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Oriented conjugates of single-domain antibodies and fluorescent quantum dots for highly sensitive detection of tumor-associated biomarkers in cells and tissues

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Abstract

Our recent results in the field of engineering and application of highly oriented conjugates of single-domain antibodies and fluorescent quantum dots are summarized. These novel conjugates proved to be excellent nanoprobes for immunolabeling of tumor-associated biomarkers on cells and tissue specimens detectable by means of flow cytometry or one- or two-photon confocal microscopy. The results are discussed in terms of the most promising future applications of these conjugates and further developments in this field.

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Keywords: quantum dots (QDs); cancer markers; single-domain antibodies (sdAbs); bioconjugates.

1. Introduction

Quantum dots (QDs) are highly fluorescent colloidal semiconductor nanocrystals that are thousands of times more photostable and 20 times brighter than organic dyes (Chan and Nie (1998)). QDs exhibit size-dependent, narrow, symmetrical emission spectra and broad excitation spectra, which allows nanocrystals of different colors to

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be excited from a single light source. Due to their superior properties, QDs are a promising fluorophores for development of diagnostic labels based on conjugates of QDs with capture biomolecules, which may be used for simultaneous multiplexed labeling (Zrazhevskiy and Gao (2013)) and ultrasensitive detection of specific biological targets (Liu et al. (2010)).

Nomenclature

Abs	antibodies
CEA	carcinoembryonic antigen
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide
HER2	human epidermal growth factor receptor 2
PMPI	p-maleimidophenyl isocyanate
QDs	quantum dots
SMCC	succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate
SdAbs	single-domain antibodies

In clinic, the development of new methods for more sensitive detection of tumor-associated biomarkers is one of cutting-edge lines of research. For this reason, special attention is paid to novel diagnostic probes with high specificity and sensitivity for cancer biomarkers that could be used for detection, imaging, and medical diagnostics. For target detection, QDs are conjugated with capture molecules, such as antibodies (Abs) (Montenegro et al. (2013) ; peptides (Chen et al. (2014)), proteins (Ag et al. (2014)), and oligonucleotides (Zhang et al. (2013)). IgG Abs conjugated to QDs have been successfully used for immunofluorescent labeling of cell-surface receptors in cell suspensions and tissues (Yang et al. (2011); Rizvi et al. (2014)). However, a comparatively large size of the conjugates may result in poor penetration into tissues and impede access to the epitopes. Some smaller Ab fragments retaining the functional activity of full-size Abs have been conjugated to QDs in order to produce a probe with smaller sizes (Mahmoud et al. (2011); Sukhanova et al. (2011)). Single-domain Abs (sdAbs), also referred to as nanobodies, are the smallest known antigen-recognizing molecules. They are derived from llama heavy-chain Abs. SdAbs are about 12 times smaller than full-size Abs, while preserving about the same affinity for antigens (Fig. 1B). We have developed (Sukhanova et al. (2012a)) and described in detail (Sukhanova et al. (2012b)) a protocol for conjugation of sdAbs with QDs in a highly oriented manner, resulting in very bright and compact conjugates, with all antigen-binding sites oriented towards the outside of the conjugate. The resultant diagnostic probes have been used for highly sensitive detection of tumor-associated antigens on the cell surface.

2. Materials and methods

2.1 Preparation of water-soluble quantum dots

QDs were synthesized as described in (Wargnier et al. (2004)), their surface properties were analyzed using an FTIR-8400S Fourier transform infrared spectrophotometer (Shimadzu). Then, the QDs were solubilized and stabilized in aqueous buffer solutions as described in (Sukhanova et al. (2012); Stsiapura et al. (2006)). Briefly, at the first stage, the QD surface was modified with DL-cysteine. At the next stage, the surface was modified with polyethylene glycol derivatives containing terminal thiol, hydroxyl, or amino groups. As a result, stable water-soluble populations of QDs with different surface compositions were obtained.

