Mixed-Dimensional Heterostructure Material-Based SERS for Trace Level Identification of Breast Cancer-Derived Exosomes

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ABSTRACT: Raman spectroscopy has capability for fingerprint molecular identification with high sensitivity if weak Raman scattering signal can be enhanced by several orders of magnitudes. Herein, we report a heterostructure-based surface-enhanced Raman spectroscopy (SERS) platform using 2D graphene oxide (GO) and 0D plasmonic gold nanostar (GNS), with capability of Raman enhancement factor (EF) in the range of $\sim 10^{10}$ via light–matter and matter–matter interactions. The current manuscript reveals huge Raman enhancement for heterostructure materials occurring via both electromagnetic enhancement mechanism though plasmonic GNS nanoparticle (EF $\sim 10^7$) and chemical enhancement mechanism through 2D-GO material (EF $\sim 10^2$). Finite-difference time-domain (FDTD) simulation data and experimental investigation indicate that GNS allows light to be concentrated into nanoscale “hotspots” formed on the heterostructure surface, which significantly enhanced Raman efficiency via a plasmon–exciton light coupling process. Notably, we have shown that mixed-dimensional heterostructure-based SERS can be used for tracking of cancer-derived exosomes from triple-negative breast cancer and HER2(+) breast cancer with a limit of detection (LOD) of $3.8 \times 10^2$ exosomes/mL for TNBC-derived exosomes and $4.4 \times 10^2$ exosomes/mL for HER2(+) breast cancer-derived exosomes.

1. INTRODUCTION

Raman spectroscopy has the ability to provide fingerprint-type identification, which is very important for medical and forensic investigation, as well as other fields.\(^1\)–\(^5\) The main disadvantage for Raman spectroscopy to be used for fingerprint recognition is that Raman scattering is very weak where only 1 out of 10 million photons is scattered.\(^6\)–\(^9\) To overcome this, several groups are working on surface-enhanced Raman scattering (SERS) spectroscopy, where the Raman intensity can be enhanced by several orders of magnitude ($10^6$ to $10^{14}$).\(^6\)–\(^15\) In case of SERS, huge enhancement of Raman intensity for adsorbed molecules on the surface occurs via the electromagnetic mechanism (EM) and the chemical mechanism (CM).\(^6\)–\(^16\) As we and others have reported, the EM exploits the excitation of localized surface-plasmon resonances (LSPR) in plasmonic zero-dimensional (0D) nanostructured materials such as Au, Ag, and Cu.\(^6\)–\(^11\)\(^20\) Because of the strong light–matter interaction via plasmon-excitation coupling, the EM enhancement factors can be on the order of $10^6$ to $10^7$.\(^21\)–\(^27\) On the other hand, the CM has capability to enhance the Raman signal by $10^4$ to $10^5$ times based on dipole–dipole interactions or charge-transfer resonances between the surface and Raman active molecules.\(^23\)–\(^31\) We and others have reported that two-dimensional (2D) nanomaterials such as graphene,\(^11\)–\(^14\)\(^19\)–\(^25\) transition-metal dichalcogenides (TMD),\(^15\)\(^17\)\(^26\)\(^27\) hexagonal boron nitride (h-BN),\(^26\)\(^27\) and others\(^26\)\(^27\) can show CM enhancement higher than $10^2$ when the laser excitation can be resonant to charge-transfer transitions.

Herein, we report huge Raman enhancements from mixed-dimensional heterostructure platform using 2D graphene oxide (GO) and plasmonic gold nanostar (GNS), which has capability for several orders of magnitude Raman enhancement via light–matter and matter–matter interactions. In our design, 2D-GO has been used been used for the SERS enhancement via the chemical enhancement mechanism.\(^14\)\(^15\)\(^18\)–\(^20\) On the other hand, anisotropic GNS has been used for huge SERS enhancement via the plasmonic enhancement mechanism and “lightning rod effect”.\(^6\)–\(^14\)\(^29\) Over the past few years, several groups have designed GO/GNS hybrid nanocomposites for SERS\(^19\)\(^23\)–\(^25\) Most of the synthetic procedure has been used is the direct growth of GNS on GO sheets, while controlling the amount of GNS on GO is...
difficult. It is now well understood that electromagnetic “hotspots” are the most important parameter to achieve huge SERS enhancement.\textsuperscript{1−10} To generate huge amount electromagnetic hotspots, we synthesized GNS using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. After that, we have performed controlled attachment of GNS on the 2D-GO surface for developing hotspots. Reported data show that because of the formation of good amount electromagnetic hotspots, we have obtained Raman EF is in the range of $\sim 10^{10}$ from GNS-attached GO-based heterostructure material, which is higher than the reported EF data for GO/GNA assembly.\textsuperscript{19,23−25}

