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Label-Free and Multiplex Detection of Antibiotic Residues in Milk Using Imaging Surface Plasmon Resonance-Based Immunosensor

Sabina Rebe Raz,*,†,‡ Maria G. E. G. Bremer,† Willem Haasnoot,† and Willem Norde‡,§

RIKILT—Institute of Food Safety, Wageningen UR, P.O. Box 230, 6700 AE Wageningen, The Netherlands, Laboratory of Physical Chemistry and Colloid Science, Wageningen University, P.O. Box 8038, 6700 EK Wageningen, The Netherlands, and University Medical Center—Groningen and University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, The Netherlands

Monitoring of antimicrobial drug residues in foods relies greatly on the availability of adequate analytical techniques. Currently, there is a need for a high-throughput screening method with a broad-spectrum detection range. This paper describes the development of a microarray biosensor, based on an imaging surface plasmon resonance (iSPR) platform, for quantitative and simultaneous immunodetection of different antibiotic residues in milk. Model compounds from four major antibiotic families: aminoglycosides (Neomycin, Gentamicin, Kanamycin, and Streptomycin), sulfonamides (Sulfamethazine), fenicols (Chloramphenicol), and fluoroquinolones (Enrofloxacin) were detected using a single sensor chip. By multiplexing seven immunoassays in a competitive format, we were able to measure all the target compounds at parts per billion (ppb) levels in buffer and in 10×-diluted milk. The assays for Neomycin, Kanamycin, Streptomycin, Enrofloxacin, and Sulfamethazine were sensitive enough for milk control at maximum residue levels as established in the European Union. The overall performance of the biosensor was determined to be comparable to that of conventional four-channel surface plasmon resonance (SPR)-based biosensors, in terms of assay sensitivity and robustness. Combining the advantages of a SPR sensor and a microarray, utilization of the biosensor described here offers a promising alternative to the existing methods and is highly relevant for multianalyte food profiling.

Antibacterial drugs are widely used in veterinary medicine to treat infectious diseases.[1−3] The fact that almost all food-production animals receive medication for some part of their lives raises public health concerns, with regard to the presence of drug residues in the food chain. Possible adverse health effects include potential allergic response (penicillins) and toxicity (Chloramphenicol), but they are mainly focused on a transfer of antibiotic resistance genes to human pathogens.[2,4,5] Although the consumer health risks of these drug residues in foods are difficult to determine, maximal residue limits (MRLs) were established. The presence of antibiotic residues in foods is controlled by several national and international organizations.[6] In this study, we targeted compounds found in dairy products, belonging to four different antibiotic groups: aminoglycosides (Neomycin (NEO), Gentamicin (GNT), Kanamycin (KAN), and Streptomycin (STR)), fluoroquinolones (Enrofloxacin (ENR)), fenicols (Chloramphenicol (CAP)), and sulfonamides (Sulfamethazine (SMZ)) (see Figure 1). In the European Union (EU), the MRLs of NEO, GNT, KAN, STR, ENR, and SMZ in milk are 1500, 100, 150, 200, 100, and 100 ng/mL, respectively.[7] For CAP, a zero-tolerance policy is applied, with a minimum required performance limit (MRPL) of 0.3 ng/mL.[3] Besides consumers health concerns, the presence of antibiotics in milk is also known to have an adverse effect on the fermentation process. Therefore, dairy industry screens incoming milk for the presence of antibiotics, to prevent contaminated milk from entering the food chain.[8]

Because milk is one of the most heavily regulated products in food industry, there is a need for a single method that can simultaneously detect numerous antibiotic classes at adequate levels.[8] Traditional monitoring methods include microbial growth inhibition assays, microbial receptor assays, enzymatic colorimetric assays, receptor binding assays, chromatographic methods, and immunoassays.[6,9−12] The milk samples are first analyzed for the presence of antibiotics using rapid and qualitative or semiquantitive screening methods and only the suspected samples are subsequently quantified and confirmed with high pressure liquid chromatography (HPLC) and mass spectrometry (MS). In the past decade, many immunoassays have been developed (mainly in

* To whom correspondence should be addressed. Fax: +31 317 417717. E-mail: sabina.rebe@wur.nl.

† RIKILT—Institute of Food Safety, Wageningen UR.

‡ Laboratory of Physical Chemistry and Colloid Science, Wageningen University.