2.2 Preparation of conjugates containing quantum dots and partly reduced antibody fragments

Conjugates of quantum dots and full-size Ab fragments were prepared as described in (Mahmoud et al. (2011)). Full-size Abs were partly reduced using 2-mercaptoethanol-amine-HCl or low concentrations of dithiothreitol, which resulted in a high yield of functionally active 75-kDa fragments. Then, the fragments were conjugated with QDs using the succinimidyl trans-4-(maleimidylmethyl)cyclohexane-1-carboxylate crosslinker (SMCC) in an oriented manner to ensure functional activity of the conjugates. Briefly, QDs were activated with SMCC for 1 h;

then, reduced Ab fragments were added to QDs and incubated for 1 h. The conjugation reaction was quenched with 2-mercaptoethanol, and the conjugates were purified on size-exclusion columns.

2.3 Preparation of conjugates containing quantum dots and single-domain antibodies

Conjugates of quantum dots and single-domain Abs were prepared as described in (Sukhanova et al. (2012b)). Briefly, QDs containing amino groups on their surface were conjugated to sdAbs containing a cysteine residue integrated into the C-terminus using the SMCC crosslinker. QDs containing only hydroxyl groups on their surface were conjugated to the same sdAbs using the p-maleimidophenyl isocyanate (PMPI) crosslinker. The conjugates obtained were purified on size-exclusion columns, their hydrodynamic sizes were measured by dynamic light scattering, and the content of sdAbs in the sdAb–QD conjugates was determined.

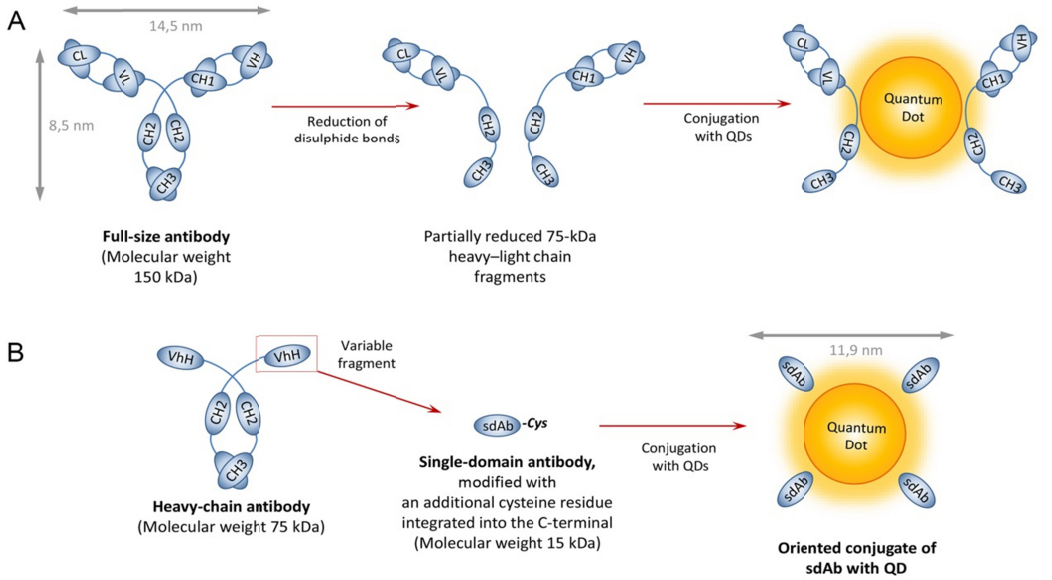

Fig. 1. (A) Schematic diagrams of a full-size antibody, partially reduced 75 kDa heavy-chain fragments of the antibody, and an oriented conjugate of a quantum dot with the fragments. (B) Schematic diagrams of a llama heavy-chain antibody and a single-domain antibody (sdAb). An additional cysteine residue has been specifically integrated into the C-terminus of the sdAb amino acid sequence, far away from the antigen-binding site. SdAbs have been covalently conjugated to quantum dots via the thiol group of the cysteine residue, which have resulted in highly oriented conjugates, with all antigen-binding sites oriented to the outside of the conjugate.

