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A preliminary study of photoacoustic/ultrasound dual-mode imaging in melanoma using MAGE-targeted gold nanoparticles

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ABSTRACT

Nanoprobes are small enough to circulate within the vasculature and can reach tumour tissues through the endothelial gap, providing a new strategy for accurate tumour monitoring and imaging-guided antitumour therapy at the molecular level. Both photoacoustic and ultrasonic imaging are noninvasive tools for cancer detection via the application of nanoprobes. In this study, a polymeric multifunctional nanoparticle probe loaded with gold nanorods (Au-NRs) and liquid perfluorocarbon (perfluorinated hexane/PFH) and conjugated to a monoclonal antibody (MAGE-1 antibody) to melanoma-associated antigens (MAGE) targeting melanoma was successfully prepared by the double emulsion and carbodiimide methods as a targeted photoacoustic/ultrasound dual-mode imaging contrast agent (MAGE-Au-PFH-NPs). Cell-targeting experiments *in vitro* showed large amounts of MAGE-Au-PFH-NPs surrounding B16 melanoma cells in the targeted group. The photoacoustic signal in the targeted group was significantly increased, and the duration was longer than that in the untargeted group *in vivo*. The photoacoustic signal of the nanoprobes was enhanced with increased Au-NR concentration in the photoacoustic experiment *in vitro*. The enhanced signal was observed by ultrasound after 808-nm laser irradiation. A cytotoxicity and biocompatibility test showed that MAGE-Au-PFH-NPs exhibited good biological safety. The MAGE-Au-PFH-NPs can be used as a photoacoustic/ultrasound dual-mode contrast agent to lay the foundation for a promising new approach for the noninvasive targeting, monitoring and treatment of tumours.

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1. Introduction

Melanoma originates from the skin, is highly malignant, and is prone to distant metastasis [1]. Therefore, early diagnosis and treatment are particularly important. The current method of pathological diagnosis is invasive and has difficulty detecting metastatic cancer. Melanoma-associated antigens (MAGE) are a specific and highly expressed family of antigens of malignant melanoma [2–4]; MAGE proteins are highly immunogenic, are considered potential targets for cancer vaccines and/or immunotherapy [5], and could also be used to prepare nanoscale probes for the molecular imaging of primary tumours and metastatic tumours and for the accurate treatment of melanoma.

Nanomedicine for the diagnosis and therapy of tumours has been developing rapidly in recent years [6]. Among the many nanoscale probes that have been explored in biomedical research, gold nanorods (GNRs/Au-NRs) have been widely used in biological testing [7], cell imaging [8], and cancer photothermal therapy [9,10] due to their strong optical extinction characteristics, high photothermal conversion efficiency and ease of biomodification [11]. Their high optical absorption coefficient allows the detection of tumour pathological features at the molecular level [12,13].

A liquid fluorocarbon emulsion has been developed for multifunctional contrast agents with nanosize scale and can be used for multimodal (ultrasound, photoacoustic, MRI, and CT) imaging and treatment [14,15]. Liquid perfluorocarbon can be vaporized via optical irradiation via a method called optical droplet vaporization (ODV) [16–19] and can be widely used in cancer biomedicine. Via the ODV method, a liquid fluorocarbon emulsion is transformed from droplets to microbubbles (phase change) [20], increasing the

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acoustic impedance of surrounding tissues and thereby enhancing ultrasound imaging.

Both photoacoustic and ultrasonic imaging are noninvasive tools for cancer detection via the application of nanoprobes [21–23]. The photoacoustic imaging (PAI) modality is a practical method for noninvasive melanoma imaging [24,25]. Ultrasound contrast agents (UCAs), such as gas-filled microbubbles, can provide simultaneous and co-localized enhancement of image contrast to facilitate disease diagnosis by highlighting tissue borders [26].

Poly (lactide-co-glycolide) (PLGA) is an FDA-approved biodegradable polymer with lactic acid and glycolic acid as degradation products, and PLGA has been widely used in nanodrug delivery systems due to its lack of toxicity and good film-forming ability [27].

This study aimed at developing a MAGE-targeted nanomolecular probe encapsulating liquid perfluorohexane (PFH) and Au-NRs and specifically targeting melanoma cells. We investigated possibilities for enhancing the performance of the prepared nanomolecular probe for photoacoustic and ultrasonic imaging in melanoma, aiming to achieve dual-mode imaging. This study therefore provides new ideas for noninvasive diagnosis and the possibility of a subsequent accurately targeted therapy for melanoma.

2. Materials and methods

2.1. Materials

Chemicals were obtained from the following suppliers: Gold nanorods (Au-NRs, 780 nm) were from Nanoseedz Ltd. (Hong Kong SAR); perfluorohexane (PFH) and poly (lactide-co-glycolide) with terminal carboxylate groups (PLGA-COOH; 50:50 polymerization ratio, molecular weight 12,000 kDa) were from Ji'nan Dai Gang Biological Engineering Co. Ltd. (Jinan, China); N-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were from Sigma-Aldrich (St. Louis, MO, USA); and the B16 mouse melanoma cell line was purchased from Punuosai Company (Wuhan, China). MAGE-1 antibody was from Bioye Company (Shanghai, China), tetramethylrhodamine isothiocyanate (TRITC)-labelled goat anti-mouse IgG antibody was from Abcam (Cambridge, UK), and the Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Japan).

