Sensing Penicillin

Volz, Esther

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Chapter 2

Aptamers for biosensing of penicillin in fungal cultures: a feasibility study

Esther Magano Volz[1,2], Richard Kerkman[1], Roel A.L. Bovenberg[1,3], Matthias Heinemann[2], Günter Mayer[4]

(1) DSM Biotechnology Center, DSM Food Specialties B.V., Alexander Fleminglaan 1, 2613 AX, Delft, The Netherlands
(2) Molecular Systems Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands
(3) Synthetic Biology and Cell Engineering, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Nijenborgh 7, 9747 AG, Groningen, The Netherlands
(4) Chemical Biology and Chemical Genetics, Life and Medical Sciences Institute (LIMES), University of Bonn, Gerhard-Domagk-Strasse 1, 53121 Bonn, Germany

Abstract

Aptamers are short nucleic acids that have attracted intense interest in the development of small molecule biosensors. Aptamer-based sensors are not only able to detect a broad range of molecular structures with high affinity and selectivity but were also shown to function in complex environments such as urine or blood serum. In recent years, chemical modifications of aptamers further extended the range of molecular targets and improved aptamer stability. However, it is unknown whether aptamers can be applied to sense small molecules in microbial cell cultures. In this study, we assessed the feasibility of DNA aptamers to detect the metabolite penicillin and assessed the stability of selected aptamer libraries in cell culture samples of the penicillin-producing fungus *P. chrysogenum*. Here, we show that the selection and nuclease vulnerability of aptamers represent significant challenges to allow for aptamer-based biosensing of metabolites in microbial cell cultures. Classical aptamer selection processes did not lead to enrichment of specific penicillin aptamers, and we could not confirm aptamer binding data of an independent study. Furthermore, we found that an endonuclease causes rapid degradation of aptamer libraries in fungal cell culture samples and that chemical modifications of the libraries are unable to prevent degradation. Our results demonstrate that the selection of aptamers targeting small molecules remains a challenging research field of its own and that further improvements of the biophysical properties of aptamers are needed before they can be applied as biosensors in cell cultures.

Introduction

Aptamers are short single-stranded RNA or DNA oligonucleotides that can recognize a great variety of small molecules with high specificity and selectivity[1,2]. The specific aptamer-target interaction can be exploited for the development of biosensors, where the aptamer functions as a biological recognition element, which induces a detectable signal upon molecule binding, with the help of a reporter system[3,4]. Once an aptamer is successfully selected *in vitro*[5], a variety of biosensors can be developed depending on the type of aptamer, the target molecule, and the envisioned application. For instance, RNA aptamers are frequently used to build synthetic riboswitches, which are intracellular mRNA switches that can regulate translation of a fluorescent protein in a ligand-dependent manner[6,7]. DNA aptamers are often used for the development of diagnostics, for which they are coupled to different materials, such as gold nanoparticles[8], fluorophores[9], or electrodes[10], which allows for a quantitative colorimetric, optical or electrochemical ligand detection, respectively.

The application fields of aptamer-based small molecule biosensors are highly diverse, from monitoring of drinking water contaminates such as toxins[11] or hormones[12] to the detection and quantification of metabolites in metabolic pathways[13] or molecules associated with the energy state of cells[14]. In recent years, selection trends of small molecule aptamers shifted significantly from RNA to DNA libraries, which is likely due to more straightforward selection procedures, higher stability, and lower synthesis costs[2,15]. Furthermore, several DNA-based sensors showed sufficient specificity and stability to detect their target molecule in complex matrices, such as urine[16,17], blood serum[18,19] or milk[20,21], thereby expanding their application spectrum. At this, aptamers are often chemically modified to obtain improved biophysical properties, such as improved stability[22,23].

Aptamers can bind numerous small molecules that vary greatly in their chemical structures and properties, ranging from small molecular dyes[24] to large glycoside structures[25]. Microbial secondary metabolites, which are not essential for growth, represent an extensive collection of complex and diverse small molecules, with many commercial applications. To obtain secondary metabolites in high quantity, microorganisms are often grown in specific metabolite-producing media, where the secondary metabolite is typically secreted into the culture from which it can be harvested and processed.

Even though a small number of DNA aptamers can bind secondary metabolites[26,27], research on their application to monitor the production of secondary metabolites in microbial cell cultures is limited so far. Given the

increasing number of DNA-based small molecule sensors and the possibility to generate highly stable aptamer variants, aptamer-based biosensors could accelerate the selection of microbial strains that produce important metabolites in high quantity. To assess this opportunity, we evaluated the ability of DNA aptamers to bind a fungal secondary metabolite and further examined the stability of aptamers in fungal cultures. For this purpose, we selected the β-lactam antibiotic penicillin which is produced by the filamentous fungus *Penicillium chrysogenum*[28]. We reevaluated the binding properties of published β-lactam aptamers and performed *in vitro* selections experiments targeting penicillin. Furthermore, we examined the stability of aptamers in fungal culture samples to assess their feasibility to monitor penicillin production using an aptamer-based biosensor.