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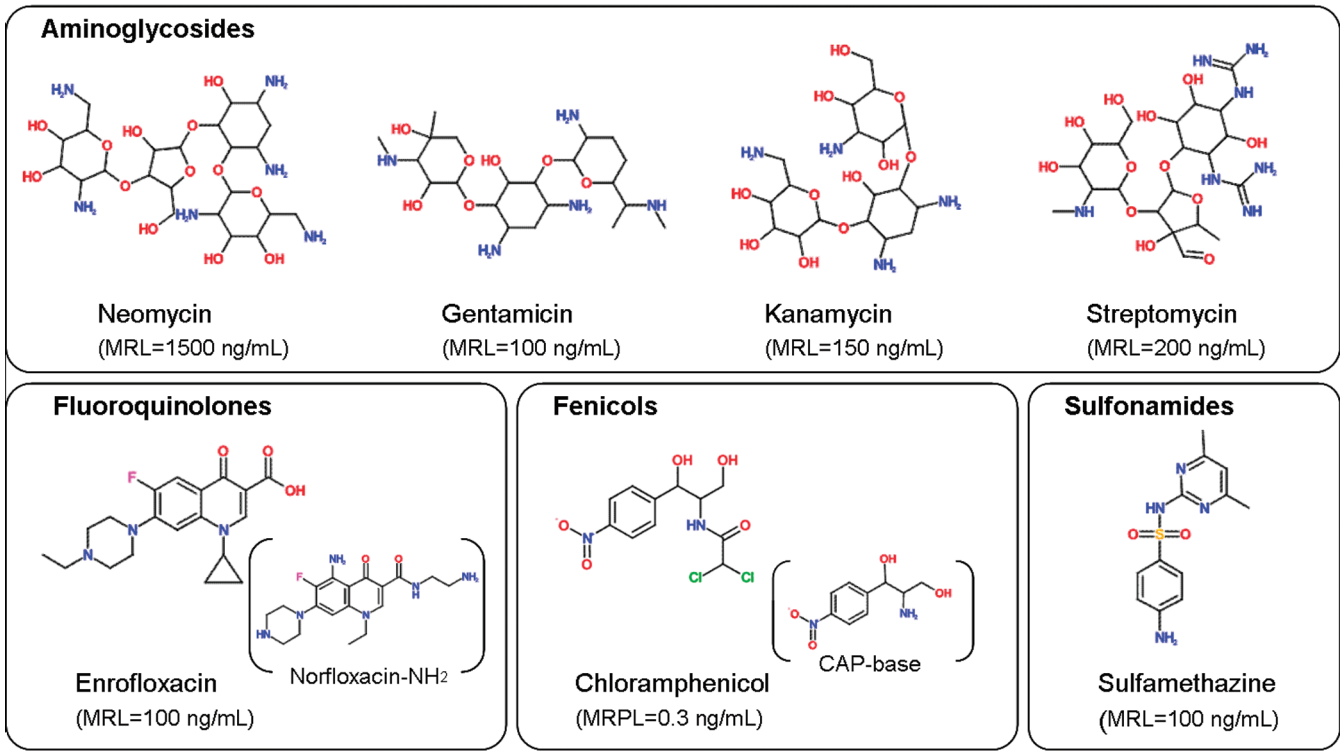

Figure 1. Scheme of the antibiotics measured in this study, their molecular structures and maximum residue limits (MRLs) or minimum required performance limit (MRPL) in milk. For fluoroquinolones and fenicols, molecular structures of the ligands used for immobilization on the sensor chip are shown in brackets.

ELISA format) to provide an alternative to unspecific and time-consuming microbial growth inhibition assays. ELISAs offer quantitive detection with high specificity toward a particular antibiotic or a group of antibiotics.[13] However, when a large number of samples must be screened for several antibiotics, they also become time-consuming and laborious. Thus, optical biosensors, based on biospecific interaction analysis (BIA), are of great interest, because the technology allows real time and automated analysis with relatively high capacity.[14,15] However, most of the SPR-based biosensors, developed so far, were targeting one compound or a family of structurally or functionally similar compounds, resulting in assays with a rather narrow detection spectrum.[16−21] The imaging surface plasmon resonance (iSPR) platform used in this study offers the possibility of multiplexing assays for several different compounds, making biosensor technology competitive with already-existing methods.[22−24] Although HPLC and MS are indispensable, in terms of confirmation, they require expensive equipment, trained personnel, and complex sample preparation steps. Using surface plasmon resonance (SPR) biosensor-based immunoassays, one can obtain robust and quantitative results with high specificity (or broad spectrum, depending on the assay) in relatively short time. In this study, we evaluated the possibility of implementing an iSPR-based biosensor for the simultaneous detection of several antibiotics in milk. The IBIS iSPR system, used in this study, was described in our previous work, where we demonstrated the possibility of high- and low-molecular-weight compounds detection via direct and competitive immunoassay.[25] Here, seven competitive immunoassays for NEO, GNT, KAN, STR, ENR, CAP, and SMZ were multiplexed on an iSPR platform and their performances were studied in buffer and in milk.

EXPERIMENTAL SECTION

Chemicals and Materials. Round sensor chips, coated with preactivated hydrogel for amine coupling (HCX) were purchased from Xantec Bioanalytics GmbH (Duesseldorf, Germany). Biacore amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-N-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride pH 8.5) and HBS-EP buffer (containing 10 mM 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid pH 7.4, 150 mM sodium chloride, 3 mM EDTA, and 0.005% (v/v) surfactant polysorbate (P20)) were purchased from GE Healthcare (Uppsala, Sweden). Twice concentrated protein printing buffer (PPBx2) was purchased from

