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Der Pharma Chemica, 2014, 6(2):167-176
(http://derpharmachemica.com/archive.html)

ISSN 0975-413X
CODEN (USA): PCHHAX

Biosynthesis and characterization of silver nanoparticles: Its use for bacterial detection

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ABSTRACT

In this study; we have designed a Silver nanoparticles (AgNps) based piezoelectric Quartz crystal microbalance (QCM) AgNPs were synthesized and were coupled with anti sera of S. typhimurium. Coupled AgNps were characterized by TEM and FITR In TEM micrographs; we have observed spherical of AgNPs at the range ~50nm diameter Developed immunosensor for S typhimurium QCM has showed good reactivity and specificity towards its antigen of S. typhimurium; also showed decrease in frequency with respect to incubation time. The developed methodology is effective; selective; inexpensive and reproducible for the detection of S. typhimurium and a further step forwards towards development of point of care technology for the detection of bacteria Moreover; we have also demonstrated that the efficacy and sensitivity of sensors could be increased by immobilization of functional nanoparticles on the surface of quartz crystal

Keywords: Nanobiosensors; Silver nanoparticles; Quartz crystal microbalance

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INTRODUCTION

The increased mortality rate due to *Salmonellosis* continues to be a major public health problem worldwide causing sixteen million annual cases of typhoid fever; one billion cases of gastroenteritis and three million deaths are estimated worldwide due to the bacterial pathogenic infection of *Salmonella* [1; 2] It also contributes to negative economic impacts due to the cost of surveillance investigation; treatment and prevention of illness; and needs urgency for an early detection of this pathogen The routine and conventional detection devices are time consuming and needs highly skilled operational method; upcoming technologies of biosensors are quick; precise and sensitive method of the pathogen-detection [3]

Bacteria are our invisible friends or invisible enemies Some bacteria aid our digestion; others destroy our poisons Still other "bugs" make us sick The ones which make us sick need attention as they can be fatal *Salmonellae* are gram negative; facultative; anaerobic bacteria of the family Enterobacteriaceae; made up of non spore forming rods; usually motile with peritrichous flagella *Salmonella* is closely related to the *Escherichia* genus and are found in both

cold and warm blooded animals including humans and in the environment They cause illness like typhoid fever; paratyphoid fever and foodborne illness [4] The capacity of *Salmonella* to produce many virulence factors contributes to its pathogenecity *S typhimurium* invade cultured epithelial [5] and macrophage cells *in vitro* and remain inside a unique membrane-bound vacuole; the *Salmonella*-containing vacuole (SCV); where they begin to replicate *Salmonellosis* can occur when infected with *S typhimurium* and produces symptoms of food poisoning like nausea; vomiting; diarrhea which can result in dehydration and even death Hence the detection of *S typhimurium* is of great clinical importance while curing these diseases *S typhimurium* can be detected by various techniques or devices but here we describe the most recent nanotechnology based nanobiosensing device for its detection

There are different immobilizing protocols which were studied in our laboratory for developing nanobiosensor devices using piezoelectric QCM Thiolization technique using *p*-aminothiophenol (ATP); was used to modify the cleaned surface of quartz crystal The crystal surface bearing SAM of ATP was utilized for immobilization of *Salmonella O antiserum factor VII* using DCC/NHS chemistry followed by appropriate blocking using bovine serum albumin (BSA) solution Finally; this developed transducer was exposed to *S typhimurium* (target analyte) for detection by recording the change in frequency of quartz crystal according to Sauerbrey equation The experimental setup and devices; protocols are in the Materials and Methods section

QCM is a piezoelectric device which can be modified to develop different immunosensors Salmain M; et al; 2011; has demonstrated that piezoelectric immunosensor could be used for direct and rapid detection of staphylococcal enterotoxin A (SEA) [6] Self assembled monolayer method (SAM) method was used for rapid detection of Bacillus anthracis [7] Immunosensor development process involves various steps The very first step in this process is the activation of quartz crystal surface The thiolization method results in activation of quartz crystal surface This modified surface is then further utilized for antibody immobilization in successive step [8] The antibody immobilization step is the key step during immunosensor development because antibody provides the specificity to immunosensor For proper immobilization of antibody and for sensitivity purpose; quartz crystal iss modified with functionalized AgNPs

This report describes the designing of Silver nanoparticles (AgNps) based piezoelectric Quartz crystal microbalance (QCM) AgNPs were synthesized and were coupled with anti sera of *S typhimurium* for developing an effective; selective; inexpensive and reproducible device for the detection of *S typhimurium*

MATERIALS AND METHODS

Chemicals and Instruments

Silver nitrate ($AgNO_3$; Sigma Aldrich; USA); methanol (CH_3OH; SRL; India); trisodium citrate ($C_6H_5O_7Na_3$; Sigma Aldrich; USA); *p*-aminothiophenol(ATP; Sigma; USA); toluene (SRL; India); Sodium hydrogen bromide ($NaBH_4$; Fisher Scientific; USA); 3;3'-dithiodipropionic acid (DTPA; Sigma; USA); N; N'- tetraoctyl ammonium bromide (TOAB; Sigma; USA); 3;3'-thiodipropionic acid (TDPA; Sigma; USA); purchased of analytical grade Double distilled (DD) water is used throughout the experiments for preparation of solutions and washing purposes

