Kinetics of Binding Interaction between Norfloxacin and Monoclonal Antibody Using Surface Plasmon Resonance

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Abstract—**Fluoroquinolones (FQs) are a group of antibiotics which have been used against both Gram-negative and Gram-positive bacteria in domestic animals. Residue of these antibiotics in food products could lead to drug resistance problem in human. As a result, surveillance detection program of these drug residues must be in practice to ensure safety of the consumers. Detections based on immunological method such as Enzyme-Linked Immunosorbent Assay (ELISA) and lateral flow immunoassay or strip test have been widely used as a screening tool in food safety applications. In this method, either an antibody with broad specificity to a group of FQs or several specific antibodies for each antibiotic are required for the assay to cover all FQs of interest. In this study, sensitivity of two monoclonal antibodies, mAb Nor132 and mAb Nor155, against norfloxacin was quantified by an antigen-captured indirect competitive enzyme linked immunosorbent assay. The sensitivity in term of 50% Inhibition Concentration (IC_{50}) of mAb Nor132 and mAb Nor155 was found to be 0.706 µg/ml and 0.072 µg/ml, respectively. Then, the binding interactions of both mAbs and norfloxacin were studied by Surface Plasmon Resonance (SPR). The equilibrium binding constants (K_D) of mAb Nor132 and mAb Nor155 were 1.152×10^{-8} M and 1.996×10^{-9} M, respectively. The results indicated that sensitivity and affinity of mAb Nor155 were higher than those of mAb Nor132. These suggested that mAb Nor155 was more suitable than mAb Nor132 for using in the development of norfloxacin detection.**

Index Terms—**norfloxacin, monoclonal antibody, ELISA, surface plasmon resonance, Biacore, antibody detection**

I. Introduction

Fluoroquinolones are a group of antibiotics extensively used against both Gram-negative and Gram-positive bacteria due to their inhibition activity of DNA gyrase, topoisomerase and other enzymes essential for bacterial DNA replication. Norfloxacin is one of fluoroquinolone antibacterial agents with a fluorine at position 6 and a piperazine ring at position 7 [1]. Consequently, norfloxacin is widely used for treatment of pathogenic microorganism infections in domestic animal. However, misuse of these antibiotics such as overdose treatment and long term use to prevent infection could lead to problems of drug residues in animal products for human consumption [2]. Unintentionally long-term consumption of these contaminated products could result in a more severe problem of antibiotic resistance in human. Therefore, to ensure its residual concentration in animal-derived foods at an acceptable level, Food and Drug Administration of many countries had set the Maximum Residue Limit (MRL) and developed the surveillance program for these drug residues in foods. The most widely used method for drug residue screening detection is based on immunological methods such as Enzyme-Linked Immunosorbent Assay (ELISA) and lateral flow immunoassay (strip test). In both methods, capability of antibody for detecting and binding to a very low amount of antigen or drug residue is crucial for the assay. Besides sensitivity, specificity which is the ability of any antibody to bind with a specific antigen is also equally important. If the antibody used in the assay could bind to many antigens, the assay might give a false positive result, thus reducing the effectiveness and reliability of the tests. In general, sensitivity and specificity of the antibodies are analyzed and compared in order to select the most suitable antibody for the assay development. However, sometimes this information is not enough for the selection. Binding affinity of each antibody to different antigens also comes into considerations. The information of affinity such as association constant and dissociation constant can be obtained by investigation of the binding kinetics between antibodies and antigens using Surface Plasmon Resonance (SPR) technique. Affinity and kinetics of molecular interaction determined by SPR is important to understand protein interactions [3].

SPR is a label-free biosensor technique for studying interactions between all classes of biomolecules and biochemical mechanisms in real time [4], [5]. The ligand

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of interest is immobilized on the surface of the sensor chip and solutions with different concentrations of the analyte are allowed to flow over it. The increase in mass associated with a binding event causes a proportional increase in the refractive index, which is observed as a change in response [6]. SPR biosensor has been used in various fields such as drug discovery, medical diagnostics, safety and environmental control [7], [8] and especially characterization of antibody-antigen interactions for approximately two decades [9], [10].

In this study, kinetics of interaction between norfloxacin and monoclonal antibodies against norfloxacin were studied. The obtained information will be useful for antibody selection and further development of fluoroquinolone detection or other applications.

