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Abstract. Biomarker-specific photothermal nanoparticles that can efficiently sense markers that are overexpressed in distinguished adenocarcinomas have attracted much interest in an aspect of efficacy increase of cancer treatment. We demonstrated a promising prospect of a smart photothermal therapy agent employing anti-epidermal growth factor receptor aptamer (Apt_{EGFR})-conjugated polyethylene glycol (PEG) layted gold nanorods (Apt_{EGFR}-PGNRs). The cetyltrimethylammonium bromide bilayer on GNRs was replaced with heterobifunctional PEG (COOH-PEG-SH) not only to serve as a biocompatible stabilizer and but also to conjugate Apt_{EGFR}. Subsequently, to direct photothermal therapy agent toward epithelial cancer cells, the carboxylated PEGylated GNRs (PGNRs) were further functionalized with Apt_{EGFR} using carbodiimide chemistry. Then, to assess the potential as biomarker-specific photothermal therapy agent of synthesized Apt_{EGFR}-PGNRs, the optical properties, biocompatibility, colloidal stability, binding affinity, and epicellial cancer cell killing efficacy *in vitro*/*in vivo* under near-infrared laser irradiation were investigated. As a result, Apt_{EGFR}-PGNRs exhibit excellent tumor targeting ability and feasibility of effective photothermal ablation cancer therapy. [DOI: 10.1117/1.JBO.19.5.051203]

Keywords: aptamer; gold nanorod; epithelial cancer; photothermal therapy; near-infrared.

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1 Introduction

Photothermal therapy that employs optical absorbing agents under light irradiation has attracted significant attention in recent years as a promising alternative to traditional cancer therapies.[1,2] Epitomical photothermal agents based on noble metal nanostructure,[1] carbon nanomaterials,[3] and copper chalcogenide nanocrystals[4] should exhibit strong absorbance in the near-infrared (NIR) region, which can penetrate tissue with sufficient intensity and could efficiently transfer the absorbed NIR optical energy into heat.[5]

One of the most promising approaches is utilizing gold nanorods (GNRs) because GNRs are strong potential photothermal therapeutic agents that can convert absorbed energy into heat and are valuable contrast agents using high absorption.[6] It has been shown that photon excitation of surface plasmon bands generates excited states in the free electrons on the surface of the GNRs; phonons are then released after relaxation of the electrons. The phonons subsequently relax and are converted to heat within 100 ps. Consequently, most photons absorbed by GNRs are transformed into heat.[7] GNR with an LSPR maximum at NIR region is considered to have a great potential for NIR laser-based therapeutic techniques.

The major goal of minimally invasive thermal treatment is to damage surrounding normal tissues as little as possible. Therefore, a targeted delivery system may surmount the nonspecific damage of photothermal therapy because it may guide photothermal agents to tumor cells and avoid the damage to normal cells. Recently, novel targeting agents, including aptamers,[8] short peptides, and other small molecules,[9] have become the new generation targeting molecules. Aptamers are small strands of DNA or RNA that specifically combine to molecular targets with high affinity.[10]

In this paper, we demonstrate biomarker-specific photothermal agent that anti-epidermal growth factor receptor aptamer (Apt_{EGFR}) as targeting agents for perceiving the cancer marker such as EGFR was conjugated with polyethylene glycol (PEG) layted GNR-based photothermal therapy system (Apt_{EGFR}-PGNRs). An EGFR is an attractive marker of cell proliferation, metastasis, angiogenesis, and blocking of apoptosis, which ultimately leads to multiple tumorgenic processes. Therefore, specific detection of EGFR is crucial for the effective treatment. For the assessment of the feasibility of Apt_{EGFR}-PGNRs to serve as a smart photothermal therapy agent, we investigated their optical properties, biocompatibility, *in vitro* binding affinities

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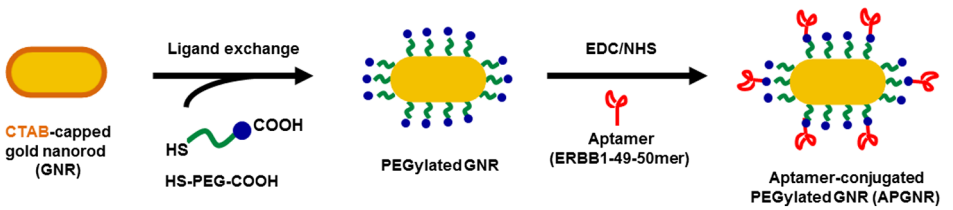

Fig. 1 Schematic illustration of the synthesis of EGFR-targetable aptamer-conjugated PEGylated GNR (Apt_{EGFR}-PGNRs).

for cancer cells, and *in vitro*/*in vivo* photothermal therapy effects. A diagram of the Apt_{EGFR}-PGNRs is provided in Fig. 1.

