Biomaterials 31 (2010) 1716–1722

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Synthesizing and binding dual-mode poly (lactic-co-glycolic acid) (PLGA) nanobubbles for cancer targeting and imaging

Jeff S. Xu [a], Jiwei Huang [a], Ruogu Qin [a], George H. Hinkle [b], Stephen P. Povoski [c], Edward W. Martin [c], Ronald X. Xu [a,*]

[a] Department of Biomedical Engineering, The Ohio State University, 270 Bevis Hall, 1080 Carmack Road, Columbus, OH 43210, USA
[b] Department of Pharmacy, The Ohio State University, OH 43210, USA
[c] Department of Surgery, The Ohio State University, OH 43210, USA

ARTICLE INFO

Article history:
Received 19 October 2009
Accepted 17 November 2009
Available online 16 December 2009

Keywords:
Cancer targeting
Dual-mode imaging
Nanobubbles
Nanoparticles
Fluorescence
Ultrasound

ABSTRACT

Accurate assessment of tumor boundaries and recognition of occult disease are important oncologic principles in cancer surgeries. However, existing imaging modalities are not optimized for intraoperative cancer imaging applications. We developed a nanobubble (NB) contrast agent for cancer targeting and dual-mode imaging using optical and ultrasound (US) modalities. The contrast agent was fabricated by encapsulating the Texas Red dye in poly (lactic-co-glycolic acid) (PLGA) NBs and conjugating NBs with cancer-targeting ligands. Both one-step and three-step cancer-targeting strategies were tested on the LS174T human colon cancer cell line. For the one-step process, NBs were conjugated with the humanized HuCC49ΔC_H2 antibody to target the over-expressed TAG-72 antigen. For the three-step process, cancer cells were targeted by successive application of the biotinylated HuCC49ΔC_H2 antibody, streptavidin, and the biotinylated NBs. Both one-step and three-step processes successfully targeted the cancer cells with high binding affinity. NB-assisted dual-mode imaging was demonstrated on a gelatin phantom that embedded multiple tumor simulators at different NB concentrations. Simultaneous fluorescence and US images were acquired for these tumor simulators and linear correlations were observed between the fluorescence/US intensities and the NB concentrations. Our research demonstrated the technical feasibility of using the dual-mode NB contrast agent for cancer targeting and simultaneous fluorescence/US imaging.

1. Introduction

Accurate assessment of surgical resection margins and recognition of occult metastatic disease within adjacent peritumoral tissues are important oncologic principles in cancer surgeries. Strict adherence to these oncologic principles is the cornerstone to minimal recurrence rates and ultimately translates into overall improvement in long-term patient outcomes. However, existing imaging tools are not optimized for intraoperative cancer imaging applications. First, existing cancer imaging modalities focus primarily on preoperative image acquisition. While preoperative imaging is helpful, it fails to provide the surgeon with real-time and cancer-specific information that may be critical for decision-making in the operating room. Second, conventional cancer imaging modalities target a single perspective of tissue structural, physiologic, and molecular signatures. Each modality contributes only a small piece of a complex puzzle in cancer development and metastasis [1]. Finally, deploying an intraoperative multimodal cancer imaging system in a clinical setting faces many challenges such as the significantly added cost and complexity, the objective validation of clinical usefulness, the coregistration between different modalities, the interpretation of multiple tissue signatures, and the data overload in image acquisition, analysis, display, and management [2].

In the past, we developed various handheld near infrared (NIR) optical devices for noninvasive detection of tumor functional anomalies associated with hypoxia and angiogenesis [3–5]. A dynamic schema was proposed to characterize changes of tumor oxygenation and hemoglobin concentration in response to external stimuli [6]. NIR optical and ultrasound (US) modalities were integrated in a single handheld probe for simultaneous characterization of compression-induced tumor structural and functional dynamics [7]. US imaging also provided the spatial guidance for inverse reconstruction of tissue optical properties in NIR diffuse optical imaging [7,8].

* Corresponding author. Tel.: +1 614 688 3635; fax: +1 614 292 7301.
E-mail address: xu.202@osu.edu (R.X. Xu).

