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Ultrasensitive detection of streptomycin using flow injection analysis-electrochemical quartz crystal nanobalance (FIA-EQCN) biosensor

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ABSTRACT

This work presents the development of an ultrasensitive biosensor for detection of streptomycin residues in milk samples using flow injection analysis-electrochemical quartz crystal nanobalance (FIA-EQCN) technique. Monoclonal antibody specific to streptomycin was immobilized on to the thiol modified gold quartz crystal surface. A broad dynamic range (0.3–300 ng/mL) was obtained for streptomycin with a good linearity in the range 0.3–10 ng/mL for PBS and 0.3–50 ng/mL for milk. The correlation coefficient (R^2) of the biosensor was found to be 0.994 and 0.997 for PBS and milk respectively. Excellent recoveries were obtained from the streptomycin spiked milk samples in the range 98–99.33%, which shows the applicability of the developed biosensor in milk. The reproducibility of the developed biosensor was found satisfactory with % RSD ($n=5$) 0.351. A good co-relation was observed between the streptomycin recoveries measured through the developed biosensor and the commercial ELISA kit. The analytical figures of merit of the developed biosensor confirm that the developed FIA-EQCN biosensor could be very effective for low-level detection of streptomycin in milk samples.

1. Introduction

Streptomycin (SRT), a member of aminoglycoside, finds wide application in modern agriculture and dairy for treatment of mastitis in cattle's (Haasnoot et al., 2003). Antibiotics such as aminoglycoside are being used either individually or in combination with other drugs for the treatment of inflammatory diseases or as feed additives. Monitoring of antibiotics is very important for safety of milk or milk products for human consumption. The presence of these residues even in minute amount can trigger potential health hazard such as allergic reactions in hypersensitive individuals, toxicity, or they can be act as potential carcinogens (Conzuelo et al., 2012). Antibiotics also give rise to an increase in the antibiotic resistance of pathogenic bacteria. Apart from the consequences for public health, economic reasons have also led to the control of antimicrobial residues in food. Milk contaminated with antibiotics may inhibit the starter cultures used in the production of other milk products (Ferguson et al., 2002). Several regulatory agencies have set the maximum residual limit (MRL) for antibiotics in milk for consumer protection. In particular, European Union (EU) has fixed the limit at 200 ng/mL for SRT in milk whereas in The United States; the tolerance limit for SRT in milk is 125 ng/mL. The Codex committee has set a combined MRL of 200 ng/mL for SRT and dihydro-streptomycin (DHSRT) (Commission Regulation (EU) No. 37/2010, 2009; Baxter et al., 2001).

A wide range of analytical methods currently exists for the detection and quantitative measurements of SRT. Microbial inhibition assay (Currie et al., 1999), enzyme immunoassay (Abuknesha and Luk, 2005) and enzyme linked immuno-sorbent assay (ELISA) (Heering et al., 1998) are commonly employed as screening tests. Conventional analytical techniques such as liquid chromatography (Ye et al., 2008) and liquid chromatography-mass spectroscopy (Zhu et al., 2008) have been described for confirmatory analysis. Although the chromatographic techniques are sensitive and specific, they are restricted to confirmatory analysis being very laborious and expensive (Wang et al., 2006). Moreover, the long and tedious sample preparation involved prior to chromatographic analysis also limit sample throughput. An effective monitoring technique for regulation of these antibiotics require specific sensitivity, rapid, reliable and economical methods able to detect them at or below the MRL levels (Conzuelo et al., 2013).

Biosensors are a recent addition to analytical instrumentation capable of performing residues testing and offer the potential for rapid, quantitative high-throughput analysis. One such testimony

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for analysis of SRT in milk is Surface plasmon resonance (SPR) based optical biosensor (Baxter et al., 2001). Compared with expensive and complicated optical instrumentation of SPR, quartz crystal microbalance (QCM) is more suitable for wide generalization and applications. Owing to its simplicity, convenience, and real time response, QCM has been increasingly explored in many fields of biological studies (Yang et al., 2009a, 2009b). The principle of QCM is based on the frequency change of the crystal that is inversely proportional to the mass change on the crystal surface under appropriate conditions. The observed mass change can be correlated to the analyte binding on the surface of crystal. QCM has the advantages of minimal electrical requirements, adaptability to microfluidic techniques, inexpensive fabrication, utility in a flow cell, and label free detection. QCM biosensor integrated in a flow injection analysis (FIA-QCM) system has the advantages in terms of reproducibility, speed of analysis, control of contact time and concentration profiling in kinetic studies (Malitesta et al., 2012).

To implement a FIA-QCM biosensor, biomolecules need to be immobilized on the crystal surface to capture target analyte. Various immobilization methods including thiolation, silanization and immobilization via cross-linking are used for binding of the biomolecules on the crystal surface. Recently antibody immobilization by way of self-assembled monolayers (SAMs) of thiolated antibody, exploiting carboxyl-amine coupling of the antibody over the SAMs of different thiols or sulfide compounds have been demonstrated (Park et al., 2004).

In this study, we present for the first time an ultrasensitive detection of SRT antibiotic in milk samples using flow injection analysis-electrochemical quartz crystal nanobalance (FIA-EQCN) biosensor. the biosensor comprised monoclonal antibody specific to SRT (mAb-SRT) immobilized on the thiol modified gold crystal surface. the broad detection range (0.3–300 ng/mL) of developed biosensor facilitates ultra-sensitive detection of SRT in milk. the remarkable features of the developed FIA-EQCN biosensor are; its precision in reproducibility and reliability. based on the state of the art, we could able to achieve the sensitive detection of SRT as low as 0.3 ng/mL in milk using FIA-EQCN, which are summarized in Table 1. this approach shows promising potential, being capable of detecting antibiotics at level below the MRL.