2.4 Labeling of cells with conjugates and flow cytometry analysis

SdAb–QD conjugates with fluorescence maxima at 625 nm that were specific for carcinoembryonic antigen (CEA) were diluted in sodium phosphate-buffered saline to a concentration of 30 μg/ml. Then, aliquots of the stock solution were added to a suspension containing 100000 MC38CEA or MC38 cells so that the stock solution was diluted 20- or 50-fold, and the mixtures were incubated for 90 min at +4°C in the dark. After the incubation, the cells were washed to remove the unbound conjugates and analyzed using an FACStar Plus flow cytometer (Becton Dickinson). The efficiency of cell labeling was evaluated by the fluorescence signal in the corresponding channel of the flow cytometer.

3. Results and discussion

3.1 Preparation of conjugates of quantum dots with antibodies

A number of effective approaches have been proposed to bind the Ab molecules to the QD surface (Bilan et al. (2015)). The methods include covalent coupling and electrostatic interactions; highly specific interactions (such as between biotin and streptavidin) have proved to be effective.

The most widely used method of covalent conjugation is based on carbodiimide-mediated coupling of primary amines to activated carboxylic acid groups. The water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) is used to activate carboxylic groups, generating a reactive intermediate that rapidly interacts with amines to form covalent amide bonds. Since the Ab molecule contains at least one amine and one carboxylic group, the method can be easily applied to almost any Ab without its preliminary chemical modifications. The conjugation proceeds randomly, which may result in undesirable cross-linking and reduction of the functional activity of the Abs. Hence, accurate selection of optimal conjugation parameters is required to produce the most biologically active product (East et al. (2011)).

Another approach to covalent conjugation, which is employed in a commercially available conjugation kit (Invitrogen), is based on reduction of disulfide bonds in full-size Abs (with a molecular weight of 150 kDa) using dithiothreitol, which results in three types of fragments with different molecular weights: light chain (25 kDa), heavy chain (50 kDa), and partially cleaved heavy–light chain (75 kDa) fragments, with only the 75-kDa heavy–light chain fragments retaining the functional activity, because they have intact antigen binding sites. The mixture of fragments was covalently attached to the QDs surface, resulting in conjugates with a functional activity strongly limited by the number of active heavy–light chain fragments in a nanoprobe. Indeed, the number of functional Abs in the commercial QD–Ab conjugates prepared according to this approach turned out to be as low as 0.076 ± 0.014 Abs/QD (Pathak et al. (2007)). In our previous study (Mahmoud et al. (2011)), we have improved the procedure for partial reduction of Abs, which allows the disulfide bonds between heavy and light chains to remain intact, while the disulfide bonds between heavy chains are reduced, resulting in a high yield of functionally active 75-kDa fragments. These active fragments were conjugated to the QDs in an oriented manner, with a preserved functional activity of the conjugate. The prepared conjugate was found to be 26 times more sensitive than the conjugate prepared from the Abs reduced according to the standard protocol.

Oriented conjugation of Abs with nanoparticles is known to improve the specificity of the resultant probe in comparison to random conjugation using carbodiimide chemistry (Puertas et al. (2011)). In our previous studies, we developed oriented conjugates of QDs and sdAbs, the smallest possible naturally occurring antigen-binding fragments known, with a molecular weight of about 13 kDa. Due to their compact size, sdAbs diffuse in tissues much better than full-size Abs and may bind sterically hindered epitopes, such as cavities (Perruchini et al. (2009)). Having the same affinity and about the same variability as full-size Abs, sdAbs exhibit a low immunogenicity, as well as better stability and capability of refolding after denaturation, which makes these Abs promising components for the development of specific fluorescently labeled probes.