Instruments used during synthesis are electronic balance (Percisa; Switzerland); magnetic stirrer hot plate (Spinot; Tarsons Products Pvt Ltd; Kolkata; India); Rotary flash evaporator (Metrex; India) etc

Synthesis of Silver Nanoparticles (AgNPs)

Chemical reduction method was used for the synthesis of bare AgNPs In this method; silver nitrate ($AgNO_3$) is chemically reduced with trisodium citrate ($C_6H_5O_7Na_3$) In a typical experiment; 50 ml of 1 x 10^{-3}M $AgNO_3$ was heated to boiling and 5 ml of 1 % trisodium citrate was added drop by drop During the reduction process; solution was stirred continuously along with heating at ~80 °C until the color changed from colorless to pale yellow Flask containing AgNPs; was then removed from the heating element and stirred until cooled to room temperature

Synthesis of Functionalized AgNPs

Phase transfer method was used for the synthesis of functionalized AgNPs AgNPs were functionalized with *p*-aminothiophenol (AgNPs@ATP) by this method The typical procedure for this synthesis involved the reduction of 25ml of (1 x 10^{-3}) M silver nitrate solution in water with 1% $NaBH_4$ aqueous solution Sodium borohydride acted as a reducing agent In the first step; the silver metal ions were transferred to organic layer by stirring the aqueous silver nitrate solution with 25 ml toluene containing N;N'-tetraoctyl ammonium bromide (TOAB); as a phase transfer

agent A biphasic system was constructed by the two solvents; water and toluene After ½ an hour of stirring at room temperature; 25 ml solution of *p*-aminothiophenol solution in toluene was added and the stirring was continued for further ½ an hour To this biphasic system; a 25 ml aqueous solution of $NaBH_4$ is added keeping the boiling condition till the color appears from colorless to brownish red The molar ratio for the Ag^+/(octyl)$_4$ NH_4^+ Br^- /thiol/$NaBH_4$ was fixed to 1:3:3:10 As the color observed; the system was allowed to cool to room temperature with continuous stirring After that AgNPs@ATP were washed with lot of ethanol A schematic representation for synthesis of functionalized nanoparticle by the phase transfer method was given in Figure (1A) along with color scheme in Figure (1B) The great advantage of this method was that the nanoparticles could be transferred into desired solvent (methanol in this case) by evaporating toluene in rotary flash evaporator and redispersing NPs

