Bimodal optical diagnostics of oral cancer based on Rose Bengal conjugated gold nanorod platform

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1. Introduction

As a typical kind of epithelial cancer, oral cancer is the sixth most common cancer in both men and women worldwide and the 5-year survival rate of patients suffering from the disease is only about 50% [1,2]. The common symptoms during the early stage of oral cancer include asymptomatic, innocuous-appearing mucosal alteration which can also be a manifestation of a variety of mucosal diseases [3]. Hence, although it may be precancerous, it is difficult to distinguish and there is an urgent need for early detection of cancer cells. The label-free sensing assay utilizes RB-GNRs as the sensing probe and by monitoring the aggregation-induced red-shift in the NIR absorption wavelength, specific and quantitative analysis of the oral cancer cell lysate is accomplished down to a detection limit of 2000 cells/mL. By employing the RB-GNRs as an imaging probe, an imaging assay is established on a home-made NIR absorption imaging system. Based on the NIR absorption by the RB-GNRs specifically conjugated with the oral cancer cells, multi-channel, rapid and quantitative detection of oral cancer cells is demonstrated. The high sensitivity and specificity of the RB-GNR platform as demonstrated by the two complementary assays provide non-invasive optical diagnostics of oral cancer cells enabling convenient screening and monitoring.

Early detection of cancer often requires time consuming protocols and expensive instrumentation. To address these limitations, a Rose Bengal conjugated gold nanorod (RB-GNR) platform is developed for optical detection of cancer cells. The GNRs are modified by poly(allylamine hydrochloride) and conjugated with RB molecules to produce RB-GNRs which exhibit strong optical absorption in the near-infrared (NIR) region, good stability in aqueous solution, low cytotoxicity, and high specificity to oral cancer cells. The label-free sensing assay utilizes RB-GNRs as the sensing probe and by monitoring the aggregation-induced red-shift in the NIR absorption wavelength, specific and quantitative analysis of the oral cancer cell lysate is accomplished down to a detection limit of 2000 cells/mL. By employing the RB-GNRs as an imaging probe, an imaging assay is established on a home-made NIR absorption imaging system. Based on the NIR absorption by the RB-GNRs specifically conjugated with the oral cancer cells, multi-channel, rapid and quantitative detection of oral cancer cells is demonstrated. The high sensitivity and specificity of the RB-GNR platform as demonstrated by the two complementary assays provide non-invasive optical diagnostics of oral cancer cells enabling convenient screening and monitoring.

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SPR properties as a result of plasmonic coupling [33]. Slight variations in the environment around the GNRs can induce significant changes in the longitudinal SPR (LSPR) peak wavelength, which is much more sensitive than the transverse SPR (TSPR) peak wavelength observed from spherical gold nanoparticles [34]. Therefore, GNRs are potentially efficient probes to sense specific target binding events on biological samples [10,35–37]. Moreover, GNRs have very strong optical extinction in the NIR LSPR band. The extinction coefficient of GNRs is about 6 × 10^9 M^−1 cm^−1 that is much larger than the value of 2.7 × 10^6 M^−1 cm^−1 of gold nanoparticles and several orders of magnitude bigger than those of traditional organic chromophores [38]. Therefore, GNRs can also be used as direct NIR absorption imaging probes in optical diagnostics [39].

Nanoparticles usually target cells by receptor-mediated binding or endocytosis and a nanoparticle surface modified with a ligand can efficiently bind with target cells. Surface functionalization of gold nanoparticles by conjugation with a specific agent such as aptamer, peptide, antibody, and protein has been proposed for diagnosis and thermo-phototherapy of cancer cells [40]. With regard to oral cancer, Rose Bengal (RB), the 4,5,6,7-tetrachloro-2′,7′-dimethyl-4H-1,3-diazolo[2,3-a]pyrazine, is a specific agent of cancer cells [41–45]. RB has been shown to have specificity to oral cancer through inhibition of DNA polymerases of oral cancer cells [41]. Based on its specificity, the RB molecule has been used as a photosensitizer in fluorescence detection of oral cancer [43]. RB has also been employed as a staining molecule to detect oral precancerous and malignant lesions clinically with colorimetric evaluation and high sensitivity and specificity of 93.9 and 73.7%, respectively, have been demonstrated [44]. Furthermore, the RB molecule is a suitable agent for nanoparticle bio-conjugation, typical photosensitizer for photodynamic therapy (PDT) [46–51], and probe for surface-enhanced Raman scattering (SERS) [52]. In this paper, we describe the method to conjugate GNRs with RB molecules and demonstrate that this RB-GNR platform is an excellent NIR-optical diagnostic tool for oral cancer.

