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ORIGINAL PAPER

Fluorescence Analysis with Quantum Dot Probes for Hepatoma Under One- and Two-Photon Excitation

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Abstract A new class of fluorescent probe produced by conjugating semiconductor quantum dots (QDs) with protein molecule is proposed as an alternative to conventional organic labels. However the fluorescence characteristics of the QD bioconjugates are not clear while they are excitied with one- or two-photon laser pulse. We synthesized specific immunofluorescent probes by linking QDs to alpha fetoprotein (AFP) antibody for specific binding alpha-fetoprotein -an important marker for hepatocellular carcinoma cell lines, and archived specific fluorescence detection with the QDs-Anti-AFP in nude mice. Then, we have analyzed the fluorescence characteristics of QDs-Anti-AFP and original QDs both under one- and two-photon excitations. The results demonstrated that QDs-Anti-AFP's fluorescent spectral and lifetime haven't varied much from that of original QDs. Moreover, QDs-Anti-AFP have exhibited higher fluorescence efficiency than QDs under two-photon examination.

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Introduction

Quantum dots (QDs) are semi-conductor particles whose radius is small or near the so-called Bohr exciton radius. QDs are proposed as alternative to conventional organic fluorophores because of distinct optical advantages [1–6]. High-quality QDs are highly stable against photobleaching and have narrow, symmetric emission spectra under repeated excitations, providing more photons and therefore higher sensitivity in measurement. In particular, the emission wavelength of QDs can be continuously tuned by changing the particle size, so that a beam of laser can excite a group of different-sized QDs. In the area of tumor detection, QDs have been made as immunofluorescent probes for cancer cells by conjugating them with protein molecules [7, 8]. In one previous study, QDs were used to link with immunoglobulin G and streptavidin to label the breast cancer marker Her2, which have proved that QDs can be very effective in cellular imaging and offer substantial advantages over organic dyes [9]. In an important improvement, PEG-coated QDs were functionalized with antibodies to prostate-specific membrane antigen and were intravenously injected into mice for tumor targeting and imaging [10].

Recently, two-photon technology has begun to be used in this area for its high three dimensional handling ability and extremely high resolution imaging ability [11]. In two-photon excitation process, the ground state atom jumps to the excited state and emits a photon after absorbing two photons, so that the excitation energy is lower than that of the emission. Moreover, because of the inverse relationship between solution scattering and laser wavelength [12, 13], two-photon

excitation can cause lower sample scattering, smaller optical bleaching and stronger sample penetrability than one-photon excitation [14, 15]. Therefore, this technology has been pursued heavily in tumor detection recently [10, 16]. However the comparison of fluorescence characteristics and efficiency between QD bioconjugates and original QDs under one- and two-photon excitations is still untouched.

In this study, we synthesized specific immunofluorescent probes by linking QDs to alpha fetoprotein (AFP) antibody for recognizing AFP specifically. AFP is the main component of mammalian fetal serum, which is synthesized by visceral endoderm of yolk sac and by fetal liver cells. The alteration of AFP blood level is an important marker for hepatocellular carcinoma, so that the accumulation and retention of AFP at the site of tumor is the basis of immunofluorescence detection and targeted therapy for hepatic cancer. Firstly, we have used the specific QDs-Anti-AFP probe to achieve active tumor targeting with an integrated optical system. Then, we have compared the fluorescence characteristics between QDs-Anti-AFP and QDs. Most important, we have further compared the fluorescence efficiency between QDs-Anti-AFP and QDs both under one- and two-photon excitations.

Materials and methods

Materials

Core-shell QDs (ZnS-capped CdSe) were synthesized by College of Chemistry & Molecular Sciences, Wuhan University, which were approximately 4 nm in diameter. Human hepatocellular carcinoma cell lines (HCCLM6) [17] were provided by Liver Cancer Institute of Fudan University. EDC (1-(3-Dimethyl-aminopropyl)-3-ethylcarbodiimide hydrochloride, $>98\%$) and NHS (N-Hydroxysuccinimide, $>98\%$) were purchased from Acros-Organics. Mouse anti-human monoclonal AFP antibody and RPMI-1640 culture medium were purchased from Sigma.