As a proof of concept, we have demonstrated that GO-GNS mixed-dimensional heterostructure-based ultrasensitive SERS is versatile for trace level fingerprint identification of exosomes derived from triple-negative breast cancer and HER2(+) breast cancer. Triple-negative breast cancer (TNBC), which lacks estrogen receptors (ER), progesterone receptors (PR), and hormone epidermal growth factor receptor 2 (HER2) protein, is usually highly aggressive and difficult to treat.\textsuperscript{32−35} Several recent reports show that exosomes can be minimally invasive biomarkers for accessing the stage for TNBC and other types of cancer.\textsuperscript{36−45} Exosomes are well-documented cell-derived vesicles which are loaded with variety of proteins, micro RNA, noncoding RNAs, and DNA.\textsuperscript{40−49} Because exosomes can be obtained easily from biological fluids in clinics, they have been considered as minimally invasive cancer biomarkers.\textsuperscript{38−43}

Recently, SERS has been used as noninvasive assay for EV study.\textsuperscript{35,39−49} For this purpose, SERS nanotags have been used as an alternative label to fluorescent dyes for exosome detection.\textsuperscript{35,39−49} For instance, Zhang et al. reported\textsuperscript{35} the design of magnetic beads and Raman reporter-based SERS for rapid capture of exosome using antibody-conjugated magnetic beads and profiling using antigen-targeting SERS nanotags. Their results show that the SERS assay is a sensitive approach for the detection of small EVs, with a limit of detection (LOD)

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{(A) TEM image shows the morphology of PEG-coated GNS. Inserted picture shows a high-resolution image which indicates that the size is $\sim 50 \pm 10$ nm. (B) SEM image shows the morphology of 2D-GO. (C) TEM image shows the morphology of mixed-dimensional heterostructures based on GO and GNS. Inserted picture shows a high-resolution image. (D) SEM image shows the morphology of mixed-dimensional heterostructure-based on GO and GNS. Inserted picture shows a high-resolution image. (E) EDX spectra from mixed-dimensional heterostructure, which indicates the presence of Au, C and O. (F) XRD spectra from mixed dimensional heterostructure, which shows the presence of (002) reflection for GO and (111), (200), (220), (3111) reflection for GNS. (G) Extinction spectra from PEG-coated GNS, GO, and mixed-dimensional heterostructure. (H) Raman spectra from only GO and GO-GNS-based mixed-dimensional heterostructure.}
\end{figure}
of $2.3 \times 10^6$ exosomes/mL. Similarly, Tian et al.\textsuperscript{44} have reported exosome identification using gold nanostar@Raman reporter@nanoshell-based SERS with an LOD of $2.7 \times 10^4$ exosomes/mL. Furthermore, Kwizera et al.\textsuperscript{47} have reported the design of a miniaturized chip platform using gold nanorods coated with QSY21 Raman reporters for phenotype profiling of small EVs, with an LOD of $2.0 \times 10^5$ exosomes/mL. For SERS-based label-free identification of exosomes, in the current manuscript, we have used 2D–0D heterostructure-based SERS for tracking of cancer-derived exosomes from TNBC and other types of breast cancer. Reported data show that heterostructure-based SERS has capability for label-free fingerprint identification of exosomes, with an LOD of $3.8 \times 10^5$ exosomes/mL for TNBC-derived exosomes and $4.4 \times 10^5$ exosomes/mL for HER2(+) breast cancer-derived exosomes. The observed very good LOD is mainly due to the formation of huge number electromagnetic hotspots on SERS substrate developed by us.

