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Using SV119-Gold Nanocage Conjugates to Eradicate Cancer Stem Cells through a Combination of Photothermal and Chemo Therapies

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Abstract

Cancer stem cells (CSCs) are believed to be responsible for the long-term growth of a tumor, as well as its metastasis and recurrence after conventional therapies. Here we demonstrated that the sigma-2 receptor was over-expressed on the surface of breast CSCs, and thus could serve as a

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biomarker for the purpose of targeting. We were able to target breast CSCs with Au nanocages (AuNCs) by functionalizing their surfaces with SV119, a synthetic small molecule capable of binding to the sigma-2 receptor with high specificity. The interiors of the AuNCs could also be loaded with an anticancer drug to be selectively delivered to breast CSCs and released in a controllable fashion. Our results demonstrate that the SV119-AuNC conjugate can serve as a new platform to carry out photothermal and chemo therapies simultaneously, eradicating breast CSCs more effectively through a synergetic effect.

Keywords

cancer stem cells; gold nanocages; SV119; sigma-2 receptor; synergetic effect

1. Introduction

As a small sub-population of cancer cells within a tumor,[1] cancer stem cells (CSCs) are believed to be responsible for the initiation of cancer, as well as its growth, metastasis, and recurrence.[1a, 1b, 2] Current procedures based upon chemo or hyperthermia therapies are known to be ineffective in eradicating CSCs.[3] In fact, recent studies indicate that the conventional chemo therapies tend to enrich the population of CSCs owing to their inherent resistance against these treatment regimes, leading to even higher rates of tumor relapse and ultimately treatment failure.[3a, 3d, 4] As a result, there is an urgent need to develop new strategies for eradicating CSCs more effectively.[1b, 5]

Nanomedicine based upon nanoparticles has the unique capability to combine different therapeutic strategies into a synergetic platform for cancer therapy.[6] To this end, Au nanocages (AuNCs) with localized surface plasmon resonance (LSPR) peaks in the near-infrared (NIR) region are particularly attractive for such a synergetic treatment due to the effective photothermal conversion.[7] In the transparent window from 700-900 nm, the incident light can penetrate deeply into soft tissues, ensuring high efficiency for hyperthermia treatment with minimum side effects.[8] By taking advantage of their hollow interiors and photothermal effect, AuNCs have also been explored as a novel class of vehicles for the delivery of anticancer drugs.[9] When combined with a smart material, the drugs loaded in the interiors of AuNCs can be released in a controllable fashion, allowing for the management of chemotherapy at the targeted site through the irradiation of a NIR laser.[9] As expected, the efficacy of such a synergetic therapy is largely determined by the ability to selectively deliver the AuNCs to the tumor site only. The most effective strategy for accomplishing this goal is to functionalize the surface of AuNCs with a ligand that binds strongly to a receptor (*i.e.*, biomarker) over-expressed on the cancer cells.[9, 10]

As a class of transmembrane proteins found in mitochondria, endoplasmic reticulum, and plasma membranes,[11] the sigma-2 receptor has received renewed interest as a potential biomarker for targeting in cancer research.[12] A high-level expression of the sigma-2 receptor has been observed in a diverse set of human and rodent tumor cells.[13] Most recently, the progesterone receptor membrane component 1 (PGRMC1) protein complex was also identified as a putative binding site for the sigma-2 receptor.[14] In this study, we demonstrate that the sigma-2 receptor can serve as a specific biomarker for breast CSCs and

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thus be employed for targeted CSC therapy.[15] Specifically, we present a facile and versatile strategy for the effective eradication of breast CSCs by combining photothermal and chemo therapies through the use of AuNCs. The AuNC itself can serve as a photothermal transducer capable of absorbing NIR light with a large cross section and then converting the light into heat with high efficiency. We can use a phase-change material (PCM) as the medium to help load the drug, and the encapsulated drug will not be released until the PCM is melted due to heating through a photothermal means. Figure 1a shows a schematic diagram of the encapsulation and release mechanisms. We demonstrated, for the first time, that the AuNCs could be derivatized with SV-119 to target breast CSCs and achieve simultaneous photothermal and chemo therapies, resulting in a synergetic effect for the eradication of breast CSCs.

2. Results and discussion

2.1 Preparation and characterization of SV119-PEG-AuNCs

The AuNCs were prepared with an outer edge length of 46 nm, a wall thickness of 7 nm (Fig. 1b), and an LSPR peak at 780 nm using the galvanic replacement reaction between Ag nanocubes and $HAuCl_4$ in an aqueous solution.[16] Heterofunctional poly(ethylene glycol) (OPSS-PEG-SVA) was reacted with SV119 and then conjugated to the surface of AuNCs *via* the Au-thiolate linkage to generate SV119-PEG-AuNCs. Upon surface modification, the LSPR peak of the AuNCs slightly red-shifted due to a minor change in refractive index on the surface (Fig. 1c). Similarly, PEGylated AuNCs (PEG-AuNCs) without SV119 group on the surface were also prepared and used as a control.

These AuNCs with an LSPR peaks around 800 nm have many attractive features for applications in nanomedicine: *i*) their porous walls and hollow interiors can be utilized for the loading and release of drugs; *ii*) they are well-suited for hyperthermia treatment due to the strong photothermal effect; and *iii*) they have an atomically flat surface that is ideal for quantitative functionalization and labeling with biomolecules or ligands.

2.2 Over-expression of the Sigma-2 receptor on MDA-MB-435 CSCs

We cultured MDA-MB-435 breast cancer cells in suspension to generate mammospheres, thus enriching the proportion of CSCs.[2e] Flow cytometry analysis was then used to determine the population with high activity of Aldehyde Dehydrogenase 1 ($ALDH^{hi}$), which is an indicator of breast CSCs. A typical value of 40% was obtained (data not shown).

