Gold Nanosphere-Deposited Substrate for Distinguishing of Breast Cancer Subtypes Using Surface-Enhanced Raman Spectroscopy

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Raman spectroscopy, as a nondestructive spectral technique, served as an efficient tool for investigating the molecular information of complex biological systems including cells. But the limitation of the technique is its low signal intensity. This inherent problem can be overcome by using surface-enhanced Raman scattering (SERS) technique. SERS can be achieved by roughening the surface of a substrate with noble metal nanoparticles. But preparation of homogenous SERS substrate with higher enhancement property is a big challenge. In this study, we report a homogenous gold (Au) nanosphere deposited ITO substrate that can significantly increase the Raman signals from analytes. By using this substrate we successfully characterize and distinguish two different sub-types of breast cancer cells. SERS method is simple, label free and non-toxic. Our newly developed Au nanosphere deposited substrate can be used as an effective platform for molecular detection, characterization, and distinguishing different cells originated from same or different organs.

**Keywords:** Gold Nanosphere-Deposited Substrate, Breast Cancer Cells, Label Free Characterization, SERS.

1. INTRODUCTION

Raman spectroscopy is a powerful analytical technique that enables a rapid, reagent-free and non-destructive analysis of living cells, but the application of Raman spectroscopy to cell-based analysis is very limited due to its weak and unstable signal. Surface-enhanced Raman scattering (SERS) phenomenon offers an exciting opportunity to overcome the critical disadvantages of this normal Raman spectroscopy. Using the SERS technique, the Raman signal can be enhanced up to 10 orders of magnitude. SERS can be achieved by roughening the surface of a substrate with noble metal nanostructures. An increased Raman signals appears due to the magnification of incident and Raman-scattered fields, an effect known as “electromagnetic enhancement.” Enhanced Raman signals are generated at the junction between two NPs, which are normally called ‘hot spots.’ For this reason, a great deal of attention has been focused on synthesis of shape-controlled SERS active structures with different morphologies including, nanospheres, nanorods or nanostars for examination of their Raman enhancing capabilities. But the use of colloidal nanoparticles for SERS has limitation due to their aggregation problem. Therefore, substrate modified with metal nanostructures is better for measuring SERS.

However, fabrication of SERS-active substrates was found to have a number of problems, including poor signal enhancement, uniformity or reproducibility and further process for removal of the template. Therefore, an advanced method for fabrication of the SERS-active surface is still required for more effective enhancement of Raman signals. Here, we report a simple, one step and template-free method for fabrication of a highly sensitive Au nanosphere deposited ITO substrate and its applications for analysis of biochemical composition of different breast cancer cell lines. Detection and characterization of cancer cells are important for personalized anticancer therapy. Among many techniques, immunofluorescence...
based techniques are more common for characterization of the cancer cells which need additional labeling of the cells, that may produce some cytotoxicity. Furthermore, fluorescence dyes have photobleaching effect. Other characterization techniques include polymerase chain reaction (PCR), but PCR needs separation, purification and amplification of nucleic acid.

In this study, we report a homogenous Au nanosphere deposited ITO substrate which possesses high signal enhancing property, and is able to label free characterization and distinguishing different breast cancer cells successfully. A simple one step electrochemical method was used to fabricate the modified ITO substrate (Fig. 1) which is non-toxic to cells, as well as the application method is non-destructive.

