Chapter 18

Electrochemical Quartz Crystal Nanobalance (EQCN) Based Biosensor for Sensitive Detection of Antibiotic Residues in Milk

Sunil Bhand and Geetesh K. Mishra

Abstract

An electrochemical quartz crystal nanobalance (EQCN), which provides real-time analysis of dynamic surface events, is a valuable tool for analyzing biomolecular interactions. EQCN biosensors are based on mass-sensitive measurements that can detect small mass changes caused by chemical binding to small piezoelectric crystals. Among the various biosensors, the piezoelectric biosensor is considered one of the most sensitive analytical techniques, capable of detecting antigens at picogram levels. EQCN is an effective monitoring technique for regulation of the antibiotics below the maximum residual limit (MRL). The analysis of antibiotic residues requires high sensitivity, rapidity, reliability and cost effectiveness. For analytical purposes the general approach is to take advantage of the piezoelectric effect by immobilizing a biosensing layer on top of the piezoelectric crystal. The sensing layer usually comprises a biological material such as an antibody, enzymes, or aptamers having high specificity and selectivity for the target molecule to be detected. The biosensing layer is usually functionalized using surface chemistry modifications. When these bio-functionalized quartz crystals are exposed to a particular substance of interest (e.g., a substrate, inhibitor, antigen or protein), binding interaction occurs. This causes a frequency or mass change that can be used to determine the amount of material interacted or bound. EQCN biosensors can easily be automated by using a flow injection analysis (FIA) setup coupled through automated pumps and injection valves. Such FIA-EQCN biosensors have great potential for the detection of different analytes such as antibiotic residues in various matrices such as water, waste water, and milk.

Keywords Quartz crystal nanobalance, Flow injection analysis, Antibiotic residues, Milk, Sulfadiazine

1 Introduction

Several biosensor techniques have been reported in recent years for detection of antibiotic residues in the milk and other biological samples. Electrochemical quartz crystal nanobalance (EQCN) is a recent advancement over the Quartz Crystal Microbalance (QCM). It is a suitable technique for a wide variety of applications. Owing to its simplicity, convenience, and real time response, QCM has been increasingly explored in many fields of biomolecular

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analysis [1, 2]. QCM measures a mass variation per unit area by measuring the change in frequency of a quartz crystal resonator when a voltage applied across a quartz crystal, it induces a mechanical stress on the crystal which causes the quartz crystal to oscillate at a specific frequency related to the voltage. The frequency of oscillation of the quartz crystal is partially dependent on the thickness of the crystal. During normal operation, all the other influencing variables remain constant; thus a change in thickness correlates directly to a change in frequency. As mass is deposited on the surface of the crystal, the thickness increases; consequently, the frequency of oscillation decreases from the initial value. With some simplifying assumptions, this frequency change can be quantified and correlated precisely to the mass change using Sauerbrey's equation. The principle of QCM detection is based on frequency changes of a crystal that are proportional to the mass changes of the crystal surface. The quantitative relationship between the frequency shifts and the mass changes of the crystal is given by the well-known Sauerbrey's equation [3].

$$\Delta f = \frac{-2\Delta m f_0^2}{A\sqrt{p_q \mu_q}}$$

Where, Δf is frequency change (Hz), Δm is the mass change (gm) on the sensor surface, f_0 is the fundamental frequency (Hz), A the electrode surface area, and ρ_q and μ_q are the density (cm^3) and the shear modulus (gm cm^{-1} s^{-2}) of the QCM sensor respectively.

QCM has the advantages of minimal electrical requirements, adaptability to microfluidic techniques, inexpensive fabrication, utility in a flow cell, and label-free detection. A QCM biosensor integrated in a flow injection analysis (FIA-QCM) system has advantages in terms of reproducibility, speed of analysis, control of contact time, and concentration profiling in kinetic studies [4].

The EQCN is a measurement system for monitoring extremely small variation in the mass of a working electrode attached to a vibrating quartz single crystal. Thin wafers cut from a quartz crystal at a specific orientation (35°15′ angle) and with precise thickness (0.166 mm), which oscillates at a characteristic fundamental frequency of 10 MHz are deployed. The working electrode is in the form of a thin metal film evaporated on one side of a quartz crystal wafer, which is then sealed to the side opening in an electrochemical cell. Any change in the mass rigidly attached to the working electrode results in the change of the quartz crystal oscillation frequency. The frequency of the working quartz crystal is measured and compared to the frequency of the standard reference quartz crystal. Hence, the frequency measurements are differential, i.e. the frequency of the reference crystal is subtracted from the frequency of the working crystal. The obtained frequency difference is then converted to an analog voltage using a frequency-to-voltage

