Biological Chemistry

The Diversity of a Polyclonal FluCell-SELEX Library Outperforms Individual Aptamers as Emerging Diagnostic Tools for the Identification of Carbapenem Resistant *Pseudomonas aeruginosa*

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Abstract: Textbook procedures require the use of individual aptamers enriched in SELEX libraries which are subsequently chemically synthesized after their biochemical characterization. Here we show that this reduction of the available sequence space of large libraries and thus the diversity of binding molecules reduces the labelling efficiency and fidelity of selected single aptamers towards different strains of the human pathogen *Pseudomonas aeruginosa* compared to a polyclonal aptamer library enriched by a whole-cell-SELEX involving fluorescent aptamers. The library outperformed single aptamers in reliable and specific targeting of different clinically relevant strains, allowed to inhibit virulence associated cellular functions and identification of bound cell surface targets by aptamer based affinity purification and mass spectrometry. The stunning ease of this FluCell-SELEX and the convincing performance of the *P. aeruginosa* specific library may pave the way towards generally new and efficient diagnostic techniques based on polyclonal aptamer libraries not only in clinical microbiology.

Since the introduction of single stranded nucleic acids as specific binding molecules in the early 1990s,[1,2] these so-called aptamers[3] and their laboratory evolution and selection process are a still exponentially growing research area, with more than one thousand publications in 2018 (Figure S1). Conceptually being used like antibodies or other biological binding molecules, but being selected completely in vitro and produced by solid phase synthesized (phosphoramidite method) as single stranded RNA or DNA molecules, aptamers can be regarded as a gift of biological chemistry to the sciences with an invaluable potential. Aptamers have opened new routes in diverse fields as molecular diagnostics, optical and electronics, sensor technology, as tools in molecular biology and therapeutics or as a novel class of pharmaceutical compounds in drug development. A typical SELEX (systematic evolution of ligands by exponential enrichment) process quickly reduces the sequence space (and thus complexity but also the utilizable diversity) available for target binding from $\approx 10^{14}$ sequences to one or very few individual molecules with 20 to 100 nucleotides without the need for laboratory animals or even physiological conditions in the experiments. Additional advantages lie in the broad repertoire of methods allowing simple and efficient optimization of stability, binding and functionality (e.g. fluorescent label, affinity label, etc.) already during their synthesis.[4–7] SELEX was described for metal ions, single proteins and up to the walls of whole cells as targets of truly impressive structural complexity.[8] Whereas it is straightforward to aim on the isolation of a single aptamer to specifically bind single proteins exposing only a very limited variety of epitopes this attempt virtually appears paradox for cells, exposing uncountable epitopes in at least hundreds of different surface structures including membrane proteins and lipopolysaccharides. Due to genetic mutations or physiological down-regulation this already existing intrinsic large range of target diversity in addition acquires a dynamic character factually excluding the possibility for distinct and reliable cell recognition with a single aptamer (Scheme 1).

Taking principles of the immune system into account for which nature has decided to involve the large sequence space and diversity of a polyclonal set of antibodies as binding mole-

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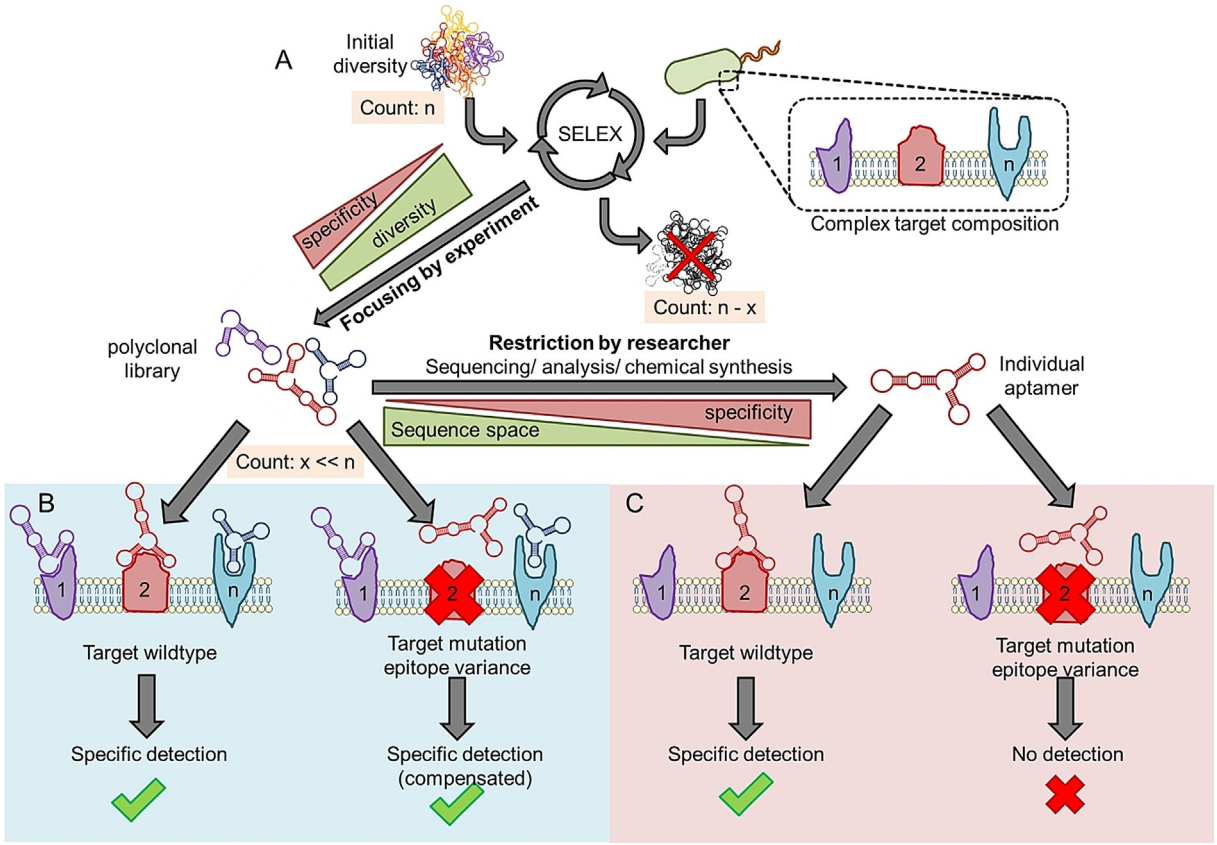
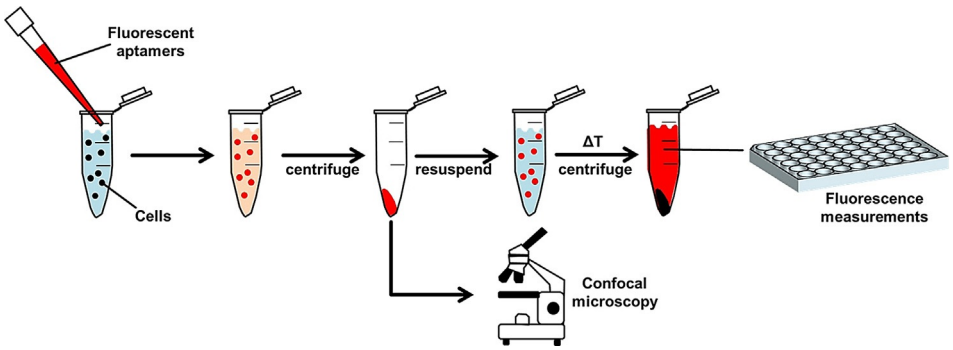


