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Highly affine and selective aptamers against cholera toxin as capture elements in magnetic bead-based sandwich ELAA

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ABSTRACT

Aptamers are single-stranded DNA or RNA oligonucleotides, which have been emerging as recognition elements in disease diagnostics and food control, including the detection of bacterial toxins. In this study, we employed the semi-automated *just in time*-selection to identify aptamers that bind to cholera toxin (CT) with high affinity and specificity. CT is the main virulence factor of *Vibrio cholerae* and the causative agent of the eponymous disease. For the selected aptamers, dissociation constants in the low nanomolar range (23–56 nM) were determined by fluorescence-based affinity chromatography and cross-reactivity against related proteins was evaluated by direct enzyme-linked aptamer assay (ELAA). Aptamer CT916 has a dissociation constant of 48.5 ± 0.5 nM and shows negligible binding to Shiga-like toxin 1B, protein A and BSA. This aptamer was chosen to develop a sandwich ELAA for the detection of CT from binding buffer and local tap water. Amine-C6- or biotin-modified CT916 was coupled to magnetic beads to serve as the capture element. Using an anti-CT polyclonal antibody as the reporter, detection limits of 2.1 ng/ml in buffer and 2.4 ng/ml in tap water, with a wide log-linear dynamic range from 1 ng/ml to 1000 ng/ml and 500 ng/ml, respectively, were achieved.

1. Introduction

Cholera is a severe diarrheal disease caused by the ingestion of *Vibrio cholerae*. Although it has been practically eliminated from high-income countries thanks to high levels of hygiene and sanitation, cholera still accounts for an estimated 95,000 deaths out of 2.9 million cases per year, with highest incidence rates in Sub-Saharan Africa and developing countries in general (Ali et al., 2015). *V. cholerae*'s toxin, cholera toxin (CT), is additionally considered a potential biohazardous agent and tool for bioterrorism (Labib et al., 2009; Rowe-Taitt et al., 2000b).

Upon ingestion of contaminated water or food, *V. cholerae* colonizes the intestinal mucosa where it secretes CT into the intestinal lumen. CT is an AB_5-type toxin composed of a donut-shaped homopentameric B subunit (CT-B, ~58 kDa) and a heterodimeric A subunit (CT-A_1 and CT-A_2, together ~27.4 kDa) (Van den Broeck et al., 2007). CT-B binds to its cell receptor GM1 and thus initiates the internalization. Once taken up into the cell, CT-A_1 is the causative agent of cholera typical watery diarrhea with severe dehydration (Sears and Kaper, 1996; Van den Broeck et al., 2007).

Apart from being a part of *V. cholerae*'s main virulence factor, (recombinant) CT-B has gained fame as a vaccine adjuvant and substance with potential immunomodulatory and anti-inflammatory properties and as a prototype to elucidate cellular physiological mechanisms (Baldauf et al., 2015; Sanchez and Holmgren, 2011).

As new serotypes emerge, and taking into account that not all *V. cholerae* O1 isolates are toxigenic, it becomes increasingly important to detect not only the secreting pathogen but also the toxin itself. CT is routinely tested for by antibody-based immunosorbent or latex agglutination assays (Almeida et al., 1990; Said et al., 1994). Other sensitive approaches for CT detection have been developed, such as electrochemical, real-time and dynamic cytotoxicity and nanoparticle-based colorimetric assays or GM1-based biosensors (Archibald et al., 2015; Jin et al., 2013; Khan et al., 2015; Rowe-Taitt et al., 2000a). Most of these methods, however, require specialized and expensive equipment.

Aptamers are currently emerging as alternative recognition elements, often in similar applications as antibodies. These single-stranded DNA- or RNA sequences are able to form defined three-dimensional structures and are thus capable of highly affine and selective interactions with their respective target molecules (Chen and Yang, 2015).

