Electronic Supplementary Information

Graphene oxide-based selection and identification of ofloxacin-specific single-stranded DNA aptamers

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Experimental

Materials and reagents

Ofloxacin, ciprofloxacin hydrochloride, enrofloxacin, norfloxacin, streptomycin sulfate and tetracycline hydrochloride were purchased from Sangon Biotech Co. Ltd (Shanghai, China). Dynabeads M-280 Streptavidin was obtained from Invitrogen Company. Graphene oxide (GO) were purchased from Suzhou Nord Derivatives Pharm-Tech Co. Ltd (Suzhou, China). Reagents for polymerase chain reaction (PCR) amplification (pfu-polymerase and deoxynucleoside triphosphate [dNTP]) were purchased from TaKaRa Bio Inc (Dalian, China).

The DNA library and PCR primers were synthesized and HPLC purified by Sangon Biotech Co. Ltd (Shanghai, China). The random DNA library (5′-TAGGGAATTCGTCGACGGATCC-N35-CTGCAGGTCGACGCATGCGCCG-3′) contained a central random sequence of 35 nucleotides, which was flanked by two primers binding sequences for PCR amplification and cloning. A forward primer (5′-TAGGGAATTCGTCGACGGAT-3′) and a reverse primer (5′-CGGCGCATGCGTCGACCTG-3′) were employed. Biotin-labeled reverse primer (5′-biotin-CGGCGCATGCGTCGACCTG-3′) was used for PCR amplification and ssDNA generation. The solution of the DNA sequences was prepared at the concentration of 100 μM for use.

All other chemicals were of analytical grade. Ultrapure water (18.2 MΩ cm) was used to prepare all aqueous solutions throughout the experiments.

***In vitro* selection**

Several rounds of *in vitro* selection were carried out to screen aptamers specific to ofloxacin. In the first round, 1 μL of 100 μM (2.44 μg) initial random ssDNA in binding buffer (20 mM Tris-HCl containing 100 mM NaCl, 2 mM $MgCl_2$, 5 mM KCl, 1 mM $CaCl_2$, and 0.02% Tween 20, pH 7.6) was denatured at 90 ^oC for 10 min, immediately cooled on ice for 10 min, then returned to room temperature (RT) for 10 min. And the ssDNA was mixed with the same concentration of ofloxacin for 1 h with mild shaking at RT. A certain population of ssDNA sequences from the initial pool can bind to ofloxacin freely in the binding buffer. Then GO were added to the solution and incubated for 2 h at RT with mild shaking. The wt. ratio of ssDNA : GO was kept at 1:1000. The free ssDNA was adsorbed on GO via π-π staking, while ssDNA bound to ofloxacin remained in solution. After centrifugation, GO was removed, whereas ssDNA bound to ofloxacin remained in solution. The solution was extracted with equal volume of phenol/chloroform/isoamylol (25:24:1), and precipitated by 1/10 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The mixture was left in the freezer at -20 ^oC for 2 h to ensure complete precipitation and then centrifuged at 10,000 rpm for 15 min at 4 ^oC. The precipitated ssDNA was washed with 75% ethanol and dissolved in 30 μL sterile water. The concentration of ssDNA was detected by spectrophotometer. After that, the obtained ssDNA was amplified by PCR. Each 25 μL of PCR reaction mixture contained 2 μL of dNTP (2.5 mM), 1 μL forward primer (10 μM), 1 μL biotinylated reverse primer (10 μM), 0.1 μL Taq DNA polymerase (2.5 $U \cdot \mu L^{-1}$), 1 μL template ssDNA (0.1 μM) and 2.5 μL PCR buffer. A standard PCR

cycle consisted of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and elongation at 72 °C for 1 min. The first cycle always had an extended denaturation step at 95 °C for 5 min. The final extension step was conducted for 5 min at 72 °C. In this way, dsDNA fragments with a biotin modification at the complementary strands were produced. The PCR products were confirmed by gel electrophoresis using 3% agarose.

In order to obtain ssDNA library for next round of selection, streptavidin-modified magnetic beads were used to separate biotinylated oligonucleotide strands from their complementary strands in PCR products. 10 μL streptavidin-modified magnetic beads were washed with binding and washing (B&W) buffer (10 mM Tris-HCl containing 1 mM EDTA, 2 M NaCl, pH 7.5) for three times and suspended in 80 μL B&W buffer. Thereafter, 20 μL PCR products were added to the beads suspension, and incubated at RT for 15 min, then washed with B&W buffer. After further incubation in 0.1 M NaOH at 37 °C for 10 min, the biotinylated strands kept bound to streptavidin-modified magnetic beads whilst the complementary non-biotinylated ssDNA was eluted from the beads. After the first round of selection, the obtained ssDNA was used as a new ssDNA pool suitable for the next round of enrichment. The concentration of ssDNA was detected by spectrophotometer.