The over-expression of the sigma-2 receptor by CSCs was confirmed by a competitive inhibition (blocking) assay involving SW120, a fluorescently labeled ligand that binds specifically to the sigma-2 receptor (Fig. 2a). The ligand was prepared by reacting dansyl chloride with the primary amine group of SV119. Breast CSCs (*i.e.*, the $ALDH^{hi}$ population) and non-CSCs (*i.e.*, the population with low activity of ALDH, $ALDH^{low}$) were separately incubated with solutions containing 10 mM SV119 at 37 °C for 1 h, treated with 50 nM of SW120 for 0.5 h, and their fluorescence intensities were then analyzed using flow cytometry. As shown in Figure 2c, the fluorescence from CSCs was significantly weakened when they were pre-treated with SV119 since SV119 displaced the binding of SW120 to the sigma-2 receptor on the breast CSCs. A similar trend was also observed in the non-CSCs

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(Fig. 2d), similar to what was found in our previous work.[10] Significantly, the relative geometric mean fluorescence intensities (GMFI) of CSCs directly cultured with SW120 was about 7.8 times higher than that of non-CSC (Fig. 2b), indicating that the sigma-2 receptor were over-expressed on the surface of the breast CSCs.

The sigma-2 receptor on the membrane of the breast CSCs belongs to a family of recycling endocytotic receptors. They are involved in tumor progression. Recently, various types of human stem cells (*e.g.*, embryonic, neural progenitor cells, amniotic fluid stem cells, bone marrow stromal cells and hematopoetic stem cells) were found to significantly express the sigma-2 receptor. The ligand for sigma-2 receptor can be taken up by clathrin-medicated endocytosis and delivered to lysosomes, as well as the cytoplasm. In this study, we found that the sigma-2 receptor was over-expressed in human breast CSCs in comparison with non-CSCs, which could thus be used for targeted delivery of drug into breast CSCs.

2.3 Targeting efficiency of SV119-PEG-AuNCs

We then evaluated if SV119 can maintain its binding affinity toward the sigma-2 receptor after conjugation to the surface of AuNCs. If it does, the AuNCs will be able to bind to the sigma-2 receptor on the cell membrane and enter the cell *via* SV119-mediated endocytosis.[10] To quantify the effect of SV119 on the cellular uptake by breast CSCs, the AuNCs with different coverage densities of SV119 (5, 12.5, 25, 50, 75, and 100%) were prepared by varying the ratio of SV119-PEG to PEG during conjugation. The photoluminescence from the AuNCs provides a convenient means to evaluate their *in vitro* targeting capability using two-photon microscopy.[17] In a typical study, the cells derived from mammospheres were incubated with SV119-PEG-AuNCs having different coverage densities of SV119 on the surface at 37 °C for 3 h, and the CSCs were then separated from non-CSCs by cell sorting. As shown in Figure 3, the photoluminescence from the AuNCs had a green color. For the CSCs incubated with the same amount of SV119-PEG-AuNCs, the cellular uptake of AuNCs increased with increasing surface coverage of SV119 and the AuNCs were evenly distributed among the CSCs. In contrast, the non-CSCs incubated with the SV119-PEG-AuNCs under the same conditions showed very little green color and few of the cells contained the AuNCs, implying that SV119-PEG-AuNCs were able to selectively target breast CSCs. The photoluminescence intensity from CSCs or non-CSCs could be used to determine the average number of AuNCs per cell.[17b] Figure 4a compares the average photoluminescence intensity of AuNCs per cell for the CSCs and non-CSCs. There exisited an optimal density of 50% for SV119, at which the AuNCs were taken up more significantly by CSCs than non-CSCs. This observation was consistent with the data obtained from the ICP-MS analysis (Fig. 4b). Again, the cellular uptake of AuNCs increased with increase in surface coverage for SV119, and SV119-PEG-AuNCs with a SV119 density of 50% gave the greatest difference between CSCs and non-CSCs. These results demonstrate that SV119 retained its binding affinity toward the sigma-2 receptor over-expressed on the MDA-MB-435 CSCs after it had been conjugated to the surface of AuNCs. The cellular uptake of AuNCs by breast CSCs was greatly enhanced after SV119 functionalization due to the receptor-ligand binding.[12] Taken together, this new class of conjugates comprised of AuNCs and a small molecule ligand specific to the sigma-2 receptor are well-suited for use as vehicles for targeted drug delivery to CSCs.

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2.4 Effective delivery of chemotherapeutic drugs into the CSCs

In our prior work, we have shown a facile and versatile strategy based on phase-change materials (PCMs) for loading hydrophobic drugs into the hollow interiors of AuNCs.[18] The PCM can serve as a carrier to help load the drug and act as a "gate-keeper" to control the release of the drug in response to temperature rise. In principle, the encapsulated drug should not be released until the PCM has been melted due to heating by a thermal or photothermal means. Since the PCM can reversibly change its physical states between solid and liquid over a narrow temperature range, it can perfectly confine drug molecules inside the AuNCs at a temperature below its melting point (in the solid state). When the local temperature is raised beyond the melting point of the PCM, it will begin to melt, and the drug will be released from the melted PCM (in the liquid state) through diffusion.