2. Materials and methods

2.1. Materials and Reagents

Anti-streptomycin monoclonal antibody-IgG fraction isolated from Mouse was purchased from M/s Abcam, Cambridge, UK. Streptomycin sulfate was procured from M/s MP Biomedicals, Santa Ana, California, USA and Streptomycin ELISA kit was procured from M/s Biooscientific Austin, Texas, USA. Sodium dihydrogen phosphate monohydrate, disodium hydrogen phosphate monohydrate, sodium chloride, 11-mercaptoundecanoic acid (11-MUA), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), N-hydroxy succinimide (NHS), glutaraldehyde solution 25%, sodium chloride, glycin and cysteaminium chloride were obtained from M/s Merck KGaA, Darmstadt, Germany. L-cysteine hydrochloride was procured from M/s Himedia laboratory, Mumbai, India. Centrifugation of milk is carried out by minispin centrifuge and for sample handling micropipettes are used. Both are procured from M/s Eppendorf, Hamburg, Germany. All the solutions were prepared in 0.22 μm membrane filtered Milli-Q system RO water, procured from M/s Millipore, Billerica, Massachusetts, USA, Seven Multi pH meter was used for pH measurements, procured from M/s Mettler Toledo, Greifensee, Switzerland. All other chemicals used were of analytical grade and used as received.

2.2. Instrumentation for FIA-EQCN biosensor

The experimental set-up used for present study is shown as Fig. 1. EQCN-700 system integrated with flow cell model FC-6 (M/s Elchema, Potsdam, New York, USA) was deployed for construction of FIA-EQCN biosensor. The gold quartz crystal (10 MHz, AT cut) was connected to the frequency-measuring unit, which contains a reference crystal with the same characteristics as the measuring crystal. The gold quartz crystal was placed in a plexiglass flow cell,

Table 1
Comparison with earlier reported biosensors for streptomycin detection.

S. no.	Matrix	Detection limit	Detection technique	Reference	Year
1.	Milk	0.3 ng/mL	FIA-EQCN biosensor	Present work	2014
2.	Milk, Honey	7 pg/mL	Electrochemical	Que et al. (2013)	2013
3.	Honey	0.1 μM	Aptasensor	Zhou et al. (2013)	2013
4.	Food	10 ng/g	HPLC	Benjing et al. (2012)	2012
5.	Food	5 pg/mL	Electrochemical immunoassay	Liu et al. (2011)	2011
6.	Honey	15.9 μg/kg	Chemiluminescence immunoassay	Wutz et al. (2011)	2011
7.	Milk	4.5 μg/L	Immunoassay	Bilandžić et al. (2011)	2011
8.	Milk	2 ng/mL and 20 ng/mL	ELISA and Immunochromatographic Assay	Wu et al. (2010)	2010
9.	Milk	100 μg/mL	TLC/Bioautographic method	Kaya and Filazi (2010)	2010
10.	Milk	1.2 ng/mL	iSPR based Immunosensor	Raz et al. (2009)	2009
11.	Meat, Juice	0.1 μg/mL	Solid-phase fluorescence immunoassay (SPFIA)	Pyun et al. (2008)	2008
12.	Milk	2.01 ng/mL	SMM–FIA	Ce et al. (2008)	2008
13.	Milk	3.0 ng/mL	ELISA	Samsonova et al. (2005)	2005
14.	Milk	5.06 μg/L	Immunoassays	Knecht et al. (2004)	2004
15.	Milk, Honey	Milk 10 μg/kg Honey 2 μg/kg	LC-MS/MS	Bruijnsvoort et al. (2004)	2004
16.	Milk	3.2 ng/mL	Immunochemical rapid test	Strasser et al. (2003)	2003
17.	Milk	65 ng/mL	Immunoassay	Haasnoot et al. (2003)	2003
18.	Milk, Honey, Meat	Milk 30 μg/kg Honey 15 μg/kg Kidney 50 μg/kg Muscle 70 μg/kg	Optical biosensor	Ferguson et al. (2002)	2002
19.	Milk	4.1 ng/mL	Optical immunobiosensor	Baxter et al. (2001)	2001
20.	Milk	0.03 mg/kg	Solid-phase extraction/Flourometric detection	Edder et al. (1999)	1999