2. Experimental section

2.1. Synthesis and characterization of RB-GNR probes

2.1.1. Materials

Chloroauric acid (HAuCl₄ 4H₂O, 99.9%), silver nitrate (AgNO₃, 99.8%), L-ascorbic acid (99.7%), sodium chloride (NaCl, 96.03%), and hydrochloric acid (HCl, 36–38%) were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China). Sodium borohydride (NaBH₄, 96%), poly(allylamine hydrochloride) (PAH, MW ~ 15,000 g/mol), and RB (95%) were obtained from Aldrich (America). Sodium chloride was used as received without purification. Ultrapure water with a resistivity of about 18.25 MΩ cm was used as the solvent in all experiments.

2.1.2. Synthesis of GNRs

The GNRs were synthesized in an aqueous solution using a seed-mediated growth method [24,28]. The 3–4 nm gold seed particles were prepared by mixing 5 mL of 0.5 μM HAuCl₄ with 5 mL of 0.2 μM CTAB. The solution was stirred vigorously followed by drop wise addition of 600 μL freshly prepared ice-cold 10 μM of NaBH₄. The seed solution was left for more than 2 h before use. In the GNR synthesis, 18 mL of 5 μM HAuCl₄ and 225 μL of 0.1 μM AgNO₃ were added to 90 mL of 0.2 μM CTAB and then 200 μL of 12 μM HCl and 10.5 μL of 10 μM ascorbic acid were added and gently swirled as the color changed from dark orange to colorless. After the color had changed, 120 μL of the CTAB-stabilized gold seed solution was rapidly injected. The resulting solution was gently mixed for 10 s and left undisturbed overnight. Finally, the GNR solution was centrifuged at 12,000 rpm for 15 min to stop the reaction. The supernatant was removed and precipitate was resuspended in ultrapure water. The GNR concentration was estimated to be about 0.65 μg/mL according to extinction coefficients for the LSPR wavelength [53].

2.1.3. Synthesis of RB-GNR probes

The GNRs were coated with PAH using a method previously described by Murphy et al. [54]. 20 mL of 10 mg/mL PAH in 1 mL NaCl and 10 mL of 10 μM NaCl were added to 100 mL of 0.65 μg/mL GNRs and stirred for several hours at room temperature. Centrifugation cycle of 10,000 rpm for 10 min was performed to remove the excess PAH and NaCl. After centrifugation in GNRs, 0.05 mM PAH-coated GNRs aqueous solution was mixed with 100 μL of 10 μM RB aqueous solution and the mixture was kept in 30 °C for at least 3 h. Afterwards, the samples underwent centrifugation at 10,000 rpm for 10 min to remove the excess RB, and redispersed in 100 μL of ultrapure water. The concentration of the RB-GNRs was about 0.5 μg/mL.

2.1.4. Characterization

Transmission electron microscopy (TEM) images were taken on a JEOL 2100 transmission electron microscope at an accelerating voltage of 200 kV. The zeta potential and hydrodynamic distribution of the samples was determined using a Zetasizer (Nano ZS90, Malvern Instruments, UK) at 25 °C. The samples were dispersed in deionized water with a concentration of 0.5 μM. Absorption spectra were taken on a TU-1810 UV-Vis-NIR spectrophotometer (Purkinje General Instrument Co. Ltd. Beijing, China). Photoruninescence emission spectra were recorded on a Hitachi F-4500 fluorescence spectrophotometer with a Xenon lamp as the excitation source.

