performance of the Pt-black templates, Raman measurements were made using both the novel Pt-black templates and commercial Au paper-based SERS substrates (RAM-SERS-AU SERS Substrate, Ocean Insight, The Netherlands – herein referred to as SERS-Au substrates). Standard silicon wafers (W-SI WAFER 06/007-P) were used to measure unenhanced Raman signals.

Nanostructured Pt-Black Template Material Characterization: SEM images were collected using a Lyra XM SEM system (Tescan, Czech Republic) at several magnifications to observe the surface topography of the Pt-black coatings. EDX spectroscopy analysis and mapping were achieved on the same SEM using a Quantax analyzer (Bruker, MA, USA). Contact angle measurements were obtained by collecting images using a VHX-1000 digital microscope (Keyence, Japan) and were analyzed using the contact angle measurement plugin (by M. Brugnara) in ImageJ (Rasband, W.S., U. S. National Institutes of Health, Bethesda, Maryland, USA).

Characterization of Substrates Using Raman Spectroscopy: The Raman spectra in this study were all acquired using a DXR3xi Raman Imaging Microscope (ThermoFisher Scientific, USA). Both 785 and 532 nm wavelength lasers were used in sample characterization and data acquisition using a 10 $\times$ objective lens. SERS-Au substrates were designed to provide optimal signal enhancement with 785 nm excitation, whereas preliminary experiments showed that the Pt-black template exhibited the strongest en-

hancement with 532 nm excitation. Thus, all measurements presented for the two SERS substrates were performed at their respective optimal excitation wavelengths (532 nm for Pt-black; 785 nm for SERS-Au). The detection limits and enhancement factors of the SERS substrates were determined using CV (C0775, Sigma-Aldrich, UK) dye, which is commonly used as a Raman reporter due to its large Raman cross-section with a well-established spectrum.^[66] The CV dye was dissolved in deionized water and samples were prepared over a concentration range of 2.5 nM–20 mM. Prior to Raman measurement, a single droplet of 10–15 μ L volume was placed on the relevant substrate (Pt-black template, SERS-Au substrate, or silicon wafer) using a pipette and allowed to dry at room temperature. For the three substrates used, Raman spectra were acquired at five different regions within the sample using the acquisition parameters shown in Table 1. All acquired Raman spectra were repeated in triplicate and then averaged.

Dynamic Light Scattering Measurements: The average size distributions of the isolated exosomes were determined using a Zetasizer advance range (Malvern, Worcestershire, UK). Exosomes isolated from fibroblast and 4T1 cell culture media were diluted (1:10 v/v) in PBS separately. Prior to the measurement, 1 mL of diluted exosomes was added to a cuvette and ten scattering measurements were recorded.

Tissue Culture: 3T3-L1 fibroblast (CL-173) and 4T1 breast cancer (CRL-2539) cell lines were purchased from LGC Ltd. (European partner of ATCC). Cells were cultured in no phenol red culture media, Dulbecco's Modified Eagle medium (DMEM) (Gibco Cat No.: 31053044), and RPMI 1640 Medium (ATCC modification) (Gibco Cat No.: A1049101), respectively, both supplemented with 10% exosome-depleted fetal bovine serum (FBS) (Gibco Cat No.: A2720803), 2 mM L-glutamine (Gibco Cat No.: A2916801), and 1% penicillin-streptomycin (Sigma-Aldrich Cat No.: P4333). Both cell lines were cultured at 37 $^{\circ}$ C, with 5% CO₂, in a humidified incubator.

Exosome Isolation: Tissue culture media were collected when cells were 70% confluent. Media were centrifuged for 30 min at 2000 $\times$ g to remove debris and cells. Total exosome isolation reagent (Thermo Fisher Cat No.: 4478359) was added to the supernatant at a ratio of 1 to 2 and refrigerated at 4 $^{\circ}$ C overnight according to the supplier's instructions. To pellet down exosomes, the sample was centrifuged at 10 000 $\times$ g at 4 $^{\circ}$ C for 1 h. The supernatant was removed, and the exosome pellet was resuspended in 100 μ L sterile 1 $\times$ PBS and stored at 4 $^{\circ}$ C.

SERS Characterization of Exosomes: To characterize exosomes using SERS, 15 μ L of exosome sample was pipetted onto the center of the substrate and air dried. Spectra of exosomes on Pt-black templates were collected using 532 nm excitation, 0.5 mW laser power, and 0.2 s acquisition time. Spectra of exosomes on SERS-Au substrates were collected using 785 nm excitation, 0.5 mW laser power, and 0.5 s acquisition time. For both substrates, spectra were averaged over ten scans in all cases. Eight spectra were collected per sample (at different regions of the substrate) to account for potential exosome concentration variation across the substrate. Three experimental replicates were performed (on different days) for both SERS substrates (using different substrates and with exosomes extracted from different cell culture batches in each case).

Pre-Processing of Spectra: Exosome spectra were trimmed to a range of 50–1800 cm⁻¹ and 400–1800 cm⁻¹ for measurements on Pt-black templates and SERS-Au substrates, respectively (as these regions contained the most prominent spectral peaks). Substrate spectra, collected using the same acquisition parameter as when the analyte was present, were subtracted from the analyte spectra. Spectra were then baseline corrected by fitting a tenth-order polynomial to the data. For exosome measurements, spectra were also normalized to the mean intensity to eliminate systemic differences between measurements for better comparison between exosome types. All pre-processing steps were performed using RStudio (version 4.1.3).

Statistical Analysis: All statistical analyses were performed using RStudio. The average spectra of individual replicates were presented as means $\pm$ standard deviation (SD). The average spectra of combined data were presented as means $\pm$ standard error on the mean (SE).

Multivariate statistical analyses—including PCA and LOOCV analysis using the random forest algorithm—were performed using the

pre-processed spectral data. For both analyses, 727 variables were selected between Raman shift 400–1800 cm^{-1} for measurements made on SERS-Au substrates while 908 variables were selected between 50–1800 cm^{-1} for Pt-black templates. PCA reduced the dimensions of the dataset and identified features that accounted for the greatest variations between sample groups. The two PCs that exhibited the greatest percentage of explained variance were plotted against one another (i.e., PC1 vs PC2) to investigate whether the exosome spectra were separable using PCA. The boundaries of exosomes derived from 4T1 cells or fibroblasts were presented as 95% confidence ellipses. LOOCV was performed using the random forest algorithm to provide a supervised classification of exosomes derived from different cell lines.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

X.L. and M.K. contributed equally to this work. X.L. contributed to the methodology, investigation, data analysis, data curation, visualization, and writing. M.K. contributed to the conceptualization, methodology, investigation, data curation, visualization, student supervision, and writing. P.K. contributed to the conceptualization, methodology, investigation, data analysis, data curation, and writing. Z.K. contributed to the methodology, investigation, validation experiments, and data analysis. A.R. contributed to the validation experiments, data analysis. E.Y. contributed to the project administration, funding, and writing. A.J.T. contributed to the conceptualization, methodology, data analysis, student supervision, project administration, and writing.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

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