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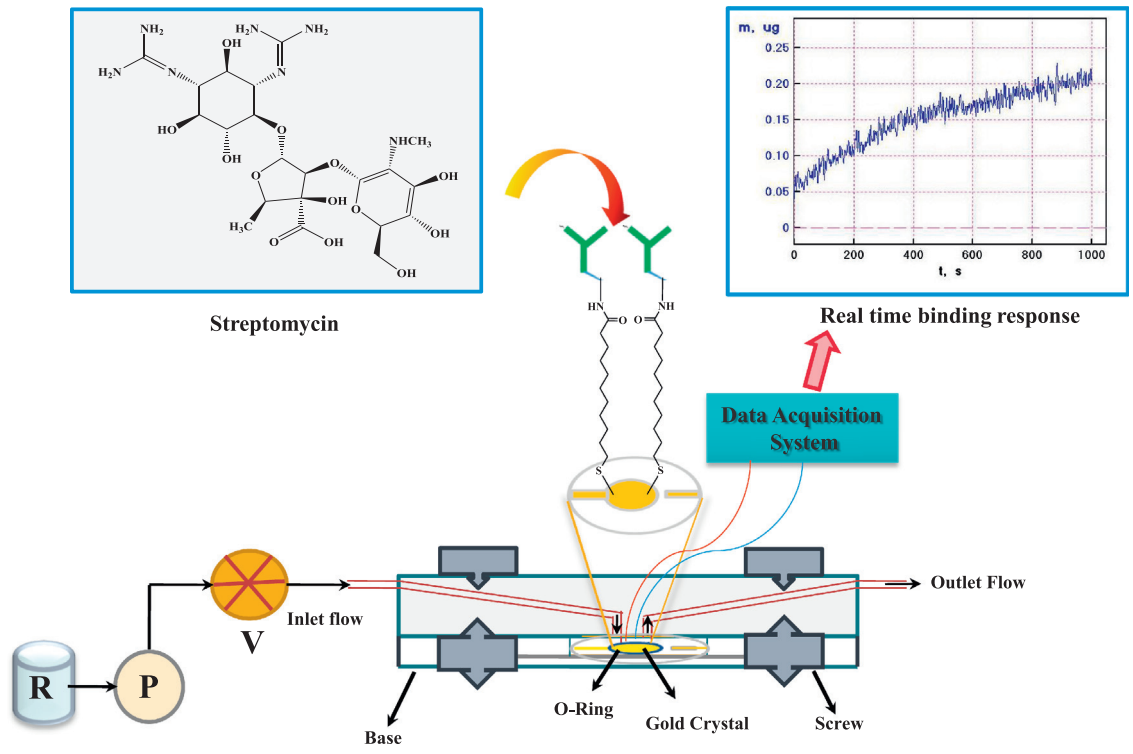

Fig. 1. Schematic of FIA-EQCN biosensor for analysis of streptomycin in milk. R – buffer reservoir, P – peristaltic pump, V – 6-port injection valve.

sandwiched between two O-rings, with its upper electrode in contact with the liquid and its lower electrode in air. The volume of the flow cell was 50 μL (Ø=8 mm and height=1 mm). A peristaltic pump (M/s Gilson Inc., Villiers-le-Bel, France) was connected through a 6-port Rheodyne injection valve (M/s, Idex health and science, Oak Harbor, Washington, USA) to the flow-cell. Samples were filled in the 6-port injection valve and injected into the carrier buffer. The measurements of direct mass change were taken using Voltascan 5 software. The EQCN-700 system has inbuilt hardware and provides the data only as mass change. The inbuilt frequency unit, which calculated the mass by using a conversion factor (1 Hz equivalent to 1 ng approx.), measured the output from the oscillator. The calculation for conversion of 1 Hz to 1 ng is provided in the Supplementary details. Though the output signal from the EQCN-700 system is in μg (auto calibrated), the mass change (Δm) is converted in to ng to impart more clarity in calibration graph. A personal computer controlled the data acquisition. The flow cell was placed in a Faraday cage to shield the crystal from external electromagnetic sources.

2.3. Biosensor preparation

The methodology employed for preparation of biosensor for SRT analysis in milk was based on multilayer assembling on the surface of gold quartz crystal. In brief, a clean gold quartz crystal was immersed overnight in 4 mM ethanolic solution of 11-MUA under ambient condition. The gold quartz crystal was gently washed with absolute ethanol to remove unbound 11-MUA residues and dried with nitrogen stream before use. For coupling of the mAb-SRT antibody, the carboxyl group of SAM on modified electrode was activated by 1:1 EDC/NHS (100 mM each) mixture for 2 h. Subsequently, the crystal was washed with membrane filtered water to remove excess EDC/NHS. Finally, solution of 10 mg/mL (1:1000 in PBS) mAb-SRT was added carefully over the activated surface of SAM followed by overnight incubation (8–12 h) at 4 °C. The coupled gold crystal was washed to remove unbound antibody and mounted on the flow cell to use in FIA mode.

2.4. Solution preparation

Phosphate buffer saline (PBS, 100 mM) was prepared by dissolving appropriate amount of Na_2HPO_4 and NaH_2PO_4 containing 137 mM NaCl and 2.7 mM KCl in membrane filtered RO water. The pH of the buffer was adjusted to 7.4 before use. For preparation of standards, known amount of SRT was dissolved in PBS and further diluted to meet the calibration standards. For analysis in milk, samples were centrifuged and diluted (1:4) in PBS. A stock solution of SRT (300 ng/mL) was prepared and further diluted to meet the calibration standards in the range of 0.001–300 ng/mL. All the solutions were freshly prepared before the experiment and stored at 4 °C when not in use. The commercial milk sample of 3.0% fat was procured from local market of Goa, India. The matrix effect on analysis of SRT in milk samples was investigated. In FIA system, fat present in the milk may lead to clogging of the fluidic channels and thus, can decrease the biosensor performance. To avoid such problems, a simple strategic approach was worked out to obtain the calibration curve for SRT in spiked milk samples. Combination of several sample pre-treatment techniques such as centrifugation, filtration, and dilution of milk with PBS was done for SRT analysis in milk. In brief, the milk samples were centrifuged at 6000 rpm for 20 min at room temperature and filtered with 0.22 μm filter (Whatman, USA) and diluted with PBS to obtain different ratios of milk and PBS (1:2, 1:4, 1:6.1:8). A known amount of SRT was spiked in diluted milk samples and it was observed that 1:4 dilution of milk sample with PBS showed maximum binding for SRT with immobilized antibody on modified crystal surface.

