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Development of an optical surface plasmon resonance biosensor assay for (fluoro)quinolones in egg, fish, and poultry meat

A.-C. Huet[a,*], C. Charlier[a], G. Singh[b], S. Benrejeb Godefroy[b], J. Leivo[c], M. Vehniäinen[c], M.W.F. Nielen[d,e], S. Weigel[d], Ph. Delahaut[a]

[a] CER Groupe, Laboratoire d'Hormonologie, Rue Point du Jour 8, 6900 Marloie, Belgium
[b] Food Research Division, Health Canada, Ottawa, Ontario, Canada, K1A 0L2
[c] Turun Yliopisto, Faculty of Natural Sciences, Tykistökatu 6 A, 6th Floor, 20520 Turku, Finland
[d] RIKILT – Institute of Food Safety, Wageningen UR, P.O. Box 230, 6700 AE Wageningen, The Netherlands
[e] Wageningen University, Laboratory of Organic Chemistry, Dreijenplein 8, 6703 HB Wageningen, The Netherlands

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ABSTRACT

The aim of this study was to develop an optical biosensor inhibition immunoassay, based on the surface plasmon resonance (SPR) principle, for use as a screening test for 13 (fluoro)quinolones, including flumequine, used as veterinary drugs in food-producing animals. For this, we immobilised various quinolone derivatives on the sensor chip and tested binding of a range of different antibodies (polyclonal and one engineered antibody) in the presence and absence of free (fluoro)quinolones. The main challenge was to detect flumequine in an assay giving good results for the other compounds. One antigen–antibody combination proved satisfactory: polyclonal antibodies raised against a dual immunogen and, on the sensor chip, a fluoroquinolone derivative. It was the first time that this concept of the bi-active antibody was described in the literature.

The assay, optimised for detection in three matrices (poultry muscle, fish, and egg), was tested on incurred samples prepared by liquid extraction followed by two washing steps. This rapid, simple method proved adequate for detecting at least 13 (fluoro)quinolones at concentrations below established maximum residue levels (MRLs). The reference molecule norfloxacin could be detected in the range of 0.1–10 $\mu g\,kg^{-1}$ in extracts of egg and poultry meat and in the range of 0.1–100 $\mu g\,kg^{-1}$ in extracts of fish. The determined midpoints of these calibration curves were about 1, 1.5 and 3 $\mu g\,kg^{-1}$ in poultry meat, egg and fish, respectively.

1. Introduction

The diverse ring structures of the quinolones (Fig. 1) share a number of common functional groups essential to their antimicrobial activity. In addition, various modifications have produced compounds with different physical, chemical, pharmacokinetic, and antimicrobial properties. For example, substitution at position 6 with a fluorine moiety markedly enhances activity against gram-negative pathogens and broadens the spectrum of activity against gram-positive

* *Corresponding author*. Tel.: +32 84 31 00 90; fax: +32 84 31 61 08.
E-mail address: ac.huet@cergroupe.be (A.-C. Huet).
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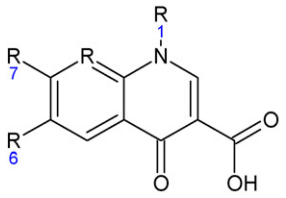


R=N in nalidixic acid, -CH or-CF in other compounds
R_1 and R_7 are different substituents
R_6 is F in fluoroquinolones

Fig. 1 – Chemical structure of quinolones and 6-fluoroquinolones.

pathogens [1]. These so-called fluoroquinolones (FQs) show efficacy against a variety of bacterial diseases and are indicated in the treatment of local and systemic diseases caused by a wide range of gram-negative and gram-positive bacteria, mycoplasmas, and chlamydiae. This wide distribution has raised concern over the possible emergence of FQ-resistant strains and resistance transfer between animal and human pathogens, especially since quinolone-resistant *Escherichia coli* strains isolated from animals and humans show the same resistance mechanisms. It is important to ensure that quinolones are used sparingly and appropriately, as highly resistant *E. coli* strains can be selected and may pass into the food chain [2].

If FQs lose their activity or are no longer available for the treatment of animal diseases, antimicrobial therapy of some diseases will be complicated. This may cause animal welfare, public health problems and economic losses. Currently there is no harmonised approach to regulating the use of FQs in food-producing animals in the different Member States of the European Union. Antimicrobial resistance should be addressed internationally, as resistant bacteria can spread via imported food. The potential risks of FQ overuse are thus realistic, and they also include a possible detrimental effect on the environment [3].

The majority of analytical methods reported in the literature for the analysis of FQs are based on chromatographic separation: liquid chromatography, gas chromatography, thin layer chromatography... Non-chromatographic methods for quinolone residue detection (immunoassays, luminescence-based assays) are quite rare [4].

Some enzyme-linked immunosorbent assays (ELISAs) [5–12] and optical-immunosensor-based assays [13,14] have been described for determining FQs in several matrices. Most of these methods were designed for determining individual quinolones, only four of them [5,9,11,12] being generic ELISAs. The current trend is no longer to develop an immunoassay for the determination of one specific FQ.

