Aptamer Selection Against Quinolones Antibiotics Using Immobilizing Free SELEX, and Studying Aptamer-Antibiotic Interaction By Spectroscopy and High-Resolution Mass Spectrometry

Maryam Kabiri
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University
Hasan Rafati
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University
Masoud Tohidfar
Shahid Beheshti University
Atousa Aliahmadi
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University
Alireza Ghassempour
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University
Zeinab Shahsavari
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University
Hassan Rezadoost (h_rezadoost@sbu.ac.ir)
Medicinal Plants and Drugs Research Institute, Shahid Beheshti University

Research Article

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Abstract

Over-consumption of antibiotics has led to antimicrobial resistance creating urgency to find fast and efficient techniques to monitor the level of antibiotics in the foods. The advantages of using aptamer for a variety of scientific challenges are becoming more abundant in different fields, making its applications more effective and cost-efficient. In this study, two aptamers against two quinolones antibiotics (Enrofloxacin and Ofloxacin) were isolated via GO-SELEX (graphene oxide assisted Systematic Evolution of Ligands by Exponential Enrichment). The interaction between the aptamer and its cognate antibiotic was investigated using spectroscopy and mass spectrometry. The obtained evidence from spectroscopy showed that the interaction of antibiotics with their aptamers resulted in a 41.98% and 22.45 % reduction in the extinction coefficient (ε) value of enrofloxacin and ofloxacin, respectively. This phenomenon could arise from the stacking interaction between the aromatic region of antibiotic and aptamer's bases. The formation of the aptamer-antibiotic complex was studied by the high-resolution ESI-Q-TOF. In the obtained mass spectra, several peaks corresponding to the aptamer-antibiotic complexes with a wide range of charge distribution were identified, which could be explained by the fact that both aptamer-ENR and aptamer-OFLO complexes were stable in the gas phase and the intact complexes survived in the vacuum. This stability could be explained by the hydrogen bonding and electrostatic interactions between the aptamers and antibiotics. As both aptamers can distinguish quinolone from other antibiotic families, these aptamers may become great recognizing elements for apta-sensors.

Introduction

Nowadays the demand for the detection of small molecules and ions is increased. These small molecules could be toxins like herbicides, pesticides, or mycotoxins (like aflatoxin and ochratoxin), or they can be drugs like veterinary drugs, or even heavy metals. Monitoring all the mentioned cases is very important for human health. Therefore a reliable and sensitive method is needed for this vital issue[1,2].

Antibiotics are one of the most important chemicals and small molecules that since their discovery human life has improved. Despite the health advantages of using antibiotics for humans and livestock but there are also some problems with over-consumption of this drug. The problem arises when these antibiotics show up in animal-derived products like meat, milk, eggs, etc. which can cause antimicrobial resistance that can put human life in danger[3,4]. According to this issue many countries have set strict policies on determining antibiotics in foods [5]. In this regard, many analytical techniques have been examined to determine antibiotics in different sample matrix accurately, but in most of the analytical techniques, the isolation of antibiotics from the sample matrix is very time-consuming and laborious[3,6,7]. Among all the developing techniques, aptamers are the promising alternative to selectively capture small molecules in the different matrix. Despite a low affinity between small molecules-aptamers compared with proteins-aptamers, aptamer-based biosensors are very suitable candidates as the diagnosing element for small molecule detection. Recently, many studies for finding specific aptamers against antibiotic molecules are in progress. These studies can help with widening the use cases for specific

aptamers associated with the target molecules and create an application for finding antibiotics faster and more cost efficient [2,4,8].

Nucleic acids have a very important role in the biological chain of life. They save hereditary information, catalyze DNA replication, DNA transcription, protein expression, and they are even involved in the communication between cells[9]. As chemically synthesis of oligo nucleic acids has been invented, some new applications have been found for these kinds of biomolecules; like recognition elements in biosensors, affinity probes in chromatography, therapeutic agents in drug delivery, etc. These biomolecules are well known as aptamers[10,11]. Aptamers for the first time in 1990 were introduced by two groups Tuerk / Gold and Ellington / Szostak[12,13].

Aptamers are single-stranded DNA, RNA (mainly from synthetic source), that could fold into a special three-dimensional structure via Watson-Crick or non-Watson-Crick interaction[14]. These molecules due to their sequences and spatial structures can have a selective affinity to a specific target. Aptamers can non-covalently interact with a wide range of molecules as the target. These targets can be biomolecules like proteins[15,16], peptides[17], and nucleic acids[18] or small molecules[1] like toxins[19–22], drugs[23,24], ions[25], and can even be whole cells[26], bacteria[27], and viruses[28]. The non-covalent interactions between aptamer and target are a combination of π-π interaction, hydrogen bonding, electrostatic interaction, and complementarity in shape[14]. Aptamers are compared with enzymes because of their specific target recognition. However, aptamers have several advantages over enzymes. Aptamers can be prepared synthetically at a lower cost. They can be easily handled due to being stable at room temperature. The most precious advantage is that they can be designed fitted for a specific target[1,9,10,29]. The aptamers are generated for a target by an iterative process named SELEX (systematic evolution of ligand by exponential enrichment). In this process, some sequences that have a high affinity to the target are separated among several sequences in a random pool. The whole of this process contains three main parts including partitioning, selection, and amplification[1,9,10].

One of the crucial steps of the SELEX process is the separation of the aptamer-target complex from the unbound DNA sequences in the random pool. The separation step can be done with two strategies; (a) immobilizing the target or pool on a stationary phase[1,24,30] (b) incubating the aptamer and target free in solution[31,32]. Although, the immobilizing method in the small molecule SELEX is very common, and in early studies about aptamers was introduced as the method for screening aptamers against small molecules, it has some disadvantages[9,29,30,33]. The stationary phase particles in these methods can create steric hindrance in the interaction between the aptamer and small molecules. Besides in the process of immobilizing small molecules on a stationary phase, the functional group of the small molecules bonded to the stationary phase is blocked and it is not exposed for interaction with aptamer. Non-specific interaction between aptamer and stationary phase is another drawback of this method[34]. Due to the mentioned disadvantages, immobilizing free methods like capillary electrophoresis, dialysis, ultrafiltration, and GO-SELEX were introduced[32,35]. The free-in-solution method has several advantages

over the immobilizing one. Particularly, there is no need for any chemical changes on the target molecule and aptamer can access the small molecule without any hindrance[9,31].

UV-Vis spectroscopy is a suited and simple technique for an investigation into the interaction between aptamer and ligand. The π-π stacking between nucleotide's bases and the aromatic region in the small molecule can cause a bathochromic shift on λ_{max} and hypochromic effect on the extinction coefficient (ε) of the small molecule. So the change in the small molecule's optical properties is a sign of the interaction between the aptamer and the small molecule[36–38].

ESI as a gentle and powerful ionization method has been established for studying the aptamer-target complex in intact form. Using ESI-Q-TOF MS compared with in-solution methods has some advantages. This method is very sensitive and there is the possibility to study each species (free aptamer and aptamer-ligand complex) separately in the same run and for this analysis, only sub-micromolar of a sample is needed. Each run can be done just in few minutes. Another valuable feature of this method is that there is no need to do any labeling, as well as the analysis by mass spectrometry does not result in a false-positive response[39–41].

