Machine Learning Approaches in Label-Free Small Extracellular Vesicles Analysis with Surface-Enhanced Raman Scattering (SERS) for Cancer Diagnostics

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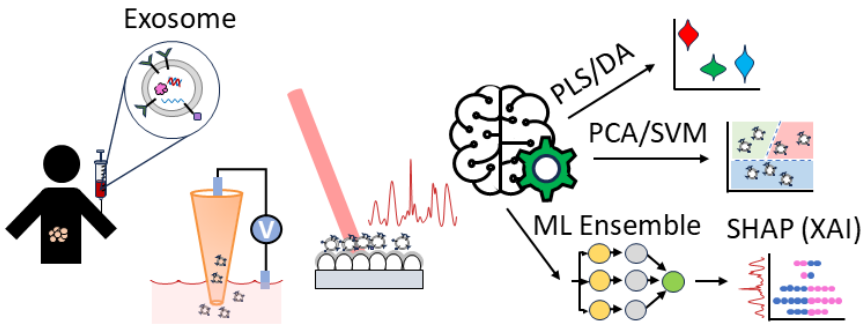
Abstract

Early diagnosis remains of pivotal importance in reducing patient morbidity and mortality in cancer. To this end, liquid biopsy is emerging as a tool to perform broad cancer screenings. Small extracellular vesicles (sEVs) found in bodily fluids can serve as important cancer biomarkers in these screenings. Our group has recently developed a nanopipette dielectrophoretic approach to extract sEVs from blood. Herein, we demonstrate the feasibility to integrate this approach with surface-enhanced Raman scattering (SERS) analysis. SERS can be used to characterized extracted exosomes through their vibrational fingerprint that changes depending on the origin of sEVs. While these changes are not easily identified in spectra, they can be modeled with machine learning (ML) approaches. Common ML approaches in the field of spectral analysis use dimensionality reduction method that often function as a black box. To avoid this pitfall, we used Shapley additive explanations (SHAP) is a type of explainable AI (XAI) that bridges ML models and human comprehension by calculating the specific contribution of individual features to a model's predictions, directly correlating model/decisions with the original data. Using these approaches we demonstrated a proof-of-concept model predictive of cancer from isolated exosomes, integrating nanopipette dielectrophoresis and SERS. This work demonstrates the use of explainable AI to perform diagnostic analysis on complex SERS data of clinical samples, while reporting interpretable biochemical information.



Introduction

Early diagnosis remains of pivotal importance in reducing patient morbidity and mortality in cancer.[1,2] Current practice involves testing with relatively invasive and costly methods only after the onset of symptoms and is further confirmed with pathology analysis of tissue biopsy, the current gold standard for cancer diagnosis.[3-5] A big issue with this diagnostic paradigm is that it is reactive rather than proactive, which causes diagnosis to arrive after the disease has progressed reducing treatment-positive outcomes. Conversely, non-invasive cancer screening is emerging as a powerful option to provide beneficial early diagnosis, as demonstrated by the popularity of protocols such as Cologuard (limited to colon cancer).[6] Thereby, there is a strong need for non-invasive methods to detect and monitor cancer.

Liquid biopsy is a non-invasive alternative to tissue biopsy to perform cancer screening.[7-10] Liquid biopsies consist of the analysis of various bodily fluids (e.g., blood, plasma, saliva, or urine samples). Bodily fluids can be collected non-invasively and contain tumor-related biomarkers. Among those biomarkers, small extracellular vesicles (sEVs), known as exosomes (i.e., nanovesicles of 30-200 nm secreted by various cell types), can be found in bodily liquids. Recently research has shown tumor-derived exosomes carry tumor-specific biomolecules, including RNA, DNA, lipids, and protein from their parent cells.[11] Because of their biochemical relation with the parent cell, exosomes have been proposed as a promising biomarker for early diagnosis and prognosis prediction for patients, thus, promoting the chances of successful and curated treatments.[12-14] These sEVs, exosomes, can be detected and quantified via Western blots, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), and sequencing techniques.[15] However, these techniques require prior knowledge of specific markers to target and can be time-consuming.

Microfluidic devices based on electrokinetics represent a category of tools designed to isolate sEVs using external electrical forces, eliminating the need for tagging or labeling specific molecules of interest.[16,17] The dielectrophoresis (DEP) phenomenon, harnessed in these devices, enables precise isolation based on the dielectric properties and size distribution of sEVs under a non-uniform electric field, offering a rapid and label-free approach. This non-uniform electric field can be generated through the application of an alternating current across an array of electrodes[18,19] or by employing an insulator-based dielectrophoretic (iDEP) strategy using obstacles such as micro-pillars in microfluidic channels.[20] The iDEP approach effectively manipulates biomolecules while maintaining device functionality, despite potential fouling effects on electrode surfaces.[21] However, the majority of existing devices in this category necessitate high operational voltage (around ~100 V/cm), posing a risk of denaturing the molecules of interest.[22] Additionally, despite their promising attributes, these devices come with inherent challenges, including high fabrication costs, the requirement for sample dilution, and the susceptibility of micro- and nano-scale channels to clogging.[23]

Our group has introduced a novel class of iDEP devices designed for the rapid and selective entrapment of nanoparticles based on their size and distinctive dielectric properties. This device, featuring an array of micropipettes, can isolate nanoparticles from small sample volumes by applying a significantly low electric field (~10 V/cm) along the

length of the pipettes.[24] The minute conical pore geometry of the micropipettes enables the isolation of sEVs without the need for sample dilution, allowing downstream analyses with uncompromised yield. Previous demonstrations have showcased the capability of our iDEP device in isolating sEVs from small sample volumes (~200 μL) of conditioned cell culture media and biofluids from healthy donors within a mere 20 minutes.[25,26]

The next step is the characterization of sEVs. sEVs from different cells can have very similar biomolecular information (e.g., protein receptors), making targeting and distinguishing cancer-related sEVs markers a challenge.[27] Using sEVs for clinical diagnostics remains a challenge, and there is a need for a more robust classification of exosome subpopulations, and exosome isolation methods.[14] Raman spectroscopy is a powerful spectroscopic technique that permits the identification of molecules through their vibrational fingerprint. These features can be used for diagnostic purposes by collecting biochemical information from different exosomes. Raman spectra are characterized by low signals making it challenging to detect significant signals from analytes at ultralow concentrations, such as exosomes. To overcome this limitation, the use of plasmonic metal nanomaterial or nanostructures can amplify weak Raman signals via surface-enhanced Raman scattering (SERS). In recent years, SERS has been shown to be able to identify exosome origins and other EVs, by incorporating multivariate analysis and machine learning algorithms as classification tools.[28-36] These tools can extract small variations in the SERS spectral profile and differentiate the exosomes.