Materials and Methods

Binding analysis of published aptamer sequences

The interaction of three ampicillin aptamers[29] with ampicillin and one penicillin G aptamer[30] with penicillin G was assessed in radioactive binding assays and Micro-Scale thermophoresis experiments, respectively (Table 1). Ampicillin was immobilized to Tosyl-activated Magnetic Dynabeads M-280 (Invitrogen), as described previously[29]. The amount of immobilized ampicillin was calculated based on a UV absorbance standard curve, which was generated by dissolving ampicillin sodium salt (Sigma-Aldrich) in 0.1 M borate buffer pH 9.5 and measuring UV absorbance at 230 nm. In total 251.2 nmol ampicillin per mg magnetic bead was immobilized. Radioactive DNA labeling and binding assays were performed as described below with an incubation time of 1 h at room temperature (RT) in selection buffer. Penicillin G sodium salt (Sigma-Aldrich) was dissolved in binding buffer (0.1 M phosphate buffer, 5 mM $MgCl_2$, pH 7.5) with a concentration of 5 µM (Run 1) or 200 µM (Run 2) and was diluted 16 times in a two-fold dilution series. The Cy-5 labeled penicillin G aptamer p8 was dissolved in water, heated for 5 min at 90 °C, cooled on ice for 3 min, transferred into the binding buffer, and incubated at RT for 15 min. Immediately after incubation, equal amounts of aptamer solution were added to the dilution series to a final concentration of 25 nM. All samples were loaded into standard-treated capillaries (NanoTemper) and measured with 40% MST infrared laser power and 60% LED laser power in a Monolith NT.115 (NanoTemper).

Table 1 List of aptamers used in this study.

Aptamer	Target molecule	Sequence (5'-3')	Labeling	Characterization method	Published Kd	Ref.
AMP4	Ampicillin	CACGGCATGGTG-GGCGTCGTG	γ-^{32}P-ATP	Radioactive Binding Assay	9.4 nM	[29]
AMP17	Ampicillin	GCGGGCGGTTG-TATAGCGG	γ-^{32}P-ATP	Radioactive Binding Assay	13.4 nM	[29]
AMP18	Ampicillin	TTAGTTGGGGT-TCAGTTGG	γ-^{32}P-ATP	Radioactive Binding Assay	9.8 nM	[29]
P8	Penicillin G	GGGAGGACGAAG-CGGAAC-GAGATGTAGAT-GAGGCTCGATC-CGAATGCGT-GACGTCTATCG-GAATACTCGT-TTTTACGCCT-CAGAAGA-CACGCCCGACA	5'-Cy5	Micro-Scale Thermophoresis	3 -12 nM	[30]

Immobilization of penicillin G to sepharose beads and magnetic beads

Penicillin G was immobilized to the surface of sepharose beads, and magnetics beads in three consecutive steps and immobilization efficiencies were determined using a fluorescently labeled penicillin antibody (Figure 2). For this, a 10 mM Penicillin G sodium salt (Sigma-Aldrich) solution was prepared with ultrapure water, incubated at RT for two days, and was subsequently freeze-dried. An amide linker was coupled to the activated carboxyl-group of penicillin G as described previously[31]. Briefly, 1 eq. (mol) deprotonated Penicillin G was dissolved in dimethylformamide (DMF), mixed with 1.2 eq. (mol) 3-(Ethyliminomethyleneamino)-N,N-dimethylpropane-1-amine hydrochloride (EDC-HCl), 1.2 eq. (mol) 1-hydroxy-benzotriazole (HOBt), incubated for 60 minutes on ice, and regularly mixed by inverting. Subsequently, 1.2 eq. (mol) amide linker with a tert-butyloxycarbonyl (BOC) protection group and 0.1 eq. (mol) 4-Dimethylaminopyridine (4-DMAP) was added and incubated at RT for 1 hour. The product was purified by preparative HPLC and was subsequently freeze-dried. For deprotection of the BOC group, water was removed using a vacuum concentrator, and the dried pellet was dissolved in 200 μL 50 v% trifluoroacetic acid (TFA)/ dichloromethane (DCM) mixture and stirred for two hours at RT. BOC removal was verified with thin layer chromatography employing a DCM/methanol mixture (9:1) and ninhydrin staining of the free NH$_2$ group. All solvents were removed in a rotational evaporator with five rounds of methanol co-evaporation, and the deprotected penicillin G-linker molecules

were re-dissolved in dimethyl sulfoxide (DMSO). The deprotonation reaction of penicillin G, the coupling reaction of penicillin G to the amide linker, as well as the deprotection of the amide linker were verified by LC-MS. Subsequently, the penicillin G - linker molecules were immobilized onto Epoxy-activated-Sepharose 6B beads (GE Healthcare) or Tosyl-activated Magnetic Dynabeads M-280 (Invitrogen) following the manufacturer's instructions. Coupling was performed for 17 hours in an overhead shaker at 50 °C or at RT, using 0.1 M borate buffer pH 9.5 as coupling buffer. In parallel, beads were coupled in the same way with DMSO to obtain negative control beads.