2.2. Cell cultures

Two oral squamous cell carcinoma (OSCC) cell lines were used in this study. The human OSCC cell line, CAL-27 (CRL-2051, ATCC), was supplied by the Affiliated Ninth People’s Hospital, Shanghai Jiashang University, Shanghai Research Institute of Stomatology, Shanghai, China. The human OSCC cell line, Tca8113 (SCC from human tongue), was purchased from China Center for Type Culture Collection (CCTC, Wuhan, China). Both the cell lines were cultured in Dulbecco’s modified eagle medium (HyClone, UT, USA) supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, California, USA) in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The protocols for obtaining human oral mucosa samples were reviewed and approved by the Ethics and Investigation Committee of the School & Hospital of Stomatology, Wuhan University. Patients with cleft lip and palate who received treatment at the Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Wuhan University were given sufficient prior information about this study, and written consent was obtained from patients (1 male and 2 female with a mean age of 1.5 years). The tissues from oral mucosa were harvested during oral surgery. The mucosal sample was put in a 15 mL conical tube containing 5 mL of serum-free Dulbecco’s modified eagle medium containing 1% antibiotic–antimycotic (Invitrogen). The samples were then pured and cells were serially passaged as previous described with only minor modifications [13]. The collected oral mucosa tissues were digested with dispase (2.4 mg/mL, Boehringer Mannheim, Indianapolis, Ind.) overnight at 4 °C. After incubation, the oral keratocytes were mechanically dissociated from the underlying connective tissue and the surface epithelium was digested further in 0.125% EDTA trypsin (GIBCO, Buffalo, N. Y.) for 10 min at 37 °C. After centrifugation at 1000 rpm for 5 min, the cell pellets were collected and resuspended in the keratocyte growth medium (KGM-2; Clonetics, San Diego, Calif.). The cultures were incubated at 37 °C under 5% CO₂ until confluence and passages 3–5 were used experimentally.

2.3. Cytotoxicity evaluation

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium reduction assay was used to measure the cytotoxicity of the RB-GNR probes on OSCC cells CAL-27. The cancer cells were distributed in 96-well plates at 1 × 10⁵ cells/well and allowed to attach overnight. In the treatment of the RB-GNR probes, the culture medium was replaced with one containing various concentrations of the RB-GNR probes from 0 to 0.45 μg/mL and incubated at 37 °C. After 4 h, the medium was removed and the cells were rinsed twice with PBS. The wells were refilled with the complete medium and the cells were incubated for another 24 h. Subsequently, the cells were incubated in a culture medium with the MTT solution (5 mg/mL) at 37 °C and 5% CO₂ for 4 h. The cells were lysed and blue formazan crystals were dissolved in 150 μL of DMSO. The optical densities (OD) were measured at 490 nm on an enzyme-linked immune sorbent assay (ELISA) plate-reader. Three wells were used for each concentration. The percentage of surviving cells was calculated using the following formula: (OD treated/OD control) × 100 and three independent experiments were conducted.

2.4. SPR sensing of cancer cell lysates

2.4.1. Preparation of cell lysates

The OSCC cell lines CAL-27, Tca8113, and primary human oral epithelial cells were plated on 10 cm diameter tissue culture dishes until confluence. The confluent cultures of all the cells were incubated with 0.25% trypsin for 2 min, carefully detached by a cell scraper, washed twice in PBS, and resuspended in deionized water at a density of 1 × 10⁶/mL. The cell lysate was frozen in liquid nitrogen and thawed at 37 °C using four freeze–thaw cycles. To remove the crude cell debris, the lysate was centrifuged at 5000 rpm for 15 min. The supernatant of the cell lysate was sampled independently by a 1.5 mL pepette and stored at −70 °C until use. The corresponding cell debris was also collected as the control sample in the sensing experiments.
2.4.2. Sensing experiments

The cell lysates of CAL-27 and Tca8113 cancer cells, and primary human oral epithelial cells were used as the sensing samples. Different volumes (10, 30, 50, 70, 100, and 150 µL) of the cell lysates with cell density of $1 \times 10^9$/mL were added to 4.5 mL of 0.5 mM RB-GNR solution. The corresponding cell concentrations were $2.2 \times 10^5$, $6.5 \times 10^5$, $1.07 \times 10^6$, $1.49 \times 10^6$, $2.33 \times 10^6$, and $3.03 \times 10^6$ cells/mL. The mixtures were incubated for about 2 h, and then their absorption spectra were measured.