2 Materials and Methods

2.1 *Materials*

Gold(III) chloride trihydrate ($HAuCl_4$), hexadecyltrimethylammonium bromide (CTAB), sodium borohydride, silver nitrate, L-ascorbic acid, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were purchased from Sigma–Aldrich (St. Louis, Missouri, USA). N-hydroxysulfosuccinimide (Sulfo-NHS) was purchased from Pierce (Grand Island, New York, USA). Thiol-poly(ethylene glycol)-carboxymethyl (CM-PEG-SH, MW 3400 Da) was purchased from Laysan Bio Inc (Arab, Alabama, USA). Dulbecco's phosphate buffered saline (PBS, pH 7.4) was purchased from Hyclone (Asheville, North Carolina, USA). The anti-EGFR DNA aptamer (50 mer, sequence: H_2N-C6-5′-d(AGTTCZZCAACCZZGAAACAGZZZZAAGCCCZZGAAAAAAGAGZAGACCA)-3′; Z is 5-(*N*-naphthylcarboxyamide)-2′-deoxyuridine (NapdU), MW: 17229.98 Da) can bind (or target) EGFR. This anti-EGFR DNA aptamer was kindly provided by Aptamer Sciences Inc. (Pohang, South Korea) and their detail information posted on www.aptsci.com, No. 41 in AptSci Aptamers and anti-EGFR antibody (Erbitux®) was purchased from Roche Pharmaceutical Ltd (Nutley, New Jersey, USA). All other chemicals and reagents were of analytical grade. Ultrapure deionized (DI) water was used for all of the synthetic processes.

2.2 *Synthesis of PEGylated Gold Nanorods (PGNRs)*

GNRs were synthesized according to the seed-mediated growth method in fresh aqueous CTAB solution. Briefly, 0.25 mL of $HAuCl_4$ (100 mM) and 7.5 mL of CTAB solution (95 mM) were mixed and then 0.06 mL of ice-cold sodium borohydride solution (100 mM) was added to the vigorously stirred solution as the seed solution and allowed to react for 2 min, after which the solution was stored at room temperature for 3 h. A growth solution containing 0.08 mL of silver nitrate solution (100 mM) and 0.5 mL of $HAuCl_4$ (100 mM) was added to 9.5 mL of CTAB solution (95 mM) under vigorous stirring. After the addition of 0.055 mL of ascorbic acid solution (100 mM), the color of the solution changed from yellow to colorless. A 0.012 mL volume of gold seed solution was introduced into the growth solution and stirred for 10 s. The product solution was kept at room temperature for 24 h without stirring and the color of the solution changed from colorless to reddish-brown. GNR solutions were centrifuged twice at 15,000 rpm for 30 min to remove excess CTAB and then re-dispersed in 5 mL of DI water. To prepare PGNRs, GNRs were coated with heterobifunctionalized PEG as a stabilizer. Thirty milligrams of CM-PEG-SH was added to 2 mL of GNRs solution (228.5 μg of Au/200 μL) and stirred for 24 h at room temperature. The mixture was centrifuged at 15,000 rpm for 30 min to remove unbound PEG molecules and re-suspended in 4 mL of PBS.

2.3 *Characterizations*

The absorbance of GNRs and PGNRs was measured using a spectrometer (Optizen 2120UV, MECASYS, Seoul, Korea). The morphologies of GNRs were evaluated using a high-resolution transmission electron microscope (HR-TEM, JEM-2100 LAB6, JEOL Ltd., Seoul, Korea). The quantity of Au in GNRs was determined using inductively coupled plasma atomic emission spectrometry (ICP-AES, Thermo Electron Corporation, West Palm Beach, Florida, USA).

