Aptamerized silica/gold nanocapsules for stimulated release of doxorubicin through remote two-photon excitation

Lih Shin Tew, Tsung-Hsi Lee, Leu-Wei Lo, Yit Lung Khung and Nai-Tzu Chen

Laboratory Service, Bagan Specialist Centre, Butterworth, Malaysia; Department of Biological Science and Technology, China Medical University, Taichung, Taiwan; Institute of Biomedical Engineering and Nanomedicine, National Health Research Institutes, Zhunan, Taiwan; Department of Cosmeceutics, China Medical University, Taichung, Taiwan

ABSTRACT
Precision-based drug delivery via remote triggering is fast becoming an attractive therapeutic design and is highly useful in complicated clinical situations that may require accurate site-delivery of drug while reducing the risk of collateral damage to surrounding healthy tissue. Of the many strategies available to achieve these desirable effects, silica/gold nano-assemblies offers a practical means to achieving these aims. Herein, as a proof-of-concept, a silica nanocapsule passivated with a gold outer nanoshell had been fabricated to deliver Doxorubicin, and this nano-assembly can be remotely triggered via two-photon excitation (TPE), even under in vivo setting. A polyethylene glycol (PEG) layer as well as AS1411 DNA aptamer had also been grafted to the surface to improve homing specificity toward MDA-MB-231 breast cancer tissue. The assembly of silica/gold nanocapsules was characterized via TEM, FTIR, and UV-Vis to validate the the nanoconstruct. Upon TPE irradiation, a higher expression level of Annexin V and Caspase-3 was observed in both in vitro and in vivo animal models. A significant reduction in tumor size on mice model was noticed after 21 days, and these results had suggested a viable nano-sized design serving as remotely triggered drug release platform based on current well-established silica nanoparticulate methodologies.

CONTACT Yit Lung Khung, yitlung.khung@mail.cmu.edu.tw, Department of Biological Science and Technology, China Medical University, Taichung 406040, Taiwan; Nai-Tzu Chen, ohnonancy@gmail.com, Department of Cosmeceutics, China Medical University, Taichung, 406040, Taiwan

© 2022 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.
This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Introduction

Stimuli-responsive nanocarriers can confer the advantage of precise on-site drug delivery while potentially reducing collateral damage to neighboring healthy tissues. Of the many types of externally triggered stimulus for drug delivery, two-photon excitation (TPE) for photodynamic cancer therapy is an appealing concept due to its lower photodamage threshold induced during administration [1–4]. Furthermore, unlike single photon irradiation, TPE is able to penetrate deeper into tissue material and its nonlinearity nature of the photon absorption of TPE permits for the restriction and localization of the focal point of the administered beam to a relatively small area [4,5], a strategy similar to the focusing from proton cancer therapy [6,7]. Hence, TPE is fast emerging as a novel strategy that can externally trigger and release photosensitive nanodrug carriers during cancer treatment while reducing the risk of collateral photodamage to surrounding healthy tissues [8]. At the receiving ends of these external triggers, the choice of mesoporous silica nanoparticle (MSN) as drug nanocarriers had often been reported due to its large volume to surface ratio that enables for high drug loading capacity as well as its biocompatibility [9–11]. So far, the chemistries for producing MSN had already been widely examined and well-understood in the literature. However, being an inert material, the nature of unmodified MSN would be non-responsive toward many external stimuli, and it would therefore be necessary to provide additional surface modifications to MSN surface that render the particles sensitive toward external stimuli [12–14].

Herein, as a proof-of-concept, a MSN-based nanocapsule with its interior loaded with cancer drug doxorubicin (DOX) was assembled with outer layer of gold nanoshell. The gold nanoshell was grafted as outer-shell due to its high photothermal efficiency from localized surface plasmon resonance (LSPR), and interestingly, this property may also provide collective chemo-photothermal effects upon exposure to TPE irradiation. Furthermore, under normal circumstances, the thin gold nanoshell would serve as a protective surface barrier for MSN while shielding the internal DOX drug load, but upon TPE irradiation, this surface gold nanoshell would exfoliate and shed due to photothermal conversion. This exfoliation event would subsequently liberate the DOX in a controlled manner. To improve on the target specificity to the nanocapsule, we had decided to passivate the gold-coated MSN nanocapsule (GNS) with DNA aptamer (AS1411) on its surface targeting MDA-MB-231 breast cancer tissue as our triggered responsive TPE delivery system to serve as a proof-of-concept study. The choice of DNA aptamer as targeting candidate was due to its high thermal stability as well as its long shelf-life compared to antibodies in general.

As shown in Figure 1, the MSN was first synthesized based on modified Stöber process. The surface of mesoporous silica core was silanized with 3-aminopropyltriethoxysilane (APTES) to achieve a surface rich in amines that can in turn serve as starting point for subsequent gold colloid deposition. These porous cavities of these amino-modified MSN were next loaded with doxorubicin through diffusive process, and the MSN nanoconstruct was then passivated with a thin outer gold layer through seed-mediated method as reported previously[15]. This ultra-thin gold nanoshell at the outer surface of MSN was then able to convert absorbed photon energy directly to heat energy under TPE irradiation [5,16,17]. The absorption of photon energy would subsequently result in the deformation and eventual exfoliation of gold shell, and this would ensure the eventual release
of loaded doxorubicin (DOX) from the MSN core. To increase the water solubility and reduce the nonspecific immunogenetic response of the GNS-DOX nanocapsule, the silica core/gold nanoshell hybrid nanocapsule carrying doxorubicin (GNS-DOX) was further modified with an underlying PEG layer via thiol linkages to the gold surface. Similar external-stimulated drug release from silica/gold nanoconstruct was reported by Cheng et al.\[18\]. The gold layer from our study was structured as an ultra-thin layer of gold shell, which capable to release the loaded drug by external-stimulated structure exfoliation, while Cheng’s study was using small size of gold particle as caps of nano-channels for control release. In addition, in our study, AS1411 DNA aptamer was exploited as targeting agent on the surface of the nanocapsule for in vivo studies to improve homing specificity. AS1411 aptamer was chemically conjugated to the PEG chain via conventional carbodiimide chemistry to provide the targeting specificity toward MDA-MB-231 breast cancer cells. It is important to reiterate that the choice of DNA aptamer over typical antibodies as targeting moieties was actually intentional from our part as DNA aptamer was known to provoke lower immune response in vivo as compared to antibodies [19,20]. It is also necessary to note that many of the underlying chemistries had been carefully selected to produce a nanoparticulate system that can be easily fabricated and with the potential for upscaling production as part of our future considerations. To validate our design, a range of different physical characterizations such as transmission electron microscope (TEM), Fourier transform infrared (FTIR), and UV-Vis spectroscopy were performed, and their results were presented here accordingly. Both in vitro and in vivo studies over a course of 42 days were also made to evaluate the overall efficiency of this TPE triggered drug delivery design based on the proposed MSN platform.

