Rose-bengal-conjugated gold nanorods for in vivo photodynamic and photothermal oral cancer therapies

Beike Wang, a,1, Jia-Hong Wang b,1, Qian Liu c, Hao Huang b,c, Ming Chen b, Kaiyang Li b, Chengzhang Li a,***, Xue-Feng Yu b,c,**, Paul. K. Chu c,***

**The State Key Laboratory Breeding Base of Basic Science of Stomatology, Huabei-MOST & Key Laboratory of Oral Biomedicine of Ministry of Education, Department of Periodontics, School and Hospital of Stomatology, Wuhan University, Wuhan 430079, China
***Key Laboratory of Artificial Micro- and Nano-structures of Ministry of Education, School of Physics and Technology, Wuhan University, Wuhan 430072, China
****Department of Physics and Materials Science, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong, China

A R T I C L E   I N F O

Article history:
Received 1 November 2013
Accepted 21 November 2013
Available online 9 December 2013

Keywords:
Gold nanorods
Rose bengal
Oral cancer
Photodynamic therapy
Photothermal therapy

Gold nanorods (GNRs) conjugated with rose bengal (RB) molecules exhibit efficient singlet oxygen generation when illuminated by 532 nm green light and high photothermal efficiency under 810 nm near-infrared (NIR) irradiation. In vitro experiments show that reactive oxygen species generated by green light and hyperthermia produced by NIR light constitute two different mechanisms for cancer cell death. The RB-GNRs also exhibit improved photodynamic efficacy by enhancing the uptake of RB by cancer cells. In vivo experiments are conducted on hamster cheek pouches to resemble the human oral cancer conditions more accurately to assess the therapeutic effectiveness. Compared to the single photodynamic therapy (PDT) or photothermal therapy (PTT), the RB-GNRs with combined PDT-PTT capabilities provide better therapeutic effects against oral cancer and have large potential in cancer treatment.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Head and neck squamous cell carcinoma is the sixth most common cancer worldwide [1] with approximately 600,000 new cases yearly [2]. Squamous cell carcinoma of the oral cavity accounts for close to 389,000 cases per year with a mortality rate of 50% [2,3]. Although standard treatments such as surgery, radiation therapy, and chemotherapy are effective, they may cause significant side effects in the patients [4]. Surgical operation on squamous cell carcinoma with wide excision margins causes unnecessary damage to adjacent or underlying anatomical structures, leading to withdrawal or social isolation as well as functional and esthetic impairment of the oral cavity and face. With regard to radiotherapy, postoperative complications such as xerostomia, osteoradionecrosis, soft tissue necrosis, and spinal cord myelitis may occur. Systemic chemotherapy is only used as an adjunctive modality for oral cancer treatment. However, the systemic toxicity, e.g. nausea, alopecia, bone marrow depression, and nephrotoxicity [5], cannot be avoided for most antineoplastic drugs. Despite recent advances in combined modality therapy, the overall 5-year survival rate of patients with head and neck squamous cell carcinoma remains at 40–50% [6] and hence, there is an urgent need to develop non-invasive modality with fewer side effects to prevent and treat lesions in patients suffering from oral cancer.

Photothermal therapy (PTT) is a minimally-invasive treatment modality for cancer employing light absorbing agents to produce photothermal damage in tumors [7–11]. On the heels of recent advances in nanoscience, various types of nanomaterials with unique optical properties have been applied to PTT for cancer, for example, gold nanoshells, magnetic nanoparticles, carbon nanotubes, semiconductor nanoparticles, and polymer composites [12]. In particular, gold nanorods (GNRs) are promising due to the ease of preparation, multi-functionalities, and excellent surface plasmon resonance (SPR) properties in the near-infrared (NIR) region [13]. Owing to strong absorption in the NIR “optical window” for biological samples, GNRs have been used to ablate tumors located deep inside body tissues [7,14,15]. On account of the enhanced

* Corresponding author. Tel.: +852 34427724; fax: +852 34420542.
** Corresponding author. Key Laboratory of Artificial Micro- and Nano-structures of Ministry of Education, School of Physics and Technology, Wuhan University, Wuhan 430072, China.
*** Corresponding author.
E-mail addresses: 156cr@hotmail.com (C. Li), yxf@whu.edu.cn (X.-F. Yu), paul.chu@cityu.edu.hk (Paul.K. Chu).
1 B. Wang and J.-H. Wang contributed equally to this work.
0412-9612/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biomaterials.2013.11.066
permeability and retention effect, long circulation periods in blood have been observed from GNRs thus suggesting their potential role as a drug delivery carrier for passive targeting of cancers. In addition, by attaching functional materials such as chemotherapeutic drugs [16–18] and photosensitizers [19–23], there is the potential to integrate PTT with other therapeutic modalities into a single nanoplatform to optimize the therapeutic efficacy and safety. In recent years, conjugating GNRs with a specific agent such as antibodies [24–31], peptides [32,33], folate [34–36], RGD [17,37], and aptamer [21,38–40] has been developed to overcome the non-specific biodistribution of GNRs in the treatment of cancer.

