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A Novel Clinically Translatable Fluorescent Nanoparticle for Targeted Molecular Imaging of Tumors in Living Subjects

Jinhao Gao^{†,‡}, Kai Chen[‡], Richard Luong[§], Donna M. Bouley[§], Hua Mao[¶], Tiecheng Qiao[¶], Sanjiv S. Gambhir[‡], and Zhen Cheng^{‡,*}

[†]Department of Chemical Biology, The Key Laboratory for Chemical Biology of Fujian Province, and State Key Laboratory of Physical Chemistry of Solid Surfaces, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China

[‡]Molecular Imaging Program at Stanford, Department of Radiology, and Bio-X Program, School of Medicine, Stanford University, 1201 Welch Road, Stanford, CA 94305-5484, USA

[§]Department of Comparative Medicine, School of Medicine, Stanford University, Stanford, CA 94305, USA

[¶]NN-Labs, Fayetteville, AR 72702-2168, USA

Abstract

The use of quantum dots (QDs) in biomedical research has grown tremendously, yet successful examples of clinical applications are absent due to many clinical concerns. Here, we report on a new type of stable and biocompatible dendron-coated InP/ZnS core/shell QDs as a clinically translatable nanoprobe for molecular imaging applications. The QDs (QD710-Dendron) were demonstrated to hold several significant features: near-infrared (NIR) emission, high stability in biological media, suitable size with possible renal clearance and ability of extravasation. More importantly, a pilot mouse toxicity study confirmed that QD710-Dendron lacks significant toxicity at the doses tested. The acute tumor uptake of QD710-Dendron resulted in good contrast from the surrounding non-tumorous tissues, indicating the possibility of passive targeting of the QDs. The highly specific targeting of QD710-Dendron-RGD₂ to integrin $\alpha_v\beta_3$ -positive tumor cells resulted in high tumor uptake and long retention of the nanoprobe at tumor sites. In summary, QD710-Dendron and RGD modified nanoparticles demonstrate small size, high stability, biocompatibility, favorable *in vivo* pharmacokinetics, and successful tumor imaging properties. These features satisfy the requirements for clinical translation and should promote efforts to further investigate the possibility of using QD710-Dendron based nanoprobe in the clinical setting in the near future.

Keywords

Nanoprobes; fluorescence; renal clearance; clinical translation; molecular imaging

The explosive development of nanotechnology has led to cross-utilization between the fields of biology and medicine, which in turn has resulted in the newly emerging research field of nanobiotechnology.^{1–4} At the forefront of nanobiotechnology is the biomedical application of quantum dots (QDs),^{5–7} yet the successful examples of clinical applications are absent due to many clinical concerns. To develop optimal nanoprobe for medical diagnosis, the

*Corresponding author. zcheng@stanford.edu.

Supporting Information. The experimental sections, structure of dendron, TEM images, DLS analysis, and hematology analysis. This material is available free of charge via the Internet at <http://pubs.acs.org>.

following features of QDs must be considered: (i) NIR emission – the NIR emitting window is important for biological optical imaging because of the low tissue absorption and scattering effects in this emission range;⁸ (ii) biocompatibility – the potential toxicity of QDs is a major concern for *in vivo* applications and biocompatible QDs are critical for clinical translation;⁹ (iii) high stability at pH 6.0–7.4 (media or serum) – the QDs should maintain chemical stability and photostability, particularly minimum non-specific binding to biomolecules in biological media;¹⁰ (iv) ultrasmall size – the small size of QDs may minimize the recognition by macrophages, thereby facilitating the rapid movement in biological media and ability to extravasate; and (v) renal clearance – the renal excretion of QDs from body after performing their task (e.g., targeted fluorescence imaging and drug delivery) may dramatically minimize the potential toxicity of QDs in the body and lower the background signal.¹¹ Although there are other key requirements, these five major items provide the basic guidelines for design of QDs as imaging probes for clinical use.