Verification of penicillin G immobilization using a fluorescently labeled penicillin antibody

Epoxy-activated sepharose beads with and without immobilized penicillin G were quenched with 1M ethanolamine pH 8.5 for four hours at 50 °C in an overhead shaker and were subsequently washed following the manufacturer's instructions. The beads were then washed with 10 mM phosphate-buffered saline (PBS) buffer pH 7.4 containing 0.1 % Tween20 and 1 mg/mL bovine serum albumin (BSA). After incubation in an overhead shaker for 30 minutes at RT, a fluorescein isothiocyanate (FITC) labeled IgG polyclonal penicillin antibody (biorbyt;orb3450) was added to a final dilution of 1:100, and the mixture was incubated for another hour at RT in the dark. After three washing steps with 10 mM PBS, 0.1 % Tween20, the FITC emission of the wash fractions, the flow-through fractions, and the beads were measured in a fluorescent reader with an excitation wavelength of 494 nm and an emission wavelength of 520 nm.

Selection strategies

A single-stranded DNA library with a random region of N=75 nucleotides was chosen for aptamer selection (5'-GCGCCAGTCTAGGGCACC-(N75)-CATTGACTCGGTGGATCC-3'). For PCR amplification and single-strand digestion a 5'phosphorylated reverse primer (5'-Phos-GGAGGATCCACCGAGTCAATG-3') and an unmodified forward primer were used (5'-GCGCCAGTCTAGGGCACC-3'). All SELEX conditions such as selection buffer, temperature and incubation time were chosen in such a way that they mimic the environmental conditions of the final application as closely as possible. The selection buffer contained 2.2 g/L $(NH_4)_2SO_4$, 5.8 g/L Na_2SO_4, 2.38 g/L KH_2PO_4, 23.4 g/L $K_2HPO_4.3H_2O$, 5 mM $MgCl_2$, 0.1 mg/mL salmon sperm DNA, pH 6.3; supplemented with 20 mL/L ml of a trace element solution containing (in g/l): $FeSO_4$-$7H_2O$, 24.84; $MgSO_4$-$7H_2O$, 0.0125; EDTA, 31.25; C_6-

$H_6Na_2O_7$, 43.75; $ZnSO_4$-$7H_2O$, 2.5; $CaCl_2$-$2H_2O$, 1.6; $MgSO_4$-H_2O, 3.04; H_3BO_3, 0.0125; $CuSO_4$-$5H_2O$, 0.625; Na_2MoO-$2H_2O$, 0.0125; $CoSO_4$-$7H_2O$, 0.625. All buffers and solutions were prepared as stocks and were filter-sterilized.

For the negative selection step, 100 µL beads were washed twice with selection buffer before ssDNA in water was added to a final volume of 200 µL. For the binding, the bead-ssDNA-mixture was incubated for 15 min at 25 °C and mixed by pipetting every 5 min. Subsequently, beads and unbound ssDNA were separated using a single-use spin column. For the first selection round, 100 µL beads with immobilized penicillin G were washed twice with selection buffer, incubated with 200 µL ssDNA from the negative selection step for 15 min at 25 °C and mixed by pipetting every 5 min. After binding, the beads were washed 4 times for 5 min with 1 mL selection buffer at RT. Bound ssDNA was eluted in a target elution step or a heat elution step in case a target elution did not result in a PCR product. For heat elution, the beads were resuspended in 4.25 M urea with 12.5 mM EDTA in water and incubated for 5 min at 80 °C. Beads and ssDNA were separated immediately afterwards and the ssDNA was put on ice. For target elution, the beads were resuspended in selection buffer containing 5 mM penicillin G and incubated for 15 min at 25 °C. Beads and ssDNA were separated immediately afterwards and the ssDNA was put on ice.

Eluted ssDNA was recovered in an ethanol precipitation step. To 200 µL eluate, 0.5 µL glycogen, 20 µL 3M sodium acetate pH5.4 and 660 µL 100% ethanol were added and incubated at -80 °C for 20 min. Afterwards, the mixture was centrifuged at 4 °C, 14 000 rpm for 15 min and the supernatant was discarded. The tube was rinsed with 100 µL 70% ethanol and centrifuged at 4 °C, 14 000 rpm for 15 min. After removal of the supernatant, the sample was incubated at 95 °C for 2 min with open lid and subsequently dissolved in 200 µL water. Recovered ssDNA was amplified in a PCR reaction applying a denaturation temperature of 95 °C for 30 s, an annealing temperature of 50 °C for 30 s and an elongation temperature of 72 °C for 60 s. After eight PCR cycles, PCR progress was analyzed on a 4 % agarose gel. In case no PCR product was visible, four more cycles were added before the PCR progress was analyzed on a 4 % agarose gel until a maximum number of 24 cycles was reached. After PCR clean-up, ssDNA was generated by incubating dsDNA with 5.66 µL 10x lambda exonuclease buffer (ThermoFisher) and 1 µL lambda exonuclease (ThermoFisher) per 50 µL eluate at 37 °C for 1 h. Digestions progress was analyzed on 4% agarose gel, and digestion was continued in case ssDNA and dsDNA were visible. ssDNA was purified using a NucleoSpin Gel and PCR Clean-up kit with NTC buffer (Macherey-Nagel). Depending on

the results of the radioactive binding assay, 7 -10 rounds of selection were performed. To increase selection pressure during the selection progress, either the number, volume or time of washing steps was increased, or the amount of ssDNA during the binding step was decreased. A summary of all the selection parameters is shown in Table 2.