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Arrayit Corporation (Sunnyvale, CA). Norfloxacin-NH_2 derivative (NOR-NH_2) was kindly supplied by Dr. Sheryl Tittlemier (Health Canada, Ottawa, Canada). Monoclonal antineomycin and antigentamicin antibodies were purchased from Biodesign (Huissen, The Netherlands). Monoclonal antichloramphenicol and polyclonal antikanamycin antibodies were purchased from Abcam (Cambridge, U.K.). The monoclonal antibody (Mab) raised against sulfamethazine (Mab 21C7) was kindly provided by the Department of Biological Regulation of the Weizmann Institute of Science (Rehovot, Israel).[26] The polyclonal antiserum (Pab CA65) raised against a norfloxacin-COOH derivative was kindly supplied by Laboratoire d'Hormonologie Animale (Marloie, Belgium). Monoclonal antibody against dihydrostreptomycin (DHS) was kindly provided by Dr. Aart van Amerongen of the Agrotechnology & Food Sciences Group (AFSG) of Wageningen UR (Wageningen, The Netherlands). The full-fat goats' milk powder (Mekkermelk from Henri Willig, Katwoude, The Netherlands) was purchased locally. The iSPR instrument, a round sensor chip holder, refractive index matching oil ($n = 1.518$), a hemispheric prism (BK7), a cuvette, and a flow cell were purchased from IBIS Technologies B.V. (Hengelo, The Netherlands). The rest of the chemicals were purchased from Sigma−Aldrich (Zwijndrecht, The Netherlands).

Sensor Chip Preparation. A Xantec HCX sensor chip was spotted with ligands using the Microgrid II contact arrayer (Apogent Discoveries, Wilmslow, U.K.) equipped with SMP3 stealth pins (TeleChem International, Inc., Sunnyvale, CA). The ligands were prepared beforehand as follows. NEO, GNT, and KAN were dissolved in water at 20 mM and immobilized in PPBx1 pH 8.5 at 10 mM final concentration. NOR-NH_2 was dissolved in 20 mM carbonate buffer pH 8.5 with 30% (v/v) dimethylformamide (DMF) and immobilized in 10 mM carbonate buffer pH 9.6 at a final concentration of 0.3 mM. CAP-base was dissolved in 100% acetic acid at a 60 mg mL^{-1} concentration and then diluted to 30 mM in 10 mM acetate buffer pH 4.5 and immobilized in PPB pH 4.5 at a final concentration of 15 mM. Dihydrostreptomycin (DHS) was solubilized and immobilized in 10 mM carbonate buffer pH 9.6 at a final concentration of 20 mM. Sulfadimidine was dissolved in 25 mM sodium hydroxide at a final concentration of 42 mM and immobilized directly. Each antibiotic was spotted in triplicate in a randomized manner over the sensor chip surface. During the spotting, 80% relative humidity was maintained and the sensor chip was left inside the instrument, to incubate for 1 h. Unreacted groups in the hydrogel were blocked with 0.5 M ethanolamine (pH 8.5) for 10 min at room temperature (RT). If not used immediately, the sensor chip was washed with reverse osmosis (RO) water, dried under a nitrogen stream, and stored at 4 °C.

iSPR Measurements. iSPR measurements were conducted using the IBIS iSPR instrument. The sensor chip was assembled with the prism using refractive index matching oil in a round chip holder. A flow cell (with a volume of 3 μL) was fixed on top of the sensor chip surface. The sample was delivered to the sensor chip surface through tubing and was pumped back and forth at a rate of 10 μL/s during the interaction (10 min). The surface was equilibrated with the HBS-EP buffer (5 min) and regions of interest (ROIs) in size of 150 μm × 150 μm were defined on the spots. The SPR angle was scanned on each predefined ROI in the range between −1.5° and + 1.5°, in steps of 50 millidegrees (one data point every 5.5 s). SPR curves were fitted automatically by IBIS software while curve parameters were limited to 20 points before and after the dip. All the measurements were performed in the "baseline mode", recording the SPR angle as a function of time. Subsequently, SPR data were analyzed using Scrubber2 software (BioLogic Software, Campbell, Australia). Raw sensorgrams were first zeroed to the baseline of the buffer before the injection and then referenced to a response of the blank spot (without antibiotic). The maximum responses were calculated for each injection from the data points collected during the dissociation phase (4 min after the sample injection stopped).

iSPR-Based Multiplexed Competitive Immunoassay. Seven competitive immunoassays for NEO, GNT, KAN, STR, ENR, CAP, and SMZ were multiplexed as follows. A freshly prepared sensor chip, microarrayed with antibiotics as described in the section "Sensor Chip Preparation", was conditioned with at least three serial injections (with a contact time of 2 min each) of regeneration solution containing 20% (v/v) acetonitrile in 10 mM sodium hydroxide until the baselines of all the spots were stable in HBS-EP buffer. To test spot-to-spot cross-contamination and antibodies cross-reactivity, each antibody was injected separately (10 min contact time) in duplicate over the surface of the preconditioned sensor chip. From this step, a final antibody dilution was selected according to the obtained responses, considering sufficiently high response without baseline buildup. Next, multistandard solutions containing all of the antibiotics previously mentioned were prepared in HBS-EP buffer at concentrations in the range of 0.032−500 ng/mL and mixed with a cocktail of antibodies containing anti-NEO (1:500), anti-GNT (1:500), anti-KAN (1:5000), anti-STR (1:100), anti-NOR (1:500), anti-CAP (1:7000), and anti-SMZ (1:2000). These mixtures were injected over the sensor chip arrayed with antibiotics in duplicate, starting with a blank solution containing only the antibody cocktail. Each cycle included sample injection (with a contact time of 10 min) and three injections of regeneration solution (with a contact time of 2 min each). For measurements in milk, 1 g of milk powder was dissolved in 9 mL of HBS-EP buffer, stirred for 0.5 h at RT, diluted by a factor of 10 in HBS-EP buffer, and filtered through a 0.45-μm HT Tuffryn acrodisc syringe filter (Pall Life Sciences, UK). Multistandard solutions in diluted milk were prepared and measured in the same way as in buffer on the same sensor chip. Relative binding (B/B_0) was calculated by dividing the response of the antibiotics-containing solution (B) by the response of the blank solution (B_0). To generate calibration curves, B/B_0 values were plotted against antibiotic concentrations. The calibration curves were fitted with a nonlinear four-parameters (4P) model, using GraphPad Prism software (GraphPad Software, Inc.) and half maximal inhibitory concentration (IC_{50}) was interpolated. In addition, immunoassays were characterized by the limits of detection (LODs) which were calculated by subtracting three standard deviations from the average maximum response of the blank solution and by the dynamic measurement ranges, which were set between $0.2B/B_0$ and $0.8B/B_0$.