As a prominent, non-invasive cancer therapy, photodynamic therapy (PDT) has been widely used in the treatment of premalignant and malignant disorders in the head and neck [41], cutaneous malignancies [42] and intraperitoneal tumors [43–45] with greatly reduced disfiguration and morbidity. By irradiation with light of a wavelength matching the absorption spectrum of the photosensitizer, the administered photosensitizer reacts with the surrounding substrates or molecular oxygen to produce reactive oxygen species (ROS) to selectively damage tumor tissues in situ. Among the various agents used in PDT, rose bengal (RB) is a well-known anionic photosensitizer with a singlet oxygen quantum yield of nearly 76% under 532 nm light irradiation [46–48]. However, as a hydrophilic photosensitizer, RB suffers from poor intracellular uptake ability and thus cannot be used to treat solid tumors [49]. Recent literatures have demonstrated the use of nanoparticles as the vehicle to conjugate with RB in order to enhance the uptake efficiency by cancer cells [50,51]. Furthermore, as an anionic water soluble xanthene dye, RB has been demonstrated to have specificity to oral cancer cells by integrating DNA polymerases of cancer cells [52]. On the basis of its specificity to oral cancer, our group has employed RB staining as a diagnostic aid to detect oral precancerous and malignant lesions by colorimetry. The sensitivity and specificity to detect epithelial dysplasia and oral squamous cell carcinoma are 93.9 and 73.7%, respectively [53]. Herein, the novel application of RB-GNRs to photodynamic and photothermal oral cancer therapies is described.

2. Materials and methods

2.1. Synthesis and characterization of RB-GNRs

2.1.1. Materials

Chloroauric acid (HAuCl₄·4H₂O, 99.99%), silver nitrate (AgNO₃, 99.8%), 6-carboxyfluorescein diacetate (CFDA, 99.0%), sodium chloride (NaCl, 96.0%), 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 300,000) were purchased from Purkinje General Instrument Co., Ltd. Beijing, China). Chloroauric acid (HAuCl₄·4H₂O, 38%), sodium chloride (NaCl, 96.0%), 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 300,000) were purchased from Purkinje General Instrument Co., Ltd. Beijing, China).

2.1.2. Synthesis of RB-GNRs

The GNRs were synthesized in an aqueous solution using a seed-mediated method [55] using CTAB as the surfactant. The detailed procedures have been reported by our group [54]. The GNRs were coated with PAH using a method described by Murphy et al. [56]. In this process, 2 mL of 10 mg/mL PAH prepared in 1 mM NaCl and 1 mL of 10 mM NaCl were mixed with 10 mL of 0.6 mg GNRs and stirred for 1 h at room temperature. The excess PAH and NaCl were removed by centrifugation at 10,000 rpm for 10 min. Afterwards, 10 μL of the 10 mM RB aqueous solution was added to 10 mL of the PAH-coated GNRs and the mixture was kept at 30°C for at least 3 h. Finally, the solution was centrifuged at 10,000 rpm for 10 min to remove the excess RB. Using the method described previously [54], the average amount of RB per GNR was determined to be about 2000.

2.1.3. Characterization

Transmission electron microscopy (TEM) was performed on the JEOL 2100F transmission electron microscope at an accelerating voltage of 200 kV and the absorption spectra were recorded on a UV-Vis-NIR spectrophotometer (TU-1810, Purkinje General Instrument Co., Ltd. Beijing, China).

2.1.4. Photothermal conversion

The photothermal conversion was measured on a homemade setup. A 1 cm path length quartz cuvette containing 2 mL of the sample was covered with a foam cap. The cuvette was clamped on the top part above the sample surface and the bottom of the cuvette was kept at about 0.5 cm above the magnetic stirrer. A fiber-coupled continuous wave semiconductor diode laser (1010 nm, 8-10W-8000, Kai Site Electronic Technology Co., Ltd. Shaanxi, China) with power density of 2.7 W/cm² and beam diameter of about 0.5 cm illuminated the cuvette. A digital thermometer (TX3001, Xintengxing, Wuhan, China) were used to monitor the temperature change. Its head was completely submerged in water and carefully prevented from direct illumination by the laser. Each sample in a cuvette was irradiated for 15 min under rigorous stirring and the temperature was recorded every 30 s. All the samples were measured three times and the average was used in the final analysis.

2.1.5. Single oxygen production

In the typical single oxygen production measurement, pure RB, PAH-GNRs, and RB-GNRs dissolved in 3 mL of water were mixed with 9, 10-anthracenediyl-bis(-methylene)dimalononic acid (ABDA, 20 μM) in a cuvette. ABDA was used as the singlet oxygen probe by taking advantage of the irreversible reaction with singlet oxygen causing reduced ABDA absorption at 380 nm [48]. The concentration of GNRs contained in the RB-GNRs and PAH-GNRs was about 0.6 μg/mL. Each sample was irradiated by green light at power density of 170 mW/cm² and the absorption spectra of ABDA was measured at different time intervals. One additional vial containing RB-GNRs not irradiated with green light served as the reference sample.

2.2. In vitro experiments

2.2.1. Cell culture

The Cal-27 (human oral squamous cell carcinoma cell line, CRL-2095, ATCC) was provided by the Affiliated Ninth People’s Hospital, Shanghai Jiaotong University, Shanghai Research Institute of Stomatology, Shanghai, China. The cells were routinely maintained in Dulbecco’s modified eagle medium (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (PBS; Gibco, Carlsbad, California, USA) in a humidified atmosphere containing 5% CO₂ at 37°C.

