IOP Conf. Series: Journal of Physics: Conf. Series **784** (2017) 012041 doi:10.1088/1742-6596/784/1/012041

Quantum Dot-Based Hybrid Nanostructures and Energy Transfer on the Nanoscale for Single- and Multi-Photon Imaging and Cancer Diagnostics

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Abstract. An ideal single-photon (1P) or multiphoton fluorescent nanoprobe should combine a nanocrystal with the largest possible 1P or two-photon (2P) absorption cross section and the smallest possible highly specific recognition molecules conjugated with the nanoparticle in an oriented manner. However, the conditions used for conjugation of typical recognition molecules (conventional antibodies, Abs) with nanoparticles often provoke their unfolding and/or yield nanoprobes with irregular orientation of Abs on the nanoparticle surface. Conjugation of smaller Ab fragments, such as single-domain antibodies (sdAbs), with quantum dots (QDs) in an oriented manner can be considered as an attractive approach to engineering of ultrasmall diagnostic nanoprobes.
QDs conjugated to 13-kDa sdAbs derived from camelid IgG or streptavidin have been used as efficient 1P or 2P excitation probes for imaging of cancer markers. The 2P absorption cross sections (TPACSs) for some conjugates are higher than 49,000 GM (Goeppert–Mayer units), which is close to the theoretical value calculated for CdSe QDs and considerably exceeds that of organic dyes. A further step in advanced QD-based cancer diagnostics has been made through implementation of efficient FRET-based imaging with 2P excitation, which has been demonstrated for double immunostaining complexes formed on the surface of cancer cells from sdAb–QD conjugates (donor) and a combination of monoclonal Abs and secondary antibodies labeled with the AlexaFluor dye (acceptor). The proposed approach permits obtaining an exceptional contrast of 2P imaging of cancer biomarkers without any contribution of cell and tissue autofluorescence in the recorded images.

1. Introduction

Conventional immunoglobulins G (IgG) have a molecular weight of 150 kDa and average sizes of 14.5×8.5×4 nm^3, which hampers their use as recognition molecules for targeted delivery of drugs, tumor imaging, and diagnosis [1]. In addition, the conditions of conjugation of monoclonal antibodies (mAbs) with labels often cause denaturation of IgG; another drawback is that mAb are attached to the nanoparticle carrier in a random orientation [2]. Smaller fragments of antibodies (Abs) conjugated with quantum dots (QDs) in a strictly predetermined orientation would be a good alternative to mAbs in designing nanoprobes of the smallest possible size [3, 4]. Single-domain Abs (sdAbs) or VhH fragments, with a molecular weight as small as 13 kDa, are the smallest known Ab fragments capable

of recognizing the corresponding antigens with a binding constant compared to that of conventional Abs [5, 6]. SdAbs are also very little prone to aggregation; they exist in the monomeric form and better diffuse into tissues than full-size IgG do [7, 8]. These advantages make sdAbs optimal recognition molecules for the use in QD-based fluorescent nanoprobes for detecting biomarkers of diseases and immunodiagnostics.

We have recently published several papers on the development of stable, highly specific, ultrasmall next-generation nanoprobes based on highly oriented conjugates of sdAbs and semiconductor QDs and their employment in cancer diagnostic platforms using flow cytometry and immunohistochemistry [3, 9, 10], as well as in multi-photon imaging of tumor cells [4]. In the first proof-of-concept experiments with the novel nanoprobes, we used carcinoembryonic antigen (CEA) as a marker [3, 4, 9]. In addition, we carried out experiments with QD-conjugated sdAbs against human epidermal growth factor receptor 2 (HER2) as nanoprobes for detecting this marker of breast and lung cancers and compared the effectiveness of these probes with that of anti-HER2 mAbs conjugated with standard organic dyes, AlexaFluor488 and AlexaFluor568 [10]. The sdAb–QD conjugates exhibited better performance in experiments with a panel of lung cancer cells with different rates of HER2 expression. In general, the sdAb–QD conjugates effectively detected the CEA and HER2 proteins. Their comparison with the conjugates of the standard fluorophores with mAbs was performed with respect to both efficiency and specificity of labeling. Confocal microscopy was used to show that the sdAb–QD conjugates specific for HER2 were considerably more effective than the standard conjugates in detecting cancer cells with a low HER2 expression rate in a mixture with primary macrophages [10].