0142-9612/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2009.11.052

Integrating NIR optical imaging and US imaging into a single handheld probe has remarkable clinical significance since both modalities are low cost, portable, with the measurement depth up to several centimeters, and with the complementary capability for imaging tissue functional and structural parameters *in vivo*. Several NIR-US hybrid systems have been tested in breast cancer clinical trials [9,10]. However, The dual-mode cancer imaging technique has not been widely accepted in a clinical setting, primarily due to the following limitations: (1) many dual-mode imaging systems have low specificity because they target cancer non-specific endogenous biomarkers such as oxyhemoglobin and deoxyhemoglobin; (2) the achievable accuracy for US guided optical imaging and tomography is limited by the mismatch between tumor morphological boundaries and tumor functional boundaries.

In order to enhance the imaging specificity, we introduced exogenous imaging agents, such as radioisotope and fluorophore, to target cancer-specific molecular biomarkers, such as TAG-72. TAG-72 is a human glycoprotein complex expressed in many epithelial-derived cancers, including colorectal, pancreatic, breast, ovarian, and gastric cancers [11]. Anti-TAG-72 antibodies, such as murine B72.3, murine CC49, and humanized HuCC49ΔC_H2, have been used to target over-expressed TAG-72 in both cancer xenograft models and tissue samples from cancer patients [12,13]. The radio-immuno-guided surgery (RIGS) technique has been developed for intraoperative cancer detection of radiolabeled anti-TAG-72 antibodies. Previous clinical trials have shown that RIGS with anti-TAG-72 antibodies detected 77%–89% of primary colorectal cancers and 78%–97% of metastatic lesions in more than 300 patients [14]. Furthermore, RIGS detected both visible gross tumors and clinically occult disease within lymph nodes in more than 70% of the cases [15]. Patients with complete removal of all tissue identified by RIGS have shown significantly longer survival than those in whom tumor tissue was unable to be removed, regardless whether all gross tumor was removed or if gross tumor was left behind [16]. Recently, humanized HuCC49ΔC_H2 antibody was conjugated with Cy7, a fluorescence cyanine, for NIR fluorescence imaging of colorectal cancers [17]. The HuCC49ΔC_H2-Cy7 conjugate successfully targeted the over-expressed TAG-72 antigen in both LS174T colorectal cancer cells and xenograft nude mice [17].

In order to enhance the imaging accuracy, we developed a dual-mode microbubble (MB) contrast agent for simultaneous imaging and seamless coregistration of tumor functional and structural boundaries using optical and US modalities [18]. The contrast agent was fabricated by encapsulating the Indocyanine Green (ICG) dye in poly (lactic-co-glycolic acid) (PLGA) MBs using a modified double emulsion method. MB-enhanced concurrent structural and functional imaging was demonstrated in a series of benchtop experiments where fluorescence and US images were simultaneously acquired as the aqueous suspension of MBs flowed through a tissue-simulating phantom at different flow rates and concentrations [18].

Although the above dual-mode MBs provide simultaneous optical and US contrasts for imaging tumor structural and functional characteristics, they can not detect cancer-specific biomarkers over-expressed in cancer cells and interstitial spaces. Since the fenestrate openings of typical tumors are within the range of 400 nm–600 nm [19], MBs with the size of several microns can not effectively penetrate the leaky tumor vasculature to target the cancer cells. To overcome this limitation, we fabricated dual-mode nanobubbles (NBs) and conjugated them with cancer-targeting ligands. This report summarizes our recent efforts on synthesizing and validating cancer-specific NB agents for optical and US imaging, with the following progresses highlighted: (1) PLGA NBs with an averaged size of 268 nm were fabricated; (2) fluorescence agents were encapsulated in NBs for simultaneous optical and US imaging; (3) NBs were conjugated with HuCC49ΔC_H2 antibody to target the over-expressed TAG-72 in many epithelial-derived cancers; (4) NB-assisted cancer targeting and dual-mode imaging were demonstrated by benchtop and cell culture models.

