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Detection of Fluoroquinolone Antibiotics in Milk via a Labeless Immunoassay Based upon an Alternating Current Impedance Protocol

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This paper describes the construction of a labeless immunosensor for the antibiotic ciprofloxacin in milk and its interrogation using an ac impedance protocol. Commercial screen-printed carbon electrodes were used as the basis for the sensor. Polyaniline was electrodeposited onto the sensors and then utilized to immobilize a biotinylated antibody for ciprofloxacin using classical avidin−biotin interactions. Antibody loaded electrodes were exposed to solutions of antigen in milk and interrogated using an ac impedance protocol. The faradaic component of the impedance of the electrodes was found to increase with increasing concentration of antigen. Control samples containing a nonspecific IgG antibody were also studied but were found to display large nonspecific responses, probably due to the antibody binding some of the large number of components found in milk. Control sensors could, however, be fabricated using antibodies specific for species not found in milk. Calibration curves could be obtained by subtraction of the responses for specific and control antibody-based sensors, thereby eliminating the effects of nonspecific adsorption of antigen. Sensors exposed to ciprofloxacin in milk gave increases in impedance whereas ciprofloxacin in phosphate buffer led to decreases, indicating the possibility of developing sensors which can both detect and differentiate between free and chelated antigen.

The principle of immunoassays was first established by Yalow and Berson[1] in 1959 and led to the development of the widely used radioimmunoassay technique, initially to determine insulin-binding antibodies in human serum, using samples obtained from subjects that had been treated with insulin. Independently within unconnected work, Clark and Lyons[2] in 1962 developed the concept of a biosensor. The original methodology involved immobilizing enzymes on the surface of electrochemical sensors so as to exploit the selectivity of enzymes for analytical purposes. Although the field has undergone continual technological developments over the last 40 years, the basic idea has remained virtually unchanged since the original design.

The incorporation of antibodies into conducting polymer films was first reported[3] in 1991. Polypyrrole films were galvanostatically polymerized onto a platinum wire substrate. When grown in the absence of a counterion, a poor polymeric film (both in appearance and electrochemical properties) was formed, suggesting that the presence of a counterion was necessary for the polymerization process to be successful. Antihuman serum albumin (anti-HSA) could be incorporated into the film; amino acid analysis of the resulting polymer using a leucine marker determined that approximately 0.1% w/w (0.2 μg) of the antibody was incorporated into the matrix. Exposure of the pyrrole anti-HSA electrode to a 50 μg mL^{-1} HSA solution for 10 min led to the formation of a new reduction peak at a potential of approximately +600 mV versus Ag/AgCl. This peak increased in magnitude after a further 30 min in the same solution, suggesting that this could be due to an antibody/antigen interaction with the polymer. Further work by the same group gave rise to reports of a reversible real-time immunosensor.[3] Other early work utilized a pulsed amperometric detection technique for other analytes, including *p*-cresol,[4] thaumatin,[5] and polychlorinated biphenyls.[6] Since this early work there has been burgeoning interest in the development of electrochemical immunosensors, as detailed in several recent reviews.[7−9]

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Antibody−antigen interactions are by their very nature complex, and it follows that the affinity reaction must be only minimally perturbed by the fabrication procedure to allow the immunosensors to display reproducible response characteristics. Previously within our group we have shown that up to 2−3 μg of antibodies for BSA and digoxin may be successfully incorporated into conducting polymer films by entrapment in a growing polypyrrole film with no detrimental effect to antibody activity.[10] Electrochemical interrogation of these films demonstrated selective interactions with the target antigens. Further work utilized an ac impedance protocol[11] as the method of interrogation for these films, and led to the development of immunosensors for digoxin and bovine serum albumin. Later work by our group studied approaches for immobilization of antibodies onto polyaniline-coated screen-printed carbon electrodes utilizing a classical avidin−biotin chemistry. This enabled the construction of immunosensors for the fluoroquinolone antibody ciprofloxacin[12] in buffer solutions. We have also demonstrated selective immunosensors for myelin basic protein,[13] prostate specific antigen,[14] and the stroke marker proteins neuron specific enolase[15] and S-100 [β].[16]

