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ARTICLE TYPE

Multifunctional Hybrid Graphene Oxide for Label-Free Detection of Malignant Melanoma from Infected Blood

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This communication reports for the first time the development of multifunctional graphene oxide for the ultra-sensitive and label-free detection of malignant melanoma from infected blood sample.

According to the American Cancer Society, around 77,000 US persons will learn that they have melanoma in 2013 and of which, 9500 will die due to malignant melanoma [1]. Since malignant melanoma cell line UACC903 is resistant to apoptosis, simple blood test for the finding of circulating UACC903 in blood can help to identify if melanoma has spread to other areas of the body[2-4]. Circulating rumor cells are known to present at extremely low abundance (1 to 10 cells in 10^5–10^6 peripheral blood mononuclear cells), and as a result, a highly sensitive label-free detection assay will be valuable [3-6]. Driven by the urgency, in this communication, we report for the first time a highly selective and label-free surface enhanced Raman spectroscopy (SERS) detection of malignant melanoma from infected blood using multifunctional graphene oxide. Multifunctional graphene oxide was developed by attaching plasmonic shell-magnetic core nanoparticle to graphene oxide surface. Since SERS is known to be unique for providing molecular fingerprinting information [7-13] and it has enhancements capability on the order of 10^8-10^{14}, we believed that the SERS is one of the most powerful tools, for the detection of rare malignant melanoma cells in whole blood. To avoid huge light scattering and auto fluorescence background from blood cells, an effective separation and enrichment is highly crucial for the detection of circulating malignant melanoma from blood using SERS [4-6]. Since magnetic properties of core-shell nanoparticles can separate bacteria and cells from complex media [4-6], here magnetic core-gold shell nanoparticle has been used for the separation of malignant melanoma cells from the whole blood by using a bar magnet. On the other hand, gold shell has been used for huge SERS plasmon enhancement [7-13]. Recently we and others have reported that metal nanoparticle attached hybrid graphene oxide can exhibit synergistic properties for SERS detection [13-20]. As a result, for the detection of extremely low abundance tumor cells using SERS probe, here we report highly sensitive label-free SERS probe using multifunctional graphene oxide. In this Raman probe, graphene oxide is used for chemical enhancement and nanopopcorn is used for plasmon enhancement, where the central sphere will act as an electron reservoir, while the tips are capable of focusing the field at their apexes[21].

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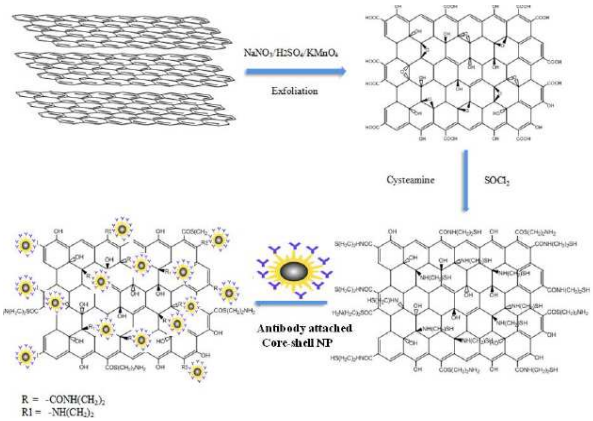


Scheme 1: Schematic representation showing our synthesis procedure for developing multifunctional graphene oxide.