**Figure 1.** Schematic illustration of GNS synthesis route via growth-mediated process and the in vivo chemo-photothermal mechanism of GNS nanocapsule through TPE irradiation, which subsequently liberates the DOX drug through nanoshell exfoliation.
Experimental section

Materials

The reagents used in this study are listed as follows: Ammonium hydroxide (NH₄OH, Sigma-Aldrich, 30–33%), Hexadecyltrimethylammonium bromide (CTAB, Alfa Aesar, 99%), Tetraethoxysilane (TEOS, 98%, Sigma Aldrich, Darmstadt, Germany), Ammonium nitrate (NH₄NO₃, Sigma-Aldrich), 3-aminopropyltriethoxysilane (APTES, 99%, Sigma-Aldrich), Doxorubicin hydrochloride (DOX, >99%, LC Laboratories), Formaldehyde (37%, Sigma-Aldrich), Thiol-polyethylene glycol-succinimidyl ester (SH-PEG-NHS, Nanocs, PG2-NSTH -5k), Tetrakis(hydroxymethyl)phosphonium chloride, (THPC, 0.96%, Sigma-Aldrich), Phosphate buffered saline (PBS, Thermo Fisher Scientific), ProLong® Diamond Antifade Mountant (Thermo Fisher Scientific), Hoechst 33,342 (AAT bioquest), and YOPRO-1 (Thermo Fisher Scientific). Unless otherwise specified, all reagents were used as received without further purification.

Preparation of amino-functionalized mesoporous silica nanoparticles (MSN-NH₂)

Mesoporous silica nanoparticles (MSN) were synthesized via a modified Stöber process. In brief, 0.58 g of cetyltrimethylammonium bromide (CTAB) was first dissolved in 300 mL of 0.17 M ammonium hydroxide (NH₄OH) at 40°C. 5 mL of 0.2 M tetraethoxysilane (TEOS) as a silica source was then added into this initial mixture. After 5 hours, 1.0 M TEOS were added, and the solution was stirred vigorously for another 1 hour. The solution was then aged at 40°C for 24 hours. MSN formed from the reaction was then collected by centrifuging at 12,000 rpm for 30 minutes and washed three times with absolute ethanol (99.5%). To remove CTAB from the MSN nanoconstruct, ion-exchange procedure with 250 mg ammonium nitrate (NH₄NO₃) in 50 mL of absolute ethanol was used at 60°C for 24 hours. The MSN solution was then centrifuged and washed with ethanol for three times at 12,000 rpm for 30 minutes to ensure complete removal of CTAB.

Prior to gold nanoshell formation, the extracted MSN was initially functionalized with 3-aminopropyltriethoxysilane (APTES) to produce amine-rich surface coverage on the MSN (MSN-NH₂). To achieve this, 25 mL of MSN in ethanol and 150 µL of APTES were added to round bottom flask and left to react overnight under vigorous stirring at 70°C. The APTES functionalized MSN were then retrieved via centrifugation and washed with copious amount of absolute ethanol.

Gold nanoshell synthesis

Similar to seed-mediated method as detailed previously in the literature [21], a two-step process was performed for the formation of gold nanoshell on MSN; (i) immobilization of ~3–5 nm of gold nanoparticles on MSN-NH₂ and (ii) the formation of gold nanoshell in the presence of plating solution and reducing agent. In the (i) step, gold nanoparticles (GNPs) of 3–5 nm in diameter were first prepared based on the method as detailed by Duff et al.[22] and Preston et al.[23]. In brief, 1.5 mL of 0.2 M sodium hydroxide (NaOH) and 1 mL of THPC, 0.96% were introduced to 45.5 mL of deionized water under vigorous stirring for 10 minutes. After that, 2 mL of 1% tetrachloroauric acid trihydrate (HAuCl₄) was added to the mixing
solution. The solution initially appeared yellow before turning dark brown after a few seconds, which was a visual indicator for formation of GNPs. The GNPs were then aged and stored at 4°C for at least 3 days prior to use. Next, attachment of the GNPs on the MSN-NH₂ was achieved following the procedures as previously reported by Pham et al.[15]. 0.5 mL of MSN with surface enriched with NH₂ moiety (3.44 mg/mL) was added into 5 mL gold colloid and was stirred vigorously for 10 minutes. The solution was then allowed to stand for 2 hours in dark, and subsequently, gold colloid attached to MSN-NH₂ (seed particles) was centrifuged and re-dispersed in 5 mL of deionized water. Prior to the formation of the thin continuous layer of gold shell on the surface of the MSN, a reducible gold salt solution (K-gold) was prepared on the previous day by adding 25 mg of potassium carbonate (K₂CO₃) to 100 mL of deionized water followed by the addition of 2 mL of HAuCl₄ followed by storage at 4°C overnight. The growth of a thin continuous layer of gold shell along the outer periphery of MSN was subsequently achieved by adding 200 μL of seed particles to different set volumes (1 to 5 mL) of K-gold. 10 μL of formaldehyde was then added quickly to reduce the K-gold, and the formation of gold nanoshell could be visually indicated by the change of color of the solution from colorless to blue.

**Doxorubicin (DOX) loading capacity**

To determine the loading capacity, a calibration curve was first obtained over the range of 2.0–125.0 μg/mL to establish the drug loading efficiency. 0.25 mL of MSN (2.5 mg/mL) was dispersed in 0.25 mL of doxorubicin (DOX) (2.5 mg/mL) and stirred for 1 hour in the dark for the process of DOX loading. The DOX loaded MSN was then washed and centrifuged at 12,000 rpm for 5 minutes, and level of DOX in supernatant was then determined using UV-Vis spectroscopy (excitation at 488 nm, emission at 590 nm) to evaluate the drug loading capacity. The drug loading capacity was calculated by the following equation:

\[
\text{Doxorubicin encapsulation efficiency} = \frac{(\text{Total DOX} - \text{Free DOX})}{(\text{Total DOX})} \times 100\%
\]

**Surface functionalization**

To facilitate the functionalization of AS1411 DNA aptamer to the surface of the gold nanoshell/DOX loaded MSN (GNS-DOX), the surface of the nanoconstruct was first grafted with PEG-NHS. In brief, 10 μL of 50 mM SH-PEG-NHS (PEG MW: 5 K from Nanocs, product number: PG2-NSTH-5k) was prepared and mixed with GNS-DOX solution. The mixture was then stirred at room temperature for 2 hours for the thiolation process that would graft the SH-PEG-NHS to the gold surface. After the thiolation process to the GNS-DOX surface that would introduce the PEG (GNS-DOX-PEG), the mixture was centrifuged to remove the excess unreacted SH-PEG-NHS. Amino-labeled AS1411 aptamer was re-solubilized in DEPC water, and a concentration of 6.16 nmoles of the aptamer was added to GNS-DOX-PEG nanoconstruct and the reaction mixture was stirred at 4°C for overnight. The DNA sequence of the AS1411 aptamer is similar to previous report from our group and as listed below [24].