In the current study two quinolone antibiotics, Enrofloxacin (ENR) and Ofloxacin (OFLO) were selected as the targets. These two antibiotics are effective against both gram-positive and gram-negative bacteria, they have widespread usage for the treatment of human and animal infections. By reflecting on the advantages of non-immobilizing methods, in this study we used GO-SELEX for screening aptamers against the target. The GO-SELEX was performed in three styles, conventional GO-SELEX, multi GO SELEX, and counter GO-SELEX. Each style was designed for a specific reason. In the initial phase, the pool and target were incubated first to provide the opportunity to find several oligo strands with the highest affinity to the target. At the final phase, the sub-pool was incubated first with GO and then the target was added. This method was carried out to select the only strands with high affinity to the target. The counter selection was done with another strategy to find the strands that can distinguish between target and structurally similar molecules[31,42]. To explore the aptamer-antibiotic interaction, the changes in optical properties of both ENR and OFLO were studied. In this regards the UV-Vis spectrum of both antibiotics before and after interaction with related aptamer were monitored. High-resolution mass spectrometry was another technique used for exploring the formation of the aptamer-antibiotic complex.

Experimental Section

Materials. The unlabeled pool and primers were synthesized by Bioneer (Daejeon, South Korea). The sequences in pool contained 72 base pairs with 16 conserved residues at the two ends as primer binding sites and 40 random base pairs in the central region.

PCR Optimization. The optimized asymmetric PCR (Figure SI-1A) was carried out using the following protocol. Each 25 µL PCR reaction contained 2 pmoles template, 25 pmoles forward primer (10 µM as initial concentration) and 1.25 pmoles reversed primer (0.5 µM as initial concentration). $MgCl_2$, dNTP's,

and Taq polymerase were added in the form of the premixed PCR buffer. The optimum PCR cycling program was as following: 4 minutes at 95°C as initial denaturing, 1 minute at 95°C, 1 minute at 63°C for annealing step, 2 minutes at 73°C, and 5 minutes final elongation at 72°C for 20 cycles. Electrophoresis was performed on 2.5% agarose gel to confirm the PCR products.

SELEX Procedure. According to the previous studies about the adsorption of oligonucleotides on the GO surface, the mass ratio of 1:100 for ssDNA:GO (GO concentration 1.25 $g.L^{-1}$) was selected for performing the SELEX[31,42]. In the first three cycles, both antibiotics (ENR & OFLO) were incubated with a denatured pool altogether to save time. At the initial step, 300 pmoles of random oligonucleotides (with estimated complexity ~ 10^{14} strands) were dissolved in the binding buffer. The prepared binding buffer contained 100 mM NaCl (as monovalent salt), 5 mM $MgCl_2$ (as divalent salt), 20 mM Tris-HCl, and pH 7.4.

The random pool was denatured by heating at 95°C for 15 minutes, then immediately cooled by placing in 4°C ice-water for 5 minutes, and, finally, they were kept at room temperature for 15 minutes to adopt the thermodynamically stable 3D structure. Subsequently, 100 nmoles of each ENR and OFLO solution were added to the denatured pool simultaneously (at the mole ratio of Pool:antibiotic, 1:300). After 120 minutes of incubation with gentle shaking at room temperature, the GO was added to the mixture. The mixture was centrifuged for 20 minutes at 15,000 *g*. The supernatant that contained the oligo strands bound to antibiotic molecules, was collected. The bound strands were precipitated by absolute ethanol and 3mM sodium acetate. The concentration of collected DNA strands was measured by Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, Co., USA). The recovered strands were used as the sub-pool for the next selection process after amplification. The second and third selection cycles were done with the same procedure.

The fourth and fifth cycles were carried out with the same style as the initial cycles but the selection of aptamers against ENR and OFLO has proceeded separately. To increase the stringency, the amount of antibiotics was decreased (at the mole ratio of pool:antibiotic, 1:200). Moreover, the incubation time was shortened to 80 and 60 minutes in the fourth and fifth cycles, respectively. Continued to increase the stringency in the sixth cycle the mole ratio of pool: antibiotic was change to 1:150.

In the seventh cycle, the counter selection was performed. The denatured pool was incubated with three counter antibiotics (CIP, SULF, and FLO) for 80 minutes. Afterward, GO was added to the mixture, and to precipitating GO the mixture was centrifuged. The precipitated-GO was washed two times with binding buffer and after resuspending in binding buffer was incubated with the main antibiotic for 60 minutes. After incubation, the mixture was spun and the supernatant containing the bound strands to antibiotic molecules was collected. The collected oligo strands were amplified and they were subjected to the next cycle selection.

From the 8th cycle, the style of selection was changed. The denatured pool was first incubated with GO for 60 minutes so most of the ssDNA's were adsorbed on GO sheets then the mixture was centrifuged. The precipitated GO sheets were washed two times by binding buffer and then they were incubated with

the main antibiotic for 60 minutes. Like before, after centrifuging the mixture, the bound oligo strand to antibiotic molecules in the supernatant were precipitated and amplified.

The 9th cycle of selection was done the same as the 8th cycle except for the amount of antibiotics that was decreased to 20 nmoles. The selection process ended in this cycle. The obtained ssDNA's were amplified by symmetric PCR and were subjected to cloning.

Cloning and Sequencing. The amplified strands from the last cycle of SELEX were inserted into the pTZ57R/T vector (Thermo Scientific CloneJET PCR Cloning Kit, United States) and the plasmids were transformed to *E. coli*. The bacteria were cultured on the solid cell culture contained IPTG/XGal and ampicillin. After two days some white colonies were checked with colony PCR. The approved white colonies were cultured in LB medium and incubated at 37°C for overnight. The plasmids of cultured bacteria were isolated using FavorPrep™ Plasmid DNA Extraction Mini Kit (Pingtung, Taiwan). As the size of the inserted oligo strands was short, for making sure that correct colonies were selected, each plasmid was amplified by both universal M13 primers and the SELEX primers separately.

The sequencing was performed using universal primers. The two candidates that each was isolated for ENR and OFLO with minimum Gibb's free energy ($\Delta G^\circ_{25^\circ C}$), were were synthesized by Metabion (Planegg, Germany).

Analyzing the Secondary Structure of Aptamers. The aptamer secondary structures were predicted using the online software Mfold (http://unafold.rna.albany.edu/)[43]. The secondary structure and Gibb's free energy were calculated at 25°C (room temperature) and the same salt condition to the binding buffer (100 mM NaCl and 5 mM $MgCl_2$).

Dissociation Constant (K_d). For evaluating the equilibrium dissociation constant (K_d) related Aptamer-antibiotic complex the ultrafiltration method was carried out as the binding assay[44]. As starting, the denatured aptamer for a final concentration of 1 μM was incubated with the increasing series of related antibiotic concentrations (0 to 120 μM) in binding buffer at room temperature for two hours.