The aim of this investigation was to develop, using surface plasmon resonance (SPR) biosensor technology, a rapid and sensitive screening method for the analysis of twelve FQs plus oxolinic acid in three matrices. SPR biosensors exploit the properties of the evanescent field and rely, for signal generation, on a change in the refractive index of the medium near the surface [15]. Results obtained on incurred samples by means of our biosensor immunoassay were compared with the results of liquid chromatography-tandem mass spectrometry (LC–MS/MS).

This work is integrated into a European project aiming to develop new technologies to screen for multiple chemical contaminants in foods (http://www.biocop.org/).

2. Experimental

2.1. Materials

The following reagents were from Biacore AB (Uppsala, Sweden): CM5 sensor chips, HBS-EP buffer (0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20), borate buffer pH 8.5, and the amine-coupling kit [*N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and 1 M ethanolamine, pH 8.5].

Sarafloxacin, ofloxacin, flumequine, norfloxacin, ciprofloxacin hydrochloride, oxolinic acid, lomefloxacin, enoxacin, and nalidixic acid were provided by Sigma (St. Louis, MO, USA). Enrofloxacin was obtained from Bayer (Leverkusen, Germany), difloxacin hydrochloride from Chemos GmbH (Regenstauf, Germany), pefloxacin from Rhone Poulenc (Vitry sur Seine, France), danofloxacin from Pfizer Inc. (New York, USA), and marbofloxacin was kindly provided by UBE Europe (Lure, France). Individual stock standard solutions (100 $\mu g\,mL^{-1}$) were prepared as follows: 10 mg standard was dissolved in 10 mL of 1 M NaOH and adjusted to 100 mL with methanol. These solutions were found stable at −20 °C for at least 10 months. Working standard solutions were prepared by serial dilution in HBS-EP buffer.

Nicotinic acid and ethoxyquin were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The following reagents were used to produce immunogens: potassium carbonate (99%), 6-bromohexanoic acid (98%), sodium hydroxide (99.9%), sodium iodide (99%), and *N*-hydroxysuccinimide (NHS, 97%), provided by Aldrich (St. Louis, MO, USA); bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), ethylenediamine (EDA), isobutylchloroformate, ethylchloroformate, triethylamine, sodium borate, and 1,4-dioxane from Sigma–Aldrich (St. Louis, MO, USA); dimethylformamide (DMF) and tributylamine from VWR international (Leuven, Belgium).

Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Sigma–Aldrich (Dorset, UK).

The following reagents were used for sample extraction: acetonitrile provided by Biosolve (Valkenswaard, the Netherlands); hexane from Merck (Haar, Germany); NaCl, $Na_2HPO_4{\cdot}2H_2O$, and KH_2PO_4 from VWR International (Leuven, Belgium).

2.2. Instrumentation

The instrument used for SPR-based measurements was an Optical SPR Biosensor System (Biacore Q) equipped with control and evaluation software (Biacore AB, Uppsala, Sweden). This instrument is a completely automatic system and has four flow channels but only one can be used at the same time.

2.3. Procedures

2.3.1. Antibody production

2.3.1.1. Polyclonal antibodies. All polyclonal antibodies were raised in rabbit by subcutaneous injection with 0.2 mg immunogen emulsified with Freund's complete adjuvant (first injection) or Freund's incomplete adjuvant (all following injections). Injections were administered on a fortnightly basis and then, from the third injection onward, at the rhythm of one injection every 28 days. Test bleeds were collected 10 days after each immunisation (from the third immunisation onward). The blood was centrifuged and the collected serum was stored at −20 °C until used.

Four different immunogens were tested in this study. Since four rabbits were injected with the same immunogen, sixteen rabbits were needed. The immunogens synthesised were as follows:

(1) *Norfloxacin hapten-BSA*: This immunogen was produced in three steps: synthesis of hapten, synthesis of ethylenediamine-modified BSA (EDA-BSA), and conjugation of hapten to EDA-BSA.
Haptens of norfloxacin (Fig. 2) containing a carboxylic acid group on the piperazinyl ring were synthesised and bound to carrier proteins in order to prepare immunogenic agents. Six grams of norfloxacin standard, 5.99 g of 6-bromohexanoic acid, 7.79 g potassium carbonate, and 0.142 g sodium iodide were added to 300 mL acetonitrile and refluxed for 8 days. The mixture was allowed to stand for a further 5 days and then the acetonitrile was removed by evaporation. The remaining white precipitate was washed with an aqueous sodium hydroxide solution and extracted with an equal mixture of ethyl acetate and hydrochloric acid. The precipitate was dissolved again in an aqueous sodium hydroxide solution and re-extracted with an equal mixture of dichloromethane and ethyl acetate. Addition of hydrochloric acid at pH 2 recrystallised the hapten as a white precipitate, which was successively washed with water, acetone, and water.
EDA-BSA was prepared for use as the hapten protein carrier, as it has been used successfully to increase the efficiency of the hapten/protein conjugation reaction [16]. It was synthesised with 300 mg BSA, 500 mg EDC, and 500 mg NHS dissolved in deionised Milli-Q purified water (Millipore, Billerica, MA). Ethylenediamine (2 mL, 5 M) was added to the solution following incubation for 4 h at 4 °C. The solution was further incubated overnight at 4 °C and then dialysed against water in 12–14-kDa tubing. The recovered cationised proteins were lyophilised and stored at 4 °C.
Haptens of norfloxacin were then conjugated to EDA-BSA by the mixed anhydride method [17]. Hapten (0.1 mmol) was dissolved in 4 mL of a 1:1 (v:v) mixture of DMF and 1,4-dioxane. After addition of 27 μL tributylamine, the mixture was stirred on ice for 10 min before adding 16 μL isobutylchloroformate. The reaction was brought to room temperature and stirred for 1 h. The resulting solution was added dropwise to an ice-cold solution of EDA-BSA as previously explained. The mixture was brought to room temperature and allowed to react overnight. The final solution was dialysed against several changes of phosphate-buffered saline (PBS) pH 7.4 (9 g NaCl, 7.78 g $Na_2HPO_4{\cdot}2H_2O$, and 0.75 g KH_2PO_4 in 1000 mL of H_2O) at 4 °C for 3 days.

