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Conjugation of biotin-coated luminescent quantum dots with single domain antibody-rhizavidin fusions

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ABSTRACT

Straightforward and effective methods are required for the bioconjugation of proteins to surfaces and particles. Previously we demonstrated that the fusion of a single domain antibody with the biotin binding molecule rhizavidin provided a facile method to coat biotin-modified surfaces with a highly active and oriented antibody. Here, we constructed similar single domain antibody–rhizavidin fusions as well as unfused rhizavidin with a His-tag. The unfused rhizavidin produced efficiently and its utility for assay development was demonstrated in surface plasmon resonance experiments. The single domain antibody-rhizavidin fusions were utilized to coat quantum dots that had been prepared with surface biotins. Preparation of antibody coated quantum dots by this means was found to be both easy and effective. The prepared single domain antibody-quantum dot reagent was characterized by surface plasmon resonance and applied to toxin detection in a fluoroimmunoassay sensing format.

1. Introduction

Single domain antibodies (sdAbs), derived from heavy chain only antibodies found in camelids (i.e. camels, llamas, and alpacas), often possess high affinity and the ability to refold and bind antigen after heat denaturation [1–4]. Because these binding elements are produced recombinantly, they can be tailored through protein engineering for specific applications. SdAbs, in general, produce very well in *Escherichia coli*; sdAb fusion constructs are also produced more readily than traditional antibody binding fragments that include both heavy and light chains. For example, expression of sdAbs as genetic fusions with peptides, or high-melting temperature proteins is a proven route towards generating highly stable molecules with enhanced utility [5–10]. Fusions have been used for a variety of purposes including: providing improved cytoplasmic production of stable sdAbs, increasing solubility of sdAbs, as well as providing oriented capture reagents [5,7,11,12].

We recently described the production and characterization of a genetic fusion between a sdAb and rhizavidin (RZ) [10], a biotin binding protein (derived from the symbiotic nitrogen-fixing bacterium *Rhizobium etli* CFN42) [13]. Unlike streptavidin, RZ putatively forms a homodimer instead of a tetramer [13,14]. RZ has a melting temperature of over 100 °C in the presence of biotin, and ~75 °C in its absence. The sdAb-RZ fusion provided the same desirable characteristics, such as providing oriented capture, as fusions with the streptavidin core protein [11]. Importantly, protein production was improved by approximately 20-fold compared to the sdAb-streptavidin core fusion [10].

Luminescent quantum dots (QDs) provide robust fluorophores that have been incorporated for applications including biosensing and imaging [15,16]. Conjugates of sdAbs with QDs, which couple stable recognition elements with robust fluorophores, have been described for detection, imaging and diagnostic applications [17–23]. Several methods for bioconjugation of proteins to QDs have been described; for instance, we have previously utilized directional conjugation of sdAbs to QDs through an extended poly histidine tail [17,20,24]. One of the previous generation of sdAb-QD reagents we tested was based on QDs made water compatible through capping with dihydrolipoic acid (DHLA). QDs functionalized with DHLA-PEG based-ligands are not as amenable to conjugation through an extended histidine tail, however they offer functionality and stability over a wider pH range [17,25]. An advantage of sdAbs is their ability to function over a wide range of conditions [26,27] including intracellular [28]. Therefore it is

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desirable to have a facile system for the directional conjugation of sdAbs to QDs functionalized with DHLA-PEG ligands that provide an increased biocompatibility. The recent development of DHLA-PEG capped QDs with a portion of the cap functionalized with biotin [29,30], in conjunction with fusions of sdAbs with RZ provides an alternate route for directional conjugates of sdAbs on QDs. A schematic illustrating both a sdAb-QD conjugate formed using DHLA-capped QDs with attachment of the sdAb through an extended histidine tail and a sdAb-QD conjugate utilizing the DHLA-PEG biotinylated QDs and a sdAb-RZ genetic fusion is shown in Fig. 1. Having a wide variety of methods to form effective sdAb-QD conjugates is advantageous as it provides researchers the ability to choose the conjugation method most appropriate for their assay or imaging conditions.

This current work focuses on ricin detection. Ricin is a 60–65 kDa highly potent toxin which consists of an A and B subunit. The A subunit is the enzymatic portion responsible for ribosome inactivation, while the B subunit binds the cell to facilitate entry of the toxin [31]. To detect ricin the sdAb, D12f, which has both high affinity and good thermal stability (Tm = 78 °C) [32], was produced as a fusion with RZ. D12f better complements the high stability of RZ than the original C8 anti-ricin sdAb used as a fusion partner with RZ, which binds the same epitope and has a high affinity for ricin, but melts ~60 °C. In addition, because we had observed sporadic degradation of constructs that utilized the llama heavy chain antibody's upper hinge as a linker, we switched to a generic10-amino acid Gly-Ser linker to join D12f to RZ. We also prepared the unfused RZ with a C-terminal hexa histidine tag (RZh), evaluated its biophysical characteristics and demonstrated its utility for use as a regenerable ligand via surface plasmon resonance (SPR) using HTE (6x-His binding) sensor chips. Nevertheless, the main objective was demonstrating the utility of the sdAb-RZ fusion by formation of a bioconjugate between the D12f-RZ and QDs that have biotins incorporated on a portion of their capping ligands. The oriented immobilization provided by the RZ on the QDs yielded a highly active sdAb that binds target effectively.