Subsequently, the free antibiotic molecules were separated from the aptamer-antibiotic complex by passing through the Amicon Ultra 0.5 mL centrifugal membrane with 3 KDa cutoff (Burlington, Massachusetts, United States). The solution in the filter was centrifuged at 15,000 *g* for 20 minutes thus approximately half of the solution has flowed through the filter. The concentration of unbound antibiotics in each filtered solution was measured using the UV-Vis spectroscopy (the external calibration curve data regarding the antibiotics is reported in table SI-3). The bound antibiotic concentration was calculated by deducting the concentration of free antibiotic from the total concentration of the initial antibiotic solution added to the mixture.

The dissociation constant at equilibrium state was determined by nonlinear regression analysis using Eq. 1. In this equation *f* is representing the saturation value, [T] is the concentration of free target at steady state, B_{max} is the maximum binding site and K_d is the equilibrium dissociation constant[45].

$f = B_{\max} \frac{[T]}{K_d + [T]}$ (Eq. 1)

The saturation binding curve was plotted using Prism 8 (GraphPad Software, CA, USA) and K_d values were computed using the same software.

Analyzing Binding Assay of Aptamers for Quinolone Antibiotics. The ultrafiltration method was performed to investigate the specificity of the two selected aptamers. In the same manner, 1 μM (as final concentration) of denatured solution of each aptamer in binding buffer was incubated with 100 μM (as final concentration) of the counter antibiotics for two hours at room temperature. After the incubation, the whole solution was passed through the Ultra 0.5 mL 3 KDa centrifugal filter. By performing this separation, the free counter antibiotic molecules were separated from those bonded to aptamers. The concentration of free counter antibiotic molecules was determined using the UV-Vis spectroscopy. By using the obtained concentration of free counter antibiotics and the initial concentration of the antibiotic solution, the fraction of bound molecules was calculated.

Investigation of the Aptamer- Antibiotic Binding Using UV-Vis Spectroscopy. 50 mM solution of each antibiotic in the binding buffer was prepared and their UV-Vis spectra were recorded using A double-beam UV-Vis spectrophotometer (model: UV-250IPC, Shimadzu Co., Kyoto, Japan) instrument. The aptamer-antibiotic complex solution was prepared by incubation of 1 μM (as the final concentration) denatured aptamer with related antibiotic (50 mM as the final concentration) in the binding buffer at room temperature for two hours. The UV-Vis spectra of antibiotics in interaction with aptamers were recorded. The blank solution for this step was the binding buffer contained an equal concentration of denatured related aptamer. The values were calculated using Beer-Lambert equation.

Investigation of the Aptamer- Antibiotic Complex Formation Using High-Resolution Mass Spectrometry. The mass spectrometry analysis was performed on high-resolution ESI-Q-TOF (model: SYNAPT/G1, High Definition, Waters, Massachusetts, USA). The MS instrument was set at negative mode. All instrument parameters were kept in a soft condition to prevent fragmentation of the aptamer-antibiotic complex. The capillary potential was set at 3.15 V. The source temperature was set at 120°C and the temperature of the ion source was kept around 350 ± 1°C. The sampling cone and extraction cone voltage were tuned at 80 V and 101.8 V, respectively. The flow rate was 50 μL/min. The mass spectra were recorded on centroid mode. All mass spectra were analyzed by MassLynx 4.1 (Waters, Manchester, UK).

The monoisotopic mass of aptamer-antibiotic complexes with all possible charges was calculated using the handbook of chemistry & physics (2016–2017)[46]. The relative mass measurement error regarding experimental mass value was calculated using the Eq. 2[47].

$$Mass Measurement Error\ (ppm) = \left[\frac{M_{Exp.} - M_{Cal.}}{M_{Cal.}}\right] \times 10^6 \text{ (Eq. 2)}$$

Where $M_{Exp.}$ is the experimental mass and $M_{Cal.}$ is the calculated mass. For assigning the aptamer-antibiotics complex peaks, only the m/z values with a relative mass error lower than 5 ppm were taken

into account.

The 10 μM solution of each aptamer was prepared separately in 150 mM ammonium acetate with pH 7 and after the denaturing process, each one was incubated for two hours with its related antibiotics. The final concentration of aptamer and antibiotics in ammonium acetate was 5 μM and 50 μM, respectively. Just before the injection, 10 μL of each sample solution was diluted in 90 μL equally premixed of 10% aqueous ammonium solution and methanol.

Results And Discussion

SELEX Procedure. In this study, GO-SELEX was carried out as an enrichment process for selecting aptamers against ENR and OFLO. GO-SELEX is a method that allows the aptamers and small molecules to interact in solution without any hindrance. The experiment design was based on the principle that the free ssDNA can adsorb onto the GO surface by π-π stacking and hydrogen bonding interactions. This circumstance provides the opportunity to remove ssDNA's from the solution to eliminating those or collecting for incubating them with a target. As both ssDNA and GO have a negative charge, the adsorption can take place in a buffer with an appropriate salt concentration. The prior studies showed the adsorption of ssDNA on GO surface in such a buffer can just be accomplished in a minute[48,49].

The used binding buffer contained 100 mM NaCl, 5 mM $MgCl_2$, and 20 mM Tris-HCl at pH 7.4. The solution with the mentioned ionic strength and pH mimics the biological condition that not only leads to the adoption of a stable spatial structure by aptamer but also this condition facilitates the adsorption of ssDNA on GO Sheets. It should be noted that the zwitterionic antibiotics ENR with pka_1 5.88 and pka_2 7.74 and OFLO with pka_1 6.06 and pka_2 8.22 exist in a neutral zwitterionic form in water and they do not bear any charge in that pH.[50] The applied GO-SELEX was operated in three styles to finding the selective and specific aptamers against ENR and OFLO. The used three styles are demonstrated in Fig. 1.

In the initial cycles of the SELEX procedure, by first incubating the aptamer and antibiotic and then adding GO to the solution, some oligo strands that had a chance to have interaction with antibiotic molecules were separated from the huge variety of strands in the random pool. The saturation percentage in the first three cycles of SELEX was between 4–9% (Figure SI-2). As in the initial cycles, all of the selection parameters were constant the saturation values did not fluctuate out of the above range. The low variation in the saturation values could arise from the efficiency of the ethanol precipitation method.

In the middle of the screening process, the counter selection was done, in which some of the antibiotics with similar molecular structures to the main antibiotics were incubated with the sub-pool, then the strands that did not have an affinity to the counter molecules were collected by GO. This process aimed to discard the strands that could be bound to similar structures. Different antibiotics were used as the counter molecules. One of them was ciprofloxacin that is a member of quinoline family antibiotics and it is structurally identical to ENR except for an ethyl group on the piperazinyl region. Other counter

antibiotics were florfenicol and Sulfabenzamide that belong to organo-fluorine and sulfonamide families, respectively.