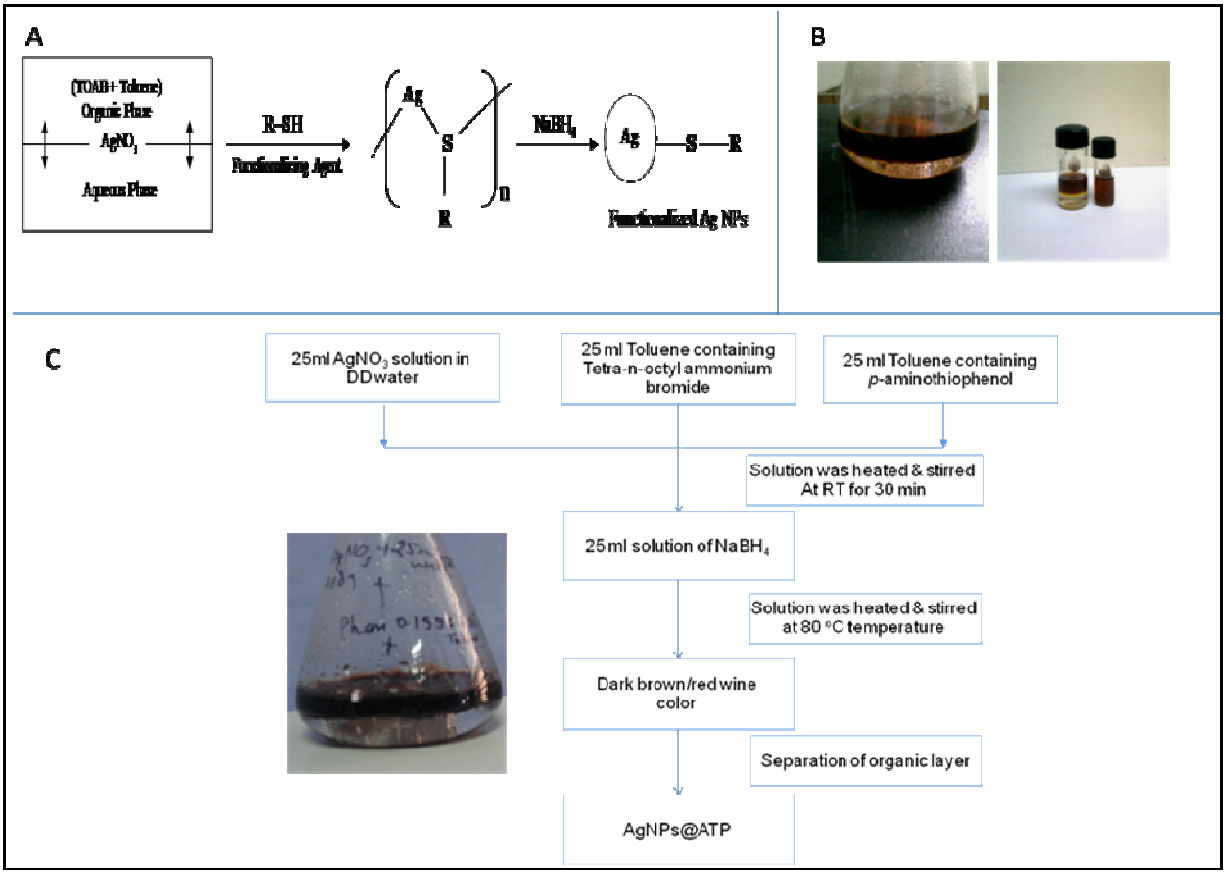

Figure 1 (A) Schematic diagram for synthesis of functionalized AgNPs by phase transfer method (B) Color scheme for the functionalized AgNPs in biphasic synthesis system (C) Flow diagram for the synthesis of AgNPs functionalized with *p*-aminothiophenol

Characterization Of Prepared AgNPs

After synthesizing AgNPs by the standard reduction method; it is important to characterize the prepared NPs Three different methodologies were used for this study which involved Transmission Electron Microscopy; Ultraviolet-Visible spectroscopy and Fourier Transform Infrared spectroscopy TEM; JEOL; Japan instrument was used for the determination of size and shape of synthesized NPs The characterization process required suspension of NPs and a thin file of NPs was developed by immobilizing them on carbon coated copper grids For this; the grid was exposed to sample for some time and then sample was run off gently from grid and allowed to dry before TEM micrograph imaging The TEM instrument with microscope JEM 1011 operated at 80 kV With the help of the software attached to the instrument; the size of prepared NPs was determined In UV-Visible spectrophotometer (UV 5704); AgNPs were optically characterized with the help of double beam Various UV spectra were collected and observed for data analysis The synthesized AgNPs@ATP in toluene were also characterized with FTIR spectroscopy Here radiation containing all IR wavelengths (eg; 5000-400 cm^{-1}) was split into two beams One beam was of fixed length; the other of variable length A data-processing technique called Fourier Transform turns this raw data into the desired result

(the sample's spectrum): Light output as a function of infrared wavelength (or equivalently; wavenumber) Since a monochromator was not used; the entire radiation range was passed through the sample simultaneously and much time was saved FT-IR instrument could have very high resolution (<0001 cm^{-1})

Preparation of QCM Immunosensor

For the detection of *S typhimurium;* the Ag and the antisera (ie *Salmonella O antiserum factor VII)* were procured from BD Also in this study; QCM was developed as piezoelectric immunosensor and QCM-200 (Stanford Research System; Inc USA) system was used for biosensor development [9] The chemicals used in this study were N-hydroxysuccinimide (NHS; SRL; India); *p*-aminothiophenol (ATP; Acros; USA); N;N'-dicyclohexyl carbodimide (DCC; SRL; India) and bovine serum albumin (BSA; Acros; USA) The transducer used was the AT-cut quartz crystal sandwiched between gold electrodes on both the sides with fundamental frequency of 5MHz (SRS; USA) The crystal surface was washed with methanol and phosphate-buffered saline (PBS); pH 74 after each step as per requirement

For the synthesis process; all the glassware was used of borosilicate glass and was purchased from Qiagen All the glassware used in the following experiments were cleaned with freshly prepared HNO_3: HCl (v/v 1:3); rinsed thoroughly with double distilled water and dried in oven To start with; it was important to have a quartz crystal with clean surface for a strong and clean interaction of the molecule Ultrasonic method was a general cleaning method which was done in a solution of detergent in deionized water In the process the crystal was exposed to 1% sodium dodecyl sulfate (SDS) detergent solution in DD water and sonicated for 2 min 30 sec pulse with on and off time setting Immediately rinsed liberally with methanol and deionized water and dry in a gentle flow of filtered nitrogen gas This cleaned surface was now ready for surface activation step SAM method was used to activate the cleaned AT-cut quartz crystal surface [10; 11] The crystal was exposed to 1×10^{-3} M methanolic solution of (ATP) and was incubated for ~12 hrs at 4 °C [12] This incubation gave a stable and well ordered monolayer on crystal surface The defined modification of the crystal surface activates the gold electrode and provides a suitable surface for the immobilization of *Salmonella O antiserum factor VII* The Ab; *Salmonella O antiserum factor VII* were attached to the crystal surface bearing SAM of ATP through amide bonding which provides specificity to the quartz crystal This immobilization of *Salmonella O antiserum factor VII* (dilution 1:6000 using 005 M PBS; pH72) [13; 14] occurs in presence of DCC and NHS (45mM/15mM) The crystal frequency was then monitored initially for ~1 hour and then incubated at 4 °C for 12 hours After immobilization of Abs on the crystal surface; the crystal was treated with 1% BSA; a blocking solution in PBS (pH 72) The crystal was incubated for ~1 h at room temperature This step was carried out to block the free sites thus avoiding any kind of non-specific interaction This crystal was then washed with PBS solution (pH 72) in order to remove traces of BSA physically attached to the gold electrode The modified crystal was then finally subjected to the suspension containing target analyte ie *S typhimurium* Ag for the detection (764 units/mg of protein); diluted (1:6000) in PBS; pH 72