To meet the requirements for *in vivo* molecular imaging applications, the QDs should emit at approximately 700–900 nm in the NIR region to minimize the problems of endogenous fluorescence of tissues and increase tissue penetration.^{12–14} Surface coating by small and neutral polymers (e.g., polyethylene glycol (PEG)) or dendrimers (e.g., dendron) may help to maintain the intrinsic properties of QDs, decrease non-specific binding, and increase blood circulation time.^{15–17} The non-specific binding to serum proteins in blood, which may be influenced by surface coating and charge of QDs, often results in a larger size and high reticuloendothelial system (RES) uptake of nanoparticles.¹¹ In general, the size of QDs should be as small as possible in both aqueous phase and biological media.¹⁸ Small QDs may be possibly cleared from the body by kidneys. Furthermore, small QDs may extravasate from blood vessels, especially leaky tumor blood vessels, and therefore delineate the tumor because of passive targeting through enhanced permeability and retention (EPR) effect.^{19–21} Also, small bioconjugated QDs may have increased specific binding to target cells *in vivo*, and thereby be retained longer for better imaging and detection.²² However, the very small QDs (e.g., ~5.5 nm in hydrodynamic diameter (HD)) may be cleared completely by the kidneys in a rapid manner,¹⁰ resulting in short blood half-lives and consequent low extravasation in tumor. *So the QDs should be carefully designed in a reasonable size to have two important features: the ability of extravasation and enough circulation time to extravasate out of tumor vessels.*¹¹

To date, there is no report in the literature addressing the success of clinical applications of QDs in targeted molecular imaging of tumor cells in patients. Here, we report for the first time that dendron-coated InP/ZnS core/shell QDs (denoted as QD710-Dendron) may be valuable for clinical use because they satisfy all of the criteria mentioned above. The InP/ZnS core/shell QDs are a type of promising NIR fluorescent probes for biomedical applications.^{23–24} After the surface coating of InP/ZnS QDs (see Supporting Information, Scheme S1) using dendron molecules the dihydrolipoic acid conjugated to a short poly(ethylene glycol) ($n = 8$; DHLA-PEG₈-COOH),²⁵ we conjugated Arginine-Glycine-Aspartic acid peptide dimers (RGD₂) with QD710-Dendron through an amide bond to form QD710-Dendron-RGD₂ conjugates (Figure 1) and tested the receptor-binding specificity. *In vivo* and *ex vivo* fluorescence imaging indicated that the QD710-Dendron-RGD₂ nanoprobe clearly imaged integrin $\alpha_v\beta_3$ -positive tumors (e.g., SKOV3 tumor) and tumor cells with high specificity, while QD710-Dendron also displayed tumor accumulation that was likely caused by passive targeting *via* the EPR effect.

The dendrimer and dendron molecules exhibit unique physicochemical and biological properties, which have great potential for use in a variety of applications, including drug delivery and surface engineering.^{26–27} The robust and small dendron molecules not only stabilize the QDs, but also minimize the size increase of coated QDs in aqueous

solution.^{28–30} Both QD710-Dendron and QD710-Dendron-RGD₂ showed good dispersion in PBS buffer that resulted in a translucent solution (Figure 2A). There was neither aggregation nor precipitation of QDs after 4 °C storage for more than 6 months. Thus, QDs exhibit long-term stability, advantageous for *in vivo* applications. QD710-Dendron had an absorption peak of approximately 680 nm and an emission maximum of 710 nm (Figure 2B). After the sample conjugation, QD710-Dendron-RGD₂ maintained the same optical and fluorescent properties, suggesting that QD710-Dendron is stable and suitable for surface modification without any changes to their fluorescent properties.