Machine learning (ML) techniques have emerged -as a transformative tool in the field of spectral analysis, where high dimensional datasets composed of hundreds of peak intensities are simplified in a meaningful way to extract patterns that enable rapid and accurate classification.[37-39] For cancer detection, a model can be built from numerous samples of both healthy and diseased patients to learn the essential relationships between a collection of peaks that can then be applied to samples of new patients for diagnostics.[40,41] Therefore, the ability of SERS to examine exosomes coupled with ML can reduce the complexity found within many spectra and can be used for the effective classification of healthy and cancerous exosomes.[41-43]

Building an ML classification model, however, can be a challenging endeavor, and it often yields misleading or inaccurate results when not meticulously tuned. One of the challenges lies in selecting the appropriate algorithm for the specific dataset, as well as balancing the model's complexity to prevent overfitting or underfitting.[37] Preprocessing steps with dimensionality reduction techniques must be carefully executed to avoid removing critical information needed for proper generalization of the model. Furthermore, failure to optimize hyperparameters, such as learning rate or tree depth, is essential for reducing the bias in a model's predictive ability. Ignoring these aspects can result in a model that appears to perform well during training but fails at generalizing the differences between samples, which can lead to misguided diagnoses for patients.

While well-tuned ML models have shown remarkable capabilities in making accurate predictions, their inherent complexity often makes it challenging to understand the reasoning behind their decisions. The most common ML applied to SERS data is dimensional reduction methods, such as partial least squares (PLS) and principal

component analysis (PCA), which do not offer a clear explanation for the classification.[44] This lack of transparency, commonly referred to as the “black box” issue of ML algorithms, is a major drawback especially with the high consequences of these tools misdiagnosing patients in healthcare applications. Shapley Additive exPlanations (SHAP) is a type of explainable AI (XAI) that bridges ML models and human comprehension by calculating the contribution of individual features to a model’s predictions.[45] SHAP is based on cooperative game theory which allows for more consistent weight assignments while also providing both local and global interpretability. This approach can be key in information-rich SERS analysis by offering a way to gain chemical insight into the classification process and link the classification to the biochemical hypothesis.

Herein, we surveyed various SERS-substrate designs to select the substrate with the highest plasmonic properties for exosome analysis. We performed extensive tuning of multiple models under two main approaches: initial transformation of the data set using dimensionality reduction tools and feature removal after model tuning to reduce the number of dimensions included in the final classification analysis (Figure 1). This present work provides useful information on how to apply and access learning models with SHAP. With SHAP, we extrapolated and identified what Raman bands significantly influenced the classification model. We then applied the optimized classification algorithm approach on SERS spectra collected from exosomes isolated from a kidney cancer patient’s blood via nanopipette dielectrophoresis. These results demonstrate a proof-of-concept model predictive of cancer from isolated exosomes. We also first demonstrate the integration of two emerging techniques for non-invasive diagnostics (i.e., nanopipette dielectrophoresis and SERS) that can potentially be used in stand-alone devices. As a further demonstration of the feasibility of this integration, we also demonstrated our predictive model using substrates fabricated with colloidal nanoparticles, easily integrated in pre-existing devices. Importantly, our work demonstrates the use of explainable AI to perform diagnostic analysis on complex SERS data of clinical samples. This approach enabled two key advances: -1- optimization of the classification model based on its correlation with specific Raman bands and not just on diagnostic accuracy and -2- classification of patients’ exosomes based on diagnosis while reporting interpretable biochemical information directly correlated to this model.

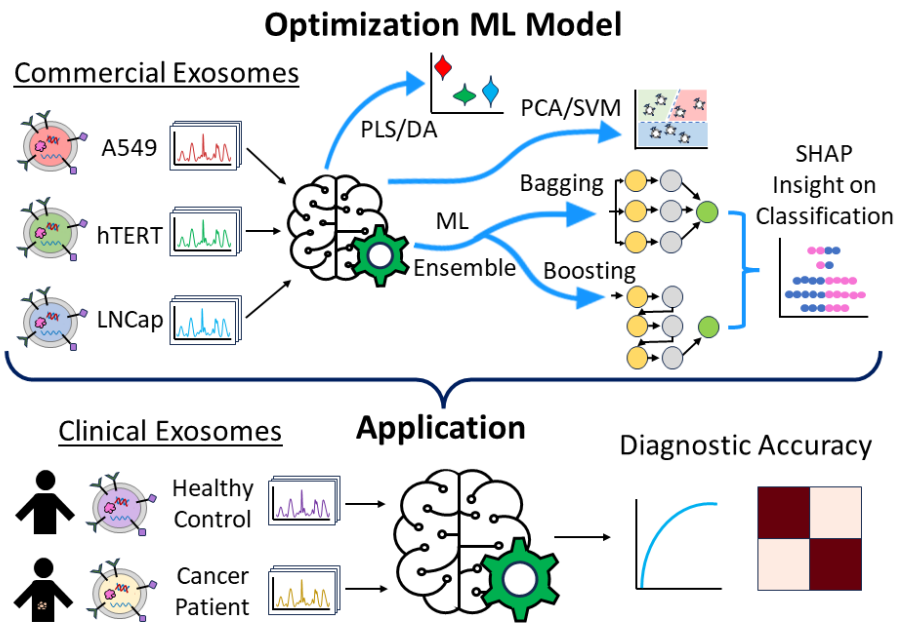


Figure 1. Schematic workflow of machine learning approach of exosome analysis for cancer diagnosis.

Experimental

Materials and chemicals

Hydrogen peroxide (H_2O_2), gold(III) chloride trihydrate ($HAuCl_4$), silver nitrate ($AgNO_3$), sodium citrate tribasic dihydrate (Na_3Cit), L(+)-ascorbic acid (AA), benzoic acid (4-MBA), and (3-aminopropyl)triethoxysilane 99% (APTES), Liposome Kit: Lipid mixture for the preparation of liposomes were purchased from Sigma-Aldrich. Ammonium hydroxide (NH_4OH) and 1N hydrochloric acid (HCl) were purchased from Fisher Scientific. Ethanol (EtOH) was purchased from Decon Labs, Inc. Phosphate-buffered saline (PBS, 10X) ultrapure was purchased from Thermo Fisher. These chemicals were used without further purification. Millipore Synergy UV-R Ultrapure (Type 1) water (DI H_2O) of resistivity = 18.2 MΩ was used in all aqueous solutions. Aqua regia solution was used to clean the stir bars. Piranha solution or RCA1 and 1N HCl was used to clean the glass and silicon slides. The cleaned slides were stored in EtOH until further use. All syntheses were performed in 20 mL disposable plastic scintillation vials purchased from Fisher Scientific. No further purification was done to chemicals before use. Silica microparticles (0.6 μm) were purchased from Bangs Laboratories, Inc. In 1 mL portions, the microparticles were centrifuged (4000xg, 10 min, 4°C) and washed 3 times with EtOH. The final pellet was redispersed in fresh EtOH to prepare a 7w/v% particle solution for use. Commercial cell-line exosomes were purchased at ATCC (i.e., A549 exosomes (CCL-185-EXM), LNCap exosomes (CRL-1740-EXM), and hTERT MSC exosomes (SCRC-4000-EXM). Commercial gold and silver SERS substrates were purchased from (Silmeco).

Small extracellular vesicles isolation from clinical samples

sEVs were efficiently isolated from biofluids without the need for pre-purification steps. Each device, comprising eight chips, accommodates eight micropipettes for high-throughput sEV extraction, allowing the extraction of 400 µL biofluid in approximately 20 minutes. The micropipettes were backfilled with 1X PBS buffer using a 33-gauge Hamilton syringe needle and positioned on the PMMA substrate. To initiate the process, the tip side of each micropipette was injected with 50 µL of biofluid, while the base side chamber received 1X PBS. Subsequently, sEVs from the biofluids were captured at the tip by applying a 10 V/cm direct current (DC) for 10 minutes, followed by release in 25 µL 1X PBS through the reversal of the applied voltage for another 10 minutes. To enhance efficiency, 1 mL biofluids underwent simultaneous processing using three iDEP devices, with parallel aliquots employed for purifying sEVs in 500 µL PBS. The isolated sEVs were stored at -80°C for subsequent analysis.