2. MATERIALS AND METHODS

2.1. Design and Characterization of GO-GNS Multi-dimensional Heterostructure. As shown in Scheme 1A–C, mixed-dimensional heterostructures were synthesized using a three-step process. As shown in Scheme 1A, in the first step, we have synthesized GNS using tetrachloro Au (III), silver nitrate, sodium hydroxide, and HEPES buffer, as we and other groups reported before.\textsuperscript{8–10,26} In our synthetic procedure, HEPES has been used as precise shape-directing agent.\textsuperscript{29} To enhance stability as well as for developing biocompatible materials, GNSs were coated with SH-PEG$_{2000}$–NH$_2$. Experimental details for the synthesis of PEG-coated GNS have been reported in the Supporting Information. After purification via centrifugation for 1 h at 3500 rpm, PEG-coated GNSs were characterized by tunneling electron microscopy (TEM), X-ray diffraction (XRD), and absorption technique.\textsuperscript{14–17} Figure 1A shows the TEM image of freshly prepared PEG-coated GNS. Figure S1A shows the TEM image of freshly prepared GNS without PEG. From TEM data, we can find that the size of bare GNS is $\sim 35 \pm 10$ nm and the size of PEG-coated GNS is $\sim 50 \pm 10$ nm. The excitation spectra from GNS, as reported in Figure 1G, shows a strong plasmon band with peak maxima for bare GNS is $\sim 500 \pm 10$ nm. The absorption peak from GO in the visible region.

![Figure 2](https://dx.doi.org/10.1021/acsomega.0c01441)  
**Figure 2.** (A) Plot shows how the Raman profile from 4-ATP varies in the presence of Si/SiO$_2$, GNS, 2D-GO, and mixed-dimensional heterostructure surfaces. (B) Plot shows how Raman profile from Rh-6G varies in the presence of Si/SiO$_2$, GNS, 2D-GO, and mixed-dimensional heterostructure surfaces. (C) Bionalyte SERS spectra from a mixture of 4-ATP ($\sim 1.4 \times 10^{-9}$ M) and Rh-6G ($\sim 1.4 \times 10^{-9}$ M) in the presence of a mixed-dimensional heterostructure surface. We have compared the bionalyte spectra with single-analyte SERS spectra for 4-ATP ($\sim 8.2 \times 10^{-10}$ M) and Rh-6G ($\sim 8.2 \times 10^{-10}$ M) separately. (D) Plot shows how Raman intensity at $1078$ cm$^{-1}$ for Rh-6G varies with the concentration in the presence of a mixed-dimensional heterostructure surface. Each data represents the average of four separate experiments. Error bars represent the standard deviation of measurements. (E) Plot shows how Raman intensity at $1078$ cm$^{-1}$ for 4-ATP varies with the concentration in the presence of a mixed-dimensional heterostructure surface. Each data represents the average of four separate experiments. Error bars represent the standard deviation of measurements. (F) FDTD simulation data show how the $(I_E^2)$ profile from a sharp branch of GNS varies with the distance for the dimer. TEM image for single three spike-based GNS particle structure has been shown in (F1), which can be simplified as a triangular shape. TEM image for another single three spike-based GNS particle structure has been shown in (F2), which also can be simplified as a triangular shape. (F3) shows FDTD simulation data, where we have taken a triangular structure for our calculation.

In the second step, we have developed graphene oxide (GO) using modified Hummer’s method, as we and others have reported before.\textsuperscript{12,14,15,18–22} For this purpose, we have used sodium nitrate, concentrated sulfuric acid, and potassium permanganate as strong oxidizing agents to synthesize graphene oxide from graphite. Experimental details have been reported in the Supporting Information. Finally, H$_2$O$_2$ (50%) was added to the mixture till there was no gas generated. The mixture turned from almost black to yellow. After purification, we have used scanning electron microscopy (SEM) and absorption spectroscopy technique to characterize 2D-GO. Figure 2B shows the SEM image of GO flakes synthesized from graphite using the Hammers method. As shown in Figure 2G, we have not observed any strong absorption peak from GO in the visible region.
Because of the lack of plasmon band in the visible region, Raman enhancement from 2D-GO surface can be mainly due to chemical enhancement. In the next step, we have developed mixed-dimensional heterostructures using GO and PEG-coated GNS. As shown in Scheme 1C, amino functionalized GNSs were covalently linked with graphene oxide nanosheets using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC)—N-hydroxysuccinimide (NHS) coupling chemistry.11−16 Experimental details have been reported in the Supporting Information. At the end, the unreacted GO and GNS were removed by centrifugation at 10,000 rpm for 30 min, followed by decantation, and then, the pellet was resuspended in nanopure water for further use. As reported in Figure 1, the morphologies of GNS-attached Graphene oxide-based mixed dimensional heterostructures were characterized by UV−vis, Raman, XRD, TEM, SEM, and energy-dispersive X-ray spectroscopy (EDX) techniques.14−17