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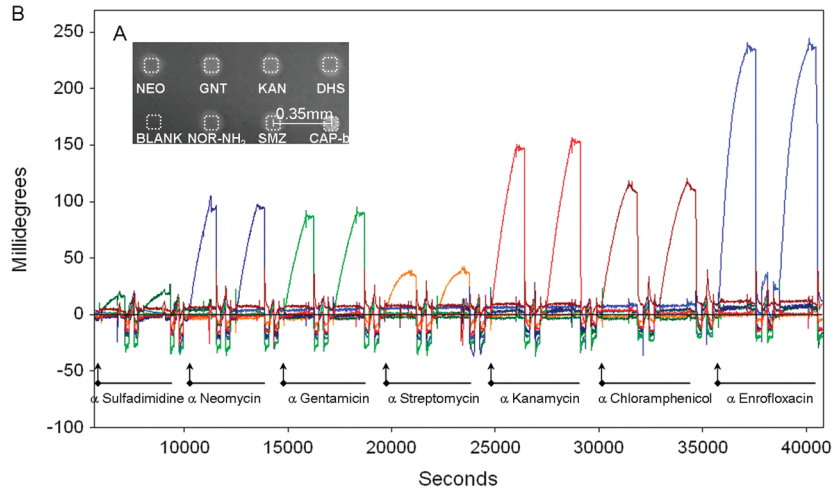

Figure 2. (A) SPR image of an antibiotics microarray, acquired immediately after spotting. Neomycin (NEO), Gentamicin (GNT), Kanamycin (KAN), Dihydrostreptomycin (DHS), Norfloxacin-NH_2 derivative (NOR-NH_2), Chloramphenicol base (CAP-base), and Sulfamethazine (SMZ) were spotted on an EDC/NHS preactivated hydrogel sensor chip. Bar shows a center-to-center separation of 0.35 mm between spots. Dotted lines show regions of interest (ROIs), where the SPR angle was measured. (B) Sensorgrams measured on seven spots containing different antibiotics during serial injections of corresponding antibodies. The start of the injection is marked with an arrow for each antibody duplicate. Between the antibody injections, the surface was regenerated twice with 20% (v/v) acetonitrile in 20 mM sodium hydroxide. The sensorgrams were zeroed to the baseline before the injection and referenced to a blank spot. The alpha symbol (α) denotes an antibody.

RESULTS AND DISCUSSION

Antibiotics Microarrayed Sensor Chip Preparation. The objective of this study was to evaluate the possibility of multiplex antibiotics detection in food using an iSPR-based biosensor with milk as a model matrix. Antibiotics from four major groups were targeted: aminoglycosides (NEO, GNT, STR, and KAN), fluoroquinolones (ENR), fenicols (CAP), and sulfonamides (SMZ) (see Figure 1).The first step in the development of an iSPR-based biosensor is a successful immobilization of target compounds on the sensor chip surface in a microarray format. When concentration assays are designed in SPR-based biosensors, maximal load of the ligand on the surface is usually preferred. Because the sensor chip should be of multiple usage in such cases, it also must withstand multiple cycles of regeneration without major losses of immobilized ligands. We chose to covalently immobilize our compounds of interest on an amine-reactive hydrogel surface, using commonly applied EDC/NHS chemistry.[17−19,27,28] Aminoglycosides and the sulfamethazine have intrinsic primary amine groups. For the fluoroquinolones assay, we used a NOR-NH_2 derivative, and for the chloramphenicol assay, we used D-(−)-threo-2-amino-1-(*p*-nitrophenyl)-1,3-propanediol (CAP-base) as ligands. The immobilization conditions of each compound were tested for compound solubility, spot formation on HCX hydrogel, and amine chemistry compatibility (data not shown). For instance, lipophilic antibiotics that required a high percentage of DMF or dimethyl sulfoxide (DMSO) for solubility were not forming spots on the hydrogel. Furthermore, using intermediate linkers to introduce a different active group to the sensor chip surface was not possible with the contact arraying technique, constraining us to a single immobilization step with a common chemistry of all the ligands. Thus, the initial number of antibiotics chosen for the assay development was narrowed to the compounds described here. NEO, GNT, and KAN were easily immobilized and formed well-defined spots. DHS and SMZ immobilization efficiencies were lower, most likely because they have less primary amine groups and thus required higher concentrations upon immobilization. Because of the presence of DMF in the NOR-NH_2 solubilization buffer, spot formation was not possible until it was sufficiently diluted (1% (v/v) DMF, final). Unlike the rest of the compounds, and in contrast to a previously published method, chloramphenicol-base was successfully immobilized in acidic buffer (pH 4.5).[19] Antibiotics immobilization and binding of the antibodies was monitored using IBIS iSPR system, which was described in our previous work.[25] Figure 2A (the smaller image, shown as an inset) shows a representative SPR image of successfully formed spots with immobilized antibiotics and a blank spot on the sensor chip surface. Although the change in refractive index that is caused by low-molecular-weight compounds is small, the spots were visible on the acquired SPR image at that stage.[29] After several regeneration cycles, when the baseline was stabilized, the image of the spots lost its intensity, indicating removal of the compounds that were noncovalently bound to the polymer. The immobilization efficiency, spot-to-spot cross-contamination, and antibody's cross-reactivity were tested by serial injections of the corresponding antibodies. Figure 2B (the larger image) shows zeroed and referenced sensorgrams of these injections, measured on seven spots of different compounds. Each antibody injection caused an increase in response on the relevant spot, indicating specific antibody binding and low cross-contamination between the spots. Some cross reactivity was observed for anti-NOR and anti-CAP (<5%). NOR-NH_2 and CAP-base sensorgrams also showed a small baseline buildup (∼10%). According to the results obtained in this experiment, final antibody dilutions were readjusted and an additional regeneration cycle was added in subsequent measurements. Sensor chip preparation is a crucial step in SPR-based biosensor assay development, because the quality of the modified sensor chip surface greatly