In multi-photon microscopy with excitation in the near-IR spectral region, the excitation band is remote from the spectral region of effective excitation of the autofluorescence of biological tissues, which makes it possible to achieve deep tissue imaging [11–13]. The main problem of multi-photon imaging is small two-photon absorption cross sections (TPACSs) of all known organic fluorophores; it is no larger than a hundred Goeppert–Mayer units (GM, 1 GM = 10^{-50} $cm^4 \cdot photon^{-1}$). Semiconductor QDs are characterized by a wide fluorescence excitation spectrum, a large absorption cross section, the possibility of exciting the fluorescence of QDs of different sizes at a single wavelength, and resistance to photobleaching [14–16]. The TPACSs of some QDs exceed 10^4 GM, which makes them optimal fluorescent labels to be used in nanoprobes for multi-photon imaging [12, 13]. Conjugates of QDs and sdAbs derived from llama IgG [17] have been used as effective probes with two-photon (2P) excitation for detecting CEA in the tissues of a normal human appendix vermiformis and colon carcinoma [3]. The TPACSs of some of the conjugates are higher than 49,000 GM, which substantially exceeds this value for organic dyes and is close to the theoretical value of 50,000 GM calculated for CdSe QDs.

Here, we summarize the results of our recent studies on single-photon (1P) and multi-photon optical characteristics of highly oriented sdAb–QD conjugates and consider their use under the conditions optimal for imaging cancer markers in terms of the absolute sensitivity of imaging and a high ratio of the sdAb–QD fluorescence signal to that of tissue fluorescence [13]. The conjugates developed in our studies are bright multi-photon nanoprobes with a high signal to autofluorescence ratio upon excitation at a wavelength of 800 nm, which allows the areas of tumor tissue overexpressing CEA to be clearly distinguished from normal tissue. The results of testing the sdAb–QD conjugates indicate their unprecedentedly high potential as new, more sensitive tools for early detection of cancer markers.

2. Materials and Methods

QDs were synthesized and stabilized in aqueous solution using the methods described earlier [14–16]. Then, they were solubilized in water according to the procedure described in [9]. In brief, the QDs were first transferred to the water phase by attaching DL-Cys (Sigma) to the surface. The resultant water-soluble QDs displayed bright-orange photoluminescence (PL) with an emission peak at 570 nm and a quantum yield close to 40% at room temperature. After that, DL-Cys on the QD surface was replaced with a mixture of thiol-containing polyethylene glycols (PEGs) with a carboxyl, hydroxyl, or

amino end group. The mixtures of the polymers used for QD solubilization were selected in such a way as to minimize nonspecific binding of QDs by cells and tissues. The treatment of the QD surface with low-molecular-weight PEGs yielded water-soluble CdSe/ZnS QDs with the smallest possible diameters. Optimal results were obtained for organic shells consisting of hydroxy-PEG and mixtures of 90% of hydroxy-PEG and 10% of amino-PEG. The samples were incubated at 4°C overnight, preliminarily purified by centrifugation with blocks of Amicon Ultra-15 filters with a cutoff of 10 kDa (Millipore), and finally purified from excess ligand by means of exclusion gel chromatography in homemade columns containing Sephadex-25 (Sigma). All the QDs (and sdAb–QD conjugates) prepared were characterized by means of dynamic light scattering (DLS) and electrophoresis using a Zetasizer Nano ZS device (Malvern Instruments, United Kingdom). The samples suspended in ultrapure water were filtered through a filter with a mesh size of 0.1 μm, and the size distribution of the particles was measured at 25°C in a small-volume quartz cuvette (Hellma ZEN2112).

