Two-photon imaging and diagnostics using ultrasmall diagnostic probes engineered from semiconductor nanocrystals and single-domain antibodies

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ABSTRACT

Semiconductor fluorescent quantum dots (QDs) have just demonstrated their numerous advantages over organic dyes in bioimaging and diagnostics. One of characteristics of QDs is a very large cross section of their two-photon absorption. A common approach to biodetection by means of QDs is to use monoclonal antibodies (mAbs) for targeting. Recently, we have engineered ultrasmall diagnostic nanoprobes (sdAb–QD) based on highly oriented conjugates of QDs with the single-domain antibodies (sdAbs) against cancer biomarkers. With a molecular weight of only 13 kDa (12-fold smaller than full-size mAbs) and extreme stability and capacity to refolding, sdAbs are the smallest functional Ab fragments capable of binding antigens with affinities comparable to those of conventional Abs. Ultrasmall diagnostic sdAb–QD nanoprobes were engineered through oriented conjugation of QDs with sdAbs. This study is the first to demonstrate the possibility of immunohistochemical imaging of colon carcinoma biomarkers with sdAb–QD conjugates by means of two-photon excitation. The optimal excitation conditions for imaging of the markers in clinical samples with sdAb–QD nanoprobes have been determined. The absence of sample autofluorescence significantly improves the sensitivity of biomarker detection with the use of the two-photon excitation diagnostic setup.

Keywords: single-domain antibodies; nanobodies; quantum dots; immunohistochemistry; imaging; diagnostics; nanoprobe

1. INTRODUCTION

Imunoflurescence diagnosis in medical practice requires high-quality images to ensure differentiation between the signals from fluorophore-labeled antibodies and tissue autofluorescence. Semiconductor quantum dots (QDs), with their unique photoluminescent properties, attract increasing attention as potential tools for biomedical applications, such as protein labeling and cell imaging. They are highly fluorescent owing to their spatially confined excited states/bandgaps. This quantum confinement allows QDs to emit fluorescence at wavelengths depending on the core size. They also exhibit (i) a broad absorption spectrum, (ii) a large absorption cross section, (iii) the possibility to excite QDs of different sizes at the same wavelength, (iv) a narrow emission spectrum, (v) a large Stokes shift, and (vi) the absence of photobleaching due to their inorganic composition. For multiphoton excitation imaging, theory predicts that a two-photon excitation (TPE) cross section may be as large as 10^4 GM (1 GM = 10^{-50} cm^4s/photon) for CdSe QDs[1-4,5], which is two to three orders of magnitude larger than those for organic fluorophores[6]. Thus, water-soluble QDs with TPE cross sections ranging from 2000 to 47,000 GM are expected to become key probes for multiphoton microscopy[6].

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Single-domain antibodies (sdAbs), very small antibody fragments (13 kDa) derived from llama IgG, are characterized by increased affinity and diffusibility[7,8], which makes ultrasmall sdAb–QD nanoprobes good candidates for immunohistochemical analysis of tissues. Conjugation of sdAbs with QDs in a highly oriented manner is considered a very attractive alternative to conventional antibodies for generation of ultrasmall targeted nanoprobes. The integration of additional cysteine residue into the C terminus of sdAbs and PMPI conjugation reaction links hydroxyl groups on the surface of QDs with exposed SH group of the sdAbs. This method provides a combination of four sdAbs per QD linked in a highly oriented manner. Sukhanova et a al.[9] recently developed sdAb–QD conjugates targeted at carcinoembriogenic antigen (CEA) and demonstrated an excellent specificity of these probes in flow cytometry. Epifluorescence microscopy showed fine immunohistochemical labeling of the target antigen with sdAb–QD probes in clinical samples, its quality being comparable with that obtained using the "gold standard" diaminobenzidine (DAB) staining.

In this study, we investigated one- and two-photon excitation imaging of appendix tissue immunostained with anti-CEA sdAb–QD_{570} conjugates. We focused on immunohistochemical detection using TPE as a way to improve the QD emission to tissue autofluorescence ratio.