The TEM and SEM images for mixed-dimensional heterostructures are reported in Figure 1C,D. Reported images clearly show that GNSs formed assembly structure on the surface of graphene oxide. Figure 1E shows the EDX data from the mixed-dimensional heterostructures, which clearly indicates the presence of C, O, and Au. XRD data from the mixed-dimensional heterostructures, as reported in Figure 1F, shows the presence of broad (002) reflection for GO. XRD data also indicates the presence of (111), (200), (220), and (3111) reflection for GNS.14,15,18−22 The excitation spectra from the mixed dimensional heterostructures, as reported in Figure 1G, shows a very broad plasmon band above 630 nm, which is due to the assembly structure of GNS on a 2D-GO surface, as we have observed in TEM and SEM images reported in Figure 1C,D. In the self-assembly of GNS, the interparticle plasmon—plasmon couplings will produce plasmonic hot spots where massive near-field amplifications will occur via light−matter and matter−matter interactions.22 The above phenomena will enable highly sensitive SERS detections via mixed-dimensional heterostructures. The Raman spectra from only GO and GO-GNS in the mixed-dimensional heterostructure are reported in Figure 1H. Reported Raman spectra shows the presence of disorder band (D band) at ~1350 cm−1 caused by the graphite edges and G band at ~1590 cm−1 caused by the in-plane vibration of sp2-hybridized carbon atoms.14,15,18−22 We have acquired Raman spectra from 10 to 15 spots and then spectra were processed with a baseline removal and averaging program. The observed enhancement of D and G bands in the presence of GNS in the mixed dimensional heterostructures is mainly due to the plasmon effect from GNS.

**2.2. Raman Experimental Details Using GO, GNS, and Mixed-Dimensional Heterostructure Substrate.** For the measurement of Raman EF, we have used the confocal Raman system with a laser excitation of 670 nm.15 For Raman data collection, we have used 100× magnification and a numerical aperture of 0.9 for our experiment.15 For Raman data collection, we have used 10 s acquisition time and 5-scan averaging, so that we could achieve a very good signal-to-noise ratio. For Raman measurement from TNBC- and HER2(+) breast cancer cell-derived exosomes, we have used a microscope attached with the confocal Raman microscopy system, to locate cluster of exosomes. Once we locate them, the laser beam was focused on cancer cell-derived exosomes cluster through the microscope object.

**2.3. FDTD Simulation for Full-Field Electromagnetic Wave Calculations.** In our calculation, the electric field intensities were simulated by using gold particle dimmer with 35 nm size for each. The amplitude of electric field was kept as 1 V/m, and courant number was taken as 0.99. We have used 670 nm wavelength, 0.001 nm mesh resolution, and 4000 fs time for our calculation, as we have reported before.12−16

**2.4. Breast Cancer Cell Culture and Exosome Isolation from Cell Culture Medium.** Triple-negative breast cancer cell line, MDA-MB-231 cells, and HER2(+) KSBR-3 cells were cultured with a culture medium suggested by ATCC, using the procedure we have reported before.12−13 When cancer cells were cultured to 60−70% confluency, we have replaced the standard culture medium with exosome-depleted medium, as reported before.13 After that cancer cells were cultured for an additional 8 h. In the next step, exosome were separated from cell media by differential centrifugation to remove cellular debris, as reported before.33 For this purpose, supernatants were collected from cell lines and centrifuged at 3000g for 25 min to eliminate cells and debris. After that we have centrifuged again at 10,000g for 30 min to eliminate microvesicles, as we have reported before.33 At the end, exosomes derived from MDA-MB-231 and SKBR3 cells separately were ultracentrifugated for 1 h and resuspended in PBS. In the next step, we have characterized exosomes using TEM, DLS, and western blot.