We have evaluated 1-tetradecanol, a fatty alcohol characterized by attractive features such as immiscibility with water, good biocompatibility, and a melting point (38-39 °C) slightly higher than the normal human body temperature (37 °C). It can also be mixed with a range of hydrophilic and hydrophobic substances. Furthermore, 1-tetradecanol is an ingredient widely used in cosmetics due to its low toxicity (oral, rat LD_{50} >5 g/kg). In a typical experiment, we loaded a mixture of doxorubicin (DOX) and 1-tetradecanol into the AuNCs, and then conjugated the AuNCs/DOX with SV119-PEG at density of 50%. After being cultured with the cells derived from mammospheres at 37 °C for 3 h, we separated and collected the CSCs by cell sorting. We further cultured the CSCs at 37 °C or 40 °C for another 2 h and the intracellular fluorescence of DOX was analyzed by flow cytometry. As shown in Figure 5, the encapsulated DOX could be released from the SV119-PEG-AuNCs/DOX when the CSCs were cultured at 40 °C. In comparison, the release of DOX at 37 °C was negligible because the DOX was entrapped by a solid PCM inside the AuNCs. In addition, culture of the breast CSCs with SV119-PEG-AuNCs at 37 °C or 40 °C did not show enhanced intracellular fluorescence in comparison with PEG-AuNCs. These results indicate that SV119-PEG-AuNCs/DOX could be used to deliver DOX into the breast CSCs more effectively when the temperature was raised to lightly pass the melting point of the PCM.

The void spaces inside AuNCs have been extensively explored for biomedical applications due to their unique capability to hold and release drugs.[18] Here we filled the hollow interiors of AuNCs with 1-tetradecanol, a PCM that has a melting point of 38-39 °C. As long as the chemotherapeutic drug is miscible with the PCM, it can be readily mixed with the PCM and loaded into the hollow interiors of AuNCs as the PCM diffuses into the AuNCs. The PCM can confine the pre-mixed drug inside the AuNCs until the local temperature is increased beyond the melting point of the PCM, resulting in controlled release of the drug into the CSCs. In addition to direct heating, the AuNCs can be irradiated with a NIR laser with its wavelength overlapping with the LSPR peak to convert the light into heat through the photothermal effect and thus trigger drug release.[18b]

2.5 Eradication of CSCs through a synergetic effect of photothermal and chemo therapies

After being incubated with SV119-PEG-AuNCs/DOX or other controls at 37 °C for 3 h, the CSCs were separated and collected from the cells derived from mammospheres by cell

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sorting. For each group, the CSCs were equally divided and placed in the wells of two 24-well plates. We irradiated the cells in one well with a diode laser centered at 808 nm from the top at a density of 0.8 W/cm^2 for 20 min,[19] and incubated the cells in another well at 40 °C for 2 h. After that, the cells in each well were seeded into 3 wells (1×10^3 cells per well) of 24-well ultra-low attachment plates and incubated at 37 °C and under 5% CO_2. We evaluated the mammosphere-formation ability and the average cell number in each resultant mammosphere 20 days after the treatment, which is considered to reflect the self-renewal ability of CSCs *in vitro*.[20] As shown in Figure 6, a combination of photothermal and chemo therapies involving SV119-PEG-AuNCs/DOX led to a significant reduction (by about 80%) for the formation of mammospheres (Fig. 6a). In addition, the cell number per mammosphere was remarkably reduced to *ca.* 20% as compared with the PBS control (Fig. 6b). In comparison, incubating the CSCs with SV119-PEG-AuNCs/DOX at 40 °C without laser irradiation only showed a minor effect on the mammosphere formation and the average cell number per mammosphere. Additionally, PEG-AuNCs or SV119-PEG-AuNCs had essentially no impact on the self-renewal capability of CSCs in comparison with PBS treatment, regardless NIR laser irradiation or incubation at 40 °C. These results indicate that a combination of SV119-PEG-AuNCs/DOX and NIR irradiation could significantly reduce the self-renewal capability of the breast CSCs *in vitro* by simultaneously generating photothermal and chemo therapeutic effects. Significantly, we observed a synergistic effect of photothermal and chemo therapies on eradicating the breast CSCs, with a combination index (*c.i.*) less than 1.

To further validate the efficacy in eradicating the breast CSCs, we performed the *in vitro* primary sphere formation assay (or *in vitro* limiting dilution analysis), which is a good measure of the frequency of cells with tumor-initiating capacity -- a key characteristic of CSC.[21] We measured the frequency of cells with tumor-initiating capacity within the ALDH^{hi} population after the cells had been treated with SV119-PEG-AuNCs/DOX or other controls under the irradiation of NIR laser or incubation at 40 °C. In a typical assay, we collected the ALDH^{hi} CSCs and cultured them with AuNCs and controls as described in the experimental section, and then calculated the frequency of cells that could regenerate mammospheres after 15 days. The frequencies were 1/11, 1/10, and 1/11 for cells cultured with PBS, PEG-AuNCs, and SV119-PEG-AuNCs, respectively. For the cells cultured with SV119-PEG-AuNCs/DOX under NIR laser irradiation, the frequency was significantly reduced to 1/231 (Fig. 7a), For cells cultured with SV119-PEG-AuNCs/DOX at 40 °C for 2 h, the frequency only dropped to 1/51 (Fig. 7b). These results indicate that a synergetic effect of photothermal and chemo therapies remarkably reduced the stemness of breast CSCs (*c.i.* < 1).

It has been demonstrated that a combination of photothermal and chemo therapies offers an synergetic approach with better efficacy in eradicating cancer cells *in vitro* and *in vivo* when compared with procedures based upon each individual therapeutic method.[6] Here we further demonstrate that the synergetic approach is a promising strategy for CSC therapy, with potential advantages such as reversal of drug resistance and reduction of drug dosage. When irradiated by NIR light, the drug-loaded AuNCs could serve as a transducer for photothermal therapy, and the anticancer drug could be released in a controllable fashion for

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chemo therapy. The stemness of MDA-MB-435 CSCs was significantly reduced after they had been incubated with SV119-PEG-AuNCs/DOX and then irradiated with a NIR laser.