Table 2 Overview of selection conditions of SELEX experiments conducted in this study

| Beads | SELEX Round | ssDNA [pmol] | Wash conditions | | | Elution |
			Number of cycles	Volume [mL]	Incubation time [min]	
	1 - 5	75	4	1	5	Heat
	6	7.5	4	1	5	Heat
Epoxy-activated sepharose	7	7.5	4	1	6	Heat
	8	7.5	5	1	5	Heat
	9	7.5	5	1	6	Heat
	10	7.5	5	1	7	Heat
	1	150	2	0.2	1	Heat
	2	13	2	0.2	1	Heat
	3	13	3	0.2	10, 5, 5	Heat
Tosyl-activated magnetic beads	4	13	2	0.2	10	Heat
	5	13	2	0.2	10	Heat
	6	13	3	0.2	10	Target
	7	12	3	0.2	10	Target

Radioactive binding assays

The amount of eluted ssDNA to beads with and without immobilized penicillin G was quantified in radioactive binding assays. For this, 10 pmol ssDNA were mixed with 10 pmol γ-^{32}P-ATP and 20 U polynucleotide kinase (NewEngland Biolabs) in 1-fold PNK reaction buffer (NewEngland Biolabs) and incubated at 37 °C for 1 h. After 1:2 dilution with distilled water, the mixture was purified with desalting spin columns (GE Healthcare). After a second purification with a NucleoSpin Gel and PCR Clean-up kit using NTC buffer (Macherey-Nagel), radioactive labeling was verified with a 10% denaturing PAGE gel. 100 µL beads w/o immobilized penicillin G were washed twice with selection buffer and incubated with 1 pmol γ-^{32}P-DNA for 15 min at 25 °C and mixed by pipetting every 5 min. Subsequently, the beads were washed three times with 100 µL selection buffer, and the flow-through fraction, the three washing fractions, and the bead fraction were analyzed in a scintillation counter detecting Cherenkov

radiation. The detected Vavilov-Cherenkov radiation (VCR) of the supernatant collected after the incubation step (fraction I), the three wash fractions (fraction II-VI) and the bead fraction after washing (fraction V) were used to determine the percentage of bound ssDNA using the following formula:

$$\text{Bound DNA [\%]} = \frac{VCR_{\text{fraction V}}}{\Sigma\ VCR_{\text{fraction I-V}}} \times 100$$

DNA stability studies and proteomics analysis

The stability of ssDNA libraries in fungal fermentation supernatant was assessed in stability experiments.

DNA libraries with a random region of N=30 were ordered with different 3' and 5' end modifications[23]. Besides an unmodified library (UM), a library with five phosphorothioated DNA bases on both ends (PS; 5'-G*G*A*G*G*CTCTCGGGACGAC-(N30)-GTCGTCCCGATGCTGCAAT*C*G*T*A*A-3') and a library with five phosphorothioated-2'-O-methyl DNA bases on both ends (PSM; 5'-mG*mG *mA*mG*mG*CTCTCGGGACGAC-(N30)-GTCGTCCCGATGCTGCAATmC*mG*T*mA*mA-3') were purchased. All libraries had a total length of 72 bp. An industrial relevant strain of *Penicillium chrysogenum* was inoculated in a glucose-limited defined medium for the production of penicillins containing the following reagents in g/l: glucose, 5.0; lactose, 36; urea 4,5; Na_2SO_4, 2,9; $(NH_4)_2SO_4$, 1,1; K_2HPO_4, 4,8; KH_2PO_4, 5.2; supplemented with 10 ml of a trace element solution containing (in g/l): $FeSO_4$-$7H_2O$, 24.84; $MgSO_4$-$7H_2O$, 0.0125; EDTA, 31.25; C_6-$H_6Na_2O_7$, 43.75; $ZnSO_4$-$7H_2O$, 2.5; $CaCl_2$-$2H_2O$, 1.6; $MgSO_4$-H_2O, 3.04; H_3BO_3, 0.0125; $CuSO_4$-$5H_2O$, 0.625; Na_2MoO-$2H_2O$, 0.0125; $CoSO_4$-$7H_2O$, 0.625. The solution was adjusted to pH 6.3. The strain was grown in a shaking incubator at 280 rpm, 25 °C. After three and seven days of fermentation, 1 mL sample was taken, centrifuged at 11000xg for 5 min and the supernatant was stored at -20 °C until further use. 7 µL DNA library (10 µM) were mixed with 63 µL medium or fermentation supernatant and incubated at 25 °C, 1000 rpm. After 0, 15 and 30 min, 20 µL sample was taken, incubated at 95°C for 5 min to denature all proteins and diluted with 13,4 µL water and 6,6 µL 6x loading dye. ssDNA stability in fermentation supernatant was analyzed in a high-throughput gel electrophoresis system (Caliper LifeSciences) using a 5k DNA chip (LabChip GXII).

Furthermore, the fermentation samples were analyzed regarding their protein content by LC-MS/MS after enzymatic protein digestion with Trypsin. All LC-MS/MS results were processed using Proteome Discoverer 1.4 Sequest

HT (Thermo Fisher Scientific, San Jose, CA, USA). Sequest HT was set up using a *P. chrysogenum* database, including protein sequences of BSA, Trypsin, and a Uniprot database containing all validated protein sequences of bacteria and fungi ("UniprotSprot100_bacteria_fungi"). Only proteins with at least two unique peptides were considered for confident identification.