II. Methods and Materials

A. Production and Purification of mAbs

1) Antibody production

Monoclonal antibody producing clone Nor132 and Nor155 were obtained from Institute of Biotechnology and Genetic Engineering (IBGE), Chulalongkorn University. Cryotube of each monoclone was taken from liquid nitrogen storage and immediately placed into a 37°C water bath until a small bit of ice left in the vials. Cells were suspended in RPMI 1640 medium and centrifuged at 1,500 rpm for 5 min to remove DMSO. Then, cell pellets were resuspended in RPMI 1640 medium supplemented with 20% FCS and cultured in 5% CO_2 incubator at 37 °C.

2) Protein purification [11]

Each monoclonal antibody (mAb) was purified using an ÄKTA affinity chromatography with HiTrap Protein G HP antibody purification column (GE Healthcare). The column was equilibrated with 2 mM phosphate buffer (PB), pH 7.0 at a flow rate of 1.0 ml/min. Prior to media loading (1 litter), unbound proteins were washed out with 30 ml PB. MAb was eluted with 0.1 M glycine-HCl buffer (pH 2.7). Then mAb was fractionally (1ml/fraction) collected in 70 µl of 1M Tris-HCl buffer (pH 9.0). Finally, fractions containing mAb was combined, aliquoted and stored at
-20 °C until further use.

B. Determination of Protein by BCA Protein Assay

Protein concentration was quantified by BCA Protein Assay kit (Pierce Company). Assay procedure was employed according to the manufacturer's instructions. Briefly, 25 µl of diluted protein samples were separately transferred into each well of microtiter plate. The BCA working reagent (WR) was prepared by mixing reagent A with reagent B (50:1, Reagent A: B) and added (200 µl) to each well. The microplate was incubated at 37 °C for 30 min and measured the absorbance at 560 nm using microplate reader. Bovine Serum Albumin (BSA) was used as standard.

C. Protein Analysis by SDS-PAGE

Gel solutions for 15% separating gel and 5% stacking gel were prepared. Antibody samples were diluted to 2 µg/ml and mixed with Next Gel® Sample Loading buffer, 4X (AMRESCO, USA) then the samples were boiled for 10 min. After that the samples and molecular weight standards were loaded (20 µl/well) onto the gels. Electrophoresis was carried out at a constant current of 25 mA per gel with 1×Tris-glycine–SDS running buffer. Following electrophoresis, gels were stained with coomassie blue and de-stained with a destaining solution.

D. Conjugation of NOR-OVA

Norfloxacin was conjugated to OVA by carbodiimide active ester method (modified from Watanabe, H., et al. (2002)). Norfloxacin (20 mg), N-hydroxysuccinimide (NHS) (10 mg) and 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (10 mg) were dissolved in dimethylformamide (DMF, 1 ml) and the solution was stirred at room temperature for 30 min. The reactant solution was added dropwise to the OVA solution (50 mg in 3 ml PBS). The solution was stirred at room temperature for 2 h and dialyzed 3 times in PBS at 4 °C. Finally, the drug-OVA solution was filtrated with 0.2 µm cellulose acetate membrane and kept at -20 °C until use.

E. Antigen-Captured Indirect Competitive ELISA [12]

The 96-well plates were coated with ovalbumin conjugated norfloxacin (NOR-OVA) (100 µl/well) at 4 °C overnight. The plates were washed three times with 10 mM phosphate buffer saline, pH 7.4 containing 0.05% Tween® 20 (washing buffer or PBST) then blocked with skim milk (300 µl/ well) at 37 °C for 1 h. The plates were washed three times with washing buffer. Various concentrations of unconjugated free norfloxacin and the monoclonal antibody of interest were added (50 µl/well) into each well and the plates were incubated at 37 °C for 2 h. After three washing steps, goat anti-mouse IgG-HRP was added (1:5000 in PBS, 100 µl/well) into each well and the plates were incubated at 37 °C for 1 h. After another three washing steps, tetramethylbenzidine substrate solution (TMB) was added (100µl/well) and the reaction was allowed to occur for 15 min in the dark at room temperature. The enzymatic reaction was stopped by adding 1 N H_2SO_4 (100 µl/well) and the absorbance was measured at 450 nm using microplate reader.

Sensitivity of the mAb was quantified in terms of 50% inhibition concentration (IC_{50}). The IC_{50} of each compound was determined using the following formula: IC_{50} = 50% (B/B_0) where B_0 and B are the average absorbance obtained from the icELISA with different concentrations of the competitors (B) and without the competitors (B_0).