3. Conclusions

In summary, we have demonstrated a new strategy for eradicating breast CSCs *in vitro* using a synergetic combination of chemo and photothermal therapies enabled by AuNCs. We were able to target breast CSCs by functionalizing the surface of AuNCs with SV119, a small molecule capable of binding to the sigma-2 receptor with high selectivity. In addition to their capacity to serve as a transducer for photothermal destruction, and the interiors of AuNCs could be loaded with an anticancer drug and then released upon exposure to the NIR laser irradiation. Our results suggest that the drug-loaded AuNCs could serve as a new platform to carry out photothermal and chemo therapies simultaneously, achieving a synergetic effect to eradicate the breast CSCs more effectively.

4. Experimental section

4.1 Cell culture

The MDA-MB-435 cells were cultured and maintained as monolayers in dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37 °C and under 5% CO_2.

4.2 Synthesis of AuNCs

The AuNCs were prepared through galvanic replacement reaction between Ag nanocubes and $HAuCl_4$ in an aqueous solution according to our previously reported protocol.[16] The stock suspension of AuNCs was prepared by dispersing them in ultra-purified water (supplied by a Milli-Q system, Millipore, Billerica, MA) at a particle concentration of 1.2 nM. The AuNCs were characterized using a transmission electron microscope (TEM) operated at 120 kV (HT7700, Hitachi, Tokyo, Japan) while their extinction spectra were recorded using a UV-vis-NIR spectrometer (Lambda 750, Perkin-Elmer, Wellesley, MA).

4.3 Conjugation of SV119 to AuNCs

SV119 was synthesized and purified according to a previously published protocol.[21] In a typical procedure, SV119 and succinimidyl propionyl PEG disulfide (SVA-PEG-OPSS, $Mw \approx 5{,}000$, Laysan Bio, Arab, AL) were dissolved in ultra-purified water and stirred at 4 °C for 24 h to generate the SV119-PEG-OPSS conjugate, which was then mixed with SVA-PEG-OPSS in different ratios at a total concentration of 0.12 mM. After introducing 1 mL of the stock suspension of AuNCs (at a final particle concentration of 0.3 nM), the mixture was stirred at 4 °C for 12 h. The suspension was centrifuged at a speed of 1.2×10^4 rpm for 8 min to remove the unconjugated PEG chains and washed three times with 1 mL ultra-purified water, and resuspended in 1 mL phosphate buffered saline (PBS, 0.02 M) to obtain SV119-PEG-AuNCs.

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4.4 Loading of AuNCs with 1-tetradecanol and doxorubicin

The stock suspension of AuNCs (0.3 mL) was centrifuged at 1.2×10^4 rpm for 8 min, washed twice with 1 mL methanol, and then dispersed in 1 mL methanol. This new suspension was then added into a solution of doxorubicin (DOX, 1 mg) in 1-tetradecanol (200 mg) held at 50 °C, followed by increasing the temperature to 90 °C to remove the methanol by evaporation. This mixture was shaken for 5 h and then centrifuged with 1 mL hot (90 °C), ultra-purified water at 1.4×10^4 rpm for 5 min to obtain the AuNCs whose interiors were loaded with 1-tetradecanol and doxorubicin. The retrieved AuNCs were washed with 1 mL cold (20 °C), ultra-purified water at least 8 times and then conjugated with SV119 as described above to obtain the SV119-PEG-AuNCs/DOX. To obtain the loading capacity of DOX in each AuNC, 0.1 mL of SV119-PEG-AuNCs/DOX was centrifuged at 1.4×10^4 rpm for 5 min and re-suspended in 1 mL methanol to fully dissolve the 1-tetradecanol and release all the DOX from the AuNCs. The concentration of AuNCs we used was about 1.2 nM and there were 0.12 pmol AuNCs. The total weight of DOX in the sample was 0.6 μg, obtained by comparing its absorbance with a calibration curve. The loading capacity of DOX was 5×10^6 g/mol AuNCs.

4.5 Culture of mammospheres

Mammospheres of MDA-MB-435 were cultured from cells derived from their monolayers as a suspension (1×10^3 cells/mL) in a medium containing serum-free DMEM/F12 (Invitrogen, Carlsbad, CA), supplemented with B27 (1:50, Invitrogen, Carlsbad, CA), 20 ng/mL EGF (BD Biosciences, San Jose, CA), 0.4% low-endotoxin bovine serum albumin (Sigma Aldrich, St. Louis, MO), and 4 mg/mL insulin (Sigma Aldrich, St. Louis, MO). The mammospheres were collected by gentle centrifugation (125 g, 5 min) and then dissociated into individual cells as described[22] prior to their culture with AuNCs.

4.6 Identification of cancer stem cells

The ALDEFLUOR kit (StemCell Technologies, Vancouver, British Columbia, Canada) was used to analyze the ALDH enzymatic activity of the mammospheres. The cells derived from the mammospheres were suspended in the ALDEFLUOR assay buffer containing the ALDH substrate (BAAA, 1 mM per 1×10^6 cells) and incubated at 37 °C for 45 min. After washing twice with the ALDEFLUOR assay buffer, the cells were analyzed by BD LSR II Flow Cytometer (BD Biosciences, San Jose, CA). The results were analyzed using FlowJo software (Treestar, San Carlos, CA). As a negative control, an aliquot of each sample was treated with 50 mM diethylaminobenzaldehyde, a specific ALDH inhibitor, and then analyzed for the ALDH enzymatic activity.