(2) *Generic-FQ-BSA*: The idea was to produce an immunogenic agent from the basic structure of quinolones. The starting product 6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid was obtained from Apollo Scientific Limited, Berdbury, UK and renamed generic-FQ (Fig. 2). The same mixed anhydride method was used to conjugate this compound to the protein carrier (BSA). The generic-FQ was dissolved at a concentration of 5 mg mL^{-1}; the solution of BSA was prepared by dissolving 100 mg BSA in 3 mL 0.1 M sodium borate. The final mixture was purified by dialysis against saline.

(3) *Flumequine-BSA*: This immunogen was produced in two steps: the carboxyl group of flumequine was converted to an acid anhydride capable of reacting with amino groups of proteins, then the hapten was coupled to BSA. The usual method of conjugation was used, but slightly modified: flumequine was dissolved at a concentration of 6.5 mg mL^{-1}, then 16 μL triethylamine was added. Finally, the reaction mixture and 11 μL ethylchloroformate were added. The final product was purified by dialysis against saline.

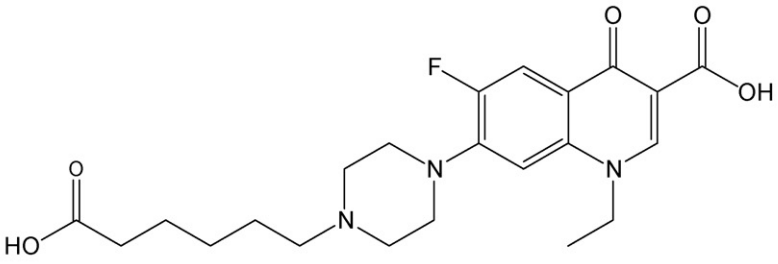
(4) *Norfloxacin hapten-flumequine-BSA*: This immunogen (Fig. 2) was synthesised from the hapten of norfloxacin (0.05 mmol) described above and from flumequine (0.05 mmol). Their conjugation to BSA was done as for generic-FQ-BSA.

2.3.1.2. Engineered antibody. An FQ antibody library was constructed by cloning of Fab fragments into a phage display vector. Only the variable-region genes of an anti-sarafloxacin monoclonal antibody were cloned as Fab fragments. Variable heavy-chain regions were random mutagenised using an error-prone polymerase chain reaction (EP-PCR). In addition, site-directed mutagenesis was carried out by targeting certain amino acids into the antigen-binding region of the antibody (Kunkel mutagenesis and splicing by overlapping extension PCR). Then the mutated variable heavy-chain regions were cloned into the wild-type Fab-vector before transformation of *E. coli* cells. Improved mutants were selected by phage display with small-sized antigen derivatives.

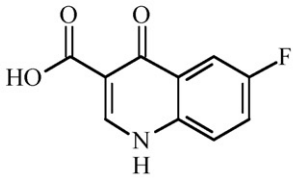
2.3.2. Preparation of biosensor chips

Four different sensor surfaces were prepared and tested in this study:

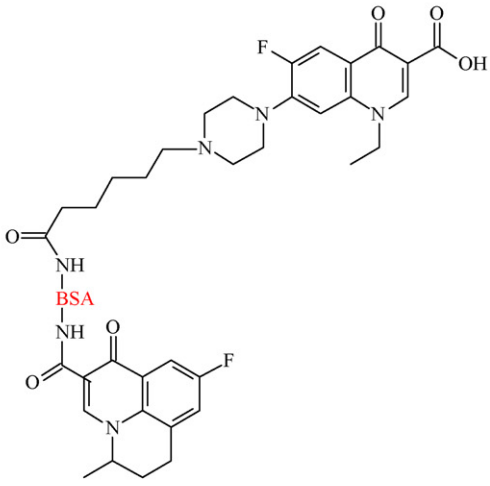
(1) *Core-FQ sensor chip*: The core-FQ derivative (Fig. 2) was immobilised by a standard amine-coupling procedure. A CM5 sensor chip was equilibrated to room temperature and 50 μL of an EDC/NHS mixture (1:1, v/v) was incubated on the chip surface for 30 min. The excess solution was removed and the surface of the chip was washed with water. The core-FQ derivative was prepared at 2.5 mg mL^{-1} in borate buffer pH 8.5 and an aliquot (50 μL) was added to the activated surface for 2 h at room temperature. The excess solution was removed and the surface of the chip



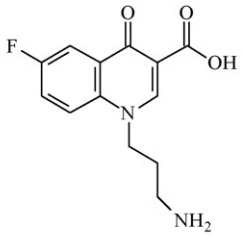
Synthesized norfloxacin hapten used to prepare the immunogenic agent norfloxacin hapten-BSA