2. MATERIALS AND METHODS

2.1 Conjugate preparation

The conjugates were prepared as described earlier[20]. We isolated llama-derived sdAbs targeted at CEA and not exhibiting a cross-reaction with the highly homologous nonspecific cross-reacting antigen (NCA). We engineered sdAbs with a high affinity to CEA (KD = 8.3 nM) via integration of an additional C-terminal cystein residue for subsequent specific site-directed conjugation of sdAbs with QDs emitting at 570 nm (QD_{570}).

The hydrodynamic diameters of the sdAb–QD_{570} conjugates (11.9 nm) were only slightly larger than the diameters of nonconjugated QDs (8.84 nm) and much smaller than that of commercially available IgG–QD conjugates (30.3 nm). Therefore, the volumes of the sdAb–QD_{570} conjugates were 16 times smaller than those of commercially available IgG–QD conjugates.

2.2 Tissue preparation

Human appendix tissues were obtained from the Department of Pathology of University Hospital Center Robert Debré (Reims, France). All samples used in experiments were retrospective specimens fixed with formalin and embedded in paraffin. The study did not influence any diagnostic procedure or therapeutic treatment. The use of these samples does not require approval by the institution's Human Subject Review Committee. 5-µm thick paraffin sections of tissue were incubated at 80°C overnight. Sections were done in non-inflammatory lesions, deparaffinized in xylene, rehydrated in a graded ethanol series (100, 95, 70, and 50%), and finally placed in water. Antigen retrieval was performed in a citrate buffer at pH 6 and a temperature of 95°C for 45 min with subsequent cooling for 30 min at room temperature. Nonspecific binding was blocked by incubating sections for 15 min in PBS containing 5% BSA at room temperature.

For tissue labeling with the conjugates of anti-CEA sdAb–QD_{570}, the slides were stained with a 1.5×10^{-8} M solution of CEA-specific sdAb–QD_{570} conjugates for 1 h in a humidified chamber at room temperature. The slides were washed three times with PBS and finally sealed.

The negative control slide was stained with a solution of QDs not conjugated with sdAbs. All other conditions were the same as in the case of slides immunostained with anti-CEA sdAb–QD_{570} conjugates.

2.3 Two-photon excitation and fluorescence imaging

Two-photon-induced fluorescence was generated by means of a mode-locked femtosecond Chameleon Ultra II laser (Coherent) with a pulse duration of 140 fs and a frequency of 80 MHz. The average laser power was 3.3 W for the excitation wavelength of 800 nm. The fluorescence emission was detected with an LSM 710 NLO

microscope (Zeiss), a 20× plan Apo dic II lens (Zeiss) with 0.45 N.A., and an MBS760+ beam splitter (Zeiss). The detected emission wavelengths ranged from 415 to 707 nm, with a wavelength resolution of 9.7 nm. The image resolution was 1024 × 1024 pixels.

RESULTS

One- and two-photon-induced fluorescence optical properties were determined for sdAb–QD_{570}. The molar extinction coefficient (ε) of QDs for one-photon excitation (OPE) was determined from the wavelength of the first excitonic absorption peak[1,10]. The TPE cross sections (σ_2) were determined in the excitation range from 700 to 900 nm to allow the evaluation of QD absorption properties under the conditions of TPE.

The values of σ_2 showed large TPE cross sections of sdAb–QD_{570} conjugates; they ranged from 10^4 to 10^5 GM units and were three orders of magnitude higher than those of the organic dye fluorescein. The OPE extinction coefficient of conjugated QDs was only two- to threefold higher than that of fluorescein (Table 1). Therefore, TPE would better perform than OPE the specific excitation of QDs in biological systems including organic fluorophores. The strong two-photon absorption by QDs can be exploited to increase the emission ratio of QDs versus tissue autofluorescence and to better detect antigenic structures in histological sections.

Table 1: TPE and OPE absorption properties of CdSe QDs (570nm).

