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An "imaging-biopsy" strategy for colorectal tumor reconfirmation by multipurpose paramagnetic quantum dots

Xiaohong Xing [a], Bingbo Zhang [b, *], Xiaohui Wang [a], Fengjun Liu [a], Donglu Shi [b, c], Yingsheng Cheng [d, *]

[a] Department of Radiology of the Tenth People's Hospital, Tongji University School of Medicine, Shanghai, 200072, PR China
[b] Shanghai East Hospital, The Institute for Biomedical Engineering & Nano Science, Tongji University School of Medicine, Shanghai, 200120, PR China
[c] The Materials Science and Engineering Program, Dept of Mechanical and Materials Engineering, University of Cincinnati, Cincinnati, OH, 45221-0072, USA
[d] Department of Radiology, Shanghai Sixth People's Hospital, Shanghai Jiaotong University, Shanghai, 200233, PR China

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ABSTRACT

Glucose transporter1 (Glut1) plays important roles in treatment of colorectal cancer (CRC) involving early-stage diagnosis, subtype, TNM stage, and therapeutic schedule. Currently, *in situ* marking and tracking of the tumor biomarkers *via* clinical imaging remains great challenges in early stage CRC diagnosis. In this study, we have developed a unique cell-targeted, paramagnetic-fluorescent double-signal molecular nanoprobe for CRC *in vivo* magnetic resonance imaging (MRI) diagnosis and subsequent biopsy. The unique molecular nanoprobe is composed of a fluorescent quantum dot (QD) core; a coating layer of paramagnetic DTPA-Gd coupled BSA (^{Gd}DTPA·BSA), and a surface targeting moiety of anti-Glut1 polyclonal antibody. The engineered ^{Gd}DTPA·BSA@QDs-PcAb is 35 nm in diameter and colloidally stable under both basic and acidic conditions. It exhibits strong fluorescent intensities and high relaxivity (r_1 and r_2: 16.561 and 27.702 s^{-1} per mM of Gd^{3+}). Distribution and expression of Glut1 of CRC cells are investigated by *in vitro* cellular confocal fluorescent imaging and MR scanning upon treating with the ^{Gd}DTPA·BSA@QDs-PcAb nanoprobes. *In vivo* MRI shows real-time imaging of CRC tumor on nude mice after intravenously injection of the ^{Gd}DTPA·BSA@QDs-PcAb nanoprobes. *Ex vivo* biopsy is subsequently conducted for expression of Glut1 on tumor tissues. These nanoprobes are found biocompatible *in vitro* and *in vivo*. ^{Gd}DTPA·BSA@QDs-PcAb targeted nanoprobe is shown to be a promising agent for CRC cancer *in vivo* MRI diagnosis and *ex vivo* biopsy analysis. The "imaging-biopsy" is a viable strategy for tumor reconfirmation with improved diagnostic accuracy and biopsy in personalized treatment.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and second in females, with over 1 million new cancer cases and 0.5 million deaths estimated to have occurred each year [1]. The stage where CRC is detected determines patients outcome, with 5-year survival rates of more than 90% for stage I disease and less than 10% for stage IV [2]. High CRC death rates can be significantly reduced by improved treatment and early detection.

Tumor markers differ from normal cells and rapidly emerge in tumor progression *via* angiogenesis, tumor cellularity, metabolism, and oxygenation which is closely related to cancer pathogenesis, invasion and metastasis [3–5]. Therefore, cancer biomarkers can be used in establishing specific diagnosis and disease prognosis. The glucose transporters1 (Glut1), as a cell surface protein with extracellular domains, has been confirmed to express a significant number of malignant tumors, including CRC, based on the immunohistochemistry (IHC) studies. Younes reported a high level of a Glut1 expression that was closely associated with an incidence of lymph node metastases in CRC [6]. Haber assessed Glut1 immunostaining in colorectal carcinoma to identify patients with poorer prognosis [7]. Sakashita suggested that Glut1 expression was positive in 18% of low-grade adenomas and in 63% of high-grade adenomas [8]. Wincewicz detected positive 58.3% Glut1 expression in

* Corresponding authors. Tel.: +86 21 65983706 819; fax: +86 21 65983706 0.
E-mail addresses: bingbozhang@tongji.edu.cn (B. Zhang), cjr.chengysh@vip.163.com (Y. Cheng).

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colorectal adenocarcinoma, while no expression in normal colorectal tissue [9]. Fenske found Glut1 expression related to potential malignant and predictor of poor prognosis [10]. These studies suggested that imaging of expression levels of Glut1 can provide an important basis for the tumor stage, tumor invasiveness and histological differentiation in order to establish personalized treatment of CRC patients.