Results and Discussion

To assess the feasibility of DNA aptamers to detect the secondary metabolite penicillin in cultures of its fungal production host *Penicillium chrysogenum*, we (i) reevaluated binding of published β-lactam aptamers to penicillin G[30] and ampicillin[29], (ii) strived to select new aptamers that bind penicillin G in fungal growth medium using SELEX and (iii) assessed the stability of aptamer libraries in fungal culture samples.

Target-binding of published aptamers could not be reproduced

Aptamers from two different studies were selected to reevaluate their interaction with β-lactam antibiotics. First, we assessed the binding properties of aptamers that were selected with beads carrying immobilized ampicillin on their surface[29]. Since the ampicillin molecules were immobilized to the beads via the amine group, we presumed that aptamers selected with those beads could bind not only ampicillin but also penicillin G, due to the high structural similarity of the molecules after immobilization (Figure 1A). We immobilized ampicillin to beads as described previously[29] and assessed the binding of three ampicillin aptamers to those beads in selection buffer using radioactive binding assays. Here, we found that the aptamers bind to a similar or better extent to beads without ampicillin then to beads with ampicillin (Figure 1B). Since those findings indicate that the chosen aptamers do not even bind to ampicillin in the tested conditions, we did not further assess their ability to bind penicillin G.
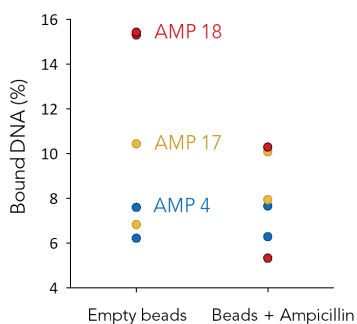
   Next, we evaluated the binding properties of a penicillin G aptamer that was selected using a SELEX approach in which the molecule is present in solution[30] and hence maintains all of its functional groups for interaction with the aptamer. The interactions of the Cy-5 labeled penicillin G aptamer to a concentration range of penicillin G in phosphate buffer were assessed in Micro-Scale Thermophoresis experiments (MST). Also, here, we did not observe a clear binding pattern and were not able to calculate a binding

constant (Figure 1C). Thus, we could neither reproduce target-binding data for the ampicillin aptamers nor the penicillin aptamer.

A   Chemical structures of ampicillin and penicillin G
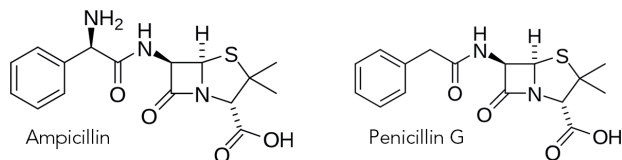
B   Binding assay ampicillin aptamers   **C**   Interaction analysis penicillin G aptamer
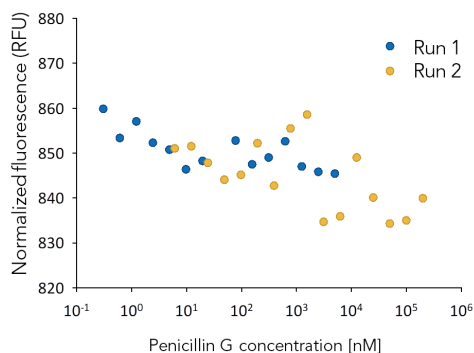

Figure 1 Binding analysis of published β-lactam aptamers to ampicillin and penicillin.
A) Molecular structures of ampicillin and penicillin G. B) Radioactive DNA binding assay of three selected ampicillin aptamers (AMP4, AMP17, AMP18). The percentage of DNA binding to beads with ampicillin and without ampicillin (empty beads) is shown. Data of two technical replicates is shown. C) Interaction of the Cy5-labeled p8 aptamer with penicillin G in two Micro-Scale Thermophoresis experiments (Run1, Run2). Data of one replicate per run is shown.

Since both, the radioactive binding assay[32], as well as MST[33], are highly sensitive methods to determine aptamer-ligand interactions, we do not expect that our chosen characterization methods are responsible for the observed lack of reproducibility. One explanation for the non-binding of the ampicillin aptamers could be the difference in buffer compositions. While selection and binding analysis of the ampicillin aptamers was initially performed in Tris-buffer[29], we used a highly complex fungal growth medium to mimic the final application environment of fungal culture. Since aptamer folding depends on several environmental conditions such as pH and ion content of the buffer, differences in binding results due to changes in buffer composition are not

unlikely[34,35]. However, an influence of buffer composition can be primarily excluded in case of the penicillin G aptamer since all binding studies were performed in 0.1M phosphate buffer containing 5 mM $MgCl_2$[30]. Even though Cy-5 labels are commonly used to characterize aptamer-ligand interactions using MST[33,36], one explanation for the observed non-binding of the penicillin G aptamer could be that the Cy-5 label attached to the 5'-end of the ssDNA influences aptamer folding and thereby prevents target binding. Since the selected aptamers did not exhibit binding in our studies, they were not considered for the further development of biosensors for the detection of β-lactam antibiotics in fungal cultures.