F. Immobilizing NOR-OVA on CM5 Sensor Chip

The amine coupling kit from Biacore was used in the immobilization. Sensor chip (CM5) was activated with freshly prepared NHS and EDC solution at a ratio of 1:1. Antigen (NOR-OVA) was diluted in 0.1 M sodium acetate buffer (pH 4.5) and immobilized for 30 min on two flow cells of a CM5 chip at 25 °C. The ligand level was calculated using the formula below:

$$R_{max} = (analyte\ MW/ligand\ MW) \times Stoichiometry \times R_L$$

where R_{max} is maximum theoretical response of the analyte for a given ligand level, and R_L is amount of ligand immobilized. The ligand was injected over the activated surface. Then, ethanolamine was used to deactivate the remaining active esters on the surface. The CM5 chip has four flow cells. In this paper, flow cell 2 was immobilized ligand while flow cell 1 as reference.

G. SPR Study of Antigen-MAb Binding

Binding kinetics of mAb Nor132 and mAb Nor155 were measured by Biacore T200. Antigen (NOR-OVA) was diluted to 0.1 µM in 0.1 M sodium acetate buffer (pH 4.5) and immobilized for 30 min on flow cells 2 of a CM5 chip at 25 °C. Then mAb (analyte) was diluted to 70-400 nM in HBS–EP+ buffer and injected with a contact time of 400 s at 10-30 µl/min and a dissociation time of 600 s. The surface was regenerated each time with 50 mM NaOH (contact time 30 s at 30 µl/min). Equilibrium binding constant (K_D) was calculated as follow: $K_D = [A][B]/[AB] = k_d/k_a$ [13].

H. Circular Dichroism Spectroscopy Analysis

The secondary structures of mAb Nor155 was determined with a CD spectropolarimeter (Model J-810, Jasco, Japan). Far-UV CD spectra were recorded at wavelengths between 190 and 240 nm using a 0.1 cm path length cell at 25 °C with a protein concentration of 0.2 mg/ml in phosphate buffered saline (PBS), pH 7.4. Each spectrum was a representative of three scans. The CD spectra were corrected for buffer contributions.

III. Results and Discussion

A. Determination of NOR-OVA and mAbs Concentration

The norfloxacin drug has a molecular weight of 319.33 Da which is unsuitable to be immobilized on the well surface of ELISA plate due to its small size. Therefore, the drug must be conjugated to a carrier protein. In this paper, the drug was conjugated to 42.7 kDa ovalbumin (OVA) using carbodiimide active ester method. Carboxyl group of norfloxacin formed peptide bond with amino groups of OVA. The concentration of NOR-OVA and mAbs quantified by BCA Protein Assay kit were shown in Table I.

TABLE I. Protein Concentrations of NOR-OVA and Purified mAbs by BCA Assay

Protein	Concentration of protein (mg/ml)
Norfloxacin-OVA	2.3464
mAb Nor132	2.1200
mAb Nor155	4.7947

After purification, the molecular weight and purity of mAb were checked by SDS-PAGE as shown in Fig 1. The samples were loaded onto gels as lane 2 and 3 which were mAb Nor132 and mAb Nor155, respectively. The result shown that the two-protein bands were found at approximately 25 kDa and 50 kDa. As compared to other report [14], [15], mAb is made up of a heavy chain of approximately 50 kDa and a light chain of approximately 25 kDa.

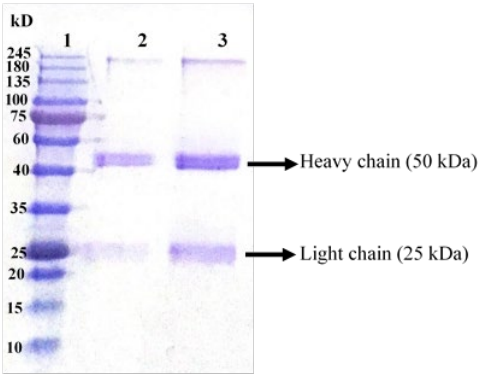

Figure 1. SDS-PAGE of purified mAbs; standard protein marker (lane1), mAb Nor132 (lane2), and mAb Nor155 (lane3).

B. Sensitivity of the Monoclonal Antibody

In the Ag-captured indirect competitive ELISA, the mAb can fully bind to the NOR-OVA conjugate immobilized on the well surface of a 96 well when no competitor is presented in the solution, thus yielding the maximum absorbance value monitored by the goat anti-mouse IgG-HRP. If any competitor is presented in the solution, the competitor can bind to the mAb. As a result, the bound mAb cannot bind to the immobilized NOR-OVA conjugate, thus yielding the lower absorbance value. The amount of the competitor which can reduce the absorbance value to 50% of its maximum value can be used to represent the sensitivity of amAb to that competitor.