Fluoroquinolones are members of the quinolone family of broad-spectrum antibiotics and comprise the majority of quinolones in clinical use, the name fluoroquinolone deriving from the fact they have a fluoro group attached to the central ring system. Antibiotics of this type have excellent tissue penetration which makes them extremely effective against bacteria that grow intracellularly such as salmonella, and this has led to their widespread use within adult patients.[17] One antibiotic within this group is ciprofloxacin (Figure S1, Supporting Information) which is a broad-spectrum antibiotic active against many bacteria including anthrax.[18] Many of these fluoroquinolones are added to farm animal feed since they can lead to greater and more rapid weight gain. Unfortunately this is also thought to have contributed to the rise of antibiotic resistant species of bacteria.[17]

The monitoring of fluoroquinolones within both food and the environment is important since these antibiotics have potential health related and environmental damaging effects. Ciprofloxacin concentrations in hospital wastewater were monitored and correlations with DNA damaging effects made.[19] HPLC techniques have been utilized to measure levels of ciprofloxacin in hospital outflow water[19] and found levels between 0.7−124.5 ng mL^{-1}, as well as displaying genotoxicity at levels as low as 5.2 ng mL^{-1}. Similar work utilized LC/MS/MS methods[20] and found wastewater ciprofloxacin levels between 0.031−5.6 ng mL^{-1} (even after treatment) with a limit of detection of 0.030 ng mL^{-1}. Levels in vivo have also been widely studied with the therapeutic ranges typically being between 0.57−2.30 μg mL^{-1} in serum and 1.26−4.03 μg g^{-1} in tissue.[21]

A horseradish peroxidase-based biosensor[18] has been developed for the detection of ciprofloxacin due to its inhibition of the oxidation of catechol. Linear responses were obtained between 0.02−65 μM with the limit of detection being 0.4 nM; however, other piperazine-based compounds could potentially interfere with this determination. We have within earlier work developed a labeless immunosensor for ciprofloxacin as a typical fluoroquinolone which utilizes screen-printed carbon electrodes modified by deposition of first, a conducting polymer (polyaniline) which is then functionalized with a biotinylating reagent.[12] Complexation of the immobilized biotin with neutravidin allows the further binding of biotinylated antibodies via standard avidin−biotin interactions (Figure S2, Supporting Information). The resultant electrodes are capable of detecting low levels of the ciprofloxacinin antigen dissolved in phosphate buffer.[12] For an immunosensor to be of practical use, however, it must be capable of the analysis of the substrate within a wide range of matrixes. Since fluoroquinonolones are widely used within the animal industry, it was decided to investigate whether ciprofloxacin could be detected in milk. The European Union has set a maximum residue limit of 100 ng mL^{-1} for enrofloxacin plus ciprofloxacin in milk.[22] Previous studies have utilized techniques such as HPLC to detect various fluoroquinolone antibodies in bovine milk[22] with a detection limit of 3 ng mL^{-1} for ciprofloxacin and LC with luminescence detection[23] to measure ciprofloxacin in milk with a linear range of 8−3500 ng mL^{-1} and a detection limit of 3 ng mL^{-1}. A number of ELISA-based tests[24,25] have also been developed to detect fluoroquinolones in milk with detection limits of several nanograms per milliliter. Recent work has also described the construction of a DNA-based sensor which when combined with a surface plasmon resonance method allows quantification of enrofloxacin between 3−20 μg mL^{-1} in milk.[26] An immunosensor for ciprofloxacin based on polypyrrole films combined with an ac impedance technique[27] has also been described with sensitivities as low as 10 pg mL^{-1}.