Preparation of Modified QCM by using AgNPs

For the biosensor development; the AT-cut quartz crystal sandwiched between gold electrodes on both the sides having fundamental frequency of 9 MHz (Maxtek; USA) was modified The change in frequency as a function of mass with respect to time was monitored by The Research Quartz crystal microbalance (RQCM; Maxtek; USA) The specificity and sensitivity of the developed biosensor depends upon the way the crystal surface was modified In order to develop SAM in the first step; the cleaned AT-cut quartz crystal was subjected to 3; 3'-dithiodipropionic acid (DTPA) solution In a typical experiment; the quartz crystal was merged in 1×10^{-3} M solution of DTPA in ethanol and incubated for ~12 hrs at 4 °C which resulted in a stable and well ordered SAM In the successive step; the modified quartz crystal was exposed to AgNPs@ATP solution in ethanol for ~12 h at 4°C The attachment of AgNPs@ATP was facilitated by the presence of DCC/NHS (45mM/15mM) to the DTPA; SAM developed by thiolization The antibody (*Salmonella O antiserum factor VII*) was immobilised on NP modified gold electrode in Phosphate Buffer Saline (PBS); pH 74 For proper immobilisation of Ab; the Abs should be incubated with the crystal for ~12 h at 4 °C After the immobilization of Ab on the NP modified crystal surface; the crystal was treated with 1% BSA; a blocking solution in PBS (pH 74) The crystal was incubated for ~1 h at room temperature This step was carried out to block the free sites thus avoiding any kind of non-specific interaction This crystal was then washed with PBS solution (pH 74) in order to remove traces of BSA physically attached to the gold electrode Finally; modified crystal was exposed to the corresponding antigen (*S typhimurium*) diluted in PBS (74; pH) for sensing

RESULTS AND DISCUSSION

Optical Characterization AgNPs

The optical characterizations of silver nanoparticles were performed with the help of double beam UV-Visible spectrophotometer (UV 5704) UV-Visible absorption spectra has provided the information about silver colloid because silver nanoparticles exhibit an intense absorption peak due to surface plasmon excitation of the conduction electrons The AgNPs was synthesized by citrate method and absorption was observed under UV range 300nm to 600nm

In Figure-2; a characteristic plasmon peak at ~420 nm indicates the existence of NPs in the system The plasmon peak and full-width of half maximum depends on the particle size As the particles were functionalized there was a shift observed in the plasmon peak and an increase was observed at ~416 nm which clearly demonstrated the presence of silver nanoparticles AgNPs@ATP Moreover; no obvious change in the peak position was observed after thirty days