QDs bioconjugation and samples preparations

We used 1.5 ml solution of high-quality oil-soluble core-shell QD590 to synthesize water-soluble QDs according to a developed procedure [18]. These activated dots modified with thioglycolic acid were dissolved in PBS (0.08 mol/l, pH 7.4) containing 50 mmol EDC and 5 mmol NHS. Then, the QDs were reacted with 20 μl mouse anti-human monoclonal AFP antibodies at room temperature in a shaking incubator for 2–4 h. The final QD bioconjugates were purified by centrifugation at 6,000 g for 10 min, and the suspension was dialyzed for 8–12 h. At last, we got the QDs-Anti-AFP probes and stored them in refrigerator at 4°C .

Using protocols approved by the Institutional Animal Care and Use Committee of Wuhan University, $\sim 10^6$ prepared HCCLM6 cells were injected into a 6- to 8-week-old nude mouse subcutaneously. Tumor growth was monitored daily until it reached the acceptable size (0.5–1 cm in diameter). Then, the synthesized QDs-Anti-AFP were injected into the tail vein at 0.4 nmol (2 ml, 0.2 μmol/L) for active targeting (4 times). The time interval between each injection was about 24 h. The mice were placed under anesthesia by injection of 3% Nembutal at a dosage of 45 mg/kg. Then the mouse was anatomized, and tumor tissues and normal tissues were collected and washed with PBS.

Primary optical systems

The fluorescence detection system is shown in Fig. 1. The pulsed laser was provided by a mode-locked Ti: Sapphire laser (Mira 900, Coherent). Samples were excited at the wavelength of 400 nm by using an optical frequency doubling system, or excited at the wavelength of 800 nm directly. The beam was focused on the samples with a lens. The fluorescence over the entire emission range was collected by a liquid-nitrogen cooled CCD-array spectrometer (ACTON, Spectrapro 2500I) along with imaging acquisition and analysis software. Two groups of samples were prepared for fluorescence detection: (1) QDs and QDs-Anti-AFP, (2) QDs-tagged tumor tissue and normal tissue in the anatomized nude mouse.

The time-correlated one photon counting (TCSPC) system [19] is also shown in Fig. 1. The QDs and the QDs-Anti-AFP were excited respectively and the subsequent fluorescence decay curves were recorded by a one-photon counting detector with a photomultiplier tube (not shown). Then,

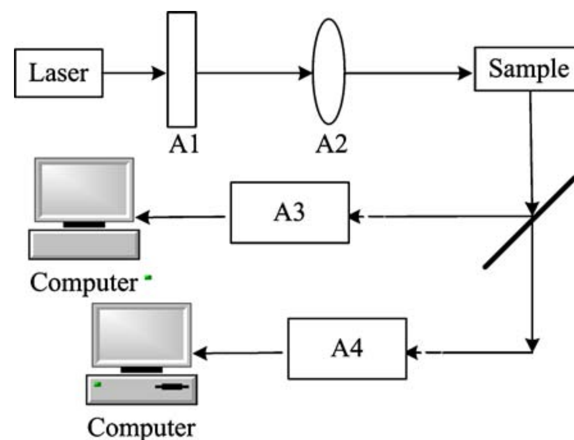

Fig. 1 Simple optical layout of fluorescence imaging system and TCSPC system: A1, optical frequency double system; A2, lens; A3, liquid-nitrogen cooled CCD-array spectrophotometer; A4, one-photon-counting detector