Scheme 1. The compensatory binding of a polyclonal aptamer library. A) SELEX based evolution of focused library and final reduction of sequence diversity to individual single aptamers by sequencing, analysis and synthesis. B) Robustness of overall detection by a focused library based on compensation of target shortfall by mutation or fluctuations of epitope abundance. C) Diminished overall target recognition by individual aptamers.

cules, we compared the potential of single aptamers and a polyclonal library evolved in a iterative whole cell SELEX process against *Pseudomonas aeruginosa* for the development of a fluorescence-based detection assay for this human pathogen including highly relevant multiresistant clinical isolates for the first time. Less complex (i.e. focused more strictly) libraries have been used in the context of cancer diagnostics in a way that mixtures of up to 2000 synthetic individual aptamers were used to create functional polyclonality.[9–11] Inspired by the FluMag-SELEX,[12] evolution of aptamers was followed by Cyanine 5 (Cy5) labelling of the aptamers and analysis of binding to whole cells as separable particles, a procedure we consequently decided to name the "FluCell-SELEX". Moreover, it was demonstrated that in contrast to single aptamers the library influenced virulence relevant cell functions and allowed the affinity based isolation and subsequent proteomic identification of bound prominent outer membrane proteins as targeted cell surface structures.

Based on a commercial library containing approximately 6×10^{14} individual aptamers with 40 randomized nucleotides (nt) flanked by two primer binding sites (23 nt each), a SELEX was performed over 16 rounds and the evolution progress was measured using fluorescent labelling, confocal laser scanning microscopy (CLSM) and fluorometric analysis of cell bound aptamers (Scheme 2, Figure 1 A).



Scheme 2. Combined analysis of aptamer evolved by FluCell-SELEX by confocal laser scanning microscopy (CLSM) and fluorimetry.

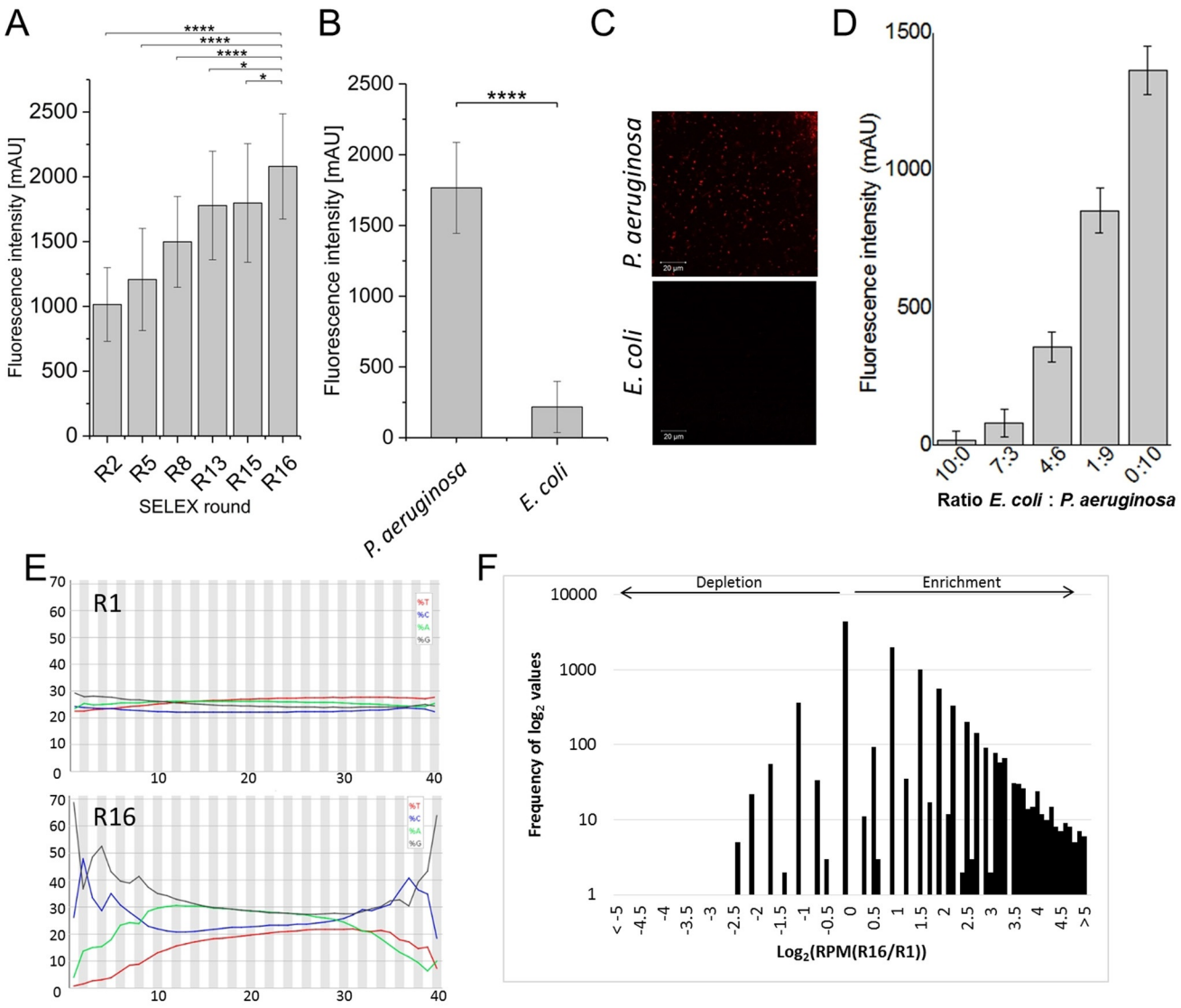


Figure 1. A) Increased binding of Cy5-labelled aptamers to *P. aeruginosa* in a FluCell-SELEX. B, C) Combined analysis of the specificity of the final polyclonal aptamer library R16 to *P. aeruginosa* cells. D) Detection of increasing amounts of *P. aeruginosa* in mixtures with *E. coli* by a Cy5 fluorescently labelled R16 polyclonal library. All experiments were performed with 2×10^8 cells and 50 nM aptamers in triplicates. The fluorescence was measured at an excitation of 635 nm and an emission of 670 nm. E) Nucleotide distribution of the library R1 and library R16. F) Sequence distribution in R16 and R1 libraries. Histogram of the relation of enrichment and depletion of individual sequences in R16 relative to the initial library R1. Statistical analysis by a t-test was performed for the enrichment of specific aptamers and the specificity of the polyclonal library R16. *P* values <0.05 were considered significant. * denotes $P<0.05$, ** <0.01, *** $P<0.001$ and **** $P<0.0001$.