In our study, sdAbs were modified with an additional cysteine residue specifically integrated into the C-terminal end of the sdAb amino acid sequence, far away from the antigen-binding site. SdAbs were covalently conjugated to QDs through the SH group of the cysteine residue, which resulted in highly oriented conjugates, with all antigen-binding sites oriented to the outside of the probe (Sukhanova et al. (2012a)) (Fig. 1B). The resultant compact probes had a hydrodynamic diameter below 12 nm and contained four molecules of sdAbs on the surface of each QD. The conjugates turned to be promising probes for immunolabeling of several biomarkers in cells and tissue specimens.

3.2 Conjugates of single-domain antibodies and quantum dots for ultrasensitive detection of tumor-associated biomarkers

In a series of our studies, we used oriented sdAb–QD conjugates for detection cell-surface cancer biomarkers in live cells and tissue specimens. We have demonstrated that the sdAb–QD conjugates can specifically bind CEA, cell surface receptor expressed by many types human tumor cells. The conjugates proved to be good candidates for immunohistochemical analysis of thick tissues: sdAbs–QD stained CEA in a more delicate manner than full-size Abs and lead to biopsy tissue labeling of an equivalent or even better quality than that obtained with “gold standard” immunohistochemical labeling of tissue sections with diaminobenzidine (Sukhanova et al. (2012a)).

Flow cytometry experiments have demonstrated that CEA-positive and CEA-negative MC38 cells can be easily identified with sdAb–QD conjugates specific for CEA. Suspensions of cells were incubated with the conjugates; then, the cells were washed to remove the unbound conjugates and analyzed by flow cytometery (Fig. 2). The experiments demonstrated a significant increase in the fluorescent signal in the case of CEA-positive cells (Fig. 2B) compared to CEA-negative cells (Fig. 2A). The higher the concentration of sdAb–QD conjugates in the incubation mixture with MC38CEA cells, the higher the signal was, whereas increasing the conjugate concentration in the mixture with MC38 cells did not lead to an increase in the fluorescent signal, which confirms the a specificity of the conjugates.

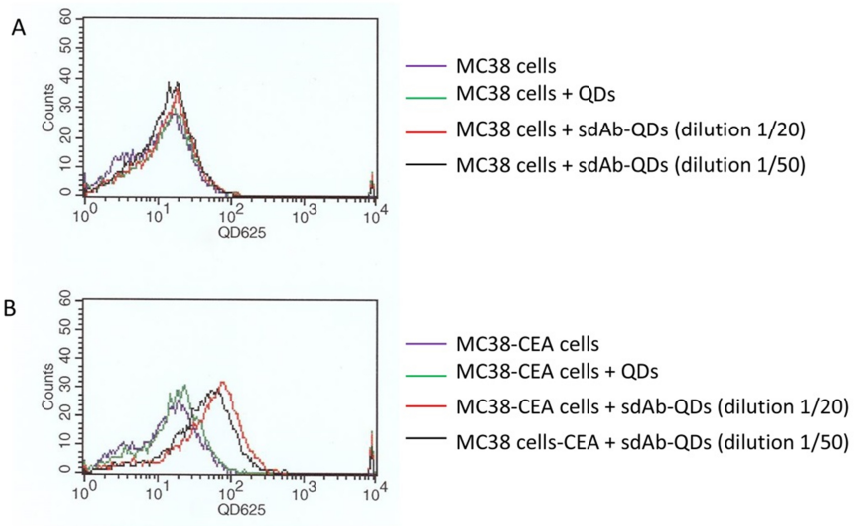

Fig. 2. Flow cytometry analysis of (A) CEA-negative and (B) CEA-positive cells labeled with conjugates of quantum dots (QDs) and single-domain antibodies (sdAbs) against CEA. Populations of cells: unlabeled cells (control, violet lines); cells incubated with unconjugated QDs (green lines); cells incubated with a stock solution of sdAb–QD conjugates diluted by 50-fold (black lines) or 20-fold (red lines).