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influences the sensitivity and robustness of the assays. Although contact printing (e.g., Microgrid) is an established method for DNA, protein, and low-molecular-weight-compound microarraying, we believe that, for SPR biosensor implementation in a microarray format, it is rather restrictive. This technology makes it difficult, and often impossible, to combine between optimal conditions for compound solubility, spot formation on the sensor chip surface, and immobilization efficiency. It also limits the high-throughput potential of the assay, because of the uniform immobilization chemistry that must be applied for all the compounds on a single sensor chip surface. Arraying methods that implement an individual fluidic spot approach, such as Continuous Flow Microspotter (Wasatch microfluidics), might have an advantage in such cases.[30]

iSPR-Based Immunoassays for Antibiotics Detection. Multiplex antibiotic detection was achieved by combining seven immunoassays on one sensor chip. The immunoassays were implemented in the competitive format, based on inhibition of antibody binding to the antibiotic immobilized on the surface by the antibiotic in solution. The higher the concentration of the antibiotic in the solution, the lower the binding of the antibody to the surface, and vice versa. Figure 3A shows two raw sensorgrams measured during injections of antibody and regeneration solution on a spot containing an antibiotic and on a blank spot, representing a single measurement cycle. Each measurement cycle lasted 40 min (including the regeneration steps) and provided 24 data points (3 for each antibiotics, plus 3 blanks). The final response after the antibody injection was calculated by first subtracting the baseline (*B*) from the response (*R*), and then subtracting the response on the blank spot from the response on the antibiotic-containing spot. Eight zeroed and referenced sensorgrams, measured on eight spots containing different antibiotics, during single injection of the antibodies cocktail (B_0) are shown in Figure 3B. Because of individual assay optimization, in terms of sensitivity, regeneration, and antibody consumption, maximum responses varied between 10 and 100 millidegrees. The necessity of three regeneration injections, because of difficulties in regeneration of NOR-NH_2 and CAP-base spots, prolonged the assay time by 50%. To shorten the assay time, alternative regeneration solutions were tested, including hydrochloric acid, acetic acid, formic acid, sodium chloride, DMF, DMSO, guanidine hydrochloride, and ethylene glycol at different concentrations. However, none was determined to be as efficient as the 20% (v/v) acetonitrile in 10 mM sodium hydroxide (data not shown).

To study the assay's performance, multianalyte standard solutions that contain NEO, GNT, STR, KAN, ENR, CAP, and SMZ were prepared both in HBS-EP buffer and in 10×-diluted milk. Standard solutions with known antibiotics concentrations (0−500 ng/mL), mixed with the antibodies cocktail, were serially injected in duplicate over the microarrayed chip with antibiotics. Figure 3C shows raw sensorgrams simultaneously measured on eight spots (NEO, GNT, DHS, KAN, NOR-NH_2, CAP-base, and SMZ) during serial injections of three standard solutions at concentrations of 0.16, 0.8, and 4 ng/mL (each duplicate is marked with an arrow). The duplicates showed good repeatability and the increase in antibiotics concentrations in the solution caused a decrease in the responses, as expected. The differences in baselines between the spots are attributed to intrinsic optical