converter and measured using a 16-bit analog-to-digital converter (ADC). The frequency shift is proportional the mass change of the working electrode. The EQCN experiments are completely computer controlled with the data logger DAQ-716 with real-time VOLTSCAN data acquisition and control system (based on a 16-bit D/A converter and 16-bit A/D converters). All the data processing, graphing, and spreadsheet reporting are done with Voltscan 5.0 software. Calculations of frequency to mass change are calculated as per the following equation:

$$\Delta m = \frac{-\Delta f \times A \times \sqrt{\mu_q p_q}}{2\left(fq^2\right)}$$

where

Δm = Mass change (gm),
Δf = Resonant frequency change (Hz),
A = Piezoelectric active surface for reaction (0.25 cm^2),
μ_q = AT-cut quartz constant (2.947 × 10^{12} g cm^{-1} s^{-2}),
ρ_q = Quartz crystal density (2.65 g cm^3),
fq = Reference frequency or resonant frequency (Hz).

In the reported EQCN 700 system, resonant frequency of the gold crystal is 10 MHz (10 × 10^6 Hz) and calculated change of 1 Hz equals 1.1040 ng.

1.1 FIA-EQCN Biosensor for Antibiotic Residue Analysis in Milk

Antibiotics are one of the most important bioactive and chemotherapeutic groups of compounds. In veterinary medicine, antimicrobial drugs are routinely used to treat mastitis in dairy cows but the efficiency of antibiotics as growth enhancers has also led to their use as supplements in animal feed. Sulfonamides and aminoglycosides are the families of chemotherapeutic widely used for therapeutic and prophylactic purposes in animal diseases. Antibiotics are being used either individually or in combination with other drugs for the treatment of inflammatory diseases or as feed additives. Toxicological data reveals that some antibiotics have anti thyroid effects in both animals and humans [5]. Sulfadiazine (SDZ) is a member of the sulfonamide or the sulfa drug family, which plays an important role in veterinary medicine by controlling many bacterial and protozoan diseases [6]. The occurrence of these antibiotic residues in food carries the risk of increasing the number of resistant bacteria and transferring antibiotic resistance genes to human pathogens. Indiscriminate use of antibiotics may produce residues in the milk, and subsequently induce allergic reactions in humans. Monitoring of antibiotics is very important for safety of milk or milk products for human consumption. The presence of these antibiotic residues even in minute amounts can trigger potential health hazards such as allergic reactions in hypersensitive individuals, toxicity, or they can act as potential carcinogens [7]. Economic reasons have also led to control of antimicrobial residues

in food. Several regulatory agencies have set the maximum residual limit (MRL) for antibiotics in milk for consumer protection. In particular, the European Union (EU) has fixed the limit for SDZ at 100 ng/mL.

2 Materials

2.1 List of the Instruments and Chemicals

1. EQCN-700 system integrated with a microfluidic flow cell, model FC-6 (Elchema, USA).
2. Gold quartz crystal (10 MHz, AT cut) (Elchema, USA).
3. Multichannel peristaltic pump (Gilson, France).
4. 6-port injection valve (Rheodyne, USA).
5. Faraday cage (Elchema, USA).
6. Micropipettes (Eppendorf®, Germany).
7. Minispin centrifuge (Eppendorf®, Germany).
8. pH meter (Mettler Toledo, Switzerland).
9. Milli-Q RO system (Millipore, USA).
10. Anti-sulfadiazine polyclonal antibody (pAb-SDZ) Ig fraction isolated from sheep (Randox, UK).
11. Sulfadiazine and sulfadiazine ELISA kit (Randox, UK).
12. Sodium dihydrogen phosphate monohydrate (Merck, Germany).
13. Disodium hydrogen phosphate monohydrate, (Merck, Germany).
14. Sodium chloride (Merck, Germany).
15. Glutaraldehyde (Merck, Germany).
16. Glycine (Merck, Germany).
17. Cysteaminium chloride (Merck, Germany).

3 Methods

3.1 Experimental Setup

EQCN-700 system integrated with a microfluidic flow cell, model FC-6 (Elchema, USA) is deployed for construction of the FIA-EQCN biosensor as presented in Fig. 1 (*see* **Note 1**). The gold quartz crystal (10 MHz, AT cut) is connected to the frequency-measuring unit, which contains a reference crystal with the same characteristics as the measuring crystal (*see* **Notes 2** and **3**). A multichannel peristaltic pump (Gilson, France) is connected through a 6-port injection valve (Rheodyne, USA) to the flow-cell. The output from the oscillator is measured by the frequency unit, which calculated the mass by using a conversion factor (1 Hz

equivalent to 1 ng). A personal computer controlled data acquisition. The flow cell is placed in a Faraday cage to shield the crystal from external electromagnetic sources.