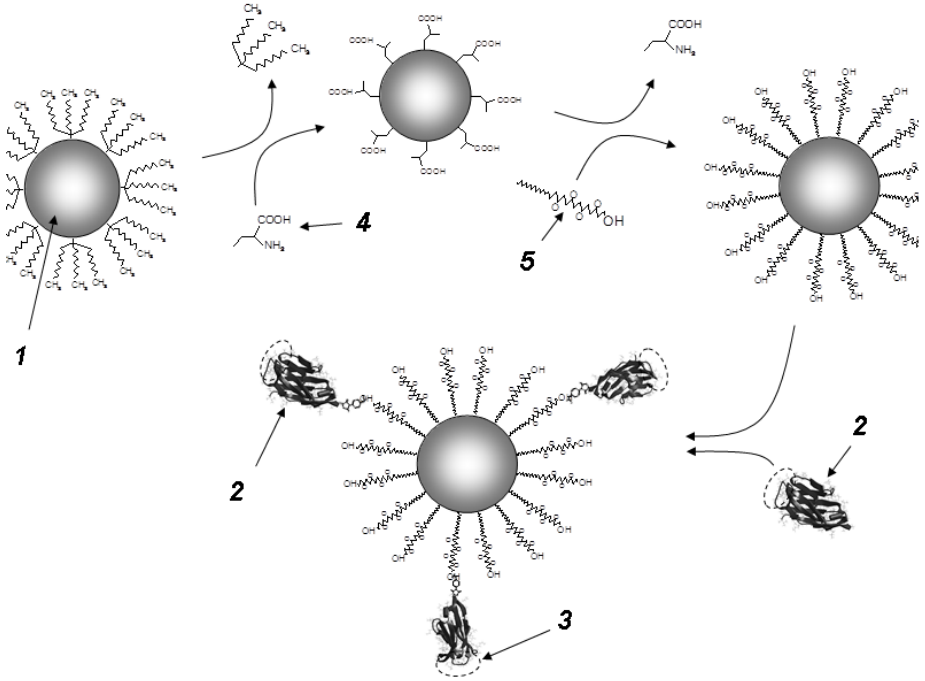

Figure 1. Schematic presentation of the main stages of obtaining water-soluble CdSe/ZnS QDs, their stabilization in aqueous solution, and highly oriented conjugation of sdAbs with these QDs, with the antigen-binding domain of each sdAb exposed to the outside and remaining functionally active.
(1) A CdSe/ZnS core/shell QD immediately after synthesis, with the surface covered with trioctylphosphine oxide (TOPO), a water-insoluble surfactant; (2) An sdAb serving as a recognition molecule; (3) The antigen-recognizing site of an sdAb (the part of the sdAb molecule that is capable of recognizing and specifically binding a disease marker); (4) A low-molecular-weight thiol-containing compound (here, cysteine) used at the first stage of nanoparticle solubilization, which is performed by replacing the hydrophobic surfactant (TOPO) on the nanoparticle surface with polar molecules; (5) Polyethylene glycol (PEG) derivatives.

The hydrodynamic sizes of the particles were calculated from the diffusion time using the Einstein–Stokes equation. The measurements were repeated 15 times for each sample (with a measurement intensity of 10 traces per measurement); the results were averaged and used for calculating the QD hydrodynamic diameter.

The sdAbs against CEA and HER2 were obtained as described earlier [5, 6]. The sdAb–QD conjugates were fabricated with the use of sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Sulfo-SMCC) (Pierce) as a water-soluble heterofunctional crosslinker using the method

described elsewhere [3]. The sdAb content of the sdAb–QD conjugates was determined spectrophotometrically using a reagent kit for protein assay (Bio-Rad Laboratories, United States); the QD content was calculated from the extinction coefficient of the first exciton band in the QD absorbance spectrum. The maximum sdAb to QD molar ratio in the conjugates was about 4:1.

Figure 1 shows the main stages of the preparation of water-soluble CdSe/ZnS QDs, their stabilization in aqueous solution, and highly oriented conjugation of sdAbs with these QDs, with the antigen-binding site of each sdAb exposed to the outside and remaining functionally active (see the caption to Figure 1 for details).

Flow cytometry of stained cells was performed by means of a FACStarPlus (Becton Dickinson, United States) or GuavaR EasyCyteTM Plus (Guava TechnologiesTM, United States) flow cytometer immediately after staining. An argon laser (488 nm) was used for exciting the fluorescence; the fluorescence intensity was measured in the spectral regions of 564–586 nm (FACStarPlus) and 570–596 nm (GuavaR EasyCyteTM Plus).

Tissue samples of the human appendix vermiformis and colon carcinoma were obtained from the Department of Pathology of the Robert Debré University Hospital Center (Reims, France). The study was not related to any diagnostic or therapeutic procedure; hence, the use of these samples did not require permission of the Committee for the Use of Clinical Material. In the control experiments corresponding to the "gold standard" of immunohistochemical labeling of tissue sections using DAB, the tissue specimens were incubated in the presence of mAbs and CEA from Ventana Medical Systems (France) (the TF 3H8-1 clone), washed with PBS, and then incubated in the presence of biotinylated anti-mouse sheep polyclonal immunoglobulin (Amersham, France) in PBS. The specimens were developed using a Dako REAL™ kit (Denmark) (peroxidase/DAB). After washing with PBS–Tween, the sections were examined under an optical microscope (Carl Zeiss, Germany).