2. Materials and methods

2.1. Materials

PLGA 50:50 (RG 502H 12,000 Da MW) was obtained from Boehringer Ingelheim (Ingelheim, Germany). Polyvinyle alcohol (PVA), methylene chloride (CH_2Cl_2), and isopropanol (C_3H_8O) were purchased from Fishers Scientific (Newton, NJ). Ultrapure deionized water was generated by NANOpure Infinity water purification system (Barnstead International, Dubuque, IA). Texas Red dye was purchased from AnaSpec Inc. (San Jose, CA). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), *N*-hydroxy succinimide (NHS), biotin hydrazide, 2-(*N*-morpholino) ethanesulfonic acid (MES), and para formaldehyde were purchased from Sigma–Aldrich (St. Louis, MO). Sodium hydroxide (1.0 N Normal solution) was purchased from Fisher Scientific (Rochester, NY). The HuCC49ΔC_H2 antibody was supplied by National Cancer Institute (Bethesda, MD). An EZ-link Micro Sulfo-NHS-Biotinylation Kit (Rockford, IL) was used to conjugate CC49 antibody with biotin. A Pierce Biotin Quantization Kit (Rockford, IL) was used to characterize the conjugation efficiency of CC49 biotinylation. Streptavidin was purchased from Biosource (Camarillo, CA). Dimethyl sulfoxide (DMSO) and IODO-GEN was purchased from Pierce (Rockford, IL). Radioactive sodium iodine-125 was supplied by Perkin Elmer Life Sciences (Shelton, CT).

2.2. Synthesizing and characterizing Texas Red NBs

PLGA NBs encapsulating Texas Red fluorescence dye were fabricated by a modified double emulsion process [18]. For the first emulsion, 0.5 mL aqueous solution of Texas Red dye (40 μM) and PVA (1% w/v) was added to 5 mL CH_2Cl_2 dissolving 125 mg PLGA. The mixture was emulsified at an Omni Ruptor 250 ultrasonic probe (Omni International, Waterbury, CT) at 90 W for 2 min in the dark. For the second emulsion, the above emulsified solution was added to 20 mL PVA solution (1% w/v), and emulsified by the ultrasonic probe at 30 W for 1 min in the dark. After the double emulsion, the suspension was added to 100 mL isopropanol solution (5% v/v) and stirred for 2 h by a magnetic stirrer at the dark to extract CH_2Cl_2. The mixture was then centrifuged by SORVALL RC-5B high speed centrifuge (Ramsey, MN) at 9000 rpm for 7 min. After centrifugation, the supernatant was discarded, and the NB precipitate was washed by deionized water. The process of centrifugation and washing was repeated three times. The washed NBs were then freeze-dried by LYPH LOCK 4.5 (Labconco Corporation, Kansas City, MO) for 36 h in the dark. Dried nanobubbles were harvested and stored in aluminum foil at 0 °C for further use.

The size distribution of Texas Red encapsulated NBs was characterized by a Dynamic Laser Scattering System (Particle Sizing Systems Inc., Santa Barbara, CA). The encapsulation efficiency of Texas Red in NBs was evaluated at the excitation wavelength of 590 nm and the emission wavelength of 618 nm by an USB4000-FL fluorescence spectrometer (Ocean Optics Inc., Dunedin, FL). To characterize the encapsulation efficiency, a calibration curve was first obtained by measuring fluorescence intensities at various Texas Red concentrations. After that, 4 mg Texas Red encapsulated NBs were dissolved in 1 mL DMSO and the fluorescence intensity of the solution was compared with the calibration curve to determine the actual amount of the Texas Red dye encapsulated in the NBs. The Texas Red encapsulation efficiency was obtained by taking the ratio between the actual amount of the encapsulated Texas Red dye and the total amount of Texas Red dye used in the process.

2.3. Biotinylating Texas Red NBs

First, for the PLGA NB's NHS-ester activation, a total of 2.5 mg Texas Red NBs was dispersed in 1.5 mL MES buffer (0.1 M, pH = 5.6). The NB suspension was transferred to a micro centrifugation tube and mixed with EDC for a final concentration of 2 mM. After that, NHS was immediately added to the suspension for a final concentration of 5 mM. The mixture was stirred for 15 min and washed by 0.1 M MES buffer at pH = 5.6 three times (each time 5 min at 7000 rpm). The precipitate was collected and re-suspended in 1.0 mL MES buffer (0.1 M, pH = 7.8).

After that, to biotinylate Texas Red NBs, biotin hydrazide was first dissolved in 0.5 mL MES buffer (0.1 M, pH = 7.8). The solution was added to the NB suspension as described above with a final biotin hydrazide concentration of 1.5 mM. The mixture was allowed to react for 2 h with gentle stirring at room temperature using a Lab-Line lab rotator (Melrose Park, IL), washed with 0.1 M PBS buffer (pH = 7.2) three times (each time 7 min at 7000 rpm), and stored at 0 °C for further use.