Multifunctional graphene oxide was synthesized using several steps process, as shown in Scheme 1**.** At first, around 20 nm size iron nanoparticles were synthesized using tri-sodium citrate and sodium borohydride, using our reported method [21]. Figure 1A shows the TEM image of iron nanoparticle. Next, the popcorn shaped magnetic iron core-plasmonic gold shell nanoparticles were synthesized using the seed-mediated growth procedure as we have reported before [21]. Hitachi 5500 Ultra high-resolution SEM microscope imaging data as shown in Figure 1B, clearly shows the formation of around 60 nm size popcorn shape core-shell nanoparticle. Energy-dispersive X-ray spectroscopy (EDX) analysis, as shown in Figure 1C, indicates the presence of both iron and gold in core-shell nanoparticle. The absorption spectra in Figure 1E clearly shows the plasmon peak at 650 nm, which also indicates the formation of gold shell**.** In next step, graphene oxide from graphite was synthesized using strong oxidizing agents as we have reported recently also as shown in Scheme 1 [13]. After that, acid chloride functionalized graphene oxide were prepared by treating –COOH functionalized graphene oxide with thionyl chloride in the presence of N,N-dimethylformamide (DMF) solvent under an argon medium. In the last step, the acid chloride group was attached with cysteamine. Figure 1E shows the broad and structureless absorption spectrum of graphene oxide, mainly due to the E_{11} and E_{22} transitions [13-20]. E_{11} and E_{22} indicate transitions between the first and second singularities located around the Fermi level. In sixth step, iron core-gold shell nanoparticles were attached to graphene oxide via free thiol linkage of cysteamine. Figure 1D shows the JEM-2100F TEM picture of multifunctional graphene oxide, which clearly shows that the popcorn shape core-shell nanoparticles are nicely

decorated on graphene oxide sheet as assembly structure. Figure 1E shows the very broad extinction spectrum for hybrid materials and it is due to the fact that, as shown in TEM image in Figure 1D, core-shell nanoparticles are in close contact. Figure 1F shows that multifunctional graphene oxide is highly magnetic and as a result, we are able to separate it by a bar magnet. Experimental details for Raman measurement, cell culture and fluorescence analysis has been described in the supporting information.