The primary oral keratinocyte cultures were established and cells were serially passaged according to the protocol previously reported [54]. The established primary oral keratinocytes were maintained in the keratinocyte growth medium (KGM-2; Clonetics, San Diego, Calif.). The cells were incubated at 37°C under 5% CO₂ until confluence and passages 3–5 were used in the experiments.

2.2.2. In vitro PDT experiments

Photodestruction of Cal-27 cells under green light irradiation in the presence of RB-GNRs, PAH-GNRs, and pure RB was assessed by the MTT assay. PAH-GNRs and RB-GNRs were diluted with DMEM containing 10% FBS to achieve a series of equivalent concentrations of 0.5, 0.7, 1, 1.5, and 2 μg/mL, and the concentrations of RB relative to RB-GNRs were 35.7, 50, 70, 107, and 143 μg/mL, respectively. The Cal-27 cells were pre-cultured on 96-well plates at 1.5 × 10⁴ cells per well for 24 h. The cells were incubated with different concentrations of pure RB, PAH-GNRs, and RB-GNRs solution as described above for 4 h. For the untreated control group, the fresh culture medium without photosensitizers was added to the wells. The cells were washed with PBS buffer three times followed by addition of Hank’s balanced salt solution (HBSS). For the PDT-treated groups, the treated cells were irradiated by a home-made light emitting diode (LED) at 530 ± 15 nm (irradiation dose rate – 170 mW/cm²) for 90 s. The dark toxicity of the pure RB, PAH-GNRs and RB-GNRs to Cal-27 cells was evaluated after incubation without irradiation. After irradiation (or not), the fresh cell culture medium was added and the cells were incubated for another 24 h. The cell viability was measured by the MTT assay as described previously [54]. The cell viability was calculated by the following formula:

\[
\text{Cell viability} = \frac{OD_{	ext{treated}} - OD_{	ext{blank}}}{OD_{	ext{control}}} \times 100\% 
\]

2.2.3. Apoptosis and necrosis assay

The Cal-27 cells were plated on 6 cm tissue dishes at a density of 1.5 × 10⁵ cells per dish. After incubation with 0.8 μg/mL RB-GNRs for 4 h in darkness, the Cal-27 cells were divided into different groups for exposure to green light (170 mW/cm²) for 90 s or not. The cells were collected at 12 and 24 h after treatment and detected for apoptosis or necrosis using Annexin V/PI double staining. Cell apoptosis or necrosis was determined by the Annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit 1 (BD Biosciences Pharmingen, San Diego, California) following the manufacturer’s instructions. The samples were analyzed by the FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using the Cell Quest software (Becton Dickinson). The Cal-27 cells without any treatment were used as the negative control for apoptosis.

2.2.4. Intracellular ROS detection

The intracellular ROS levels in the PDT-induced Cal-27 cells were monitored by staining with the fluorescent dye, 2, 7-dichlorodihydrofluorescein diacetate (H₂DCFDA, Sigma–Aldrich, St. Louis, USA) following the manufacturer’s protocol. The experiment was based on the interaction between the endogenous H₂DCFDA and intracellular ROS resulting in the formation of the fluorescent compound, 2, 7’
-dichlorofluorescin (DCF). After growing to 70%–80% confluence, the pre-seeded cells were incubated with RB-GNRs (0.8 μM) for 4 h at 37 °C with 5% CO2. Subsequently, all the cells were incubated with 20 μM of H2DCFH-DA dissolved in the free-serum DMEM for 30 min at 37 °C. After washed with PBS thrice, some RB-GNRs treated cells were irradiated by green light (170 mW/cm²) for 90 s as the PDT-treated group. The positive control cells were prepared by incubation with Roseup solution diluted with high-glucose Dulbecco’s modified eagle’s medium (DMEM) in 1:100 for 30 min at 37 °C. The PDT-treated group was harvested at 5, 15, 30, and 60 min after treatment whereas the RB-GNRs without light irradiation were collected immediately without further incubation. Intracellular ROS generation detected by H2DCFDA was monitored on the FACS Calibur at the excitation wavelength of 488 nm and emission wavelength range of 515–545 nm. The results were analyzed by the Cell Quest Software.

2.2.5. In vitro PTT experiments

The cell viability following PTT at different time points was determined by trypan blue assay and MTT assay. The trypan blue assay was used to determine the immediate cell viability following PTT treatment, while MTT was employed to monitor the long-time viability (at 24 h post PTT). The cells were dispersed on 96-well plates at a concentration of 1.5 × 10⁴ cells per well for 24 h and exposed to the RB-GNRs solution (0.8 μM) for 4 h (or without RB-GNRs). After washed with PBS 3 times, the treated cells were irradiated by an 810 nm continuous wave NIR laser (8.16 W/cm², VELAS30B-CHEESE, Gigaa, China, Wuhan) for 1.5 or 5 min (or not). To assess the transient cell viability, the trypan blue assay was performed immediately after PTT following the manufacturer’s instructions. The images showing the staining results were acquired by inverted microscopy. For the long term cell viability, the MTT assay was carried out 24 h after PTT treatment as previously described and the cell viability was calculated as a percentage of the untreated control cells.