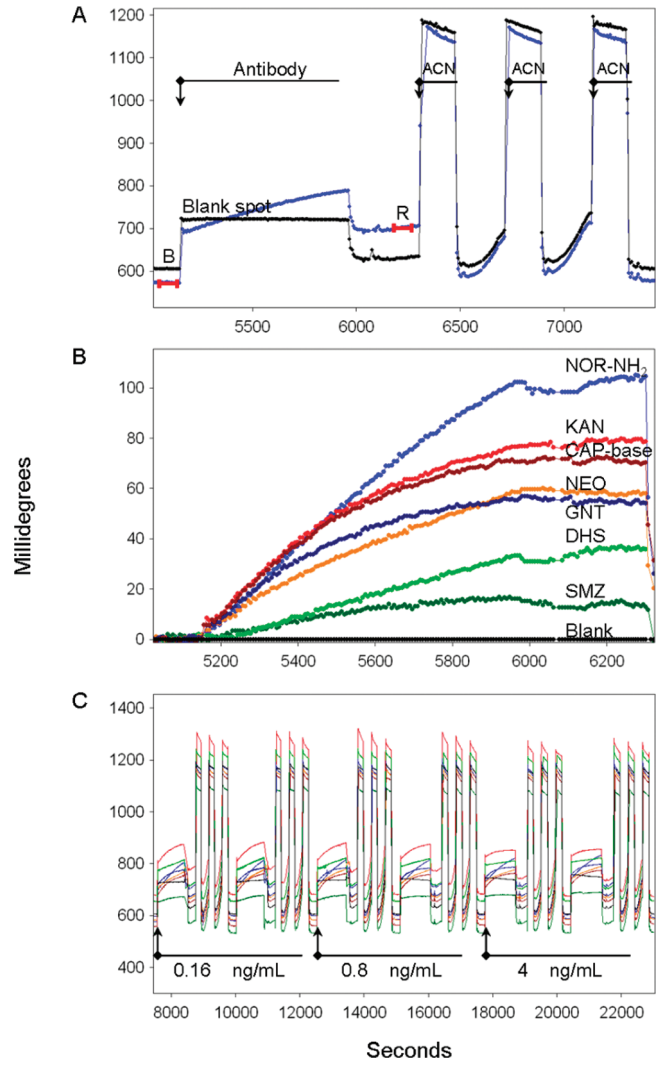

Figure 3. (A) A single measurement cycle. Raw sensorgrams measured on a spot containing antibiotics and on a blank spot during antibody injection and three injections of regeneration solution (20% (v/v) acetonitrile in 20 mM sodium hydroxide). Start of each injection is marked with an arrow. Final response in each measurement cycle was calculated as follows. Baseline (*B*), measured before injection start, was subtracted from a maximum response (*R*), measured in the dissociation phase and the final response measured on the blank spot was subtracted form the final response measured on the spot containing antibiotics. (B) Zeroed and referenced sensorgrams of a single injection of the antibodies cocktail, measured simultaneously on 8 spots (Neomycin (NEO), Gentamicin (GNT), Kanamycin (KAN), Dihydrostreptomycin (DHS), Norfloxacin-NH_2 derivative (NOR-NH_2), Chloramphenicol base (CAP-base), Sulfamethazine (SMZ), and blank spots) without the presence of antibiotics (B_0). (C) Raw sensorgrams measured simultaneously on NEO, GNT, KAN, DHS, NOR-NH_2, CAP-base, SMZ, and blank spots during duplicate injections of multianalyte standard solutions (0.16, 0.8, and 4 ng/mL) mixed with corresponding antibodies. The start of injection of each concentration duplicate is marked with an arrow.

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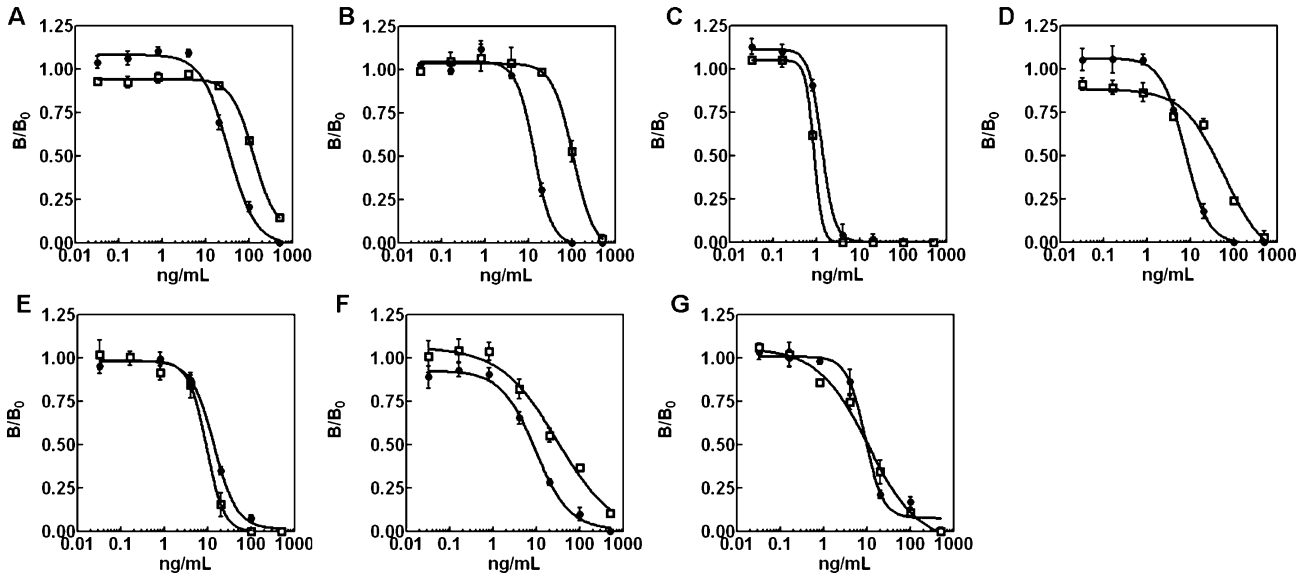

Figure 4. Multianalyte calibration curves (●) in buffer and (□) in 10×-diluted milk of seven antibiotics: (A) Neomycin, (B) Gentamicin, (C) Kanamycin, (D) Streptomycin, (E) Enrofloxacin, (F) Chloramphenicol, and (G) Sulfamethazine. Multianalyte standard solutions that contain antibiotics at concentrations in the range of 0−500 ng/mL were mixed with a cocktail of corresponding antibodies and injected in duplicate over the sensor chip, microarrayed with antibiotics. The relative binding (B/B_0) was calculated by dividing a response (B) of each concentration by a response obtained in a solution without antibiotics (B_0). Solid lines show curves fitted with the four-parameters (4P) model. Error bars represent the standard deviations between three different sensor chips.

