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Detection of carcinoembryonic antigen using single-domain or full-size antibodies stained with quantum dot conjugates

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ABSTRACT

Compact single-domain antibodies (sdAbs) are nearly 13 times smaller than full-size monoclonal antibodies (mAbs) and have a number of advantages for biotechnological applications, such as small size, high specificity, solubility, stability, and great refolding capacity. Carcinoembryonic antigen (CEA) is a tumor-associated glycoprotein expressed in a variety of cancers. Detection of CEA on the tumor cell surface may be carried out using anti-CEA antibodies and conventional fluorescent dyes. Semiconductor quantum dots (QDs) are brighter and more photostable than organic dyes; they provide the possibility for labeling of different recognition molecules with QDs of different colors but excitable with the same wavelength of excitation. In this study, the abilities for specific detection of CEA expressed by tumor cells with anti-CEA sdAbs biotinylated *in vitro* and *in vivo*, as well as with anti-CEA mAbs biotinylated *in vitro*, were compared using flow cytometry and the conjugates of streptavidin with QDs (SA-QDs). The results demonstrated that either *in vitro* or *in vivo* biotinylated anti-CEA sdAbs are more sensitive for cell staining compared to biotinylated anti-CEA mAbs. The data also show that simultaneous use of biotinylated sdAbs with highly fluorescent SA-QDs can considerably improve the sensitivity of detection of CEA on tumor cell surfaces.

Conventional organic dyes are commonly used as fluorescent markers in biomedical studies, including diagnostics and biological imaging. These applications require specific targeting of different biomolecules, for which purpose numerous antibody-based labeling strategies have been developed. The possibility of conjugation of a fluorescent probe with an antibody (Ab)[1] allows both specific recognition of the target and quantitative detection of the associated fluorescent signal. Detection of different fluorescent signals simultaneously would offer the possibility of multiplexed analysis and a better understanding of supramolecular systems and biological processes. Routine fluorescence-based immunolabeling strategies depend on four main parameters, which impose limitations on their practical use: the affinity of Abs, the optical properties of the fluorescent probes, the labeling method, and the size of the resultant conjugate composed of the Ab, fluorescent probe, and optional linker molecules. Conjugates of single-domain Abs (sdAbs) and quantum dots (QDs) are new interesting tools which can reduce some shortcomings of the immunolabeling and detection techniques, such as the large size and poor optical properties of fluorescent probes [1–3].

Most organic dyes generally used as fluorescent probes suffer from photobleaching, a low brightness above background fluorescence, a wide overlap between the absorption and the emission spectra of different dye molecules, etc. These shortcomings severely limit the use of organic dyes for detection of rare events or multiplexed imaging and analysis. In contrast, semiconductor QDs are characterized by an exceptionally bright, stable fluorescence and, hence, are particularly interesting as tools for biological imaging and diagnostics [4]. Moreover, different QD populations

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[1] *Abbreviations used:* CEA, carcinoembryonic antigen; CDRs, complementary determining regions; Fab, antigen-binding fragment; HcAbs, heavy-chain antibodies; IPTG, isopropyl-β-D-thiogalactopyranoside; mAbs, monoclonal antibodies; QDs, quantum dots; SA-QD, conjugate of streptavidin with quantum dot; ScFv, single-chain variable fragment; sdAbs, single-domain antibodies.

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can be excited at the same wavelength which may be very far from their respective emission bands, depending on the QD core size and composition [5,6]. Since the first successful transfer of inorganic QDs in aqueous solutions by Bruchez [7] and Chan [8], these fluorescent nanoprobes have been among the most exciting and promising tools for fluorescent-based biological applications, such as microarray analysis [9], immunohistochemistry [10], single-particle tracking [11], flow cytometry [12], cancer research [13], and drug targeting [14,15]. The use of QDs in different biological applications requires solubilization and chemical functionalization of the QD surface and their conjugation with recognition molecules that are responsible for specific interaction of QD conjugates with biological targets.

Abs are characterized by high specificity and avidity of interaction with specific target molecules, which makes them efficient capture and detection tools for numerous biological applications. However, their complex structure, fragility and large size limit their applications. The standard conjugation procedures for attachment of a fluorescent probe to Ab may lead to conformation changes in the Ab molecular structure and decrease in antigen recognition capacity. Moreover, Abs have a high molecular weights (150 kDa) and large average size of the full-length molecule ($14.5 \times 8.5 \times 4$ nm) [16] and, hence, are not absolutely suitable for targeting. To overcome this shortcoming, small and compact antibody fragments, such as $F(ab')_2$ (100 kDa), Fab (50 kDa), and ScFv (25 kDa) with comparable specific recognition capacities are used (Fig. 1A–D). Despite the obvious advantages of such relatively small capture molecules, their biochemical properties, sophisticated production procedures, and a high production cost make them poorly available.