MRI is a powerful noninvasive medical tool for tumor diagnosis with impressive anatomic resolution and tissue penetration, but it is limited by low sensitivity and cell specificity [11]. Clinical MRI contrast agents (CAs) include Gd (III) chelates, e.g., Gd-DTPA-BMA, Gd-DOTA, and other small molecular Gd-based CAs. Their structures are stable, but with poor relaxivity, specificity, and retention time in blood stream [12,13]. A promising solution to improving MRI sensitivity and specificity can be achieved by conjugation specific CAs with anti-tumor biomarker antibodies. Upon administration of these specific CAs, biomarker expression levels and distribution of the diseased tissues on CRC tumor can be effectively tracked by the targeted and CAs-enhanced imaging techniques [14]. It should be note that in clinical cancer diagnosis the suspicious lesion tissues in MRI are often removed for conclusive diagnosis using the immunohistochemical (IHC) analysis [8,15]. It is also used to identify the tumor type, degree of malignancy, metastasis and recurrence [6,16]. There is, therefore, a great need to search for multipurpose tumor-specific CAs with MRI moieties and tissue molecular profiling moieties.

In this study, integrated gadolinium (Gd) -functionalized quantum dots (^{Gd}DTPA·BSA@QDs) were designed and synthesized as MRI CAs and IHC signal reporter. ^{Gd}DTPA·BSA, a paramagnetic metal ion biomolecular complex, was used as a moiety of T_1-weighted MRI CAs and the reaction sites for conjugation of targeting ligands such as peptides, antibodies, and DNA. QDs were used as moieties of fluorescent reporters in IHC for tissue biopsy. QDs have uniform size and shape, narrow emission peak, high quantum yield (QY), and photo and chemical stability [17–19]. Due to their special properties, QDs have been widely used as nanoprobes for biomedical labeling such as *in vitro* cancer molecular pathology [20,21]. As fluorescent probes for immunohistochemistry assay, QDs have significant advantages over conventional fluorophores [21]. High quality core/shell semiconductor QDs were synthesized in this study following a previously reported hot-injection method [22,23].

^{Gd}DTPA·BSA was prepared by conjugation of BSA and diethylenetriaminepentaacetic dianhydride (DTPAA), and chelation with Gd^{3+}. For integration of two imaging moieties, hydrophobic QDs were surface engineered from organic to the aqueous phase in the presence of ^{Gd}DTPA·BSA aqueous solution under ultrasonication [24]. The as-prepared ^{Gd}DTPA·BSA@QDs exhibited good water-dispersibility, high relaxivity and strong anti nonspecific binding. Upon conjugation with anti-tumor polyclonal antibody (Glut1) [25], the resulting ^{Gd}DTPA·BSA@QDs-PcAb were investigated for *in vitro* and *in vivo* tumor targeted imaging. Furthermore, the expression and distribution of Glut1 on tumor site was investigated in tissue biopsy with the fluorescent reporter of ^{Gd}DTPA·BSA@QDs-PcAb.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and materials

Cadmium oxide (99.99%), selenium powder (99.99%), zinc oxide (ZnO, 99.99%), sulfur (99.98%), octadecylamine (ODA, 90%), oleic acid (OA, 90%), 1-octadecene (ODE, 90%), tri-n-octylphosphine oxide (TOPO, 90%) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC•HCl) were purchased from Sigma–Aldrich. Diethylenetriaminepentaacetic acid dianhydride (DTPAA, 95%), dimethyl sulfoxide (99.8%), gadolinium (III) chloride hexahydrate (99.9%) were purchased from Alfa Asear. Glut1 antibody was purchased from Millipore Corporation. Bovine serum albumin (BSA) was purchased from Beijing Dingguo Biotechnology. Trisodium citrate dehydrate, trichloromethane, acetone, sodium hydrogen carbonate, argon was purchased from local suppliers. Deionized water (18.2 MΩ cm resistivity at 25 °C) was used in this study. All the chemicals were used without further purification.

2.2. Methods

2.2.1. Preparation of hydrophobic surfactant-capped core/shell QDs

Hydrophobic surfactant-capped QDs were synthesized with minor modifications according to previously published procedures [22,23]. The CdSe core synthesis was carried out as follows. Separately, CdO (0.6 mmol), OA (0.8 mL) and ODE (8 mL) were heated to 150 °C in an argon atmosphere in a three-necked flask. After dissolution of CdO, the solution was cooled to room temperature, followed by adding TOPO (1 g) and ODA (2.5 g), and the mixture was heated again to 260 °C. At this temperature, a stock solution (3.6 mmol of Se powder dissolved in 2 mL of TOP) was rapidly injected into the reaction chamber (containing a Cd precursor) to start nucleation until the color change to red. Addition of ethanol into the solution resulted in a precipitate which was washed with acetone for several times and then dried for use.