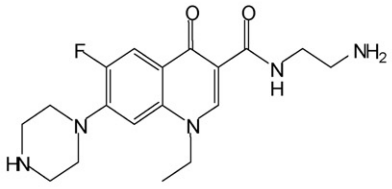
Generic-FQ used to prepare the immunogenic agent generic-FQ-BSA and also to prepare the generic-FQ sensor chip



The immunogenic agent: norfloxacin hapten-flumequine-BSA



Core-FQ derivative used to prepare the core-FQ sensor chip



Norfloxacin-NH_2 derivative used to prepare the norfloxacin-NH_2 sensor chip

Fig. 2 – Structure of norfloxacin hapten, generic-FQ, norfloxacin hapten-flumequine-BSA, core-FQ derivative, norfloxacin-NH_2 derivative, used in this study.

was washed with water. Unreacted sites were deactivated with 1 M ethanolamine (50 μL) added to surface for 0.5 h at room temperature.

(2) *Norfloxacin-NH_2 sensor chip*: It was decided to immobilise norfloxacin hapten instead of norfloxacin hapten-BSA to the chip in order to avoid any potential interaction between the antibody and the BSA carrier protein. The norfloxacin hapten used to make the immunogen was not suitable, because the presence of COOH groups at either end could lead to improper positioning or low accessibility of antibody to the immobilised hapten. For this reason a second norfloxacin hapten was produced with an amino-terminal group. This norfloxacin-NH_2 hapten (Fig. 2) was then immobilised on the chip. Its synthesis and immobilisation have been described in detail [18].

(3) *Dual norfloxacin/flumequine sensor chip*: Both flumequine and norfloxacin were immobilised via their respective carboxyl groups on the carboxylated sensor surface. The procedure used has been described for enrofloxacin [13] and flumequine [14]. Briefly, the chip surface was activated with a mixture of NHS and EDC, followed by amine coupling with ethylenediamine (Sigma–Aldrich, Bornem, Belgium). Norfloxacin and flumequine were esterified with NHS in the presence of EDC. A 1:1 (v:v) mixture of the resulting solutions was applied to the activated surface. To the washed chip, ethanolamine was added before a last washing step.

(4) *Generic-FQ sensor chip*: Generic-FQ (Fig. 2) was immobilised on a CM5 chip as described above: activation of the surface with EDC/NHS, preparation of an amine surface with ethylenediamine, injection of generic-FQ at a concentration of 5 mg mL^{-1} after activation with NHS and EDC, chip surface blocking with ethanolamine.

After immobilisation, the chips were stored between analyses over desiccant at 4 °C in a sealed container.

2.3.3. *Biological samples and extraction procedure*

Negative samples of chicken muscle, fish, and egg were chosen from our routine sampling procedures after LC–MS/MS confirmatory analysis. The samples were spiked with 50 μL working standard solutions or with HBS-EP buffer for the blank and incubated for 10 min at room temperature.

Chicken muscle samples were prepared by homogenising chicken breast tissue with an appropriate machine. Fish samples were prepared by homogenising skin and muscle in natural proportions. Egg samples were prepared by homogenising the whole egg.

A 2-g portion of known negative homogenate was weighed into a 50-mL tube. Acetonitrile (8 mL) was added to the tube. Each tube was immediately shaken manually to mix its contents and then all tubes were vortexed for 30 s before vigorous head-over-head shaking for 10 min. After centrifugation at 3000 rpm for 10 min at 4 °C, all of the supernatant (poultry meat and egg matrices) or a 2-mL aliquot thereof (fish matrix) was transferred into a glass tube. The supernatant was evaporated to dryness at 50 °C under a gentle stream of nitrogen. The residue was reconstituted in 1 mL of PBS pH 7.4 and mixed on a vortex mixer for 30 s. Then 1 mL hexane was added to all tubes, and the tubes were vortexed for 30 s. After centrifugation (10 min, 3000 rpm), the hexane layer and any traces of emulsion at the interface were removed. The washing step with hexane was repeated once again. Just before application to the 96-well plate, the samples were quickly vortexed and centrifuged at 3000 rpm for 10 min at 4 °C.

2.3.4. *Cross-reactivity determinations*

The cross-reactivity (CR) of the antibody towards various FQs was calculated from the relevant calibration curves as IC_{50} (norfloxacin)/CR_{50} (competitor) × 100, where IC_{50} is the concentration at midpoint of the standard curve and CR_{50} is the concentration of competitor required to cause the same decrease in signal as obtained with norfloxacin used at the IC_{50} concentration.

2.3.5. *The optimised biosensor immunoassay*

The optimised method emerging from this study is as follows. Antiserum raised against norfloxacin hapten-flumequine-BSA (Section 2.3.1.1) and freshly diluted 1:100 in HBS-EP buffer is mixed 25%:75% with final extract from poultry muscle, fish, or egg. Each sample is injected over the surface of the core-FQ sensor chip at a flow rate of 25 μL min^{-1} for 210 s. Report points are recorded 10 s before the start of injection and 30 s after each injection. The chip surface is regenerated with a 5 μL injection (flow rate: 10 μL min^{-1}) of 10 mM NaOH prepared from 1 M sodium hydroxide (VWR International, Leuven, Belgium) with HPLC grade water (Acros organics, Geel, Belgium).