Absorption property / label	sdAb–QD_{570}	Fluorescein
ε (λ_{exc} = 457.9 nm)	200 000	80 000
σ_2 [GM] (λ_{exc} = 700 nm)	54000	25
σ_2 [GM] (λ_{exc} = 800 nm)	24000	60
σ_2 [GM] (λ_{exc} = 900 nm)	22000	25

Immunohistological examination of human appendix was performed to reveal CEA in epithelial cells by means of anti-CEA sdAb–QD_{570}. Figure 1 shows TPE and OPE images of CEA on deparaffined slides. Each area of each slide was successively analyzed using the TPE (800 nm) and OPE (457.9 nm) laser lines; there was no significant photo bleaching during any analysis. TPE clearly reveals a bright yellow fluorescence of sdAb–QD_{570} conjugates (Figs. 1A, 1B) in several cell types, including epithelial cells from the lumens of the appendix and epithelial crypts. In addition, mononuclear cells of the conjunctive tissue were stained with sdAb–QD_{570} conjugates. Figure 1B shows a magnified epithelial crypt section where both apical and basal poles of the cytoplasm, but not the nucleus, of the cells were stained. Serial slides from the same appendix were prepared to perform imaging using sdAb–QD_{570} conjugates (Fig. 1A) and free QDs (Fig. 1C) as a control. Epithelial cells in a negative control slide (Fig. 1C) exhibit no yellow emission of QDs, which agrees with the absence of affinity of free QDs for epithelial cells, and a very weak green autofluorescence.

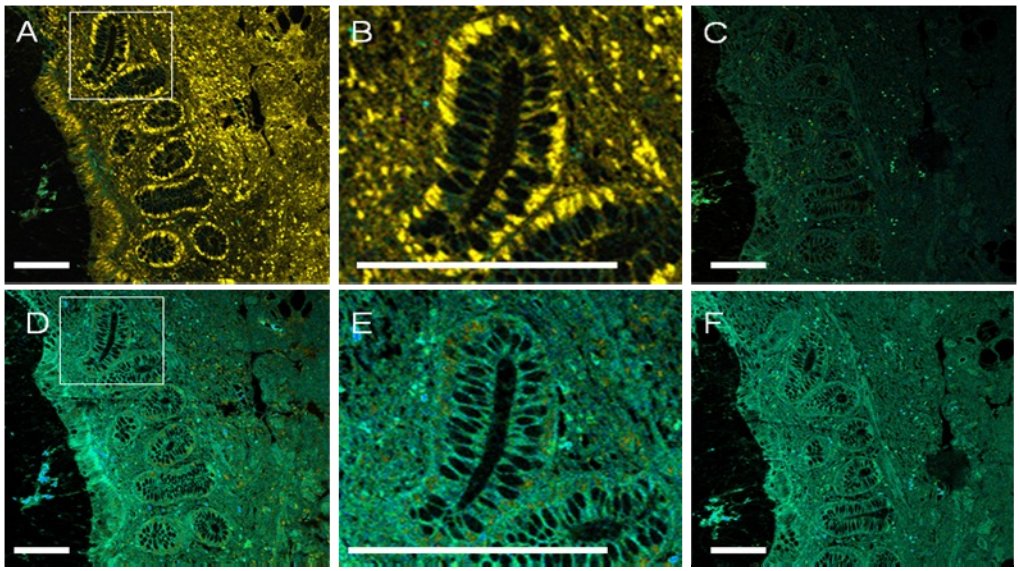

Fig. 1. Immunostaining of appendix epithelial crypts with sdAb–QD_{570} conjugates. CEA is revealed in yellow with anti-CEA sdAbs covalently linked to QD_{570}. The slides were viewed at the (A, B) TPE wavelength (800 nm) and (D, E) OPE wavelength (457.9 nm). Panels (C) and (F) show control slides treated with free QD_{570} and viewed at the (C) TPE and (F) OPE wavelengths. The scale bar is 100 μm.