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3. Result and discussion

3.1. Optimization of experimental variables

3.1.1. Surface modification

Earlier reported protocols for SAMs were used for optimization of surface modification to immobilize mAb-SRT on gold quartz crystal, with slight modifications. 11-MUA (4 mM) (Park et al., 2004), cysteaminium chloride (10 mM) (Silva et al., 2010) and L-cysteine (10 mM) (Zhang et al., 2009) were tested. The concentrations of different surface modifiers and cross-linkers were optimized and mAb-SRT antibody was attached to the surface. The SAMs based on 11-MUA showed the maximum mass change against mAb-SRT coupled gold quartz crystal for SRT standard which confirms the maximum binding. Thus, further experiments were carried out using 11-MUA. Fig. 2a represents the binding responses obtained with different SAM's.

3.1.2. Antibody dilutions and binding capacity of the gold crystal

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 with minimum physical steric hindrance (Ahmed et al., 2013). To determine optimum antibody concentration and to check the maximum binding efficiency, different dilutions of mAb-SRT were injected into the FIA system on the SAMs modified crystal surface. The mAb-SRT antibody was diluted with PBS as 1:100, 1:500 and 1:1000. As shown in Fig. 2b, it was found that 1:1000 dilution of

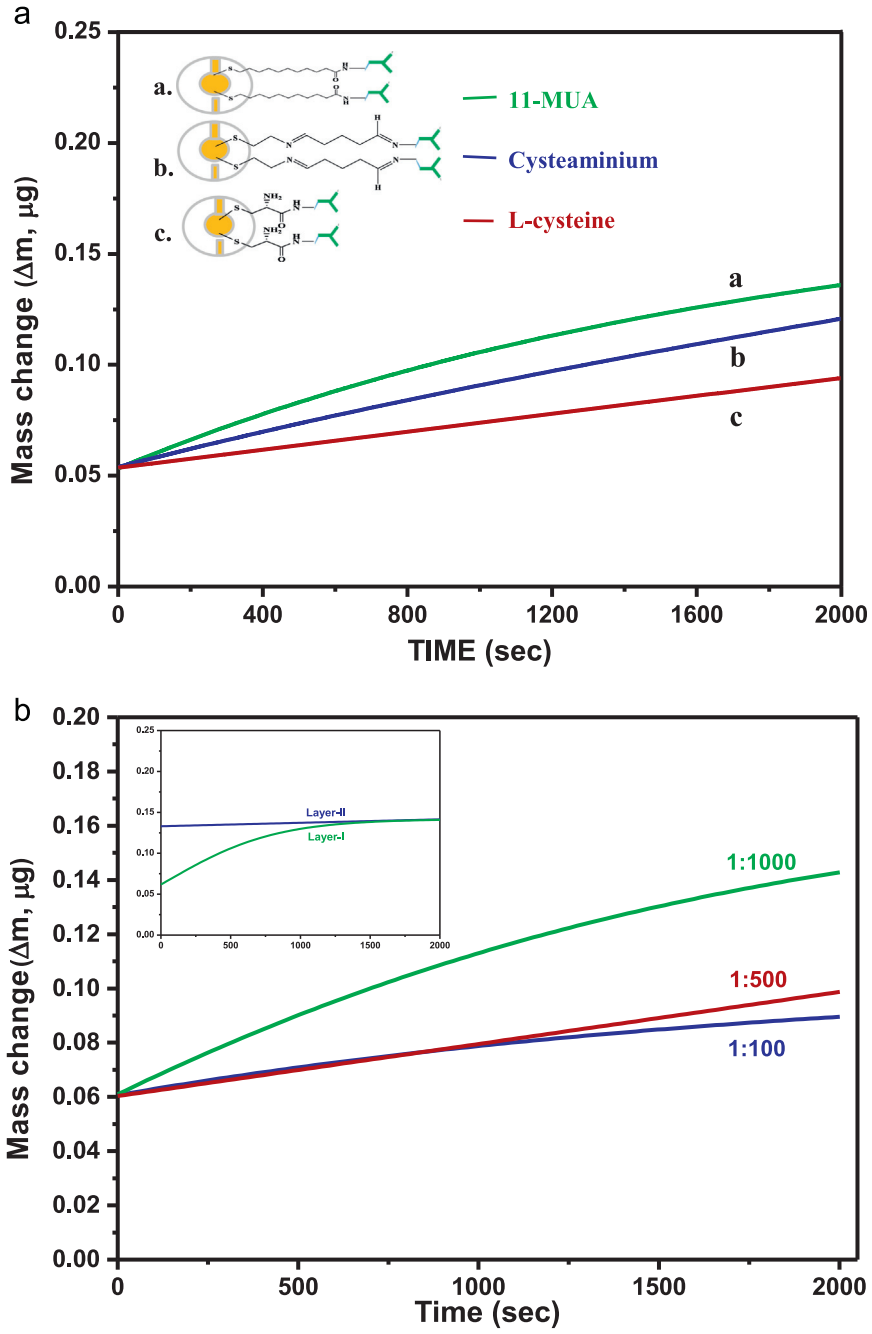

Fig. 2. (a) Optimization of different Self Assembled Monolayer's. Inset: representation of different thiols attached to the gold quartz crystal. (b) Optimization of antibody dilutions for analysis of streptomycin. Inset: binding capacity of the gold crystal through different layers of antibody. Carrier buffer – PBS (100 mM), pH 7.4, flow rate 0.1 mL/min.