EXPERIMENTAL SECTION

Sodium dihydrogen orthophosphate, disodium hydrogen orthophosphate, sodium chloride, hydrochloric acid, were obtained from BDH (Poole, Dorset, U.K.). Potassium chloride was obtained from Fisher Scientific UK Ltd., Loughborough, U.K.. Aniline,

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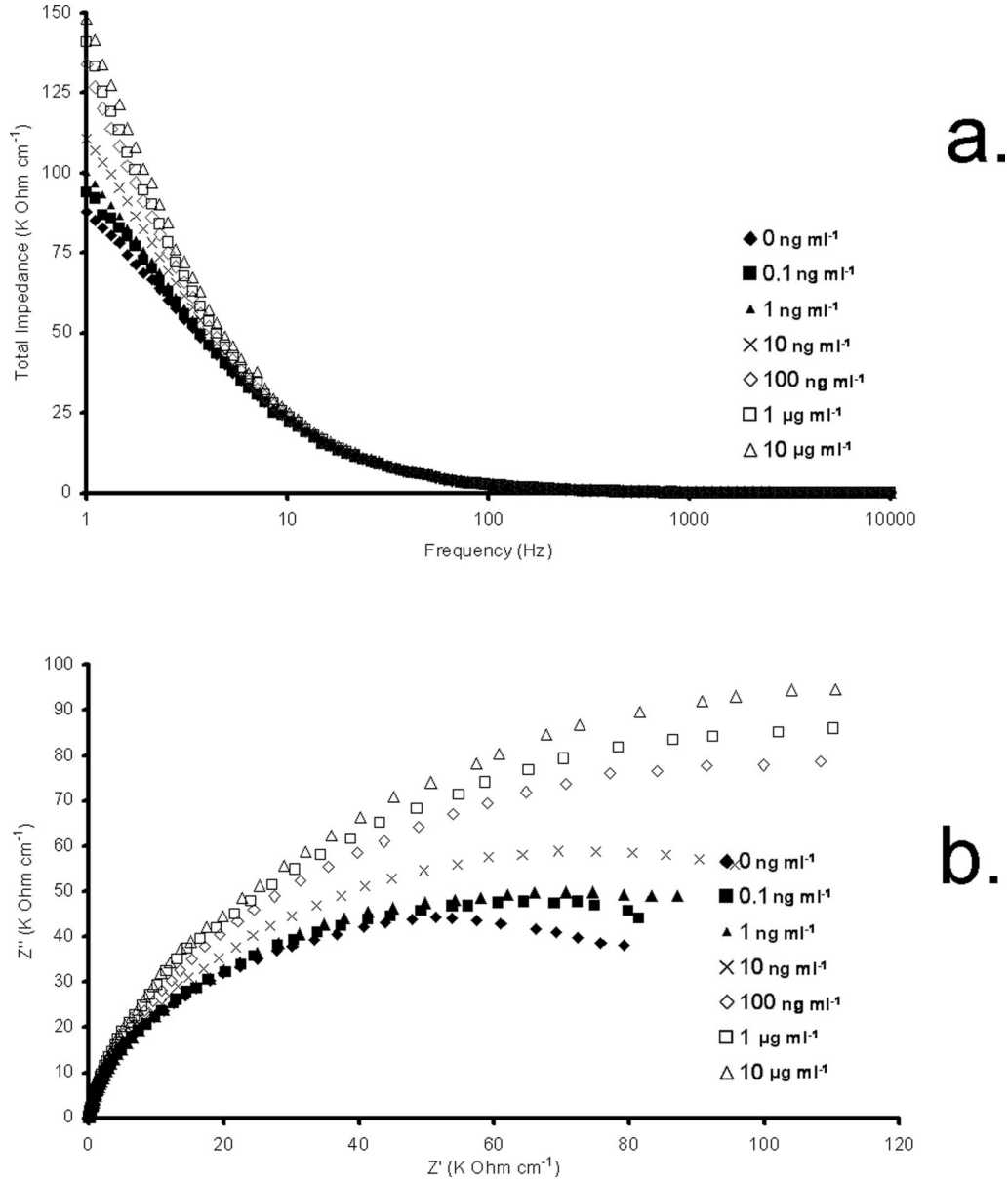