4.7 Inductively coupled plasma mass spectrometry (ICP-MS) analysis of AuNCs

The cells derived from mammospheres were seeded in 6-well ultra-low attachment plates at a density of 1×10^6 cells/well and incubated at 37 °C and under 5% CO_2 overnight. The cells were centrifuged at 1×10^3 rpm for 5 min and re-suspended in a culture medium containing the PEG-AuNCs or SV119-PEG-AuNCs with different coverage densities of SV119 on the surface and incubated at 37 °C for 3 h. The AuNCs in all groups were kept at a final particle concentration of 0.2 nM. After washing three times with 1 mL cold PBS, the

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cells were labeled using the ALDEFLUOR kit. The CSCs ($ALDH^{hi}$ population) and non-CSCs ($ALDH^{low}$ population) were separated by BD FACSAria III cell sorter (BD Biosciences, San Jose, CA). The collected cells were freeze-dried, and 300 μL aqua regia was added to completely dissolve the cells and AuNCs. The Au content was measured by ICP-MS (NexION 300Q, Perkin-Elmer, Waltham, MA), converted to the number of AuNCs, and finally normalized to the cell number.

4.8 Flow cytometry analysis of cells

SW120 was synthesized and purified according to a previously published protocol.[23] It was dissolved in DMSO to prepare a stock solution at a concentration of 100 μM. Prior to cell labeling, 0.5 μL of the stock solution of SW120 in DMSO was diluted with 1 mL culture medium. In this case, the effect of the trace amount of DMSO on cell viability was negligible. The cells derived from mammospheres were seeded in 6-well ultra-low attachment plates and incubated overnight as described above. The medium was replaced with the culture medium containing SV119 (10 μM) or SV119-PEG-AuNCs (0.12 nM). After 1 h pre-incubation at 37 °C, the medium was replaced with the culture medium containing 50 nM of SW120 together with 10 μM SV119 or 1.2 nM SV119-PEG-AuNCs for another 0.5 h. The CSCs were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter as described above, and then suspended in 200 μL of paraformaldehyde solution (1%) for analyses using BD LSR II Flow Cytometer. The data were analyzed using FlowJo software.

4.9 Two-photon confocal microscopy analysis of AuNCs

The cells derived from mammospheres were incubated with SV119-PEG-AuNCs at 37 °C for 3 h, which had different coverage densities of SV119 on the surface. The AuNCs in all groups were kept at a final particle concentration of 0.2 nM. The CSCs and non-CSCs were labeled using the ALDEFLUOR kit and separately collected using the BD FACSAria III cell sorter as described above and washed three times with 1 mL cold PBS. The cells were covered with a cover slip and sealed with mounting medium. They were kept in dark and imaged within the next few hours. The imaging was performed using the upright Zeiss LSM 510 system (Carl Zeiss, Oberkochen, Germany).

4.10 Assay of targeted drug delivery

The cells derived from mammospheres were incubated at 37 °C for 3 h with mPEG-AuNCs, SV119-PEG-AuNCs, and SV119-PEG-AuNCs/DOX (DOX dose: 1 μg/mL), respectively. The AuNCs in all groups were kept at a final particle concentration of 0.2 nM. After washing twice with cold PBS, half of the cells in each group were incubated at 40 °C for 2 h, and the remaining cells were incubated at 37 °C for 2 h. The CSCs were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter as described above, and then resuspended in 200 μL of paraformaldehyde solution for analyses using the BD LSR II Flow Cytometer. The data were analyzed using FlowJo software.

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4.11 Assay of mammosphere formation

The cells derived from mammospheres were incubated at 37 °C for 3 h with PBS, PEG-AuNCs, SV119-PEG-AuNCs, and SV119-PEG-AuNCs/DOX (DOX dose: 1 μg/mL), respectively. The AuNCs in all groups were kept at a final particle concentration of 0.2 nM. The CSCs were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter as described above. Half of the cells in each group were irradiated using 0.8 W/cm^2 NIR light for 20 min,[9] and the rest was cultured at 40 °C for 2 h. The cells were then seeded in 24-well ultra-low attachment plate at a density of 1×10^3 cells/well, in 3 identical replicates, and cultured for 20 days prior to counting. Mammospheres larger than 100 μm in diameter were counted under the microscope. These mammospheres were then digested into individual cells and the number of cells was determined using the cell counting plate.

4.12 Assay of mammosphere regeneration

The cells derived from mammospheres were incubated at 37 °C for 3 h with PBS, PEG-AuNCs, SV119-PEG-AuNCs, and SV119-PEG-AuNCs/DOX (DOX dose: 1 μg/mL), respectively. The AuNCs in all groups were kept at a final particle concentration of 0.2 nM. The CSCs were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter as described above. Half of the cells in each group were irradiated using 0.8 W/cm^2 NIR light for 20 min,[9] and the rest was cultured at 40 °C for 2 h. The CSCs were diluted by two times successively (from 128 cells/well to 1 cell/well) and seeded into the ultra low attachment 96-well plate containing 100 μL mammosphere growth medium, in 8 identical replicates. After 15 days of culture, the number of wells containing mammospheres larger than 100 μm was counted under the microscope. The frequency of CSCs with mammosphere regeneration ability was calculated using the Extreme Limiting Dilution Analysis software.[24]

4.13. Statistical analysis

Triplicate data were analyzed using oneway ANalysis Of VAriance (ANOVA) on the significance level of $p < 0.05$. Significant differences are indicated by asterisks in the Figures.