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mAb-SRT showed the maximum binding on optimized monolayer surface. However, antibody dilution of 1:100 and 1:500 showed minimum binding which agrees with the hypothesis that too high a density of bioreceptors can actually hinder binding because of steric hindrance at high concentration. Recently, Holford et al. has also demonstrated that a high concentration of antibody decreased biosensor sensitivity, as it did not facilitate bioreceptor binding on the surface, probably due to steric hindrance (Holford et al., 2013). Hence, all the further experiments were carried out using 1:1000 dilutions. Simultaneously, experiments were carried out to study the maximum binding capacity of modified crystal with mAb-SRT. Optimized dilution of antibody solution in buffer was passed to the modified crystal surface and real time binding response was recorded. Saturation of the modified SAMs layer on crystal surface was observed during the second run of antibody as evident from Fig. 2b (inset).

3.1.3. *Effect of influential parameters of FIA system*

Different influential parameters like phosphate concentration, pH of the carrier buffer and flow rate of the FIA system were optimized before the experiment was performed. Measurements were carried out with different phosphate concentrations (10, 50, 100 and 200 mM) and pH (6.8–7.6) of PBS for 10 ng/mL SRT. It is evident from supplementary Fig. S1 that the 10 ng/mL SRT showed the maximum binding in 100 mM PBS, similarly, maximum binding was found at pH 7.4 as presented in supplementary Fig. S2. The effect of flow rate on SRT binding on modified crystal surface, immobilized with mAb-SRT was tested. The SRT standard (10 ng/mL) was passed over the mounted crystal in different flow rates (0.01, 0.1, 0.5 and 1 mL/min). As shown in supplementary Fig. S3, flow rate of 0.1 mL/min provides sufficient contact time for binding of SRT to immobilized mAb on gold crystal, thus gives the maximum response. These optimized parameters (phosphate concentration 100 mM, pH 7.4 and flow rate 0.1 mL/min were used throughout the experiment.

3.2. *Surface characterization of mAb-SRT immobilized gold crystal*

Fourier Transform InfraRed (FT-IR) spectroscopy was applied to measure the surface properties of the AT-cut gold quartz crystal coupled through 11-MUA SAMs via the carbodiimide cross-linking reaction. The surface was characterized before and after coupling of mAb-SRT at each step during the experiment. Spectra were recorded using IR Affinity-1 (M/s SHIMADZU Corp., Kyoto, Japan) with attenuated total reflectance (ATR) attachment Specac Diamond ATR AQUA. The spectra were recorded using 40 scans at 4 cm^{-1} resolution collected under vacuum condition. The FT-IR spectra are shown in Fig. 3. FT-IR spectrum of bared gold surface is shown as Fig. 3(a). Spectrum of gold surface treated with 11-MUA is shown as Fig. 3(b) where it showed the C–H Stretches in asymmetric and symmetric mode at 2925 cm^{-1} and 2848 cm^{-1}. The broad region from 2500–3500 cm^{-1} with out-of-plane bending for O–H at 935 cm^{-1} confirming the presence of COOH group of 11-MUA. The broad region with asymmetric and symmetric Stretches at 1676 and 1619 cm^{-1} confirm the presence of C=O of acid. Two-associated peak for C–H deformation(scissoring) was found to be at 1488 and 1448 cm^{-1}. Three associated peak for long chain carbon of 11-MUA appears at 684, 635 and 537 cm^{-1}. As shown in Fig. 3(c), after immobilization of Ab on gold crystal, new absorption bands appears at 1645 and 1552 cm^{-1} which confirm the presence of amide bond (CO-NH) in between 11-MUA and mAb-SRT. The presented FT-IR spectra confirms the attachment of mAb-SRT to the modified surface of gold quartz crystal.

3.3. *Biosensor performance in PBS and milk*

The mAb-SRT immobilized gold quartz crystal was mounted in Plexiglas flow-cell and degassed PBS was passed until the constant baseline was achieved. Freshly prepared SRT standard in PBS (0.001–300 ng/mL) were injected into the FIA-EQCN system, starting from lower to higher concentration. The response signals of different concentration were recorded using the data acquisition system (DAQ-716) and plotted as mass change (Δm) vs. concentrations. The obtained data from the Voltascan 5 software was treated using OriginPro 8 software and the data of best fit was plotted. In the developed FIA-EQCN biosensor, results were obtained as mass change resulting from the real time binding of SRT to the mAb-SRT immobilized via the SAMs on the quartz crystal. The mass change (Δm) is converted in to ng to impart more clarity in the calibration graph. The background value (50 ng/mL) is