Figure 1. (a) Bode and (b) Nyquist plots of a typical ciprofloxacin-specific antibody modified electrode exposed to ciprofloxacin.

polyclonal IgG (rabbit), the biotinylation kit (part no. BK101), biotin 3-sulfo-*N*-hydroxysuccinimide, avidin, and bovine serum albumin (BSA) were obtained from Sigma-Aldrich, Gillingham, Dorset, U.K. Ciprofloxacin hydrochloride was purchased from UQUIFA (Barcelona, Spain). All water used was obtained from a Purelab UHQ Deioniser (Elga, High Wycombe, U.K.). Commercial screen-printed carbon electrodes identical to those used in previous work (Figure S3, Supporting Information) containing carbon working and counter electrodes and an Ag/AgCl reference electrode were obtained from Microarray Ltd., Manchester, U.K. The surface area of the working electrode was 0.2178 cm^2. Semiskimmed bovine milk was purchased from a local supermarket.

Phosphate Buffered Saline (PBS) stock solution (pH 7.4) was prepared containing 0.14 mol L^{-1} NaH_2PO_4, 0.52 mol L^{-1} Na_2HPO_4, and 0.0051 mol L^{-1} NaCl. Aniline buffer (pH 1−2) was prepared containing 0.5 mol L^{-1} KCl, 0.3 mol L^{-1} HCl, and 0.2 mol L^{-1} aniline.

Polyclonal antiserum (As 171) was a gift from AMRg and was raised (in rabbit) against 1-(3- mercaptopropyl)-6-fluoro-7-(piperanicyl)-1,4-dihydro-4-oxo-quinoline-3-carboxylic acid coupled to horseshoe crab hemicyanin. The preparation of the immunogen and of the antibodies has been described elsewhere.[36] The antibody was supplied in raw serum. Monoclonal antibody to prostate specific antigen (PSA 30 mAb, isotype IgG1, product number 323-01) with sodium azide preservative was supplied by Canag Diagnostics, Ltd. (Gothenburg, Sweden).

For antibody biotinylation, the procedure outlined in the BK101 kit was followed (see manufacturer's instructions for details). Biotinylated antibodies were kept frozen in aliquots of 200 μL at a concentration of 1 mg mL^{-1} until required.

Cyclic voltammetry (Sycopel Potentiometer, Sycopel Scientific, Tyne and Wear, U.K.) was utilized to deposit polyaniline films on the carbon electrodes. Screen-printed carbon electrodes were placed in aniline buffer and cycled from −200 to +800 mV versus Ag/AgCl for approximately 20 cycles (occasionally this was varied

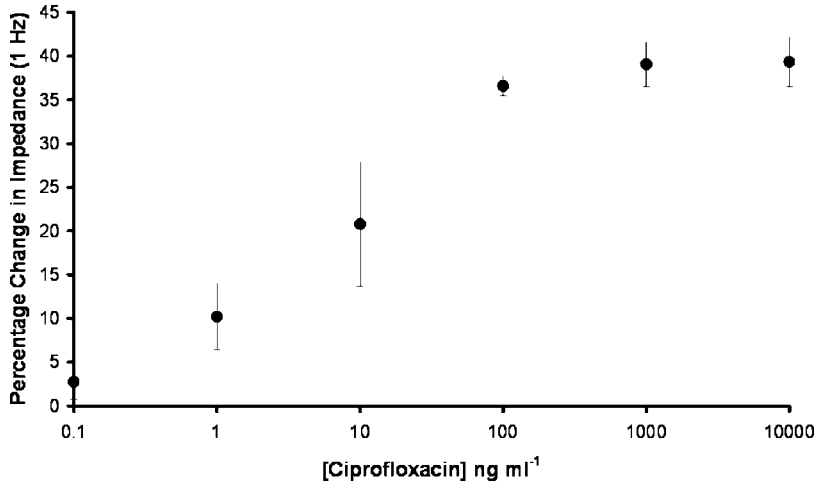

Figure 2. Calibration curve for anticiprofloxacin modified electrodes.