The resulting library R16 after 16 rounds was suited to efficiently label *P. aeruginosa* cells, whereas *E. coli* cells as the negative control delivered only marginal signals in the combined assay (Figure 1 BC) and allowed to discriminate both bacteria mixed in different ratios (Figure 1 D). In addition R16 proved its specificity for *P. aeruginosa* also in comparison to a set of other human bacterial commensals or pathogens (Figure S2).

SELEX rounds R1 and R16 were analyzed by Illumina sequencing using individual index sequences according to the protocols by Tolle and Mayer[13] (Figure S3) resulting in 5.3 million reads, with 2.6 million reads for round one and 2.7 million reads for round 16. In R1 the nucleotide distribution was even demonstrating that the initial library was composed almost ideal with 25% of each nucleotide (Figure 1 E, upper panel), whereas the GC content was elevated in R16 indicating drastic alterations in the library composition after 16 rounds of selection (Figure 1 E, lower panel). R16 and R1 were composed of 1.8 and 2.1 million individual sequences, respectively. The enrichment profile of R16 showed a significant increase of individual sequences compared to R1 with 31 to 6 individual sequences enriched more than 10 fold (11.31 fold) ($\log_2=3.5$) to more than 32 fold ($\log_2>5$) (Figure 1 F). Subsequently the sequences were compared and ranked according to their abundance using the FASTAptamer toolbox.[14] The ten most frequent sequences were selected and estimated as relevant when clustering within a Levenshtein distance of 16 was possible. This resulted in sequence families with more members than one individual sequence. The rationale behind this is that

a sequence from a true evolutionary selection process with multiple amplifications by the Taq polymerase with an error rate of 10^{-5} for base pair substitutions and 10^{-6} for frameshift errors,[15] virtually demands to possess close sequence relatives within the family. Accordingly round R1 harbored only individual sequences, whereas round R16 among the ten most abundant sequences as lead sequences delivered eight clusters with 5 (cluster 4) up to >200 (cluster 2) individual members, which were further investigated. Again, the ten most abundant sequences within a cluster or all representatives of a cluster could be aligned using the CLC workbench alignment algorithm[16] and used to build consensus sequences for each cluster (Figure S4). Additionally, secondary structure simulations of the aligned sequences (73 in total) were run on the Mfold webserver for nucleic acid folding and hybridization prediction[17] also delivering the folding enthalpy of the putative structures as the base to judge the theoretical stability as a measure for the probability of their experimental occurrence. Seven aptamers (Figure 2A) met the criteria of: i) high stability (i.e. low enthalpy), ii) secondary structure formation predominantly within the randomized region of the aptamer, and iii) without extensive participation of bases of the primer binding sites in secondary structure.[18] Except aptamer C4R2 all chemically synthesized individual aptamers proved to be functional for specific labelling of *P. aeruginosa* cells and allowed to discriminate them from *E. coli* controls in CSLM. This failure of C4R2 in the microscopic analysis may be explained by its enthalpy close to zero and the methodological fact that in microscopy a focused laser beam is used leading to thermal unfolding of the molecule and its release from the target during analysis. This is supported by the results of the fluorescence measurements where C4R2 showed comparable binding and allowed, like the other aptamers, labelling of *P. aeruginosa* cells. In contrast to microscopy these measurements uncouple binding from analysis by heat denaturing and separation of target cells and aptamers prior to analysis (Scheme 2) and results in a fluorescence signal as a measure of the labelling efficiency for C4R2 of approximately 60% relative to R16 values as the maximum thus representing the median within the test series (Figure 2B). Implicitly considering fluorescence measurements of R16 to be normally distributed, the statistically relevant range for reliability of two (+/−) standard deviations is between 105.8% and 94.2% of R16 values (red dotted lines in Figure 2B). Among the individual aptamers only C1R1 reaches this degree of labelling efficiency within the reliability of R16, whereas already the second best aptamer decisively misses this mark with 65% of R16. The preparation of binding competent aptamers normally requires an experimental step of thermal denaturation and renaturation (refolding) resulting in molecules which are activated to bind their target structures.[19–21] The binding of both the non-activated (scrambled) R16 and C1R1 was drastically limited to 34% and 18% of their heat activated counterparts (Figure 2C). As expected for a mixture of molecules R16 did not show a distinctive dissociation constant with k_D values from 1 to 213 nM and a Hill coefficient of $n=1.1$ when fitted with a Hill equation. In contrast for C1R1 the k_D was 31(±2) nM and a Hill coefficient of $n=5.1$ which is typical for a cooperative binding mode[22] suggesting that C1R1 requires relatively high concentrations to obtain full experimental functionality (Figure 2D). Commonly the limit of detection (LOD) for cells or individual proteins is estimated by quantifying the aptamer binding to a decreasing amount of target.[23–25] Both, the library R16 and C1R1, thereby delivered fluorescence signals above the detection limit for the reference strain *P. aeruginosa* PAO1, which was calculated as three standard deviations from the mean blank value. With 250 cells as the lowest measured cell number a reasonable limit of detection for diagnostic applications was achieved (Figure 3E).

Fast and reliable detection of pathogens in clinical microbiology may be regarded as a prototypic application of aptamers in the future not only arising from their high specificity alone but also from their potential to be optimized for different assay systems. Emerging multiresistance of bacterial pathogens represents a global threat to human health which has recently triggered an insistent alert by the WHO to brace clinical sciences and the healthcare system for the resulting challenge and the fight against fatal infections.[26] Among the three most critical bacteria in this alert was *P. aeruginosa*, which is considered one of the most hospital acquired infections with mortality rate of up to 50%.[27] Genetic mutation and physiological adaptation can cause resistance also towards so called last resort antibiotics like carbapenems and thus dramatically increase the mortality risk in intensive care units.[28] Successful management of life threatening infections requires techniques for fast and reliable detection of the pathogen on the species level independent from the occurrence of different strains or variants. To compare the potential of the polyclonal library R16 with the aptamer C1R1 labelling of different *P. aeruginosa* strains was performed. The set of 51 tested clinical *P. aeruginosa* isolates included strains, with intermediate resistance against ciprofloxacin, against Gentamicin or showing multiresistance including resistance against carbapenem. *P. aeruginosa* PAO1 is a worldwide used type or reference strain and served as the reference strain and was used here also to generate the library R16. The fluorescence signals resulting from incubation of *P. aeruginosa* PAO1 cells with the fluorescent R16 or C1R1 were each defined to represent 100% of labelling efficiency and served as references for the labelling precision of R16 and C1R1. The precision of R16 reached values between 35% to 99% with a median for all 51 strains of 82% demonstrating that with this polyclonal serum also clinical isolates could be identified with high fidelity. Compared to this with 1.8% to 95% and a median of only 27% the precision of the detection by C1R1 was drastically reduced and characterized by fluctuations between the individual clinical isolates indicating variations in the amount of the (unknown) molecular target for C1R1 in these strains (Figure 3). This suggests that the higher precision of the polyclonal library may result from the compensation of the reduced C1R1 binding to its target by other individual aptamers from the larger available sequence space of the library to other targets on the cell surfaces. In *P. aeruginosa* the composition of the cell wall is tightly regulated and not only depends on the genetic configuration of the respective

