Paper-based plasmonic platform for sensitive, noninvasive, and rapid cancer screening

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Abstract
Surface-enhanced Raman scattering (SERS) fingerprints of individual molecules offer the possibility of multiplexing as well as cancer screening. A highly sensitive, noninvasive, and rapid cancer screening platform encompassing exfoliative cytology and paper-based SERS technology is described. The SERS substrate which consists of plasmonic gold nanorods (GNRs) adsorbed on a piece of filter paper forms the flexible and three-dimensional heterogeneous scaffold for cancer screening. Different and reproducible SERS spectra are obtained from normal and cancerous cells due to specific biomolecular changes in cancerous cells. A diagnostic algorithm based on the ratio of the spectra values is adopted to distinguish between cells exfoliated from 20 normal and cancerous tissues, and a high sensitivity of 100% and specificity of 100% are achieved by I_{1600}/I_{1440} (peak ratio of signals at 1600–1440 cm\(^{-1}\)) and I_{1440}/I_{1340} (1440–1340 cm\(^{-1}\)), which is better than I_{1600}/I_{1340} (1600–1340 cm\(^{-1}\)) with a sensitivity of 70% and specificity of 60%. The combination of exfoliative cytology and paper-based plasmonic technology enables highly sensitive, rapid, and non-invasive cancer screening and has large clinical potential.

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1. Introduction
Cancer is the leading cause of death in the developed world and ranks second in developing countries. Approximately 12.7 million kinds of cancer have been diagnosed and 7.6 million people died of cancer worldwide every year (Jemal et al., 2011). Delay in diagnosis contributes to the poor living quality of patients and low survival rates (Ait Ouakrim et al., 2013). In spite of extensive research devoted to early cancer detection and diagnostics (Wang et al., 2013), many cancer screening techniques are still not very effective and new screening techniques targeting early cancer detection is urgently needed.

Exfoliative cytology is a rapid and noninvasive method to sample pathologic cells from suspicious lesions for microscopic examination by pathologists and epithelial brushings or scrapings have been used in cervical cancer screening for decades (Wandre et al., 2011). However, conventional cytology is considered unfavorable in some types of tumors due to the limited diagnostic ability and low sensitivity. The drawbacks can be attributed to inadequate cell sampling and subjective cytologic interpretation and diagnosis. Recently, molecular cytomorphometry and DNA aneuploidy analysis of these exfoliated cells have emerged to enhance the utility of exfoliative cytology (Weigum et al., 2010) and these improvements have stimulated renewed interest in cytology for clinical diagnostics of some diseases including cancer (Belgami and Shetty, 2013).

The efficacy of Raman scattering techniques in the diagnostics of cancer such as renal tumors (Zhuang et al., 2013), hepatocellular carcinoma (Salameh et al., 2013), lung cancer (McEwen et al., 2013), cervical cancer (Feng et al., 2013), and oral cancer (Kah et al., 2007) has been investigated. For example, Raman scattering conducted in biopsy in vitro has demonstrated the feasibility of differentiating normal and malignant tissues in oral cancer with good specificity and sensitivity of over 95% (Deshmukh et al., 2011). Healthy, malignant, and premalignant lesions can be discerned by Raman scattering performed in vivo (Singh et al., 2012b).

In these studies, the principal component analysis (PCA) provides good differentiation of the lesions, since normal tissues produce...
lipid-dominated spectra and malignant tissues yield protein-dominated ones (Patel et al., 2011). However, invasive and massive biopsy is generally needed to obtain the adequate amount of sample on account of small scattering cross section and direct illumination of the suspicious lesion with an intense laser beam may not be preferred.

Surface-enhanced Raman scattering (SERS) provides the capability of single molecule detection (Kneipp et al., 1997) and owing to the high sensitivity, inherent molecular specificity, and narrow bandwidth, SERS is an excellent diagnostic tool for cancer (Vendrell et al., 2013). For example, specific DNA sequences and mutations have been checked for genetic errors by SERS (Hu and Zhang, 2012) and ultrasensitive detection of proteins and RNA has been demonstrated (Li et al., 2012; Yuen et al., 2010). Moreover, immunoassays employing SERS labels have been established to selectively target and quantify biomarkers to facilitate early diagnosis in vitro (Lee et al., 2011c; Lin et al., 2011b; Yuen et al., 2010) and canerous cells have been detected from periphery blood and other media (Lee et al., 2011b). The multiplexing ability of molecular imaging with encoded SERS nanoparticles has been utilized in living cells and animal tumor models (Jokerst et al., 2012) and ultrasensitive detection of proteins and RNA has been demonstrated (Li et al., 2012; Yuen et al., 2010). Moreover, immunoassays employing SERS labels have been established to selectively target and quantify biomarkers to facilitate early diagnosis in vitro (Lee et al., 2011c; Lin et al., 2011b; Yuen et al., 2010) and cancerous cells have been detected from periphery blood and other media (Lee et al., 2011b). The multiplexing ability of molecular imaging with encoded SERS nanoparticles has been utilized in living cells and animal tumor models (Jokerst et al., 2012) and ultrasensitive detection of proteins and RNA has been demonstrated (Li et al., 2012; Yuen et al., 2010).