slightly to ensure the same quantity of polyaniline was deposited on each electrode). Deposition was terminated at +800 mV to ensure the polyaniline remained in its conducting form. After deposition, electrodes were rinsed in water.

A volume of 30 μL of biotin-sulfo-NHS (10 mg mL^{-1} in water) was placed on the polymer-coated working electrode surface for 24 h. The sensors were rinsed with copious water, and 30 μL of avidin (10 μg mL^{-1} in water) was placed on the working electrode for 1 h followed by rinsing in water. Then 30 μL of biotinylated antibody (1 mg mL^{-1} in water) was added followed by rinsing. Finally, nonspecific interactions were blocked by BSA (10^{-6} M in PBS); the sensors may be used at this point, however, if opted, can be stored in PBS at 4 °C for up to 24 h or dry at 4 °C for several weeks.[14−16]

Alternating current impedance measurements were performed using an ACM Auto AC DSP frequency response analyzer. Antigen solutions were prepared by initially dissolving ciprofloxacin in PBS, then diluting to the required concentration of antigen in 30 mL of milk. A range of concentrations was utilized; we set our minimum level at 0.1 ng mL^{-1} with an upper limit of 10 μg mL^{-1}. The sensors were first interrogated without antigen addition. Following this, each sensor was exposed to the required antigen concentration for 30 min, rinsed well with deionized water, and then subjected to impedance interrogation in fresh milk. Three electrodes were used for each measurement. A frequency range from 10 kHz to 1 Hz was measured, with a peak amplitude of 5 mV and a dc offset of +400 mV against Ag/AgCl.

RESULTS AND DISCUSSION

Deposition of Polyaniline. The voltammograms for the deposition of polyaniline were as found in earlier work[12,13] and imply a steady in situ formation of polymer at the electrode surface. As the number of scans increases, peaks appear between +350 and 400 mV vs Ag/AgCl corresponding to the oxidation and reduction of surface bound polyaniline, with the current passed increasing with the number of scans due to the increase in polyaniline thickness and coverage of the electrode.

Impedance Profiles of the Electrodes. A series of Bode (Figure 1a) and Nyquist curves (Figure 1b) were obtained for the sensors after exposure to various levels of ciprofloxacin in milk. As can be seen, there is a steady increase in the impedance of the electrodes with increasing antigen concentration. This was unexpected since our work on ciprofloxacin in phosphate buffer (containing 10 mM ferri/ferrocyanide) clearly demonstrates that binding of the antigen leads to a drop in impedance. As can be seen, we see much larger increases in impedance at the lower frequencies. Therefore it was decided that changes in impedance at 1 Hz would be used as a measurement of antigen binding.

The Nyquist plots display the two components of the impedance spectra: the real (Z') component where the impedance in phase with the ac potential waveform is measured and the imaginary (Z'') where the measured impedance is 180° out of phase. Both of the individual components contribute to the total impedance of the cell. However it is important to differentiate between these so that the individual components of the composite impedimetric response may be identified and quantified.

In this system, the Z' (real) component of the impedance can be seen to steadily increase with decreasing frequency. In the baseline plot, the Z'' (imaginary) component increases to a maximum value before falling as the frequency approaches 1 Hz. Similar behavior appears to occur as antigen binding increases except that for the higher concentrations of ciprofloxacin the maximum value is beyond our frequency range. This type of impedance spectrum is indicative of a surface-modified electrode system where the electron transfer is slow and the impedance is controlled by the interfacial electron transfer.[28]

In previous pieces of work within our group we have demonstrated that although both the imaginary and real components increase, the increase in the real component dominates the total increase in the impedance.[11−13] Although in this case and in previous work,[11−13] changes in both real and imaginary components are visible, once again the real component is the major component of total impedance and perhaps more importantly we have repeatedly found that the real component offers far greater reproducibility in comparison to the imaginary contribution.