Core/shell QDs were synthesized as follows. CdSe nanocrystals dissolved in 10 mL of hexane were mixed with 1.5 g of ODA and 5.0 g of ODE in a 25 mL three-neck flask. The flask was then pumped down at room temperature with argon atmosphere for 30 min to remove air at 100 °C for another 5–10 min to remove hexane from the system. Subsequently, the system was switched to argon flow and the reaction mixture was further heated to 240 °C for injections of Cd, Zn and S resource solution using the method described by Li [26]. After reaction, the raw products were separated by acetone precipitation followed by centrifugation. In this study, CdSe/CdS^{2ML}/$Cd_{0.75}Zn_{0.25}S$/$Cd_{0.5}Zn_{0.5}S$/$Cd_{0.25}Zn_{0.75}S$/ZnS^{2ML} core/shell QDs were provided for further experiment. Herein, ML is the abbreviation of monomolecular layer.

2.2.2. Preparation of ^{Gd}DTPA·BSA complex

The synthesis of ^{Gd}DTPA·BSA complex was according to a previously published protocol with minor modifications [27]. Briefly, 5 g of BSA was dissolved in 75 mL of 0.1 M $NaHCO_3$ (pH 8.5) solution. 5 g of DTPAA dissolved in 25 mL of dry DMSO was then added to the BSA solution. The pH value of mixture solution was adjusted to 8.5 by using 1 M NaOH. The solution was stirred for 2 h at room temperature and dialyzed against 5 × 4 L of deionized water. Subsequently, 2.5 g of $GdCl_3 \cdot 6H_2O$ was dissolved in 25 mL of 0.1 M Na-acetate buffer (pH 6.0) and added drop wise to the above BSA-DTPA solution to produce ^{Gd}DTPA·BSA complex, while keeping pH at 6.5. The solution was further stirred for 24 h at room temperature. Redundant Gd^{3+} were removed through dialyzing ^{Gd}DTPA·BSA against 5 × 4 L of citrate buffer (0.1 M, pH 6.5) and 5 × 4 L of deionized water. Finally, the solution of ^{Gd}DTPA·BSA was frozen-dry from liquid to solid for further use.

2.2.3. Preparation of ^{Gd}DTPA·BSA@QDs

The hydrophilic ^{Gd}DTPA·BSA-coated QDs was synthesized according to our previously reported procedure [24]. Core/shell QDs/chloroform solution was transferred into a clean syringe for injection. The mole ratio of ^{Gd}DTPA·BSA to QDs was kept at 500. Typically, the weighted ^{Gd}DTPA·BSA (30 mg) was completely dissolved in 4 mL of deionized water in a 10 mL beaker. The beaker was placed under an ultrasonic cell crusher with an ultrasonic booster. The top of the booster was ~0.5 cm lower than the liquid level of ^{Gd}DTPA·BSA water solution. The top of the long syringe needle was placed next to that of the booster. The QDs/chloroform solution was slowly injected into the ^{Gd}DTPA·BSA water solution with ultrasonication at 300–500 W pulsed every 10 s for an interval of 10 s. The emulsion-like solution was treated by a rotary evaporator to remove chloroform. In the last step, ^{Gd}DTPA·BSA@QDs were purified by ultracentrifugation at 100,000 × g for 15 min to remove the remains of ^{Gd}DTPA·BSA. The purified product was dispersed in borate saline buffer (50 mM, pH 8.2) and stored at 4 °C for further study.

2.2.4. Preparation of targeted dual-signal ^{Gd}DTPA·BSA@QDs-PcAb nanoprobes

^{Gd}DTPA·BSA@ QDs were conjugated with antibodies by using EDC·HCl as the cross-linker. The dual-signal ^{Gd}DTPA·BSA@ QDs were reacted with antibodies at a QDs/Glut1PcAb/EDC·HCl molar ratio of 1:10:4000 in borate saline buffer (50 mM, pH 8.2) with continuously stirring for 2 h at room temperature. The final bioconjugates were dispersed in phosphate buffered saline (PBS, 0.01 M, pH 7.4, 0.5% BSA, 0.02% sodium azide) after purifying by ultracentrifugation at 100,000× g for 15 min and washed with 0.01 M PBS (pH 7.4) twice.

2.2.5. In vitro relaxation rate and MRI

The longitudinal and transverse relaxation times of ^{Gd}DTPA·BSA and ^{Gd}DTPA·BSA@QDs were determined by using the 1.41 T minispec mq 60 NMR analyzer (Bruker, Germany) at 37 °C. The relaxivity values of r_1 and r_2 were calculated by fitting the $1/T_1$ and $1/T_2$ relaxation time (s^{-1}) versus Gd^{3+} concentration (mM) curves. The *in vitro* MR images of the ^{Gd}DTPA·BSA@QDs samples were obtained using a MRI system (MesoMR23-060H-I; Shanghai Niumag Corporation, China). The measurement conditions were as follows: T_1-weighted sequence; multi-