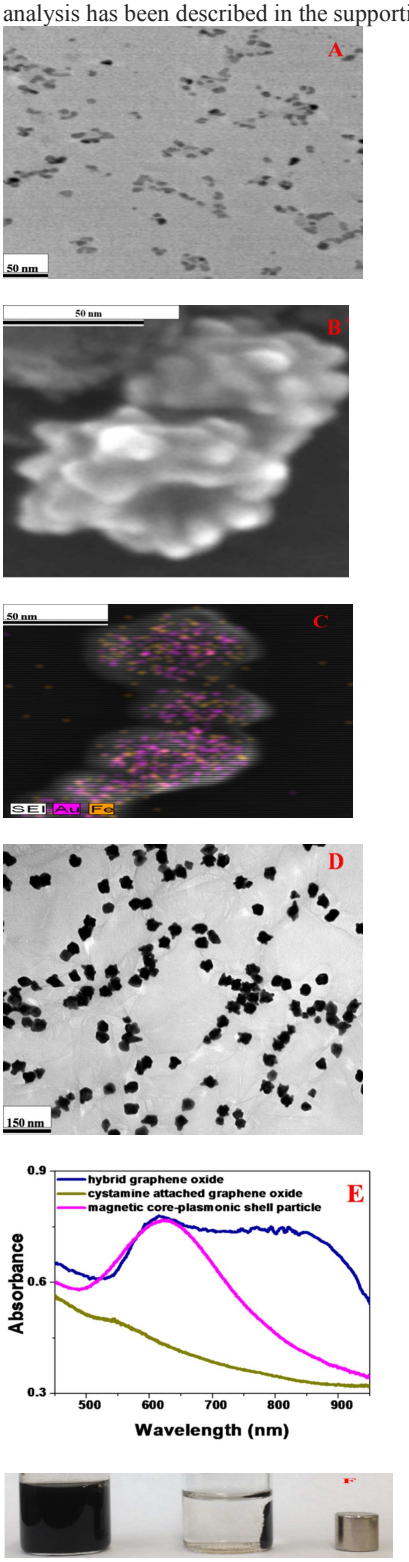


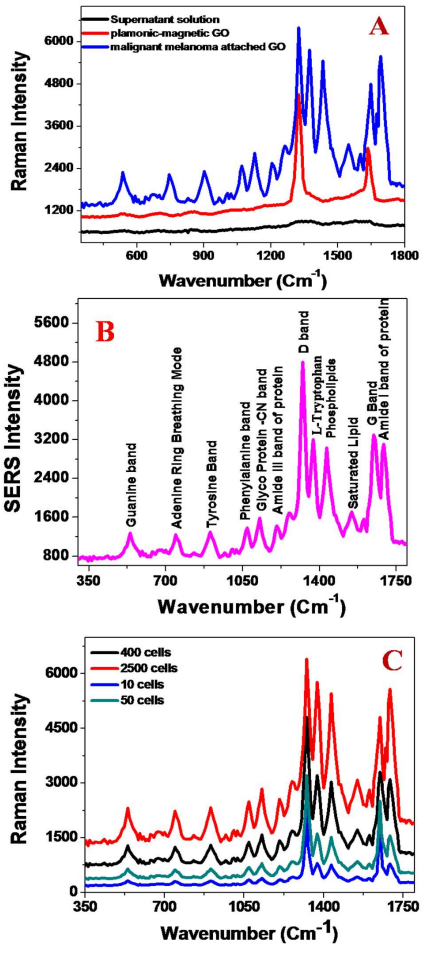
Figure 1: A) TEM image shows the morphology of iron magnetic nanoparticle. B) SEM image shows the morphology of iron core-gold shell popcorn shape nanoparticle. C) EDX mapping analysis of core-shell nanoparticle, D) TEM picture shows the morphology of magnetic core-gold shell nanoparticle attached hybrid graphene oxide. E) Extinction spectra of core-shell nanoparticle, graphene oxide and hybrid graphene oxide. F) Photograph shows hybrid graphene oxide is highly magnetic, and as a result, we can separate them by a bar magnet.

The tumor-associated ganglioside GD2 is known to be uniformly expressed in most melanomas [2-3,22], and as a result, anti-GD2 antibody attached multifunctional graphene oxide development is necessary for the separation of malignant melanoma UACC903 cell from the pool of other cells. For this purpose, we have first modified the core-shell nanoparticle, with anti-GD2 antibody, using the reported method [6,21]. After that, we have attached core-shell nanoparticle with graphene oxide via free thiol linkage of cysteamine. To understand that whether our multifunctional graphene oxide can be used for malignant melanoma analysis in the settings close to clinical diagnosis, malignant melanoma UACC903 cells were spiked at various densities into the suspensions of citrated whole rabbit blood. After 90 minutes of gentle shaking, we incubated 100 µL hybrid graphene oxide with 2 mL of malignant melanoma UACC903 cells infected blood sample. After 30 minutes of incubation at room temperature under gentle shaking, we used a bar magnet to separate & enrich malignant melanoma UACC903 cells attached with hybrid graphene oxide. After that, the supernatant blood sample was carefully removed with a pipette. Next, suspensions of the multifunctional graphene oxide attached malignant melanoma UACC903 cells and supernatant were characterized using TEM, Raman, fluorescence and enzyme-linked immunosorbent assay kits analysis, as shown in Figure 2A-2I. As shown in the Figure 2I, using enzyme-linked immunosorbent assays, we found no GD2 in the supernatant, indicating that malignant melanoma UACC903 cells are separated by a magnet. On the other hand, for the immunosorbent assays results, we noted that GD2 is present in the cell suspension that had attached to the multifunctional graphene oxide. The above immunosorbent experiments clearly indicate that the cells were malignant melanoma UACC903 cells. Our experiments, as shown in Figure 2I, indicate that the recovery of malignant melanoma UACC903 cells was about 99%. As shown in Figure 2A, our Raman experimental data show no SERS signal from supernatant, which also indicates that no multifunctional graphene oxide was present in the supernatant. On the other hand, we have seen very nice SERS signals from the cells which is attached with multifunctional graphene oxide and were separated by a magnet. As shown in Figure 2B, strongest Raman band seemed to consists of the graphene oxide D-band at 1340 cm^{-1} and a G-band at 1612 cm^{-1}, amide I band of protein, C=C stretching band of lipids and pyrrole ring vibration of L-tryptophan [3,12]. Other SERS bands can be assigned easily to tyrosine, phenylalanine, glycol protein, Amide-III and nucleic acids bands as reported in the literature [3,12]. Our experimental data, as shown in Figure 2B, clearly show that multifunctional graphene oxide based SERS can be ideal for the development of diagnostic assays for UACC903, malignant melanoma cell.