The gold quartz crystal is placed in the plexiglass flow cell, sandwiched between two O-rings, with its upper electrode in contact with the liquid and its lower electrode in the air. The diameter of the quartz wafer is 14 mm and that of the working electrode is 5 mm. The volume of the flow cell is 50 μL. The peristaltic pump is connected in such a way that it reduced the pressure exerted on the crystal. Samples are placed in the 6-port injection valve and injected into the carrier buffer through a 100 μL sample loop. The samples are passed over the biofunctionalized gold coated quartz crystal

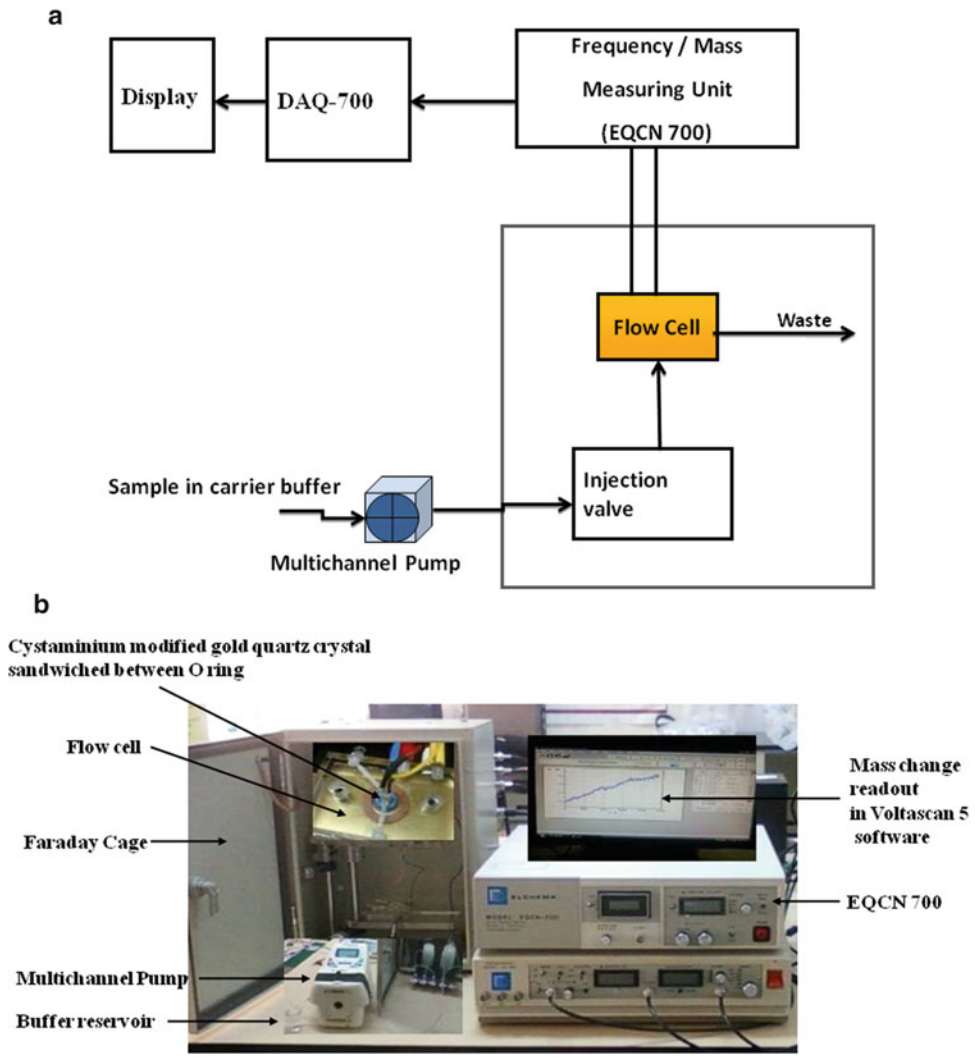

Fig. 1 (**a**) Schematic of FIA-EQCN and (**b**) Photograph of FIA-EQCN biosensor for antibiotic residue analysis. (**c**) Photographic procedure for FIA-EQCN operation