2. Materials and methods

2.1. Construction SdAb-RZ fusions with Gly-Ser linker

The D12f-L10-RZ was constructed by first inserting the RZ into the *XhoI* site of a pET22b expression vector in which the D12f sdAb sequence had been cloned into *NcoI-NotI* sites (D12f-pET22b); this vector includes a C-terminal 6xHis tag [32]. The RZ fragments flanked with a *XhoI* site at both ends were amplified from the original vector using PCR and inserted to the *XhoI* site within D12f-pET22b. D12f-RZ [33] then served as a template to insert a 10 amino acid Gly-Ser linker (L10, GGGGSGGGGS) using the Quikchange II mutagenesis kit and minor modifications to the manufacturer's protocol (Agilent Technologies; Santa Clara, CA). Mutagenesis was achieved using the forward primer, 5′-GCGGCCGCACTCGAGGGCGGTGGCGG-TAGCGGCGGTGGCGGTTCTTTTGATGCGTCCAATTTTAAA-3′, and its reverse complement sequence as the reverse primer. In brief, low annealing temperature (47 °C) and 18 amplification cycles were employed and the rest of procedures were unchanged from the manufacturer's protocol, to obtain the mutated plasmid. All the clones were confirmed by sequencing (Euofins Genomics; Huntsville, AL). The mutant protein is termed D12f-L10-RZ. All restriction enzymes and T4 DNA ligase were purchased from New England Biolabs, Inc. (Ipswich, MA).

2.2. Construction of unfused RZ with a C-terminal hexa histidine tag (RZh) in pET22b(+)

The rhizavidin fragment was excised from D12f-RZ using the restriction enzyme *XhoI*. Gel purification of the excised fragment was performed with QIAquick Gel Extraction Kit (QIAGEN). Using

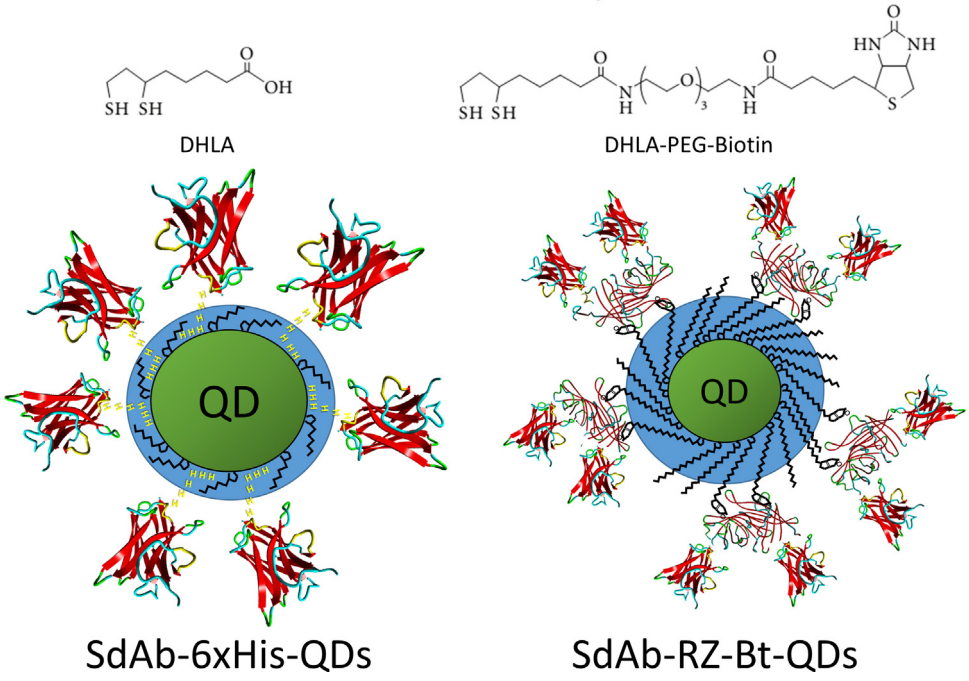

Fig. 1. Schematic of sdAb-QDs prepared previously, through an extended histidine tail on the sdAb and through the current method utilizing biotinylated QDs and sdAb-RZ. The left side shows a DHLA-capped QD onto which sdAb have been conjugated through an extended histidine tail. The right side shows a QD capped with 80% DHLA-PEG550-OMe and 20% DHLA-PEG400-biotin onto which sdAb-RZ are conjugated through the RZ-biotin interaction. The structure of the sdAb is from PDB:4W70 [40] and the RZ structure from PBD:3EW2 [14]. The components are not drawn to scale.