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In vitro selection and characterization of DNA aptamers recognizing chloramphenicol

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ABSTRACT

Chloramphenicol (Cam), although an effective antibiotic, has lost favour due to some fatal side effects. Thus there is an urgent need for rapid and sensitive methods to detect residues in food, feed and environment. We engineered DNA aptamers that recognize Cam as their target, by conducting *in vitro* selections. Aptamers are nucleic acid recognition elements that are highly specific and sensitive towards their targets and can be synthetically produced in an animal-friendly manner, making them ethical innovative alternatives to antibodies. None of the isolated aptamers in this study shared sequence homology or structural similarities with each other, indicating that specific Cam recognition could be achieved by various DNA sequences under the selection conditions used. Analyzing the binding affinities of the sequences, demonstrated that dissociation constants (K_d) in the extremely low micromolar range, which were lower than those previously reported for Cam-specific RNA aptamers, were achieved. The two best aptamers had G rich (>35%) nucleotide regions, an attribute distinguishing them from the rest and apparently responsible for their high selectivity and affinity ($K_d \sim 0.8$ and 1 μM respectively). These aptamers open up possibilities to allow easy detection of Cam via aptamer-based biosensors.

1. Introduction

Veterinary drugs are often administered to farm animals for therapeutic and prophylactic purposes (Prescott, 2008; Winckler and Grafe, 2001). However, only a small percentage of the ingested antibiotics are metabolized by the animals, leaving a significant proportion to be either accumulated in their tissues or excreted and emitted into the environment (Boxall, 2010). The occurrence of antibiotics in the environment endorses antibiotic resistance and raises food safety issues since antibiotic resistance can be transmitted to humans via the food chain (Girardi and Odore, 2008). One such antibiotic is chloramphenicol (Cam). Cam inhibits translation, by binding prokaryotic ribosomes and blocking the peptide bond formation (Jardetzky, 1963). Although an effective antimicrobial drug, Cam has lost favour due to serious side effects such as aplastic anemia (Burton et al., 1988), leukaemia (Smith et al., 2000) and gray baby syndrome (Mulhall et al., 1983). To protect consumer health, maximal residue limits of veterinary medicinal products in foodstuffs of animal origin have been established according to European Union regulation (Regulation (EU) No. 37/2010). More specifically, in the case of Cam, it is a forbidden substance, and a minimum required performance limit of 0.3 μg/kg (Decision 2002/657/EC) has been designated, in particular as a decision limit for products coming from outside the European Union.

For fast and sensitive detection of Cam residues in food, feed and environment, chemically analytical methods do exist (Gantverg et al., 2003), but they have limitations in terms of costs incurred by the sophisticated equipments and the highly trained personnel required. The conventional affinity-based assays, which use enzymes or antibodies as recognition elements (Fodey et al., 2007), are more cost-effective, but lag in terms of detection time. Thus, there is a pressing need for rapid, accurate and economic alternative recognition elements. Aptamers fulfil these requirements and have therefore emerged as a viable option. Aptamers are artificial nucleic acids ligands (DNA or RNA) able to specifically recognise a given target, ranging from small molecules (Mann et al., 2005) to whole cells (Cerchia and de Franciscis, 2010). The name *aptamer* derives from the Latin word *aptus* meaning *to fit*, and the Greek word *meros* meaning *part* or *portion*, referring to the folding properties of single-stranded nucleic acids, responsible for their specific three-dimensional structures. This flexibility of aptamers results in

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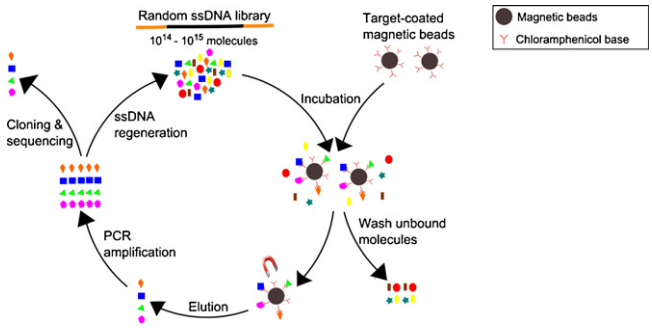

Fig. 1. Aptamer selection procedure (SELEX).

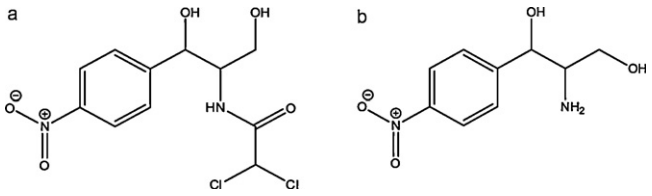

Fig. 2. Chemical structures of (a) chloramphenicol (Cam) and (b) Cam-base – the precursor to Cam, which was used for immobilization purposes.

their high affinity and specificity for their targets. Aptamers, also known as 'chemical antibodies' (Song et al., 2008), are selected from combinatorial libraries of synthetic nucleic acid which can contain more than 10^{15} different sequences (Ellington and Szostak, 1990). They are commonly evolved *in vitro* via a selection procedure known as Systematic Evolution of Ligands by EXponential enrichment (SELEX) (Tuerk and Gold, 1990). This iterative process consists of binding and elution steps of ligands in contact with the molecule of interest and amplifying (via PCR) the resulting ligands (Fig. 1). In this application of nucleic acids in biotechnology, the recognition is based on the three-dimensional structure of the aptamer, more than in its sequence. An advantage of aptamers is that they avoid the use of animals for their production, which additionally enables them to be generated against toxic targets. Thus, they are innovative, animal-friendly alternatives to antibodies, making them more appealing and ethical. They are also cost-effective, reproducible and can be produced faster than antibodies (Van Dorst et al., 2010). An added benefit is their superior stability which makes them more suited for measuring targets in complex food and environmental matrices.