2.5. NIR absorption imaging of cancer cells

2.5.1. NIR absorption imaging system

A home-made NIR absorption imaging system was set up in a dark room in order to prevent stray light. An LED (16 V, 5.0 W, 800 nm ± 10 nm) serves as the light source and the image is recorded by a CCD camera (MTV-1881EX, MINTRON) equipped with a 790–810 nm band-pass filter to eliminate scattering from other wavelengths. The LED and CCD are placed, respectively, on the bottom and top of a transparent sample stage in 180° geometry. The system control and image acquisition are via computer software written by ourselves.

2.5.2. Imaging experiments

The CAL-27 cancer cells and primary human oral epithelial cells were used as the imaging samples. The cells were distributed in 96-well plates with different concentrations of $1 \times 10^4$, $2 \times 10^4$, $3 \times 10^4$, and $4 \times 10^4$ cells/well, and incubated with 0.45 mM RB-GNR probes at 37 °C for 2 h. The probe targeted cells were allowed to attach to the plate, and the cells were rinsed twice with PBS to remove the unbound probes. 200 µL of water were added to increase the optical path. The 96-well plate was then examined by using the NIR absorption imaging system described above, and the water sample was used as the basement. Under software control, the NIR absorption data were acquired and transferred to the output pseudo-color images, and data representing “LSRP absorption intensity” of the samples.

3. Results and discussion

3.1. Preparation and characterization of RB-GNR nanoparticles

In this work, RB is used as the specific agent and conjugated on GNRs to obtain the RB-GNR probes (RB-GNRs) for oral cancer diagnostics. The TEM image of the synthesized RB-GNRs is shown in Fig. 1. It can be seen that the average diameter and length of the RB-GNRs are $13 \pm 2$ nm and $52 \pm 4$ nm, respectively. However, their average hydrodynamic diameter is about 60 nm (inset in Fig. 1) which is larger than the dimensions measured by TEM possibly due to the contributions from the polyelectrolyte and RB coating. The average hydrodynamic diameter is about 60 nm (inset in Fig. 1) which is larger than the dimensions measured by TEM possibly due to the contributions from the polyelectrolyte and RB coating. The results demonstrate that the RB-GNRs have the suitable size and good dispersibility in an aqueous solution.

Fig. 2 schematically illustrates the preparation of the RB-GNR probes. The original CTAB-coated GNRs are synthesized by using a well known seed-mediated growth method utilizing the CTAB surfactant [24]. A cationic polyelectrolyte (PAH) is used to modify the surface of the GNRs to obtain PAH-coated GNRs (PAH-GNRs). The PAH-GNRs were mixed with RB molecules in an aqueous solution for 3 h to facilitate absorption of RB on the GNR surface. After the PAH-GNRs + RB solution is centrifuged, the supernatant containing excess RB is removed, and the RB-GNRs in the precipitate is collected and re-dispersed in an aqueous solution to obtain the RB-GNRs. To demonstrate the effects of PAH in the synthesis of the RB-GNRs, a control experiment in which the original GNRs are directly mixed with RB without PAH modification is performed. The GNRs + RB solution is centrifuged and the supernatant and precipitate were collected.

Fig. 3a and b show the absorption spectra of the samples prepared in the GNRs + RB and PAH-GNRs + RB experiments. As shown in Fig. 3a, the characteristic RB absorption bands at 510 and 551 nm are observed from the GNRs + RB solution before centrifugation. However, no obvious RB absorption peaks can be found from the precipitate of the GNRs + RB solution after centrifugation, demonstrating that little or no RB is conjugated to the GNRs without PAH modification.

In contrast, in the PAH-GNRs + RB experiment, RB absorption bands can be observed from both the PAH-GNRs + RB solution and precipitate from the PAH-GNRs + RB solution after centrifugation (see Fig. 3b). The results clearly indicate RB is coated on the PAH-GNRs. Compared to the PAH-GNRs and pure RB, the TSPR peak of the RB-GNRs blue-shifts slightly and the RB absorption band red-shifts slightly. This is induced by plasmonic-molecular resonance coupling in which molecules near the plasmonic nanoparticles exhibit light absorption around the plasmon resonance peak [55]. Therefore, such plasmonic-molecular resonance coupling confirms successful coating of RB molecules on the PAH-GNRs.