5’ GGTGGTGGTGTTGTTGGTGTTGTTGGGCCGGGCCCCC 3’
Nanoparticle characterization

Transmission electron microscope (TEM) (JEOL JEM-2010) was performed to determine the morphology and size of synthesized MSN and GNS. The nanoparticles were loaded on copper grids prior to TEM analysis. Fourier transform infrared (FTIR) spectra were used to examine and confirm on the surface chemistry of the MSN nanocapsules as well as the GNS.

Aptamer specificity assay

In this work, non-cancerous breast epithelial cell MCF-10A and breast cancer cell MDA-MB-231 were used to evaluate the aptamer specificity. MCF-10A cells were cultured in mammary epithelial cell basal medium (MEBM), while MDA-MB-231 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium. Both media were supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. 2 × 10^4 cells were seeded to glass-bottom cell culture dish 24 hours prior examination and labeled with Cy5-tagged AS1411 aptamer at concentrations of 0, 17, 34, and 68 pmoles. After washing and fixation, cells were counterstained with Hoechst 33,342 for nucleus and Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate (WGA488) for cell membrane. Cellular images were then captured with fluorescence microscopy (Olympus BX51).

Cytotoxicity assay of GNS loaded with DOX

The cytotoxicity profile of the GNS loaded with DOX toward MDA-MB-231 cells was evaluated using the cell meterTM colorimetric cell cytotoxicity assay kit (AAT Bioquest, Sunnyvale, CA, USA). In summary, MDA-MB-231 cells were cultured in 37°C with 5% CO₂ and upon reaching 80% confluence, the cells were treated with trypsin after washing with PBS for 3 times. The detached cells were then centrifuged at 1000 rpm for 5 minutes, and the remaining cell pellets were re-suspended with pre-warmed growth medium before seeding on a new 96 well culture plate at a concentration of 1000 cells per well. After 24 hours incubation at 37°C, the cells were then exposed to gold nanoshell (GNS) loaded with DOX at a concentration of 25, 50, 75, and 100 µg/mL in serum free RPMI-1640. After 4 hours of incubation, the nanoparticles containing media were discarded and 100 µL of fresh medium was added into each well for another incubation period of 24 hours. Subsequently, 20 µL of cell meterTM assay solution was added into each well, and the solution was gently shaken for 30 seconds. The absorbance changes at 570 and 605 nm were then recorded with a microplate reader after another 4 hours of incubation, and the readings were used to determine for cell viability in each well.

ICP-MS sample preparation

MDA-MB-231 was plated to 60 mm dishes at a density of 1.6 × 10^6 cells, and after 24 hours of incubation, the cells were treated with 100 µg/mL of DOX loaded GNS in serum-free medium for another 4 hours. After the cellular uptake phase of the DOX-loaded GNS, the cells were trypsinized and carefully digested with 100 µL fresh aqua regia for 24 hours. The digested samples were then diluted to a final volume of 1000 µL with deionized water prior to ICP-MS analysis.
**Photostability of GNS**

To evaluate the photostability of the GNS nanoconstruct, a custom built NIR laser scanning two-photon microscopy was utilized (Integrated Stem Cell Center, China Medical University Hospital). First, 50 µL of GNS was carefully aliquoted and sealed between two glass slides, and the sample was then exposed to two-photon irradiation (TPE at 800 nm) for few seconds. After that, the sample was collected and analyzed with ultraviolet-visible (UV-Vis) spectroscopy.

**In vitro cytotoxicity of GNS alone and loaded with DOX with Two Photon Irradiation**

MDA-MB-231 cells were first seeded at a density of $1.6 \times 10^6$ in 60 mm dishes and incubated overnight. To obtain the in vitro cytotoxic profile, the medium was removed and cells were gently washed with PBS for 3 times. After that, the cells were treated with 100 µg/mL GNS and GNS loaded with DOX separately in a serum-free medium for 4 hours at 37°C. Cells incubated without GNS in serum-free medium were used as control for this experiment. The cells were then washed with PBS for three times to remove the excess GNS and replenished with fresh serum-free medium. YOPRO-1 was added into medium prior to irradiation by the two-photon laser microscopy at 850 nm with output power of 46 mW. Cell images were finally captured by fluorescence microscopy (Olympus BX51) and fluorescence intensity (excitation at 488 nm and emission at 520 nm) of YOPRO-1 was acquired at 541 nm ± 55 nm. The fluorescence intensity was subsequently quantified by ImageJ software.

**In vivo TPE-mediated chemo-photothermal therapy**

All experiments involving the use of animals were performed in accordance with the guidelines of the Management Group of Animal Experiments (MGAE) in Taiwan (Animal protocol number: CMUICUC-2017-352-2). In brief, 4–6 weeks old female nude mice (nu/nu; 20–25 g; NARLabs Taiwan) were anesthetized by isoflurane inhalation and subcutaneously inoculated with MDA-MB-231 cells ($2 \times 10^6$ cells in 0.1 mL PBS) into the lower flank. The tumor-bearing mice were randomly divided to five groups for different treatments: control, Laser, GNS-DOX, GNS with laser (GNS-Laser), and GNS-DOX with laser (GNS-DOX-Laser). Each of the group had 9 mice for the purpose of monitoring tumor growth curve, survival fraction, and tumor immunohistochemistry studies. In vivo chemo-photothermal therapy experiments were performed when the tumor volume reached approximately 150–200 mm$^3$. The nude mice were anaesthetized with isoflurane inhalation and received intra-tumor injections of sonicated nanoparticles at a concentration of 16 mg/kg in 100 µL of saline solution with non-treated tumor mice serving as our control groups. At the 1-hour post-injection mark, the tumors in mice were irradiated using a Ti:Sapphire femtosecond amplifier (Thorlabs, USA). An average power of 200 mW was measured and applied to all mice with exposure time of 20 minutes. Three mice from each group were sacrificed after 24 hours post-irradiation for histological evaluation. The tumors were sliced to 5 um thickness and stained with Hoechst 33,342 for nucleus and also two apoptosis
reagents – Annexin V (early apoptosis indicator) and Caspase-3 (late apoptosis indicator). The rest of the mice (n = 6) were monitored 21 days for tumor growth and 42 days for survival curve. It is important to note that all mice were euthanized for humane endpoint when the tumor reached a volume of 1500 mm³ so as to reduce unnecessary suffering from the animal.