2.4 *Preparation of EGFR-Targetable PGNRs*

To conjugate Apt_{EGFR} and Ab_{EGFR} with PGNRs, EDC (2.9 μmol), Sulfo-NHS (2.5 μmol), and 200 μL of Apt_{EGFR} and Ab_{EGFR} (0.003 μmol) were added to 2 mL of the PGNR (13.77 μg of Au/2 mL) solution and reacted at 4°C for 6 h. After the reaction, side products were removed by centrifugation at 15,000 rpm for 30 min and re-dispersed in 4 mL of PBS.

2.5 *Quantification of Targeting Molecules in EGFR-Targetable PGNRs*

The conjugated aptamers from Apt_{EGFR}-PGNRs were quantified using the Take3 module for single-strand DNA detection and quantification. Ten microliters of Apt_{EGFR}-PGNRs was pipetted on the Take3 module and absorption was measured using multifunctional reader at a wavelength of 260 and 280 nm. Furthermore, the immobilized antibodies from Ab_{EGFR}-PGNRs were quantified using the bicinchoninic acid (BCA) assay (Pierce, Grand Island, New York, USA) for the colorimetric detection and quantification of total protein. This method combines the reduction of Cu^{2+} (BCA reagent B) to Cu^{1+} by protein in the biuret reaction with the colorimetric detection of the cuprous cation (Cu^{1+}). Twenty-five microliters of Ab_{EGFR}-PGNRs was pipetted into a 96-well plate and then 200 μL of working reagent was added to each well; the well contents were mixed by shaking on a plate shaker for 30 s. The plate was covered and incubated at 37°C for 30 min and cooled at room temperature. Absorption was measured using enzyme-linked immunosorbent assay (ELISA) at a wavelength of 562 nm.

2.6 *Evaluation of Photothermal Ability of EGFR-Targetable PGNRs*

To investigate the photothermal effect induced by NIR laser irradiation of Apt_{EGFR}-PGNRs and Ab_{EGFR}-PGNRs, 1 mL of Apt_{EGFR}-PGNR and Ab_{EGFR}-PGNRs (50 μg of Au/mL) solution and DI water (as a control) were prepared in glass vials. Each solution was exposed to an NIR coherent diode

laser (808 nm, 0.5 W cm^{-2}, UM30K, Jenoptik, Jena, Germany) for 5 min, and the elevation of the solution temperature was monitored by a thermocouple (187 true rms multimeters, Fluke, Everett, Washington, USA).

2.7 *Assessment of Biocompatibility for EGFR-Targetable PGNRs*

Cell viabilities for target cells treated with EGFR-targetable PGNRs were quantified by a colorimetric assay based on the mitochondrial oxidation of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) using a cell proliferation kit (Roche, Germany). The epidermoid carcinoma A431 cell line was obtained from the American Tissue Type Culture (ATCC, USA) and cells were plated at a density of 10^4 cells/mL in a 96-well plate and incubated at 37°C under a 5% CO_2 atmosphere. The cells were incubated for 24 h with Apt_{EGFR}-PGNRs or Ab_{EGFR}-PGNRs (6 μg of Au/100 μL) and then rinsed with 100 μL of PBS (pH 7.4, 1 mM). The cells were then treated with 10 μL of freshly prepared MTT and incubated for an additional 4 h before treatment with 100 μL of solubilization solution (10% sodium dodecyl sulfate in 0.01 M HCl). After 24 h, the plate was assayed using an ELISA (Spectra MAX 340, Molecular Devices, California, USA) at an absorbance wavelength of 575 nm and a reference wavelength of 650 nm.

2.8 *Targeting Ability of EGFR-Targetable PGNRs*

To determine the cellular affinities of EGFR-targetable PGNRs, A431 and MCF7 cells (8×10^5 cells/well) were, respectively, treated with Apt_{EGFR}-PGNRs or Ab_{EGFR}-PGNRs for 1 h at 4°C to avoid nonspecific binding. The treated cells were washed three times with PBS to eliminate unbound PGNRs. The washed cells were detached using trypsin and dissolved in aqua regia for 12 h at 120°C. The amount of Au isolated in the cells was measured using ICP-AES.