Table 1. The clinical sEVs, also known as exosomes, used in this work.

Subject/ Sample ID	Sample Type	Gender/ Age at collection	Race	Primary Diagnosis	Histological Subtype	Source
URS400/ URS400ST1SE1	Serum	Female/ 43 yrs	Black or African American	Kidney Cancer	Clear Cell Papillary Renal Cell Carcinoma	University of Cincinnati Biorepository
ISERS10 ML 37363-04/ ISERS10 ML 37363-04	Serum	-	-	Healthy Donor	Singal Donor Human serum of the Clot	Innovative Research

The study was carried out in accordance with the principles of the Declaration of Helsinki under an approved protocol of the institutional review board of the University of Cincinnati (IRB# 2015-2364). Written informed consent was obtained and documented for all patients; samples were deidentified for patient confidentiality. Two of the urine samples were from the same patient. sEVs harvested from hTERT-immortalized mesenchymal stem cells and A549 NSCLC cell lines were purchased from ATCC Inc. For the rest of the paper, the kidney cancer sample will be denoted as a cancer patient and the healthy donor as a healthy control.

Gold seeds synthesis

Gold citrate-capped nanoparticles (12 nm, 0.1 nM) were synthesized via a modified Turkevich method.[46] In brief, 100 mL of deionized (DI) water and 200 µL of 0.5 M $HAuCl_4$ were vigorously stirred and brought to a boil for 10 s. Afterwards, 15 mL of 1% Na_3Cit was added to the boiling solution. The solution continued to boil for 15–30 min, while the total volume was maintained. Afterward, the resulting gold citrate-capped nanoparticle (AuNPs) solution was cooled to room temperature (r. t.), filtered through a 0.22 µm

nitrocellulose membrane, and stored at 4°C until ready for use. Extinction profile and transmission electron microscopy (TEM) images can be found in Figures S1 and S2.

Nanostars synthesis

The silver-coated gold nanostars were synthesized following a modified Vo-Dinh group synthesis.[47] Briefly, a plastic scintillation vial of 10 mL of DI H_2O was stirred (700 rpm, r. t.), then 10 µL 1 N HCl, followed by 493 µL of 5.08 mM $HAuCl_4$ were added to the vial. After 5 seconds, 100 µL of 0.1 nM (12 nm) AuNPs were added. After 10 seconds, $AgNO_3$ (50 uL) of various concentrations (2 or 3 mM; samples named S10, and S15 relating to the $AgNO_3$ final concentration, 10 µM and 15 µM, respectively) quickly followed by 50 µL of 0.1 M AA. After this step, gold nanostars (AuNS) were synthesized and ready for immediate use. For silver-coated gold nanostars (AuNS-Ag), additional steps are required. After the addition of 0.1 M AA, the solution was allowed to stir for 30 s. Lastly, 0.1 M $AgNO_3$ (20, 50, or 100 µL; samples named Ag2, Ag5, or Ag10 relating to the various final concentrations, 0.20, 0.50, and 1.0 mM, respectively) was added, immediately followed by 10 µL of NH_4OH. The resulting NS solution continued to stir for 60-90 s to ensure the silver coating stabilized the gold nanostars. The final solution was transferred and incubated at room temperature for 2 h, idle.

The aged AuNS-Ag were centrifuged in ~5.5 mL portions (1500 xg, 15 min, 4°C) and concentrated by 10 times. The 10X concentrated s AuNS-Ag were stored at 4°C until ready for use. The AuNS and AuNS-Ag nanoparticles were characterized via UV-vis spectroscopy and transmission electron microscopy (TEM) (Figure S1-S3).

Substrate fabrication

To prepare solid-state SERS substrates, microscope glass slides were used as the base. First, the microscope slides were cut to 8 x 25 mm. First, the slides were cleaned in base, $NH_4OH/H_2O_2/H_2O$ (1:1:5, RCA1), for 1 h with gently swirling every 15 min. Then RCA1 solution was replaced with 1 N HCl and soaked for another hour. Lastly, the slides were washed three times with ultrapure water (18 MΩ), once in fresh EtOH, and stored in fresh EtOH until use. The clean glass slides were removed and allowed to air dry. Immediately after drying, 10 µL of washed 7w/v% silica microparticle in EtOH solution was dispensed onto the surface. The cleaned slide was rotated to form a silica coating on the surface. Afterward, a layer of silver was deposited on top of the silica via the Denton Discovery 24 Sputtering System at a base pressure of $1e^{7}$ to $6e^{-8}$ Torr, deposition pressure 5.38 – 5.32 mTorr, and deposition speed of 10 Å/s. A Crystal sensor was used to measure the silver thickness. Three different silver film thicknesses were tested (i.e., 50, 125, and 200 nm). The metallic film over nanospheres (MeFON) was characterized via SEM and reflectance spectroscopy (Figures S4 and S5).

Silicon wafers were used as the base for preparing colloidal nanoparticles (i.e., AuNPs, S15, S10-Ag2, S10-Ag5, and S10-Ag10) SERS substrates. First, the silicon wafers were cut to 4 x 8 mm. The slides were cleaned with acid HCl/H_2O_2 (4:1, piranha) for 1 h, with gentle swirls every 15 min. Then, the slides were washed three times with ultrapure water

(18 MΩ), and 3 times with fresh EtOH, and stored in fresh EtOH until use. To attach the colloidal nanoparticles to the silicon surface, we applied 2 different methods: (1) drop cast and (2) incubation. For drop cast, first, the cleaned, cut silicon wafers were incubated in fresh 10% APTES at 70°C for 1h. Afterward, the APTES functionalized silicon was rinsed in with EtOH, DI H_2O, then EtOH. The washed substrate was dried at room temperature (10 min). Then, 50 µL of the 10X concentrated nanoparticles were dispensed on top of the APTES functionalized silicon. The nanoparticles were dried onto the surface overnight before use. For the incubation method, the cleaned glass was incubated in fresh 10% APTES under the same conditions as the previous method. Afterward, the APTES fictionalized silicon wafer was rinsed with EtOH, DI H_2O, EtOH, and then H_2O. The rinsed slide was placed in a clean microcentrifuge tube with 10X concentrated nanostars and incubated in the nanostars solution for 2 h. Lastly, the resulting nanoparticles silicon substrates were quickly rinsed with DI H_2O, and dried at r. t., and ready for use. Colloidal nanoparticles SERS substrates were characterized via scanning electron microscopy (SEM) imaging (Figure S2 and S3). Commercial gold (Au) and silver (Ag) substrates were purchased and used to compare with the lab-made substrates (Figure S6).