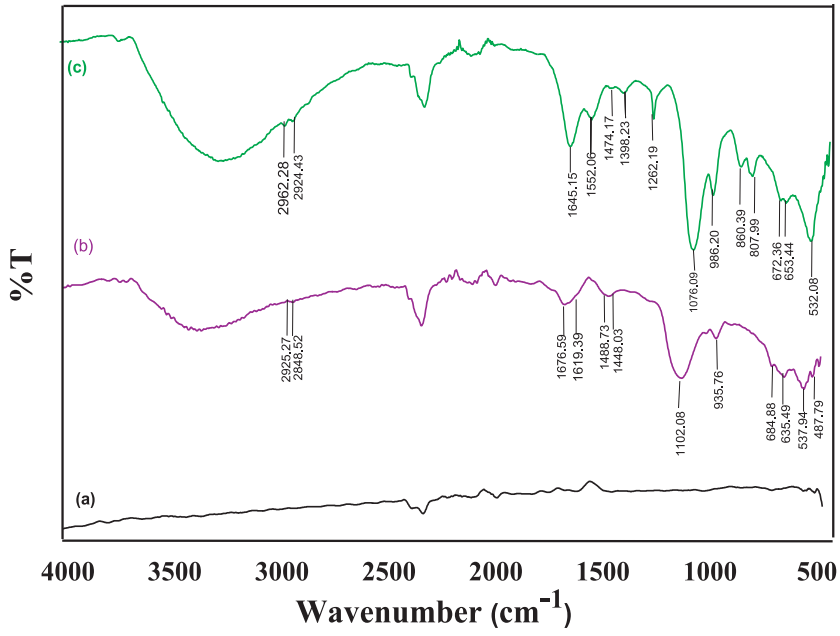

Fig. 3. FTIR characterization of immobilized antibody on modified gold crystal. The spectra's were recorded using 40 scans at 4 cm^{-1} resolution collected under vacuum condition.