**3. RESULTS AND DISCUSSION**

**3.1. Determine the Raman Enhancement Factor from Mixed-Dimensional Heterostructures.** As we have discussed before, the Raman scattering signal has to be enhanced by several orders of magnitude before it can be used for fingerprint identification application.9−10 As a result, we have measured Raman EF for 2D-GO, GNS, and mixed-dimensional heterostructure using 4-aminothiophenol (4-ATP) and Rh-6G as the Raman probe. For the measurement of Raman EF, we have used confocal Raman system (Horiba Scientific) with a laser excitation of 670 nm and 4−6 mW power.15 For Raman data collection, we have used 100× magnification and a numerical aperture of 0.9 for our experiment.15 Before SERS experiment, mixed-dimensional heterostructure substrates were immersed in the different concentrations of 4-ATP or Rh-6G for 2 h, and that SERS substrate was dried under N2 flow. The distribution of GNS monomers, dimers, or clusters is not uniform throughout the substrate as reported in the TEM and SEM images for mixed-dimensional heterostructures. As a result, to obtain high-quality data, we have acquired Raman spectra from 10 to 15 spots, and then, the spectra were processed with a baseline removal and averaging program. The variation of relative standard deviation (RSD) values at different spots for GO-GNS substrate is ~10.5% for 4-ATP and ~13.1% for Rh-6G on the GNS-GO surface. To keep the experimental condition same, for each Raman experiment, we have acquired Raman spectra from 10 to 15 spots for GO, GNC, or GO-GN substrates, and then, the spectra were processed by baseline removal and averaging. Because several Raman probes have Raman active bands near D and G bands of GO, which can interfere with the actual intensity measurement, we have subtracted the Raman spectra of each Raman probe with substrates from Raman spectra of only substrates without probes. In every case, we have acquired Raman spectra from 10 to 15 spots, and then, spectra were processed with a baseline removal and averaging program.
Raman spectra from different concentrations 4-ATP on GNS, GO, and mixed dimensional heterostructure surfaces is reported in Figure 2A. Raman peaks from 4-ATP on different surfaces are dominated by $a_1$ and $b_2$ vibrational modes. As shown in Figure 2A, the observed $a_1$ modes are for strong electromagnetic enhancement mechanism.8–13 Part of the huge Raman enhancement in the presence of a GNS surface can also be due to the chemical enhancement effect caused by the charge-transfer process happening on the ATP-GNS surface.11–20

On the other hand, using reported data in Figure 2A, we have estimated the average Raman EF for mixed-dimensional heterostructure to be $1.4 \times 10^{10}$. We have observed excellent Raman EF from the mixed-dimensional heterostructure surface because of the strong electromagnetic as well as strong chemical enhancement capability of heterostructure. As reported in Figure 1C,D, in case of mixed-dimensional heterostructures, GNSs formed dimer and higher aggregates on the GO surface. As a result, the electromagnetic field experienced by the 4-ATP in “hot spot” formed by dimers or higher aggregates is much stronger than the field it will experience in monomer. Because of the above fact, a synergistic mechanism is expected in case of mixed-dimensional heterostructure material. Figure 2E shows how the Raman signal at $1078$ cm$^{-1}$ varies with the concentration of 4-ATP in the presence of a mixed-dimensional heterostructure surface. We have used the following equation to determine the limit of detection (LOD).11–20

$$\text{LOD} = 3\sigma/S$$

In this equation, $\sigma$ is the standard deviation of the blank and $S$ is the slope of the calibration curve. Standard deviation of blanks was determined from the baseline noise of SERS substrates without analytes over the range of Raman peak of interest. For this purpose, we have recorded 10–15 spectra from substrate in the absence of the analyte. Using the concentration-dependent data as reported in Figure 2E and eq 4, we have estimated the limit of detection (LOD) to be $3.1 \times 10^{-13}$ M for the 4-ATP Raman probe in the presence of a mixed-dimensional heterostructure material.