SERS measurements

The commercial cell-line exosomes and clinical exosomes' concentrations and sizes were evaluated via nanoparticle tracking analysis (Figure S7). From this, 30 µL of 10^9 particles/mL of exosomes in 1X PBS was dispensed on SERS substrate and allowed to dry overnight, r. t. The Raman and SERS spectra were obtained using a lab-built Raman microscope. Inverted dried samples were tested on a 50 mW 633 nm laser (PD-LD) at 20x objective in a Nikon Eclipse Ti Raman microscope with MaxLine, RazorEdge Dichroic, EdgeBasic; Semrock filters, and a Princeton Instruments Pixis100 CCD coupled with Isoplane160. Data were collected via LightField software under 10-second exposures, 25 acquisitions, and a minimum of 50 spectra per substrate in a 0.25π mm^2 area at 5mW power. Commercial exosomes from ATCC were tested on 4 substrates per exosome type. Clinically isolated exosomes were tested on 6 substrates per exosome type. For the colloidal substrate, cell-line exosomes and clinically isolated exosomes were tested on 2 substrates per exosome type.

The SERS spectra obtained from the solid-state substrates range contained 359-2767 cm^{-1} with 1024 pixels. Next, the spectra were smoothed and background-subtracted using a Savitzky–Golay filter, with a 40-point window and first-order polynomial, on MATLAB 2021b, followed by normalized by dividing it for its integral (area under the curve of all Raman peaks) to avoid bias due to concentration differences. For the SERS spectra obtained by the colloidal-state substrates, the spectra range from 550-2767 cm^{-1} with 950 pixels. Smoothing, background removal, and normalization were performed the same way.

Spectral classification using predictive and explainable machine learning

The characterization of cell-line commercial exosome spectra using the solid-state SERS substrate was refined through a series of iterative tests, involving dimensionality reduction techniques and machine learning (ML) classifiers (python version 3.9.10). An Nvidia GeForce GTX 1060 GPU was used for analysis. The ultimate goal was to assess the performance of the final model in distinguishing between clinically isolated spectra of healthy and cancerous exosomes. Initially, Partial Least Squares Discriminant Analysis (PLS-DA) was conducted, determining the optimal number of PLS components through 10-fold cross-validation runs to minimize the mean squared error (scikit package version 1.3). Following dimensionality reduction, multiclassification was carried out using both linear (LDA) and quadratic (QDA) discriminant analysis. Additionally, Principal Component Analysis with Support Vector Machines (PCA/SVM) was employed to reduce the dimensionality of the dataset, which contained intensities of 984 pixels between 408-2720 cm^{-1}, based on explained variance ratios. Finally, linear, cubic, and radial basis function kernels for SVM were applied to the reduced data set to compare classification performances.

In addition to the initial data transformation using PLS or PCA, the reduction of the high-dimensional dataset was also achieved through a process of eliminating peaks that were determined to be unnecessary for various trained classifiers (PyCaret package version 3.0.4). A comprehensive evaluation of 15 classifiers (Table S1) was carried out using a stratified 10-fold cross-validation approach. The scoring metrics collected include model accuracy, AUC, precision, recall, and F1-score. Taking the top 5 performing classifiers, based on the area under the curve (AUC), hyperparameter tuning was executed through 100 iterations, resulting in a total of 1,000 runs per classifier using RandomGridSearch, again basing optimization on the AUC. The best models after hyperparameterization were then selected to compute feature importance using SHapley Additive exPlanations (SHAP). Subsequently, the top 5% of pixels/features determined by each classifier were retained and employed for the best classifier(s) to identify the pixels that are most characteristic of changes between classes. Here, we implemented a typical 70/30 train/test split. The most successful model(s) were then applied to clinically isolated exosome spectra to test the reliability of the prediction based on commercial cell-line samples for the clinical samples, and the interpretation of results was completed through SHAP beeswarm plots. This procedure was iteratively executed for the dataset utilizing solid-state and colloidal-state SERS substrates.

Results and Discussion

Our long-term goal is to integrate SERS detection and exosome extraction in a single device. In this work, we optimize ML methods for SERS classification of exosomes. We performed the optimization on commercial cell-line exosomes and tested the methods on clinical exosome samples extracted via nanopipette dielectrophoresis. Both initial optimization and testing on clinical samples were performed using solid-state fabricated SERS substrates. These substrates (i.e., metallic film over nanospheres or MeFON) have

superior SERS signal amplification and were used for initial optimization. However, these substrates are not easily integrated into a device. To this end, we then used the optimized ML methods to test the exosome samples on the colloidal-state fabricated substrate (i.e., immobilized nanostars) demonstrating their efficacy. For both solid- and colloidal-state fabrication, we analyzed a series of substrate options to select the substrate with the highest signal, which was best suited for exosome classification (Figure S8 and S9).

SERS classification of cell-line derived exosomes

As test data for this analysis of classification methods, we tested different cell-line derived exosomes and controls. We collected SERS spectra on MeFON substrates (Figure 2A). To avoid concentration bias in the SERS spectra, the spectra were normalized by dividing them by their integral (area under the curve of all Raman peaks). In Figure S10, we show the spectra of exosomes compared to liposomes and PBS controls, which show observable differences. However, when visually comparing the exosome spectra, there are only subtle variations (Figure 2B). The Raman Shift at 1000, 1030, 1200, and 1700 cm^{-1} are lower or higher normalized intensity for LNCap compared to A549 and hTERT. A549 and hTERT averaged spectra show slight differences at 1540 cm^{-1}, where the signal is higher and more present in A549, and at 1320 cm^{-1}, where a small signal can be observed in hTERT and not in A549. To classify these spectra, we tested several ML methods to extract the variance in these exosome spectra and accurately classify the cell-line commercial exosomes. The goal of this study was to identify an ML method ideal for cancer diagnostics, defined as a method with high accuracy while conserving testable chemical insight.

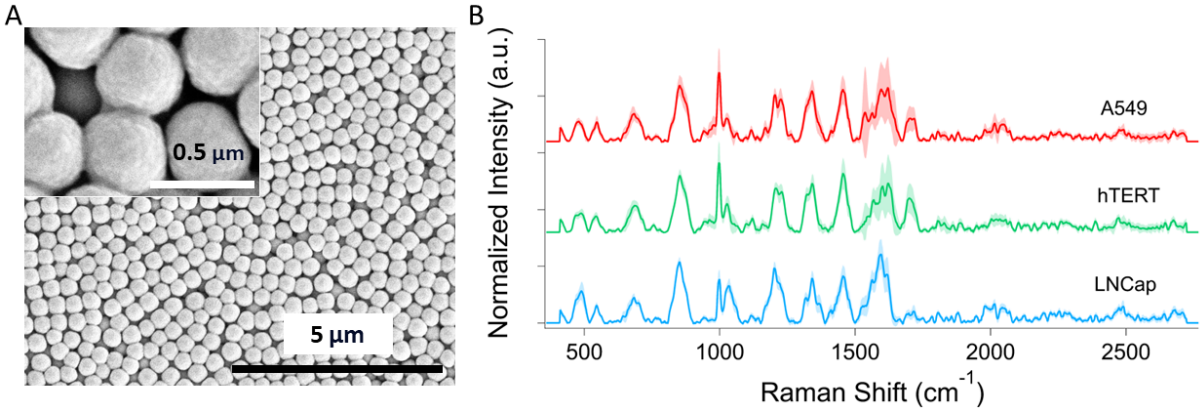


Figure 2. Exosome detection via label-free SERS on solid-state fabricated substrates. (a) SEM images of the SERS solid-state substrate metallic film over nanospheres (MeFON). (B) Averaged normalized SERS signals of commercial cell-line exosomes of A549 (red, top), hTERT (green, middle), and LNCap (blue, bottom).