In this study, we engineered specific DNA aptamers that bind Cam with high affinity. To our knowledge, there are no reports on Cam-specific DNA aptamers and we present here the first results of the *in vitro* selections of novel single-stranded DNA (ssDNA) aptamers that bind to Cam with high molecular recognition. In the past, Cam-specific RNA aptamers had been isolated mainly to study RNA–antibiotic interactions (Burke et al., 1997). However, DNA aptamers have the advantage of being more stable than RNA aptamers which are susceptible to nuclease attack, and additionally, they can be isolated faster than RNA aptamers since the steps of transcription and reverse transcription are not required in the SELEX procedure. Thus, given the unprecedented advantages brought by DNA aptamers, the highest binding aptamer(s) selected in this study could be incorporated as recognition elements in aptamer-based biosensors to allow easy detection of Cam. This can find broad applications in environmental and food monitoring.

2. Materials and methods

2.1. DNA library and primers

The DNA library and PCR primers were custom-synthesized and HPLC purified by Eurogentec (Belgium). The random DNA library (5′- AGCAGCACAGAGGTCAGATG -N40-CCTATGCGTGCTACCGTGAA-3′) contained a central randomized sequence of 40 nucleotides (N40) flanked by 20-nucleotide constant regions (Mendonsa and Bowser, 2004). The set of primers that was used for the library were: – forward: 5′- AGCAGCACAGAGGTCAGATG-3′ and reverse: 5′-TTCACGGTAGCACGCATAGG-3′. Biotin labelled 5′reverse primer was used in PCR reactions for the synthesis of biotin-labelled double-stranded DNA (dsDNA) products in order to enable strand separation via streptavidin beads. The library and primers were dissolved in Milli-Q water (Millipore, Belgium) to get appropriate template concentrations and stored at −20 °C.

2.2. Immobilization of antibiotics onto magnetic beads

Tosylactivated dynabeads M-280 (Invitrogen, Belgium) were used as immobilization supports and were handled according to the manufacturer's protocol. For the immobilization process we used D-threo-(1R, 2R)-1-p-Nitrophenyl-2-amino-1, 3-propanediol (Cam-base) (Sigma–Aldrich, Belgium), which is the precursor to Cam (Fig. 2). Briefly, 4 mg of Cam-base was dissolved in 1 mL of 0.1 M borate buffer, pH 9.5, and was incubated to react overnight with approximately 2×10^9 tosylactivated beads by gentle rotation and tilting at 37 °C. The unreacted tosyl groups on the beads were then blocked with a Tris containing buffer (0.2 M Tris, pH 8.5). The beads were washed, resuspended in PBS buffer and stored at 4 °C until use. A separate aliquot of magnetic beads coated only with Tris, was also prepared for counter selection steps.

The Cam coupling was qualitatively verified by infrared (IR) spectroscopy by obtaining spectra of the target itself, the target-coated beads and the Tris-coated beads using an ATR-FT IR spectrometer (Vector 22, Bruker). The IR spectra were recorded at a resolution of 4 cm^{-1} and were averaged over 100 scans. The unbound targets were quantitatively estimated by UV–vis spectrophotometry by using a nanodrop (ND-1000 Spectrophotometer, Nanodrop Technologies, Inc.). The bound target concentration was calculated as the difference of initial and unbound concentrations by measuring the solutions at a wavelength of 278 nm.

2.3. In vitro selection of aptamers for Cam

Cam-specific aptamers were selected via an affinity selection procedure illustrated in Fig. 1. For each SELEX round, 1×10^8 coated beads were used. A homogeneous suspension of the target-coated beads was made by vortexing for approximately 1 min. Before each SELEX round, the beads were washed 8 times in binding buffer (BB) (100 mM NaCl, 20 mM Tris–HCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, 0.02 Tween 20, pH 7.6) and finally resuspended in 100 μL of BB. For the first round of SELEX, 36 μg of DNA library was added to 400 μL of BB. Before its application in the binding reaction, the ssDNA library was denatured (unfolded) in BB at 90 °C for 10 min, immediately cooled at 4 °C for 15 min, followed by a 5 min incubation at room temperature (RT). Finally the ssDNA solution (400 μL) was added to the target-coated beads solution (100 μL) and incubated at RT for 30 min with tilting and rotation. The unbound oligonucleotides were removed by five washing steps using 500 μL BB each time. The bound oligonucleotides were eluted from the Cam-coated magnetic beads, by incubating the target-DNA complex in 200 μL elution buffer (EB) (40 mM Tris–HCl, 10 mM EDTA, 3.5 M urea, 0.02% Tween 20, pH 8.0) at 80 °C for 10 min with gentle shaking, followed by magnetic separation of beads and ssDNA recovery. This process was repeated four times in order to retrieve