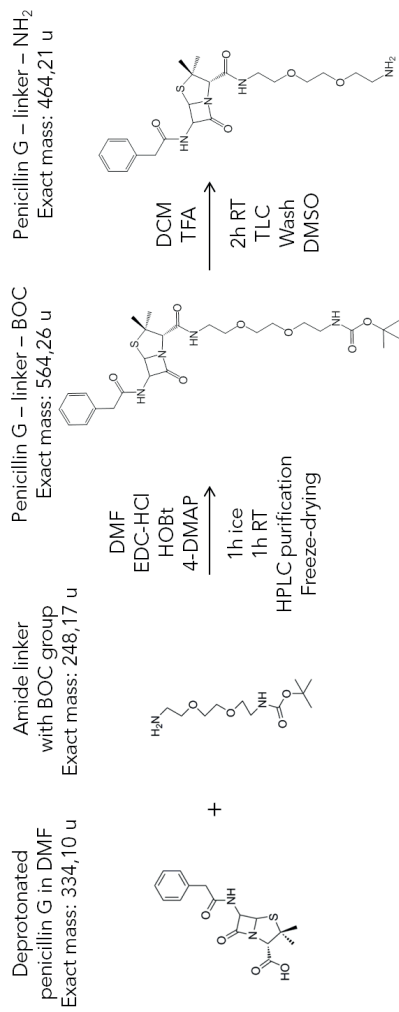## Non-specific DNA enrichment in SELEX experiments

An *in vitro* selection technique named SELEX was applied[5] to select for aptamers that bind to penicillin G in fungal growth medium. Briefly, an extensive library of oligonucleotides is exposed to beads that carry the target molecule penicillin G on their surface. Oligonucleotides that do not interact are removed by washing. Subsequently, bound sequences are eluted from the beads by heat or an excess of penicillin G and amplified by PCR to start the next selection round. In each round the selection stringency is increased by altering binding, wash or elution conditions (Table 2) to finally enrich for individual aptamers that bind penicillin with high affinity. The progress of DNA enrichment is analyzed after different selection rounds by calculating the percentage of eluted DNA that binds to the beads in radioactive binding assays.

First, we needed to immobilize penicillin G to the surface of selection beads. For this, a new workflow was generated in which we first coupled an amide linker to penicillin G to improve the spatial availability of penicillin G on the bead surface (Figure 2A), before we attached the penicillin-linker-molecule to either epoxy-activated sepharose beads or tosyl-activated magnetic beads and assessed the penicillin G immobilization efficiency using a fluorescently labeled penicillin antibody (Figure 2B).

Here, we found an increase in fluorescence when penicillin G was immobilized at 50°C compared to control beads or coupling at room temperature (Figure 3A). Compared to sepharose beads, magnetic beads showed a higher initial fluorescence level for the control beads and a smaller absolute increase when penicillin G was immobilized.

Next, two SELEX experiments were performed with either sepharose beads or magnetic beads to which penicillin G was immobilized at 50°C. An increase in bound DNA with an increasing number of selection rounds was observed

A Chemical modification of penicillin G with an amide linker arm

Deprotonated
penicillin G in DMF
Exact mass: 334,10 u

+

Amide linker
with BOC group
Exact mass: 248,17 u

Penicillin G – linker – BOC
Exact mass: 564,26 u

DMF
EDC-HCl
HOBt
4-DMAP
→
1h ice
1h RT
HPLC purification
Freeze-drying

Penicillin G – linker – NH₂
Exact mass: 464,21 u

DCM
TFA
→
2h RT
TLC
Wash
DMSO

B Immobilization of penicillin G to selection beads

Epoxy-activated
sepharose beads

Tosyl-activated
magnetic beads

H₃BO₃
→
17 h
50 °C / RT

Penicillin G immobilized to
beads with an amide linker

Verification of immobilization with
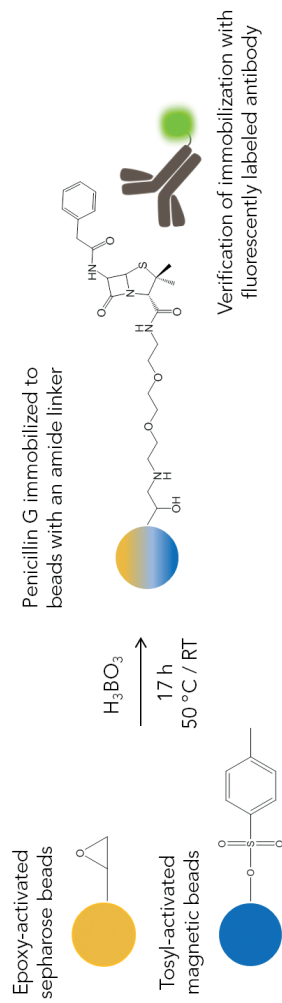fluorescently labeled antibody

Figure 2 Schematic overview of a new workflow for the immobilization of penicillin G to the surface of selection bead. A) Chemical modification of penicillin G with an amide linker arm in three consecutive steps, including HPLC purification and removal of the BOC group. B) Coupling of the modified penicillin G to two different selection beads and analysis of the immobilization with a fluorescently labeled penicillin antibody.

for the SELEX performed with sepharose beads, whereas no trend was seen for the magnetic bead SELEX (Figure 3B,C). However, we found that DNA did not only bind to beads with penicillin G but to a considerable amount also to empty beads in both experiments.