Next, to visualize the malignant melanoma cells by fluorescence microscope, we have performed a different set of experiments where we have modified the multifunctional graphene oxide with anti-GD2 antibody and methylene blue attached DNA. Figures 2E–H, show that the multifunctional graphene oxide was bound only to malignant melanoma UACC903 cells and not blood cells. Similarly, as shown in

Figure 2D, the TEM images also show that malignant melanoma UACC903 cells are attached with multifunctional grapahene oxide. All the above reported experimental data clearly show that multifunctional graphene oxide is highly selective for binding with the malignant melanoma UACC903 cells, which over-expresses GD2.

Figure 2C clearly shows nice SERS peak can be observed even in the presence of 10 malignant melanoma UACC903 cells/mL. This huge SERS sensitivity obtained using multifunctional graphene oxide is mainly due to the several factors and these are as follows: 1) in multifunctional graphene oxide platform, graphene oxide itself can enhance the Raman signal by chemical enhancement; 2) very high plasmonic SERS enhancement by plasmonic shell nanoparticle due to the aggregated assembly structure; 3) core-shell nanoparticles are in close contact in multifunctional graphene oxide, which is a very good condition to achieve huge SERS enhancement. Due to the formation of the aggregated structure, the 785 nm excitation and scattered light is coupled into surface plasmon modes of the hybrid graphene oxide substrate. The above coupling is highly responsible for the huge enhancement of SERS signal from malignant melanoma cells.



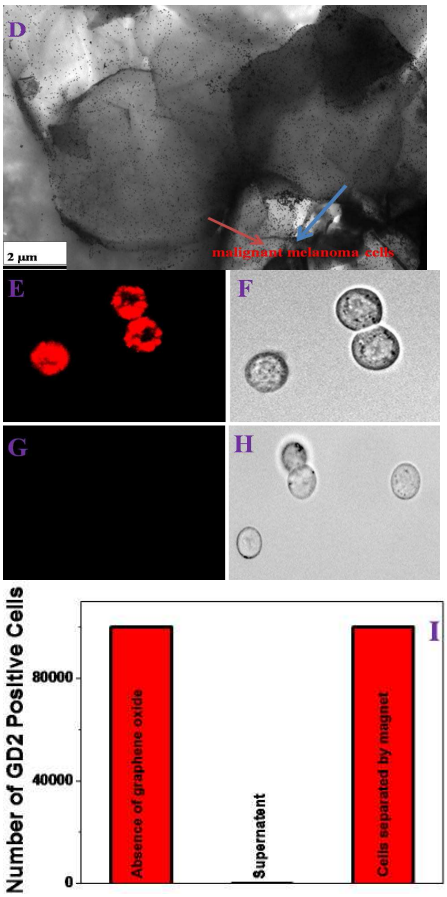


Figure 2: A) Plot showing SERS enhancement of Raman signal from malignant melanoma cells at 785 nm excitation, in the presence of hybrid graphene oxide. B) Plot shows SERS intensity from malignant melanoma UACC903 cells conjugated hybrid graphene oxide after magnetic separation. C) Plot shows SERS intensity variation from different concentrations of malignant melanoma UACC903 cells attached hybrid graphene oxide. D) TEM image shows aggregation of malignant melanoma UACC903 cells inside the bio-conjugated hybrid oxide sheet after the addition of 1000 UACC903 cells. Arrows are indicating malignant melanoma UACC903 cells. E) Fluorescent images of hybrid graphene oxide attached malignant melanoma UACC903 cells, after separation from blood sample using a magnet. F) Bright-field image of the same cells after magnetic separation. G) Fluorescence image of supernatant after magnet separation. H) Bright-field image of same supernatant after magnetic separation. I) Number of GD2 positive cell before and after magnetic separation, measured by immunosorbent assay.

In conclusion, in this communications, we have reported the development of multifunctional plasmonic-magnetic graphene oxide for the ultra-sensitive and label-free detection of malignant melanoma UACC903 cells from infected blood sample. We have shown that the sensitivity of the label-free plasmonic-magnetic graphene oxide based SERS assay can be as low as 10 cells /mL. Given the simplicity and the high sensitivity of the developed SERS assay, we believe that, this label-free assay can become a new method of choice for early stage CTC detection in clinical environment.

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