A Chameleon Ultra II femtosecond mode-synchronization laser (Coherent, Germany) with a pulse duration of 140 fs, frequency of 80 MHz, and 2P excitation wavelength from 700 to 1000 nm was used for excitation of 2P fluorescence. The mean power of the laser was 3.3 W at an excitation wavelength of 800 nm. An Ar^+ laser (Coherent, Germany) was used for 1P excitation in the visible range (wavelength, 457.9 nm) at an excitation power of 10 mW. The fluorescence was recorded using an LSM-710 microscope (Carl Zeiss, Germany) with a 20× Plan Apo DIC II lens (0.45 N.A.) and an MBS760+ beam splitter. The image resolution was 1024 × 1024 pixels. The QD fluorescence from the stained specimens upon 2P laser excitation was absolutely stable throughout the irradiation period. The radiation intensity was permanently controlled during the experiments; photodegradation was not observed. In experiments with excitation in the UV spectral region (350–400 nm), the sections were examined under an Axiovert-200 fluorescence microscope (Carl Zeiss, Germany) with a low-frequency filter (450 nm).

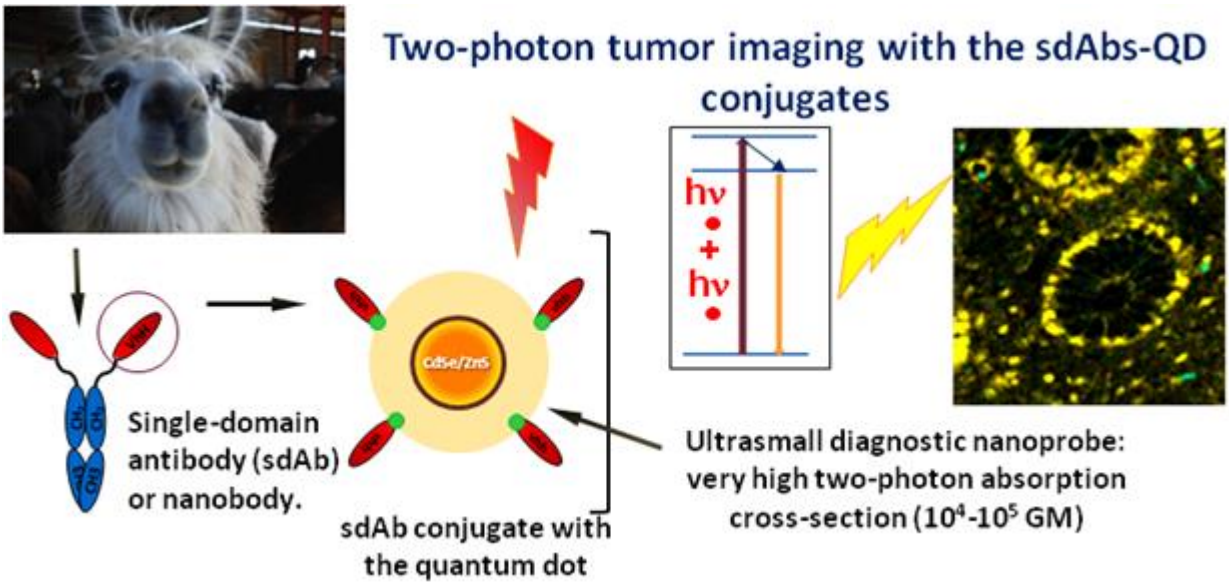

Figure 2. On the left, schematic description of the fabrication of a diagnostic nanoprobe by conjugating single-domain antibodies (sdAbs or VhH fragments) with semiconductor CdSe/ZnS quantum dots (QDs); on the right, the use of the nanoprobe for multi-photon imaging of a cancer marker.

3. Results and Discussion

SdAbs with high affinities for CEA and HER2 (KD ≈ 8.3 nM) were obtained by integrating an additional C-terminal cysteine residue [9] for subsequent site-specific oriented conjugation of the sdAbs with QDs. CdSe/ZnS QDs with a quantum yield of about 70% were transferred to water phase by replacing the TOPO/TOP ligands on their surface with DL-Cys. After that, we prepared a series of compact QDs with a controlled surface charge coated with three-functional low-molecular-weight PEG polymers. This procedure provides stable, compact PEG-functionalized QDs with a hydrodynamic diameter <9 nm and a quantum yield of about 50%.