Herein, the merits of exfoliative cytology and SERS are combined to establish a sensitive, rapid, and noninvasive platform for cancer screening. Scheme 1 depicts the schematic diagram of the fabrication process of the paper-based plasmonic SERS substrate and the examination process in exfoliative cytology for oral cancer patients. Gold nanorods (GNRs) constitute the plasmonic nanostructures due to their strong surface plasmon resonance (SPR) extinction in the near-infrared (NIR) region, high stability, and low toxicity (Sun et al., 2008). The SERS substrate is produced conveniently by dipping a piece of common filter paper into the aqueous solution containing a high concentration of GNRs (Lee et al., 2010) and exfoliative cytology is subsequently performed to screen oral cancer. Oral cancer is chosen in this work because it is a typical epidermal cancer and the sites are easily accessible for direct examination without special techniques such as endoscopy. By collecting the exfoliative cells from the mouth of a patient and putting them on the plasmonic paper, cancer screening can be performed by SERS. Owing to the structural and metabolic changes, the exfoliated cells from the normal and cancer tissues yield different SERS spectra which can then be used to differentiate these two kinds of tissues with high specificity and sensitivity.

2. Experimental details

2.1. Preparation of plasmonic paper-based SERS substrate

The GNRs were synthesized in an aqueous solution using a seed-mediated growth method by using Hexadecyltrimethylammonium bromide (CTAB) as the surfactant and the detailed procedures have been reported previously (Wang et al., 2013). The GNRs were washed with distilled water twice to remove excessive CTAB. The concentration of the GNR solution was estimated to be about 3.25 nM according to the extinction coefficient at the LSPR wavelength (Orendorff and Murphy, 2006). A piece of common laboratory filter paper (2 × 2 cm²) was immersed in the culture dish containing 1.5 mL of the GNR solution for 24 h. Afterwards, the filter paper was removed from the GNR solution and rinsed thoroughly with distilled water, dried, and kept at 50% relative humidity and 23 °C before further experiments.

2.2. Characterization of GNRs

Transmission electron microscopy (TEM) images were taken on a JEOL 2010 transmission electron microscope at an accelerating voltage of 200 kV and absorption spectra were acquired on a TU-1810 UV–vis spectrophotometer (Purkinje General Instrument Co. Ltd, Beijing, China). Scanning electron microscopy (SEM) was conducted on Hitachi S4800 at an accelerating voltage of 10 kV. Raman scattering was performed on a Renishaw Invia Raman microscope equipped with a 785 nm NIR laser as the excitation source.

2.3. Preparation of cultured cells

The oral squamous cell carcinoma (OSCC) cell line CAL-27 (CRL-2095, ATCC) was supplied by the Affiliated Ninth People’s Hospital, Shanghai Jiaotong University, Shanghai Research Institute of Stomatology, Shanghai, China. The cells were cultured in Dulbecco’s modified eagle medium (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, California, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The protocols for oral keratinocyte culture were similar to the previous method (Hiroshima et al., 2011) and the oral mucosa collection was approved by the Ethics and Investigation Committee of the School & Hospital of Stomatology, Wuhan University. The surgically excised oral mucosa tissues from the patients with informed consent were digested with dispase (2.4 mg/mL, Boehringer Mannheim, Indianapolis, Ind.) overnight at 4 °C. The oral keratinocytes were mechanically dissociated from the underlying tissue by gentle agitation and the digested cells were collected. The cells were then seeded onto the filter paper and kept for 24 h.