For the initial benchmark classification, we use commonly adopted dimensionality reduction methods. We applied partial least squares (PLS) to the commercial cell-line

exosome spectral data, which included A549, hTERT, and LNCap samples measured using MeFON. The determination of the optimal number of components for reduction was based on minimizing the mean squared error (MSE) across 10 stratified cross-validation runs, testing a range from 1 to 40 components. The lowest MSE (0.097) was achieved with 9 components, resulting in an acceptable R^2 value of 0.849 (Figure S11). A visual examination of the first two components revealed a clear differentiation among the three classes, with a slight division observed with hTERT (Figure S11). We applied linear (LDA) and quadratic (QDA) discriminant analysis to distinguish between the three classes using the reduced 9-dimensional space. Notably, LDA outperformed QDA across all evaluation metrics (Table S2), achieving a validation set accuracy of 99.6%, while QDA exhibited a significantly lower accuracy of 36.3% (Table S2). The classification results from LDA were summarized as a confusion matrix (Figure 3). The model was able to predict each exosome accurately.

We employed a similar reduction method, principal component analysis (PCA), on the cell-line exosome data to explore an alternative transformation of the original dataset. By retaining the first 20 components, we could explain less than 60% of the total variance present in the original dataset. Visualizing the first two components (PC1: 18.2%, PC2: 12.9% variance explained), we observed clustering of data points within each class, as depicted in (Figure S12). However, there was a small cluster with overlap between A549 and hTERT, which was expected given the similarity between the average spectra and their known protein expression of CD63 when compared to LNCap. To leverage as much variance as possible, we applied Support Vector Machines (SVM) with various kernels that enabled modeling in higher-dimensional spaces using the first 20 PC components. We found that the radial basis function kernel outperformed the polynomial and linear kernels, although all kernels produced scores above 90% (Table S3), and the confusion matrix summarized the classification results in Figure 3. There was a 6% misclassification for A549 exosomes and no misclassification for hTERT and LNCap exosomes from the validation set.

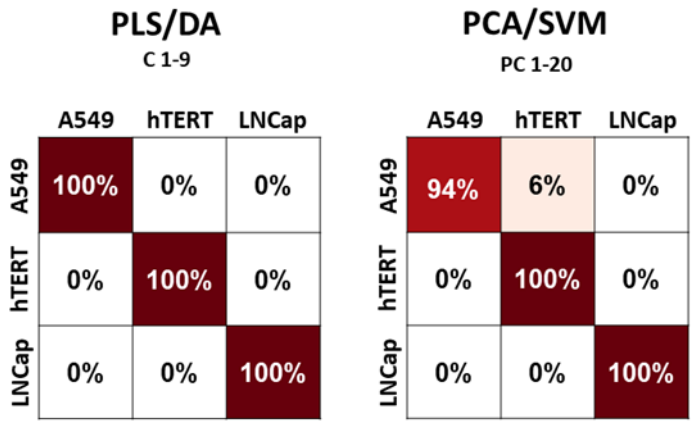


Figure 3. Confusion Matrix from PLS/DA and PCA/SVM classifications on commercial cell-line exosomes, A549, hTERT, and LNCap. PLS/DA shows a perfect classification among the

exosomes. PCA/SVM demonstrates 6% misclassification of A549 with hTERT, but overall high performance.

In summary, our analysis revealed PLS/LDA to be the most effective model for dimensionality reduction of the commercial cell-line spectra data. PCA/SVM with the radial basis function kernel showed successful application to the cell-line and clinical data. However, it's worth mentioning that the PCA/SVM approach could only account for approximately 60% of the data's total variance when using 20 components, which perpetuates the concerns about this method's application on future data.

Explainable ML algorithms comparison for exosome classification

While PCA and PLS showed high accuracy in exosome classification, the initial dimensionality reduction performed strongly reduces the interpretability of the classification model. To bolster our confidence in building a versatile model capable of classifying both the cell-line and clinical spectra, we transitioned from the initial dimensionality reduction approach to utilizing the normalized peak intensities in our classification (i.e., model applied to the Raman data with XAI). In this context, our objective was still to avoid the risk of overfitting a model with more features than observations. Additionally, our final goal is to produce a model for cancer diagnostics. Conserving interpretable Raman chemical information could be key in the clinical translation of this method, by offering a testable hypothesis for the classification model.

In this approach, we adopted a strategy of feature selection by identifying and eliminating features that were deemed nonessential for distinguishing healthy from cancer spectra. To determine the significance of each pixel or feature, we harnessed the power of SHAP, an XAI algorithm grounded in game theory that assigns weights to features based on their contributions to the model's classification. Through this process, we fit a finalized model that exclusively incorporated the most crucial features, those that had the greatest influence in discerning between the various spectra.

Our initial step involved conducting multiclass classification using a diverse set of 15 classifiers (Table S1). Here, we implemented a stratified k-fold cross-validation approach with a value of 10 and collected various metric scores. After rigorous evaluation, we identified the 5 top-performing classifiers, which included Light Gradient Boosting Machine (LGBM), Extra Trees (ET), CatBoost (CAT), Random Forest (RF), and Gradient Boosting (GB) based on their excellent average accuracy, AUC, recall, precision, and F1-score.

It is worth noting that each of these classifiers possesses a range of parameters whose optimal settings can heavily depend on the nature of the input data. To account for this variability, we conducted a thorough RandomGridSearch optimization process for the top 5 algorithms. This involved running RandomGridSearch for each algorithm 100 times, considering 1,000 runs per classifier, including cross-validation iterations. Table S4 provides comprehensive details on the final optimized settings for each algorithm.