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Immobilized on magnetic beads (MBs), columns or in microfluidic devices, they are used as capturing agents in diagnostics or for target purification (Citartan et al., 2016). Aptamers have been selected against a range of bacterial toxins, including *Clostridioides difficile* binary toxin, toxin A and toxin B, anthrax protective antigen, botulinum neurotoxin type A light chain and staphylococcal enterotoxin B (Chang et al., 2016; Choi et al., 2011; DeGrasse, 2012; Ochsner et al., 2013). Bruno and Kiel (2002) developed aptamers against cholera toxin, but neither sequences were disclosed nor dissociation constants (K_D) determined.

In this study, we applied the recently in-house developed *just in time*-selection to identify aptamers that bind to CT-B with high affinity and selectivity. This method has already been used successfully to identify aptamers against food allergens and small molecules (Fischer et al., 2016a; Fischer et al., 2016b; Hünniger et al., 2014). *Just in time*-selection is a semi-automated two-step SELEX protocol. During the first step, which is called FISHing, aptamer pool incubation with target-coated MBs, washing and aptamer elution are performed in an automated way using a robotic magnetic separator. The magnetic separator uses a magnetic comb to move the MBs in a deep well plate and finally into a heatable elution strip. In comparison to the conventional method, this holds the advantage of the hands-on time and risk of contamination being greatly reduced and of the possibility to select against up to 12 targets in parallel (Hünniger et al., 2014). Amplification of the aptamer pool and strand separation are accomplished during the second step called BEAMing. BEAMing comprises an emulsion PCR (emPCR) in which the reverse primers are covalently coupled to MBs. DNA is amplified in small compartments in an emulsion, with each compartment containing only one to few ssDNA templates. In comparison to conventional PCR, emPCR has the advantage of reducing PCR bias and minimizing the occurrence of PCR by-products (Schütze et al., 2011; Shao et al., 2011). The use of reverse primer-MBs simplifies the strand separation. After the emulsion is broken following PCR, the enriched aptamer pool can be eluted directly from the reverse primer MBs by applying heat while the reverse strand remains covalently coupled. A time-consuming enzymatic digestion or costly strand separation using streptavidin-coated MBs as it is usually done in SELEX can hence be omitted.

After 12 rounds of selection, we identified eight unique aptamer sequences of which 5 shared a large common sequence motif. All aptamers were characterized concerning their target affinity. One representative of the common motif family and the three other aptamers were tested for cross-reactivity towards other proteins and one aptamer was finally selected to develop an enzyme-linked aptamer assay (ELAA). After thorough assay optimization, the assay was successfully applied to detect CT-B from spiked tap water.

2. Materials and methods

2.1. Expression of and purification CT-B

CT-B was produced as a monomer containing an amino-terminal 6-His-tag (amino acids 22–124 according to Acc. No. **AND74811.1**). For this, 50 µl of chemically competent *Escherichia coli* BL21 cells were thawed on ice, mixed with 0.08 µg plasmid DNA containing VC-CTBX gene (Genscript, Piscataway, NJ, USA) in sterile water and incubated on ice for 30 min. Cells were heat shocked for 20 s at 42 °C and cooled for 1 min on ice. One milliliter of Luria-Bertani (LB) broth was added and incubated under mild shaking for 30 min at 37 °C. Cells were then spread on LB agar plates containing 100 mg/l ampicillin and incubated o/n at 37 °C.

For protein expression, 100 ml of LB broth containing 100 mg/l ampicillin were inoculated with a single colony and incubated o/n at 37 °C under mild shaking. This pre-culture was used to inoculate a larger amount of LB broth containing 100 mg/l ampicillin, which was then again incubated at 37 °C under shaking until an optical density at 600 nm (OD_{600}) of ~0.5 was reached. Flasks were transferred to 16 °C and 2 mM IPTG was added after 30 min of shaking to induce protein expression. After 40 h, cells were pelleted for 30–40 min at 4000 rpm and 4 °C, washed with 0.9% (w/v) saline and pelleted again.