Figure 2 shows the percentage increase in Z' (measured at 1 Hz) across a range of antigen concentrations. As can be seen, there is a steady increase in the real component of impedance as antigen concentration increases up to a concentration of about 100 ng mL^{-1}, above which concentration there is a tendency

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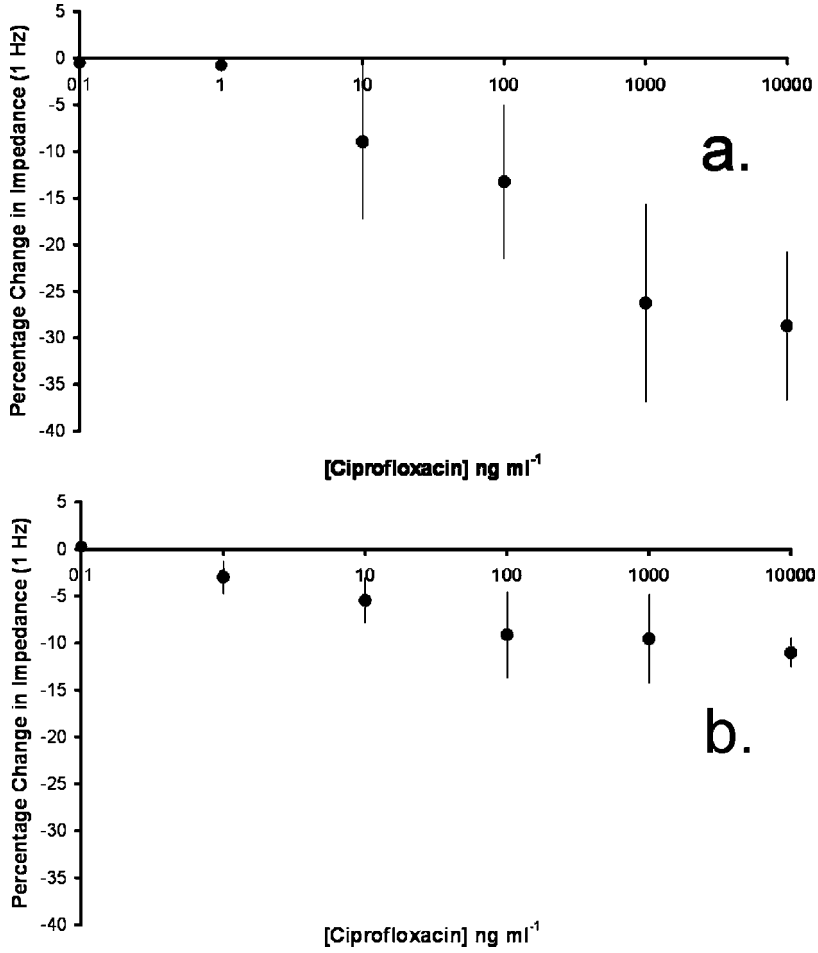

Figure 3. Control calibration curves for (a) IgG modified electrodes and (b) PSA modified electrodes.

toward a plateau, possibly indicating saturation of the specific binding sites. It is possible that any further changes in impedance beyond this level could simply be due to nonspecific interactions. Between a concentration range of 1−100 ng mL^{-1}, there is a near linear correlation of the impedance change with the $\log_{10}$ of concentration ($R^2 = 0.96$).