2. EXPERIMENTAL DETAILS
2.1. Materials
Hydrogen tetrachloroaurate (III) trihydrate (HAuCl$_4$·3H$_2$O), Polyethylene glycol (PEG), Phosphate Buffer Saline (PBS) and 4-mercaptobenzoic acid (4-MBA) were purchased from Sigma Aldrich. Roswell Park Memorial Institute (RPMI) 1640 media, Fetal bovine serum (FBS), antibiotics (Penicillin and Streptomycin), and trypsin (0.05% trypsin, 0.53 mM EDTA-4Na) were collected from Gibco (Invitrogen, Grand Island, USA). Deionized water (DIW) and ethanol sequentially, and then by basic piranha solution (1:1:5, H$_2$O$_2$:NH$_3$:H$_2$O) for 30 min at 80 °C. Finally, the substrates were cleaned by DIW and then dried under a N$_2$ stream to obtain a clean ITO surface. Au nanospheres were electrochemically deposited on ITO substrates (20 mm × 20 mm) using different concentrations of HAuCl$_4$ (0.4 to 0.7 mM) aqueous solution containing PEG (20 µM) as a surfactant. The potential was maintained at −1.3 V (vs. Ag/AgCl) and the deposition temperature was controlled for maintenance at 25 °C in an electric-heated thermostatic water bath. Different deposition time was applied to observe any changes in the formation of nanostructures. In order to remove any surfactant adsorbed on the Au nanosphere-deposited ITO surface, the substrates were rinsed in DIW and then boiled for 5 min with isopropyl alcohol. The active area for electrochemical deposition of Au nanospheres was 10 mm × 10 mm, and their surface morphologies were analyzed by a scanning electron microscope (SEM). A cell culture chamber unit with the dimensions of 1 cm × 1 cm × 1 cm (width × length × height) was attached to the Au nanosphere/ITO surface using polydimethylsiloxane (PDMS).

2.2. Fabrication of Au Nanospheres on ITO Substrate
ITO-coated glass substrates were cleaned by sonication for 2.2. Fabrication of Au Nanospheres on ITO Substrate
analytical grade reagents. All other chemicals were used throughout the experiment. All other chemicals were analytical grade reagents.

2.2. Fabrication of Au Nanospheres on ITO Substrate
ITO-coated glass substrates were cleaned by sonication for 15 min using 1% Triton X-100 solution, deionized water (DIW) and ethanol sequentially, and then by basic piranha solution (1:1:5, H$_2$O$_2$:NH$_3$:H$_2$O) for 30 min at 80 °C. Finally, the substrates were cleaned by DIW and then dried under a N$_2$ stream to obtain a clean ITO surface. Au nanospheres were electrochemically deposited on ITO substrates (20 mm × 20 mm) using different concentrations of HAuCl$_4$ (0.4 to 0.7 mM) aqueous solution containing PEG (20 µM) as a surfactant. The potential was maintained at −1.3 V (vs. Ag/AgCl) and the deposition temperature was controlled for maintenance at 25 °C in an electric-heated thermostatic water bath. Different deposition time was applied to observe any changes in the formation of nanostructures. In order to remove any surfactant adsorbed on the Au nanosphere-deposited ITO surface, the substrates were rinsed in DIW and then boiled for 5 min with isopropyl alcohol. The active area for electrochemical deposition of Au nanospheres was 10 mm × 10 mm, and their surface morphologies were analyzed by a scanning electron microscope (SEM). A cell culture chamber unit with the dimensions of 1 cm × 1 cm × 1 cm (width × length × height) was attached to the Au nanosphere/ITO surface using polydimethylsiloxane (PDMS).

2.3. Measurement of SERS Property of the Au Nanosphere-Deposited ITO Substrate
For measuring SERS property adequate quantity of 4-MBA (6 µM) solution was added to the Au nanosphere-deposited ITO substrate to cover the whole surface area, and incubated at room temperature for 12 hours for proper immobilization through self assembly process. To prevent drying the substrates were kept in covered petridishes and sealed with paraffin. After incubation the substrate was washed with ethanol and dried under nitrogen stream. Then SERS signal was measured from the 4-MBA immobilized substrate using 785 nm wavelength NIR laser.

2.4. Culturing of Cells
Two breast cancer cell lines (MDA-MB-231 and MCF-7) were obtained from ATCC (Manassas, VA). The cells were cultured at 37 °C in RPMI 1640 medium supplemented with 10% heat inactivated FBS and 1% antibiotics (penicillin and streptomycin) in a humidified atmosphere of 95% air with 5% CO$_2$. The cells were grown in TC-grade Petri dishes. At 80% confluence the cells were sub-cultured at a density of 1 × 10$^5$ cells/ml on culture plates, and then incubated for 72 hours. After every 48 hours the old medium was removed and fresh medium was added.