Scheme 1. Schematic diagram illustrating the fabrication of the paper-based SERS substrate and SERS examination of the exfoliated cells obtained from oral-cancer patients.
connective tissue and the surface epithelium was digested further in 0.125% EDTA trypsin (GIBCO, Buffalo, N.Y.) for 10 min at 37 °C. The cell pellets were collected and resuspended in the keratinocyte growth medium 2 (KGM-2, Clonetics, San Diego, Calif.) and cells at passages 3–5 were used experimentally. The cells were harvested and fixed in 4% paraformaldehyde for 15 min. After washing twice, the cells were stored in PBS at 4 °C.

2.4. Cell exfoliation

The cell samples were collected from 10 normal healthy individuals and 10 oral cancer patients. The healthy individuals showed no sign of epithelial abnormality in the oral cavity. The eligibility criteria included patients presented with a visible oral lesion, leukoplakia, or erythroplakia and who needed surgical biopsy for suspicious oral cancer. This study was reviewed and approved by the Ethics and Investigation Committee of the School & Hospital of Stomatology, Wuhan University. The clinical samples were collected from the lesion site or surgically excised tissues using similar protocols. The specimens were collected using a cytology brush while brushing firmly against the epithelial surface back and forth 10–15 times and applying a moderate pressure until pinpoint bleeding was attained. The cells were then released from the brush, suspended in Thin-Cytologic Test storage fluid with vigorous agitation for 15–30 s, and collected by negative pressure filtration. After fixation for 20 min, the medium was removed by centrifugation at 2000 rpm for 5 min. The cells were washed twice in the PBS buffer and re-suspended in PBS at 4 °C for long-term storage.

2.5. Optimization and detection procedures

Prior to performing SERS, the medium was removed by centrifugation at 2000 rpm for 5 min. The cells on the bottom of the centrifuge tube were suctioned out and placed on the SERS substrate. They were washed twice with water gently. The laser beam was positioned through a Leica imaging microscope objective lens (50 ×). The laser power illuminating the sample was about 50 mW and an integration time of 60 s was adopted. The spectra were acquired from five different locations on the sample and the results from five measurements were averaged and presented after baseline subtraction referenced to the control sample.

3. Results and discussion

The paper-based SERS substrate was fabricated by loading GNRs as the plasmonic nanostructures on a piece of commercial filter paper and the GNRs were synthesized by a seed-mediated approach with CTAB as the capping agent (El-Sayed, 2001). Fig. 1A reveals that the average diameter and length of the GNRs are 13 ± 2 nm and 49 ± 3 nm, respectively. After immersing the filter paper in the GNR solution for 24 h, a dark red color is observed from the paper indicating successful incorporation of the GNRs (Fig. 1B). Furthermore, a nearly 60% drop in the extinction intensity is observed from the GNRs. As shown in Fig. 1C, there are two characteristic peaks at ~524 and ~782 nm in the extinction spectra of the GNR solution corresponding to the transverse SPR band and longitudinal SPR band of the GNRs, respectively. The GNR-loaded paper yields a similar extinction spectrum but with a slightly broader and blue-shifted SPR band. There is also increased scattering from the filter paper.

Fig. 2A and B shows the surface morphology of the bare filter paper and plasmonic paper (loaded with GNRs), respectively. It can be observed that large quantities of nanorods adsorb on the paper surface but there is no large-scale aggregation of the nanorods. Cellulose has a large number of hydroxyl groups, which are accessible for attachment to positively charged species (Samir et al., 2005). The colloidal GNRs can randomly assemble on the filter paper possibly due to the electrostatic interaction between the positively charged GNRs (CTAB-capped) and negatively charged filter paper which is mainly composed of cellulose (Lee et al., 2011a). There is no noticeable change in the GNR density on the plasmonic paper and only a small amount of GNRs are released to water under vigorous rinsing, suggesting strong adhesion strength and good stability (Fig. S1).

The SERS effect of the plasmonic paper has been investigated by loading a typical NIR Raman molecule: 3,3′-Diethylthiatricarbocyanine iodide (DTTCI) (Fig. S2). A great Raman enhancement and a monotonic increase in the intensity with DTTCI concentration can be observed. Furthermore, based on the SERS intensity of the 1020 cm⁻¹ band, the enhancement factor rendered by the plasmonic paper is calculated to be about 2 × 10⁶ using the method previously reported (Lee et al., 2010). Similar observations have been made by other research groups (Abbás et al., 2013; Holthoff et al., 2009; Cheng et al., 2011; Lee et al., 2011a; Ngo et al., 2013; Yu and White, 2013). Not only is this technique economical and environmentally friendly, but also the use of a plasmonic paper-based substrate enables the detection of biomarkers in complex physiological fluids (Tian et al., 2012; Chen et al., 2013;
Nergiz et al., 2013). Hence, its potential as a cancer screening method is further investigated by examining the exfoliated cells.