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Aptamer	*P. aeruginosa*	*E. coli*	Structure	Enthalpy ΔG [kcal/mol]	Randomized sequence (5′→3′)	Frequency [RPM]	Fold Enrichment
C1R1				-2.49	GCGCGCGAGATTAA CCCCCCAATGCTGC ACCGAGCCACGA	51.9	-
C2R1				-2.08	GCGGCAGGGAAGG ACTATGTGGGTGAA AGGAGTGCGCGGG	44.05	209x
C2R2				-2.65	GCAGCGGGATGGG GTAAATGGTGGCGA GAGGCGTCGGGGG	28.75	-
C4R2				-0.1	GCGGGTTGACTAGT ACATGACCACTTGA GTCGCTTGAACT	20.03	97x
C6R3				-1.33	GCGGGGAGAGGCG AAAGAAGCTGGGAT GGAAGGGCGTAGG	16.8	-
C10R5				-3.98	GCAGCCACAGCAGA GACGGGAAGGGCC AGGGTTGAGCGGG	7.28	35x
C10R6				-2.99	GCGGCGGTGGGGC TTTCGGTGATTTGG GCGGTTTGGCGGG	7.09	-

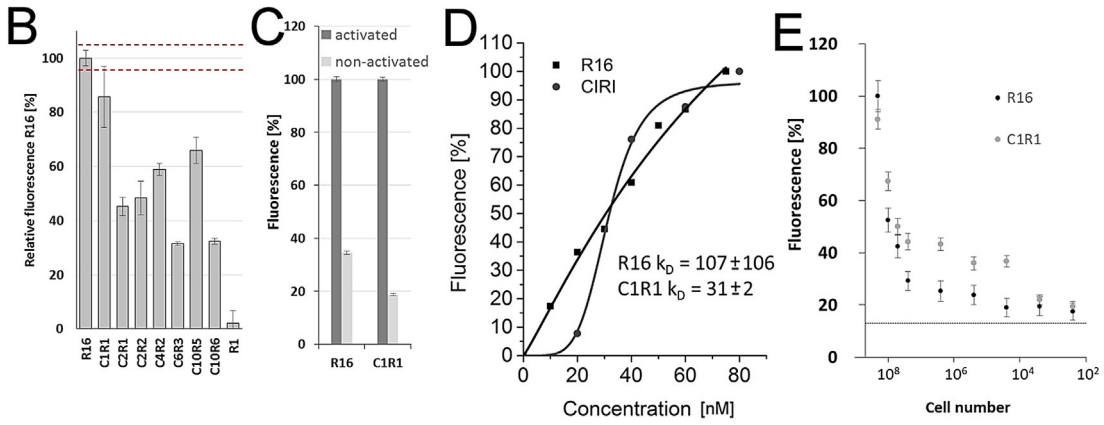


Figure 2. A) Qualitative characterization of selected individual Cy5-labelled aptamers (excitation: 635 nm) by CLSM specificity testing, secondary structure simulation, folding enthalpy, sequence, frequency of the aptamer within the library R16 and the enrichment in comparison to R1. B) Binding of individual aptamers (50 nM) to *P. aeruginosa* PAO1 (2×10^8 cells) compared to the polyclonal library R16. Red lines indicate the range of two standard deviations as a threshold for reliability of a normally distributed measurement. C) Binding comparison of activated correctly folded library R16 and C1R1 in contrast to non-activated misfolded library R16 and C1R1 at equal aptamer concentrations (50 nM) and cell numbers (2×10^8 cells). D) Binding curves of different concentrations of the polyclonal library R16 and the individual aptamer C1R1 to a standardized amount of cells (2×10^8). E) limit of detection estimation using 50 nM aptamer solutions with decreasing amounts of *P. aeruginosa* cells. The horizontal line represents the detection limit of the measurement calculated as three standard deviations from the mean of the blank value.

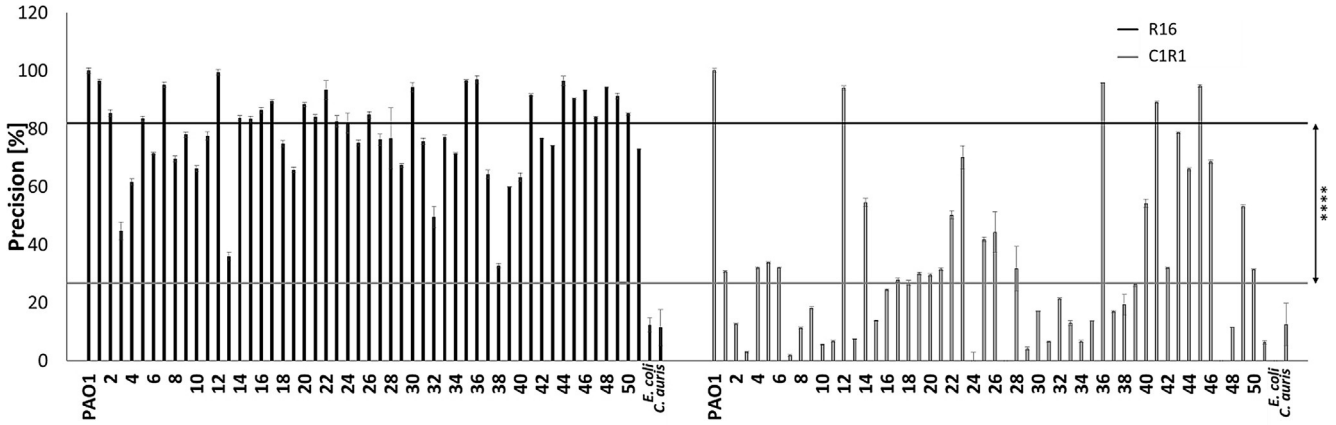


Figure 3. Precision of R16 and C1R1 in the recognition of clinical isolates ($n = 51$) as a measure of the accuracy. The accuracy of identification of clinical isolates as *P. aeruginosa* measured as the precision of matching fluorescence signal strengths relative to *P. aeruginosa* PAO1 as a reference strain. The 51 clinical isolates contained strains resistant to clinically important antibiotics including carbapenem. *E. coli* and the pathogenic yeast *Candida auris* (*C. auris*) served as negative controls. Samples were adjusted to 2×10^8 cells and detected with 50 nM of the respective aptamers. The lines at 82% (R16) and 27% (C1R1) represent the median of the precision of all clinical isolates for the polyclonal library R16 and the individual aptamer C1R1. Statistical analysis of the medians by a t-test thereby revealed a significance level of $P < 0.0001$ (****).