The core sizes of QD710-Dendron and QD710-Dendron-RGD₂ are about 5 nm in diameter, based on transmission electron microscopy (TEM) (see Supporting Information, Figure S1). We then used both gel-filtration chromatography (GFC) and dynamic light scattering (DLS) to determine the HDs of QD710-Dendron and QD710-Dendron-RGD₂. The HD of QD710-Dendron is about 11.8 nm, and the HD of QD710-Dendron-RGD₂ is slightly larger at about 12.0 nm (Figure 2C; see Supporting Information, Figure S2). Importantly, the HDs of QD710-Dendron and QD710-Dendron-RGD₂ did not change during the incubation with mouse serum (Figure 2D), indicating that QD710-Dendron and QD710-Dendron-RGD₂ do not show significant non-specific binding to serum proteins, which is extremely important for *in vivo* applications. Because there are only a few number of carboxylate groups (~20) and limited RGD₂ molecules on the surface coating of QDs, QD710-Dendron and QD710-Dendron-RGD₂ may have very low protein absorption within blood vessels and tissues,²² consequently decreasing the probability of phagocytosis by macrophages and reducing the accumulation of QDs in RES organs, which would significantly increase the selectivity and efficiency of nanoprobes *in vivo*. Moreover, QD710-Dendron showed excellent fluorescence stability under the pH range from 6.0 to 9.0 (Figure 2E), although an approximately a 25% decrease in fluorescent intensity occurred at pH ~ 5.0 after 1 day, which is most likely due to the low dispersability and slow aggregation of carboxyl QDs in acidic conditions. The fluorescent intensity of QD710-Dendron and QD710-Dendron-RGD₂ remained at about 90% after being incubated in mouse serum for 1 day (Figure 2F), which is acceptable for *in vivo* fluorescence imaging.

Before the QDs can be ready for clinical translation, a better understanding of their *in vivo* behavior is needed to minimize their potential toxicity after administration.⁹ Because the microPET analysis is not suitable for long-term biodistribution study of isotope-labeled QDs,³¹ we therefore tested the long-term biodistribution of QD710-Dendron *in vivo* by inductively coupled plasma mass spectroscopy (ICP-MS).³² Using healthy BALB/c mouse (age and gender) as a model, 1 nmol of QD710-Dendron (5-fold higher than that used in fluorescence imaging study) was injected via the tail vein into each mouse and the biodistribution of indium (In) element was obtained by ICP-MS. One day following administration, approximately 60% of QD710-Dendron was eliminated from the body based on the ICP-MS results. Organ/tissue accumulation was found to be highest in the liver, spleen, and kidney (Figure 3A). These findings suggest that QD710-Dendron accumulates in tissues containing high density of macrophages (e.g., liver and spleen); and the possibility of both renal and hepatobiliary clearance of QD710-Dendron *in vivo*, which was further confirmed by ICP analysis of urine and feces samples after administration (data not shown). Sequentially, the QD710-Dendron that had been accumulated in all organs/tissues significantly decreased and appeared to be cleared from the body within a period of 1 week, and almost completely cleared 10 weeks post-administration such that the content of In in the most of the organs was undetectable by ICP-MS (Figure 3A). This encouraging data indicate the absence of long-term retention of QD710-Dendron in the body, suggesting QD710-Dendron is promising for clinical translation. Moreover, there were no statistically significant differences between the body weights of control and treated mice throughout the

study as all mice continued to gain weight in a similar fashion during the *in vivo* treatment time (Figure 3B).

Complete necropsies and hematology were performed on humanely euthanized control and treated mice in order to evaluate if the QD710-Dendron was potentially toxic. The exposure dose in mice was about 1 μg In/g mouse weight, which is comparable to the previous study on the toxicity of InP.³³ Examination of major organs (heart, lung, liver, kidney, spleen, and bone marrow) was performed 1 day, 1 week, and 10 weeks post-injection (p.i.) of QD710-Dendron (1 nmol). Gross evaluation and histopathology revealed no organ abnormalities or lesions in control or QD-treated mice (Figure 3C). Furthermore, abnormalities in red or white blood cells or serum chemistry that might indicate organ damage or inflammation were not detected (see Supporting Information, Figs. S3, S4). These results are consistent with a recently published report using cadmium-based QDs.³² Although it is a pilot study and more extensive tests are needed,^{34–35} the systematic animal toxicity evaluation shown here suggests that QD710-Dendron is highly biocompatible in an *in vivo* model and may be amenable to clinical translation.