2.3. In vivo studies in a DMBA-induced hamster tumor model

2.3.1. Establishment of animal model

Male Syrian Golden hamsters (Mesocricetus auratus, 6–8 weeks old, 150–200 g, 42 animals) were purchased from Wuhan Institute of Biological Products Co., Ltd. (Wuhan, China). The animals were maintained under standard housing conditions. All animal experiments were approved by the ethics committee of Wuhan University in accordance with institutional animal use and care regulations. A 0.5% solution of 7, 12-dimethylbenz(a)anthracene (DMBA, Sigma, St. Louis, USA) in acetone was applied topically to the left cheek pouch mucosa three times a week for 14 weeks to induce oral carcinomas. The longest and shortest dimensions of the tumors were measured daily by digital calipers. When the tumors reached about 5.0 mm in the longest dimension, the tumor-bearing hamsters were chosen for in vivo studies.

2.3.2. In vivo photodynamic-photothermal therapy

The excitation light source for PDT was a 532 nm solid state diode laser (MGL-H-532-500 mW, Bluesun Lighting Co., Ltd. Guangdong, China) with power density of 1.79 W/cm² and the irradiation time was 10 min. The excitation source for PTT was an 810 nm CW NIR light with power density of 17.86 W/cm² (spot size = 6 mm) and the irradiation time was 5 min. The selected tumor-bearing animals were randomized into six groups (n = 7 per group). The hamsters in group 1 (G1) were maintained in the natural state without treatment. The hamsters in group 2 (G2) were intratumorally injected with sterilized 10 mM PBS solution (200 μL per 50 mm³ tumor volume) followed by sequential NIR light and green light irradiation 4 h after injection. The hamsters in group 3 (G3) were intratumorally injected with RB-GNRs
(363 μg/mL, 53 nm/mL, 200 μL per 50 mm³ tumor volume) and did not undergo light irradiation. The hamster in groups 4 (G4) were intratumorally injected with RB-GNRs followed by green light irradiation 4 h after injection. The hamster in groups 5 (G5) were intratumorally injected with RB-GNRs followed by NIR light irradiation 4 h after injection. The hamster in groups 6 (G6) were intratumorally injected with RB-GNRs followed by sequential green light and NIR light irradiation 4 h after injection. The temperature during PTT was measured by an infrared camera thermographic system (infrared thermal tomography HBT-2A, Hao Bo Technology Co., Ltd., Wuhan, China). Thermal images of the RB-GNRs were acquired from the treated tumor-bearing hamsters during NIR light irradiation. The temperature change was analyzed by an infrared thermal tomography analysis system.

In the in vivo tumor volume measurements, all the hamster groups were returned to animal housing after treatment. The tumor volumes were monitored daily by digital calipers from the starting day to 10 days following treatment. The tumor volume was calculated by the formula 

\[ V = \frac{1}{2} (L \times W^2) \]

where \( L \) is the length (longest dimension) and \( W \) is the width (shortest dimension). The increased tumor percent (Ti) was calculated by the formula: 

\[ Ti(\%) = \frac{(DP - DC)}{DC} \times 100\% \]

where DC and DP are the tumor volumes before and after treatment, respectively.

2.3.3. TUNEL assay

All the tumor tissues from all hamsters were collected 24 h after treatment. They were fixed in formalin, embedded in paraffin, and sectioned and stained using the TUNEL technique on the In Situ Cell Death Detection Kit (Roche Applied Science, Germany). The experimental procedures were in accordance with the manufacturer's instructions. DAPI was used to stain the sections in darkness to label the apoptotic cells and cellular DNA. The fluorescence images were acquired on the Leica DM4000B fluorescence microscope (Leica, Nussloch, Germany).

2.4. Statistical analysis

The data were expressed as means ± standard deviation. The one-way ANOVA followed by the post-Turkey comparison tests and Student–Newman–Keuls test were used for statistical analysis with \( p < 0.05 \) being considered statistically significant.

3. Results and discussion

3.1. Photothermal and photodynamic properties of RB-GNRs

The RB molecules are conjugated with GNRs to obtain RB-GNRs for oral cancer therapy. The original CTAB-coated GNRs are synthesized by using a seed-mediated growth method [55] and a cationic polyelectrolyte (PAH) is adopted to modify the surface of the CTAB-coated GNRs to produce the PAH-coated GNRs (PAH-

![Fig. 4. Low magnification (left) and high magnification (right) TEM images of Cal-27 oral cancer cells treated with (a) RB-GNRs and (b) GNRs using the same concentration of 0.8 μM for 1 h. The red arrows point to the cytosolic vesicles containing the samples. (c) Quantification of the mean GNR numbers per oral cancer cell. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image-url)
GNRs). The PAH-GNRs are mixed with RB in an aqueous solution for 3 h to absorb RB onto the PAH-GNRs surface. The conjugation mechanism between RB molecules and PAH-GNRs is described in our previous paper [54]. Fig. 1a compares the absorption spectra of PAH-GNRs, pure RB, and RB-GNRs. The PAH-GNRs show a transverse SPR peak at ~522 nm and longitudinal SPR peak at ~802 nm. The pure RB shows absorption bands at 510 and 551 nm. Compared to the PAH-GNRs, the longitudinal SPR peak of the RB-GNRs redshifts to 813 nm due to the altered local environment [57,58]. The TEM image of the as-synthesized RB-GNRs depicted in Fig. 1b shows that the average diameter and length of the RB-GNRs are 13 ± 2 nm and 52 ± 4 nm, respectively. These values are suitable for good dispersion in an aqueous solution.