strain but also on environmental conditions.[29] Strains isolated from cystic fibrosis patients showed major alterations in the expression pattern of proteins involved in surface related cellular functions like drug resistance, chemotaxis and motility.[30] In addition, mutation or deletion of the gene for the outer membrane protein D (OprD) has been described as a molecular reason for carbapenem resistance and increased virulence.[31] Growth conditions like the medium composition, temperature or growth phase significantly influence the membrane proteome also when Gram-negative bacteria are cultivated in vitro.[32,33] When cultivated in a complex medium *P. aeruginosa* shows the typical bacterial growth phases including the lag-, exponential growth, early stationary and stationary phases (Figure 4A) represented by characteristic changes in the growth rates (Figure 4B). Both, R16 and C1R1 could label cells isolated from all growth phases, however, fluorescence signals

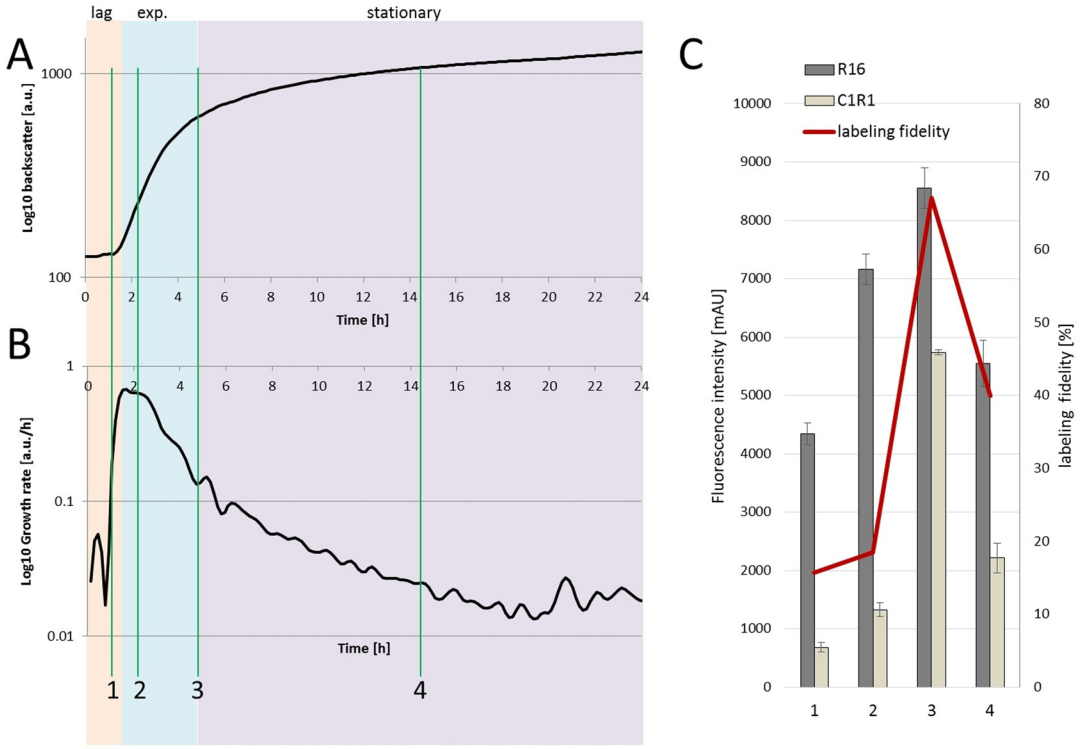
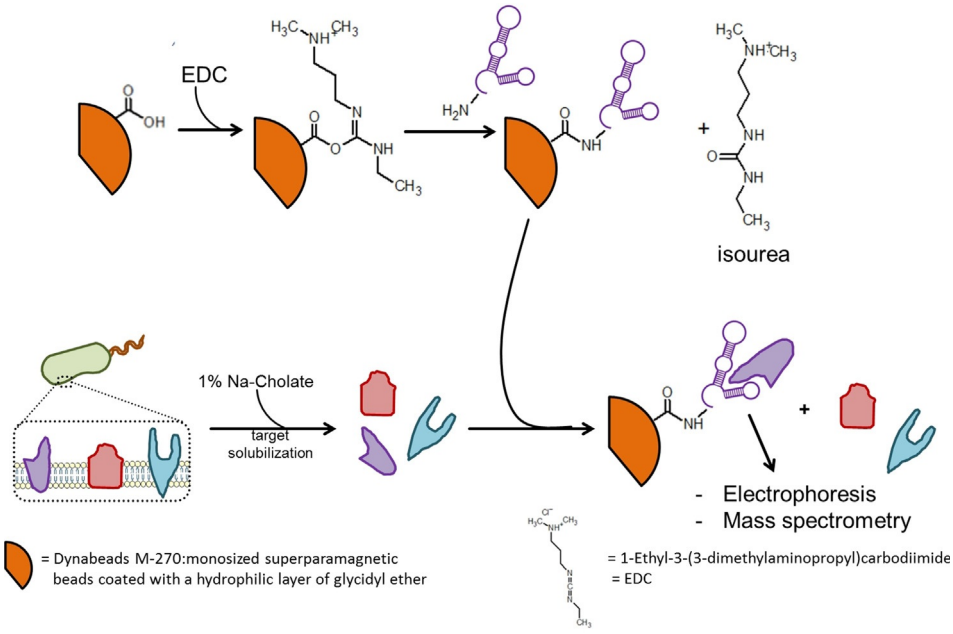


Figure 4. A, B) Growth of *P. aeruginosa* cultures. A) Light backscatter as a measure for cell density. Lag-, exponential growth and stationary phase are highlighted. B) Growth rate of *P. aeruginosa* as backscatter per hour. C) Labelling fidelity of *P. aeruginosa* cells (red line) for different growth phases derived from the fluorescence signal of C1R1 relative to R16. At each point cells were adjusted to 2×10^8 and incubated with 50 nM Cy5-labelled aptamers.

of R16 where particularly higher compared to C1R1. Recognition was most efficient for both with cells from the early stationary phase. Here, C1R1 labeling approaches the level of R16 and the quotient of C1R1 and R16 dependent fluorescence signals as a measure of the labeling fidelity towards *P. aeruginosa* cells reaches its maximum (Figure 4C). Interestingly cells from early stationary phases were also used for the SELEX process and the initial characterization of individual aptamers, respectively. The lack of labeling fidelity of C1R1 not only represents a consequence of a specific growth phase dependent cell surface but also qualifies this fixed experimental fashion of the SELEX as a "snap-reading method" which, although it delivers functional individual aptamers, bears the risk of a loss of labeling efficiency towards all cells which do not exactly match the original growth phase dependent cell wall or outer membrane composition. The higher uniformity of labeling using R16 may be again supportive for the view that the larger sequence space of a polyclonal library and the resulting higher diversity of aptamers binding not only to one specific but to multiple targets compensates for fluctuations in the amount of particular cell surface targets and consequently guarantees increased experimental flexibility and thus higher performance in microbiological detection applications.