**Statistical data analysis**

Statistical analysis was performed using Origin software (OriginLab, USA). Data were summarized as means ± standard deviation, and the statistically significant differences between 2 groups were being assessed using an unpaired Student’s t-test. Statistical significance is denoted as follows: * represents a p value ≤ 0.05, ** represents a p value ≤ 0.01. All experiments were conducted in triplicates under otherwise specified.

**Results and discussion**

In the initial stages of this work, the focus had been on the assembly and the synthesis of the MSN core that serves as the template for the growth of the gold outer layer. The fabrication methodologies of the MSN core was relatively straightforward and well established, and as shown in Figure 2, a series of transmission electron microscope (TEM) images had revealed the near-spherical shape of amino-terminated mesoporous silica core (Figure 2a).

The average diameter of mesoporous silica core as determined by ImageJ was found to be approximately 84.90 ± 10.90 nm (N = 150). The subsequent self-assembly of gold colloids toward nucleation sites of amino-terminated silica surface was easily visualized from the TEM images (Figure 2b), and over time, this would gradually proceed toward the formation of a thin gold layer in the presence of the K-gold solution and reducing agent. Different ratios of K-gold (see experimental section) to seeding concentration was initially attempted so as to determine and establish the most optimal protocol to attain complete surface gold coverage in our setup as well as to study the evolution of plasmon absorption from the all resultant GNS-DOX. As shown in Figure 2b, there was a large quantity of highly dispersed gold colloids evenly distributed along the outer periphery of mesoporous silica core right at the onset of the reaction and these colloidal “nodules” help to facilitate for the subsequent coagulation of seed particles that eventually forms an uniform gold layer on the surrounding surface of the MSN. The ensuing representative TEM images of the progressively growing seed particles at different ratios of K-gold to seed (1:1, 2:1, 3:1 and 4:1) are shown in Figure 2c–f, while the evolution of the plasmon absorption spectra for different K-gold-to-seed ratio is illustrated in Figure 2g. The nanoparticle was observed to possess the nominally interconnected gold network on the MSN surface (Figure 2c) at a seed ratio of 1:1, while the plasmon absorption spectra had shown an extinction peak at 645 nm. Gold nanoshell produced with K-gold to seed ratio of 3:1 had a profile of having complete outer-shell layer formation, although the interior MSN cavities were still observable from the TEM, and this had suggested that the amount of K-gold solution might still be insufficient to form a thick layer on the surface. Finally, at a ratio of 4:1, a complete layer of thin gold shell around the core
was obtained in consistent fashion, and the TEM had also suggested complete passivation while its respective plasmon absorption extinction peak at 805 nm also being recorded. Overall, as the gold layer thickens on the surface of the MSN, the plasmon absorption peak of gold tends to red-shifted toward the near-infrared (NIR) region during the growth of nanoshell, and this observation was consistent with previous findings in the literature [25]. On the basis of the surface coverage as presented from TEM analysis as well as the plasmon absorption profile, we had decided upon GNS-DOX formation with the K-gold to seed ratio of 4:1 as our main delivery nanoconstruct in this study as under these conditions, the production of the nanocapsule with complete surface gold passivation on the MSN surface was most consistent.

Figure 2. TEM images of (a) MSN, (b) THPC-gold colloids seeded on MSN. Growth of gold nanoshell with different plating K gold solution-to-seed ratio: (c) 1:1 (d) 2:1 (e) 3:1 and (f) 4:1. (g) Normalized UV-Vis absorption spectra of gold nanoshell. Scale Bar: 50 nm.
To confer targeting specificity onto the GNS, AS1411 DNA aptamer specific to surface nucleolin receptors was conjugated to the surface of the nanoconstruct. It is important to note that since its first appearance in 90s [26], AS1411 DNA aptamer sequence had been examined by many groups had been used extensively for the targeting of surface nucleolin receptors on various cancer cell lines [27–29]. Although our approach in this work was to administer the nanoconstruct directly to the tumor site followed by photo-irradiation, the presence of the AS1411 was included to provide a reinforcing bonus on improvements in terms for the rate of uptake in principle. This point was well-illustrated by our ICP-MS data in Figure 3c. Furthermore, considering that cancer is typically a complicated disease condition that would often rely on range of different therapeutic strategies in order to treat or arrest the growth, it is therefore necessary to employ a wide range of different anti-cancer strategies in a collective fashion. It is under these considerations that the inclusion of AS1411 was selected with the intentions to help improve on the uptake of the nanoparticles. The attachment of AS1411 aptamer was achieved via conventional thiol grafting of a PEG chain carrying a distal N-hydroxysuccinimide (NHS) group to the gold surface, which was quickly followed by carbodiimide conjugation between the NHS and amine-tagged AS1411 DNA aptamer. The functionalization of PEG to the gold surface and the subsequent DNA aptamer conjugation were confirmed with Fourier transform infrared (FTIR) spectroscopy. As shown in Figure 3a, GNS-PEG FTIR spectrum had exhibited a noticeable peak at 1071 cm$^{-1}$, which was in turn assigned to the C–O–C stretching vibration from the PEG chain. Furthermore, the spectrum had exhibited peaks at 1589 cm$^{-1}$ and 2886 cm$^{-1}$, which were attributed to the stretching mode for C = O as well as the aliphatic C–C contribution, respectively, and this was indicative of organic grafting on the surface. After conjugation with amino-labeled AS1411 aptamer, the peak for tertiary amide had shifted from 1589 cm$^{-1}$ toward 1538 cm$^{-1}$, and this had suggested a successful attachment of aptamer to PEG chain through amide coupling to the surface of the nanoconstruct. Appearance of band at 1580 cm$^{-1}$ denotes in-plane stretching vibrations of pyrimidine and pyridine [30], thus suggesting the presence of DNA aptamer on the surface of the gold nanoshell. Interestingly, thermogravimetric analysis (TGA) performed on all the nanocapsules (Figure 3b) had shown that in the absence of the gold coating, MSN nanocapsules were unable to retain the doxorubicin loaded in the interior core cavities, while the mass of gold nanoshell-coated MSN remained relatively unchanged for temperatures of up to 600°C (melting point of gold = 1064°C). From these data, we had shown that the presence of the gold nanoshell could help to serve as a barrier against the leakage of DOX within the core of the MSN nanocapsule prior to the administration of the photoirradiation.