Consequently, the selection with sepharose beads resulted in a non-specific enrichment of DNA, meaning that a fraction of enriched DNA species binds to empty beads and beads with immobilized penicillin G. In the SELEX with magnetic beads, no DNA enrichment was observed. The lack of DNA enrichment for the magnetic bead selection could be explained by a lower amount of immobilized penicillin G compared to sepharose beads, as indicated by the antibody assay. This is likely since sepharose beads have a notably higher density of active chemical groups (19-40 µmol/mL) than magnetic beads (1-2 µmol/mL) and thus allow to immobilize more penicillin G on their surface. One explanation for the non-specific DNA enrichment during the sepharose SELEX could be that the penicillin G molecule does not display sufficient functional groups after immobilization via the carboxyl group to allow for a strong structural DNA recognition. As a consequence, the DNA-penicillin interaction is not strong enough to completely differentiate from the DNA-bead interaction. The lack of epitopes and functional groups that allow for stronger aptamer binding is a well-known challenge during the selection of aptamers for small molecules[1,15].

Consequently, we found that (i) penicillin G can be immobilized to sepharose beads using our presented workflow, that (ii) DNA is enriched after 10 rounds of selection with sepharose beads, but that (iii) the selection method chosen here does not allow to discriminate between specific penicillin G and non-specific bead binding sequences. Hence, the SELEX method applied in this study is not suitable for the selection of aptamers targeting penicillin G.

## Endonuclease causes rapid degradation of aptamer libraries in fungal culture samples

Next to specific binding to penicillin G, aptamer stability in fungal cultures would be the central requirement to exploit aptamers as penicillin biosensors to accelerate the selection of high penicillin-producing fungal strains. Therefore, the stability of DNA aptamers in fungal culture samples was assessed in parallel to aptamer binding studies. To mimic penicillin production conditions, the fungus *Penicillium chrysogenum* was grown in a defined medium and cells were separated from their supernatant after 3 and 7 days of fermentation. Subsequently, the stability of DNA aptamers was assessed by incubating one unmodified and two chemically modified aptamer libraries (Figure 4A) with

fungal culture supernatant obtained after 3 and 7 days or medium without fungal growth (Day 0) as negative control. After incubation for 0, 15 or 30 minutes, the size of the aptamer libraries was analyzed by gel electrophoresis. Here, all three aptamer libraries showed clear signs of degradation when incubated with fungal culture samples for 15 or 30 minutes, whereas no degradation was observed when incubated with medium alone (Day 0) (Figure 4B). Thus, neither the modified nor the unmodified DNA libraries are stable in fungal culture samples.
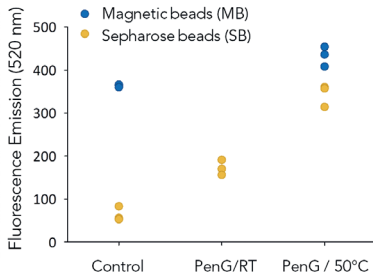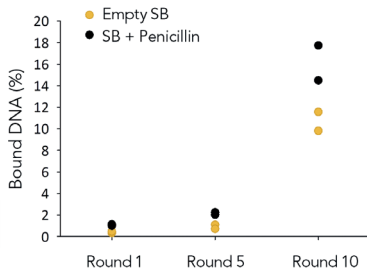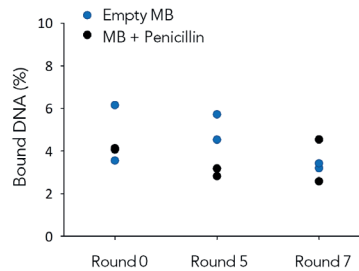
Figure 3 Selection of penicillin G aptamers by SELEX. A) Validation of penicillin G immobilization efficiency to magnetic beads (blue) and sepharose beads (yellow) using a fluorescently labeled penicillin antibody. Data of three technical replicates is shown. B) Radioactive DNA binding assay to sepharose beads with (black) and without (yellow) penicillin after 1, 5, and 10 rounds of SELEX. Data of two technical replicates is shown. C) Radioactive DNA binding assay to magnetic beads with (black) and without (blue) penicillin after 0, 5, and 7 rounds of SELEX. Data of two technical replicates is shown.