In the final stages of selection, the denatured pool was first incubated with GO. Having washing GO with binding buffer, the target molecules were incubated with the washed GO together with the adsorbed ssDNA's on its surface. Under this condition, only strands that have a high affinity to antibiotic molecules detach from the GO surface, and by refolding they interact with antibiotic molecules. It can be said that in some cases the affinity to the target predominates over the interaction with GO, by nearing the target to the oligo strands on the GO surface, the interaction between these two molecules induced refolding of aptamer and results in detaching of the strand from the GO surface[48,49]. This process of selection has some advantages. By performing this process, the dsDNA that may exist in the solution would be eliminated and the only strands that have a high affinity to the target will be selected.

From the results (Figure SI-2) it can be seen that in SELEX cycles the most fluctuation in saturation values is contributed to changing the selection strategy or increasing the pressure on the enrichment process like descending the aptamer concentration or shortening the incubation time.

Cloning and Sequencing. The selected sequences at the end of the SELEX procedure were cloned by Thermo Scientific PCR Cloning Kit. Having extracted the plasmid, two different PCR reactions were carried out to pick the right strands for sequencing. These PCR reactions were performed in two series, one with universal M13 primers and another with SELEX primers. In the former, it was expected to see the PCR products with 227 base pairs and in the latter with the SELEX primers, it was expected to see the PCR products with 72 residues. The product of these two kinds of PCR was run on 1% agarose gel and those plasmids that had two different and specific bands in front of the 100 and 300 base pairs band of the ladder, were selected for sequencing. The procedure is demonstrated in Fig. 2. The image of the agarose gel is represented in Figure SI-1B.

The NA sequence of the selected aptamers associated with the quinolone antibiotics is presented in table SI-1. The aptamers related to Enrofloxacin and Ofloxacin were named ENR-APT and OFLO-APT, respectively.

The analysis of the random part related to each aptamer was shown that the random part of ENR-APT and OFLO-APT contained 57.5% and 72.5% GC residues, respectively. Both aptamers were analyzed using the Pairwise Sequence Alignment online software from European Bioinformatics Institute (EMBL-EBI) site. The result of this analysis demonstrated that there was a 38.1% similarity in the paired format between the random parts of two aptamers (Table SI-2).

Analyzing the Secondary Structure of Aptamers. The secondary structure of both aptamers was illustrated in figure SI-3. The predicted Gibb's free energy for ENR-APT and OFLO-APT were − 16.68 $kcal.mole^{-1}$ and − 20.30 $kcal.mole^{-1}$, respectively. The predicted secondary structure of both ENR-APT and OFLO-Apt have the stem-loop (hairpin) patterns that two and three bulges can be seen in the stem part of

ENR-APT and OFLO-Apt, respectively. In both aptamer's secondary structures two mismatches between G and T can be seen in the stem region and both of them have a single strand segment at the 5' and 3' end.

Dissociation Constant (K_d). To evaluate the level of dissociation tendency of aptamer-antibiotic complex, the equilibrium filtration method was carried out. This assay was performed in the light of the assumption that the ratio of aptamer-antibiotic binding in solution is 1:1. The calculated K_d were 12.025 ± 0.007 μM and 6.98 ± 2.44 μM, corresponding to aptamer-ENR and aptamer-OFLO complexes, respectively. The saturation binding curve plot is represented in Fig. 3.

Analyzing Binding Assay of Aptamers for Quinolone Antibiotics. The result of the specificity binding experiments showed that both aptamers had a similar affinity to the three fluoroquinolone family antibiotics; enrofloxacin, ciprofloxacin, and ofloxacin. This result is depicted in Fig. 4. By contrast, both aptamers recognized the quinolone antibiotics from the sulfabenzamide and florfenicol.

As it is demonstrated in Fig. 5 all the three quinolone antibiotics have a quinolinemonocarboxilic scaffold and all the functional groups that interact with aptamers (e.g. the fluoroquinolone scaffold and piperazinyl region) in the used quinolone antibiotics are identical, then it is not far from the mind that both aptamers showed affinity to all of them. Also, the indiscrimination of both aptamers showed that the bulky ethyl and methyl groups do not affect the interaction between antibiotics and aptamers. This evidence clarified that most probably the complementarity in shape has not an effective role in the interaction between the isolated aptamer and its target.

Despite large biomolecules (like; proteins, etc.) that have high diversity in their functional groups which result in very specific interaction with aptamers, the lack of various epitopes in small molecules leads to lower affinity and lower specificity between them and the aptamers[14]. There are several reported cases in which the introduced aptamer could not distinguish between structurally very similar small molecules[39,51–55]. For instance, Stojanovic et al. have reported an aptamer that could not distinguish between some cinchona alkaloids. They raised the hypothesis that the aromatic motif is the main recognized part of these small molecules therefore their aptamer responded to alkaloid structures with a similar aromatic region[53]. In another study, Gülbakan et al. presented a cocaine aptamer that could bound to another alkaloid even stronger than its main target. Regarding another aptamer related to the adenosine, they represented that this aptamer had the affinity to several analogs of adenosine with the same hydroxyl group on the sugar ring. They expressed that the prior studies confirmed the important role of these hydroxyl groups in the interaction with aptamer[39].

So far in this study, it has been proposed that indiscrimination of aptamers between the quinolone antibiotics arises from the similarity of antibiotic structures. But it should be noted that the alignment between the sequences of two aptamers revealed that there is a 38% similarity in their nucleotides. Another hypothesis that was brought up by this fact, is the similar nucleotides of both aptamers could be located in the binding site. But this needs further investigation to find out which part of the aptamer has the most important role in the interaction with antibiotics. Since both isolated aptamers can distinguish

quinolone antibiotics from the other class of antibiotics significantly, they have the potential to be used as the recognition element in apta-sensors to monitor the amount of this family of antibiotics in the different matrices.

Investigation of the Aptamer- Antibiotic Binding Using UV-Vis Spectroscopy. To study more about the antibiotic-aptamer complex and to trace changing the small molecule's optical properties, an UV-Vis spectroscopy assay was performed. In this regards, a solution of each antibiotic in the absence of aptamer and in complex with aptamer was prepared and the value of both ENR and OFLO was determined. The comparison of the obtained value related ENR and OFLO, in the absence and in the interaction with aptamer, revealed that 41.98 ± 0.18 % and 22.45 ± 0.40 % reduction happened in value associated to complex formation between ENR and OFLO with their related aptamers, respectively. And also 3 and 1 nm red shift was observed in λ_{max} of ENR and OFLO, respectively (Fig. 6). This finding confirmed that the aromatic region of both antibiotics was involved in the interaction with the aptamer strand.

The aromatic region, carboxylic group, carbonyl, and flour of quinolone antibiotics, are placed on a flat surface and the flat region of small molecules can be located in a coplanar position with the aptamer bases. As a result of this coplanar position, they can interact with each other by π-π stacking [56]. As a consequence of this interaction, less energy is needed for electronic excitation so hypochromic effect and bathochromic shift appear in small molecule's UV-Vis spectrum. Previous studies claimed that in some cases the extinction coefficient of the small molecule could be reduced 40%-60%[36,57].