The OPE (457.9 nm) laser line (Figs. 1D, 1E) revealed a predominant green autofluorescence emission from a deparaffined tissue section. The slide analyzed using sdAb–QD_{570} conjugates displays a punctual yellow emission from QDs, which was largely masked by the green autofluorescence. These data show a considerable advantage of TPE over OPE in terms of detecting QD fluorescence in tissues with intense autofluorescence.

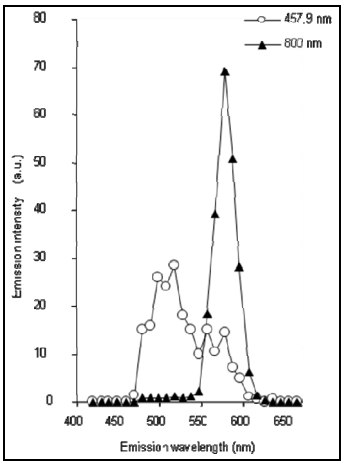

Fig.2. Emission spectra of an appendix immunostained with sdAb–QD_{570} conjugates; the spectra from the square areas in Figs. 1A and 1D obtained using OPE (457.9 nm) and TPE (800 nm), respectively, are compared.

Figure 2 shows the spectra of TPE- and OPE-induced emissions from the same marked area (squares in Figs. 1A and 1D) of an appendix tissue specimen as revealed by means of sdAb–QD_{570}. In the case of the 800-nm TPE excitation, the emission spectrum is entirely accounted for by the 570-nm QD emission, without the 450- to 580-nm autofluorescence (Fig. 2, ▲). In the case of the 457.9-nm OPE excitation, the emission spectrum is mainly accounted for by intense 480- to 580-nm autofluorescence (Fig. 2, ○). For this area, the ratios of the QD fluorescence intensity to the autofluorescence intensity are 20 and 0.5 for TPE and OPE, respectively. This

considerable enhancement of QD emission allows their detection in histological specimens with bright autofluorescence.

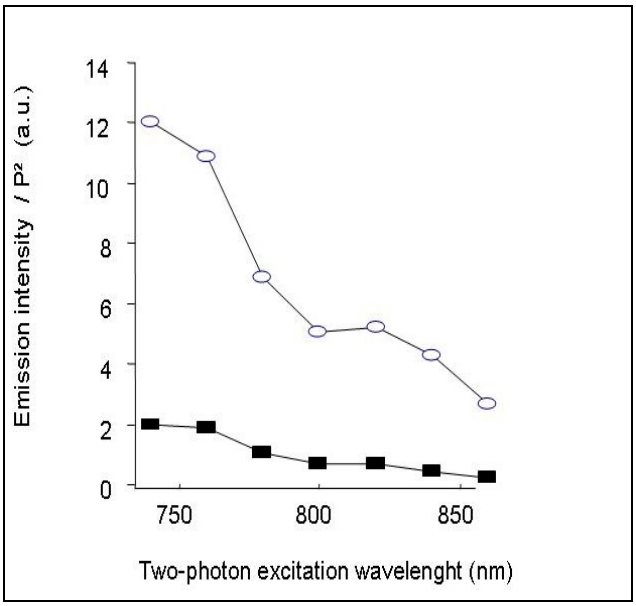

Fig.3. Emission intensity in an appendix immunostained with sdAb–QD_{570} conjugates as a function of the two-photon excitation wavelength. The QD emission (570 nm,○) and tissue autofluorescence emission (500 nm,■) from the same area of the histological specimen are compared.

The TPE excitation wavelength was varied to determine its influence on both QD fluorescence and autofluorescence intensities (Fig. 3). The sdAb-QD_{570} fluorescence emission corrected for the squared excitation power was obtained from the same area of the histological section. The absence of photodegradation had been previously validated. The 740-nm excitation caused intense QD emission due to a high σ_2 of QDs. The increase in the excitation wavelength from 740 to 860 nm caused a fourfold decrease in the sdAb–QD_{570} emission (570 nm) and a tenfold decrease in the autofluorescence emission (450–520 nm). Thus, excitation at 860 nm ensures a better differentiation between QD fluorescence and autofluorescence of the tissue than excitation at 740 nm.