The absorption and fluorescence spectra of the supernatants in the GNRs + RB and PAH-GNRs + RB experiments are depicted in Fig. 3c and d. Compared to those acquired from the original RB, the absorption and fluorescence intensity of RB in the supernatant of the PAH-GNRs + RB solution after centrifugation decreases by 81% and 79%, respectively. Most of the RB is absorbed by the PAH-GNRs and only a small amount of RB residues can be found in the supernatant. In contrast, in the GNRs + RB experiment, the absorption and fluorescence intensity of RB respectively decreases by only 14% and 15%, respectively, providing evidence that only a small amount of RB is coated on the GNRs without PAH modification [54]. The results further confirm that the RB has been successful conjugated onto the PAH-GNRs via the interaction between the RB and PAH. Furthermore, by estimating the amount of RB absorbing on the PAH-GNRs, the number of RB molecules attached to each single GNR is determined to be about 2000.

![Fig. 1. TEM image and hydrodynamic distribution (inset) of RB-GNRs.](image1)

![Fig. 2. Schematic illustration of the synthesis of RB-GNRs.](image2)
The zeta potential is measured to determine the surface charge of GNRs in the aqueous solution (Fig. 4). The zeta potential of the original CTAB-coated GNRs is 43.9 mV. After modification with positively-charged PAH, the zeta potential of the PAH-GNRs increases to 56.0 mV. After negatively-charged RB is conjugated to the GNRs, the zeta potential of the RB-GNRs declines to 27.3 mV. The zeta potential of RB-GNRs is still positive but close to zero, demonstrating that the GNRs are covered by RB partly and corresponding to higher cell transmission than PAH-GNRs [56].

According to the above results, the mechanism of the conjugation between RB and GNRs can be concluded as follows. Owing to the negatively-charged carboxylic group (−\(\text{COO}^-\)) and aromatic hydroxyl group (−\(\text{OH}\)) of RB (see Fig. 2), the RB molecules can conjugate with the positive-charged GNRs via electrostatic interaction [57]. Therefore, the RB can be conjugated to the original CTAB-coated GNRs (see Fig. 3c). Similarly, the RB can also be conjugated to the positively-charged PAH-GNRs by electrostatic interaction. However, more RB molecules are conjugated to the PAH-GNRs than original CTAB-coated GNRs with the similar zeta potential (see Fig. 3), suggesting that there is another interaction on the PAH-GNRs. Considering the carboxylic group (−\(\text{COO}^-\)) of RB and amine groups (−\(\text{NH}_2\)) of PAH, RB can also be bound to PAH-GNRs by covalent bonds generated in the condensation reaction.

These two processes constitute the mechanism of the conjugation of RB to PAH-GNRs to finally obtain the RB-GNR probes.

The stability of the RB-GNR probes in the aqueous solution is examined (see Fig. 5). Fig. 5a shows the absorption spectra of the RB-GNRs dissolved in the aqueous solution for different time at room temperature (\(\sim 25^\circ\text{C}\)). After standing for one week, both the GNR LSPR band and RB absorption band of the probes are almost unchanged. This demonstrates the stability of the RB-GNR probes.

Fig. 4. Zeta potentials: (1) original CTAB-coated GNRs, (2) PAH-coated GNRs, and (3) RB-GNRs.
unchanged, demonstrating good stability of the RB-GNR probes in the aqueous solution. After standing for three weeks, a little red-shift in the LSPR band can be found and it is probably due to tiny aggregation of the probes. Fetal bovine serum (FBS) is used as a competitive protein to further assess the stability of conjugation between the RB and PAH to the GNRs.

Fig. 5b shows the absorption spectra of the RB-GNR probes dissolved in aqueous solutions with 0%, 1%, and 10% FBS and kept at 37°C for 4 h. Although a small tail is found at long wavelengths, no obvious change is observed from both the LSPR peak wavelength and RB absorption band, demonstrating little influence from the FBS protein on the RB conjugation. The increased UV absorption is ascribed to the larger FBS content. All in all, the experimental results demonstrate the RB-GNR probes have good and long-lasting stability even in a complex environment with high protein concentrations.

The cytotoxicity results are shown in Fig. 6. The cell viability declines as the concentration of RB-GNRs increases. It may be caused by a rise in the free RB on the probes or change in the intracellular osmotic pressure. The high viability values determined from the cells indicate that even a high sample concentration of 0.45 nM does not generate much cytotoxicity. Such concentration is much higher than the highest concentration used below for cell sensing and imaging.