2.3.6. *FQ incurred sample materials*

Incurred FQ-containing sample materials were obtained from an animal experiment described in a recent publication [19]. Conventional microbiological screening and LC–MS/MS confirmation were used to analyse the incurred sample. These residue concentrations in the final incurred samples were used to evaluate our method.

3. Results and discussion

To develop a single efficient biosensor immunoassay for the entire class of FQ antibiotics, it was necessary to optimise both the antibodies used (which must give highly selective binding with sufficient cross-reactivity within the class) and the drug immobilised onto the CM5 chip surface. Four immunogens and one engineered antibody were synthesised, and three different FQ derivatives were immobilised on the sensor surfaces (for syntheses and chip preparation, see Sections 2.3.1 and 2.3.2). In order to facilitate the understanding of the following section, Table 1 summarizes the different approaches investigated for the development of the new FQ SPR assay.

3.1. *Flumequine challenge*

Wang et al. [12] compared several papers describing the production of FQ antibodies. The authors think that the FQs not containing a substituent on the piperazinyl ring make the best haptens for producing a broad-specificity antibody, and most of the antibodies obtained from these haptens were of the generic nature. It is the reason why norfloxacin was selected as first hapten.

Table 1 – Schematic representation of different approaches investigated in this study

	Antibody	Sensor chip	Observations
Preliminary approach	Polyclonal, norfloxacin hapten-BSA	Norfloxacin-NH_2	No detection for flumequine
First approach	2 Polyclonals, norfloxacin hapten-BSA + flumequine-BSA	Dual norfloxacin/flumequine	Low sensitivity
Second approach	Polyclonal, generic-FQ-BSA	-Generic-FQ -Core-FQ -Norfloxacin-NH_2	-No binding -No binding -No inhibition
Third approach	Engineered	-Generic-FQ -Core-FQ -Norfloxacin-NH_2	-No binding -No binding -Inhibition but low CR for flumequine and marbofloxacin
Last approach	Polyclonal, norfloxacin hapten-flumequine-BSA	-Generic-FQ -Core-FQ -Norfloxacin-NH_2	-Low binding -Best inhibition -Inhibition

Sera from rabbits injected with the immunogen norfloxacin hapten-BSA, evaluated previously by ELISA [11] were first re-evaluated with the norfloxacin-NH_2 sensor chip. Binding was observed, and the cross-reactivity of the sera towards other FQs was estimated with respect to norfloxacin (Section 2.3.4). Significant cross-reactivity was found with difloxacin, sarafloxacin, marbofloxacin, danofloxacin, enrofloxacin, and ciprofloxacin, but not with flumequine. As flumequine is licensed in Europe for use in both poultry and livestock [2] and as it is the drug most frequently administered to broilers in the Netherlands [14], we focused on devising an assay that would detect flumequine in addition to the other derivatives.

The first approach tested was the use of a dual sensor chip, carrying both norfloxacin and flumequine on the surface of the same flow cell (Section 2.3.2). The antibody solution used was a mixture of specific anti-flumequine antibody (flumequine-BSA) and generic antibody (norfloxacin hapten-BSA). The problem with this approach was its low sensitivity: it was not possible to detect the FQs in matrices at their corresponding maximum residue levels (MRLs).

We then raised antibodies against the basic structure of the FQs (Fig. 1), hoping thus to cover their entire range. Rabbits were injected with BSA-coupled generic-FQ (Fig. 2 and Section 2.3.1.1), and a new chip surface was also prepared with this molecule (Section 2.3.2). The serum showed interaction neither with the generic-FQ sensor chip nor with the core-FQ sensor chip, whereas low binding but no inhibition was observed with norfloxacin-NH_2 chip.

Our third approach was to test an engineered antibody, the advantage of such development means that it is possible to engineer different properties into the single chain variable fragment. These directed evolutions could increase binding strength and broaden binding recognition for selecting improved binders. This engineered antibody (Section 2.3.1.2) failed to interact with the generic-FQ sensor chip and with the core-FQ sensor chip, whereas low binding was observed with the norfloxacin-NH_2 chip. The antibody showed high specificity towards sarafloxacin and difloxacin (100% and 109%, respectively), medium cross-reactivity (14–35%) with five other FQs, and low cross-reactivity with marbofloxacin and flumequine (0.5% and 6%, respectively). Thus, this alternative proved unsatisfactory for both flumequine and marbofloxacin.

As none of these approaches solved the flumequine problem and as using the right immunogen is crucial, we opted for synthesising a new type of immunogen.

3.2. *Production and testing of bi-active antibodies*

In the above experiments, the best results were obtained with antisera raised against either flumequine-BSA (for flumequine) or norfloxacin hapten-BSA. We therefore developed a new immunogen, called norfloxacin hapten-flumequine-BSA (Fig. 2), combining features of both of these preparations. Into the norfloxacin hapten we introduced a carboxylic acid group at position 7, so as to preserve the carboxylic group at position 3, which is common in FQs, as a possible epitope. This approach also makes this side of the molecule more accessible. The carboxyl group of flumequine was converted to an acid anhydride capable of reacting with amino groups on the protein (BSA). The BSA molecule has 35 active amino groups, and it seems that both antigens were conjugated (ratio 1:1) to different NH_2 groups on the same BSA molecule.