A second major aspect in aptamer research in general is the claim to develop novel pharmaceutically active components. Specific individual aptamers with antimicrobial effect have been evolved for Salmonella typhimurium[34] but could not be isolated from a whole cell SELEX against *P. aeruginosa*.[35] To adopt its full pathogenic potential *P. aeruginosa* requires different physiological functions associated with virulence, among them cellular motility and its capability to form elaborate biofilm architectures which especially represent a major burden in hospitals and predominantly in intensive care units. Different modes of motility exist which are mediated by appendices of the cell surface like type IV pili and the flagellum.[36–38] Flagella dependent swimming motility and thus flagella function can be measured quantitatively simply in a dedicated assay based on soft agar plates as a haze of growth surrounding the point of inoculation.[36,38] Compared to nonspecific reactions towards single stranded herring sperm control DNA (HS) *P. aeruginosa* cells were significantly impaired in swimming when they were confronted with R16 on agar plates at the onset of cellular movement, whereas C1R1 almost failed to inhibit motility (Figure 5A). This effect of R16 showed a dose dependency and appeared to be drastically reduced after extended periods of incubation (Figure 5BC). *P. aeruginosa* produces extracellular DNAses for the utilization of DNA as a nutrient source.[39] Enzymatic degradation and inactivation of the aptamers is the probable explanation for the recovery of motility between day one and three of the experiment. Inhibition of biofilm formation in microtiter plates (Figure 5D), however, was less pronounced with only marginal differences between R16 and C1R1 (Figure 5E).

With the experimental setup of the FluCell-SELEX using whole cells as targets it is undisputed, that molecular targets of the isolated aptamers predominantly should reside on the cell surface. To prove this a pull-down assay using aptamers on magnetic particles was designed. A whole cell membrane preparation from *P. aeruginosa* harboring membrane proteins from both the inner and the outer membranes was used to solubilize membrane proteins with sodium cholate as a gentle detergent preserving their native structures.[40] The aptamers were functionalized by introduction of an amino group to their 5'-ends by PCR using amino modified primers. The functionalized aptamers were then coupled to magnetic particles by carbodiimide conjugation using 1-ethyl-3-(3-dimethylaminopro-



Scheme 3. Functionalization of mono-sized superparamagnetic beads with aptamers to generate an affinity matrix for the purification of membrane proteins and subsequent identification of aptamer targets from solubilized bacterial membranes.

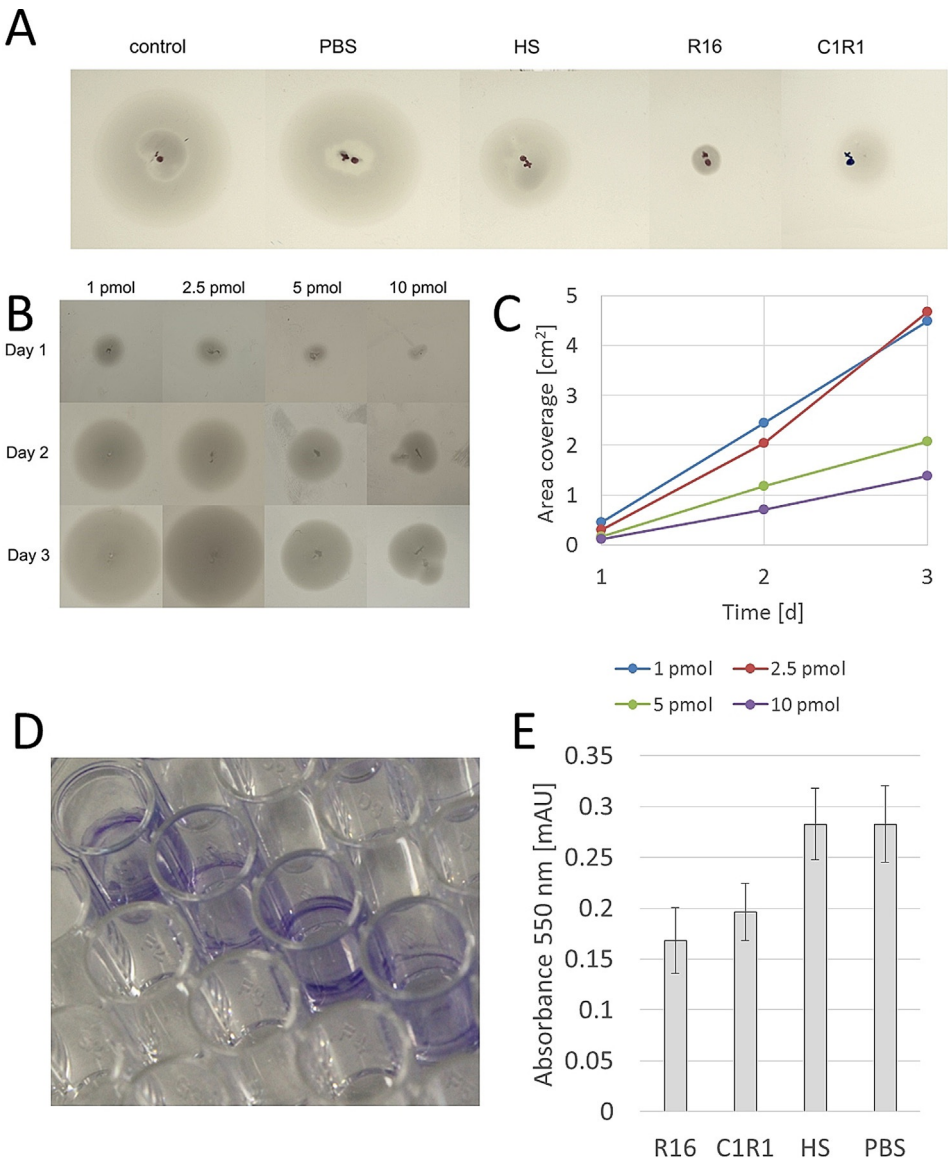


Figure 5. A) Influence of 2.5 pmol R16 and C1R1 in close proximity to a colony on the swimming motility of *P. aeruginosa* after 24 h. B) Swimming motility inhibition of R16 at different concentrations over time. C) Area coverage of swimming colonies quantified from B). D) Typical *P. aeruginosa* biofilm stained with crystal violet in a 96 well plate. E) R16 and C1R1 dependent Inhibition of *P. aeruginosa* biofilm formation using single stranded herring sperm DNA (HS) as a non-binding control (aptamers and non-binding control were applied at equal concentrations of 50 nM.

pyl)carbodiimide (EDC) activated carboxylic acid coated beads to achieve a zero length carboxyl to amino crosslinking (Scheme 3) as was verified in a PCR reaction with the functionalized particles as the template (Figure 6 A). Subsequent incubation of the membrane preparation with the aptamer modified magnetic beads allows the isolation of the solubilized target structures, which could then be separated by electrophoresis and identified by mass spectrometry. In contrast to C1R1, the R16 dependent pull-down assay delivered three dominant distinctive bands in the electrophoresis as analyzed by densitometry upon staining with Coomassie brilliant blue G250 (Figure 6 B C).