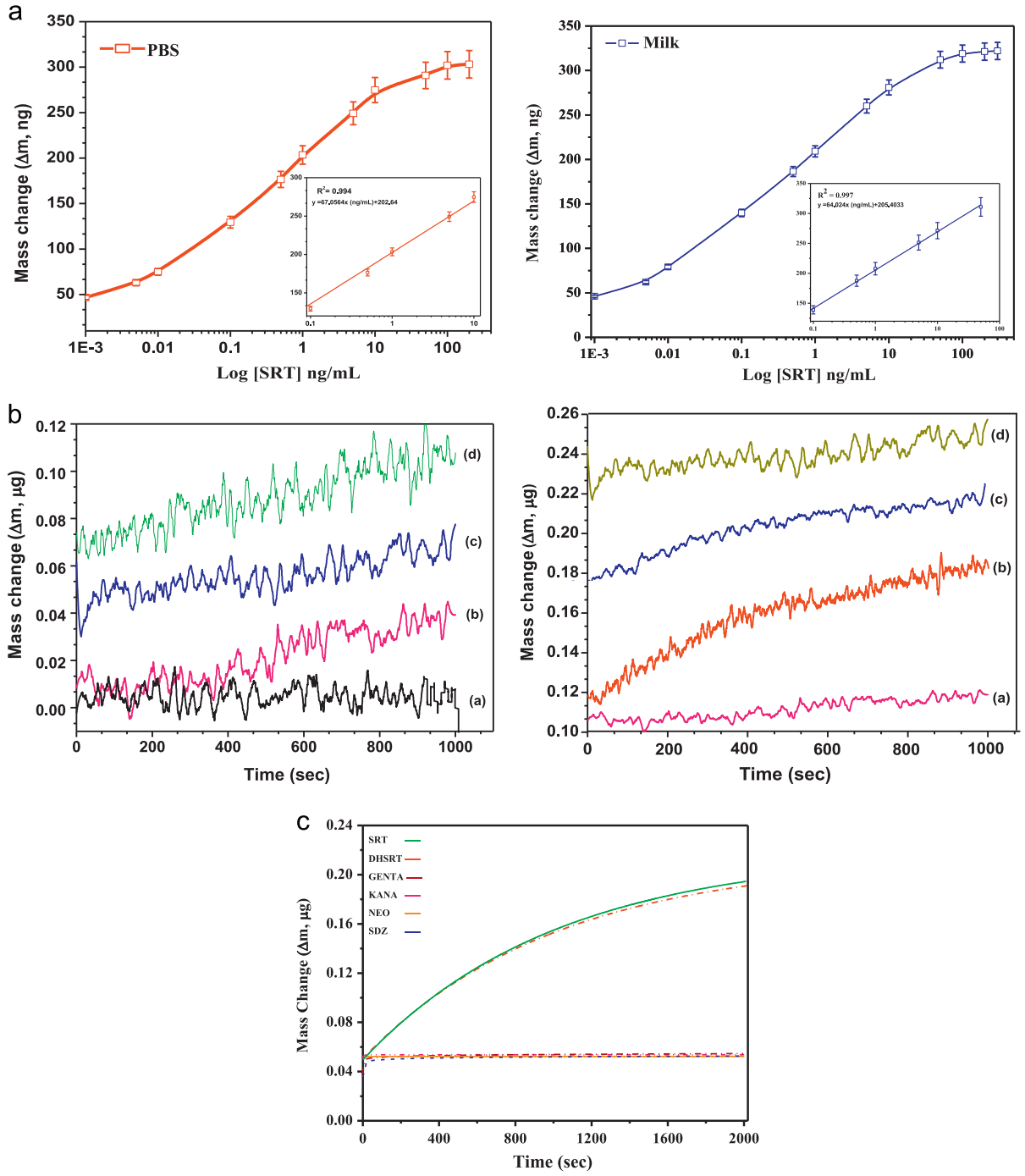

Fig. 4. (a) (i) Logarithmic representation of calibration graph for streptomycin analysis in PBS. Inset: linear fit data representation for streptomycin analysis in PBS. (ii) Logarithmic representation of calibration graph for streptomycin analysis in milk. Inset: linear fit data representation for streptomycin analysis in milk. (b) (i) Actual data graph for (a) response of functionalized gold crystal (b) response of mAb-SRT immobilized gold crystal (c) response of 0.001 ng/mL SRT spiked milk sample (d) response of 0.005 ng/mL SRT spiked milk sample. (ii) (a) Response of 0.01 ng/mL SRT spiked milk sample (b) response of 0.1 ng/mL SRT spiked milk sample (c) response of 0.05 ng/mL SRT spiked milk sample (d) response of 1 ng/mL SRT spiked milk sample. (c) Cross reactivity of streptomycin with other antibiotics. Carrier buffer – PBS (100 mM), pH 7.4, flow rate 0.1 mL/min.

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. Good dynamic range was obtained for SRT in PBS from 0.3 to 300 ng/mL with linear range of 0.3–10 ng/mL. The correlation coefficient (R^2) of the biosensor performance in PBS was 0.994 with % R.S.D. ($n=3$) 0.23–2.13 and the linear equation was $y=67.0564x$ (ng/mL)+202.64. Furthermore, the usefulness of the developed biosensor was also evaluated in the milk sample by analyzing 1:4 diluted milk with PBS. Known concentrations of SRT (0.001–300 ng/mL) were spiked in the diluted milk samples to construct a calibration graph. A good dynamic range was obtained in SRT spiked milk from 0.3 to 300 ng/mL with linear range of 0.3–50 ng/mL. The correlation coefficient of the developed biosensor in milk was found to be 0.997, with % R.S.D. ($n=3$) 0.13–1.10 and the linear equation was $y=64.024x$ (ng/mL) +205.4033. Based on the calculation of baseline peak to peak noise root mean square (RMS) i.e. 6.7 and the sensitivity of the calibration curve i.e. 67.0564 ng/mL for PBS and 64.024 ng/mL for milk, the minimum

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limit of detection for SRT in PBS and milk was determined as 0.3 ng/mL. The obtained results are presented as Fig. 4a(i) and (ii) for PBS and milk respectively. Sensitivity and the detection range of the developed biosensor is attributed to the affinity interaction between the antibodies and the antigen, since the specific monoclonal antibody against SRT was immobilized on the SAM surface which selectively graft the target analyte (SRT). To support the ultrasensitive detection of the developed biosensor, screen shot for real time binding of mAb-SRT to SRT (0.1 ng/mL and 1 ng/mL) have been provided in the supplementary section as supplementary Figs. S4 and S5 respectively. Actual readings were plotted for baseline of functionalized gold crystal and mAb-SRT immobilized crystal together with response of 0.001 and 0.005 ng/mL SRT and presented as Fig. 4b(i). For comparison, actual interaction data of 0.01, 0.1, 0.5 ng/mL and 1 ng/mL SRT spiked milk were also plotted and presented as Fig. 4b(ii). Obtained real time mass change (Δm) and cumulative mass change values for baseline (functionalized gold crystal and mAb-SRT immobilized crystal) and SRT (0.001, 0.005, 0.01, 0.1, 0.5 and 1 ng/mL) spiked milk have also been included in the supplementary section as supplementary Table ST3. The dissociation constant (K_d) for SRT spiked in PBS and milk with immobilized mAb-SRT on modified gold crystal was determined using ligand binding kinetics in sigma plot 11.0.0 and found to be 1.33×10^{-10} M and 1.161×10^{-10} M for PBS and milk respectively.

3.4. Cross reactivity

Cross-reactivity studies were performed to check the group specificity of the developed biosensor against the most structurally similar molecule dihydrostreptomycin (DHSRT) and other three non-targeted aminoglycosides namely gentamycin (GENTA), neomycin (NEO) and kanamycin (KANA). Apart from aminoglycosides, one sulfonamide e.g. sulphadiazine (SDZ) was also tested for cross reactivity, which is commonly used in veterinary medicine and may present in milk (Mishra and Bhand, 2012). Cross-reactivity was tested in 1:4 diluted milk samples using 10 ng/mL standard solutions of above mentioned antibiotics. mAb-SRT immobilized crystal was fixed on flow cell and solutions of different antibiotics were passed individually through the FIA system at the flow rate of 0.1 mL/min. As shown in supplementary Table ST1, very high cross-reactivity was obtained for SRT and DHSRT (100% and 93.8% respectively) on mAb-SRT immobilized gold crystal, which contains more related structure with SRT. Other aminoglycosides e.g. GENTA, KANA and NEO showed no significant cross reactivity with mAb-SRT antibody and obtained results were less than 0.1% for all the three aminoglycosides. Similarly, no cross-reactivity was observed with SDZ. Fig. 4c shows the real time response for cross reactivity with different antibiotics. Obtained results demonstrate that there is no significant interference produced by any other non-targeted antibiotics, which may commonly be present in the milk. The results indicate the remarkable specificity of the developed FIA-EQCN biosensor.