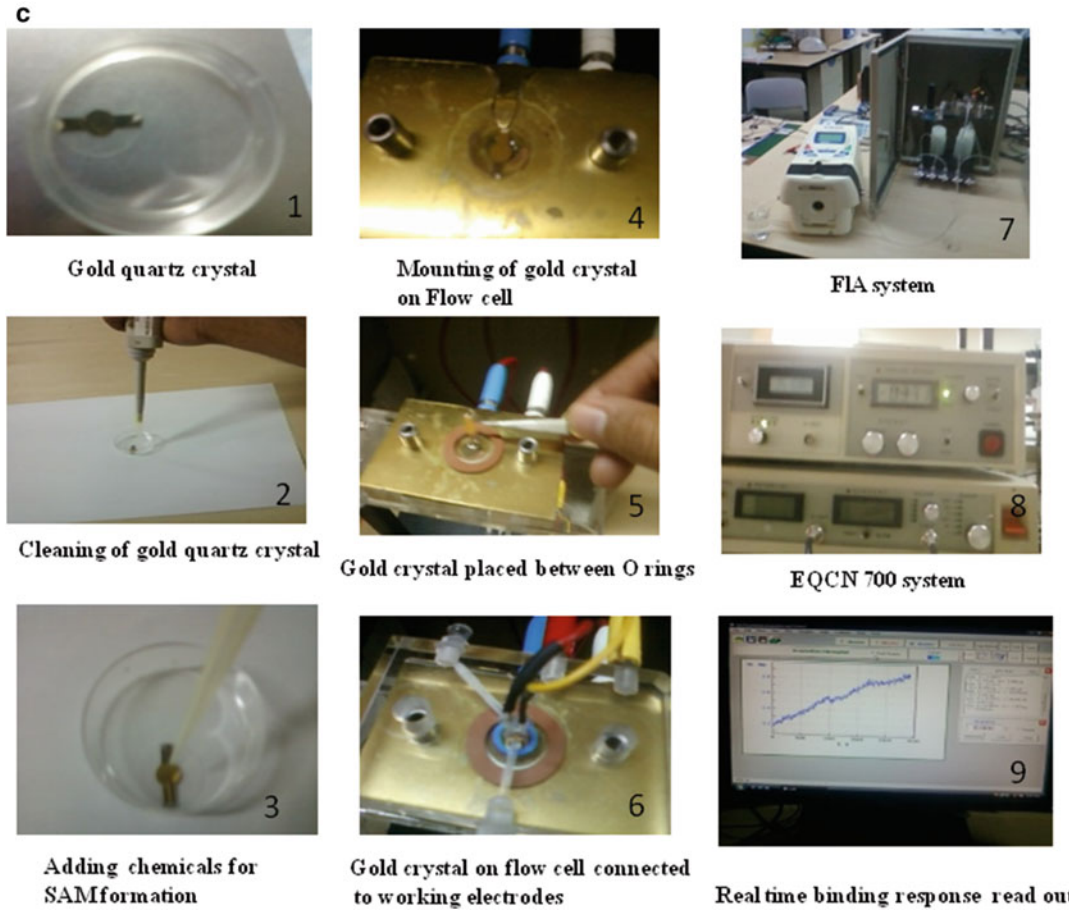

Fig. 1 (continued)

surface through the flow cell using a flow rate 0.5 mL/min in static mode and 0.1 mL/min in semi-automated mode. Real time responses are recorded until equilibrium. To attain a steady state equilibrium, about 2 mL of sample is used at the flow rate of 0.1 mL/min. The capacity of the working area (area under O-ring) of the flow cell is 40 μL at equilibrium.

3.2 Solution Preparation

Phosphate buffered saline (PBS, 100 mM) is prepared by dissolving appropriate amount of Na_2HPO_4 and NaH_2PO_4 containing 137 mM NaCl and 27 mM KCl in membrane filtered RO water. The pH of the buffer is adjusted to 7.4 before use. For preparation of standards, known amounts of antibiotics are dissolved in PBS and further diluted to meet the calibration standards. For analysis of milk, samples are centrifuged and diluted (1:4) in PBS. All solutions are freshly prepared before the experiment and stored at 4 °C when not in use (*see* **Note 4**).

3.3 Formation of SAMs

Immobilization of biomolecules on the sensing surface is an important consideration in the construction of an efficient biosensor. Several immobilization and surface modification strategies are used for biomolecule attachment. One such approach is to use cross-linking via Self-assembled monolayers (SAMs). To prepare a FIA-QCM biosensor setup, biomolecules need to be immobilized on the crystal surface to capture target analyte. Various immobilization techniques have been reported for binding of biomolecules on the crystal surface, including adsorption and covalent cross linking via thiolation, silanization or phosphonation. Recently immobilization has been demonstrated using SAMs of thiolated antibody, exploiting carboxyl amine coupling of the antibody over the SAMs of different thiol or sulfide compounds [8]. SAMs of organic molecules are molecular assemblies formed spontaneously on surfaces by adsorption and self-organization into large ordered domains. SAMs are created by the chemisorption of head groups onto a substrate from either the vapor or liquid phase followed by a slow organization of tail groups. The head groups assemble on the substrate, while the tail groups assemble far from the substrate. Areas of close packed molecules nucleate and grow until the surface of the substrate is covered in a single monolayer. Moreover, SAMs provide versatility and novel properties on surfaces and interfaces by conjugating biomolecules to facilitate a low-cost method of fabricating biosensor devices with improved activity, stability and selectivity [9]. Binding of biomolecules using cross-linkers is a popular approach of chemical modification and can be achieved using compounds like glutaraldehyde. One such testimony of the glutaraldehyde cross linking based SAM has been used in this work and procedure is explained in the next section (*see* **Note 5**).