We further validated that QD710-Dendron could be cleared through renal system by fluorescence imaging and fluorescence spectra analysis. Fluorescent signals originating from QDs in urinary bladder were observed 30 min after QD710-Dendron was administered to mice (Figure 4A), and the fluorescence was also noted in urine collected 90 min after administration. No fluorescent signals were detected in untreated mice urinary bladders or in voided urine (Figure 4A, B). This result was further confirmed by optical and fluorescent spectra analysis (Figure 4C, D). Although there are many factors that could affect the pharmacokinetics of QDs, size plays a crucial role in *in vivo* behavior.¹¹ QDs with larger HDs (>20 nm) normally end up within the RES (i.e., phagocytosed by macrophages within the liver, spleen, lymph nodes, and bone marrow) with long-term exposure in the body, resulting in higher potential for long-term toxicity. Smaller-sized QDs tend to be possibly cleared *via* urinary excretion, thereby reducing the potential toxicity of QDs and making clinical translation more viable.

To test the *in vivo* fluorescence imaging capabilities of QD710-Dendron based nanoprobes to detect tumors, both QD710-Dendron-RGD₂ and QD710-Dendron were prepared to a final concentration of 1 μM in PBS, and injected *via* tail vein (200 μL per mouse) into athymic nude mice bearing subcutaneous SKOV3 tumors. The fluorescent signals derived from both QD710-Dendron-RGD₂ and QD710-Dendron appeared in tumors 1 h p.i., and tumors (arrows) were readily distinguished from surrounding tissues after 4 h in both groups (Figure 5). The tumor uptake of QD710-Dendron with visible contrast from surrounding tissues indicated the possibility of EPR effect for the suitably small-sized QD710-Dendron,^{11,36} but the tumor fluorescent intensity dramatically decreased over time (Figure 5B). By contrast, in the mice injected with QD710-Dendron-RGD₂, the tumor contrast was still apparent even after 24 h (Figure 5A), indicating that the highly specific targeting of QD710-Dendron-RGD₂ to integrin $\alpha_v\beta_3$ -positive SKOV3 tumor induced the long-term retention of QDs in the tumor site.^{37–38}

Using the Living Image[®] software, the changes in fluorescent signal over pre-defined regions of interest (ROI) were assessed (see Supporting Information, Figure S5). After approximately 5.5 h p.i. of QD710-Dendron-RGD₂, the fluorescent signal of tumor reached the maximum and then slightly decreased over time (tumor-to-background ratios were 1.51 ± 0.05 , 1.70 ± 0.16 , 2.72 ± 0.03 , 3.61 ± 0.15 , 4.06 ± 0.27 , 3.53 ± 0.21 , 3.11 ± 0.15 , 2.04 ± 0.15 , and 1.83 ± 0.38 at 0.5, 1, 4, 5, 5.5, 6, 18, 24, and 28 h p.i., respectively, $n = 3$). Importantly, the tumor-to-background ratio remained at approximately 2 even after 24 h administration. This strong and specific targeting will be useful for long-term diagnosis and

treatment monitoring. For the injection of QD710-Dendron, the fluorescent signal of tumor was relatively low and then decreased significantly ($P < 0.05$) over the time, and there was little to no tumor contrast after 24 h (see Supporting Information, Figure S5). The rapid change of tumor fluorescent signal in the mice injected with QD710-Dendron may be due to a weak interaction of passive targeting.³⁶

To validate the highly specific targeting of QD710-Dendron-RGD₂ to integrin $\alpha_v\beta_3$ and the EPR effect of ultrasmall QD710-Dendron, two time points, 4 h and 24 h, were chosen for the *ex vivo* experiments. The tumors and major organs were collected to acquire fluorescence images under the same conditions as *in vivo* imaging immediately. *Ex vivo* fluorescence imaging further confirmed the obvious fluorescent signal in SKOV3 tumors of mice injected with QD710-Dendron-RGD₂ or QD710-Dendron at 4 h (Figure 6A). At 24 h, the fluorescent signal in SKOV3 tumors of mice injected with QD710-Dendron-RGD₂ remained high with excellent contrast, whereas there was virtually no fluorescent signal in the tumor of mice injected with QD710-Dendron (Figure 6A). The results were consistent with *in vivo* fluorescence imaging. The fluorescent signal in kidneys was extremely high at 4 h, which was consistent with the biodistribution of QD710-Dendron and is consistent with a scenario of renal excretion. The ROI signal integration analysis on the *ex vivo* fluorescence images was then performed to semi-quantitatively study the uptake ratio of QDs in each organ. At 4 h p.i., the ROI analysis showed that the tumor uptakes of QD710-Dendron-RGD₂ and QD710-Dendron under the same condition were high with 19.5 ± 2.2 %ID/g and 20.8 ± 3.5 %ID/g, respectively (Figure 6B). By comparison, at 24 h p.i. the tumor uptakes of QD710-Dendron-RGD₂ and QD710-Dendron were significantly different ($P < 0.05$); they were 7.2 ± 1.5 %ID/g and 1.1 ± 0.2 %ID/g, respectively (Figure 6C).