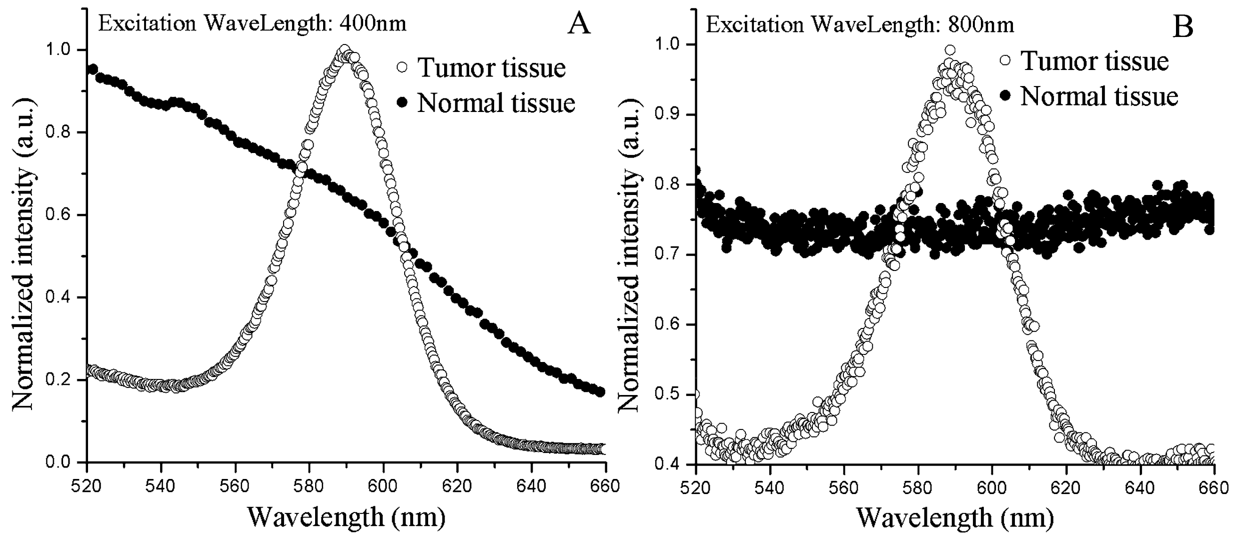

Fig. 2 **A** Fluorescence emission spectra of tumor and normal tissue excited at 400 nm. **B** Fluorescence emission spectra of tumor and normal tissue excited at 800 nm

the fluorescence lifetimes were calculated by fitting the decay curves.

Results and discussion

Specific binding ability

To investigate the specific binding ability of QDs-Anti-AFP to hepatoma, we used them to target AFP for active targeting [10, 20]. Under one-photon excitation, the QD fluorescence in tumor can be clearly distinguished from tissue autofluorescence, which exhibits only a linearly decreasing autofluorescence in normal tissue (Fig. 2A). In contrast, under two-photon excitation, the fluorescence of QDs-tagged tumor tissue is 'pure' QD signal without autofluorescence at 520 nm, while the autofluorescence of normal tissue hold in a nearly level state (Fig. 2B). These results demonstrated that QDs-Anti-AFP can recognize hepatoma specifically. Moreover, two-photon excitation is better for tumor fluorescence detection for getting 'pure' QD signal through eliminating tissue autofluorescence.

Fluorescence characteristics comparison

QDs-Anti-AFP and original QDs were excited by 800 nm two-photon laser under different exposure times for spectral matching. It is showed in Fig. 3A that QDs and