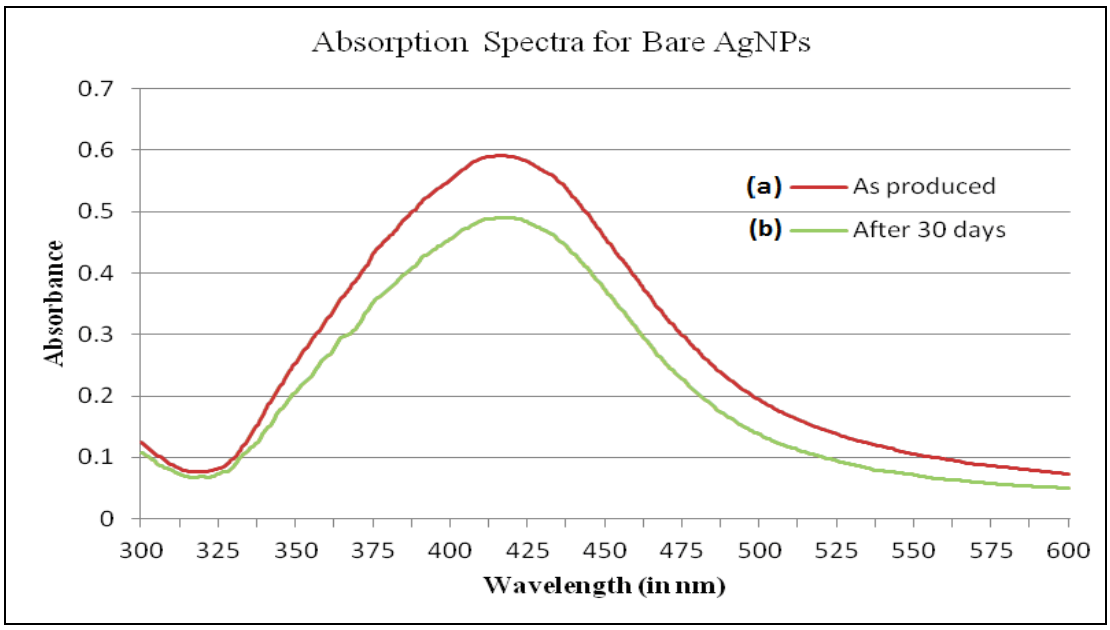

Figure 2 Analysis of bare AgNPs and AgNps coupled at two time periods

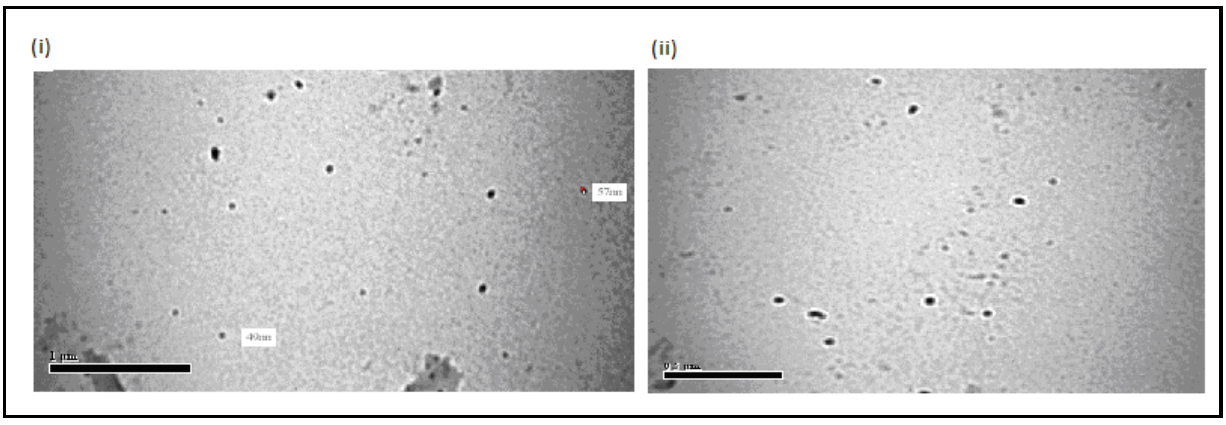

Figure-3(i) TEM micrograph of the silver nanoparticles functionalized with *p*-aminothiophenol at lower magnification (ie as indicated X3000C) (ii) TEM micrograph of the silver nanoparticles functionalized with *p*-aminothiophenol at higher magnification (ie as indicated X5000C)

Characterization of AgNps by Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM; JEOL; Japan) instrument was used for characterization of AgNps The liquid sample of nanoparticles was used to characterize Figure 3(i) & 3(ii); depicted TEM micrographs of the silver nanoparticles functionalized with *p*-aminothiophenol.

The TEM images demonstrated that the size of the spherical AgNPs was ~50nm diameter which was appropriate for our study A shining boundary was also observed at the NP surface; due to adsorption of the *p*-aminothiophenol to NP surface The use of AgNPs@ATP led to the advantages in developing physical attractions (dipole-dipole interaction/charge interaction) in between antibodies as well as negatively charged gold electrode due to the exposure of –COOH group of 3; 3'-dithiodipropionic acid perpendicular to the surface In addition to this; functionalization step had led to prevention in NPs agglomeration due to steric forces (Figure 4) As the amino group lied perpendicular to the nanoparticles surface; an envelope was developed around the nanoparticle with a partial positive charge The repulsion force developed due to same charge in between the two nanoparticles which helped to kept them apart

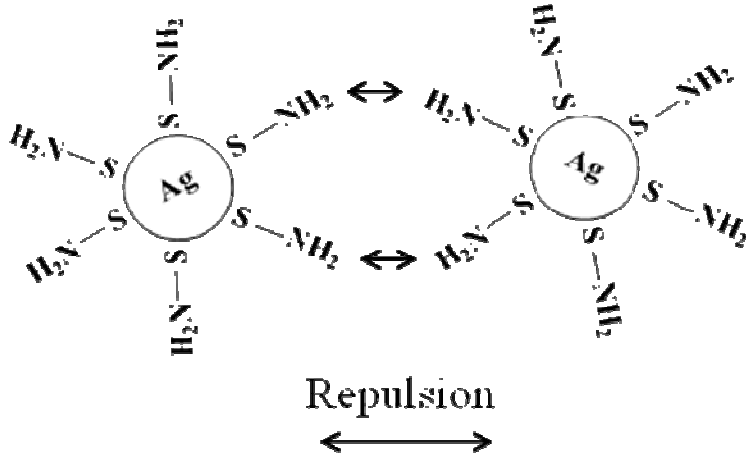

Figure 4 Repulsive force developed between functional NPs imparted by –NH_2 group