The conjugation reaction using Sulfo-SMCC is the standard procedure for fabrication of batches of QD conjugates. This reaction links accessible amino groups on the QD surface with sulfhydryl groups of biomolecules. The PMPI conjugation reaction links hydroxyl groups on the QD surface with the exposed sulfhydryl group of the sdAb (Figure 1). Thus, both reactions ensures selective and oriented attachment of sdAbs to the QD surface specially functionalized for these reactions via the only accessible Cys residue introduced into their C-terminal domain. All the conjugated sdAb molecules are bound with the QDs via the same functional groups and have the same orientation relative to the QD surface.

DLS studies have shown that the conjugation of the QDs obtained in this study with sdAbs yields maximally compact nanoprobes with a very small hydrodynamic diameter (about 11.9 nm). They are only slightly larger than unconjugated QDs (8.84 nm) and are much smaller than IgG–QD conjugates (30.3 nm) [3, 9]. Thus, the ultrasmall diagnostic probes developed are nine times smaller than conjugates of full-size mAbs with nanoparticles of the same diameter.

The conjugates developed were originally used for flow cytometry analysis of mixtures of CEA-positive and CEA-negative MC38 cells in human blood serum, which demonstrated the possibility of detection of very small numbers of cancer cells in cell populations containing tumor and normal cells [3, 9]. The method used in our studies allows less than 1% of CEA-positive cells to be detected among a large excess (99%) of CEA-negative MC38 cells, which confirms a high specificity of flow cytometry using sdAb–QD conjugates. Figure 2 schematically shows the experimental procedure of multi-photon imaging with the use of sdAb–QD conjugates. We have found that excitation at 920 nm

is a good compromise solution ensuring both the necessary intensity of QD fluorescence and strong suppression of tissue autofluorescence, with an ideal QD fluorescence to autofluorescence ratio higher than 50.

4. Conclusions

Recently, we have developed a next-generation ultrasmall diagnostic nanoprobe consisting of the smallest possible functionally active Ab fragments (13 kDa) highly affine for CEA (KD = 8.3 nM), which are termed single-domain antibodies (sdAbs) or "nanobodies", conjugated with a compact semiconductor CdSe/ZnS quantum dot (QD) in a strictly predetermined orientation [3–5, 9, 10]. The high degree of orientation is ensured by site-specific conjugation of the QD with sdAbs via an additional cysteine residue specifically integrated into the C-terminal domain of the sdAb amino acid sequence. The nanoprobes developed carry four identically oriented sdAb antibody molecules on the surface of each QD and have a hydrodynamic diameter smaller than 12 nm.

Experimental results have shown that the sdAb–QD nanoprobes specifically bind CEA, a cell receptor expressed by cells of many human tumors. We have also demonstrated that sdAb–QD conjugates are stable, retain their specificity for CEA-expressing tumor cells in human blood serum, and exhibit excellent sensitivity and specificity in quantitative flow-cytometric detection of cancer markers and rare biomarker-expressing cells. A high diffusion capacity of sdAbs used for fabrication of these ultrasmall nanoprobes makes them good candidate tools for immunohistochemical analysis of thick tissue sections impermeable for conventional IgG. Our data show that sdAb–QD conjugates label antigens more precisely than conventional Abs do, ensuring the quality of labeling in biopsies equal to or better than that of the "gold standard" immunohistochemical diagnosis using the DAB chromogen.

We have also demonstrated that the use of two-photon excitation in immunofluorescent diagnostics with the use of sdAb–QD conjugates provides the best results in detecting cancer markers in human histological specimens. This excitation mode allows the high brightness and stability of QD fluorescence to be combined with a high ratio of QD fluorescence to tissue autofluorescence. The sdAb–QD conjugates used in our studies [3, 7, 9] have proved to be a substantially better alternative to the conjugates of conventional monoclonal antibodies with organic fluorophores. These conjugates are bright and specific labels that make it possible to trace tumor cells in bulk tissues and detect target antigens at unprecedentedly low concentrations [2]. However, their effectiveness should be confirmed in model experiments on animals before the novel nanoprobes are used in clinical practice.

Acknowledgments

This study was supported by the Federal Targeted Program for Research and Development of the Ministry of Education and Science of the Russian Federation (grant no. 14.584.21.0012, contract no. RFMEFI58415X0012). We are grateful to Vladimir Ushakov for assistance in preparation of this manuscript.

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