To understand how the Raman EF values vary with different Raman probes, we have also performed same set of experiments with the Rh-6G Raman probe. Figure 2B shows the SERS spectra from Rh-6G at different concentrations on the GNS, 2D-GO, and mixed-dimensional heterostructure surface. The observed Raman modes from Rh-6G at 376 cm$^{-1}$ is due to the N–C–C bending modes of the ethylamine group of the Rh-6G ring. Other observed prominent Raman modes are at 615 cm$^{-1}$ due to the C–C–C C ring in-plane bending, 778 cm$^{-1}$ due to the C–H out-of-plane bending, 1181 cm$^{-1}$ due to the C–H in-plane bending, 1366 cm$^{-1}$ due to the C=N stretch, 1507 cm$^{-1}$ due to the aromatic C–C stretching, and 1603 and 1650 cm$^{-1}$ due to the C=N stretch, as we and others reported before.8–14 Using eqs 1–3 and reported data in Figure 2B, we have estimated the Raman EF to be $3.8 \times 10^{10}$ for mixed-dimensional heterostructures, $2.6 \times 10^{10}$ for GNS, and $2.1 \times 10^{10}$ for 2D-GO. Although the Raman EF values vary a little bit for different Raman probes, the orders of magnitude remain the same. In both cases, we have observed $10^3$ higher Raman EF from the mixed-dimensional heterostructure surface with respect to GNS, and it is due to the strong electromagnetic as well as chemical enhancement capability of the mixed-dimensional heterostructure surface. Figure 2D shows how the Raman signal at 1507 cm$^{-1}$ varies with the concentration in the presence of a mixed-dimensional heterostructure surface. Using Figure 2D and eq 4, we have determined the LOD, which is estimated to be $2.5 \times 10^{-13}$ M.
for a Rh-6G Raman probe in the presence of a mixed-dimensional heterostructure material. Because we have observed very high sensitivity, to demonstrate that the observed Raman signals is coming from a few molecules, we have performed SERS experiment using a bianalyte Raman probe. Le Ru et al.\textsuperscript{16} have proposed that simultaneous use of two analyte molecules is necessary to determine whether the observed SERS signals are from the single or few molecules nature.

Figure 3C shows the bianalyte SERS spectra from a mixture of 4-ATP ($\sim 1.4 \times 10^{-9}$ M) and Rh-6G ($\sim 1.4 \times 10^{-9}$ M) in the presence of a mixed-dimensional heterostructure surface. By comparing the bianalyte spectra with single-analyte SERS spectra for 4-ATP and Rh-6G separately, as reported in Figure 3C, we can conclude that the observed SERS signal from bianalyte is a mixture of SERS spectra from individual Raman probes. The above observation clearly evidenced that the SERS signal comes from a very small number of molecules.

To understand hot spot effects on SERS, we have performed the finite-difference time-domain (FDTD) simulation.\textsuperscript{11,14,15,30,31} As shown in Figure S1, several GNS structures developed by our group have three spikes and they can be simplified as a triangular structure where three corners can act as spikes for the lighting rod effect, as shown in Figure 2F(1,2). For FDTD calculation, we have used a simple triangular structure with three corners as spikes, as shown in Figure 2F(3). For the simulation, we have used a triangular gold particle with 30 nm length for each side. We have used 670 nm wavelength, 0.001 nm mesh resolution, and 4000 fs time for our calculation, as we have reported before.\textsuperscript{12–16} Figure 2F shows how that the square of field enhancement ($|E|^2$) in sharp branch for GNS varies with distance for dimer. Reported data clearly show that more than 2 orders of magnitudes higher field enhancement in hot spot position than that of an individual particle. Because the Raman EF ($|E|^4$), our FDTD simulation data indicate that there is a possibility of 4 orders of magnitude Raman enhancement in hot spot position on the heterostructure. As we have discussed before, the distribution of GNS monomers, dimers, or clusters is not uniform and as a result, the measured EF value will vary at different spots for the mixed-dimensional heterostructure surface. As a result, the experimental average EF values reported in this manuscript are lower than expected theoretical values calculated by FDTD simulation.

Because adsorption affinity, molecular aggregation under dry condition, and other several factors can alter the estimated EF values from mixed-dimensional heterostructure, we have measured the average EF values on the heterostructure surface using Rh-6G as a Raman probe, when they have been prepared in different batches. Experimental data reported in Figure S3 show that the variation of RSD values for substrates made in different batches is $\sim 15.5\%$ for Rh-6G. For this measurement, we have performed 10 different spots in each substrate and averaged it. Similarly, we have determined the variation of RSD values for substrates made in different batches for 4-ATP and it is $\sim 14.1\%$.