2.2. Cell culture

B16 mouse melanoma cells were cultured in T75 flasks containing Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% foetal bovine serum and 1% penicillin and streptomycin (antibiotics) and incubated at 37 °C under 5% CO2, with medium changes every 2–3 days. For all the experiments, the cells were harvested using 0.25% trypsin solution and were then resuspended in fresh medium before plating.

2.3. Animal experiment

All the animals (male BALB/c nude mice and BALB/c mice: ~20 g, 4–6 weeks) were purchased from the Experimental Animal Center of Chongqing Medical University. All the experiments and procedures were performed under guidelines approved by the Institutional Animal Care and Use Committee of Chongqing Medical University. B16 cells were suspended in PBS (1×10^6 B16 cells in 100 μL of PBS per mouse) and then injected subcutaneously into the flanks of the BALB/c nude mice to establish tumour-bearing mice. The BALB/c mice were used for the biosafety evaluation of nanoparticles (NPs).

2.4. Preparation and characterization of MAGE-Au-PFH-NPs

Untargeted nanoparticles (Au-PFH-NPs) were prepared by the double emulsion method. A 200-μL aliquot of PFH was added to 200 μL of Au-NR solution and sonicated for 1 min (duty ratio 5:5 and 103 W) in an ice bath to obtain a white primary emulsion. Next, 50 mg of PLGA-COOH was added to 2 mL of dichloromethane, followed by thorough mixing and shaking using a sonicator (model VCY-500, Shanghai, China) until the mixture was completely dissolved. The abovementioned primary emulsion was added to the PLGA dichloromethane solution with continuous sonication for 4 min to obtain a milky multi-emulsion. Then, 5 mL of 4% PVA was added to the multi-emulsion and then homogenized (XHF-D, Zhejiang, China) for 5 min; next, 10 mL of 2% isopropanol was added to solidify the surface of the NPs. The suspension was stirred for 3 h to volatilize the dichloromethane, centrifuged three times (10,000 rpm, 8 min) using a high speed refrigerated centrifuge (Eppendorf, Hamburg, Germany), and then washed with PBS. To modify the Au-PFH-NPs, EDC and NHS were added and incubated at 4 °C and then shaken for 1 h (EDC:NHS molar ratio (N/P) = 1:3; PLGA:EDC molar ratio (N/P) = 1:10). After centrifugation and three washes with PBS, the NPs were reconstituted in MES buffer (0.1 M, pH = 8). Then, 10 μL of MAGE antibody (MAGE antibody: PLGA molar ratio (N/P) = 1:1) was added and incubated overnight at 4 °C; afterward, the sample was centrifuged and washed with PBS three times. The targeted nanoparticles (MAGE-Au-PFH-NPs) were obtained and stored at 4 °C for future use.

The size and uniformity of the NPs were observed by transmission electron microscopy and scanning electron microscopy. The size was measured by a Malvern laser particle size analyser (Malvern, England). A MAGE-Au-PFH-NP suspension at a concentration of 25 mg/mL underwent PAI scanning at different wavelengths ranging from 680 nm to 970 nm (interval = 5 nm) to determine the maximum absorbance for optimized PAI by a Vevo LAZR Photoacoustic Imaging System (Vevo LAZR, Toronto, Canada). A UV spectrophotometer (FEI Nova 450, USA) was employed to measure the entrapment efficiency of Au-NRs.

2.5. Cytotoxicity in vitro

The B16 cells reached the logarithmic growth phase and were seeded into 96-well plates and incubated for 24 h. RPMI 1640 medium was used to dilute the NPs to five different concentrations: 1.57 mg/mL, 3.125 mg/mL, 6.25 mg/mL, 12.5 mg/mL, and 25 mg/mL, and 100 μL of RPMI 1640 medium was used for the control group. The NPs at five concentrations were added to the culture wells and then incubated for another 24 h. The typical CCK-8 assay was used to evaluate the cell viability of each group. Five replicates were used for each group.

2.6. Targeting abilityin vitro

To assess the MAGE-1-antibody connection in NPs, 1 mL of MAGE-Au-PFH-NPs and Au-PFH-NPs was transferred to two separate Eppendorf (EP) tubes and diluted with MES buffer (0.1 M, pH = 8) to 2 mL. Next, 20 μL of TRITC goat anti-mouse secondary antibody was added to these two tubes and incubated in an ice bath in the dark for 4 h with shaking. The mixture was then centrifuged and washed three times, dissolved in 2 mL of PBS, transferred to two confocal dishes and observed under an inverted fluorescence microscope.

To assess the targeting ability of NPs *in vitro*, MAGE-Au-PFH-NPs and Au-PFH-NPs were all treated with DiI fluorescent dye in the first step of synthesis before the sonication. B16 were seeded in confocal laser dishes at 1×10^5 and co-incubated with dyed

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