3.4 Immobilization of Anti-sulfadiazine Antibodies

The methodology employed for anti-sulfadiazine polyclonal antibody (pAb-SDZ) immobilization is based on multilayer assembly on the gold surface of the piezoelectric transducer gold quartz crystal. The gold quartz crystal is gently washed with absolute ethanol to remove any dust particles present on the surface and dried with nitrogen stream before use. The cleaned gold quartz crystal is incubated for 12 h in an aqueous solution of 15 mM cystamine chloride (CYST) (*see* **Notes 2** and **3**). The crystal is then washed with double distilled water and incubated for 30 min in (2.5 % v/v) glutaraldehyde solution. The crystal is then washed with PBS to remove residual glutaraldehyde. The crystal is finally exposed to the pAb-SDZ solution (1:500 dilution of 10 μg/mL) for 1 h and rinsed with PBS. To block reminiscent aldehyde groups, the modified crystal surface is exposed to glycine solution (4 % w/v) for 30 min and subsequently washed with PBS. Schematic for immobilization of pAb-SDZ is shown as Fig. 2. Antibody immobilized gold quartz crystal might be regenerated by cleaning it with piranha solution; However the binding efficiency of the gold quartz crystal may reduce upon reuses (*see* **Note 6**).

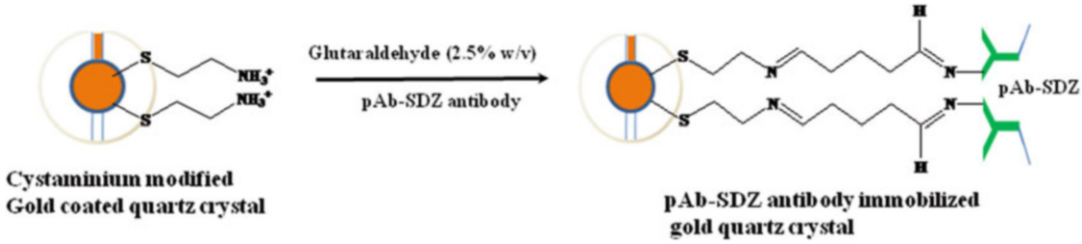

Fig. 2 Schematic representation of immobilization procedure for anti-sulfadiazine polyclonal antibody (pAb-SDZ) through cystaminium modified gold coated quartz crystal

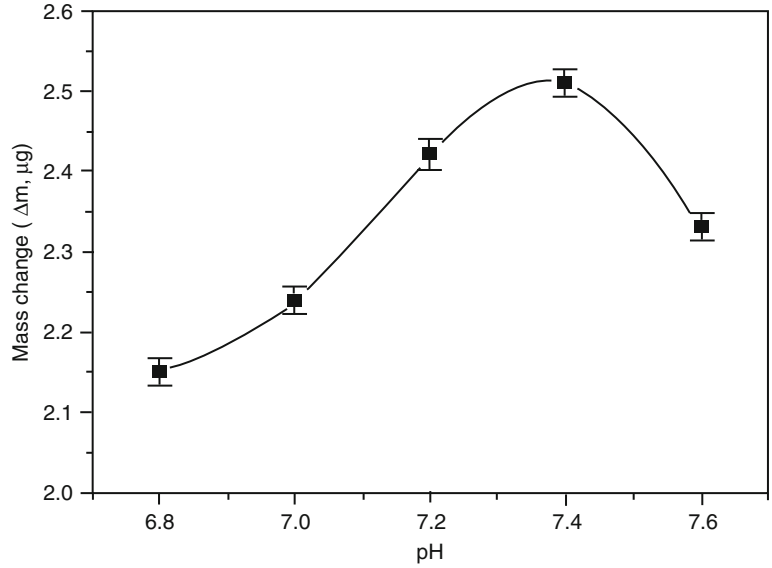

Fig. 3 Effect of pH of PBS on the response of FIA-EQCN biosensor for 100 ng/mL SDZ (PBS 100 mM, flow rate 0.5 mL/min)

3.5 Optimization of the FIA-EQCN System for SDZ Analysis

3.5.1 Effect of Influential Parameters of FIA System

Various parameters like pH, ionic strength of the carrier buffer and flow rate of the FIA system are optimized. PBS of different pH (6.8, 7.0, 7.2, 7.4, 7.6) and ionic strength (10, 50, 100, and 200 mM) is prepared and measurements are carried out against 100 ng/mL SDZ by passing the standard antibiotic solution over the mounted gold quartz crystal. Based on the responses obtained from the EQCN biosensor, graphs are plotted for mass change verses the pH and Ionic strength of the PBS and presented below.

