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Aptamer-based trapping of phytosphingosine in urine samples

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ABSTRACT

Usually, small molecules like single metabolites used in clinical diagnostic can be quantified by instrumental approaches like LC–MS or bioanalytical techniques using antibodies or aptamers as selective receptors. The present work comprises the generation of aptamers with an affinity towards the medically relevant metabolite phytosphingosine via the previously reported *just in time*-Selection approach (Hünniger et al., 2014). The whole approach could be seen as a proof of concept to extend the existing *just in time*-Selection protocol for selection towards small molecules with dissociation constants in the low nanomolar range. Moreover it is conceivable that the shown methods could be quickly adapted to further scopes. Aptamers could be applied for clean-up or concentration processes prior to further analysis. As an example, we used the selected aptamers towards phytosphingosine bound to magnetic particles for affinity enrichment in both selection buffer and urine samples. As an outcome, enrichment factors of up to 9-fold (selection buffer)/4-fold (urine samples) were achieved by this approach.

1. Introduction

Various small chemical compounds like metabolites up to a molecular mass of 2000 Da are of great importance as markers in medical diagnosis (Blaas et al., 2011). For instance, metabolites or degradation products thereof are often used in therapy monitoring or as key substances to tackle the state of health. Thus, their role in analytical applications is rapidly increasing. On the other hand, the authentication of food and commodities for food production based on small marker molecules, e.g. metabolites, becomes more and more important. Single small molecules can be quantified by instrumental approaches e.g. by LC–MS or by bioanalytical techniques using antibodies or aptamers depending on (i) the chemical structure and the physical properties, (ii) the technical configuration and (iii) the education and training of the staff (Hanzlick and Gowitt, 1991; Roth and Breaker, 2009; Wink, 1999; Zhang et al., 2009). If metabolites, contaminants or residues are embedded in a complex matrix or are only present in low concentrations a clean-up and/or enrichment step using trapping techniques might be appropriate.

Aptamers are short single stranded DNA or RNA oligonucleotides, which are usually selected from a randomized oligonucleotide library via the SELEX (systematic evolution of ligands by exponential enrichment) process. Due to their single stranded conformation, they are able to form distinct three dimensional structures, which are the basis for highly affine interactions – comparable to these of antibodies – to several target molecules (Ellington and Szostak, 1990, 1992; Stoltenburg et al., 2007; Tuerk and Gold, 1990). Aptamers, once generated, can be used for a wide range of applications, like detection, sensing, labeling or trapping, of e.g. spores or molecules with different physical or chemical properties (Chi et al., 2011; Fischer et al., 2015; Hünniger et al., 2015; Kim et al., 2009; Levy et al., 2005; McCauley et al., 2003; Ng and Adamis, 2006; Savla et al., 2011).

The present work presents the suitability of the *just in time*-Selection process for small molecules. Furthermore a trapping application in urine via aptamer-linked magnetic particles was developed exemplary. So far only aptamers towards proteins gained via *just in time*-Selection were published. The present work demonstrates herein the novel proof of concept, that this selection method is also suitable for other target classes than proteins (Hünniger et al., 2014). The target chosen for the present work (phytosphingosine) is known as a medical biomarker in urine for chronic kidney disease and chronic renal failures. Thereby a manifestation includes changes in renal detoxification capacity, deregulation of salt and water balance and altered endocrine functions (Zhao, 2013; Zhao et al., 2012). Furthermore phytosphingosine is indicated as potential ovarian cancer biomarker in urine (Chen et al., 2012). Classical sphingolipid analysis is performed

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using thin layer chromatography, high-performance liquid chromatography, immunochemical and coupled mass spectrometry methods. Thus low detection limits could be achieved especially by mass spectrometry, but these technologies are in terms of costs and time highly demanding. As the concentration of biomarkers in real samples were often near the detection limits of instrumental approaches, a trapping/enrichment prior to the analysis can be useful to gain a valuable diagnostic tool.

Generally, trapping applications e.g. with antibodies are known to be important for routine analysis as they allow a rapid clean-up of samples. Considering the directive 2010/63/EU on the protection of animals used for scientific purposes, the demand for alternatives for antibodies is continuously increasing. The physical properties of aptamers (e.g. binding constants) are comparable to antibodies. Aptamer driven trapping enables the enrichment of target compounds such as pesticides, antibiotic residues, vegetative cells or spores, which could not be detected without an enrichment step which can be time consuming (Hünniger et al., 2015). The presented enrichment technique, which is based on aptamer-linked magnetic particles, offers a rapid and easy to handle application demonstrated in spiked urine samples and could be seen as proof of concept for aptamer-based biomarker enrichment in real samples.

2. Materials and methods

2.1. Selection of aptamers towards phytosphingosine

Selection of aptamers with an affinity towards phytosphingosine (Santa-Cruz Biotechnology Inc., Purity 98%) was performed using *just in time*-Selection (Hünniger et al., 2014). For defined interaction conditions the following buffer conditions were used for aptamer selection: 0.5 mM EDTA, 1 M NaCl, 5 mM Tris-HCl, pH 7.5 (Hünniger et al., 2015). The aptamer pool for the first selection round consisted of the following sequence and included a randomized part comprising of 40 bases: 5′-CATCCGTCACACCTGCTC-$(N)_{40}$-GGTGTTGGCTCCCGTATC-3′ (Thermo Fisher Scientific Inc., Darmstadt, Germany). For the first selection step, the so called FISHing, the metabolite was immobilized on the surface of carboxylated magnetic particles (SiMAG-Carboxyl, chemicell GmbH, Berlin, Germany) regarding the manufactures protocol to enable the following semi-automated steps as described elsewhere (Hünniger et al., 2014). FISHing includes both several washing respectively discarding steps and a final elution step for each round. The resulting eluate contains the enriched aptamers. This semi-automated process was performed using the KingFisher Duo (Thermo Fisher Scientific Oy, Vantaa, Finland).