Nonspecific interactions could potentially interfere with immunosensor performance. We have previously described the utilization of control electrodes containing a nonspecific IgG antibody.[11−13] These permit the subtraction of unspecific interactions from the specific binding response and also may help to increase the stability and reliability of these sensors when applied to complex mixtures such as milk, other foodstuffs, or clinical samples. Therefore an identical set of immunosensors were fabricated utilizing a nonspecific IgG antibody in place of the specific ciprofloxacin antibody. The commercial IgG sample was taken from the same species as the specific antibody was raised (rabbit) in order to make the match as relevent as possible. Results for these electrodes were obtained in exactly the same way, and the calibration plot is shown (Figure 3a). As can be seen, there is a very high decrease in impedance for the nonspecific antibody. However further experiments showed that these results are a function of the time of exposure rather than ciprofloxacin concentration, i.e., a repeat of the experiment where the electrodes were exposed just to milk without antigen gave an identical plot. A possible explanation for this is that since milk is such a complex mixture of components and IgG is a mixture of different antibodies, some of the antibodies will specifically bind to certain species in milk.

In order to test this theory, another set of control electrodes were utilized, where instead of nonspecific IgG antibody, a specific antibody for an antigen not normally found in milk was utilized. Figure 3b shows the results upon exposure to ciprofloxacin in milk for electrodes fabricated using a specific antibody for prostate specific antigen (PSA), used in earlier work.[14] Results for these electrodes showed that although there are nonspecific interactions, between the ranges of 0.1−100 ng mL^{-1}, they comprise a minor component of the detected response and in fact lead to a drop rather than a gain in impedance, as was found within our earlier work on ciprofloxacin in PBS.[12] Although PSA has been found in human milk,[29] the median levels were well below 1 ng mL^{-1} and also as all readings are referenced to electrodes after exposure to bovine milk containing zero ciprofloxacin, any effect to due binding of "bovine PSA" would cancel out. Also our previous work has shown antibodies can differentiate between similar compounds from different species, for example, they can differentiate between bovine and human serum albumin.[30]

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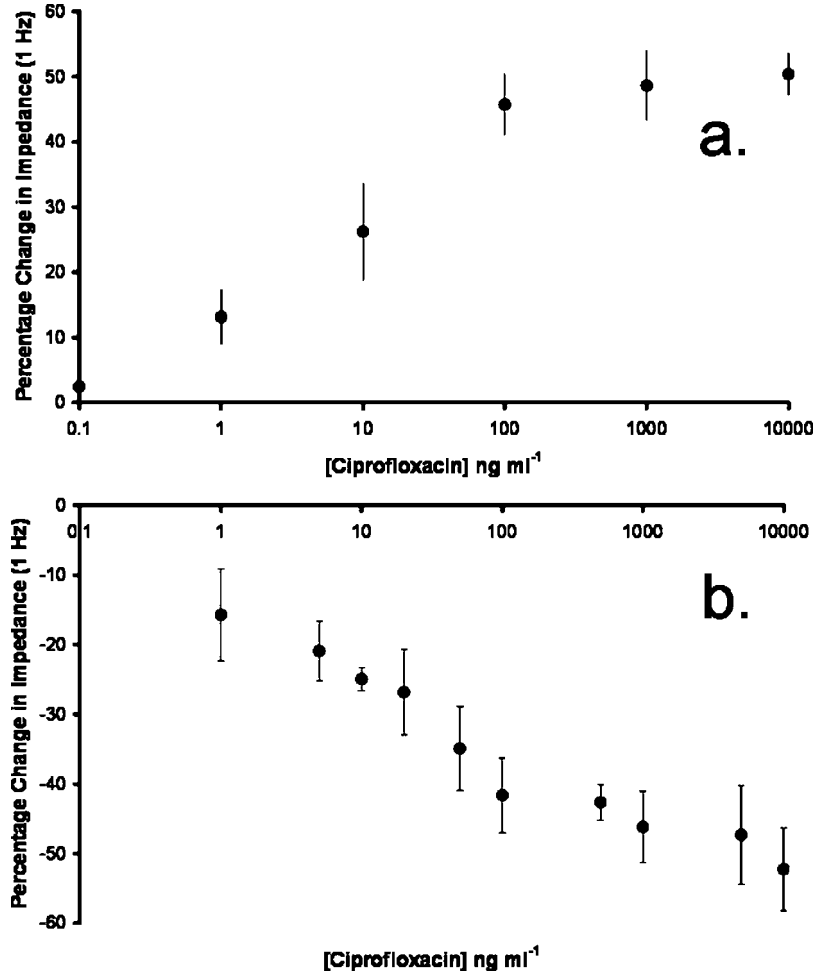