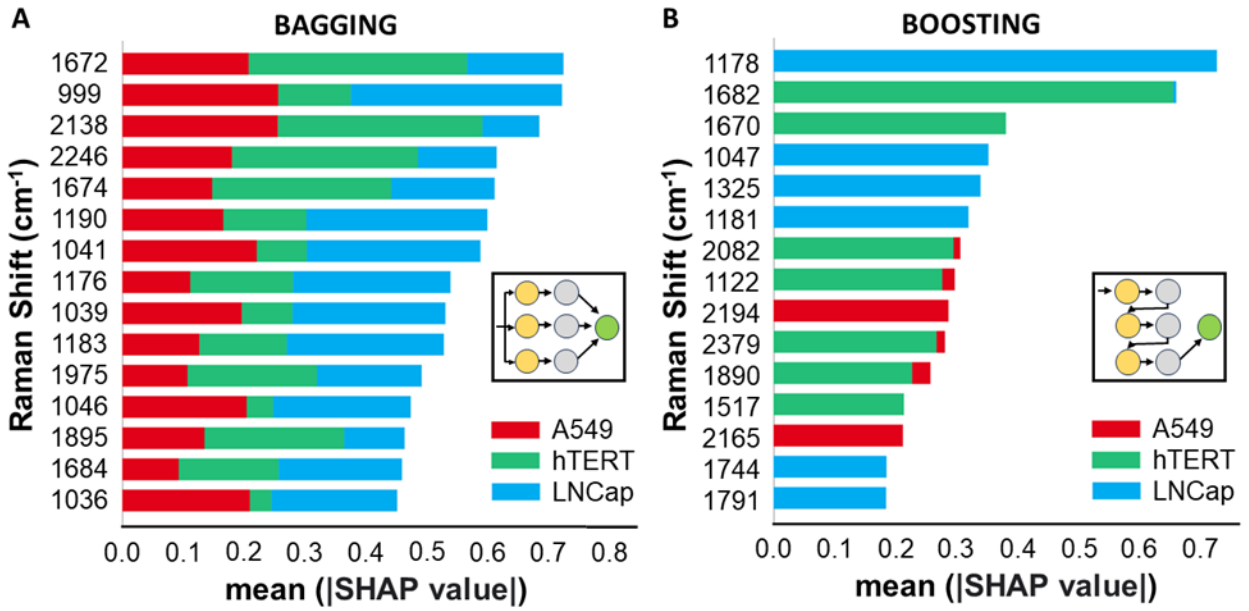


Figure 4. SHAP barplot from Bagging and Boosting Classifiers. SHAP barplot showing the most important features, calculated by the mean absolute SHAP value, and colored based of the feature's importance per exosome for (A) the bagging algorithm Extra Trees (ET) Classifier and (B) the boosting algorithm Light Gradient Boosting (LGBM) Classifiers. Bagging algorithm demonstrates balanced contributions from each exosome in the classification. Boosting algorithm demonstrates unbalanced contribution from each exosome and leads to lower quality and bias towards one or two out of the three exosomes.

Utilizing SHAP, we quantified the importance of every spectral pixel for each classifier. An additional facet that the SHAP analysis illuminates is how specific exosomes benefit from certain special pixels, as important weights are calculated per class. Intriguingly, we uncovered a disparity in how bagging classifiers (ET and RF) and boosting classifiers (LGBM, CAT, and GB) were trained with the commercial cell-line data. The two bagging classifiers, which rely on averaging from multiple decision trees, recognized the top features as crucial for all three cell-culture exosomes. In contrast, the boosting classifiers, which learn through sequential predictions by implementing loss functions, predominantly identified features as important for only one or two exosomes. While it's not surprising for features to play a pivotal role in characterizing one or two specific exosomes, observing that boosting algorithms were completely dependent on spectral pixels that are significant for only one or two exosomes led us to consider bagging algorithms as offering a more balanced classification approach (Figure 4). Given these insights, we decided to focus our final classification efforts on ET and RF classifiers. To further optimize and reduce dimensions, we employed SHAP to construct models using only the top 5% of the most important spectral pixels. Leveraging the top 5% of features (49 pixels), of which exact pixels differed slightly between ET and RF, we achieved remarkable results by using a

70/30 train and test split. We found RF reaching an accuracy of 96.2%, while ET demonstrated an exceptional accuracy of 99.7%, as detailed in Table S5 and Figure S13. We decided to move forward with ET.

The ET confusion matrix for the classification of exosomes is shown in Figure 5, for direct comparison with PLS and PCA results. As can be observed, the accuracy of classification is comparable with the dimensionality reduction methods, although slightly lower. However, the small decrease in accuracy is counterbalanced by a high degree of confidence in the model and by conserving the chemical insight through the classification process. As an example, the SHAP analysis shown in Figure 4 was used to determine the appropriate ML method (i.e., bagging) by looking at the model, rather than simply based on the accuracy of the results (effectively removing part of the black box). In addition, while PLS offers some visualization of the correlation between the classification model and the original data with the VIP scores (Figure S14), the actual PLS classification is not represented by the VIP scores, which reduces the interpretability of these results. With SHAP, an XAI analysis, we can rank specific Raman bands based on their importance to the classification, conserving a high degree of chemical insight, which is a major advantage of Raman.

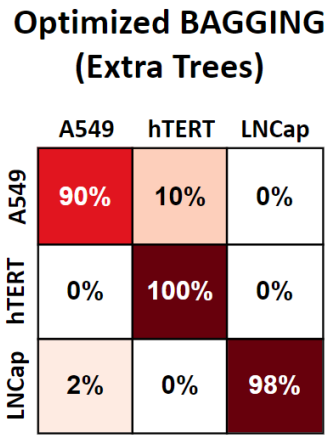


Figure 5. Confusion Matrix from the optimized Extra Trees Bagging algorithm classification on commercial cell-line exosomes, A549, hTERT, and LNCap. Compared to Figure 3, the Extra Trees demonstrate some misclassification compared to PLS/DA and PCA/SVM. However, Extra Trees can utilize SHAP, an XAI, to extrapolate and identify specific chemical insight to the classification.

SERS classification of exosomes extracted from clinical samples

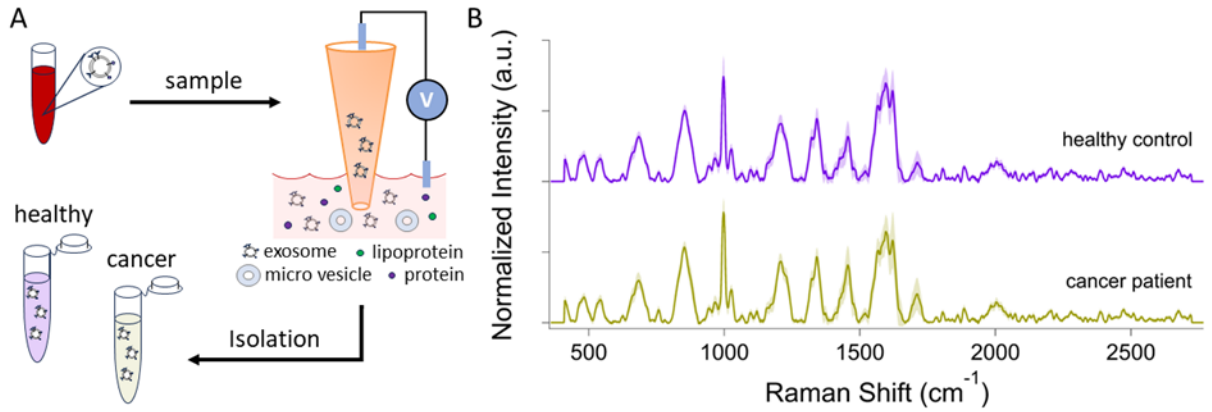


Figure 6. (A) Schematic of clinical exosomes isolation via nanopipette dielectrophoresis. (B) Averaged normalized SERS signals of clinical exosomes from healthy control (purple, top) and cancer patient (yellow, bottom).