Efficient singlet oxygen generation is a prerequisite to the application of photosensitizers to PDT. Here, the singlet oxygen generation capability of RB-GNRs under 532 nm light excitation is evaluated by a chemical method using ABDA as the detection probe. In this method, ABDA reacts irreversibly with singlet oxygen to yield endoperoxide that decreases the ABDA absorption band at 380 nm [48]. Photoxidation of ABDA in the presence of PAH-GNRs, RB-GNRs, and pure RB with the same power density is monitored for 12 min. The solution of RB-GNRs has the same RB concentration as the pure RB solution and same GNRs concentration as the PAH-GNRs. As shown in Fig. 2, the absorption intensity of ABDA in the RB-GNRs solution decreases by approximately 92%, while only 15% degradation is observed without light excitation or without RB (PAH-GNRs). In contrast, the absorption intensity of ABDA with pure RB only decreases by 85%. The comparison indicates that RB-GNRs have enhanced ability to release singlet oxygen compared to pure RB at the same RB concentration. The enhancement may be attributed to enhanced absorption arising from the transverse SPR of the GNRs [59].

The strong surface plasmon resonance (SPR) of the PAH-GNRs in the NIR region leads to good photothermal properties. The temperature elevation experiments (Fig. 3) conducted in the aqueous solutions of PAH-GNRs and RB-GNRs, and pure water (control sample) under 810 nm light irradiation were performed to compare the photothermal efficiency. After irradiation for 14 min, the temperature of PAH-GNRs and RB-GNRs increased by 40.6 and 43.3 °C, respectively, whereas that of water only increased by 2.2 °C. The results indicate that the GNRs can rapidly and efficiently convert NIR light into thermal energy. Furthermore, by means of a reported method [60], the photothermal conversion efficiency of RB-GNRs is measured to be about 21% [61]. Compared to PAH-GNRs, better photothermal performance is achieved from the RB-GNRs because the longitudinal SPR peak is closer to the center wavelength (810 nm) of the light.

3.2. Specificity test of RB-GNRs to oral cancer

Fig. 4 shows the TEM images of Cal-27 oral cancer cells incubated with the RB-GNRs (a) and GNRs (b) using the same concentration of 0.8 nM for 1 h. More particulates with a large mass thickness contrast can be observed from the cytosolic vesicles of Cal-27 cells treated with RB-GNRs compared to GNRs. Cell viability (CV) of pure RB (a), PAH-GNRs (b), and RB-GNRs (c) with (+) and without (−) 532 nm light irradiation. (d) CV+CV− vs concentration of pure RB, PAH-GNRs, and RB-GNRs.

![Fig. 5. PDT effects of RB-GNRs on Cal-27 cells: (a–c) Cal-27 cell viability (CV) of pure RB (a), PAH-GNRs (b), and RB-GNRs (c) with (+) and without (−) 532 nm light irradiation. (d) CV+CV− vs concentration of pure RB, PAH-GNRs, and RB-GNRs.](image-url)
the RB-GNRs treated Cal-27 cancer cells than the GNRs treated ones. By quantifying the GNR number per cell, the lower bar in chart (c) shows a significant difference in the GNR amounts between the RB-GNRs treated cells and GNRs treated ones (presented as mean ± SD obtained from three parallel experiments). Compared to the GNRs, the RB-GNRs can be more easily uptaken into the oral cancer cells thus suggesting the RB-GNRs have better specificity than the GNRs without RB coating. It is known that RB has specificity to oral cancer by integrating DNA polymerases of cancer cells [52,53]. Furthermore, Fig. S1 illustrates a higher specificity of RB to oral cancer cells than normal mucosal epithelial cells, suggesting that the specificity of the RB-GNRs is probably attributed to the RB component.

3.3. In vitro PDT

3.3.1. Cytotoxicity induced by PDT

Fig. 5 compares the cell viability of Cal-27 treated with pure RB, PAH-GNRs and RB-GNRs. After irradiation with 532 nm light for 90 s, the RB-GNRs induce significant cytotoxicity to the Cal-27 cancer cells in a dose-dependent manner at concentrations between 0.5 and 2 nM (p < 0.01). Without green light irradiation, the RB-GNRs show no toxicity at a concentration of 1 nM and only limited cytotoxicity at 1.5 and 2 nM, corresponding to 27% and 34% of the cells being killed, respectively. Each constituent of the RB-GNRs (RB and PAH-GNRs) was administered to the cancer cells at corresponding concentrations with or without green light exposure (see Fig. 5b and c). Neither pure RB nor PAH-GNRs show phototoxicity to Cal-27 cells at the corresponding concentrations. Furthermore, the ratio of cell viability (CV) of the Cal-27 cells with green light irradiation to that without green light irradiation (CV+/CV−) is shown in Fig. 5d. Compared to pure RB and PAH-GNRs, RB-GNRs induce significantly smaller CV+/CV− at any concentration. The results suggest that RB-GNRs can induce significant phototoxicity to the Cal-27 cells. Furthermore, the phototoxicity of RB-GNRs is much higher than that of pure RB. Additional experiments show that pure RB with a higher concentration also exhibits efficient phototoxicity to Cal-27 cells under green light irradiation, as shown in Fig. S2 in Supporting Information, which is similar phototoxic effect of RB-GNRs on Cal-27. It is known that the PDT effect of pure RB molecules is often limited by high hydrophilicity [62]. Here, by conjugating RB onto the GNRs, the RB-GNRs not only increase the PDT efficiency (Fig. 2), but also might improve the PDT induced cytotoxicity by enhancing the uptake of RB into the cancer cells via the GNRs as the vehicle. Hence, RB-GNR is a promising photo-therapeutic agent for oral cancer cells under green light irradiation.