Figure 4. (a) Corrected calibration curve for anticiprofloxacin modified electrodes in milk (Figure 2 − Figure 3b) and (b) for comparison purposes the calibration curve for ciprofloxacin in phosphate buffer previously reported.[12] All data points are means of three electrodes; error bars give a measure of the reproducibility of the system.

Figure 4a shows the subtracted responses (Figure 3a − Figure 2). As can be seen this demonstrates linearity between the response and the $\log_{10}$ of ciprofloxacin concentration between 0.1−100 ng mL^{-1} ($R^2 = 0.98$). Above 100 ng mL^{-1}, the receptors appear to be saturated. Figure 4b displays our earlier results for ciprofloxacin in PBS (containing 10 mM ferri/ferrocaynide)[12] overlaid over those for milk. As can be seen, the results are very similar in that they give linear ranges with very similar gradients in the region 0−100 ng mL^{-1} and above 100 ng mL^{-1} appear to be saturated. However they are of opposite nature; adsorption of ciprofloxacin from milk gives an increase in Z' whereas from PBS a decrease is observed. One potential explanation for this is that in milk a different ciprofloxacin species is being adsorbed. It has been reported that in the presence of calcium ions, many fluoroquinolone antibiotics, including ciprofloxacin, readily form calcium chelate complexes.[31,32] These complexes are usually dimeric in nature with two ciprofloxacin units complexed to a single calcium ion.[31] Binding of ciprofloxacin in either its free or chelated form over similar concentration ranges is occurring, as shown by responses compared to control samples, with the great structural differences between the free and complexed forms of the antigen leading to different effects on the ac impedance results. The effects of adsorption of various moieties on the ac impedance behavior is highly complex and not well understood. For example in previous work by our group on DNA-based sensors, adsorption of cDNA stands has been shown to lead to drops in the ac impedance of our electrodes whereas adsorption of noncDNA leads to an increase.[33,34] Unfortunately control experiments where calcium ions were added to PBS were not possible since addition of calcium to the buffer cause immediate precipitation of calcium phosphates.

These results are of interest since they show that it may be possible to differentiate between complexed and free ciprofloxacin. Ongoing work is attempting to determine whether the complexed and uncomplexed forms can be differentiated. This is of interest since complexed form of ciprofloxacin is inactive, for example, the FDA recommends that ciprofloxacin is not taken with dairy products, calcium fortified juices, or calcium containing antacids[35] since they may lower adsorption of the drug.

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CONCLUSIONS

We have demonstrated the construction of an immunosensor for the antibiotic ciprofloxacin using a combination of screen-printed electrodes coated with conducting polyaniline and immobilized antibodies. The electrodes were then exposed to solutions of the antigen in milk. Interrogation of the electrodes by ac impedance demonstrated the detection of the antigen. Linear increases of the impedance change with the $\log_{10}$ of concentration ($R^2 = 0.98$) was observed between concentrations of 0.1−100 ng mL^{-1}. The sensor not only bound the antigen from PBS or milk but the ac response was dependent on the matrix the ciprofloxacin was adsorbed from, possibly due to formation of a dimeric calcium chelated form of the antibiotic in milk. This is especially of interest, since free and chelated ciprofloxacin have very different adsorption characteristics when ingested.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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