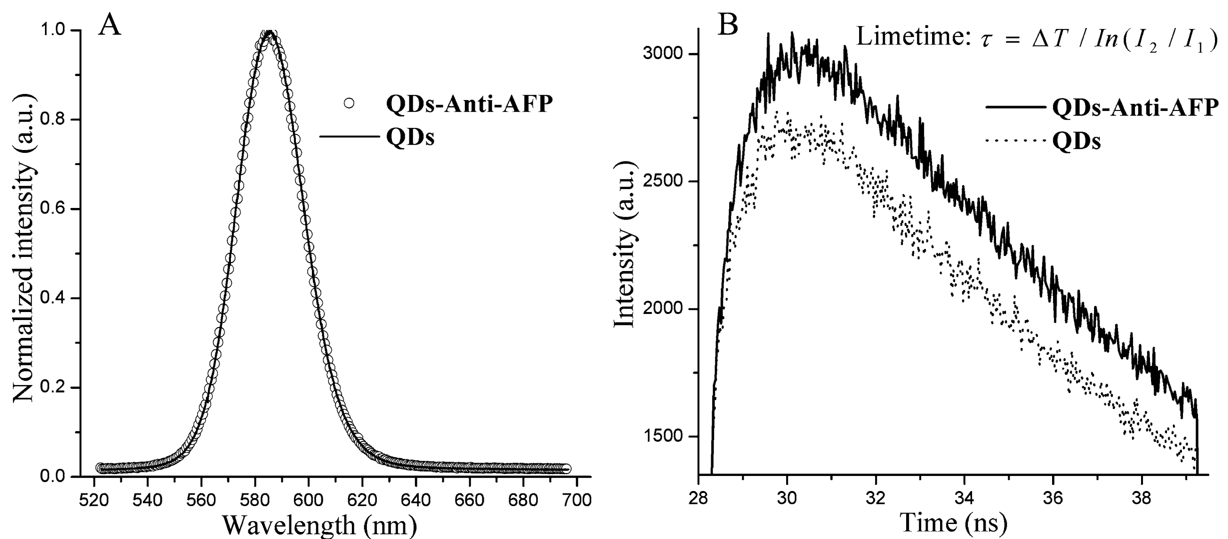

Fig. 3 **A** Fluorescence emission spectra of QDs and QDs-Anti-AFP excited at 800 nm. **B** Fluorescence decay curves of QDs and QDs-Anti-AFP as measured by TCSPC under 800 nm two-photon excitation

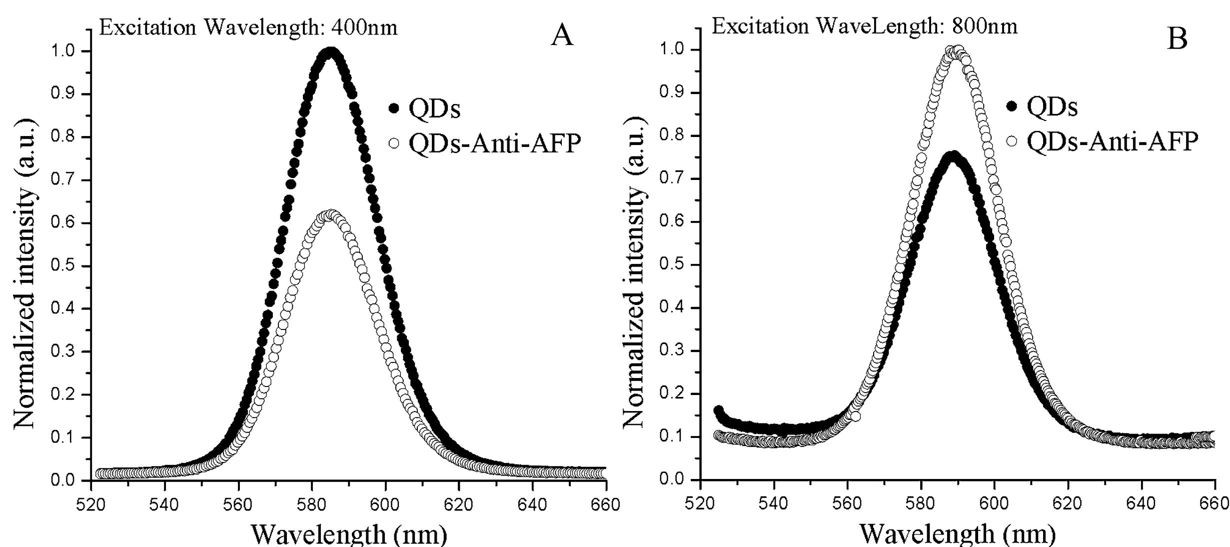

Fig. 4 **A** Fluorescence emission spectra of QDs and QDs-Anti-AFP excited at 400 nm under the same exposure time. **B** Fluorescence emission spectra of QDs and QDs-Anti-AFP excited at 800 nm under the same exposure time