Figure 3 confirms that at pH 7.4, 100 ng/mL showed the maximum binding. Similarly, the maximum binding of SDZ to pAb-SDZ is observed for ionic strength corresponding to 100 mM PBS (Fig. 4).

The effect of flow rate on the binding of SDZ to the immobilized antibody (pAb-SDZ) on the modified crystal surface is also tested. A known amount of SDZ standard solution (100 ng/mL) is

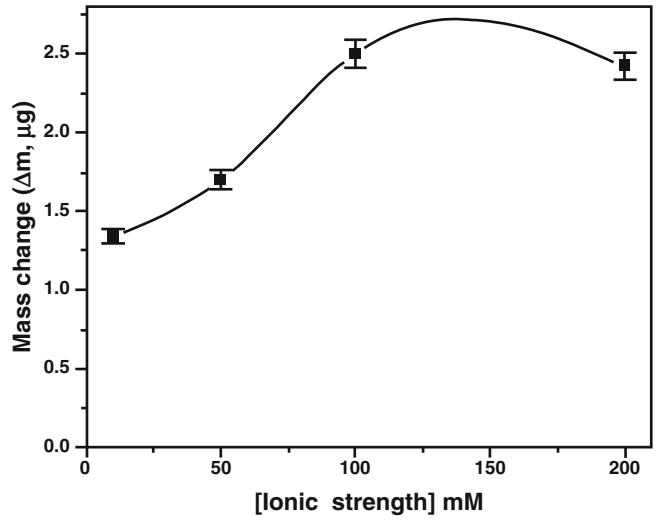

Fig. 4 Effect of ionic strength of PBS on the response of FIA-EQCN biosensor for 100 ng/mL SDZ, flow rate 0.5 mL/min

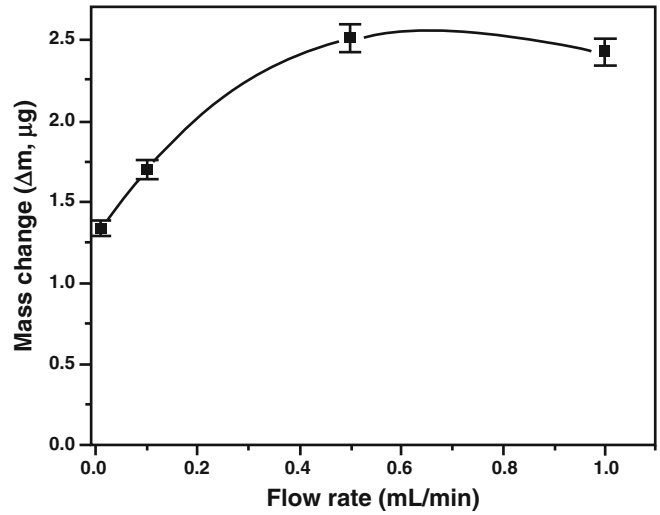

Fig. 5 Effect of flow rate of PBS on the response of FIA-EQCN biosensor for 100 ng/mL SDZ. PBS 100 mM, pH 7.4

passed over the mounted crystal at different flow rates (0.01, 0.1, 0.5 and 1 mL/min). The optimum flow rate, corresponding to the maximum binding response, is found at 0.5 mL/min as presented in Fig. 5. This flow rate also provided sufficient contact time for binding of SDZ to the immobilized antibody. The optimized parameters (pH 7.4, ionic strength 100 mM, and flow rate 0.5 mL/min) are used throughout the experiment for further analysis of SDZ.

3.5.2 Optimization of Antibody Dilutions for pAb-SDZ

It is very important to optimize the concentration of antibody (bioreceptors), which critically affects their conjugation onto the electrodes as well as their ease of access toward analytes [10] (*see* **Note 7**). To determine the optimum antibody concentration and to check the maximum binding efficiency, different dilutions of pAb-SDZ are injected into the FIA system on the different SAMs-modified crystal surfaces. The pAb-SDZ antibodies are diluted with PBS at 1:100, 1:500, 1:1000, 1:2000, and 1:2500. As shown in Fig. 6, a 1:1000 dilution of pAb-SDZ showed the maximum binding on the optimized monolayer surface. Hence, further experiments are carried out using 1:1000 dilutions of antibodies. Other dilutions of antibody with PBS showed less binding as compare to 1: 1000 dilutions, which agree with the hypothesis that too high or low density of bio-receptors can actually hinder antigen antibody binding due to steric hindrance resulting from high concentrations.