To test our substrates and ML analysis on a real application, we collected SERS spectra from exosomes extracted from healthy and cancer blood samples (Figure 6A). Blood samples were collected in kidney-cancer and healthy individuals, and then exosomes were extracted via nanopipette dielectrophoresis, which permitted to obtaining of clean exosome samples.[33] The exosomes were analyzed by the same process as the commercial cell-line exosomes, on MeFON SERS substrates by drying on the substrate surface before SERS measurements. To avoid concentration bias in the SERS spectra, the spectra were normalized by dividing them by their integral (area under the curve of all Raman peaks) (Figure 6B). Since we found consistent success with bagging algorithms in cell-line exosomes' data classification, we exploited this analysis to evaluate their capability to distinguish between spectra obtained from clinical samples. We maintained the same parameters that were optimized for the cell-line dataset, ensuring a fair and valid comparison of the model's performance across different datasets. The extended analysis of the clinical data exclusively used ET. For ET, a reduced features set comprising the top 5% (49 pixels) yielded an accuracy of 91.9% with the 70/30 data split. Figure 7 shows the confusion matrix results for the validation set and an area under the curve (AUC) of 0.95, with a sensitivity of 95.7% and a specificity of 97.9%. For completeness, we also show the results for the RF model (Figure S15), the second best-performing bagging model, including the PLS/DA (Figure S16, Table S6) and PCA/SVM (Figure S17, Table 7) models. RF, PLS/DA, and PCA/SVM had an AUC of 0.90, 1.0, and 0.97, respectively. PLS/LDA continues to reveal high-performance modeling scores; however, revealed a low R2 of 0.353 and is not statistically relevant. In addition, the initial fit of the reduced space was suboptimal, which raises concerns about the method's broader applicability. As for PCA/SVM, the first 20 components explain less than 60% of the data's total variance, which perpetuates the concerns about this method's application on future data. Both PLS and PCA approaches are not highly statistical. Though ET's classification accuracy is

slightly lower, it is an explainable learning method. This slight difference can be counterbalanced by conserving the chemical insights and applicability of SHAP to identify and extrapolate the most important insights in the overall classification.

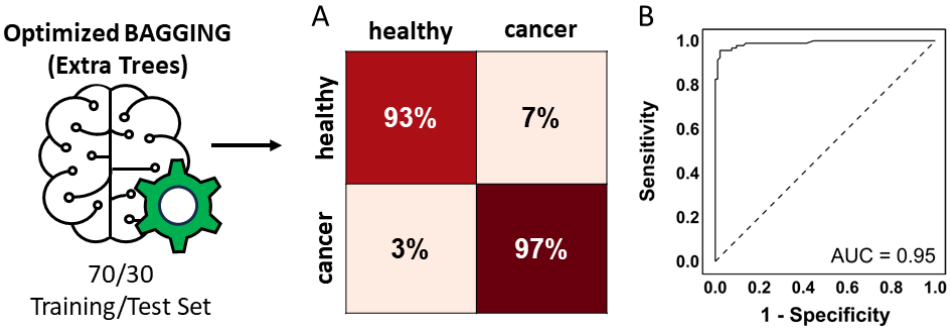


Figure 7. The application of the Optimized ML approach of Extra Trees Bagging on clinical exosomes and the diagnostic accuracy. (A) The confusion matrix from the clinical exosomes, heathy control (healthy) and cancer patient (cancer) show high classification, and the (B) ROC Curve had an area under the curve (AUC) of 0.95.

Important Raman spectral features from SHAP

From the previous section, the ET classifier was employed on the clinical exosomes. SHAP was further used to evaluate the ET's performance by calculating the contributions of each spectral pixel to the model's prediction. This process allows specific identification of Raman signals that significantly influence the classification model. Figure 8A shows a SHAP beeswarm plot of Raman shift (Raman bands) and relative SHAP values. We limit the plot to the top 5% pixel based on SHAP importance. We also bracketed together pixels/shifts that are relative to the same peak in the spectrum for clarity. High absolute SHAP values signify more important Raman shifts for the classification among the top 5%. Figure 8A also shows the colors ranging from red to blue express whether the signals are on average higher or lower, respectively. Figure 7B highlights the 5% important Raman shift in the averaged spectra and with the most significant Raman shifts in red, to get a clear representation of the model on the collected spectra.

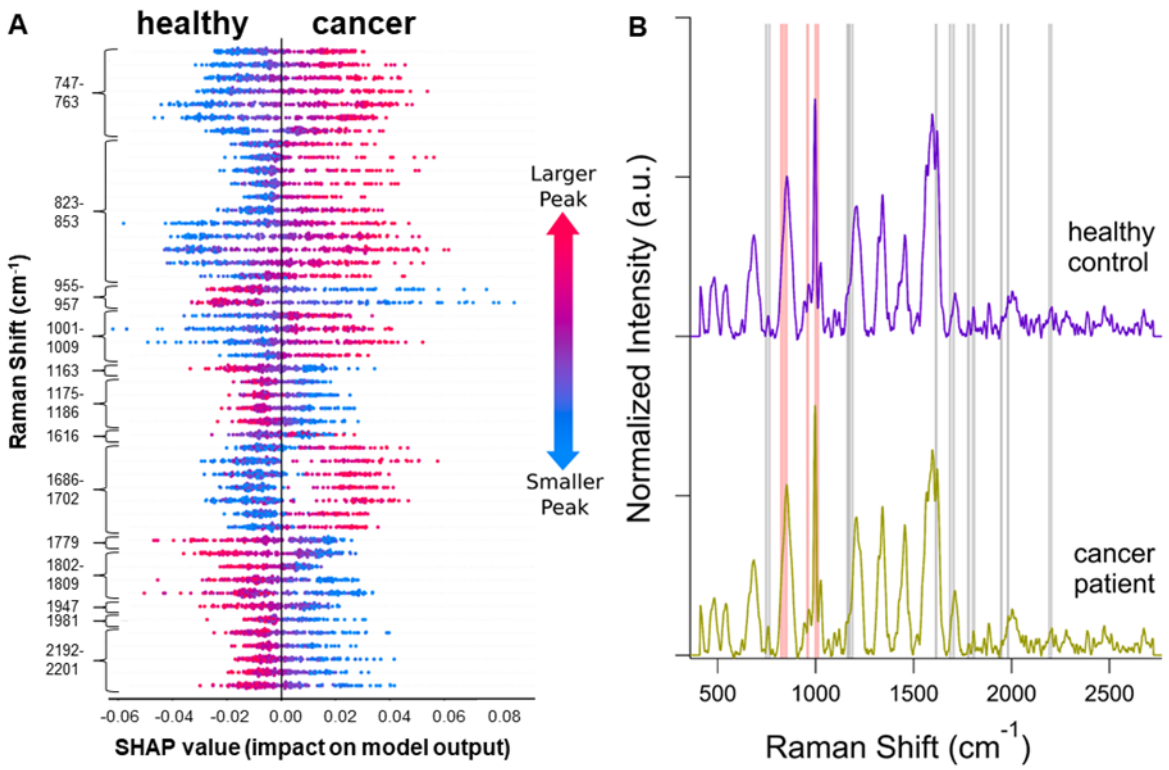


Figure 8. SHAP beeswarm plot (A) from the Optimized ML approach of Extra Trees Bagging on clinical exosomes and the results connection to the spectra (B). The SHAP beeswarm plot shows the top 5% of the most important Raman Shifts in the classification of the clinical exosomes on MeFON. (B) Averaged normalized SERS signals of clinical exosomes from healthy control (purple, top) and cancer patient (yellow, bottom). The gray and red highlighted regions are the top 5% most important in the classification. The red regions are the highest influence among the top 5%.