Cell pellets were resuspended in buffer A containing 50 mM Tris-HCl, pH 8, 500 mM NaCl, 15 mM imidazole, 15% glycerin (v/v) and 0.02% NaN_3 (w/v) and cells were disrupted using a French press (Constant Systems Limited, Low March, UK). After centrifugation (1 h, 15000 rpm, 4 °C), the protein was purified from the supernatant by affinity chromatography using a Ni-NTA column with a ÄKTAPrime plus system (GE Healthcare, Little Chalfont, UK) and an imidazole gradient from 15 to 800 mM. Protein fractions were visualized on self-casted 15% Tris-glycine polyacrylamide gels by SDS-PAGE with Coomassie staining. To ensure the highest possible purity of CT-B, the fractions with highest protein content at the expected size and least impurities were again purified using Size-Exclusion Chromatography (Superdex200, GE Healthcare) in 50 mM Tris-HCl, pH 8, containing 500 mM NaCl and 0.02% NaN_3 (w/v). Purity of the obtained fractions was again checked by SDS-PAGE. The purest fractions were concentrated using Amicon Ultra 5 centrifugal units (Merck KGaA, Darmstadt, Germany) and stored at 4 °C until further use.

2.2. Verification of identity and homogeneity

In order to verify the identity and homogeneity of the expressed protein, Western Blot and Dynamic Light Scattering (DLS) were performed.

2.2.1. Western blot

Western Blot was performed principally as previously described (Towbin et al., 1979). Briefly, proteins were separated by SDS-PAGE on 15% self-casted Tris-glycine polyacrylamide gels and blotted onto a nitrocellulose membrane (Merck) for 2 h at 120 mA. After drying, the membrane was blocked twice for 15 min with blocking and washing buffer (BWB; 50 mM Tris-HCl, 150 mM NaCl, 0.5% Tween 20, pH 8) and incubated for 2 h with primary anti-CT antibody (1:2000 in BWB; Sigma-Aldrich, St. Louis, Missouri, USA). After washing with BWB three times for 5 min, the membrane was incubated with HRP-conjugated secondary antibody (1:20000 in BWB: Agilent, St. Clara, CA, USA). Color was developed after another three washes by addition of 3,3′,5,5′-tetramethylbenzidine in ethanol/citrate buffer (50 mM citric acid monohydrate, 100 mM Na_2HPO_3, pH 4.5) and 0.05% H_2O_2 (v/v). The reaction was stopped by rinsing the membrane with demineralized water.

2.2.2. DLS

A solution of 1 mg/ml CT-B was analyzed by DLS measurements via a Specrolight 300 instrument (XtalConcepts, Germany) to determine its dispersity and the respective hydrodynamic radius (Rh) values in order to verify its pentameric conformation and homogeneity. In a quartz glass cuvette, 15 µl of sample solution in 50 mM Tris-HCl, 500 mM NaCl, 0.02% NaN_3 (w/v) at pH 8.0 were exposed to a 120 mW red-light laser (660 nm). Light scattered by the particles in solution is detected at an angle of 90°. The scattering intensity is autocorrelated after short periods of time in order to determine the decay time distribution and the corresponding diffusion coefficient using the CONTIN algorithm (Provencher, 1982). Finally, the Stokes-Einstein equation allows calculating Rh. A series of 20 measurements each with a length of 20 s was recorded and accumulated.

2.3. SELEX library and primers

Unmodified, biotinylated and amine-C12-modified DNA oligonucleotides were purchased from Invitrogen (Invitrogen, Carlsbad, USA) and amine-C6-modified oligonucleotides were purchased from Sigma-Aldrich. The sequence of the library was: 5‘-CATCCGTCACACCTGCTC-(N_{40})-GGTGTTGGCTCCCGTATC-3“. The forward primer was 5”CATC