Before evaluating the efficacy of our proposed nanoconstruct in animal model, it was necessary to conduct physical and biological profiling assay to determine the in vitro cellular uptake of nanoconstruct, drug release profile, as well as other post-thermal exfoliation behaviors. Generally, AS1411 aptamer would gain cellular entry via nucleolin receptor that were typically overexpressed in cancer cells, while the surface of non-cancerous cells does not normally present them in large quantities [31,32]. Derenzini et al. had described the expression of nucleolin as inversely proportional to the cell doubling time, and hence, the level of nucleolin is usually presented at higher levels in cancer cells compared to normal healthy cells [33]. In this study, GNS-PEG (without aptamer) and GNS-PEG-Apt (with aptamer) was introduced to MDA-MB-231 cancer cells
to determine the uptake efficacy of the nanoconstruct model under in vitro conditions. Inductively coupled plasma mass spectrometry (ICP-MS) was then used to quantify the absolute amount of gold that was internalized within MDA-MB-231 cells and the results are presented in Figure 3c. ICP-MS analysis had indicated that the total amount of gold internalized by MDA-MB-231 cells after the aptamer conjugation was significantly higher (approximately 8 fold higher) than that of GNS-PEG. This suggested that the presence of the aptamer had help improve on the level of entry into MDA-MB-231 cells, and its presence had increased the overall efficiency for cellular internalization. Next, we tested the aptamer specificity by co-incubating Cy5-tagged aptamers with non-targeted MCF-10A (non-cancerous) cell lines and MDA-MB-231 cells (cancerous), respectively, and observed the differences in uptake through confocal microscopy visualization (Figure 3d). It was obvious that Cy5-tagged aptamer (red) were found to be more prevalent within the cytoplasm of MDA-MB-231 cells after 2 hours of incubation even at 17 pmoles concentration (Figure 3d). Furthermore, the visualization of intracellular uptake was observed to have increased with rising concentrations of the aptamer. In contrast, MCF-10A cells did not shown any noticable uptake from confocal visualization, even at high concentration (68 pmoles) of aptamer. From these studies, we had demonstrated the usefulness of AS1411 DNA aptamer as a targeting moiety on the surface of the nanocapsule. Furthermore, in view of the fact for low uptake of the nanoparticles in the absence of
the AS1411 due to the underlying PEG layer, the author felt that a direct comparison between non-functionalized nanoparticle and functionalized nanoparticle would not be meaningful due to non-binding/aggregation effects even as observed during the course of our experimentation.

Next, the assembled GNS nanocapsules were subjected to deliberate two-photon excitation (TPE) for evaluation of photoluminescence and photostability of GNS. The structures of GNS before and after TPE at 850 nm were first analyzed by UV-Vis spectroscopy. The original plasmon resonance peak of GNS at 800 nm had disappeared after TPE (Figure 4a), and a blue shift of plasmon resonance peak to 570 nm was observed as a result. This finding was in full agreement with previous findings as reported by Aguirre et al. [34]. Furthermore, it is also necessary to note that another possible explanation for this blue-shift may be due to the fragmentation of the gold nanoshell resulting in a plasmon adsorption at a lower wavelength [35]. Representative TEM images from pre-irradiation (Figure 4b) and post irradiation had also shown a near-complete exfoliation/deformation of the gold shell, as shown in Figure 4c. Scanning transmission electron microscope (STEM) line cross-section scan profiles of the GNS before (Figure 4d) and after (Figure 4e) TPE irradiation at 850 nm were obtained via STEM (JEM-2100 F with EDX). Energy-Dispersive X-ray Spectroscopy (EDX) was performed across each of selected nanoparticle to provide detailed chemical profiling across the surface of the assembled nanoparticle. As shown in Figure 4d, the line graph had indicated that higher gold (Au) signal was mostly confined at outer periphery of the nanocapsule at both distal ends, and this was interpreted as thickening of the gold layer from contours of the nanocapsule, while higher silica (Si) signal was mostly localized at the center of the nanocapsule. Upon TPE irradiation, these signals disappeared (Figure 4e) and fragmentation of GNS into smaller gold colloid was observed, hence suggesting that exfoliation had indeed occurred. Halas’ group had previously described that the deformation of GNS to a more thermodynamically stable morphology after TPE was mainly due to rapid lattice temperature raising to 1064°C to reach the melting point of bulk gold [34], and this was way above the melting point of nanoparticulate gold [36]. Similarly, our irradiation process had also resulted in the formation and reorganization of small gold aggregates, as shown in Figure 4c, and this had correlated well to the previously observed plasmon resonance peak at 570 nm. In conjunction, two-photon induced photoluminescence (TPIL) profile of GNS was determined and plotted as a function of time (as shown in Figure 4f). GNS nanocapsule had shown strong photoluminescence signal under TPE, but rapid decays of this signal of 0.79 a. u. and 0.44 a. u were also recorded during the first 2 seconds of exposure at 0.5% and 1% of power laser irradiation, respectively, as well as comparing against a non-irradiated control sample. Over time, the luminescence intensities had gradually decreased and finally plateauing at 0.68 a. u. and 0.37 a. u after 10 seconds of TPE exposure.

The loading capacity of our MSN-NH₂ was experimentally determined to be closed to 29.87% on an average, and to evaluate the release profile of these construct, we performed drug release under a range of different pH and the released DOX was detected using UV-Vis spectroscopy. Due to the fact that the beam width of the TPE laser setup is generally very narrow, the exposure on a given area would only exfoliate nanoparticles found within the area of exposure. It would not be possible to provide an accurate release profile as only a fraction of the nanoparticle would be exposed to the photoirradiation.
Therefore, the release behavior of DOX from mesoporous silica-core uncoated with gold nanoshell (MSN-NH$_2$) and GNS-Dox was evaluated under different pH values (pH 5 and 7) to represent status of after and before the gold shell exfoliation. As shown in Figure 4g, in the absence of gold coating, the initial burst release from MSN-NH$_2$ was found to be significant at pH 5 compared to pH 7 and the cumulative amount of released DOX reached approximately ~3 μg after 24 h at pH 5. However, no DOX was detectable from GNS-DOX at both conditions, pH 5 and pH 7 (realistic physiological setting in both in vivo and in vitro experimentation), thus indicating zero premature release which is suggestive of the efficient gold barrier stopping unwanted leakages. The line for GNS-DOX, pH 5 (green) was not observable in the Figure 4g due to similarity to the line for GNS-DOX at pH 7. These findings are relevant in the context of cancer tissue setting as physiological environment of cancer tissues are often found to be more acidic. This was also highly encouraging as we had also shown that in the presence of gold passivated layer, we were able to resist and arrest the burst release of DOX nature from on the onset.