3.5. Recovery studies from spiked milk samples

The potential application of developed FIA-EQCN biosensor has been tested for the detection of SRT in the diluted milk samples. Initially, all the milk samples were assumed to be free of SRT. Known amount of SRT (1 ng/mL) was spiked in the diluted milk samples from the prepared stock solution. Based on the response signals recorded from FIA-EQCN biosensor, recoveries were calculated. Supplementary Table ST2 shows the % recovery obtained for SRT spiked milk samples. The recoveries obtained (98–99.33%) from the developed biosensor indicates the potential application of the FIA-EQCN biosensor for the ultra-sensitive detection of SRT in milk sample

3.6. Precision of FIA-EQCN biosensor performance

Precision of developed biosensor was tested by determining intra and inter run analysis on the same modified gold quartz crystal immobilized with mAb-SRT antibody and with the diverse sets of immobilized crystals. A known amount of SRT

Table 2
Analytical performance and validation of FIA-EQCN biosensor.

Repeatability of FIA-EQCN SRT biosensor on same crystal

SRT concentration [ng/mL]	Response-1 (Δng)	Response-2 (Δng)	Response-3 (Δng)	Response-4 (Δng)	Response-5 (Δng)	S.D. ± Mean (n=5)	% R.S.D.
10	279.461	280.951	280.1	278.91	281.2	280.12 ± 0.9	0.351

Reproducibility and performance of FIA-EQCN SRT biosensor on different crystals

Sensors	SRT Concentration [ng/mL]	Response-1 (Δng)	Response-2 (Δng)	Response-3 (Δng)	S.D. ± Mean (n=3)	% R.S.D.
Sensor-1	10	279.40	280.94	280.10	280.15 ± 0.7	0.2781
Sensor-2	10	280.65	281.20	279.64	280.496 ± 0.7	0.282
Sensor-3	10	279.60	280.34	281.34	280.43 ± 0.8	0.310
Sensor-4	10	281.56	280.40	280.00	280.654 ± 0.8	0.290
Sensor-5	10	279.84	281.781	282.94	281.52 ± 1.5	0.5581

Comparison of FIA-EQCN SRT biosensor with commercial kit

Milk sample	SRT added [ng/mL]	SRT found (n=3)		Mean ± S.D. (n=3)		% RSD		% Recovery	
		FIA-EQCN	Commercial kit	FIA-EQCN	Commercial kit	FIA-EQCN	Commercial kit	FIA-EQCN	Commercial kit
Milk-1	10.0	10.03	10.02	10.03 ± 0.06	10.02 ± 0.1	0.601	1.60	100.3	100.2
Milk-2	10.0	9.971	9.871	9.971 ± 0.1	9.871 ± 0.1	1.502	1.31	99.70	98.70
Milk-3	10.0	10.02	10.06	10.02 ± 0.09	10.061 ± 0.1	0.901	1.781	100.21	100.61
Milk-4	10.0	10.13	10.11	10.13 ± 0.1	10.11 ± 0.1	1.381	1.281	101.30	101.10

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concentration (10 ng/mL) was spiked in the diluted milk sample and successively analyzed for biosensor performance ($n=5$). The baseline was corrected before each analysis and mass change (Δm) was calculated based on the obtained responses. The biosensor showed a good reproducibility in Δm values with Standard deviation of 280.13 ± 0.9 and calculated % R.S.D ($n=5$) 0.351 within the same crystal. Similarly, the biosensor performance was tested on diverse sets of immobilized crystals. Satisfactory results were obtained for SRT analysis in diluted milk samples when analyzed using different sets of biosensor. The % R.S.D. ($n=5$) was found in range of 0.2781–0.5581. The obtained results were summarized in the Table 2. These results indicate the precision and reproducibility of the biosensor thus can be used as potential tool for analysis of SRT in milk samples.

3.7. Validation of FIA-EQCN biosensor

The results obtained from the FIA-EQCN measurements were cross-validated against commercial ELISA kit. Different diluted milk samples were spiked with a known amount of SRT (10 ng/mL) and analyzed through the developed biosensor and commercial ELISA kit. Analysis was performed to determine % recovery for obtained results. The recoveries obtained from the FIA-EQCN biosensor (99.70–101.30%, % R.S.D. ($n=3$) 0.601–1.502) was more or less similar to the results obtained from the ELISA kit (98.70–101.10%, % R.S.D. ($n=3$) 1.281–1.781). It is evident from the Table 2 that the obtained results from both the methods show good co-relation in terms of sensitivity and reproducibility. The developed biosensor is reliable and as precise as the commonly used ELISA method.

4. Conclusion

A FIA-EQCN biosensor was developed and demonstrated for analysis of SRT residues in milk samples. Specific monoclonal antibody against SRT was immobilized on the gold crystals after SAMs formation. The developed biosensor could detect an ultra-trace level of (0.001 ng/mL) in milk samples. A good reproducibility was obtained with Standard deviation of 280.13 ± 0.9 and calculated % R.S.D. ($n=5$) 0.351 within the same crystal and % R.S.D. ($i=5$) in range of 0.2781–0.5581 on diverse set of crystals. Precision of these results showed the reproducibility and reliability of the biosensor. The obtained recoveries (98–99.33%) for SRT spiked milk samples encourage the application of developed biosensor towards the quantification of SRT in milk sample. Validation of the presented biosensor against commercial ELISA kit (Biooscientific, USA) also proofs that developed biosensor provides much sensitive detection (0.3 ng/mL) as compare to the commercial kit (5 ng/mL). The developed biosensor could complete the analysis in less than 17 min. and exhibited a great potential for automated analysis. This approach may become practical for routine automated analysis for detection of SRT in milk samples.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.09.033.

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