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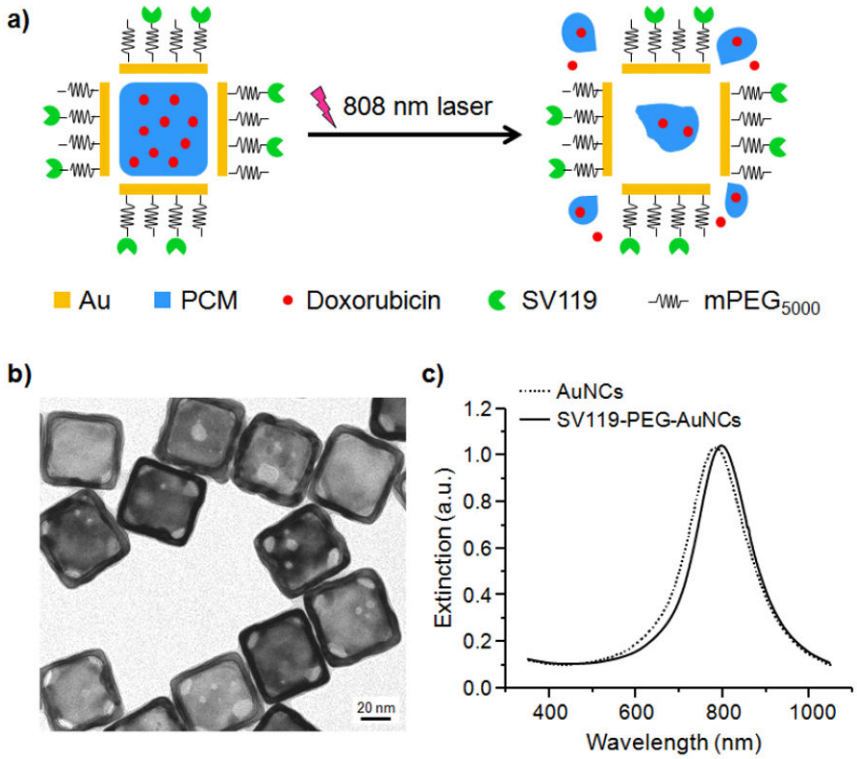

Figure 1.

(a) Schematic illustration showing the loading and then release of doxorubicin from AuNCs through the assistance of a phase-change material (PCM). (b) A typical TEM image of the as-synthesized AuNCs and (c) UV-vis-NIR spectra of the AuNCs and SV119-PEG-AuNCs in aqueous suspensions.

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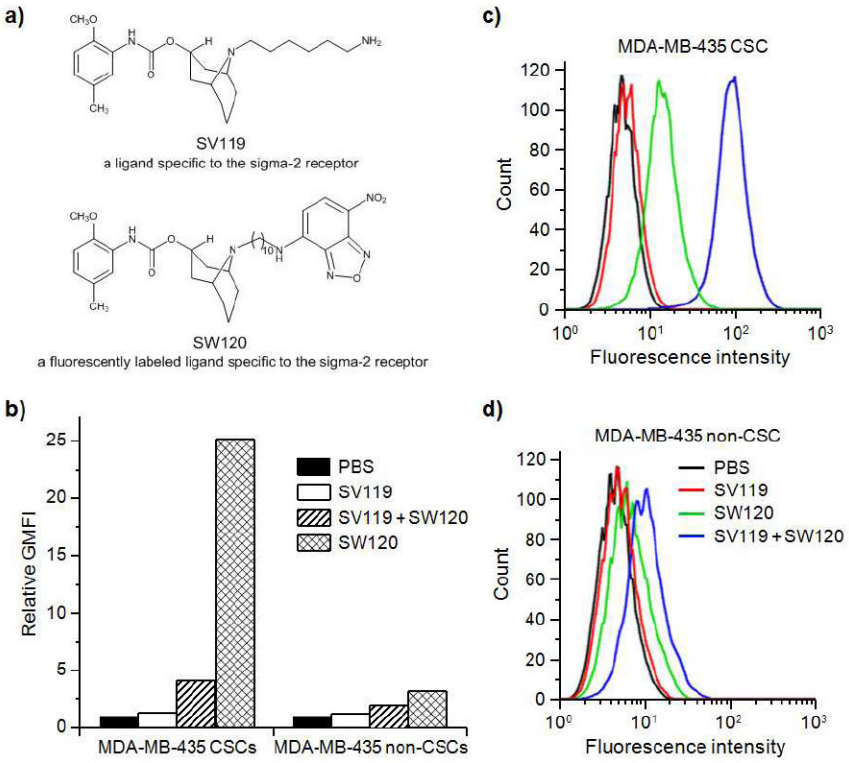

Figure 2.

(a) Chemical structures of SV119 and SW120 that bind specifically to the sigma-2 receptor. (b) The relative geometric mean fluorescence intensities (GMFI) recorded from the CSCs and non-CSCs. (c) Flow cytometry analyses of the CSCs that were pre-treated with SV119 for 1 h and then incubated with SW120 (50 nM) for 0.5 h. Cells incubated with PBS were used as a control. (d) Flow cytometry analyses of the non-CSCs that were pre-treated and incubated using the same protocol as in (C). The CSCs ($ALDH^{hi}$ population) and non-CSCs ($ALDH^{low}$ population) derived from the mammospheres were labeled using the ALDEFLUOR kit and separately collected using the BD FACSAria III cell sorter.