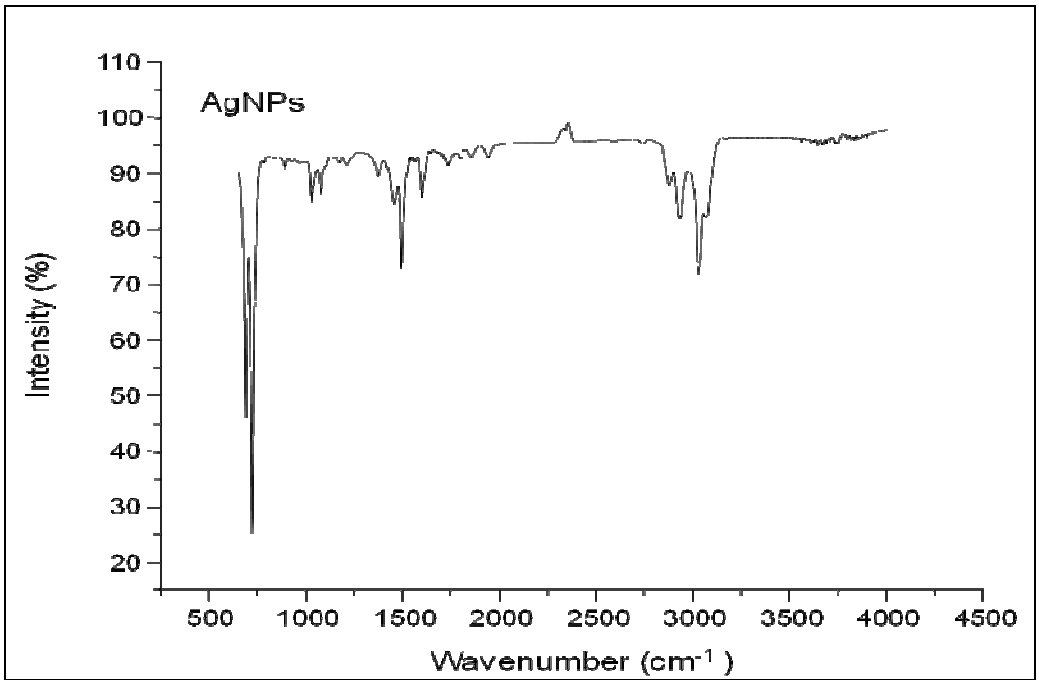

Figure 5 FTIR spectra for AgNPs@ATP; showing peak at 750 cm^{-1}

Fourier Transform Infrared Spectroscopy (FTIR)

The synthesized AgNPs@ATP in toluene was also characterized with FTIR spectroscopy (Figure 5) The main peaks in the FTIR spectra for AgNPs confirm their existence The peak at 750 cm^{-1} represents the substituted benzene ring The peaks in the range 1450-1500 cm^{-1} were for aromatic C=C bond The peak at about 1600 cm^{-1} showed the presence of the primary amino group The infrared bands of thiol were usually medium to weak intensity because of small dipole moments of S-H bond The only useful group wavenumber for thiol is the S-H stretching vibration; which was found at 2590-2560 cm^{-1} Absence of a peak at ~2550 cm^{-1} confirmed the absence of free S-H from the free thiol and peak at about 3025 cm^{-1} could be perhaps attributed to the thiolization