Raman spectra are acquired from the normal cultured cells (oral keratinocyte) and cancerous cultured cells (CAL-27) on the paper-based SERS substrate (Fig. 3). Excellent signal-to-noise ratios are achieved from both the cancer and normal cultured cells on the plasmonic substrate, but the Raman signals acquired from both types of cultured cells on the bare paper are quite weak.

It should be noted that in our experiments, the incident laser power is low (50 mW) and the wavelength is in the NIR region (785 nm) in order not to cause substantial cell damage. Owing to the unique SPR properties, the signals can be amplified and the cells must be in direct contact with the substrate. The experiments performed by placing the suspension containing the fixed cells on the substrate directly were similar to those adopted in previous studies (Kah et al., 2007; Premasiri et al., 2005). After drying, only the properly oriented molecules close to the SERS active substrate, presumable a fraction of the cell surface components, are enhanced (Picorel et al., 1992). However, the fixed cells have lost the integrity of cell membrane after their permeabilization with detergents (Darzynkiewicz et al., 1992). Some intracellular components such as fragments of DNA attached to the nuclear matrix might contact with the plasmonic paper.

The SERS spectra in Fig. 3 show different vibrational fingerprints from the normal and cancer cultured cells consistently compared to results obtained from former studies (Ghanate et al., 2011; Singh et al., 2012a). When healthy, epithelial cells are rich in adipose, the normal cultured cells show an intense dCH$_2$ band at 1440 cm$^{-1}$ and relative weak DNA peak at 1340 cm$^{-1}$. The broad peak width in the amide I region between 1550 and 1655 cm$^{-1}$ and the ester band at $\sim$1744 cm$^{-1}$ suggest a large concentration of lipid (Fig. 3A). On the contrary, in the tumorous cells, there is loss in the architectural arrangement of different layers indicating loss of the lipid features. In addition, these cells have large amounts of surface and receptor proteins as well as large nuclei, enzymes, antigens, and antibodies which tend to produce protein and DNA dominated-spectra. Hence, the oral cancer cells yield an intense DNA band at 1340 cm$^{-1}$ but relatively weak dCH$_2$ band at 1440 cm$^{-1}$. The broad peak width in the amide I region between 1550 and 1655 cm$^{-1}$ and broad peaks in the amide III region (1200–1350 cm$^{-1}$) suggest high concentrations of
protein and DNA (Fig. 3B). The clear differences in the peaks and intensity existing between the two types of cultured cells make the paper-based SERS substrate practical in cancer screening.

The excellent SERS effects enable the establishment of an oral cancer screening method. 60% of the patients and healthy people are male and their average age is 52 (37–70 and 40–67 years of age for patients and healthy people, respectively) and so there is very little influence from the gender and age (Table S1). Diagnosis is made based on surgical biopsy of the lesion site by oral and maxillofacial pathologists.

The paper-based SERS technique can be used to differentiate normal and cancer conditions. Small amounts of exfoliated cells are collected from the cancerous tissues and normal tissues from the patients (Table S1) and put on the SERS substrate to evaluate the efficacy. Similar to the previous study (Salameh et al., 2013), the exfoliated cells from the normal tissue show a relatively intense dCH2 band at 1440 cm$^{-1}$ indicative of increased lipid content. On the other hand, the exfoliated cells from the cancerous tissue yield an intense DNA band at 1340 cm$^{-1}$ and broad amide I band between 1200 and 1350 cm$^{-1}$ due to larger amount of protein and DNA (Fig. 4). That is, the exfoliated cells from the normal tissue yield lipid-dominated spectra while those from the cancerous tissue give rise to protein-dominated and DNA-dominated spectra. The corresponding Haematoxylin and Eosin staining results show a larger nucleo-cytoplasmic ratio and nuclear pleomorphism in the cells from the cancer tissue than normal one and hence, it is possible to use the spectra to diagnose the existence of tumors.