2.9 *In Vitro Photothermal Ablation Study*

A431 cells (EGFR overexpressing) and MCF7 cells (EGFR-deficient) were obtained from ATCC. Both A431 and MCF7 cells (10^5 cells/well) were, respectively, incubated with APT_{EGFR}-PGNRs or Ab_{EGFR}-PGNRs (20 μg Au) at 37°C for 12 h on thin glass cover slides in a 48-well plate. The cells were rinsed with PBS (pH 7.4, 1 mM) and 1 mL of phenol red free Dulbecco's modified Eagle medium was added to each well. For the laser irradiation experiment, the cells were exposed to an NIR coherent diode laser for 10 min and washed with PBS. Then, the distribution of live cells was observed using an optical system microscope (Olympus BX51, Japan) after cellular staining with 500 μL of calcein AM (1 μM, Molecular Probes, Grand Island, New York, USA) for 30 min.

2.10 *In Vivo Photothermal Therapy*

To prepare tumor-bearing xenograft mice model, collected A431 cells (5×10^6 cells) suspended in 50 μL of PBS (pH 7.4, 1 mM) were subcutaneously injected into the proximal thigh region of male BALB/c-nude mice, 5 to 8 weeks of age, obtained from the Institute of Medical Science (University of Tokyo). All experiments were conducted with the approval of the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Two weeks after transplantation, xenograft mice were anesthetized and 36 μg of APT_{EGFR}-PGNRs was intravenously injected into the tail vein and comparative therapeutic efficacy was evaluated using control group of mice (PBS injection). After 4 h, the tumor site was exposed to an NIR coherent diode laser for 10 min. During the period under observation, the length of the minor axis ($2a$) and the major axis ($2b$) of each tumor was measured using a caliper. Each tumor volume was then calculated using the formula for a prolate spheroid ($(4/3)\pi \times a^2 b$).

3 Results and Discussion

3.1 *Synthesis and Characterization of EGFR-Targetable PGNRs*

We synthesized GNRs using a seed-mediated growth method for photothermal ablation of EGFR-expressed cancer cells. To prepare the gold seed nanoparticles, CTA-AuBr4 complex was formed by mixing $HAuCl_4$ solution and CTAB solution and then gold nuclei were assembled by adding sodium borohydride as a strong reduction agent. These ion nuclei agglomerated to form individual gold seed nanoparticles. Subsequently, Ag ions in silver nitrate were then deposited on the {1 1 0} surface of gold seed nanoparticles. GNRs grew on additional crystal sides such as the {1 0 0} surface and the {1 1 1} surface when using CTAB and ascorbic acid as reducing agents because the larger energy barrier from Ag ion attachment causes much slower growth of gold on the {1 1 0} surface leading to rod-shaped nanostructures.

It is essential that cationic CTAB from GNRs was eliminated because the release of cationic molecules from GNRs can induce cytotoxicity by disrupting cellular membrane. Thus, CTAB bilayer on GNRs was replaced with heterofunctional PEG (COOH-PEG-SH) as biocompatible stabilizer and the gold-thiol reaction was initiated by the oxidative addition of the S-H bond to the gold, followed by the reductive elimination of the hydrogen as well as linker to conjugate Apt_{EGFR}.

For guiding PEGylated photothermal therapy agent to epithelial cancer cells, anti-EGFR aptamer (Apt_{EGFR}) and antibody (Ab_{EGFR}) as targeting moiety were conjugated to the surface of PGNRs. Apt_{EGFR}-PGNRs and Ab_{EGFR}-PGNRs were synthesized by conjugation between the primary amine ($-NH_2$) groups of targeting moiety and the carboxylic acid groups ($-COOH$) of the PGNRs using EDC/NHS chemical conjugation. Consequently, the Apt_{EGFR} and Ab_{EGFR} were covalently attached to the outer ends of the PEG monolayer via carboxylic acid bonds. Moreover, to quantify the aptamer and antibody, Apt_{EGFR}-PGNRs and Ab_{EGFR}-PGNRs were calculated by Take3 module and a BCA assay kit, respectively. As the result, approximately 30 equivalent targeting moieties were successfully conjugated on the surfaces of PGNRs.