3.3.2. Apoptosis caused by PDT

It has been reported that apoptosis may be the preferred mechanism of cell death induced by RB mediated PDT [63]. To determine whether the cytotoxicity to Cal-27 cells induced by PDT of the RB-GNRs is due to the apoptosis induction, Annexin-V/PI double staining is performed. After incubation with 0.8 nM RB-GNRs for 4 h in darkness, the Cal-27 cells were irradiated with green light for 90 s (or not) and collected at 12 and 24 h for detection. As shown in Fig. 6, the total apoptotic cells of Cal-27 cells at 12 and 24 h after exposure of green light are 19% and 26.3%, respectively, which are significantly higher than that without exposure to green light (p < 0.05). Meanwhile, the Cal-27 cells without undergoing green light irradiation show no dramatic increase in the total apoptotic cells including both early and late apoptotic cells at 12 or 24 h compared to the control group. The results indicate that the high cytotoxicity induced by RB-GNRs mediated PDT can mainly be attributed to apoptosis induction.

3.3.3. ROS studies

The ROS production in the Cal-27 cells is further investigated to determine the cause of induced apoptosis after the PDT treatment (Fig. 7). After 532 nm green light irradiation for 90 s, the intracellular ROS level in the cancer cells incubated with RB-GNRs is significantly higher than that in darkness over time. In contrast, negligible DCFH fluorescence is observed from the cancer cells.

![Fig. 6. PDT Effects of RB-GNRs on apoptosis of Cal-27 cells with 532 nm light irradiation. Representative images of flow cytometry analysis in Cal-27 cells with different treatment: (a) Without treatment; (b, c) 12 h (b) and 24 h (c) after RB-GNRs incubation and without light irradiation; (d, e) 12 h (d) and 24 h (e) after RB-GNRs incubation and with light irradiation. The incubation time is 4 h and irradiation time is 90 s.](image-url)
without photoactivation. Notably, the strongest green fluorescence of DCFH (about 94.67%) is observed 5 min after the PDT treatment and fluorescence diminishes progressively during the next 60 min. Fig. 7f exhibits the differences in the ROS levels among these samples in comparison with the control sample (p < 0.01). The excessive ROS production followed by a progressive recession is coincident with the observation of the RB-mediated PDT with time [63], indicating that the ROS generation by RB-GNRs stems from the RB molecules. The evidence suggests that excessive ROS generation in the cancer cells after the PDT treatment may be the main reason for the induced apoptosis. It should be noted that other pathways for cell death may also be considered and this issue will be explored in more details in the future.

3.4. In vitro PTT of RB-GNRs

The photothermal effects of RB-GNRs on oral cancer cells under NIR light irradiation are further explored. Fig. 8 depicts the transient (a–f) and long-time (g) cell viabilities of Cal-27 cells after different treatment using the trypan blue assay and MTT assay, respectively. In the trypan blue assay, the dead cells are stained blue while the viable cells cannot be stained for their ability to get rid of trypan blue. The trypan blue assay is utilized to determine the cell viability immediately following PTT and it can be regarded as transient cell viability. Fig. 8g shows the quantified cell viability of Cal-27 cells at 24 h after the treatment and it can be regarded as long-time cell viability. Compared to the untreated cells (a), higher cell viability can be observed from the cells with light irradiation only (b, c) and RB-GNRs incubation only (d). There are no stained blue cells and is similar viable cell quantification (no significant differences, p > 0.05). The RB-GNRs or NIR light irradiation are almost nontoxic to the Cal-27 cells. In contrast, when the cells incubated with the RB-GNRs are subjected to NIR light irradiation, light exposure time-dependent cytotoxicity is observed from groups e and f. The cells after undergoing 5 min of NIR light irradiation (f) display the highest cytotoxicity, as evidenced by almost complete trypan blue staining and significantly lower cell viability (10.9%, p < 0.0001). Nearly 40% of the stained blue cells and 46% cell viability can be observed from group e after 1.5 min NIR light irradiation, indicating that inadequate NIR light exposure leads to a minor cell killing and ongoing cell death after 24 h. The results demonstrate that the RB-GNRs also impose the PTT effects on oral cancer cells under NIR light irradiation, making it possible to kill cancer cells by combined photodynamic-photothermal therapy.

3.5. Combined PDT-PTT with RB-GNRs for oral cancer in vivo

3.5.1. Hamster cheek pouch animal model

In vivo experiments are performed using hamster cheek pouches model. The hamster cheek pouch model has been one of the most widely accepted oral cancer models due to the similarity with tumorigenesis and progression of human oral cancer [64]. In contrast, the xenografts model generally lacks natural progression of diseases. By adopting the hamster cheek pouch model, the natural recovery
history of human oral cancer after treatment with RB-GNRs can be more effectively gauged. In our experiments, 0.5% DMBA in acetone was applied to the left cheek pouch mucosa three times a week for 14 weeks. In addition, topical (intratumoral) injection is considered the more suitable way of drug administration than the systemic one (intravenous or intraperitoneal) in the case of oral cancer.