To further quantitatively assess the cancer screening capability, the ratios of the SERS peaks obtained from normal and cancerous cells are determined. According to the results illustrated in Fig. 4, the SERS peak intensities at 1340, 1440, and 1600 cm$^{-1}$ show obvious differences between the normal and cancerous cells. An empirical diagnostic algorithm based on the ratios of $1600/1440$ cm$^{-1}$ ($I_{1600/1440}$ for protein and lipid), $1440/1340$ cm$^{-1}$ ($I_{1440/1340}$, lipid and DNA), and $1600/1340$ cm$^{-1}$ ($I_{1600/1340}$, protein and DNA) is adopted. Fig. 5A shows that $I_{1600/1440}$ of the normal exfoliated cells varies between 0.08 and 0.80 ($0.59 \pm 0.2$, $n = 10$), $I_{1440/1340}$ between 1.00 and 4.47 ($1.63 \pm 1.03$, $n = 10$), and $I_{1600/1340}$ between 0.39 and 1.10 ($0.87 \pm 0.33$, $n = 10$). With regard to the cancerous cells, $I_{1600/1440}$ varies between 1.16 and 5.54 ($2.52 \pm 1.49$, $n = 10$), $I_{1440/1340}$ between 0.38 and 0.9 ($0.61 \pm 0.20$, $n = 10$), and $I_{1600/1340}$ between 0.93 and 2.88 ($1.62 \pm 0.61$, $n = 10$). Statistics analyses reveal significant differences for the three ratios with $I_{1600/1440}$ ($p < 0.01$), $I_{1440/1340}$ ($p < 0.01$), and $I_{1600/1340}$ ($p < 0.01$), thus corroborating that feasibility of this technique in oral cancer screening.

To determine the specificity and sensitivity of the cancer screening method, the scatter plot is obtained from exfoliated normal and cancerous cells. As shown in Fig. 5B, the decision line ($I_{1600/1440} = 1.10$, $I_{1440/1340} = 0.95$) as the diagnostic threshold separates the cancerous group from the normal one with high sensitivity and specificity. However, the dotted line for $I_{1600/1340}$ ($I_{1600/1340} = 1$) fails to differentiate cancerous cells from normal ones in the 20 patients with a sensitivity of 70% and specificity of 60%. Hence, it is assumed that the cells are cancerous if $I_{1600/1440}$ is larger than 1.10 and $I_{1440/1340}$ is less than 0.95, as compared to that $I_{1600/1440}$ is less than 1.10 and $I_{1440/1340}$ is more than 0.95 for normal cells. This simple but effective diagnostic algorithm has been developed in previous studies. The ratio of intensities at 1455 and 1655 cm$^{-1}$ has been used to classify precancerous and normal conditions (Han et al., 2009). To classify colorectal cancer and normal serum

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![Fig. 4. Raman spectra acquired from the exfoliated cells: (A) normal tissue and (B) cancerous tissue on the plasmonic paper-based SERS substrate (left) and corresponding Haematoxylin and Eosin staining (right).](image-url)
samples, the ratio of the SERS peak intensity at 725 cm\(^{-1}\) for adenine to that at 638 cm\(^{-1}\) for tyrosine has been shown to be significantly higher in cancer serum than normal serum with a sensitivity of 68.4% and specificity of 95.6% (Lin et al., 2011a). In our experiment, the higher cancer screening ability may be due to the greater SERS enhancement (Premasiri et al., 2005) and reliable samples (Wandeur et al., 2011).

Clinically all suspicious lesions should be subjected to surgical biopsy and pathologic evaluation but it may be unnecessary and even invasive. Besides, pathologic evaluations tend to be subjective and sometimes unreliable due to considerable inter- and intra-examiner variations in the histopathologic diagnosis. Rose Bengal staining malignant tissues has been used in cancer screening by dentists based on the color change (Du et al., 2007). The results demonstrate that paper-based SERS in conjunction with exfoliative cytology is a convenient, fast, and cost effective tool for the non-invasive detection and screening of oral cancer. Our next step will be to conduct more detailed prospective studies to verify the reliability of this new cancer detection method.

4. Conclusion

A paper-based plasmonic substrate is proposed for non-invasive and effective cancer screening. Different and reproducible SERS spectra are obtained from normal and cancerous cells. By comparing the peak intensities \(I_{1600}/1440\), \(I_{1440}/1340\) and \(I_{1600}/1340\), cells exfoliated from cancerous and normal tissues can be discerned due to the molecular changes in the cancerous cells. \(I_{1600}/1440\) and \(I_{1440}/1340\) effectively differentiate cancerous cells from normal ones from 20 patients with a sensitivity of 100% and specificity of 100%, compared to \(I_{1600}/1340\) with a sensitivity of 70% and specificity of 60%. The results demonstrate that paper-based SERS in conjunction with exfoliative cytology is a convenient, fast, and cost effective tool for the non-invasive detection and screening of oral cancer.

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Appendix A. Supplementary material

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