Table 1. iSPR-Based Multiplex Immunoassay Characteristics and Comparison to Previously Reported SPR-Based Immunoassays and ELISAs for the Detection of Antibiotics in Milk

		iSPR-Based Multiplex Immunoassay				SPR-Based Immunoassay	ELISA
target compound	matrix	goodness of 4P fit,[a] R^2	curve steepness (mL/ng)	signal inhibition of 1 MR(P)L in milk[b]	IC_{50} (ng/mL)[c]	IC_{50} (ng/mL)	IC_{50} (ng/mL)
Neomycin	buffer	0.9909	−1.4	56%	33 ± 0.5	70 (ref 17)	1 (ref 33), 0.098 (ref 34), 70[g] (ref 35)
	milk[d]	0.9945	−1.7		123.2 ± 0.7	150 (ref 17)	
Gentamicin	buffer	0.9916	−2.1	0% (>3.7 MRLs)[e]	13.4 ± 1.2	40 (ref 17)	0.92 (ref 36), 2[g]
	milk[d]	0.9895	−1.8		105.4 ± 15	70 (ref 17)	
Kanamycin	buffer	0.9971	−2.9	100%	1.2 ± 0.2	20 (ref 17)	2, 5 (ref 37)
	milk[d]	0.9992	−4.5		0.9 ± 0.07	40 (ref 17)	
Streptomycin	buffer	0.9892	−1.1	32%	18.9 ± 1.2	60[f] (ref 17)	2[g] (ref 35)
	milk[d]	0.9944	−1.2		50.1 ± 1.1	140[f] (ref 17)	
Enrofloxacin	buffer	0.9953	−1.6	55%	13.7 ± 2.1	3.4[f] (ref 18)	2[g] (ref 35)
	milk[d]	0.9895	−2.1		9.2 ± 1.1		
Chloramphenicol	buffer	0.9905	−1.1	0% (>22 MRPLs)[e]	9.3 ± 1	5.7, 2.3 (ref 19)	0.2[g] (ref 35)
	milk[d]	0.9742	−0.7		30.3 ± 1.7	9, 4.2 (ref 19)	
Sulfamethazine	buffer	0.9880	−2.1	49%	8.9 ± 1.2	8[f] (ref 16)	0.1[g] (ref 35)
	milk[d]	0.9882	−0.8		10.1 ± 1.3		

[a] Goodness of the four-parameter model fit to the calibration curve. [b] Calculated from the four-parameter fitted calibration curve. [c] Half inhibitory value interpolated from the four-parameter fitted calibration curve, averaged between three different sensor chips. [d] In the iSPR-based immunoassay, 10×-diluted milk was used. [e] Antibiotic concentration that should be present in milk, to produce 10% signal inhibition. [f] Immunoassay is based on the same antibody; [g] Approximated value from the calibration curve presented in the product information.

settings of the iSPR instrument and were not observed to affect the sensitivity of each spot.[25]

For each compound, calibration curves in buffer and in 10×-diluted milk were plotted using relative binding values (B/B_0) as a function of antibiotic concentration (expressed in units of ng/mL) (see Figure 4). The curves were fitted with the 4P model, and IC_{50} values were interpolated. The goodness-of-fit (R^2) and the steepness of the curves are shown in Table 1. For most of the assays, the 4P model fit the data well, both in buffer and in milk. The steepness of the curves for the NEO, GNT, and STR assays did not change much in milk; however, it decreased in CAP and SMZ assays and increased in KAN and ENR assays. Also, note that some of the curves shifted toward the right, to the higher-concentrations range, and some remained unchanged (see Figure 4). Figure 5 shows a summary of the multiplexed assay's characteristics (IC_{50}, LOD, and dynamic range values) in buffer and in milk. We did not observe an uniform influence of the milk on all the immunoassays, indicating that matrix effects, in this case, are most likely due to interaction of milk components with the antibodies or with antibiotics on spots. The effects of different food matrixes on the biosensor performance, as well as its applicability to raw milk samples, will be the focus of further research.