3.5.2. Thermographic analyses of RB-GNRs in vivo

The in vivo photothermal effects of RB-GNRs on the tumor are first investigated thermographically under 810 nm NIR light irradiation. Fig. 9 shows the infrared thermal images of the tumor intratumorally injected with the RB-GNRs. Under NIR light irradiation, the temperature of the tumor increases rapidly from 33.5 to 43.5 °C during the first 60 s (see Fig. 9e) and reaches 53.5 °C after 5 min (data not shown). In contrast, the temperature of the tumor intratumorally injected with PBS only reaches ~38 °C after 60 s (Fig. 9e) and remains at 41 °C after 5 min (data not shown). Since a temperature over 43 °C can selectively destroy cancer cells due to its lower heat tolerance caused by poor blood supply [65], the results show the potential effectiveness of RB-GNRs in oral cancer ablation as PTT agents.

3.5.3. Combined PDT-PTT treatment for oral cancer in vivo

To assess the anticancer efficacy of RB-GNRs combined PDT and PTT in vivo, the DMBA-induced tumor bared hamsters are divided into six different treatment groups (G1-G6, n = 7 in each group) and the change in the tumor is measured for 10 days post different treatment. All the tumors have similar sizes with the longest dimension of about 5.0 mm before treatment. The different groups are described as follows: G1: Tumors without treatment; G2: Tumors injected with 10 mM PBS as control and irradiated by NIR and green light; G3: Tumors injected with RB-GNRs but without light irradiation; G4: Tumors injected with RB-GNRs and irradiated by green light; G5: Tumors injected with RB-GNRs and irradiated by NIR light; G6: Tumors injected with RB-GNRs and irradiated by NIR and green light. The concentration of RB-GNRs is 53 nM and injection dose is 200 μL per 50 mm³ tumor volume. They are irradiated with 810 nm NIR light and 532 nm light for 5 and 10 min, respectively.

Fig. 10a and b shows the typical photographs and average size variation of the tumors in groups G1–G6 at different time points (days) after the corresponding treatment. G1–G3 show uninhibited tumor growth and there is no significant difference in the tumor
growth rates ($p > 0.05$), indicating that tumor growth is not affected by light illumination (green plus NIR light) without agent injection or RB-GNR injection without irradiation. On the other hand, therapeutic effects are observed from G4-G6. After the PDT-only treatment (G4), a 46.5% tumor inhibition rate is observed on the 10th day, whereas after the PTT-only treatment, a 65.5% tumor inhibition rate is shown. The tumor growth rate decreases progressively during the initial 3 days but reverses from the 4th day due to rapid growth of the remaining cancer cells. In addition, there is no significant difference in the tumor growth rates between G4 and G5. In contrast, after the combined PDT-PTT treatment (G6), a 95.5% tumor inhibition rate is achieved on the 10th day, demonstrating its excellent antitumor efficiency superior to that in all the other groups ($p < 0.001$ for G1-G3 and $p < 0.01$ for G4 and G5). The combined PDT and PTT treatment employing RB-GNRs is thus the most effective modality for oral cancer therapy. In fact, with regard to the therapy for superficial oral cancer, PDT alone often suffers from the limited penetrating depth of 532 nm light into biological tissues, while the therapeutic effect of PTT alone may be compromised by the uneven distribution of heat stemming from nonuniform light illumination.

Here, the in vivo results confirm that the combination of PDT and PTT can complement each other thereby providing better synergistic therapeutic effects against oral cancer.

3.5.4. TUNEL assay in tumor sections

The anticancer efficiency of tumors in G1–G6 is further analyzed by a TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling) assay. The TUNEL assay is generally utilized to detect apoptosis in tumor tissues. Fig. 10c displays the results of TUNEL immunofluorescence staining. No or few TUNEL-positive cells (shown in green (in the web version)) are observed from G1-G3, confirming that light irradiation without injection or RB-GNR injection without light irradiation have little effect on tumor growth. More TUNEL-positive cells are found in G4 and G5. The TUNEL signals are larger and distributed at deeper sites of the tumor tissue in G4 than G5, implying that NIR light provides PTT treatment to deeper tissue sites than green light (PDT treatment). With regard to G6 which undergoes combined PDT-PTT treatment, colocalization of nuclei (DAPI staining, shown in blue (in the web version)) and TUNEL-positive apoptotic cells
(green) in the tumor sections are significantly higher than any other group. All the TUNEL results correspond well with the trend of tumor growth rates illustrated in Fig. 10b.