In 1993, Hamers-Casterman et al. found, in Camelidae, a new form of IgG-like antibodies, so-called heavy-chain antibodies (HcAbs), which lack the two light chains [17]. Since this discovery, a number of researchers have produced and isolated sdAbs consisting of the variable domains of these HcAbs alone (Fig. 1E and F) [18]. sdAbs have several important advantages over mAbs due to such characteristics as small size and weight, high stability, solubility, and expression rate [19–21]. These tiny antibodies (about 13 kDa) exhibit the same affinity and about the same variability as "classical" mAbs; they can also recognize and bind very small antigens, such as haptens, for example [22]. Consisting of about 110 amino acids, sdAbs include three distinct complementarity determining regions (CDR) ensuring the antigen recognition. Several recent studies have shown that sdAbs exhibit (i) a better tissue diffusion capacity than conventional Abs [23], (ii) a low immunogenicity, (iii) high thermal and chemical stabilities, and (iv) the ability to refold and recover their binding capacities after heat denaturation [24,25]. Most importantly, the relatively simple sdAb structure allows the amplification and straightforward cloning of the corresponding genes, without requiring enzymatic cleavages of the Ab (as with the Fab fragment and $F(ab')_2$ fragment), bicistronic constructs (as with the Fab fragment), or artificial linker peptide (as with the single-chain Fv fragment). This permits direct cloning of large sdAb repertoires from immunized camelids. The characteristics of sdAbs allow them to be produced with a high yield by prokaryotic (lactobacilli [26] and *Escherichia coli* [27]) and eukaryotic (*Saccharomyces cerevisiae* [28]) microorganisms as well as by cell lines obtained from multicellular organisms (Chinese hamster ovary cells [29]). Thus, due to their unique properties, these antibodies are promising components of specific fluorescently labeled probes for biological and medical applications.

This study was focused on the possibility of detecting human carcinoembryonic antigen (CEA), a tumor-associated glycoprotein expressed in a variety of cancers, on MC38 and MC38-CEA cells with the use of biotinylated sdAbs or mAbs. Detection of CEA was carried out through interaction of biotinylated sdAbs or

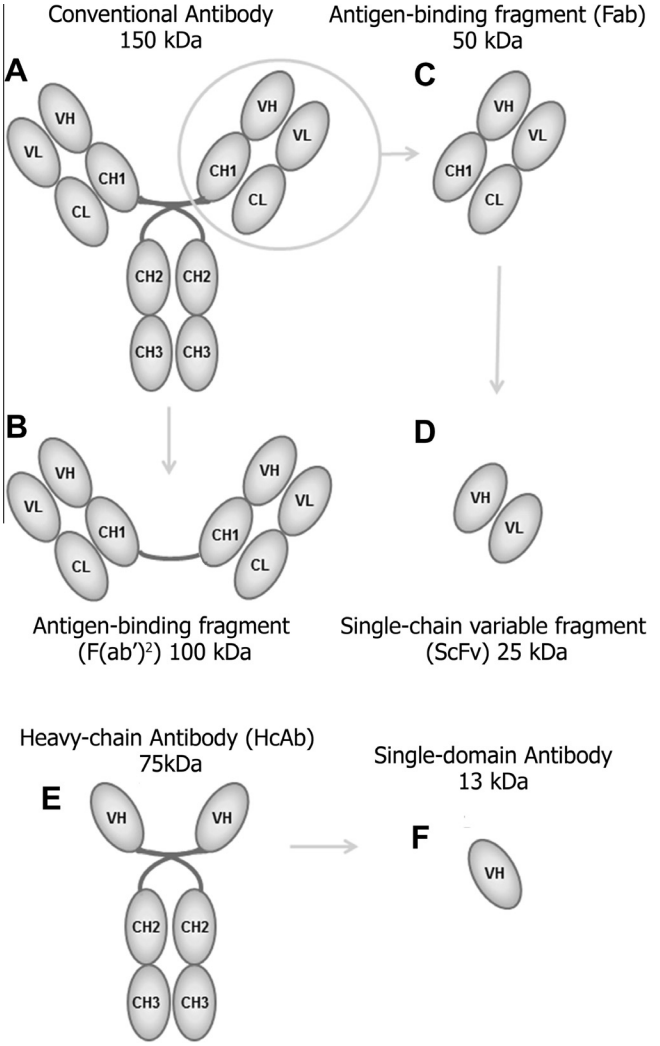

Fig.1. Schematic diagrams of conventional and camelid antibodies. (A) A classical IgG and its derivatives: (B) $F(ab')_2$, (C) Fab, and (D) ScFv. (E) Camelid heavy-chain antibody. (F) Single-domain antibody.

mAbs with streptavidin-QD conjugates (SA-QDs) followed by flow cytometry analyses of formed complexes. First, we optimized the protocol for *in vitro* biotinylation of both mAbs and sdAbs. In addition, the recombinant origin of sdAbs allows their *in vivo* site-directed enzymatic biotinylation by addition of a short peptidic tag that is recognized and modified by the *E. coli* enzyme BirA during the sdAb biosynthesis. Finally, we compared the antigen detection efficiencies by *in vitro* and *in vivo* biotinylated Abs. The results demonstrated that either *in vitro* or *in vivo* biotinylated anti-CEA sdAbs are more sensitive for cell staining compared to biotinylated anti-CEA mAbs. Considering that anti-CEA mAbs and sdAbs recognize different epitopes [31], we suggested that the more sensitive cell staining with biotinylated sdAbs is a result of a higher affinity of sdAbs compared to mAbs. The results of this study allow us to conclude that sdAbs are more sensitive than mAbs and very easy to use, and their combination with highly fluorescent QDs provides additional specific advantages in the immunodetection.

Materials and methods

Materials and equipment

All general chemicals were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Sulfo-NHS-biotin, sulfo-NHS-LC-biotin,