Four rabbits were hyperimmunised with this immunogen. All four showed an immune response as of the first bleeding, as shown by a high relative response upon injection over the chip surface (flow rate at 5 $\mu L\,min^{-1}$ for 10 min) of sera diluted ten fold in HBS-EP buffer. The response of the generic-FQ chip was low (below 420 response unit – RU), so we gave up this combination. The norfloxacin-NH_2 chip gave a higher response than the core-FQ chip (7813–8981 RU vs. 1121–1636 RU). We then performed preliminary experiments with the bi-active antibodies to test the ability of free competitor FQs in buffer to inhibit the response. Despite its lower response to the bi-active antibodies, the core-FQ chip showed higher sensitivity in these tests and was thus retained for further development.

3.3. *Optimisation with free fluoroquinolones in buffer*

A first optimisation of the retained Biacore Q assay was done with the four diluted antisera (raised against norfloxacin hapten-flumequine-BSA) and with standard solutions of norfloxacin, flumequine, and sarafloxacin (concentration range: 0–200 $ng\,mL^{-1}$) in HBS-EP buffer injected separately on the core-FQ sensor chip. Midpoint calibration curves were cal-

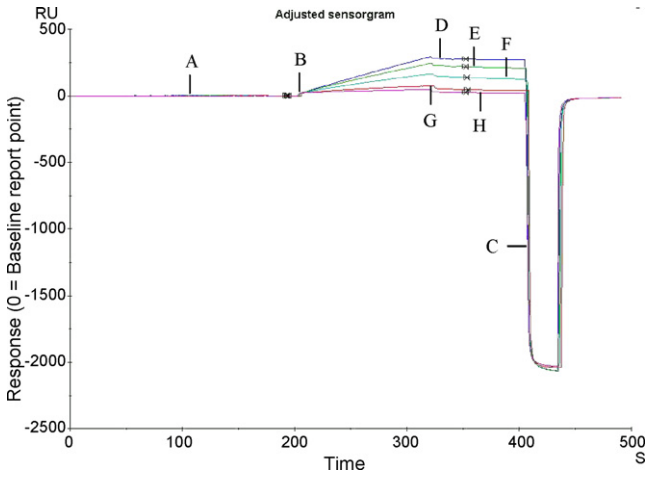


Fig. 3 – Superimposed sensograms obtained for norfloxacin calibrants in buffer. (A) Baseline response; (B) calibrant and antibody injection; (C) regeneration solution; (D) 0.1 ng mL^{-1} norfloxacin response; (E) 0.5 ng mL^{-1} norfloxacin response; (F) 1 ng mL^{-1} norfloxacin response; (G) 2 ng mL^{-1} norfloxacin response; (H) 4 ng mL^{-1} norfloxacin response.

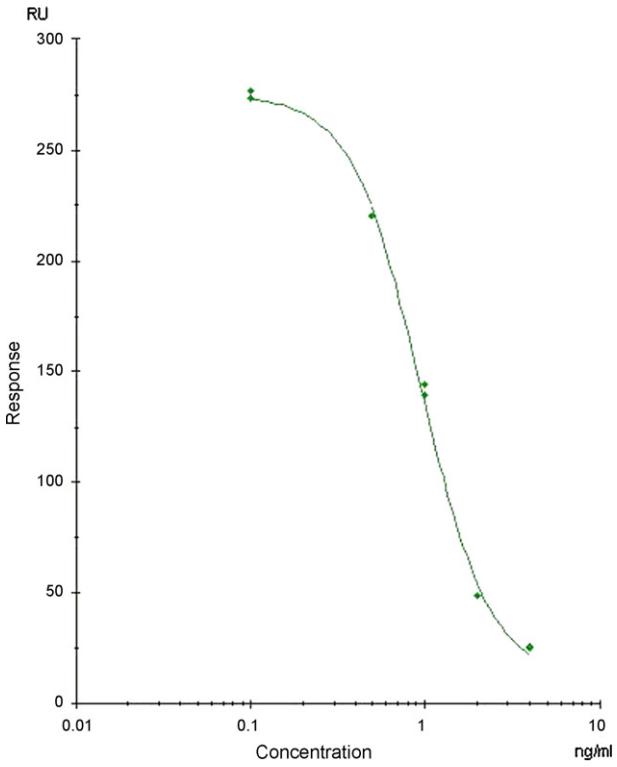


Fig. 4 – Typical calibration curve of norfloxacin in buffer constructed with antibody raised against norfloxacin hapten-flumequine-BSA and core-FQ sensor chip.

culated with the BIAevaluation software, and the serum giving the best performance was retained for further experiments.

Under optimal assay conditions, a fixed concentration of the diluted antibody was mixed with reference calibrant and injected. Norfloxacin present in solution inhibits binding to the core-FQ sensor chip, and response is therefore inversely proportional to the norfloxacin concentration. Each analysis cycle is recorded in the form of a sensogram that monitors, in real time, changes in SPR response with time, thanks to the report points. Fig. 3 illustrates the superimposed sensograms obtained with a set of calibration standards, including regeneration of the sensor surface to remove bound antibody. A typical standard curve for norfloxacin in this optimised assay is shown in Fig. 4.