3.6 Calibration of SDZ in Buffer and Milk

In order to calibrate the analytical system, the pAb-SDZ immobilized gold quartz crystal is kept in a Plexiglas flow-cell and sandwiched between two O-rings as presented in experimental setup. Degassed PBS solution is passed using the peristaltic pump via FIA manifold until a constant baseline is achieved. SDZ standard solutions in the concentration range 10–200 ng/mL are prepared in carrier buffer and are injected into the FIA-EQCN system. The response signals are recorded using a data acquisition system connected to the resonator and is plotted as mass change versus concentration of SDZ. All the samples are injected in triplicate. A good linear response is obtained for SDZ binding in the range 50–200 ng/mL. The linear fit data shows a good correlation

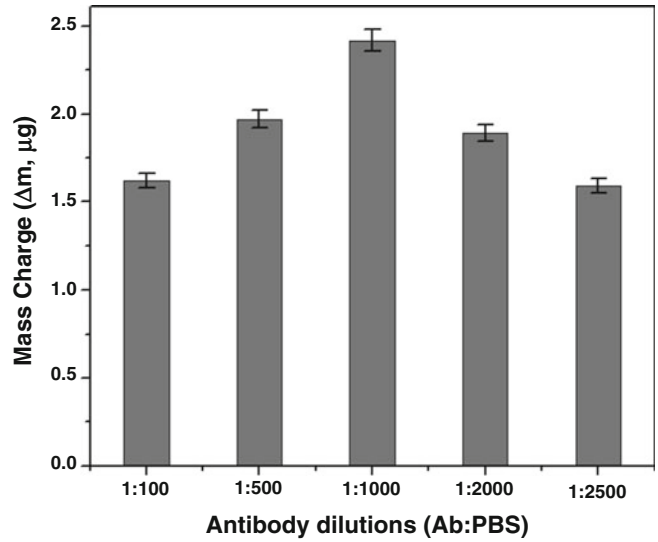

Fig. 6 Effect of antibody dilution on the response of FIA-EQCN biosensor for 100 ng/mL SDZ. Carrier buffer—PBS (100 mM), pH 7.4, flow rate 0.5 mL/min

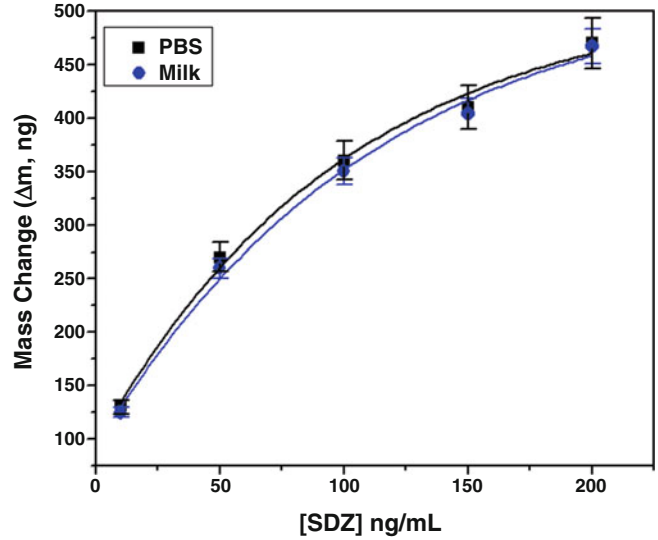

Fig. 7 Calibration graph for analysis of SDZ in PBS and milk

between immobilized pAb and SDZ [R^3 = 0.98 % RSD (n = 3) 0.83] (*see* **Note 8**). Furthermore, the usefulness of the developed biosensor is also evaluated in the fresh pasteurized diluted milk samples by analyzing a 1:4 dilution of milk with PBS. Known concentrations of SDZ (10–200 ng/mL) are spiked in the diluted milk samples to construct a calibration graph (*see* **Note 9**). A good dynamic range is obtained from 10 to 200 ng/mL with a linear range 50–200 ng/mL. The correlation coefficient (R^3) of the developed biosensor in the milk is found to be 0.98, with % RSD (n = 3) 0.80. The minimum limit of detection for SDZ in PBS and milk is found to be 10 ng/mL. Calibration of the biosensor in PBS and milk is presented as Fig. 7 whereas linear range of the biosensor in PBS and milk is presented as Fig. 8.