A   Chemical modification of aptamer libraries for enhanced stability
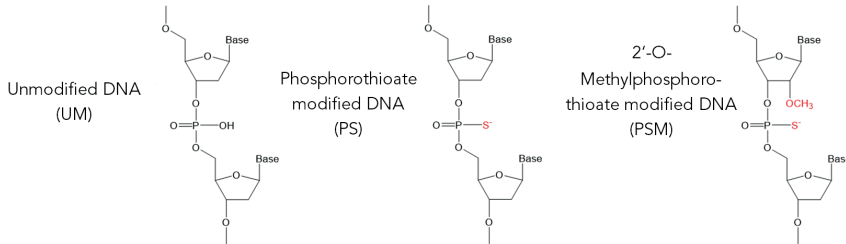
B   Stability of aptamer libraries in fermentation samples
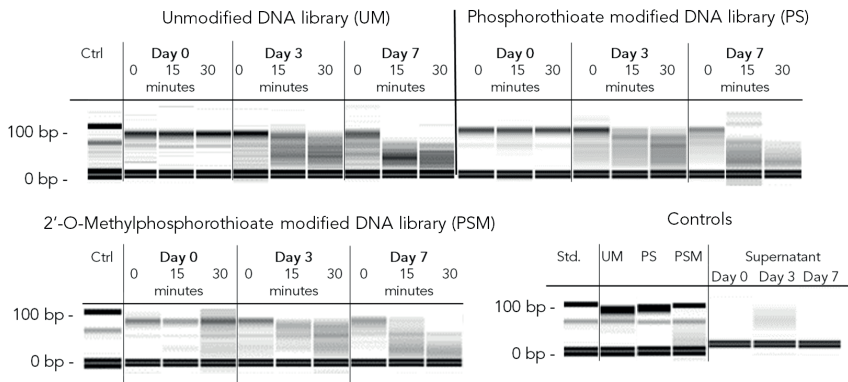
C   Nucleases found in fermentation samples

Accession	Description	Amino acids	MW [kDa]	% of total assigned proteins		Uniprot ID	Molecular function as described by Uniprot
				Day 3	Day 7		
Pc06g00430	Strong similarity to ribonuclease T2 precursor rntB – A.oryzae	257	28.2	0.22	0.12	P10281	Ribonuclease T2 activity
Pc13g09490	Strong similarity to ribonuclease TRV – T. viride	405	44.9	0.05	0.05	P24657	Ribonuclease T2 activity
Pc13g03800	Strong similarity to precursor of mitochondrial nuclease Nuc1 – S. cerevisiae	327	35.9	0.05	0.04	P08466	RNAse and DNAse activity (endo- and exo-deoxyribonuclease activity)

Figure 4 Stability assessment of chemically modified DNA libraries in fungal cultures. A) Chemical structures of DNA libraries with modifications depicted in red. B) Gel electrophoresis images of DNA libraries and controls after 0, 15 or 30 min of incubation with fungal culture supernatant obtained after 3 or 7 days of fermentation and medium without fungus (Day 0). C) List and description of nucleases found during the protein analysis of fungal fermentation supernatant samples.

To investigate the cause of the degradation further, we analyzed which proteins are present in fungal culture supernatant obtained after 3 and 7 days using a high-throughput LC-MS/MS method. We detected three proteins with strong similarity to nucleotide hydrolyzing enzymes, so-called nucleases (Figure 4C). According to the protein database UniProt, two of them have ribonuclease T2 activity, indicating that they are solely active on RNA[37]. However, the third enzyme has a strong similarity to the nuclease Nuc1, which acts on RNA and DNA as both endo[38]- and exonuclease[39]. Even though chemical end-capping was shown to increase aptamer stability by increasing exonuclease resistance[40], a significantly smaller stabilizing effect was reported for endonucleases[23]. Thus, we assume that the endonuclease Nuc1 causes the rapid degradation of DNA aptamer libraries in fungal fermentation samples, implying that DNA aptamers are not stable and cannot be chemically stabilized to function in fungal fermentation supernatant.

Conclusion and Outlook

In this study, we assessed the potential of DNA aptamers to sense penicillin in fungal cultures to determine whether aptamer-based sensors can be developed to monitor penicillin formation. To this end, we performed different experiments to first, assess the ability of DNA aptamers to bind to penicillin and second, to evaluate the stability of different aptamer libraries in fungal culture samples. The reevaluation of published β-lactam aptamers showed no target-binding in radioactive and thermophoretic binding assays, which might be attributed to the fact that different buffers were used for aptamer selection and in the binding assays[34]. Thus, our results suggest that aptamers are preferably selected directly in the conditions of the planned application and underline that a reevaluation of aptamer binding under final conditions is essential.

   To generate new aptamers that could bind penicillin in a buffered fungal culture medium, the SELEX method was applied using sepharose and magnetic beads with immobilized penicillin and an aptamer library in solution. However, no penicillin specific DNA enrichment was found under the conditions employed. Alternative selection methods, such as capture SELEX, in which the aptamer library is immobilized to beads and the molecular ligand is presented in solution[41,42] could circumvent non-specific DNA enrichment and hence represent an exciting alternative for selections targeting small molecules.

Finally, we found that standard, as well as chemically modified aptamer libraries, are degraded in fungal culture supernatant which is likely due to both endo and exonuclease activity. Measures to prevent DNA degradation in fermentation broth could include the addition of specific nuclease inhibitors[43] or the use of modified aptamers, such as locked nucleic acids[44], or nucleic acid-like synthetic polymers, such as XNA aptamers[45].

Taken together, our results show that the selection of aptamers for small molecules is a highly complex and challenging process and that current aptamer modification methods are insufficient to prevent aptamer degradation by endonucleases in fungal cultures. Furthermore, our data suggest that the selection of aptamers against β-lactam antibiotics is especially challenging due to the limited number of functional groups that are available for DNA interaction. Our feasibility assessment contributes to revealing the technical boundaries of the development and application of aptamers-based small molecule biosensors.

Acknowledgment

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