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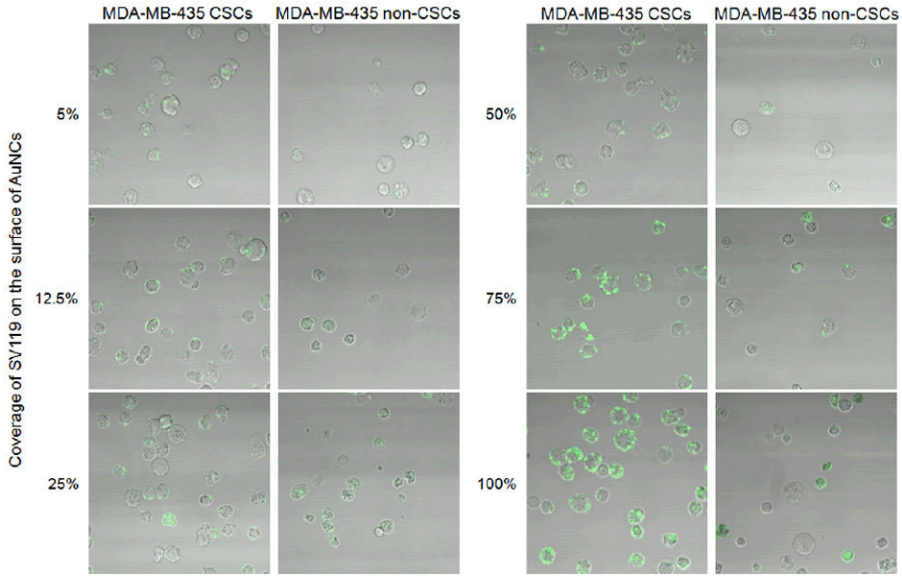

Figure 3.

Two-photon fluorescence confocal images of the CSCs and non-CSCs after incubation with SV119-PEG-AuNCs with different densities of SV119 for 3 h. The CSCs ($ALDH^{hi}$ population) and non-CSCs ($ALDH^{low}$ population) derived from the mammospheres were labeled using the ALDEFLUOR kit and separately collected using the BD FACSAria III cell sorter. The green color represents the photoluminescence from the AuNCs.

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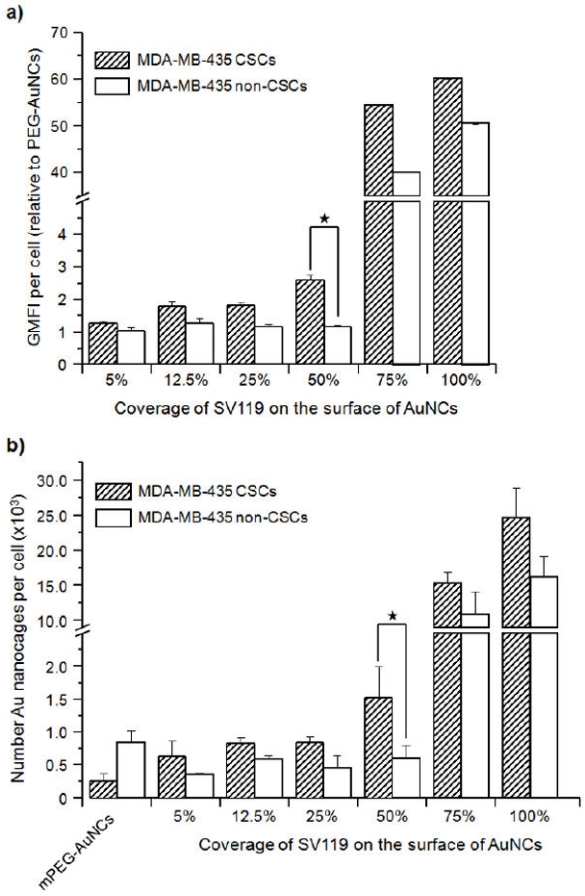

Figure 4.

(a) Geometric mean fluorescence intensities (GMFI) of the CSCs and non-CSCs after incubation with SV119-PEG-AuNCs with different densities of SV119 for 3 h. respectively. (b) Uptakes of AuNCs by the CSCs and non-CSCs after incubation for 3 h, where the AuNCs had different coverage densities of SV119 on the surface. The CSCs ($ALDH^{hi}$ population) and MDA-MB-435 non-CSCs ($ALDH^{low}$ population) derived from the mammospheres were labeled using the ALDEFLUOR kit and separately collected using the BD FACSAria III cell sorter. * indicates $p < 0.05$.

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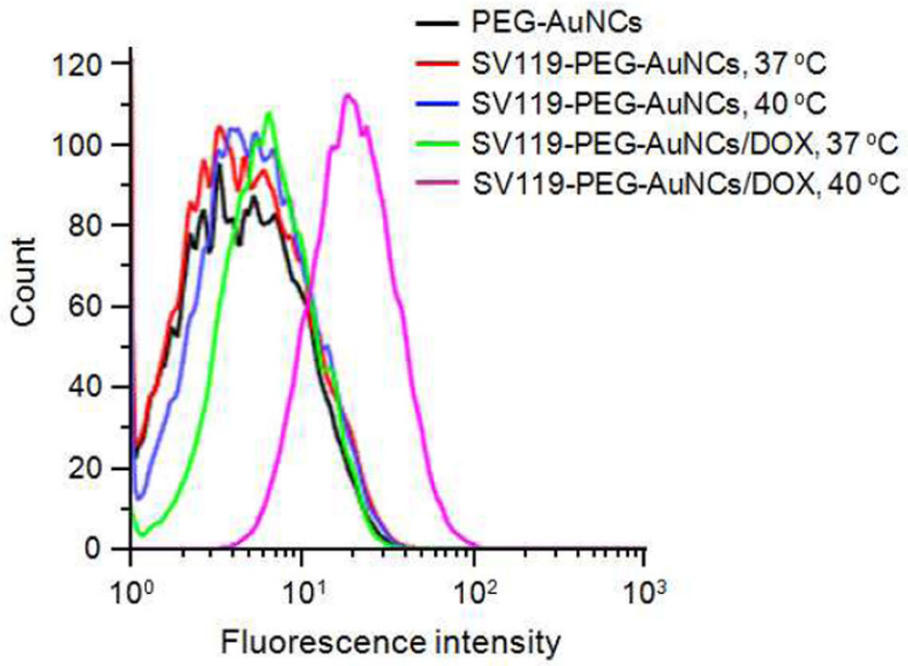

Figure 5.