To better showcase the chemical information offered by SERS and SHAP analysis, we summarize the top 5% features in the ET classification in Table 2 which includes the band assignment and relative chemical component. The Raman features with the highest SHAP values (red regions in spectrum, Figure 7B) were attributed to ring system from Tyrosine (Tyr, 823-853 cm^{-1}), C-C stretching (955-957 cm^{-1}) likely from the α-helix backbone in proteins, and Phenylalanine (Phe, 1001-1009 cm^{-1}). Tyr- and Phe-associated regions display a high-intensity Raman peak in the cancer spectra and lower intensity in the healthy spectra (as determined from the SHAP plot, Figure 7A). The region associated with C-C stretching shows the opposite behavior, with intensity higher in the healthy spectra. In addition, higher Raman intensity was from the Tryptophan's ring system (Trp, 747-763 cm^{-1}) and Amide I (1686-1702 cm^{-1}) were observed for the cancer spectra. Other features highly significant for the model were assigned to Tyr ring breath (1163 cm^{-1}), C-H bend (1175-1186 cm^{-1}), and C=C stretching (1616 cm^{-1}) from Tyr, Phe, and proteins. The Raman shift of other features may be assigned to C=O, C-H and C≡C stretching. These latter spectral regions had lower Raman intensities in the cancer

spectra and higher intensities in the healthy spectra. Overall, our results suggest Raman signal from Tyr, Phe, Trp, and protein significantly contributed to the model's prediction. In our results obtained from cell-line derived exosomes (Figure 4), we can see similar results with protein-associated features being the most important for model prediction (specifically, Phe and Amide I). This agreement determined using the SHAP analysis is a further validation of the robustness of this model.

Table 2. Important Raman features that made significant contributions to clinical exosome classification via Extra Trees (ET) on metallic film over nanospheres (MeFON).

Raman Shift (cm^{-1})	Assignment	Molecular components	Reference
747-763	symmetric ring breath	Trp, proteins	48, 49
823-853	v(C–C) proline, ring breath	Tyr, proteins	48, 49
955-957	v(C-C) stretching	α-Helix backbone, proteins	48, 49
1001-1009	v(C-C) skeletal, ring breath	Phe, proteins	48, 49
1163	ring breath	Tyr, proteins	49
1175-1186	v(C-H) bend	Tyr, Phe, proteins	48, 49
1616	v(C=C), stretching	Tyr, Trp, proteins	48, 49
1686-1702	Amide I, turn and bands	proteins	48, 49
1779, 1802-1809	v(C=O)	lipids	40
1947, 1981	v(C-H)	unassigned	-
2192-2201	v(C≡C)	lipids	41

Exosome classification on SERS substrates fabricated with colloidal nanostars

To extend our results toward our long-term goal, we used the optimized ML methods to examine SERS spectra obtained from substrates fabricated with colloidal nanoparticles. These substrates were fabricated by incubating functionalized silicon substrates in a suspension of concentrated silver-coated gold nanostars (NS) (Figure 9A), previously optimized (Figure S9). This fabrication method is more suitable to be added to a potential exosome-extraction device to automate this type of analysis. However, colloidal fabrication of SERS substrates is often characterized by lower signal enhancements, which could reduce classification accuracy. To determine feasibility, we tested the classification of clinical samples with these substrates. We applied the ET classifier on the normalized spectra to find similar scoring metrics found with the solid-state substrate classification. Results for the classification are shown in Figure 9.

Figure 9B shows the spectra obtained with these substrates on clinical exosome samples. These spectra were significantly different from previous results with MeFON, mostly due to the different background signal and signal-to-background ratio for the exosome signal. A blank spectrum is shown in the SI (Figure 18), to compare with the exosome signal from the figure. Figure 9C, D, the ET classification shows high accuracy for the identification of exosomes from cancer patients, as shown in the confusion matrix of the

validation set and in the ROC curve, showing an AUC of 0.94. The accuracy for NS substrates was below what was observed using MeFON. In addition, compared to results obtained with MeFON substrates, the SHAP analysis showed an overall weaker classification, with the top 5% exhibiting smaller magnitudes of importance overall (Figure S19). More importantly, the top 5% spectral peaks collected differed in regions as well as trends found in the MeFON, solid-state substrate (Figure 8). This result is likely due to the lower intensity of peaks associated with the exosomes, with some peaks not observed with NS substrates. Overall, these results show the superiority of MeFON substrates but also demonstrate the feasibility of performing classification with NS substrates. We believe that further optimization of NS substrates could lead to robust SERS classification in exosome-extraction devices.

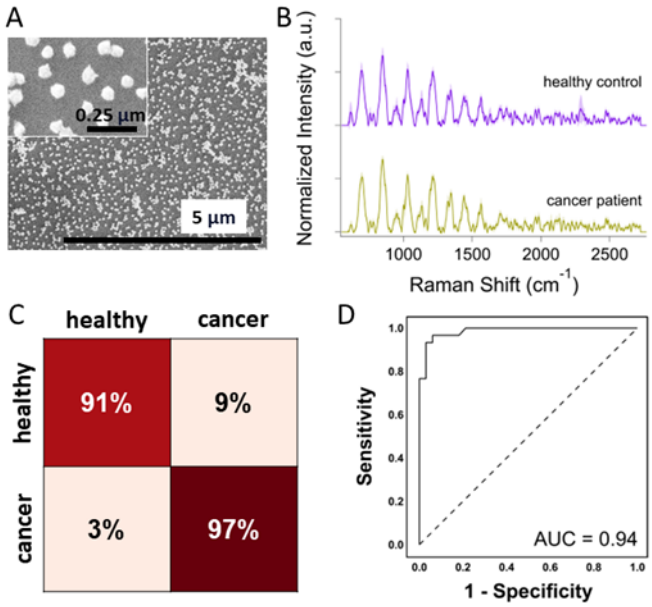


Figure 9. Exosome detection via label-free SERS on colloidal-state fabricated substrates and diagnostic accuracy. (A) SEM images of silver-coated gold nanostars (NS) on silicon. (B) Averaged normalized SERS signals of clinically isolated exosomes from human blood. The Extra Trees (ET) Bagging classification performance is summarized in the confusion matrix (C) and ROC curve (D).

Conclusion

In summary, we study the use of ML methods for exosome classification from exosomes derived from cell-lines and extracted from clinical samples. This study was performed by first evaluating different classification methods (PLS/LDA, PCA/SVM, and ET/SHAP) for cell-line-derived exosomes. These studies show high classification accuracy for all methods but revealed how PLS and PCA are not statically relevant in the clinical diagnostic and are not explainable methods. The results show the superiority of XAI, which through SHAP analysis permitted to selection of the most robust model (bagging

classifer, ET) and to directly relate spectral features to the classification. The optimized ET/SHAP model was then used on exosomes, sEVs, extracted from clinical samples, showing high diagnostic accuracy. These results show the potential for SERS analysis to be used for non-invasive cancer diagnostics. Finally, we tested clinical samples on substrates fabricated with NS, proving that the classification was possible also on this type of cheap and versatile substrates. In conclusion, we demonstrated the integration of nanopipette dielectrophoresis with SERS, including with colloidal NS that can be easily integrated in a potential device. We also demonstrated the use of XAI to perform diagnostic analysis on complex SERS data of clinical samples, showing how this approach enables optimization and interpretation of the model due to its direct association to the input spectral data.

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