Based on the signal intensity and sequence coverage as measures for the abundance of the detected peptides and the completeness of the identified protein sequence the identified proteins were the outer membrane proteins (omp) OprF, OprM and OprD (Table 1).

These are the most prominent omp in *P. aeruginosa* and known to be involved in various physiological functions. OprF as the most abundant porin not only facilitates diffusion of small molecules but is also involved in maintaining membrane

Table 1. Proteins identified by mass spectrometry.

Protein	Description	MW [kDa]	Unique peptides	Sequence coverage [%]	Band
OprD	outer membrane porin	48.36	8	67	1
OprM	multidrug transporter	52.598	16	47.2	1
OprF	outer membrane porin	37.639	18	83.1	2, 3

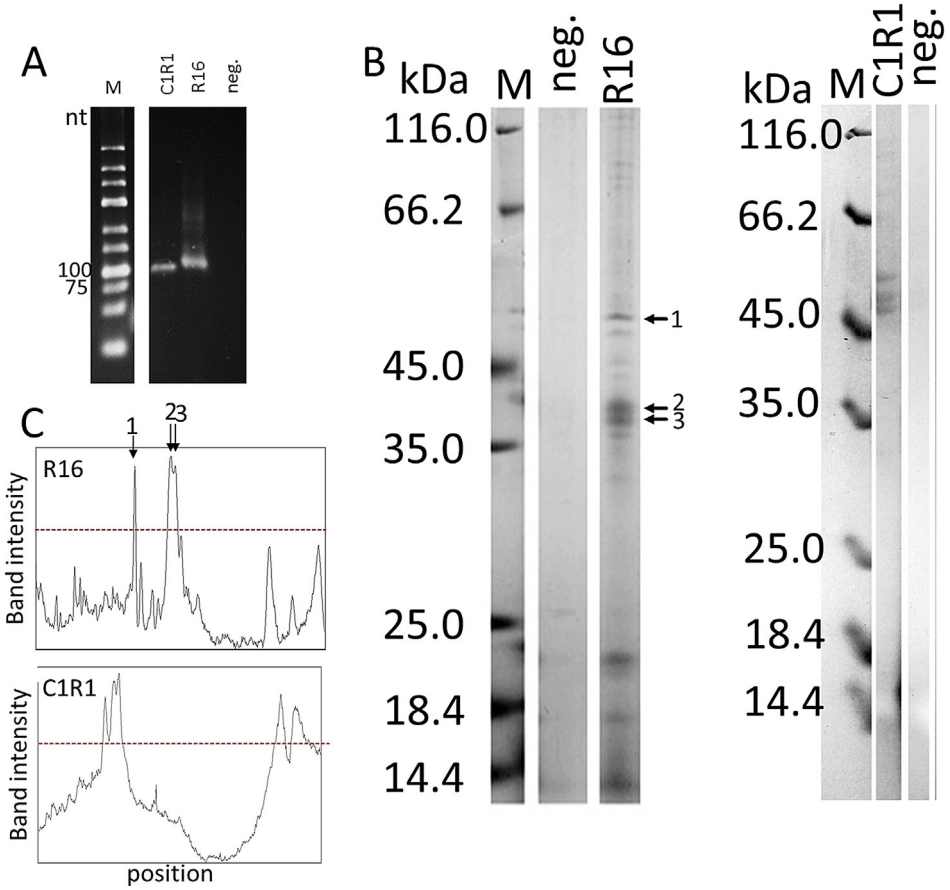


Figure 6. A) Verification of magnetic particle functionalization with aptamers. Products of a PCR with aptamer loaded particles as templates. B) Electrophoretic separation of purified R16 and C1R1 targets. C) Band intensity profile of the SDS-gel displayed in B).

integrity, adhesion to host cells and establishment of acute and chronic infections as nicely reviewed by Chevalier et al. 2017.[29] As a part of major efflux pumps OprM mediates antibiotic resistance,[41] whereas OprD mediates uptake of carbapenem explaining resistance towards this important antibiotic of OprD mutants.[31] It is also known that these Omp are differently expressed in clinical isolates.[41,42] Significant alterations in cell surface composition and abundance of available targets explain why the broader specificity of R16 causes a higher flexibility of labelling and fidelity of detection for different strains and isolates compared to C1R1. The reduced signal fluctuations as a consequence of compensated binding deficiencies of single aptamers by recognizing alternative targets then ensure sufficiently robust measurements for the save identification of pathogenic *P. aeruginosa* strains. Such an increased performance qualifies the use of polyclonal aptamer libraries as an ideal new strategy in diagnostics and sensor technology. Large scale production of polyclonal aptamer libraries at an industrial level, however, then requires efficient biotechnological routes typically based on in vitro enzymatic processes. This option was tested by successive reamplification of R16 demonstrating that the superior quality of binding of R16 were passed down at least for four generations without noteworthy losses (Figure S5). An alternative to a true library produced by reamplification would be mixtures of individual aptamers originating from chemical synthesis which would unify the advantages of polyclonality and the possibility of reproducible chemical synthesis which can be regarded as a key advantage of the aptamer technology. Such a concept has been successfully demonstrated by Domenyuk et al. in the context of breast cancer where they used mixtures of 2000 individual aptamers.[9,10] On the way towards (focused) libraries as a product for diagnostics of pathogenic bacteria this would be a reasonable and promising next step in the development. We believe that the potentially outstanding performance and their biotechnological availability paves the way for the introduction of polyclonal aptamer libraries as the next generation of truly robust molecular binding mediators in general. We envision the development of novel sophisticated optical and electronical sensors for the use in clinical diagnostics as new powerful tools to strengthen healthcare in the fight against the global threat of multiresistant pathogens.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antibiotics · antigens · aptamers · biological activity · biosensors