We then sought to determine the specificity of the assay by testing different dilutions of the following FQs (injected concentrations: 0, 1, 5, 10, 25, 50, 100 and 1000 ng mL^{-1} in HBS-EP): norfloxacin, sarafloxacin, difloxacin, ciprofloxacin, enrofloxacin, flumequine, danofloxacin, marbofloxacin, pefloxacin, enoxacin, lomefloxacin, ofloxacin, oxolinic acid, and nalidixic acid. The cross-reactivities calculated (Section 2.3.4) from the calibration curves are shown in Table 2. The cross-reactivity ranged from 41% to 115% for the compounds possessing a piperazinyl moiety and was lower for all three compounds lacking this moiety (flumequine, oxolinic acid, and nalidixic acid). Although low, the cross-reactivity recorded for flumequine (7%) proved acceptable.

Two quinolone-related substances were also included in the specificity test: ethoxyquin and nicotinic acid. These two compounds were prepared at high concentration (50 μg mL^{-1} in HBS-EP) and injected as above. As no binding inhibition was observed, we conclude that the antiserum raised against norfloxacin hapten-flumequine-BSA specifically recognizes the studied class of antibiotics.

3.4. *Biosensor immunoassay detection in matrices*

Three matrices were selected for this work: egg, fish, and poultry meat. To simplify routine analysis, a common sample preparation procedure was developed for the three matrices (Section 2.3.3). The extraction procedure is slightly different for the fish matrix: after centrifugation of the acetonitrile-treated homogenate, only a 2-mL aliquot of the supernatant is evaporated to dryness (instead of the whole supernatant). This reduces a matrix effect we observed with fish of different origins when we used the entire supernatant. The SPR-assay conditions described in Section 2.3.5 offer the optimal performance for each matrix. The background response due to nonspecific binding of matrix constituents, as measured by replacing antibody with running buffer, was only 35 ± 8 RU.

Calibration curves were constructed by adding norfloxacin to FQ-free fish, egg, and chicken muscle (Fig. 5). The norfloxacin concentration in the standards prepared ranged from 0.1 to 10 μg kg^{-1} (0.1, 0.5, 1, 2.5, 10 μg kg^{-1}) for egg and poultry samples and from 0.1 to 100 μg kg^{-1} (0.1, 1, 2.5, 5, 100 μg kg^{-1}) for fish samples. The IC_{50} measured in the optimised assay was 3.1 μg kg^{-1} for fish, 1.5 μg kg^{-1} for egg, and 1 μg kg^{-1} for chicken muscle.

The decision limit (CCα) of the assay was calculated as the concentration corresponding to the average instrumental response for at least twenty negative samples minus three times the standard deviation. The decision limits were therefore estimated at 0.13, 0.29 and 0.30 μg kg^{-1} for chicken muscle, egg and fish, respectively.

Table 2 – IC_{50} values and cross-reactivities (CR) between FQs in buffer and in the matrices obtained with antibody raised against norfloxacin hapten-flumequine-BSA and core-FQ chip

	Buffer		Fish		Egg[b]		Chicken muscle	
	IC_{50} (ng mL^{-1})	CR (%)	IC_{50} (μg kg^{-1})	CR (%)	IC_{50} (μg kg^{-1})	CR (%)	IC_{50} (μg kg^{-1})	CR (%)
Norfloxacin	1.8	100	3.1	100	1.8	100	2.3	100
Sarafloxacin	3.6	50	7.3 (30)	43	2.3	77	4.1	56
Difloxacin	2.5	72	5.1 (300)	61	1	173	2 (300)	114
Ciprofloxacin	1.6	115	3.5 (50)[a]	91	1.2	151	2.2 (50)[a]	104
Enrofloxacin	1.8	101	2.7 (50)[a]	116	0.8	237	1.6 (50)[a]	144
Flumequine	26	7	68.1 (600)	5	16.1	11	34.2 (400)	7
Danofloxacin	1.7	104	3.3 (100)	95	0.9	191	2.2 (200)	105
Marbofloxacin	1.9	96	3.2	98	1.2	148	1.7	138
Pefloxacin	2.7	66	3.2	99	0.6	287	2.1	109
Enoxacin	2.1	86	7.6	41	3.3	54	4.1	57
Lomefloxacin	2.2	83	5	63	4	45	3.1	74
Ofloxacin	1.7	106	2.8	112	1	187	1.6	146
Oxolinic acid	4.4	41	11.6 (100)	27	3	60	7 (100)	33

The corresponding MRL value is indicated as ().

[a] Sum of ciprofloxacin and enrofloxacin = 100 μg kg^{-1}.

[b] Not for use in animals for which eggs are produced for human consumption.

To test the applicability of the optimised assay to other poultry meats, norfloxacin calibration curves were prepared with chicken, duck, and turkey meat. The results obtained were similar in all three cases: the relative response measured for unfortified samples was 399 RU for chicken, 404 RU for duck, and 423 RU for turkey. The respective IC_{50} values were 0.9, 0.9, and 0.8 μg kg^{-1}.

Table 2 shows the cross-reactivities calculated for the 13 different (fluoro)Qs added (separately) to the fish, chicken, and egg matrices. The calibrants selected for this test were added at 0, 0.25, 1, 2.5, 10, 25, 100 and 1000 μg kg^{-1} concentration. The European Commission has established MRLs for some of these compounds, and these values are included in the table [20]. The cross-reactivities observed were similar to those obtained by adding the compounds to buffer. The table shows that our optimised assay can detect at least 13 of the most widely (mis)used (fluoro)quinolones at levels below their established MRLs in fish, egg, and chicken muscle.