2.5. Raman Spectroscopy
For SERS experiment the cells were cultured on the chamber-attached glass substrate at a concentration of 2 × 10$^4$ cells/ml and incubated for 48 hours. Then, the biochemical composition of living MCF-7 and MDA-MB-231 cells were investigated by Raman spectroscopy using Raman NTEGRA spectra (NT-MDT, Russia). Before measuring SERS spectra from cell, SERS map of the cells

Figure 1. Schematic diagram for preparation of substrate; (a) bare ITO (b) gold (Au) nanosphere deposited ITO (c) immobilization of cancer cells on the modified substrate and measurement of SERS spectra.
were made by selecting an area on the basis of the size of the cells with 32 × 32 point number. Raman spectra were recorded using NIR laser emitting light at a 785 nm wavelength. Ten scans of 1s from 600 cm\(^{-1}\) to 1800 cm\(^{-1}\) were recorded and the mean of these scans was used for making a curve.

3. RESULTS AND DISCUSSION

3.1. Fabrication of Au Nanosphere-Deposited Substrate and Its Plasmon Absorption

Au nanospheres were fabricated by deposition of Au from HAuCl\(_4\) aqueous solution in the presence of PEG as a surfactant using electrochemical deposition method (Fig. 1). Figure 2(a) shows a topographic SEM image of the Au nanospheres fabricated on the ITO surface representing the uniform distribution of nanospheres with about diameter of 30 to 60 nm. Figure 2(b) demonstrates the UV-vis. spectra of the Au nanosphere-deposited ITO substrate. The substrate exhibited a strong surface plasmon absorption bands at 534 nm which is characteristic from Au spherical nanostructures.\(^{11}\) Since the enhancement of surface electric field depends on the surface Plasmon excitation, Au nanospheres strongly absorbed the energy and scatter electromagnetic field. For this reason, our Au nanosphere-deposited ITO substrate produced a high enhancement of Raman signals.

3.2. Study of SERS Property of the Au Nanosphere-Deposited ITO Substrate

To study the SERS property of the substrate, a Raman reporter 4-MBA (6 \(\mu\)M) was immobilized on the substrates prepared with different concentration of HAuCl\(_4\) and with different deposition time, and SERS spectra was measured on the substrate. Before measuring SERS spectra, a SER map was prepared from the 4-MBA immobilized substrate (Fig. 2(c)). The SERS map is showing homogenous distribution of hotspots. The SERS spectra of the 4-MBA molecule were measured with 1 s exposure time. The strong Raman band at 1076 and 1586 cm\(^{-1}\) are due to aromatic ring breathing mode.\(^{12}\) Among many substrates, the substrate prepared with 0.5 mM of HAuCl\(_4\) with 40 s deposition time showed highest SERS intensity (Fig. 3). The SERS result (Fig. 2(d)) demonstrated that the 4-MBA layer on the Au nanosphere-deposited ITO substrate produced a significant increase of intensity of the overall spectra.

3.3. SERS Spectra of Living Breast Cancer Cells

Two major enhancement factors are involved in SERS mechanism;\(^{13}\) one is electromagnetic enhancement and the other is chemical enhancement called as “charge-transfer effect.” Among two mechanisms the electromagnetic enhancement plays a key role in the SERS effect.\(^{14}\) The Au nanostructures allow the excitation of the Au nanosphere’s surface plasmon which could be increased the concentration of the electric field density. Due to the difference in dielectric constant between the Au nanostructure’s surface and the surrounding media, the electromagnetic energy density of the Au nanostructures could be a source for the electromagnetic enhancement that mainly contribute to the SERS effect. Moreover, Au nanospheres have high value of surface roughness due to its structural characteristics and finally induce the changes in electric field that can radiate both in a parallel and a perpendicular direction to the surface. Thus, if an incident photon falls on the roughened surface, there may be excitation of the
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Figure 4. Detection of SERS spectra from MCF-7 cells immobilized on the Au modified substrate; (a) Bright field image of an MCF-7 cell. (b) SERS map image of the cell, scanning area 23 μm. (c) Measured SERS spectra of a MCF-7 cell (solid line), Raman spectra of a MCF-7 cell (dash line), and laser exposure time 1 second.

plasmon resonance of the metal nanostructures and as a result scattering occurs.