### 3.2. SPR sensing assay for oral cancer cells

#### 3.2.1. Sensing mechanism

The SPR bands of the GNRs result from the collective oscillation of surface electrons and so the resonance frequency of the SPR bands depends strongly on the dielectric properties, local environment, and aggregation of GNRs [34]. Furthermore, as a specific agent of oral cancer cells, the RB molecules on the GNRs conjugate with the protein or nucleic acid from the cancer cell lysate to form TSPR.
inducing aggregation of the RB-GNR probes. Based on this mechanism, the immunoassay for detection of oral cancer cells based on the RB-GNR platform is established. The assay is label free and the experimental protocol is very simple. As shown in Fig. 7a, a certain volume of the cell lysate aqueous solution is added to the RB-GNR solution. The mixture is incubated for 2 h and the absorption spectra are acquired on a UV–Vis–NIR spectrophotometer. An obvious red-shift is observed from the LSPR peak of the mixture containing the RB-GNRs and cancer cell lysate, as shown in Fig. 7b. The inset TEM image in Fig. 7b suggests that the red-shift can be ascribed to the end-to-end assembly of the RB-GNRs thereby confirming the specificity of the RB molecules to oral cancer cells. The results also indicate that the LSPR peak wavelength is a sensitive parameter to monitor target specific binding of the RB-GNRs onto the cancer cells. The red-shift in the LSPR peak is much bigger than that of the TSPR peak, suggesting that the assay based on the LSPR of the GNRs has higher sensitivity than traditional sensing assays based on TSPR of gold nanoparticles.

3.2.2. Specificity

The specificity of the sensing assay is assessed. Two sets of RB-GNRs are treated with the same amount of the lysate from the cancer cells and normal cells, respectively. As shown in Fig. 8a and b, the LSPR peak of the RB-GNR probes display an obvious red-shift of ~32 nm after reacting with the lysate of cancer cells, but only a small red-shift of ~7 nm is observed in the case of oral normal cells (see Fig. 8b). The large difference in the red-shifts demonstrates the specificity of the sensing assay to cancer cells. The specificity of the RB-GNR probes is further evaluated using the CTAB-coated GNRs and PAH-coated GNRs as control samples. As shown in Fig. 8c and d, almost no red-shift is observed from the two control samples after treatment with the lysate of cancer cells. As it is known that RB has high specificity to oral cancer [41], and has been used as an effective agent for detection of oral precancerous and malignant lesions with colorimetric evaluation [44]. Here, the RB-GNR probes have specificity in cancer cell sensing attributable to the specificity of the coated RB molecules.

3.2.3. Quantitative sensing

Quantitative sensing experiments are conducted on two cancer cell lines of CAL-27 and Tca8113 at different concentrations of cell lysates, and the cancer cell debris with cell membranes and organelle membranes serve as the control. As shown in Fig. 9a and b, a much larger red-shift is observed from the LSPR peak for the cancer cell lysate than the cell debris. The red-shifts also increase linearly with increasing cancer cell concentrations in the range from $2.2 \times 10^3$ to $30.3 \times 10^3$ cells/mL. The detection limit defined here as the lowest concentration in the sensitivity regime is about 2000 cells/mL. In contrast, no significant signal appears from the control samples (cell debris), verifying that the effect of nonspecific interactions on the RB-GNRs is minimal. Similar results are obtained from the other oral cancer cell line Tca8113, thus furnishing strong evidence that this sensing method is suitable for different oral cancer lines. All in all, this label-free sensing platform based on RB-GNR probes is specific, sensitive, non-invasive, economical, and convenient and suitable for quantitative optical diagnostics of oral cancer cells.

3.3. NIR absorption imaging assay for oral cancer cells

3.3.1. Principle of NIR absorption imaging system

NIR light penetrates deeply into cells and tissues. The strong optical absorption by the RB-GNRs in the NIR region enables not
only SPR sensing but also absorption imaging [39]. In our experiments, an NIR-optical absorption imaging system is established to perform the bio-imaging. As shown in Fig. 10a, an LED serves as the light source and the image is recorded by a CCD camera equipped with a 790–810 nm band-pass filter. The LED and CCD are placed, respectively, on the bottom and top of a transparent sample stage in 180° geometry. The system control and image acquisition are via computer software written by ourselves.