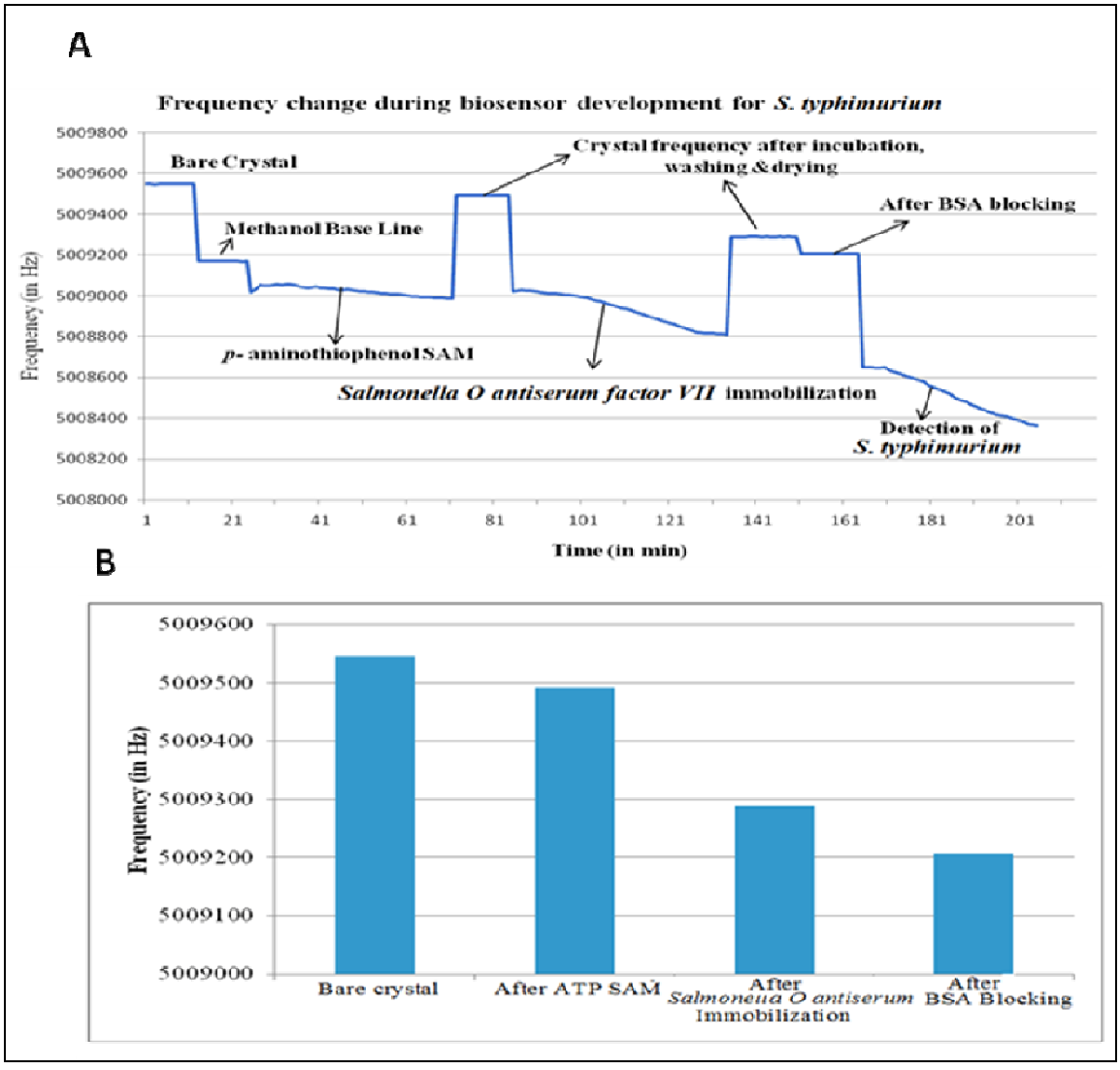

Figure 6 (A) Biosensor development using QCM for the detection of *S typhimurium* (B) Bar graph for the average frequency monitored after each step in dry state

Detection of *S typhimurium* using basic QCM device

The initial step in the development of QCM based immunosensor was to achieve a thiolated crystal surface Thiolization not only led to surface activation but also modification in favour of antibody immobilization Attachment of ATP molecules decreased the average frequency of bare quartz crystal (500954659 Hz) The modification was done by using ATP because thiol group showed greater affinity towards the gold and second; the developing SAM film activates the gold electrode by providing partial positive charge to crystal surface as the $–NH_2$ lies perpendicular to the surface This partial positive charged surface was a better site for antibody immobilization

Development of the SAM on gold electrode led to decrease in the frequency Average frequency monitored after ATP modification was about 500949251Hz (Figure-6A)

This ATP modified crystal was exposed to *Salmonella O antiserum factor VII* (diluted in PBS; pH 72); in order to make the biosensor surface specific for *S typhimurium* Activation by coupling agents (DCC/NHS) was shown to enhance the stability of the coating and facilitate the formation of a suitable intermediate to condense antibodies reproducibly and densely at the SAM; leading to high sensitivity and good precision of the developed immunosensor The average frequency of the crystal after incubation with *Salmonella O antiserum factor VII* for ~12h at 4°C is recorded and found 50092886 Hz

The immobilization of antibody on ATP modified crystal left some active sites and crystal surface free These free sites and surface area was available for foreign molecule and/or for target entity This led to false signaling due to attachment of *S typhimurium* directly to crystal surface/free sites This false signing could be minimized by blocking the free sites with 1% BSA The average frequency of crystal after this modification was 500920737 Hz The average frequency after each step can be summarized in the graphical form as shown below in (Figure-6B)

Total decrease in frequency after SAM development with respect to bare crystal was 5408 Hz The frequency shift after *Salmonella O antiserum factor VII* immobilization with respect to bare crystal is 16391 Hz This shift is 8123 Hz after blocking of free sites with BSA compared with Ab modified crystal.

For the detection of *S typhimurium*; the quartz crystal modified with *Salmonella O antiserum factor VII* is exposed to *S typhimurium* suspension (target molecule) diluted (1:6000) in PBS; pH 72; initially a small change in frequency was observed Initially a small response rate was observed perhaps due to initiation of binding process but after ~5 min frequency decreases sharply and ultimately it reached to a minimum value This decrease in frequency directly infers the attachment of *S typhimurium* to the crystal surface In this way; an immunosensor against *S typhimurium* was developed by using *Salmonella O antiserum factor VII* immobilized on quartz surface through SAM of p-aminothiophenol

Detection of *S typhimurium* by modified QCM with AgNP

The process of biosensor development starts with quartz crystal surface modification with DTPA This leads to a SAM on the gold electrode This SAM film activates the gold electrode by developing the partial negative charge on its surface as the –COOH lies perpendicular to the surface The modification was done by using DTPA because thiol showed the greater affinity towards the gold and secondly; the partial negatively charged surface; due to –COOH groups; also provides better site for partial positively charged (AgNPs@ATP) Development of the SAM on gold electrode leads to decrease in the frequency Average decrease in Δf is about 4426 Hz This decrease in frequency with respect to the mass was plotted with the help of QCM (Figure-11 —◇— line curve)

When AgNPs@ATP was applied to the modified gold electrode with DTPA; there was an attraction force developed due to greater affinity of the partially negative charged –COOH group towards the partially positively charged $–NH_2$ group Such attraction helped silver nanoparticles to hold on the gold surface The modification of AgNPs by ATP developed a stable and passive layer about nanoparticle surface; which prevents their oxidation during the experiment

During the course of the attachment of functionalised AgNPs to the thiol modified quartz crystal; an average decrease in Δf is 6690 Hz noticed (—□— line curve; Figure-7) Immobilisation of silver nanoparticles provides a larger surface area along with a partial positive surface This partially positively charged surface also provided a better site for partially negative charged antibody for the attachment to the surface This NP modified crystal was exposed to PBS buffer solution containing *Salmonella O antiserum factor VII* Ab; in order to make the biosensor surface specific for *S typhimurium* antigen The average frequency change of about 9020 Hz is recorded (—X — curve; Figure-7)