As an alternative to the release of DOX, we recorded temperature changes (ΔT) from gold nanoshell (GNS) in water under continuous wave (CW) laser irradiation (800 nm, 15 minutes) to profile the photothermal conversion efficiency as this physical effect may contribute additional anti-cancer therapeutic property from our nanocapsule (Figure 5). Water, as our negative control, was poor in converting photon energy to thermal energy as there was only
12.5°C increase in temperature that was recorded in the absence of the nanocapsule. By contrast, the GNS in water solution at 0.5 mg/ml exhibited an increment of 25.9°C after 15 minutes of laser irradiation. Interestingly, when the concentrations of GNS reaches 1.0 mg/mL and 1.5 mg/mL, the temperature raised up from room temperature to 58.2°C and 59.8°C, respectively, at the 15 minute mark. This observation indicated that the photon energy as absorbed by GNS was responsible in converting photon energy to heat, and this may also provide additional beneficial effects arising from photothermal therapy (PTT) in conjunction to our chemotherapeutic (DOX) approach for the treatment against cancer cells in the following section. Moreover, we did not notice any significant changes in temperature on MSN (1.5 mg/ml) without gold nanoshell coating, thus indicating that gold nanoshell was solely responsible for the elevation in temperature.

As mentioned earlier, the heating effects from TPE-mediated PTT alone as well as the chemo-photothermal therapy was subsequently evaluated through the deliberate introduction of GNS without DOX loading (GNS-PEG-Apt) and GNS with DOX loading (GNS-DOX-PEG-Apt) to MDA-MB-231 cells in the presence of YO-PRO-1. First, it is necessary to note that YO-PRO-1 detects for early cellular apoptosis marker, as it is a nuclear marker that binds specifically to dying mammalian cell types and is extremely useful in detecting early cell death. As illustrated in Figure 6a and b, control MDA-MB-231 cells, before and after 850 nm TPE, the fluorescence intensity of YOPRO-1 was undetectable, hence suggesting that the cancer cells remained viable and healthy. However, GNS-PEG-Apt (Figure 6a) treated cells exhibited a dramatic increase in fluorescence intensity of YO-PRO-1 after exposure to TPE. At the equivalent concentration, GNS-DOX-PEG-Apt exhibited higher light intensity (Figure 6b). The higher light intensity in this experiment group might be attributed to the diffusion of DOX from the mesoporous channel owing to the filter (541 nm ± 55 nm) used in this experiment setup, which was able to capture the fluorescence emitted from DOX (excitation at 480 nm and emission at 590 nm) upon the exfoliation of GNS gold outer layer. Furthermore, cytotoxicity level of GNS nanocapsule was evaluated. Cell viability was determined via MTT assay on cells treated with 100, 200, and 400 μg/mL of GNS-DOX-PEG-Apt in the absence of TPE triggering. Equal amount of free DOX was used as for comparison. There was no notable cytotoxicity to the cells, as shown in Figure 6c, thus suggesting that cytotoxicity from gold nanocapsule was negligible and the gold nanoshell was effective in preventing passive diffusion from the MSN core.

To evaluate the mechanism of the cell apoptosis, caspase-3, a cell death signal molecule that is highly expressed in cells undergoing apoptosis was stained accordingly. By staining the MDA-MB-231 cells with the Hoechst 33,342 and Caspase-3 under identical conditions, fragmentation of chromatin and typical apoptotic condensation was observable for MDA-MB-231 cells treated with GNS-PEG-Apt and GNS-DOX-PEG-Apt. This observation had further reinforced the notion of TPE-mediated PTT also led to cell death via apoptosis. Interestingly, DOX was also detectable (red) when examining DOX-GNS-PEG-Apt treated MDA-MB-231 cells under confocal microscope (see Figure 6d). These findings further confirmed the release of DOX from mesoporous channel upon exfoliation of GNS mediated by TPE. However, elucidating the efficacy of the synergistic chemo- and photothermal therapy by comparing to either types of therapy alone was technically
challenging for in vivo studies at this stage and would certainly form the basis of future work pertaining to the effectiveness of collective chemo- and photothermal therapy with the presence of GNS-DOX-PEG-Apt.

In our in vivo study, intratumoral injection of gold nanocapsule was selected as mode of delivery in accessing the in vivo efficacy of TPE-mediated synergistic chemo-photothermal therapy. In this study, mice were treated with 5 different conditions: control, Laser, GNS-DOX, GNS-Laser, and GNS-DOX-Laser. 9 nude mice for each group were implanted with $2 \times 10^6$ MDA-MB-231 breast cancer cells and served as our animal models. When the tumor volume reached approximately 150–200 mm$^3$, mice were subsequently anaesthetized and administrated with targeting GNS nanocapsules. After an hour of administration, the tumors sites were then irradiated with 850 nm femtosecond laser pulses for exposure energy of 200 mW for 20 minutes. From our experience, a 20 minutes exposure time was necessary to attain the desired TPE effects due to the thick subcutaneous tissue of the skin of the mice and hence this exposure time differs slightly from the in vitro experiments. Three mice from each group were sacrificed at 24-hour mark post-irradiation for immunohistochemistry evaluation. The rest of the mice (6 mice for each group) were monitored the tumor size and mice survival fraction for 3 and 6 weeks after treatment. Non-treated mice were used as control group. In Figure 7a, we had investigated the apoptosis-related indicators on the treated tumors to elucidate the

![Figure 5](image-url)
cytotoxicity mechanism. Both Annexin V (red) and caspase-3 (green) were used as early and late apoptosis indicators, respectively [37–39], while Hoechst 33,342 were also used to counterstained cellular nucleus. The tumors were harvested and sectioned into 8 mm thick slices 24 hours post-irradiation. In the control tumor, as well as the laser and GNS-DOX tumor sections, there were no Annexin V and caspase-3 fluorescence observed. This had indicated that there was no significant cell death after 20 minutes TPE excitation or GNS nanocapsule treatment. Similarly, GNS incubated cells in Figure 6c had also shown no evidence of cell death, which then further lead us to conclude that the cytotoxicity level from gold nanocapsules were negligible. GNS-Laser tumor section had showed Annexin V and caspase-3 signals due to the TPE-mediated PTT. However, GNS-DOX treated MDA-MB-231 cells excited under two-photon illumination at 850 nm exhibited high cell death levels, as indicated by the presence of Annexin V and caspase-3 (Figure 7a). Furthermore, it was observed that the tumor growth of mice group receiving the TPE irradiation alone without drug loading were only slightly delayed (Figure 7b, green line). However, with the combination of TPE irradiation and GNS-DOX (Figure 7b, yellow line), these collective effects had help to reduce tumor growth to 73% by day 21, and this was mostly attributed