The RB-GNRs exhibit better biosafety both in vitro and in vivo and the corresponding PTT-PDT treatment is minimally invasive and non-destructive. The in vitro results shown in Fig. 5 indicate that the RB-GNRs have low toxicity to Cal-27 cancer cells within the concentration range without light. In the in vivo experiments, the hamster do not show toxic effects after intratumorally injected with RB-GNRs. The results suggest that the combined PTT and PDT therapy using RB-GNRs can be repeated without tissue toxicity and there remains the possibility of treatment by other methods.
4. Discussion

Most of the previous studies on in vivo therapy of nanomaterials have been performed on subcutaneously grown tumors of the xenografts model. To the best of our knowledge, the therapeutic effects of RB-GNRs in hamster cheek pouch grown tumors have not been investigated. Compared to the xenografts model, this animal model is more accurate for human oral cancer and better illustrates the actual therapeutic effects.

In our in vivo studies, intratumoral (topical) injection of RB-GNRs is consider the more suitable strategy of drug administration than the intravenously or intraperitoneal (systemic) one in oral cancer therapy. In recent years, direct intratumoral delivery methods have attracted considerable attention in in vivo experiments on the potential clinical utility of nanomaterials [66–74]. For example, Huang et al. have suggested that for photothermal cancer therapy, the preferred route of GNRs administration was intratumoral injection instead of intravenous injection [71]. The advantages of topical administration over systemic routines are discussed in the following. First of all, the antitumor activity of anticancer drugs may be enhanced by direct intratumoral injection [72,75–77] thereby providing a high local concentration and limited injectable dose of the anticancer drugs [71]. Direct intratumoral injection can reduce the systemic drug levels and decrease the incidence of side effects commonly associated with systemic therapy [78]. Secondly, by avoiding the rapid reticuloendothelial system (RES) clearance [68] caused by systemic injection, topical administration is promising in achieving prolonged action periods for its low metabolic rates, thus facilitating patient compliance and comfort. Thirdly, it can circumvent the various physiological barriers to tumor drug delivery common to most solid tumors. It is also a considerable approach in tumor drug administration is promising in achieving prolonged action periods for its low metabolic rates, thus facilitating patient compliance and comfort. Thirdly, it can circumvent the various physiological barriers to tumor drug delivery common to most solid tumors. It is also compounded by simultaneous drug excretion during the drug transportation process [79–81]. Fourthly, it is more suitable for oral cancer which is a kind of superficial, visibly accessible external lesions compared to internal cancer such as lung cancer and liver cancer. Hence, we select intratumoral injection in our in vivo experiments also because it does not add other risk factors affecting the therapeutic outcome. It should be noted the specificity of RB-GNRs to cancer cells is important to intratumoral injection. Even in the context of intratumoral injection, the specificity can improve the distribution patterns of nanoparticles inside the tumor cells, as shown in Fig. 4, thereby improving the therapeutic efficacy.

The current PDT-PTT treatment is demonstrated to be safe and effective for oral squamous cell carcinoma offering advantages over conventional treatments in terms of improved organ functions and cosmetic appearance. The anatomy of the oral and orpharynx allows good exposure of the tumor to light and the lesions occurring here are usually superficial and involve a rather big area. Despite the limited room at this anatomic site, a lot of organs and tissues are critical to the normal functions and cosmetics. Consequently, tumors are difficult to be treated with conventional therapies without morbidity. In our in vivo experiments, with the merits of tumor specificity, minimal invasive treatment, and light-guided treated area, the tumors after the PDT-PTT treatment are nearly cleared with preservation of normal tissues and more importantly, the treatment can be repeated as often as necessary. Another advantage is that under certain circumstances, the combined photothermal and photodynamic therapy does not require general anesthesia and can be performed in outpatient clinics.

5. Conclusion

Multifunctional RB-GNRs exhibit efficient singlet oxygen generation under 532 nm light irradiation and high photothermal efficiency when illuminated by 810 nm NIR irradiation. Significant anticancer effects are observed from the RB-GNRs during combined photothermal and photodynamic therapy, as confirmed in vitro and in vivo using hamster cheek pouch as the animal model. Compared to existing oral cancer treatment methods, the combined PDT-PTT treatment utilizing RB-GNRs offers several advantages. First of all, the RB-GNRs exhibit efficient PDT-PTT effects in vivo and the combined PDT-PTT produces better therapeutic effects than PDT or PTT alone. Secondly, RB is specific to oral cells, thus allowing the RB-GNRs to preferentially accumulate in the cancer cells to overcome the nonspecific delivery of heat by GNRs while sparing normal cells. Thirdly, compared to pure RB, RB-GNRs show remarkably enhanced PDT efficacy caused by enhanced uptake of RB by the cancer cells. Fourthly, the RB-GNRs exhibit excellent cytocompatibility and the combined PTT-PDT treatment is minimally invasive and non-destructive. Because of these advantages and good performance, the multifunctional agent presented here has large potential in cancer treatment applications.

Acknowledgments

The work was financially supported by the Wuhan Science and Technology project No. 20026002084, the Natural Science Foundation of China (NSFC) No. 51372715, Hong Kong Research Grants Council (RGC) General Research Funds (GRF) No. 112510 as well as City University of Hong Kong Applied Research Grants (ARG) Nos. 9667066 and 9667069. We wish to thank Wuhan Gigaa Optronics Technology Co., Ltd. for kindly providing the 810 nm continuous wavelength (CW) laser.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.11.066.

References


Kim JY, Choi WJ, Kim M, Tae G. Tumor-targeting nanogel that can function independently for both photodynamic and photothermal therapy and its synergy from the procedure of PDT followed by PTT. J Control Release 2014;175:233–43.