Investigation of the Aptamer- Antibiotic Complex Formation Using High-Resolution Mass Spectrometry. All the advantages of mass spectrometry that were mentioned before, required us to apply this method for more investigation into the formation of the aptamer-antibiotic complex. In this regard, each aptamer was incubated with its related antibiotic in ammonium acetate buffer with pH 7. Even though the monovalent ions (Na^+) and divalent ions (Mg^{+2}) in binding buffer help in adopting the spatial folding of oligonucleotides by decreasing the electrostatic repulsion between the phosphates groups in the polyanionic backbone of oligonucleotides strands, they also cause the aggregation of DNA strands in the droplets formed in the ion source which results in the suppression of oligonucleotides peaks in the spectrum. For this reason, ammonium acetate is used instead of others salts. In the gas phase, ammonium ions transfer one proton to oligo strands and become neutral species in form of the gas. By proton transferring, the salt interfering is eliminated moreover results in neutralizing some phosphate groups in oligonucleotide strands and also by reducing the negative charge and decreasing repulsion between phosphate groups, the oligo strands can adopt the stable 3D structure[39,40,58].

The recorded MS spectra are shown in Figs. 7 and 8. In MS spectra related to Aptamer-ENR and Aptamer-OFLO complexes, 22 and 27 peaks with wide charge distribution were identified, respectively (tables SI-4 and SI-5). All the recognized peaks were at the ratio of 1:1 between aptamer and antibiotics.

Gülbakan et al. made the hypothesis that the aptamer strands with less negative charge have more impact spatial structure in the gas phase and this could result in the formation of a more stable complex with the ligand[39]. But our obtained results were not inconsistent with their hypothesis and several peaks corresponding to aptamer-antibiotic complexes with a wide range of charge distribution were observed in mass spectra. The charge distribution of complexes is represented in figure SI-4.

Keller et al. previously presented some evidence stating that in the vacuum and in the absence of solvent competition, the hydrogen bonding and electrostatic interaction between aptamer and target become stronger. So in the aptamer-target complex where the H-bonding and electrostatic interaction is the predominant kind of interaction, they are more stable in the gas phase. But the π-π interaction would be the last non-covalent bonding that survives in the gas phase. In general, the kind of interaction between small molecules and aptamers will determine the stability of their complex in the gas phase[41]. As in both obtained mass spectra of Aptamr-ENR and Aptamer-OFLO complexes, several peaks were identified. It can be concluded that the formed complexes were stable in the gas phase and this stability in the gas phase can reflect the contribution of H-bonding or electrostatic interaction between aptamer and antibiotic.

The obtained evidence by specificity test, UV-Vis spectroscopy, and mass spectrometry suggested the predominant interaction between the isolated aptamer and the quinolone antibiotics are H-bonding, π-π interaction, and electrostatic interaction. In our opinion, every study and research about the interaction between aptamers and small molecules can lead to wider usage of the science of aptamers and help design more efficient Apta-sensors to be used for enhancing the quality of human health and even in environmental protection.

Declarations

Corresponding Authors

Hassan Rezadoost- Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Iran. Email: h_rezadoost@sbu.ac.ir

Hasan Rafati- Department of Pharmaceutical Engineering, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran. Email: h_rafati@sbu.ac.ir