Histological analysis of sections of tumors injected with QD710-Dendron-RGD₂ and QD710-Dendron contained many fluorescent foci representative of QDs (Figure 6D, E). To investigate the microscopic location of QDs in the tumors, anti-CD31 immunostaining of tumors was performed to visualize tumor vasculature. Fluorescence overlay images confirmed the presence of QD710-Dendron-RGD₂ both inside and outside tumor vessels (Figure 6F; see Supporting Information, Fig S6), indicating that QD710-Dendron-RGD₂ not only specifically binds to vascular $\alpha_v\beta_3$ but also extravasates and interacts with $\alpha_v\beta_3$ expressed on tumor cells. The major reason for the extravasation of QD710-Dendron-RGD₂, which is dramatically different from the previous reports by using QDs of HDs larger than 20 nm,^{39–42} may be its relative small HD *in vivo*.²² The microscopic imaging after immunostaining further confirmed the high specificity of QD710-Dendron-RGD₂ to integrin $\alpha_v\beta_3$ in tumor vasculatures and cells. The small HDs of nanoprobe *in vivo* play a key role in the successful targeted molecular imaging of tumor cells.²² However, we did not observe QD fluorescent signal in the tumor slides of mice injected with QD710-Dendron after CD31 immunostaining (Figure 6G), presumably due to the weak interaction of QD710-Dendron in the tumor interstitial space resulting in the cleanout during the complicated procedure of immunostaining.⁴²

In summary, we have characterized and validated a novel indium based fluorescent nanoparticle, QD710-Dendron, as an excellent nanoplatform for *in vivo* fluorescence imaging. QD710-Dendron possesses several significant desirable features: NIR emission, encouraging biocompatibility with living subjects, high stability in biological media, reasonable size with potential passive targeting and renal clearance. These characteristics make QD710-Dendron based nanoprobe a viable candidate for clinical translation. Moreover, these important traits may be used as some of the basic guiding criteria for the design considerations of nanoprobe in many applications of targeted molecular imaging. After surface modification using dimeric RGD peptide as a targeting motif, the small and targeted QD710-Dendron-RGD₂ shows highly specific binding to integrin $\alpha_v\beta_3$ -positive

tumor vasculature and cancer cells in living subjects. Importantly, it may be possible to conjugate other disease-specific biomolecules with QD710-Dendron for targeting and detection of different desired targets, especially for the *in vivo* targeted molecular imaging of biomarkers present on tumor cells.^{43–46} Overall, QD710-Dendron has great potential to become a useful nanoplatform for development of many nanoprobes for pre-clinical biomedical research (e.g., image-guided surgery) and many clinical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The structure and synthesis of QD710-Dendron-RGD₂ conjugate. QD710-Dendron with carboxylate terminal group was conjugated with RGD dimer by carbodiimide coupling.

Figure 2.