DISCUSSION

Since 1998, QDs have been used in immunofluorescence for the detection of a variety of proteins in tissue specimens and in isolated cells by means of flow cytometry. Current immunostaining protocols usually use fluorescent nanoprobes consisting of either (i) biotinylated antibodies conjugated with streptavidine-coated QDs or (ii) mAbs covalently linked to QDs. Streptavidin-coated QDs combined with either primary or secondary biotinylated antibodies can be used for detecting any protein[11,12]. Streptavidin-coated QDs are suitable tools for biomarker imaging because they are easy to use. Multiple staining faces significant problems due to the cross reactivity of streptavidin-coated QDs with different biotinylated antibodies[13,18]. This method is time-consuming, because dual protein staining requires a six-step protocol, such as IgG1/IgG2-biotine/SA-QD1 followed by IgG3/IgG4-biotine/SA-QD2[14,15]. The second way involves conjugation chemistry to link QDs to mAbs by different methods[12,16]. Immunostaining with mAb–QD conjugates allows multiplex imaging, as demonstrated by simultaneous detection of four tumor biomarkers in prostate cancer cell lines[17]. However, molecular targeting could be disturbed because of the large size of the conjugates limiting their penetration into the specimens and the irregular orientation of mAbs on the surface of QDs.

A new generation of ultrasmall nanoprobes has been engineered by our group through conjugation of QDs with sdAbs derived from llama IgG against CEA[9]. In addition, the immunohistochemical imaging of CEA in

formalin-fixed appendix tissue specimens embedded in paraffin with the use of UV excitation has proved to be of the same quality as the reference standard (DAB chromogenic staining). Ultrasmall sdAb–QD conjugates constitute an excellent alternative as tools for immunofluorescence due to easier diffusion into tissues and antigenic site accessibility. In addition, QD conjugation to sdAbs allows one to use their good optoelectrical properties, particularly in the case of the two-photon excitation mode.

The OPE imaging of QDs (UV or visible excitation) has been used in many studies to reveal antigens in formalin-fixed, paraffin-embedded histological specimens. Since semiconductor QDs are characterized by intense two-photon absorption, we have used TPE of sdAb–QD nanoprobes for immunohistochemical labeling of appendix. The TPE cross sections of QDs, the key parameter for assessing two-photon absorption, are higher than 10^4 GM[1,2], which is three orders of magnitude higher than those of conventional probes[1,3-6] and six orders of magnitude higher than those of intrinsic fluorophores, such the reduced NADH[21]. An empirical nonlinear correlation (power relationship) between the TPE cross section and the core diameter of QDs has been reported[1,22]. Thus, the high absorption capacity of QDs makes them excellent imaging tools for the use in the two-photon mode, in particular, in biomedical studies. We used sdAb–QD nanoprobes to leverage the large TPE cross-section of QDs for more efficient imaging of antigen structures. The increase in the QD emission by TPE is accompanied by a considerable decrease in the background autofluorescence mainly accounted for by biological fluorophores, such NADH and flavins[3,5]. The ratio of the QD fluorescence intensity to the background intensity was about 40 times higher for the 800-nm TPE than for the 457.9-nm OPE. Since autofluorescence is largely attributed to intrinsic NADH, the difference between te intensities of two- and one-photon-induced fluorescence is explained by a much larger TPE cross section of QDs compared to NADH.

CONCLUSION

Two-photon excitation of QD fluorescence is a very promising method for increasing the absorption of the nanocrystals with large cross sections. The use of TPE allows the high brightness and robustness of QD fluorescence to be combined with autofluorescence extinction, thereby providing a strong alternative to conventional fluorophore staining as a method for the detection of antigenic structures in biological media with a high autofluorescence and in tissue sections embedded in paraffin. Finally, TPE of QD emission applied to immunochemistry is a suitable way to almost completely suppress autofluorescence of tissues.

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