Flow cytometry analyses of the CSCs that were pre-treated with SV119-PEG-AuNCs/DOX for 3 h and then incubated at 37 °C or 40 °C for another 2 h (as a control, the cells were also incubated with SV119-PEG-AuNCs or PEG-AuNCs). The CSCs ($ALDH^{hi}$ population) derived from the mammospheres were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter.

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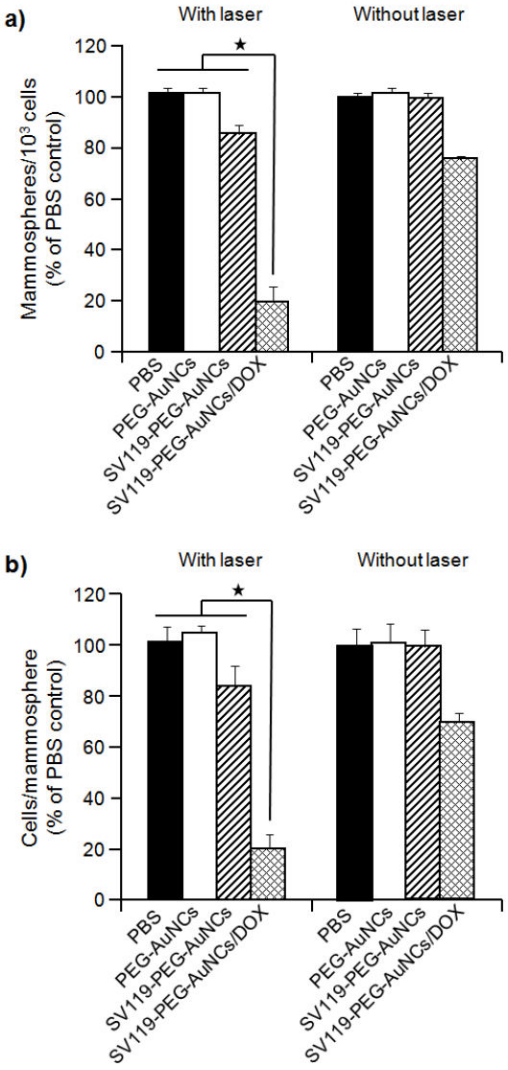

Figure 6.

SV119-PEG-AuNCs/DOX significantly affected the stemness of CSCs. Quantification of (a) the mammosphere-forming ability of the CSCs and (b) the relative average cell number per mammosphere on day 20 after incubation with PBS, PEG-AuNCs, SV119-PEG-AuNCs, and SV119-PEG-AuNCs/DOX, followed by treatment with 808 nm laser (0.8 W/cm^2) for 20 min or incubation at 40 °C for 2 h. The CSCs (ALDH^{hi} population) derived from the mammospheres were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter. * indicates $p < 0.001$.

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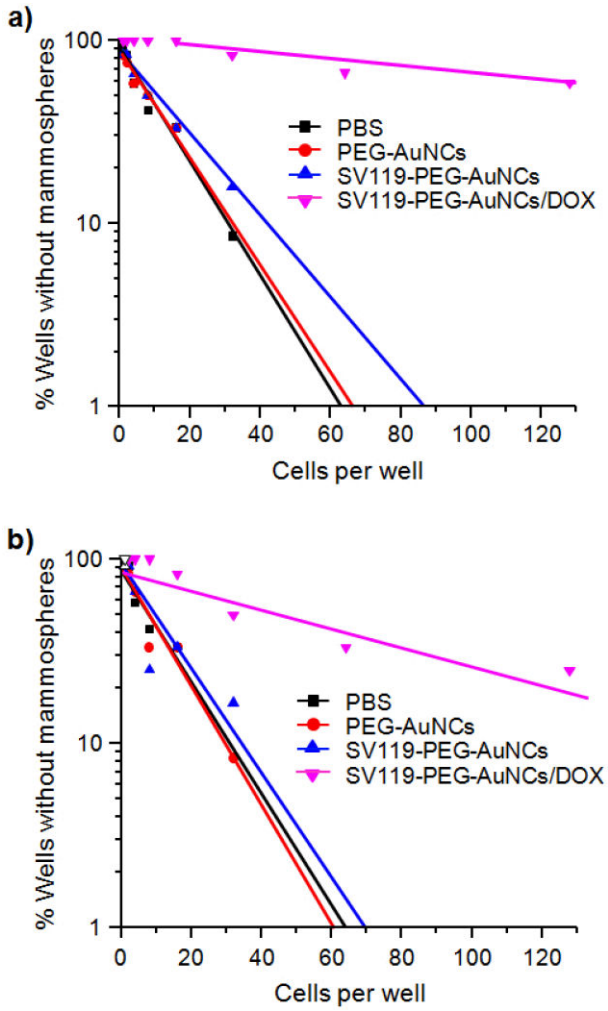

Figure 7.

Results of the *in vitro* limiting dilution assays performed with the CSCs pre-treated with PBS, PEG-AuNCs, SV119-PEG-AuNCs, and SV119-PEG-AuNCs/DOX, respectively, followed by (a) treatment with 808 nm laser (0.8 W/cm^2) for 20 min or(b) incubation at 40 °C for 2 h. The CSCs (ALDH^{hi} population) derived from the mammospheres were labeled using the ALDEFLUOR kit and collected using the BD FACSAria III cell sorter.