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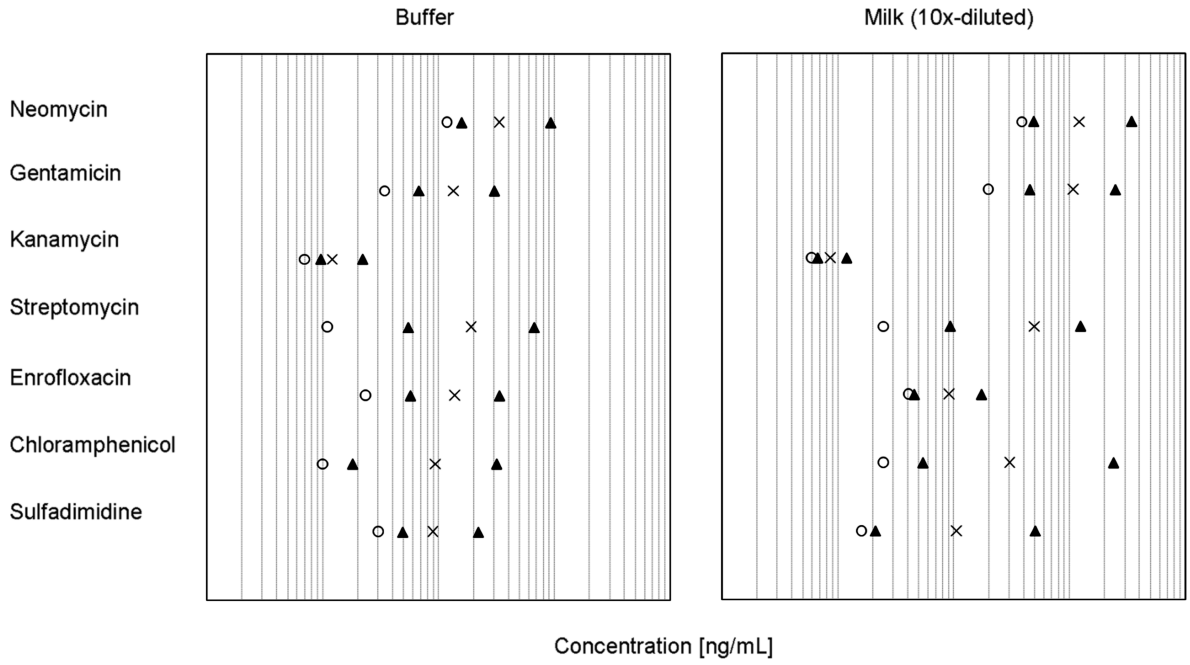

Figure 5. Scheme showing (○) the limit of detection, (▲) the dynamic range, and (×) the IC_{50} value of Neomycin, Gentamicin, Kanamycin, Streptomycin, Enrofloxacin, Chloramphenicol and Sulfamethazine multianalyte iSPR-based biosensor assay in buffer and in 10×-diluted milk. The limit of detection was calculated by subtracting three standard deviations from the responses obtained in blank solutions without antibiotics. The dynamic ranges of the arrays were set in the range of 20%−80% inhibition. IC_{50} values were interpolated from 4P fitted curves.

The immunosensor showed ppb-level sensitivity for the target compounds, both in buffer and in 10×-diluted milk. NEO, KAN, STR, ENR, and SMZ assays were sensitive enough for antibiotic residues detection in milk at MRL levels. However, CAP and GNT assays required more than 20 MRPLs and 4 MRLs, respectively, of antibiotics to be present in milk, to produce 10% signal inhibition. To improve CAP assays sensitivity, we tested a different antibody (rabbit Pab 426 raised against CAP-HS BSA), which was shown to be sensitive in the 0.03−2.2 ng/mL range in ELISA.[31] However, despite the big improvement in sensitivity ($IC_{50\ buffer} = 0.37$ ng/mL), this antibody could not be utilized in the multiplex format, because of cross-reactivity with the rest of the compounds (data not shown). The overall performance of the immunosensor, described here, was determined to be comparable to previously described Biacore 3000-based immunosensors (Table 1).[16−18,32] In comparison to ELISA, iSPR-based immunoassays showed approximately 10-fold lower sensitivity (see Table 1).[33−35] However, the high sensitivities of the ELISA method are unnecessary for the detection of antibiotics in milk at MRLs levels.

In principle, the iSPR platform used in this study can be extended to a maximum of 56 multiplexed assays (using a 56-spot microarray). The use of antibodies with broad cross-reactivity range toward a family of compounds can extend the screening range even further. In our case, cross-reactivity of anti-KAN with Kanamycin B and Tobramycin, anti-SMZ with another 17 sulfonamides, and cross-reactivity of anti-ENR with another four fluoroquinolones broadens the potential screening range of the assay to 30 antibiotic residues.[18,32]

CONCLUSIONS

In this study, we showed an implementation of an imaging SPR-based biosensor to quantitative measurements of antibiotic residues in milk. Seven model compounds (Neomycin, Gentamicin, Kanamycin, Streptomycin, Enrofloxacin, Chloramphenicol, and Sulfamethazine) were simultaneously detected via multiplexed competitive immunoassay, in buffer and in milk, using one sensor chip. Neomycin, Kanamycin, Streptomycin, Enrofloxacin, and Sulfamethazine assays were sensitive enough for milk control at MRL levels. The overall performance of the microarray biosensor based on iSPR was comparable to that reported for conventional Biacore-based biosensors with four flow channels. Efficient immobilization of ligands in a microarray format on the sensor chip, and sensitivity and specificity of the antibodies, played a crucial role in the utilization of a multiplex immunoassay on the iSPR platform. The method described here enabled rapid, simultaneous, and label-free detection of model compounds from four different types of antibiotics: aminoglycosides, fluoroquinolones, fenicols, and sulfonamides, without sample preparation, opening a door to automated and high-throughput food analysis.

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