During the subsequent BEAMing, the amplification of the enriched aptamer fraction takes part (Schütze et al., 2011). This step also includes a strand separation to obtain single stranded aptamers again. After 15 selection rounds the resulting enriched aptamer pool was ligated using TOPO TA cloning kit (Thermo Fisher Scientific Inc., Darmstadt, Germany) and transformed into *E. coli* XL1 cells. The plasmids were isolated using the QIAprep Spin miniprep kit (Qiagen GmbH, Hilden, Germany). Afterwards the cloned aptamers were sequenced by GATC Biotech AG (Constance, Germany) using M13 forward primers. The obtained sequences were analyzed regarding their percent identity matrix (Clustal Omega and Gentle) and secondary structures were predicted using mfold, considering the buffer specifications (Peyret, 2000; Zuker, 2003). Furthermore, the dissociation constants were determined via fluorescence assay (see below) to gain information about the interaction behavior of aptamers and target. Thereby a low dissociation constant is striven indicating a good interaction.

2.2. Characterization of selected aptamers via fluorescence assay

The target beads used for selection were also used for determination of dissociation constants using a fluorescence assay. The fluorescently labeled aptamers (labeling protocol see supplementary material (SM)) were diluted in a total volume of 90 μL to 20 nM, 40 nM, 60 nM, 80 nM, 100 nM, 200 nM, and 400 nM with binding buffer. Afterwards, 10 μL of target beads (10 mg/mL) were added to each dilution and the resulting solution was mixed and incubated for 30 min under agitation and exclusion of light. The solutions were centrifuged (10 min at 18,400 × *g*) and the supernatant was removed. The resulting pellet, containing the bound fluorescently labeled aptamers, was washed once with 100 μL binding buffer to remove unspecific bound aptamers. Afterwards, the solution was centrifuged (10 min at 18,400 × *g*), each pellet was resuspended in 100 μL ddH_2O and used directly for fluorescence measurement using the SpectraMaxx®2 (extinction 485 nm, emission: 525 nm, Molecular Devices Analytical Technolgies GmbH, Ismaning, Germany). The obtained fluorescence data were fitted nonlinear (Hill-fit) using OriginPro 9.0G and the dissociation constants were extrapolated at $V_{max}/2$.

2.3. Magnetic trapping and enrichment of phytosphingosine

First, the aptamers towards phytosphingosine (phyto-Apt-1) were linked to magnetic particles (SiMAG-Carboxyl, chemicell GmbH, Berlin, Germany) according to the manufacturerís protocol. The linked particles were washed twice and resuspended using binding buffer. To ensure that aptamers exhibit their specific three-dimensional structure, which is of prime importance for successful trapping, the aptamer-linked magnetic particles were unfolded for 5 min at 95 °C. Afterwards, the solution was cooled down quickly to 4 °C to inhibit unspecific hybridization. Subsequent trapping was performed in (i) binding buffer and (ii) four different urine samples gained from test persons at the Hamburg School of Food Science. The urine samples were buffered prior to the trapping with 5-fold binding buffer. 10 μL of the linked particles were incubated for 30 min under rotation with 1 μL phytosphingosine (100 ppm) in a total volume of 1000 μL binding buffer or buffered urine (containing 20% binding buffer). Subsequently, the reaction mixture was centrifuged (2 min at 10,000 × *g*), the supernatant was discarded, the particle pellet, containing the bound targets, was washed with 1000 μL binding buffer and resuspended in 100 μL methanol. Finally, a heat elution (5 min at 75 °C) was performed and the hot supernatant, including the targets, was transferred into a new reaction tube. The trapping technique is schematically shown in Fig. 1. The concentrations of phytosphingosine before and after trapping were analyzed by LC-ESI–MS/MS. To gain valid enrichment factors and to proof the suitability of the developed LC-ESI–MS/MS method, samples without trapping were also analyzed by LC-ESI–MS/MS.

2.4. Monitoring of phytosphingosine trapping via LC-ESI–MS/MS

Liquid chromatography was performed on a PRP™-C18 HPLC Column (5 μm, 150 mm × 2.1 mm i.d., Hamilton Company, Reno, NV, USA) at 20 °C with a flow rate of 200 μL/min using an Agilent 1260 Infinity Quarternary LC System (Agilent Technologies, Waldbronn, Germany). The mobile phase A was water and B methanol, both containing 0.1 % formic acid. The gradient elution is shown in SM Table S1. The injection volume for all samples was 5 μL. For detection a triple quadrupole-MS/MS API 2000 (Applied Biosystems, Darmstadt, Germany) equipped with a turbo ion spray source was used, operating in positive ion mode with the following mass spectrometer settings: ion spray voltage = 4500 V; temperature = 450 °C; ion source gas 1 = 50 psi; ion