3.7 Cross-Reactivity of SDZ

Cross-reactivity studies are performed in PBS using standard solutions (100 ng/mL) of different sulfonamides. Most common sulfonamides like sulfadiazine, acetyl-sulfadiazine, sulfathiazole, sulfamethazine, sulfaquinoxaline, and sulfamethoxazole are tested for cross-reactivity with sulfadiazine-specific pAb. Very high cross-reactivity is obtained for sulfadiazine and acetyl sulfadiazine, which are more structurally related to sulfadiazine, and are recognized by the antiserum with a cross-reactivity of 100 and 92 %, respectively. Sulfathiazole and sulfamethazine shows cross-reactivity of 1.2 and 0.2 % respectively. Sulfaquinoxaline and sulfamethoxazole shows less than 0.1 % cross-reactivity. In addition to sulfonamides the aminoglycoside streptomycin (SRT, 100 ng/mL), which may be present in the milk samples, is also tested for cross-reactivity with SDZ [11]. No cross-reactivity is observed. Results are compiled and presented in Table 1.

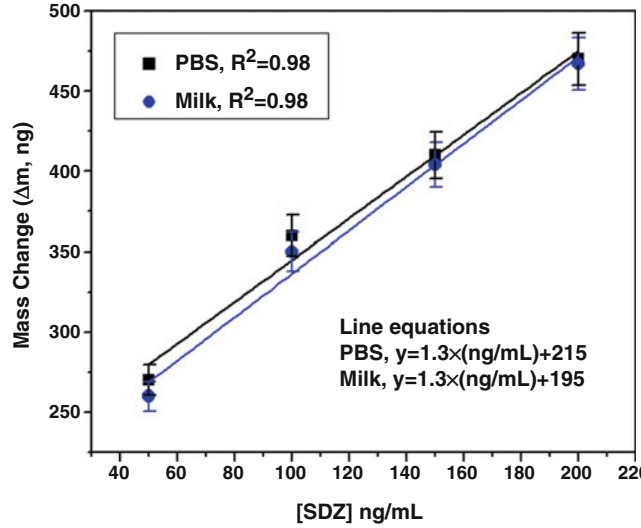

Fig. 8 Linear fit data representation for SDZ analysis in PBS and milk

Table 1
% Cross-reactivity of sulfadiazine with other antibiotics

Sulfonamides	% cross-reactivity
Sulfadiazine	100
Acetyl-sulfadiazine	92
Sulfathiazole	1.2
Sulfamethazine	0.2
Sulfaquinoxaline	<0.1
Sulfamethoxazole	<0.1
Aminoglycosides	
Streptomycin	0.00

3.8 Recovery Studies from SDZ Spiked Milk Samples

The potential application of the developed FIA-EQCN system for detection of SDZ in the milk is tested. All the milk samples are initially assumed as SDZ free. Known amounts of the SDZ standard solution are added to centrifuged, diluted, and filtered milk and are analyzed in the FIA-EQCN system. Based on the response signal, recoveries are calculated. Table 2 shows the recovery results obtained from milk samples. Recoveries in the range of 98.6–100.5 % are obtained. This ensures the potential application of the developed biosensor for SDZ analysis in the milk samples.

Table 2
Recovery studies of sulfadiazine from spiked milk samples

SDZ added (ng/mL)	SDZ found (ng/mL) mean ($n = 3$)	Mean ± S.D.	% RSD	% Recovery
100	98.06	98.06 ± 0.40	1.10	98.6
100	99.02	99.02 ± 0.26	0.96	99.2
100	100.05	100.05 ± 0.10	0.21	100.5

4 Notes

1. EQCN-700 integrated with the FIA system from M/s Elchema, Potsdam, USA has been used for construction of the biosensor. The Voltascan 5 software of EQCN-700 has an in-built auto conversion factor to calculate mass change from frequency change.
2. SAM-coated gold quartz crystal should be used within a few days. Otherwise, it should be stored in a vacuum desiccator.
3. Quartz crystal coated with metals other than gold are also available and can be deployed based on the application.
4. Unless otherwise stated, all the solutions should be prepared in R.O. water that has a resistance higher than 18.2 MΩ and a total organic content of less than 5 parts per billion.
5. In SAMs, activated –COOH groups are hydrolyzed and inactivated in the presence of water. Therefore, the sensor surface is washed with phosphate buffer after adding the activators such as EDC (1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide)/*N*-Hydroxysuccinimide (NHS) (*see* **Note 2**).
6. Piranha solution reacts violently with many organic materials and should be handled with extreme care.
7. We have used a kind of antibody that is recommended for ELISA. Numerous competitive reagents are available from other commercial sources (or antibody mimics).
8. In FIA-EQCN biosensor, results are obtained as mass change resulting from the real time binding of SDZ to the pAb-SDZ immobilized via the SAMs on the quartz crystal. The background value is exhibited from the interaction of the antibody coupled quartz crystal with the carrier buffer (PBS 100 mM, pH 7.4) at a flow rate of 0.1 mL/min.
9. Calibration of the FIA-EQCN biosensor is plotted after calculating the mass change for each concentration of SDZ. Response may vary for repetitive experiments; however, the mass change value should be same for specific concentration.

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