3.2. Mixed-Dimensional Heterostructure-Based SERS for Label-free Identification of TNBC- and HER2(+) Breast Cancer-Derived Exosomes. Inspired by the huge Raman EF, we attempted to explore whether mixed-dimen-
sional heterostructure-based Raman can be used for tracking different cancer-derived exosomes from TNBC and HER2(+) breast cancer cells. Exosomes were derived from triple-negative breast cancer cell line, M.D. Anderson Metastasis Breast cancer (MDA-MB)-231 cells, and HER2(+) type Sloan-Kettering Breast Cancer (SKBR) 3 cells separately using the procedure we have reported recently. For this purpose, cells were cultured first separately in a standard culture medium and then with an exosome-depleted medium. At the end, exosome was separated by centrifugation to eliminate cellular debris and microvesicles.

The TEM image reported in Figure 3A indicates that the size of freshly separated MDA-MB-231 cell-derived exosomes varies between 200 ± 50 nm. Table 1 shows the DLS measurement data which also indicate similar size for TNBC-derived exosomes, which are between 180 ± 60 nm. We have also performed the western blot test, which indicates that CD63 proteins are overexpressed by exosomes which are derived from MDA-MB-231 cells. Similarly, Figure 3D shows the TEM image of SKBR3 cell-derived exosomes, where the size varies between 180 ± 60 nm. DLS measurement also indicates similar size for SKBR-3 derived exosomes, which are between 170 ± 60 nm. The western blot test indicates that HER2 is overexpressed by exosomes derived from SKBR3 cells.

For Raman measurement from TNBC- and HER2(+) breast cancer cell-derived exosomes, we have used a microscope attached with the confocal Raman microscopy system, to locate cluster of exosomes. Once we locate them, the laser beam was focused on cancer cells derived exosomes cluster through the microscope object. To acquire reproducible Raman spectra, we have measured the SERS data from 10 to 15 spots and then we have performed the baseline removal and averaging. Figure 3B shows Raman spectra from TNBC-derived exosomes in the presence of a mixed-dimensional heterostructure. The observed vibrational Raman bands can be assigned to the spectral contributions of mainly lipids, protein, and nucleic acids as reported in Table 2.40–43 As reported in Figure 3B, we have observed several lipid bands, and these are at ~1605 cm⁻¹ due to the ergosterol, ~1454 cm⁻¹ due to (CH₂, CH₃) in acyl chain, ~1260 cm⁻¹ due to (==CH₂) in acyl chain, and ~1014 cm⁻¹ due to tryptophan. Similarly, we have also observed one protein peak at ~880 cm⁻¹ due to tryptophan. As a result, after proper engineering design, heterostructure-based SERS may have excellent prospects for highly sensitive analysis of exosomes.

4. CONCLUSIONS

In conclusion, our finding reveals that mixed-dimensional heterostructure-based Raman substrate has the capability to be used for trace level tracking of cancer-derived exosomes. Reported experimental data show that mixed-dimensional heterostructure using 2D-GO and plasmonic GNS exhibits very high Raman EF, which is ~10⁷ via combined EM and CM enhancement mechanism. Simulation data indicate that...
because of the formation of hot spots by GNS through sharp edge on the 2D-GO surface, more than 2 orders of magnitudes higher field enhancement occurs, which can enhance the Raman signal by 4 orders of magnitude. We have demonstrated that mixed-dimensional heterostructure-based SERS can be used for the identification of exosomes derived from TNBC-type MDA-MB-231 cells and HER2(+) type SKBR3 breast cancer cells via their fingerprint Raman bands. Reported data shows that the LOD is $3.8 \times 10^2$ exosomes/mL for TNBC-derived exosomes and $4.4 \times 10^5$ exosomes/mL for HER2(+) breast cancer-derived exosomes. Our reported data show the potential of mixed-dimensional heterostructure-based SERS for cancer biomarker identification. Because of the high heterogeneity of exosomes as well as biological fluids, it is very important to understand how Raman spectra for exosome vary from different sources before it can be used for clinics.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c01441.

Detailed design and characterization of mixed-dimensional heterostructure; other experimental details; TEM image showing the morphology of freshly prepared bare GNS without PEG; XRD spectra from PEG coated GNS; and plot showing how the Raman EF from mixed-dimensional heterostructure surface varies with samples made in different batches (PDF)

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**Notes**

The authors declare no competing financial interest.

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