Sensitivity of mAb Nor132 and mAb Nor155 was determined in terms of 50% inhibition concentration (IC_{50}) by Ag-captured indirect competitive ELISA. The lower IC_{50} means the higher sensitivity. The IC_{50} values of mAb Nor132 and mAb Nor155 were found to be 0.7055 µg/ml and 0.0716 µg/ml, respectively (Fig 2.). This result indicated that both mAbs were sensitive enough to detect norfloxacin residual at the MRL currently enforced at 0.02-0.1 µg/ml.

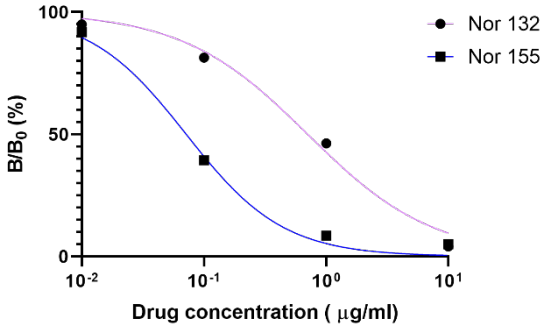

Figure 2. Dose-response curve of the monoclonal antibody analyzed by Ag-captured indirect competitive ELISA.

C. Binding Kinetics Studies via SPR

SPR signals related to mAbs concentration and binding ability of mAbs were shown in Fig. 3. The association

time was from 0-400 s, then the dissociation time was started when the injection was stopped, and only buffer was flowing. The SPR sensorgram of mAb Nor155 (Fig. 3B) was higher SPR signals and equilibrated faster than mAb Nor132 (Fig. 3A). The kinetic of mAb Nor132 and mAb Nor155 were calculated by the Biaevalution software using a 1:1 Langmuir binding model (Table II). The kinetic association constants (k_a) of mAb Nor132 and mAb Nor155 are higher than the kinetic dissociation constants (k_d). These data indicated the strength of interaction between antibody and antigen [16]. The equilibrium binding constants (K_D) of mAb Nor132 and mAb Nor155 were 1.152×10^{-8} M and 1.996×10^{-9} M respectively. It has been reported that low K_D value indicates high affinity between the analyte and the ligand [16]. Therefore, the result suggested that mAb Nor155 bound to norfloxacin better than mAb Nor132 did. Therefore, mAb Nor155 should be selected for development of norfloxacin detection based on antigen-antibody binding methods.

TABLE II. KINETIC PARAMETERS OF BINDING BETWEEN NORFLOXACIN AND MABS

mAbs	k_a (1/Ms)	k_d (1/s)	K_D (M)
Nor132	2.281×10^{4}	2.628×10^{-4}	1.152×10^{-8}
Nor155	2.206×10^{5}	4.401×10^{-4}	1.996×10^{-9}