Notes

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Figures

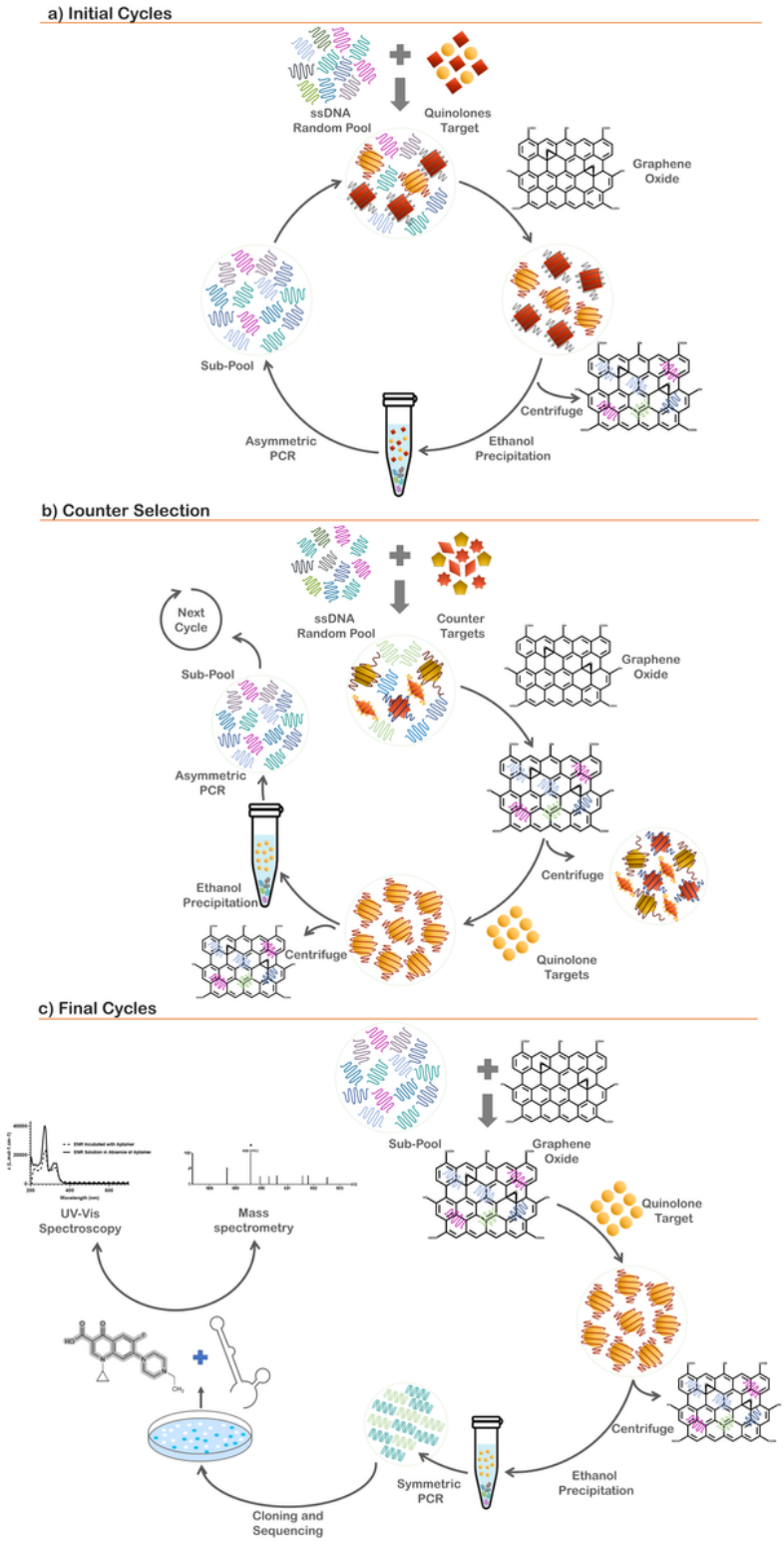

Figure 1

The three different performed styles of GO- SELEX

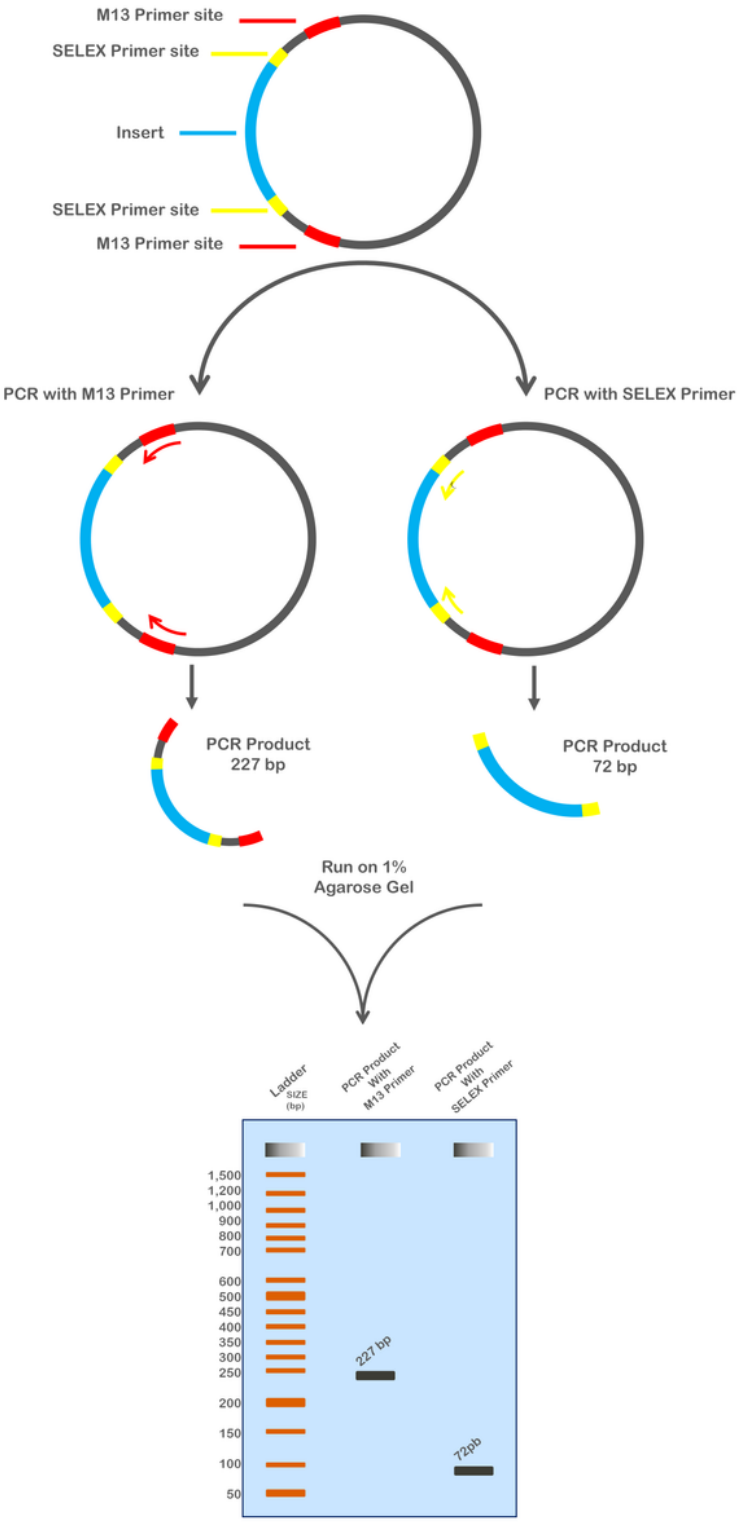

Figure 2

The PCR procedure of extracted plasmid using two different primers

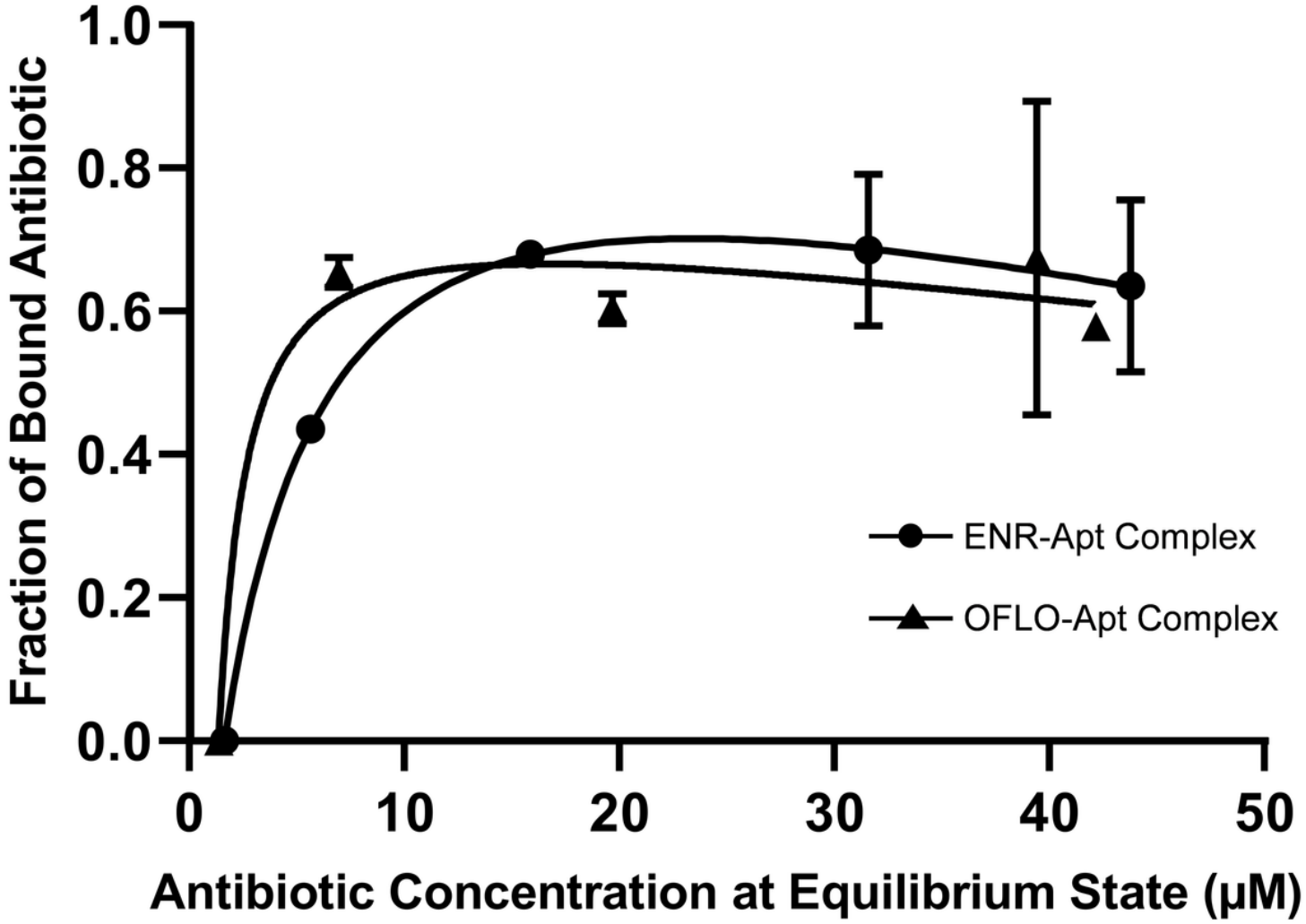

Figure 3