Figure 7 shows decrease in the frequency as a function of mass with respect to time for a number of steps monitored by using QCM During Ab-Ag interaction study; it is observed that initially there was a small change in Δf as there was initiation of binding process but after some time Δf decreases sharply and ultimately it reached to a minimum value (—△— curve) when almost all the binding sites get occupied by corresponding *S typhimurium* Ag The average decrease in Δf is 61523Hz When the crystal was exposed to a non-complementary antigen; it was noted that

there was no appreciable decrease in the Δf as the non-specific antigen was unable to bind with the *Salmonella* Ab (—○—curve) Only about 373 Hz change was observed during this interaction which was negligible as compared to interaction between *Salmonella* antibody with corresponding *Salmonella* antigen Thereby; confirming the specificity of antibody along with the specificity of developed immunosensor

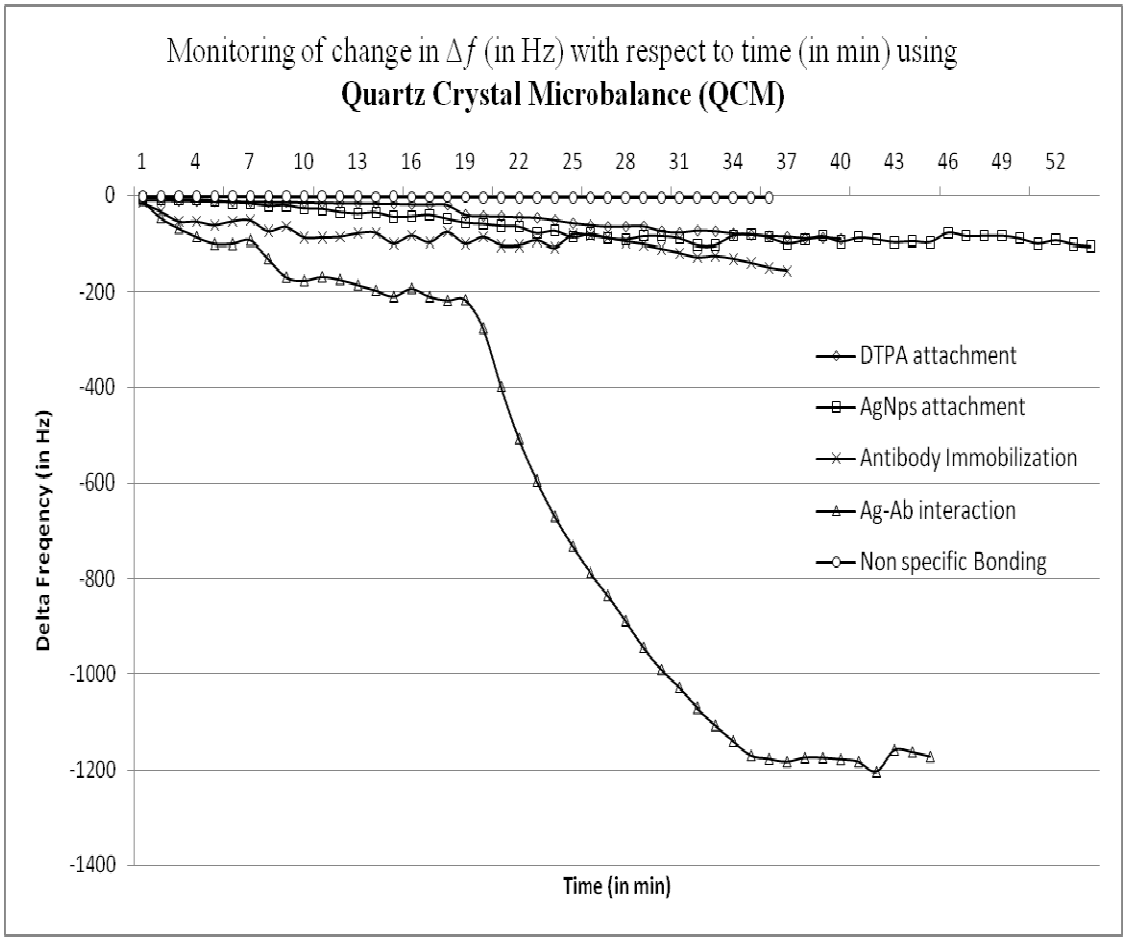

Figure-7 Monitoring of change in delta frequency using QCM for a number of steps: Curve shown by the —Δ— represents the strong interaction in between *Salmonella O antiserum factor VII* and its corresponding Ag ie *S Typhimurium* Whereas —○— curve clearly shows non specific bonding

CONCLUSION

The study demonstrates that QCM based nanoimmunosensor could be modified using nanoparticles and can be used for the detection of *S typhimurium* Antibodies specific to *S typhimurium (Salmonella O antiserum factor VII)* were immobilized onto a self assembled monolayer and biosensor was developed on thiolated quartz crystal surface for the detection of *S typhimurium* The developed methodologies were effective; selective and reproducible for the detection of *S typhimurium* Future plan of this study is to develop a clinical trial for assessing the sensitivity/specificity of the technique and results will be compared with traditional techniques like ELISA The data presented and methodology developed is of immense potential for regulatory authorities along with other concerned scientists and organizations These methodologies may be used for the detection of other pathogens by appropriate functionalization and may be used for the analyses in various national and international laboratories These types of biosensors can be studied not only for bacterial detection but should be initiated for other chemical detection as well thereby highlighting the need of further research work in this area Advancement in nano-biosensors could be a step forward towards development of potable point of care devices

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