As shown in Fig. 10b, the pseudo-color images in the inset obtained by the system reveal NIR absorption signals (790–810 nm) from the RB-GNR probes at different concentrations. The sum of the digital color value of all pixels is calculated to represent the LSPR absorption intensity in the quantitative analysis. As shown in Fig. 10b, the calculated the LSPR absorption intensity is proportional to the concentration of the RB-GNR probes between 5 and 40 pM. Comparing the data with those obtained by spectrophotometer (Figs. S2a and S2b), it can be concluded that the NIR absorption imaging system is capable of indicating the amount of GNR-targeted samples.

### 3.3.2. NIR absorption imaging assay for oral cancer cells

Based on the imaging system described in the previous section, an imaging assay is established to detect oral cancer cells. As shown in Fig. 11a, different amounts of CAL-27 cancer cells and normal cells are cultured on a 96-well plate with the medium containing a certain amount of the RB-GNR probes with an overdose (final concentration = 0.45 nM) for 2 h. The samples were rinsed with PBS twice to remove the unbound probes and 200 μL of ultrapure water were added. Considering the specificity of the probes to the cancer cells, the absorption intensity of the bounded RB-GNR probes can be used to indicate the amount of the cancer cells. Fig. 11b shows the pseudo-color images obtained from the samples containing different amounts of the cancer cells. It can be seen that the absorption intensity increases obviously with cancer cell quantities, while much weaker absorption signals are obtained in the normal cells, demonstrating the specificity of the imaging assay for oral cancer cells. In addition, Fig. 11c reveals a linear correlation between the LSPR absorption intensity $I_{790-810}$ and the amount of cancer cells $C$ between 10 and $50 \times 10^3$ cells/mL $I_{790-810} = 0.58C + 29.5$. The results demonstrate that the imaging assay containing the RB-GNR probes can detect cancer cells quantitatively. It should also be noted that these ten samples of cells are assessed simultaneously, indicating that it is a multi-channel detection method in which multiple samples can be analyzed at the same time. Hence, this method is particularly useful for rapid and high-throughput early cancer screening.
3.4. Discussion

The above results show the sensing and imaging assays employing the RB-GNR platform for the optical diagnostics of oral cancer. Both of these two assays are based on the NIR absorption signal from the RB-GNR probes, however have different detection objects and are according to distinct mechanism. The sensing assay uses the red-shifted wavelength in the LSPR absorption band of the probes to indicate the amount of the cancer cell lysate in the aqueous solution; while the imaging assay use the LSPR absorption intensity of the probes to indicate the amount the cancer cells. The results indicate that these two assays are specific and quantitative. The sensing assay is more sensitive and suitable for accurate optical diagnostics, whereas the imaging approach which offers multi-channel and rapid detection is more suitable for screening examinations of a large number of cases.

The advantages of the established assays are further demonstrated by comparing to traditional diagnostics methods. Firstly, it is known that the currently used non-invasive cancer diagnosis techniques include ELISA, micro-satellite analysis, and high performance liquid chromatography (HPLC), all of which need labor-intensive procedures. In contrast, the two assays described here are label-free and convenient and are thus more efficient for screening examinations of oral cancer. Secondly, fluorescence spectroscopy which is the most commonly used detection technique in immunoassays requires moderately expensive instruments, and fluorophores are easily susceptible to photodestruction. In contrast, our assays are based on the NIR-optical absorption signals from the GNRs. Such metal nano-materials are not prone to photodestruction, and the instruments required are much cheaper.

4. Conclusion

RB-GNRs are produced synthesized by conjugating RB molecules and GNRs using a special technique. The materials exhibit good dispensability, low cytotoxicity, strong LSPR absorption band in the NIR region, and specificity to oral cancer cells. Oral cancer cells are tested using the RB-GNR platform which can be used as both a sensor and imaging probe. The sensing assay is more sensitive and accurate, whereas the imaging approach offers multi-channel and rapid detection. Both modes are highly-efficient, and convenient, and this RB-GNR platform has immense potential in cost-effective diagnostics of oral cancer.
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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2013.02.012.

References