Throughout assay development, the same flow cell was used in over 200 analytical cycles without any apparent loss of activity being observed.

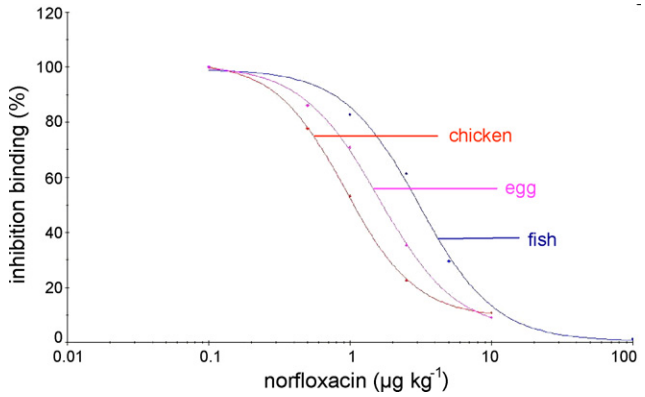


Fig. 5 – Biosensor calibration curves for norfloxacin obtained after extraction from fish, egg and chicken muscle.

The team of Haasnoot et al. [14] has developed a specific biosensor immunoassay for detecting flumequine in broiler serum and muscle. Mellgren and Sternesjö [13] have described an optical immunobiosensor assay for determining enrofloxacin and ciprofloxacin (its main metabolite) in bovine milk. These two publications were limited to one or two residue(s) within the FQ family.

Marchesini et al. [18] have described a procedure for identifying FQs in chicken muscle by LC electrospray time-of-flight MS. They combined two biosensor immunoassays: multi-FQ screening for detection of five FQs (norfloxacin, sarafloxacin, difloxacin, enrofloxacin, ciprofloxacin) and specific screening for flumequine. This approach is very sensitive but requires sophisticated instrumentation.

We compared the SPR biosensor performance versus the four generic ELISAs mentioned in the introduction. Holtzapple et al. [5] have produced monoclonal antibodies recognizing sarafloxacin (10 μg kg^{-1}), difloxacin and trovafloxacin with a similar CR plus three other FQs with very low CR (inferior to 3%). Bucknall et al. [9] have developed generic immunoassay for bovine milk and sheep kidney. The antibody raised against norfloxacin has shown a CR percentage between 40% and 143% for three FQs whereas the range for seven other FQs was only included between 1% and 18 %. The ELISA developed by our team [11] was able to detect a broad range of 15 FQs but with a lower sensitivity. The assay's detection capabilities for most of the compounds were inferior to 10 μg kg^{-1}. An indirect ELISA using monoclonal antibodies has recently been described [12] featuring high CR (35–100%) with 12 of 14 FQs and detecting these FQs below their MRLs.

3.5. *Comparing our SPR biosensor assay with LC–MS/MS*

As explained in Section 2.3.6, FQ-free and incurred FQ-containing samples of chicken muscle, egg, and fish were produced. The following (fluoro)quinolones were selected as

Table 3 – Comparison of instrument performance after analyses achieved from both methods: biosensor screening and confirmatory LC–MS/MS method

Chicken muscle	
Flumequine (400)	
83[a]	119[b]
312[a]	341[b]
Difloxacin (300)	
154[a]	161[b]
215[a]	219[b]
Enrofloxacin (100)	
48[a]	49[b]
96[a]	98[b]
Eggs	
Flumequine (200*)	
67[a]	101[b]
151[a]	235[b]
Oxolinic acid (50*)	
25[a]	17[b]
Fish	
Flumequine (600)	
256[a]	249[b]
508[a]	495[b]

The values are expressed in $\mu g\,kg^{-1}$. (): MRL value; (*): target value.
[a] LC–MS/MS assay.
[b] SPR assay.

representatives of the entire family of quinolone antibiotics and used for the production of incurred sample materials: flumequine, difloxacin, and enrofloxacin for incurred chicken meat, oxolinic acid and flumequine for incurred eggs, and flumequine for incurred fish. Non-compliant materials containing two residue levels (0.5 and 1 MRL or target) were analysed by both LC–MS/MS [19] and our optimised biosensor assay. Each incurred sample was simultaneously extracted with its corresponding calibration curve. The data generated by the two analytical procedures, presented in Table 3, were in good agreement giving a degree of correlation ($r^2 = 0.96$) and slope of 0.96.

4. Conclusion

The present work has resulted in an effective analytical screening procedure combining simple sample preparation with a sensitive SPR optical biosensor assay. The procedure is designed to produce qualitative data and to detect at low levels a wide range of (fluoro)quinolones in poultry meat, fish, and egg samples. Non-compliant samples can subsequently be analysed by confirmatory methods allowing residue identification and quantification. According to the criteria specified in European Commission Decision 2002/657/EC [21] for qualitative screening methods, a complete validation is scheduled to prove the usefulness in routine analyses.

This study revealed an innovation on the production of generic polyclonal antibodies able to recognize several compounds showing different chemical structures. For the first time, the concept of the bi-active antibody was experimentally used and its efficiency was proved.

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