Figure 6. (a) MDA-MB-231 cells treated PBS, aptamer-labeled GNS, and aptamer-labeled GNS-DOX were irradiated with TPE and exposed YO-PRO-1, while the fluorescence intensities are quantitative in (b). (c) Cell viability of cells via MTT assay after treatment with GNS-DOX-PEG-Apt and equivalent concentration of free DOX. (d) Fluorescence images of cells treated GNS without DOX loading and GNS with DOX loading. Cell images were acquired through difference channels: DOX (red), caspase-3 (green), Hoechst 33,342 (blue). Scale bar: 25 μm.
to the photothermal effect as well as the release of DOX from the nanocapsule. This effect was much more profound compared to that of GNS-laser (mere photothermal effect) without any drug loading (Figure 7b, pink line). The tumor size of nude mice with GNS-Laser treatment was 134.9% on 21st day, thus indicating the synergistic chemo- and photothermal as compared to photothermal therapy alone. However, tumor growth was registered at 284% for day 21 for the control mice group without any treatment (Figure 7b, orange line). Similar observations were made for the group of mice administered with GNS-DOX without TPE irradiation as the tumor size had reached 266% at day 21 (Figure 7b, blue line). Due to humane reasons, the growth study ended at day 21 as the tumor size had reached an unmanageable volume that can cause discomfort to the mice in the control group. However, the mice studies were subsequently monitored up for 42 days to determine the animal survival curve. The tumor mice were eventually sacrificed for the humane endpoints at the end of the experimentation. The data from Figure 7c had indicated that the mice receiving GNS-DOX with TPE had the longest survival time and highest survival fraction when compared to the control or against any separate treatments such as TPE laser, GNS-DOX or GNS with TPE irradiation. Thus, we can conclude that based on the result of animal studies, we were able to obtain good survivability as well as the arrestment of tumor growth via TPE irradiation of administered GNS-DOX

![Images](image-url)
nanocapsule. And more importantly, there was relatively low photodamage or toxicity observed in our experimentation with TPE irradiation and from our administration of the nanocapsule in the absence of TPE irradiation.

Conclusion

TPE for cancer therapeutic strategy is a relatively new and novel concept, and herein, we have demonstrated that DOX loaded MSN nanocapsules with an outer gold nanoshell protective barrier (GNS-DOX) can serve as TPE triggered delivery system that is highly cell/tissue specific. There are several advantages arising from our proposed delivery platform. First, we had shown that due to the gold nanoshell barrier, we can ensure that there would no passive or unwanted leakage of DOX during administration, and this helped to reduce the overall toxicity to neighboring tissue region. By implementing the gold nanoshell at the outer periphery of the MSN, we were able to deliver drug in situ in a control manner and shield the drug from further losses. Second, we added DNA aptamer to the surface of the gold nanoshell, and this enables for the tissue specific targeting and this was as shown from our results obtained from the MCF-10A studies earlier. Finally, by conferring the gold nanoshell to the surface, MSN can subsequently be triggered by external TPE, and this forms the very basis of our design of a smart delivery system.

Cancer is one of the most challenging diseases and often requires collective strategies to help impede and arrest its growth. In view of this, the authors had reported a smart anti-cancer system and encompassed the virtues from various different approaches to finally achieve to a nanoparticulate system that can be remotely triggered via TPE. In this manuscript, we had systemically characterized both the chemical and the physical properties of the MSN-GNS nanocapsule, and we have performed both in vitro and in vivo studies using MDA-MB-231 breast cancer cells. We had shown that through the site administration of the nanocapsule, we were about to externally trigger the release of DOX via TPE to impede and arrest cancer growth over a period of 42 days, and these preliminary results were highly encouraging. We envisaged that future work would entice the expansion and loading of other drug candidate as well as the tailoring of the MSN system for other cancer cell types.

Abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| DOX          | Doxorubicin                                      |
| MSN          | Mesoporous silica                               |
| TPE          | Two-photon excitation                            |
| APTES        | 3-Aminopropyltriethoxysilane                     |
| Apt          | DNA Aptamer (AS1411)                             |
| PEG          | polyethylene glycol                              |
| GNS          | Gold nanoshell coated mesoporous silica nanocapsules |
| GNS-PEG      | Pegylation on the gold nanoshell coated mesoporous silica nanocapsules |
Acknowledgments

We would like to thank the Integrated Stem Cell Center, China Medical University Hospital (CMUH) for providing access to the two-photon fluorescence microscopy.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

The study was supported by the Ministry of Science and Technology, Taiwan (MOST 107-2113-M-039-003-MY2, MOST 107-2113-M-039-002-MY2 and 109-2221-E-039-013-MY2) and China Medical University (CMU107-N-20, CMU110-S-02 and CMU109-MF-24). We thank Integrated Stem Cell Center, China Medical University Hospital (CMUH) for providing two-photon fluorescence microscopy.

Author contributions

N.T.C and Y.L.K. had both conceived and designed the study for this manuscript and provided all necessary resources for the completion of this work. L.S.T and T.H.L had produced the MSN nanocapsule in bulk while most physical characterization had been performed by L.S.T while T.H.L had assisted in the animal studies. L.W.L had helped collaborated with various aspects of the TPE reactions. Y.L.K. had written the bulk of the manuscript in the physical characterization/interpretation as well as discussion and N.T.C had written all cellular studies while the rest of the authors had contributed to parts of their respective segments.

References

[1] Hopt A, Neher E. Highly nonlinear photodamage in two-photon fluorescence microscopy. Biophys J. 2001;80(4):2029–2036.
[2] Huang HY, Yu BL, Zhang PY, et al. Highly charged ruthenium(II) polypyridyl complexes as lysosome-localized photosensitizers for two-photon photodynamic therapy. Angew Chem-Int Ed. 2015;54(47):14049–14052.
[3] Mekaru H, Lu J, Tamanoi F. Development of mesoporous silica-based nanoparticles with controlled release capability for cancer therapy. Adv Drug Deliv Rev. 2015;95:40–49.
[4] Shen YZ, Shuhendler AJ, Ye DJ, et al. Two-photon excitation nanoparticles for photodynamic therapy. Chem Soc Rev. 2016;45(24):6725–6741.
[5] Zhao TT, Yu K, Li L, et al. Gold nanorod enhanced two-photon excitation fluorescence of photosensitizers for two-photon imaging and photodynamic therapy. ACS Appl Mater Interfaces. 2014;6(4):2700–2708.
[6] Allen C, Borak TB, Tsuji H, et al. Heavy charged particle radiobiology: using enhanced biological effectiveness and improved beam focusing to advance cancer therapy. Mutation Res Fundamental Mol Mechanisms Mutagenesis. 2011;711(1–2):150–157.
[7] Koooy HM, Clasie BM, Lu HM, et al. A case study in proton pencil-beam scanning delivery. Int J Radiat Oncol Biol Phys. 2010;76(2):624–630.
[8] Fox JL, MacFarlane M. Targeting cell death signalling in cancer: minimising ‘Collateral damage.’ Br J Cancer. 2016;115(1):5–11.
[9] Mura S, Nicolas J, Couvreur P. Stimuli-responsive nanocarriers for drug delivery. Nat Mater. 2013;12(11):991–1003.