[1] A. D. Ellington, J. W. Szostak, *Nature* **1990**, *346*, 818 – 822.
[2] C. Tuerk, L. Gold, *Science* **1990**, *249*, 505 – 510.
[3] L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas, J. J. Toole, *Nature* **1992**, *355*, 564 – 566.
[4] G. Mayer, *Angew. Chem. Int. Ed.* **2009**, *48*, 2672 – 2689; *Angew. Chem.* **2009**, *121*, 2710 – 2727.
[5] S. Ni, H. Yao, L. Wang, J. Lu, F. Jiang, A. Lu, G. Zhang, *Int. J. Mol. Sci.* **2017**, *18*, 1683.
[6] S. L. Edwards, V. Poongavanam, J. R. Kanwar, K. Roy, K. M. Hillman, N. Prasad, R. Leth-Larsen, M. Petersen, M. Marušič, J. Plavec, J. Wengel, R. N. Veedu, *Chem. Commun.* **2015**, *51*, 9499 – 9502.
[7] A. Vater, S. Klussmann, *Drug Discovery Today* **2015**, *20*, 147 – 155.
[8] T. K. Sharma, J. G. Bruno, A. Dhiman, *Biotechnol. Adv.* **2017**, *35*, 275 – 301.
[9] V. Domenyuk, Z. Gatalica, R. Santhanam, X. Wei, A. Stark, P. Kennedy, B. Toussaint, S. Levenberg, J. Wang, N. Xiao, R. Greil, G. Rinnerthaler, S. P. Gampenrieder, A. B. Heimberger, D. A. Berry, *Nat. Commun.* **2018**, *9*, 1219.
[10] V. Domenyuk, Z. Zhong, A. Stark, N. Xiao, H. A. O'Neill, X. Wei, J. Wang, T. T. Tinder, S. Tonapi, J. Duncan, T. Hornung, A. Hunter, M. R. Miglarese, J. Schorr, D. D. Halbert, J. Quackenbush, G. Poste, D. A Berry, G. Mayer, M. Famulok, D. Spetzler, *Sci. Rep.* **2017**, *7*, 42741.
[11] T. Hornung, H. A. O'Neill, S. C. Logie, K. M. Fowler, J. E. Duncan, M. Rosenow, A. S. Bondre, T. Tinder, V. Maher, J. Zarkovic, Zenyu Zhong, M. N. Richards, X. Wei, M. R. Miglarese, G. Mayer, M. Famulok, D. Spetzler, *Nucleic Acids Res.* **2020**, *48*, 4013 – 4027.
[12] R. Stoltenburg, C. Reinemann, B. Strehlitz, *Anal. Bioanal. Chem.* **2005**, *383*, 83 – 91.
[13] F. Tolle, G. Mayer, *Methods Mol. Biol.* **2016**, pp. 77 – 84.
[14] K. K. Alam, J. L. Chang, D. H. Burke, *Mol. Metab.* **2015**, *4*, e230.
[15] P. Keohavong, W. G. Thilly, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 9253 – 9257.
[16] "CLC Workbench 7.6.2," can be found under https://www.qiagenbioinformatics.com/, **n.d.**.
[17] M. Zuker, *Nucleic Acids Res.* **2003**, *31*, 3406 – 3415.
[18] T. T. Le, O. Chumphukam, A. E. G. Cass, *RSC Adv.* **2014**, *4*, 47227 – 47233.
[19] S. Wildner, S. Huber, C. Regl, C. G. Huber, U. Lohrig, G. Gadermaier, *Sci. Rep.* **2019**, *9*, 1111.
[20] Y. Wang, Y. Luo, T. Bing, Z. Chen, M. Lu, N. Zhang, D. Shangguan, X. Gao, *PLoS One* **2014**, *9*, e100243.
[21] F. Pfeiffer, F. Tolle, M. Rosenthal, G. M. Brändle, J. Ewers, G. Mayer, *Nat. Protoc.* **2018**, *13*, 1153 – 1180.
[22] J. N. Weiss, *FASEB J.* **1997**, *11*, 835 – 841.
[23] J. G. Bruno, T. Phillips, M. P. Carrillo, R. Crowell, *J. Fluoresc.* **2009**, *19*, 427 – 435.
[24] J. G. Bruno, T. Phillips, *J. Biomed. Eng. Med. Devic.* **2016**, *1*, 103.
[25] Y. V. V. A. Kumar, R. M. Renuka, J. Achuth, M. Venkataramana, M. Ushakiranmayi, P. Sudhakar, *Front. Pharmacol.* **2018**, *9*, 271.
[26] WHO, Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery and Development of New Antibiotics, **2017**.
[27] Y. Zhang, X. L. Chen, A. W. Huang, S. L. Liu, W. J. Liu, N. Zhang, X. Z. Lu, *Emerging Microbes Infect.* **2015**, *5*, e27.
[28] C. Willyard, *Nature* **2017**, *543*, 15.
[29] S. Chevalier, E. Bouffartigues, J. Bodilis, O. Maillot, O. Lesouhaitier, M. G. J. Feuilloley, N. Orange, A. Dufour, P. Cornelis, *FEMS Microbiol. Rev.* **2017**, *41*, 698 – 722.
[30] K. S. Kamath, D. Pascovici, A. Penesyan, A. Goel, V. Venkatakrishnan, I. T. Paulsen, N. H. Packer, M. P. Molloy, *J. Proteome Res.* **2016**, *15*, 2152 – 2163.
[31] D. Skurnik, D. Roux, V. Cattoir, O. Danilchanka, X. Lu, D. R. Yoder-Himes, K. Han, T. Guillard, D. Jiang, C. Gaultier, F. Guerin, H. Aschard, R. Leclercq, J. J. Mekalanos, S. Lory, G. B. Pier, *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 20747 – 20752.
[32] R. Pieper, S. T. Huang, J. M. Robinson, D. J. Clark, H. Alami, P. P. Parmar, R. D. Perry, R. D. Fleischmann, S. N. Peterson, *Microbiology* **2009**, *155*, 498 – 512.
[33] G. NandaKafle, A. A. Christie, S. Vilain, V. S. Brözel, *Front. Microbiol.* **2018**, *9*, 762.
[34] O. S. Kolovskaya, A. G. Savitskaya, T. N. Zamay, I. T. Reshetneva, G. S. Zamay, E. N. Erkaev, X. Wang, M. Wehbe, A. B. Salmina, O. V. Perianova, O. A. Zubkova, E. A. Spivak, V. S. Mezko, Y. E. Glazyrin, N. M. Titova, M. V. Berezovski, A. S. Zamay, *J. Med. Chem.* **2013**, *56*, 1564 – 1572.
[35] J. Soundy, D. Day, *PLoS One* **2017**, *12*, e0185385.
[36] G. A. O'Toole, R. Kolter, *Mol. Microbiol.* **1998**, *30*, 295 – 304.
[37] F. Rosenau, S. Isenhardt, A. Gdynia, D. Tielker, E. Schmidt, P. Tielen, M. Schobert, D. Jahn, S. Wilhelm, K. E. Jaeger, *FEMS Microbiol. Lett.* **2010**, *309*, 25 – 34.
[38] S. Wilhelm, A. Gdynia, P. Tielen, F. Rosenau, K. E. Jaeger, *J. Bacteriol.* **2007**, *189*, 6695 – 6703.
[39] H. Mulcahy, L. Charron-Mazenod, S. Lewenza, *Environ. Microbiol.* **2010**, *12*, 1621 – 1629.
[40] R. E. W. Hancock, H. Nikaido, *J. Bacteriol.* **1978**, *136*, 381 – 390.
[41] G. Horna, M. López, H. Guerra, Y. Saénz, J. Ruiz, *Sci. Rep.* **2018**, *8*, 16463.
[42] S. Ranjitkar, A. K. Jones, M. Mostafavi, Z. Zwirko, O. Iartchouk, S. W. Barnes, J. R. Walker, T. W. Willis, P. S. Lee, C. R. Dean, *Antimicrob. Agents Chemother.* **2019**, *63*, e01718.

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