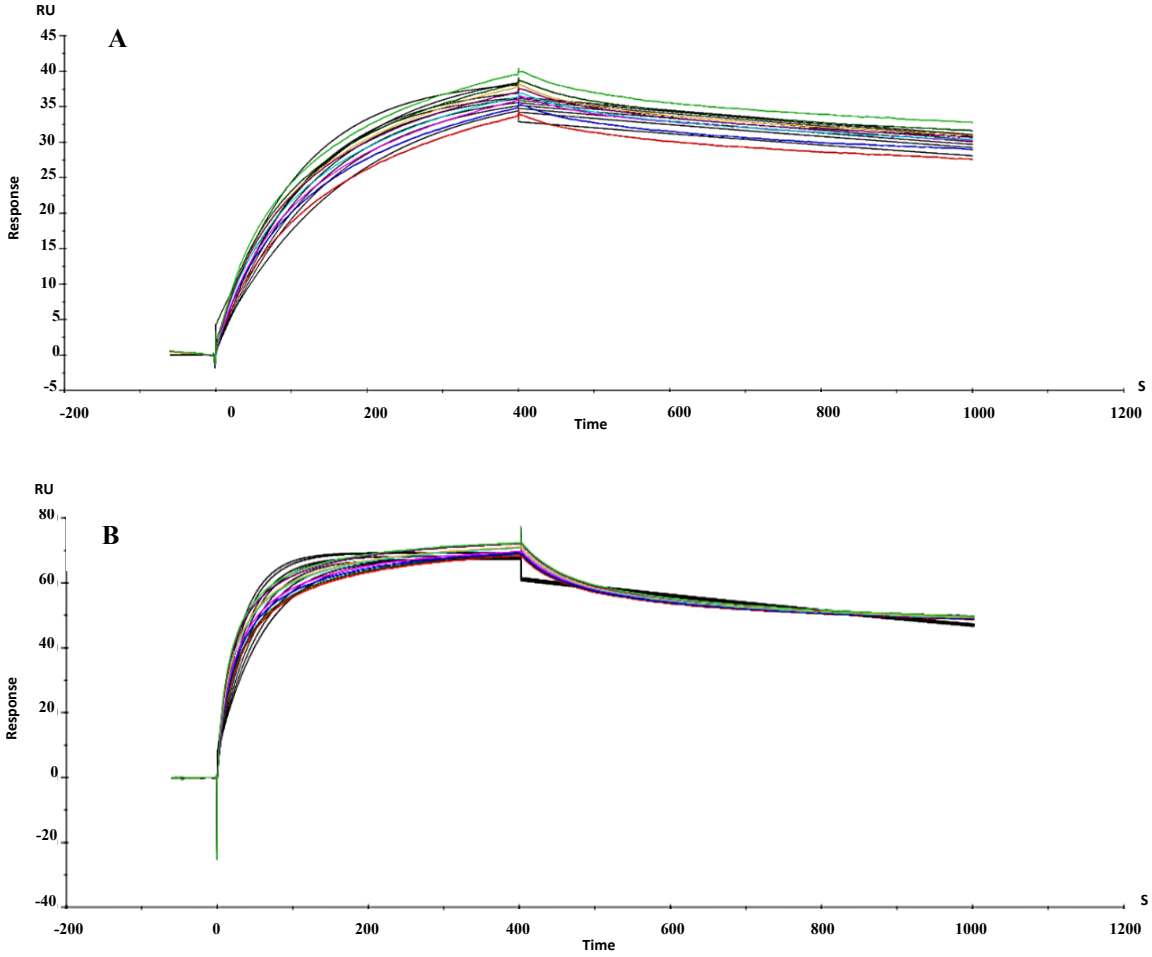

Figure 3. SPR sensorgrams of mAbs at different concentrations; (A) concentration of mAb Nor132 at 240, 280, 320, 340, 360, 380, 400 nM and (B) concentration of mAb Nor155 at 70,80,90,100,110,120,130,140 nM on CM5 sensor chip which Nor-OVA was immobilized and the association and dissociation times were 400 and 600 s, respectively.

D. Conformational Analysis of mAb Nor155 Using CD Spectroscopy

In general, CD spectroscopy has been used to study the conformation of protein in solution. Far-UV CD spectra were recorded at wavelengths between 190 and 240 nm and were directly related to the asymmetrical packing of the intrinsically chiral peptide bond groups of the secondary structure of the protein [17]. Far UV CD data of mAb Nor155 dissolved in PBS buffer (Fig. 4.) and the secondary structure analysis of mAb Nor155 (Table III) was analyzed by the CDNN software. The analysis showed that the predicted secondary structure elements of the mAb Nor155 consist of β-sheet 61%, β-turn 5.1% and random coil 25.2%. These results are resembled with the reported structure of antibody catumaxomab which included α helical 6.2%, β-sheet 37.5%, β-parallel 5.4%, β-turn 18%, and random coil 34.9% [18]. According to Joshi, et al. (2014), the secondary structure elements in IgGs consists of the antiparallel β-sheet, β-turns and

random coil conformations. Moreover, α- helices are found in some bends of mAb.

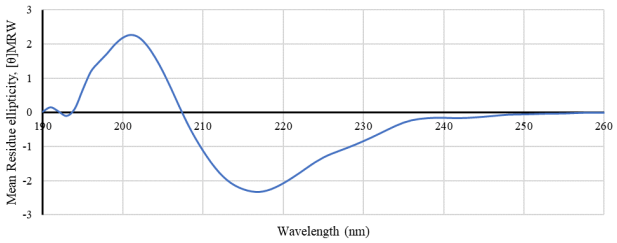

Figure 4. Far UV CD spectra of the mAb Nor155.

TABLE III. SECONDARY STRUCTURE ANALYSIS OF MAB NOR155 IN PBS BUFFER

%Helix	%Strand (Beta sheet)	%Turn	%Unordered (Random coil)	Total (%)
0	69.6	5.1	25.2	99.9

IV. CONCLUSION

Sensitivity of mAb Nor155 and mAb Nor132 was high enough to detect norfloxacin at the currently enforced maximum residue limit. SPR study indicated that the binding of mAb Nor155 to norfloxacin was stronger than that of mAb Nor132. Therefore, mAb Nor155 should be selected for applications in norfloxacin detection.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Patamalai Boonserm, Songchan Puthong conducted the research; Sajee Noitang, Thanaporn Wichai supported and facilitated for equiptment utilization; Patamalai Boonserm, Pongsak Khunrae analyzed the data; Kittinan Komolpis and Sarintip Sooksai advised and wrote the paper; all authors had approved the final version.

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