The second round of selection was then carried out from the above obtained secondary ssDNA library with the repeated process of the first selection round. After each round of selection, ssDNA was precipitated with ethyl alcohol and the concentration was determined. The selection was repeated for several rounds.

The recovery of ssDNA was calculated by determining the concentration of ssDNA before and after each round of selection. The selection rounds were repeated until the recovery of ssDNA reached saturation.

For GO counter-SELEX, the prepared ssDNA from the former round of selection was dissolved in 200 μL binding buffer and denatured at 90 ^{o}C for 10 min. Then the mixture of ciprofloxacin, enrofloxacin and norfloxacin (ciprofloxacin : enrofloxacin : norfloxacin=1:1:1) was added and allowed for incubation for 1 h with mild shaking at RT. After that, GO were added to the solution and incubated for 2 h at RT with mild shaking. The wt. ratio of ssDNA : GO was kept at 1:1000. The free ssDNA was adsorbed on GO via π-π staking, while ssDNA bound to ciprofloxacin, enrofloxacin or norfloxacin remained in solution. The supernatant after centrifugation was discarded, and the precipitated GO was washed for 3 times with binding buffer. Thereafter, ofloxacin was added to GO suspension and incubated for 1 h at RT with mild shaking. After centrifugation, the supernatant was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in sterilizing water. The concentration of ssDNA was determined.

Cloning, sequencing and structural analysis

The recovered ssDNA from the last round of GO-SELEX was amplified with non-biotinylated primers. Then the PCR products were cloned into vector pMD-19T. *Escherichia coli* JM109 cells were used for transformation. 21 positive clones were picked and sequenced (Sangon Biotech Co. Ltd., Shanghai, China). Sequence

alignments were performed using DNAMAN software. Mfold software was used to predict the lowest free energy shapes and analyze the secondary structures of the aptamers.

Determination of the dissociation constant (K_d)

The dissociation constants of the sequenced aptamers were determined by equilibrium filtration method.[1] Firstly, 200 μM ofloxacin and different concentrations of aptamers ranging from 0 to 1.4 μM were mixed in 200 μL binding buffer and incubated at RT for 30 min with mild shaking. Then the solution was put into ultrafiltration centrifugal tube with the cutting molecular weight of 3,000 Da, and centrifuged at 12,000 rpm for 8 min. The filtrate through the membrane contained only free ofloxacin, which was measured by UV-vis spectroscopy at 293 nm. The concentration of ofloxacin can be calculated from the calibration curve.

The dissociation constant was calculated by fitting the data points through the non-linear regression analysis with the following equation[2] and GraPad Prism 5.0 software:

$$y = B_{max} \cdot [\text{free ssDNA}]/(K_d + [\text{free ssDNA}])$$

where y represents the degree of saturation, B_{max} represents the maximum number of binding sites, and [free ssDNA] is the concentration of unbound ssDNA.

Specificity evaluation of the aptamers

To investigate the specificity of the screened aptamers, the aptamers were

incubated with ciprofloxacin, enrofloxacin, norfloxacin, streptomycin and tetracycline for 1 h, respectively. Then GO was added to the solution and incubated for another 2 h at RT with mild shaking. The wt. ratio of ssDNA : GO was kept at 1:1000. The free ssDNA was adsorbed on GO, which was then removed by centrifugation. Whereas ssDNA bound to different antibiotics remained in solution, which was then extracted with equal volume of phenol/chloroform/isoamylol (25:24:1), precipitated by the addition of 1/10 volumes of 3 M sodium acetate and 2.5 volumes of ethanol for 2 h at -20 ^{o}C. ssDNA pelleted was washed with 75% ethanol. Then the concentration of ssDNA was measured by spectrophotometer after dissolving. The recoveries of the aptamers were calculated and used to evaluate their specificity.

Notes and references

1. C. Andriole and V. Andriole, *Mediguide Infect. Dis.*, 2001, **21**, 1-5.

2. J. H. Niazi, J. L. Su and B. G. Man, *Bioorgan. Med. Chem.*, 2008, **16**, 7245-7253.