The saturation binding curve related aptamer-ENR and Aptamer-OFLO complexes

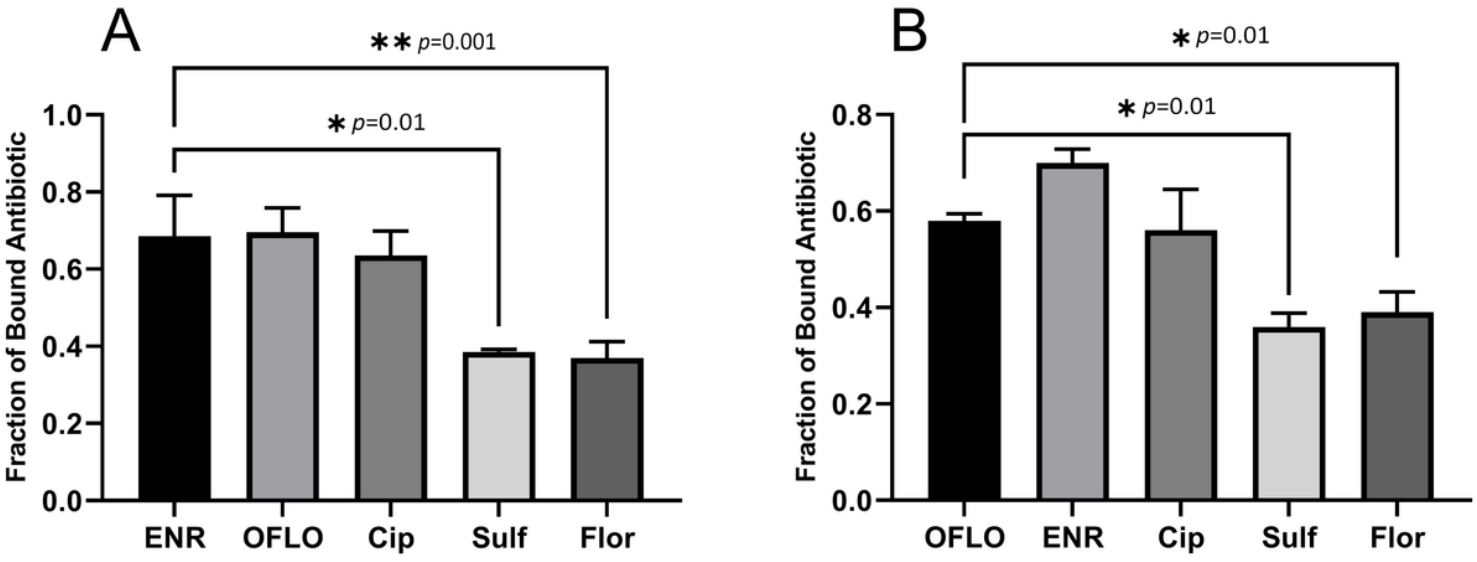

Figure 4

The result of specificity assay regarding (A) ENR-APT (B) OFLO-APT

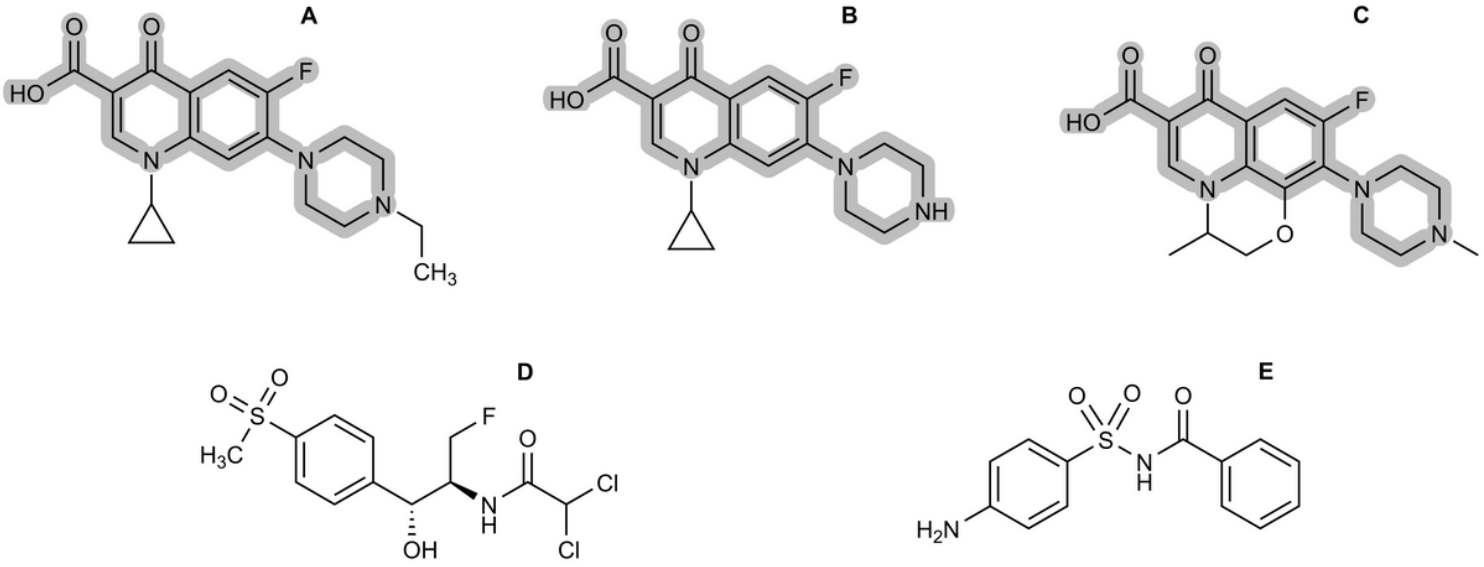

Figure 5

The chemical structures of (A) Enrofloxacin (B) Ciprofloxacin (C) Ofloxacin (D) Florfenicol (E) Sulfabenzamide