Biochemical compositions of the living MCF-7 and MDA-MB-231 breast cancer cells were studied using the SERS spectra based on 785 nm NIR laser within the spectral range from 600 cm$^{-1}$ to 1800 cm$^{-1}$. Before measuring SERS spectra SERS map images was prepared from the cells (Figs. 4(a), (b), 5(a), (b)). The SERS spectra of the living cells consist of a series of bands corresponding to all biochemical substances found in the cells (Figs. 4(c), 5(c)). The tentative assignments of the measured SERS spectra from MCF-7 and MDA-MB-231 cells are presented in Table I. Biochemical compositions of the living MCF-7 breast cancer cells. The cells composed of high content of tyrosine and nucleic acids (Raman band at 820 and 1490 cm$^{-1}$ respectively). Figure 5 shows the contents of biomolecules in MDA-MB-231 breast cancer cells. These cells composed of high content of phenylalanine and fatty acids (Raman band at 615 and 1158 cm$^{-1}$ respectively). On the other hand, the intensity of Raman signal measured from the cells immobilized on the bare ITO (Figs. 4(c), 5(c)) are very low and non-distinguishable. The experimental results indicate that SERS can be used successfully to obtain molecular information from cells. Therefore, using the Au nanosphere deposited ITO substrate, it is easy to know the biochemical composition of the cells and easy to distinguish cells based on their chemical contents.

4. CONCLUSIONS

In this study, we introduced a highly homogenous gold nanosphere-deposited ITO substrate which can be prepared by simple one step electrochemical deposition method. The modified substrate can significantly increase Raman signals from analytes, and able to label-free characterize and distinguish different breast cancer cells based on biochemical composition detected by SERS. Compared to

<table>
<thead>
<tr>
<th>Raman bands (cm$^{-1}$)</th>
<th>Tentative Molecular origin type</th>
<th>Cell type</th>
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<tbody>
<tr>
<td>615</td>
<td>Phe Proteins</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>670</td>
<td>T-G Nucleic acids</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>712</td>
<td>C-N str. Lipids</td>
<td>MCF-7</td>
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<tr>
<td>780</td>
<td>DNA (T) Nucleic acids</td>
<td>MDA-MB-231</td>
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<tr>
<td>820</td>
<td>Tyr Proteins</td>
<td>MCF-7</td>
</tr>
<tr>
<td>936</td>
<td>C-C backbone Proteins</td>
<td>MDA-MB-231</td>
</tr>
<tr>
<td>960</td>
<td>C-C str, α-helix Proteins</td>
<td>MCF-7</td>
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<td>1003</td>
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<td>1126</td>
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<td>C-H str. Proteins</td>
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<tr>
<td>1413</td>
<td>Deoxyribose: stretching COO-</td>
<td>MDA-MB-231</td>
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<tr>
<td>1471</td>
<td>C-H def./bending</td>
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<tr>
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<tr>
<td>1566</td>
<td>Amide-II Proteins</td>
<td>MCF-7, MDA-MB-231</td>
</tr>
</tbody>
</table>

Table I. Comparison of the chemical compositions between MCF-7 and MDA-MB-231 cells on the basis of their Raman bands.
bare ITO substrate, Raman signals from Au nanosphere-deposited ITO substrate showed significantly increased signals confirming its better ability for enhancement of optical signals from cells. Au nanosphere-deposited ITO surface are non-toxic and able to sensitive detection of biochemical composition in living cells without cellular damages. Our study based on SERS technique using Au nanosphere-deposited ITO substrate can be successfully applied for sensitive biochemical characterization and distinguishing cancerous or other cells originated from same or different organ which has potential application in personalized anti-cancer therapy.

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References and Notes

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