Synthesized Apt_{EGFR}-GNRs and Ab_{EGFR}-PGNRs' morphology were evaluated by TEM [Fig. 2(a)]; these GNRs had an aspect ratio (length/width) of 4.0. Then, to investigate the optical properties, their absorption spectra were evaluated using a spectrometer, in which two main absorption bands were apparent; a transverse absorption band at 520 nm and longitudinal band at 800 nm resulting from the coherent electronic oscillation. The coherent electronic oscillation along the long axis had an intensity of 3.6 times higher than that of the transverse absorption band [Fig. 2(b)]. Therefore, the GNRs exhibit suitable optical characteristics for use as photothermal agents because they absorb light strongly in the NIR region. To investigate the photothermal characteristics of Apt_{EGFR}-PGNRs and

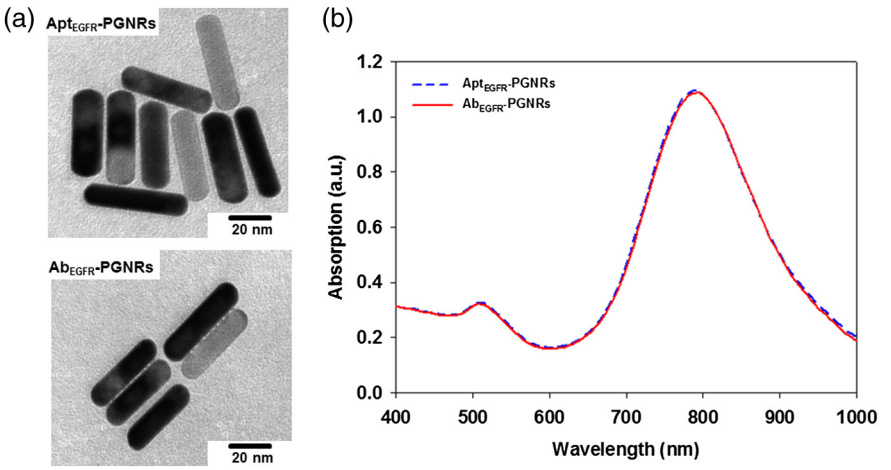

Fig. 2 (a) TEM images and (b) absorption spectra of Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs.

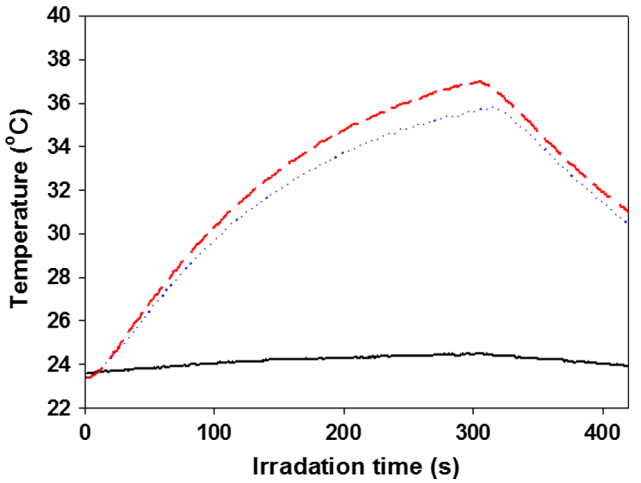

Fig. 3 Temperature profiles for Apt$_{EGFR}$-PGNRs (red, long dash), Ab$_{EGFR}$-PGNRs (blue, dot) (50 μg/mL), and water (black, solid) irradiated by NIR laser (808 nm) with a power density of 0.5 W cm^{-2} for 5 min. NIR laser was turned off after 5 min.

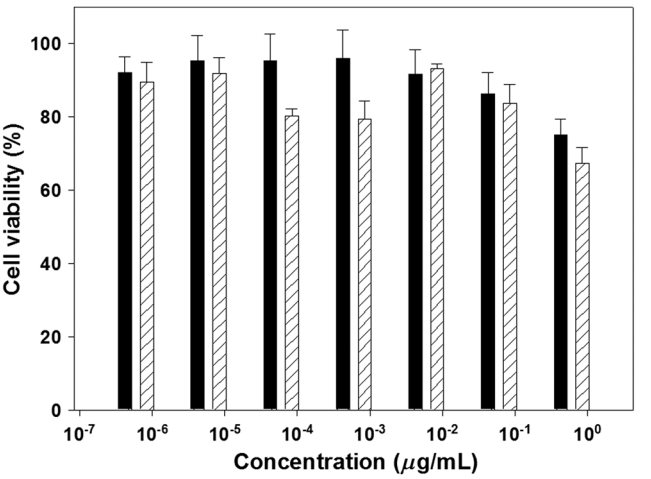

Fig. 4 Growth-inhibition assay by MTT assay for A431 cells treated with Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs (6 μg/mL).