The Characterization and stability analysis of QD710-Dendron and QD710-Dendron-RGD₂. (A) Optical image of QD710-Dendron and QD710-Dendron-RGD₂ in aqueous solution. (B) UV-Vis absorbance and fluorescence spectra of QD710-Dendron and QD710-Dendron-RGD₂ in aqueous solution. GFC analysis of QD710-Dendron and QD710-Dendron-RGD₂ in (C) PBS buffer and (D) mouse serum (incubation at 37 °C for 4 h) at the absorbance of 420 nm. Molecular weight markers M1 (blue dextran; 2,000 kDa, 31.1 nm HD), M2 (thyroglobulin; 669 kDa, 18.0 nm HD), M3 (γ-globulin; 158 kDa, 11.9 nm HD), and M4 (ovalbumin; 44 kDa, 6.13 nm HD) are shown by arrows. (E) Stability analysis of QD710-Dendron in different pH media at 5, 6, 7, 8, and 9, respectively. (F) Serum stability analysis of QD710-Dendron and QD710-Dendron-RGD₂ over 24 hours.

Figure 3.

Biodistribution and toxicity analysis of QD710-Dendron. (A) *In vivo* biodistribution of QD710-Dendron over a period of 10 weeks in Blab/C mice. The indium concentration in the organs was determined at different time points after tail-vein injection of QD710-Dendron (1 nmol) using ICP-MS ($n = 3$). (B) Change in body weight of mice injected with QD710-Dendron compared with PBS control ($n = 4$). There is no statistically significant difference in the mass change between control and QD710-dendron injected mice over a period of 10 weeks. The error bars represent standard deviation (SD). (C) Representative organ histology of PBS buffer and QD710-Dendron treated animals (1 day, 1 week, and 10 weeks respectively). Organs were stained with heamatoxylin and eosin. The data shows that the major organs (e.g., heart, lung, liver, kidney, spleen, and bone marrow) did not exhibit any significant microscopic lesions. Scale bar (a to x), 100 μm .

Figure 4.

Renal clearance of QD710-Dendron. (A) Fluorescence imaging of BALB/c mice (ventral) before and after tail-vein injection of QD710-Dendron (200 pmol) or PBS at 30, 60, and 120 min, respectively. The fluorescent signal originating from QD710-Dendron in the bladder (labeled as B) was visible, while there was no fluorescent signal in control mice. (B) The fluorescence imaging of urine samples collected after 90 min p.i. of QD710-Dendron or PBS, indicating that the urine from the mice injected with QD710-Dendron had a strong fluorescent signal. (C) UV/Vis absorption and (D) fluorescence emission spectra of the urine samples collected after 90 min p.i. of QD710-Dendron or PBS show the characteristic absorption peak and emission maximum of QD710-Dendron.

Figure 5.

In vivo NIR fluorescence imaging. The dorsal images of SKOV3 tumor-bearing (arrows) mice (L, left side; R, right side) injected with (A) QD710-Dendron-RGD₂ (200 pmol) and (B) QD710-Dendron (200 pmol) at 0.5, 1, 4, 5, 5.5, 6, 8, 24, and 28 h, respectively. The incidental high fluorescent signals in other body parts (arrowheads) might have originated from regular rodent food in stomach and feces in intestine.

Figure 6.

Ex vivo analysis. (A) *Ex vivo* NIR fluorescence imaging after 4 h and 24 h p.i. of QD710-Dendron-RGD₂ and QD710-Dendron, respectively. In each image: 1, tumor; 2, muscle; 3, heart; 4, lung; 5, liver; 6, spleen; 7, kidney (left); 8, kidney (right); 9, intestine; 10, femur. **b,c**, ROI analysis of major organs in *ex vivo* fluorescence imaging after (B) 4 h and (C) 24 h p.i. of QD710-Dendron-RGD₂ and QD710-Dendron, respectively ($n = 3$). The error bars represent standard deviation (SD). * $P < 0.05$ being significantly different compared with the mice injected with QD710-Dendron. Microscopic images of frozen tumor slices from SKOV3 tumor-bearing mice injected with (D) QD710-Dendron-RGD₂ and (E) QD710-Dendron. Immunofluorescence staining (CD31, green) of frozen tumor slices from SKOV3 tumor-bearing mice injected with (F) QD710-Dendron-RGD₂ and (G) QD710-Dendron. DAPI stained the nucleus in tissue slices. Arrow indicated the QD fluorescence signal in tumor vessel; arrowhead suggested the extravasation of QDs from the vessel. Scale bar (D to G), 10 μ m.