QDs-Anti-AFP have the same narrow and symmetric emission spectra and their emission peaks are all at 590 nm with no red shift trailing. QDs-Anti-AFP in our study exhibit the same excellent spectral characteristics as original QDs, which is similar to another QD bioconjugates in one previous study [21]. In addition, the two samples excited by 400 nm one-photon laser get nearly the same fluorescence spectra (not shown) as those in Fig. 3A.

Fluorescence lifetime is an "intrinsic contrast" parameter to discriminate fluorescent signals from different samples [19, 22]. The fluorescence decay curves of QDs and QDs-Anti-AFP were obtained by TCSPC (Fig. 1) and the lifetimes were calculated according to the given formula (Fig. 3B): the lifetimes of QDs and QDs-Anti-AFP are 13.6 ns and 13.0 ns respectively under two-photon excitation. The nearly same values of these two samples further demonstrate that QDs-Anti-AFP haven't changed the intrinsic fluorescent characteristics of original QDs. In addition, similar results were recorded under one-photon excitation (data not shown).

Fluorescence efficiency comparison

To compare the fluorescence efficiency of QDs-Anti-AFP and original QDs under different excitations, we prepared solutions of QDs and QDs-Anti-AFP at the same optical densities at 400 nm and excited them under the same exposure time. Then, we obtained the fluorescence emission spectra by getting the average value after multi-times measurement.

Under one-photon examination, the immune antibodies in QDs-Anti-AFP solution cause much scattering and absorption of the light [12]. In contrast, no immune antibodies exist in QDs solution to weaken the light. So the fluorescence efficiency of QDs is higher than that of QDs-Anti-AFP (Fig. 4A). But under 800 nm two-photon excitation, the medium scattering and absorption can be neglected because of the inverse relationship between solution scattering and laser wavelength [12, 13]. We believe that the quantum size effects and surface effects cause a different result from above [23–25]. In fact, opposite to one-photon situation, the fluorescence efficiency of QDs-Anti-AFP is higher than that of QDs under two-photon excitation (Fig. 4B). Along with the reduction of quantum particle size, the quantum specific surface area and surface phase atomicity increase, which lead to the surface defect of superficial atom coordinate bond, unsaturated bond and floating bond. In this situation, the superficial atom is extremely unstable and is very easy binding to other atomic union. This surface defect affects the quantum luminescence properties and causes nonlinear optics effects. In previous studies, ZnS has ever been used to modify the surface of QDs [26] and thioglycolic acid has been used to grow complex passivation layer on the QDs [27] to increase fluorescence quantum yield. Similarly, the AFP antibodies in our study play the same role to QDs: they modify the surface of QDs and reduce the unsaturated bond and floating bond. Therefore the fluorescence efficiency of QDs-Anti-AFP is higher than that of QDs under two-photon excitation. In addition, the nearly same fluorescence lifetimes of QDs and QDs-Anti-AFP under one- or two-photon excitation indicate that there is no need to consider the effect of fluorescence lifetime in fluorescence efficiency comparison.

Conclusions

The indirect immunofluorescence method we adopt is based on the combination of AFP antibody and core-shell QD590.

The synthesized QDs-Anti-AFP serve as secondary antibody to combine with specific AFP (as primary antibody), and the bioconjugates can specifically recognize hepatoma in nude mice. The research reveals that the QD bioconjugates maintain the fluorescence characteristics of original QDs and have higher fluorescence efficiency than QDs under two-photon excitation. We note that some remaining issues should be investigated before the probes are practically used [10, 28–30]. We expect this new mark probe will open a new road for cell activity research and make great significance contribution for life science development.

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