Ab$_{EGFR}$-PGNRs, we measured the temperature change of solutions under NIR laser irradiation (Fig. 3). Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs solutions with NIR light increased the temperature from 24°C to 36°C, which is sufficient to induce cell damage. However, the change in the temperature of distilled water as a control was small (23.5 to 24°C). These results indicated that Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs can be used as efficient plasmon resonants for hyperthermic treatment of cancer.

3.2 *Assessment of Cytotoxicity for EGFR-Targetable PGNRs*

We next determined the *in vitro* cytotoxic effects of both Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs using the MTT assay; the results are shown in Fig. 4. Over 80% of A431 cells (1×10^4) treated with Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs in the concentration range of 10^0 to 10^{-6} mg/mL remained viable, indicating that Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs are biocompatible even at high nanoparticle concentrations. These results indicate that the innocuously surface-modified GNRs are biocompatible.

3.3 *In Vitro Targeted Photothermal Ablation of EGFR-Expressed Cancer Cells*

To evaluate the targeting abilities of Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs to EGFR *in vitro*, the affinities against A431 (EGFR over-expressing) and MCF7 cells (EGFR-deficient) were examined by measuring the Au concentration in cells using ICP-AES. As shown in Fig. 5(a), 7.5-fold higher Au concentration for A431 cells treated with Apt$_{EGFR}$-PGNRs was confirmed compared to MCF7 cells.

In particular, cellular affinity against in A431 cells exhibited the distinction between Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs. Apt$_{EGFR}$-PGNRs had an approximately 3.5-fold higher cellular affinity capability compared to the Ab$_{EGFR}$-PGNRs, whereas the amount of Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs taken up was little different in MCF7 cells due to the expression degree of EGFR. These data demonstrated that Apt$_{EGFR}$-PGNRs were efficiently taken up by the EGFR-expressing target A431 cells due to the specific binding affinity of Apt$_{EGFR}$.

We next evaluated the *in vitro* photothermal ablation capacity of Apt$_{EGFR}$-PGNRs and Ab$_{EGFR}$-PGNRs for A431 and MCF7 cells upon NIR laser irradiation ($\lambda = 808$ nm, 20 W cm^{-2}), respectively. Cell viabilities were evaluated by staining with

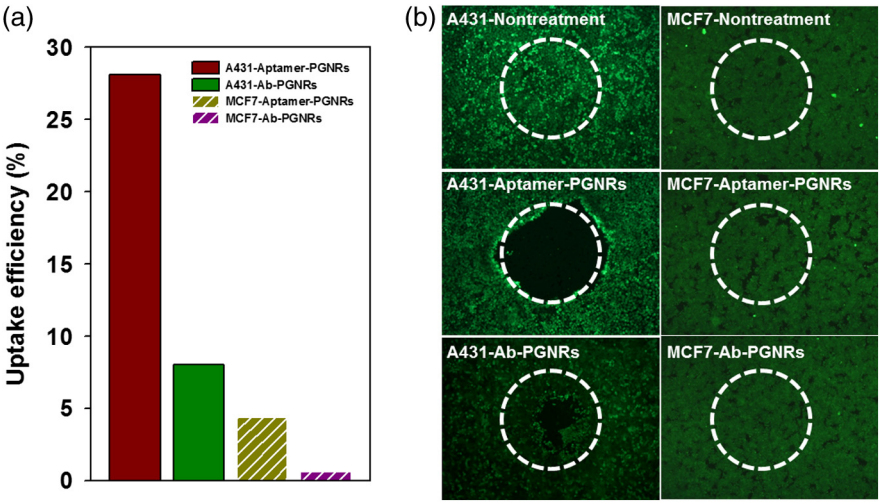

Fig. 5 (a) Cellular uptake efficiencies for A431 and MCF7 cells after treatment with Apt_{EGFR}-PGNRs and Ab_{EGFR}-PGNRs (each data point represents the mean value for $n = 5$). (b) Fluorescence microscopic images of A431 and MCF7 cells stained with calcein AM after NIR laser irradiation ($\lambda = 808$ nm, 20 W cm^{-2}); laser only as a control, Apt_{EGFR}-PGNRs and Ab_{EGFR}-PGNRs, respectively. White dotted circle indicates laser beam location.