[10] Slowing Il, Vivero-Escoto JL, Wu CW, et al. Mesoporous silica nanoparticles as controlled release drug delivery and gene transfection carriers. Adv Drug Deliv Rev. 2008;60 (11):1278–1288.

[11] Vallet-Regí M, Balas F, Arcos D. Mesoporous materials for drug delivery. Angew Chem-Int Ed. 2007;46(40):7548–7558.

[12] Biju V. Chemical modifications and bioconjugate reactions of nanomaterials for sensing, imaging, drug delivery and therapy. Chem Soc Rev. 2014;43(3):744–764.

[13] Feng X, Fryxell GE, Wang LQ, et al. Functionalized monolayers on ordered mesoporous supports. Science. 1997;276(5314):923–926.

[14] Meng HA, Liong M, Xia TA, et al. Engineered design of mesoporous silica nanoparticles to deliver doxorubicin and P-Glycoprotein siRNA to overcome drug resistance in a cancer cell Line. Acs Nano. 2010;4(8):4539–4550.

[15] Pham T, Jackson JB, Halas NJ, et al. Preparation and characterization of gold nanoshells coated with self-assembled monolayers. Langmuir. 2002;18(12):4915–4920.

[16] Gao L, Vadakkan TJ, Nammalvar V. Nanoshells for in vivo imaging using two-photon excitation microscopy. Nanotechnology. 2011;22(36):365102.

[17] Chen NT, Tang KC, Chung MF, et al. Enhanced plasmonic resonance energy transfer in mesoporous silica-encased gold nanorod for two-photon-activated photodynamic therapy. Theranostics. 2014;4(8):798–807.

[18] Cheng B, He HC, Huang T, et al. Gold nanosphere gated mesoporous silica nanoparticle responsive to near-infrared light and redox potential as a theranostic platform for cancer therapy. J Biomed Nanotechnol. 2016;12(3):435–449.

[19] Ireson CR, Kelland LR. Discovery and development of anticancer aptamers. Mol Cancer Ther. 2006;5(12):2957–2962.

[20] Song KM, Lee S, Ban C. Aptamers and Their Biological Applications. Sensors. 2012;12 (1):612–631.

[21] Wang YC, Rheumae E, and Lesage F, et al. Synthetic methodologies to gold nanoshells: an overview. Molecules. 2018;23(11):1–28.

[22] Duff DG, Baiker A, Edwards PP, et al. Hydrosol of gold clusters. 1. formation and particle size variation. Langmuir. 1993;9(9):2301–2309.

[23] Preston TC, Signorell R. Growth and optical properties of gold nanoshells prior to the formation of a continuous metallic layer. Acs Nano. 2009;3(11):3696–3706.

[24] Tung J, Tew LS, Hsu YM, et al. A novel 4-arm DNA/RNA nanoconstruct triggering rapid apoptosis of triple negative breast cancer cells within 24 hours. Sci Rep. 2017;7(1). DOI:10.1038/s41598-017-00912-3

[25] Brito-Silva AM, Sobral RG, Barbosa-Silva R, et al. Improved synthesis of gold and silver nanoshells. Langmuir. 2013;29(13):4366–4372.

[26] Kochetkova M, Iversen PO, Lopez AF, et al. Deoxyribonucleic acid triplex formation inhibits granulocyte macrophage colony-stimulating factor gene expression and suppresses growth in juvenile myelomonocytic leukemic cells. J Clin Investig. 1997;99(12):3000–3008.

[27] Sakhtianchi R, Darvish B, Mirzaie Z, et al. Pegylated magnetic mesoporous silica nanoparticles decorated with AS1411 Aptamer as a targeting delivery system for cytotoxic agents. Pharm Dev Technol. 2019;24(9):1063–1075.

[28] Jing YY, Cai MJ, Zhou LL, et al. Aptamer AS1411 utilized for super-resolution imaging of nucleolin. Talanta. 2020;29(13):4366–4372.

[29] Yazdian-Robati R, Bayat P, Oroojalian F, et al. Therapeutic applications of AS1411 aptamer, an update review. Int J Biol Macromol. 2020;155:1420–1431.

[30] Urena FP, Gomez MF, Gonzalez JI, et al. A new insight into the vibrational analysis of pyridine. Spectrochim Acta A Mol Biomol Spectrosc. 2003;59(12):2815–2839.

[31] Otake Y, Soundararajan S, Sengupta TK, et al. Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA. Blood. 2007;109(7):3069–3075.
[32] Soundararajan S, Chen WW, Spicer EK, et al. The nucleolin targeting aptamer AS1411 destabilizes bcl-2 messenger RNA in human breast cancer cells. Cancer Res. 2008;68(7):2358–2365.

[33] Derenzini M, Sirri V, Trere D, et al. The quantity of nucleolar proteins nucleolin and protein B23 is related to cell doubling time in human cancer-cells. Lab Invest. 1995;73(4):497–502.

[34] Aguirre CM, Moran CE, Young JF, et al. Laser-induced reshaping of metallodielectric nanoshells under femtosecond and nanosecond plasmon resonant illumination. J Phys Chem B. 2004;108(22):7040–7045.

[35] Soule S, Allouche J, Dupin JC, et al. Design of Ag-Au nanoshell core/mesoporous oriented silica shell nanoparticles through a sol-gel surfactant templating method. Microporous Mesoporous Mater. 2013;171:72–77.

[36] Inasawa S, Sugiyama M, Yamaguchi Y. Laser-induced shape transformation of gold nanoparticles below the melting point: the effect of surface melting. J Phys Chem B. 2005;109(8):3104–3111.

[37] Darzynkiewicz Z, Juan G, Li X, et al. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry. 1997;27(1):1–20.

[38] van Engeland M, Nieland LJW, Ramaekers FCS, et al. Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure. Cytometry. 1998;31(1):1–9.

[39] Mancini M, Nicholson DW, Roy S, et al. The caspase-3 precursor has a cytosolic and mitochondrial distribution: implications for apoptotic signaling. J Cell Biol. 1998;140(6):1485–1495.