In the study, the conjugates with other type of QD have been used for highly sensitive quantitative detection of CEA on the surface of cells by means of flow cytometry. Different amounts of CEA-positive cells in a mixture with CEA-negative cells were identified by staining with sdAb–QD conjugates. As a result, even 1% of CEA-positive cells could easily be discerned against a background of 99% of CEA-negative cells, thus confirming the high specificity detection with sdAb–QD conjugates.

We also used the approach of oriented conjugation described above to develop ultrasmall, bright probes based on QDs and sdAbs against human epidermal growth factor receptor 2 (HER2), a surface receptor overexpressed in many types of cancer (Rakovich et al. (2014)). The ability of the conjugates to label breast and lung cancer cell lines was compared with that of anti-HER2 monoclonal Abs conjugated to the conventional organic dyes Alexa Fluor 488 and Alexa Fluor 568. With the use of confocal microscopy it was shown that sdAb–QD probes were superior over conventional organic dyes in detection of cancer cells with a low HER2 expression rate.

Another advantage of QDs over organic dyes is very large values of two-photon absorption cross-section, which determines the probability of simultaneous absorption of two photons and subsequent transition to an excited state. This value for QDs increases nonlinearly with their size, approaching the theoretical value of 50 000 Goeppert-Mayer units, which several orders of magnitude exceeds that of organic dyes (Hafian et al. (2014)). Due to this advantage, QDs are the best fluorescent labels to be used for multiphoton imaging. This technique enables deep imaging of biological tissues, because the excitation band is far from the spectral region of tissue autofluorescence efficient excitation. Recently we performed two-photon imaging of CEA in normal human appendix and colon carcinoma tissues with the use of sdAb–QD conjugates. We have demonstrated that tissue imaging with two-photon excitation leads to a very high signal to noise ratio due to a bright signal and an extremely low autofluorescene of

the tissues, which allow the detection of sdAb–QD conjugates more than three times deeper in tissue sections (26 μm) than it is possible using single-photon excitation (8 μm). Thus, sdAb–QD conjugates have proved to be a superior alternative to conjugates of conventional organic dyes in their application to imaging biomarkers in biological fluids and tissue sections displaying intense autofluorescence. We suppose that the conjugates developed are useful for detecting low concentrations of antigens and for tracking cells deep within tissues by means of multiphoton microscopy. Table 1 summarizes the most interesting prospects for future applications and further development of the conjugates described.

Table 1. Prospects for future applications and further developments of the sdAb–QD conjugates.

In vitro applications	In vivo applications	Bioanalytical assays	Combined imaging techniques
Membrane receptor labeling	Tumor detection and diagnostics	Fluorescent staining in ELISA	Epifluorescent, single- and miltiphoton microscopies
		Planar microarrays	
Intracellular labeling	Single-cell tracking	Suspension microarrays	Fluorescence lifetime imaging microscopy
Single molecule tracking	Photodynamic therapy	Cell sorting	Ultrahigh-resolution imaging

4. Conclusions

Compact highly oriented sdAb–QD conjugates is a superior alternative to conjugates of conventional organic dyes traditionally used as diagnostic probes and to commercially available conjugates of QDs with conventional full-size Abs. These novel nanoprobes have proved to be particularly efficient for detection of the cell-surface cancer biomarkers CEA and HER2 both on live cells and on fixed tissue specimens. Due to unique spectral properties of QDs in combination with high specificity of compact sdAbs, it is possible to detect small quantities of CEA-positive cells in a mixture with CEA-negative cells by means of flow cytometry. Immunohistochemical staining of thick tissues with sdAb–QD probes display the same or even better quality than that obtained with the "gold standard" immunohistochemical labeling used in clinical analysis. Since QDs are characterized by the highest values of two-photon absorption cross-sections, promising prospects are opened for sdAb–QD applications in multiphoton microscopy for deep tissue imaging and highly sensitive detection of low concentrations of antigens. We believe that the approach described here will be a great stride towards highly sensitive multiplexed detection of numerous of biomarkers for precise clinical analysis.

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