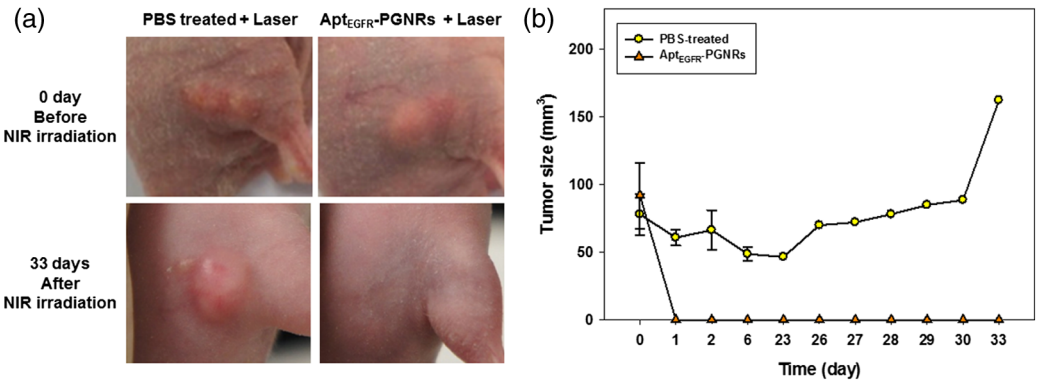

Fig. 6 (a) Comparative photothermal therapeutic efficacy study for tumor-bearing animal model with NIR laser irradiation ($\lambda = 808$ nm, 2.5 W cm^{-2}) after PBS-treated as a control and Apt_{EGFR}-PGNRs, respectively. (b) Measurement of tumor growth of mice treated with PBS (yellow cycle) and Apt_{EGFR}-PGNRs (orange triangle) for 33 days.

calcein AM after 10 min of NIR laser treatment (a membrane permeable green fluorescent cell marker that is hydrolyzed by endogenous esterase and consequently emits fluorescence in the cytoplasm of live cells). In agreement with the cell uptake results, significant cell death (dark hole) was apparent only in A431 cells treated with Apt_{EGFR}-PGNRs compared to Ab_{EGFR}-PGNRs [Fig. 5(b)], whereas none of the other control groups showed distinct damage to the cancer cells because of the difference in cellular uptake efficiency. Therefore, these results demonstrated the feasibility of Apt_{EGFR}-PGNRs as a photothermal agent.

3.4 *In Vivo Photothermal Therapy*

We tested the *in vivo* photothermal ablation potential of Apt_{EGFR}-PGNRs using tumor-bearing xenograft mice. EGFR-overexpressing A431 cells were transplanted into the proximal thigh region of the nude mice and Apt_{EGFR}-PGNRs were intravenously injected into the tail vein. Apt_{EGFR}-PGNRs treated group and PBS-treated group as a control were subsequently exposed to NIR laser irradiation ($\lambda = 808$ nm, 2.5 W cm^{-2}) for 10 min. As a result, tumors injected with Apt_{EGFR}-PGNRs were shown to be mostly ablated the next day, whereas no antitumor effect was observed in the PBS-treated group (Fig. 6). Furthermore, no apparent tumor mass was detected in any of the three mice in the Apt_{EGFR}-PGNRs treated group until day 33. These *in vivo* results imply that Apt_{EGFR}-PGNRs may serve as an efficient photothermal agent.

4 Conclusion

In summary, we described the development of Apt_{EGFR}-PGNRs and the *in vitro* and *in vivo* evaluation of their functionalities in photothermal therapy of epithelial carcinoma. Consequently, the advantageous features of Apt_{EGFR}-PGNRs allowed us to obtain positive therapeutic results demonstrating the utility of this nanoprobe design in future biomedical applications.

Acknowledgments

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