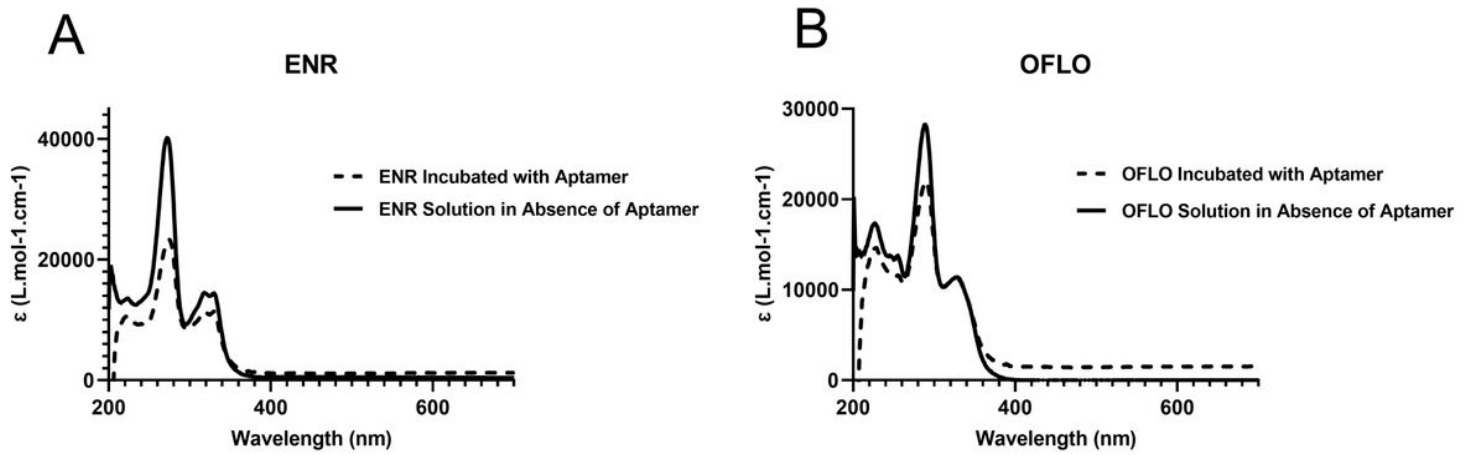

Figure 6

The UV-Vis spectra of antibiotics (A) Enrofloxacin (B) Ofloxacin – Before the incubation with aptamer (solid line) and after incubation with aptamer (dashed line)

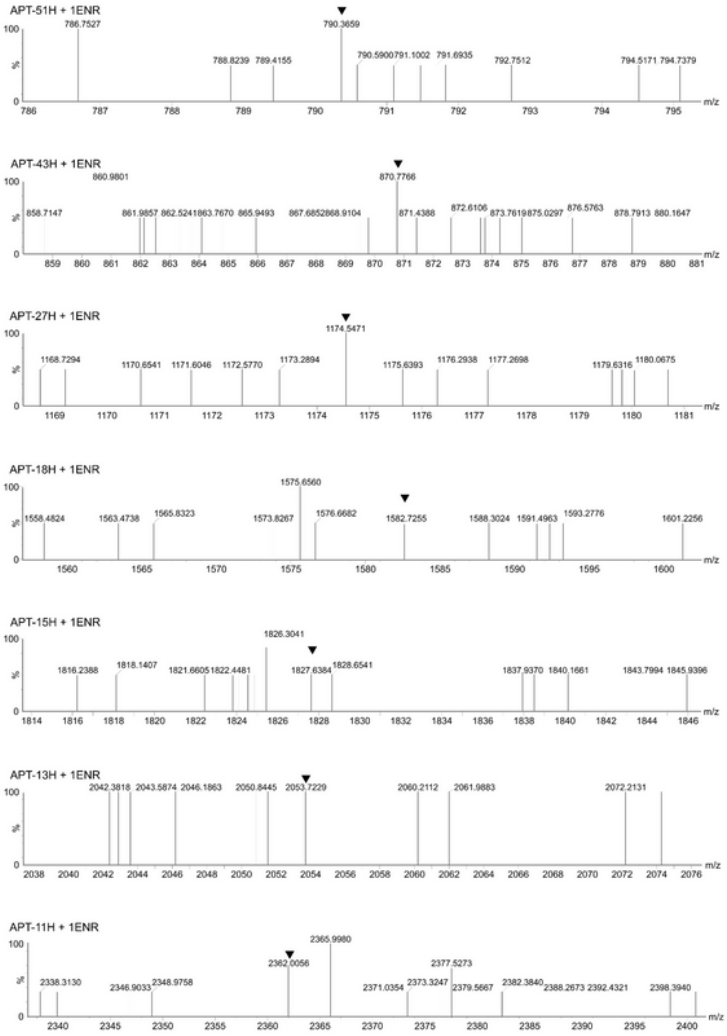

Figure 7

The Mass spectra of aptamer-ENR complex

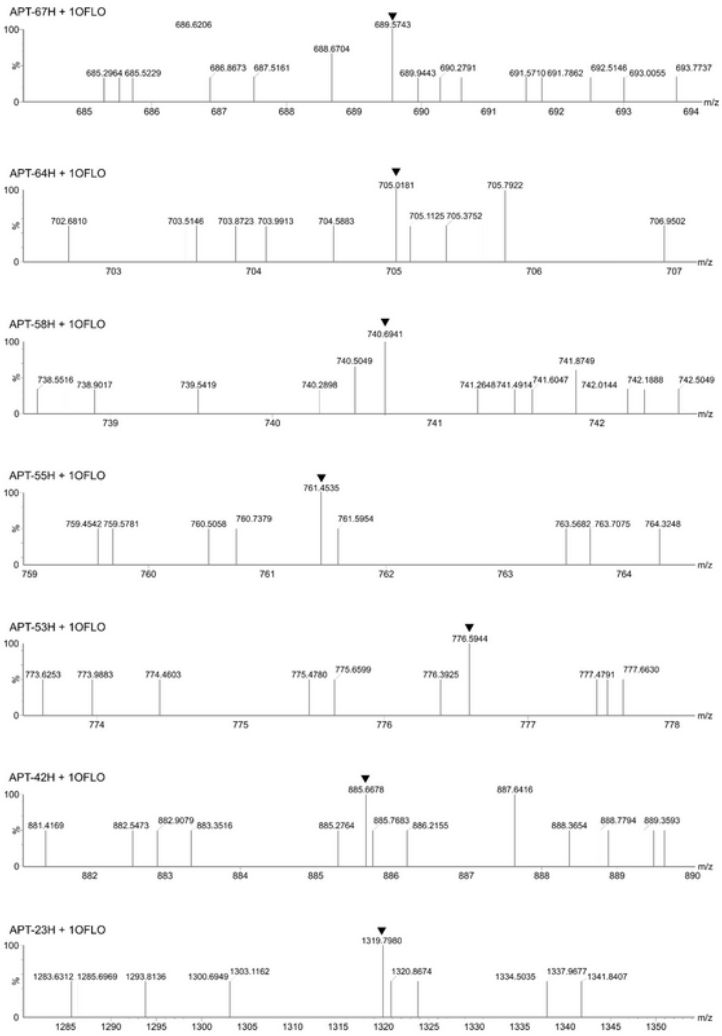

Figure 8

The Mass spectra of aptamer-OFLO complex

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