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SYNTHESIS OF POLYSTYRENE NANOPARTICLES AND THEIR APPLICATION FOR DETECTION OF *INFECTIOUS BURSAL DISEASE VIRUS* **(*IBDV*)**

Abstract

Polymeric nanoparticles are the nanostructured polymers and are more economic, easy to prepare, less toxic and multipurpose as compared to metallic nanoparticles. The investigation undertaken represented the preparation of polystyrene nanoparticles through chemical induced micro–emulsion polymerization of styrene. The negatively charged emulsifier, sodium dodecyl sulfate (SDS) was used to stabilize the polystyrene nanoparticles. The characterization of prepared polystyrene nanoparticles was done by TEM and FTIR. The resultant nanoparticles were spherical in shape, uniformly distributed and had an average diameter of 75 nm. Polystyrene Nanoparticles (PSNPs) were immobilised on AT cut quartz crystal to enhance the binding site and efficacy of Quartz Crystal Microbalance (QCM) based nanobiosensor. The immobilisation of PSNPs on quartz crystal has been studied by SEM. PSNPs have been

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tested for surface modification during the development of QCM based biosensor for detection of *infectious bursal disease virus* (**IBDV**). The effect of concentration of suspension containing PSNPs in methanol was also studied. The detection of bacteria/virus was done by developed nanobiosensor and crossed check with RK_2 antigen (specific to Cd-Rhizobacteria). The observed signal of detachment of Antigen – Antibody complex from the crystal surface is the beauty of present work.

Keywords**:** Polystyrene nanoparticles, Quartz Crystal Microbalance (QCM), Biosensor, IBDV

I. INTRODUCTION

Nanotechnology came forward with various nanomaterials in the field of infectious disease diagnosis using biosensors [1]. This technology includes various nanomaterials such as nanoarrays, nanopores, nanoparticles (NPs) based immunoassays, quantum dots, etc. Traditional methods of diagnosis take time. Advanced and more reliable devices namely nanobiosensors are being used nowadays for rapid, real-time and label-free detection [2]. These biosensors achieve their specificity through immunoassays[3]. Antibody-based immunoassays in piezoelectric nanobiosensors are well developed for the purposes of diagnosis and detection of infectious microorganisms [4, 5]. Genetically modified organisms were successfully detected using QCM based DNA biosensor [6]. In another study, a non – labelled QCM biosensor for detection of microorganism using both carbohydrate and lectin binding has been developed [7].

In nanoparticles, metal nanoparticles especially gold NPs and semiconductor quantum dots get large attention. But, in recent polymeric NPs get attension in development of immunoassays [8]. Among the available polymers, polystyrene (PS) has been preferemtialy used in medical fields due to its low cost and high practicality. PS is commonly used in the field of diagnostics due to its optical properties and non-toxic nature. However, it had several drawbacks such as poor chemical resistance, difficulty in controlling surface properties and its hydrophobic nature which caused problems in its applications. Therefore, surface modification is necessary to improve wettability, adhesion, biocompatibility and topography. The surface properties of untreated PS can be easily modified by ion beam treatment, plasma treatment, UV/ozone treatment and graft polymerization.

A simple immunoassay can be used for different application in medical diagnostics and in study of protein – protein interaction. Darain et.al. have developed an immunoarray using polystyrene for

detection of horse IgG. Specific antibody was immobilised on gold coated polystyrene surface using thiol chemistry and silanization [9, 10]. For ELISA application a carboxylated haptens supported on polystyrene has also been studied for pH and reaction time study [11].

Polymerisation of styrene via photo-initiated micro – emulsion technique was reported by P.L. Kuo and N.J. Turro. The effect of concentration of initiator on degree of polymerisation and rate of polymerisation was explored. The polydispersity index was found in narrow range of 1.05 – 1.08 with particle size 30 – 60 nm [12]. J. Jang & H. Ha reported micro – emulsion technique for preparing hollow PS nanoparticles using a copolymer matrix. The size of NPs was fond dependent on surfactant concentration and weight ratio, $[Surfactant]/[Monomer]$ [13]. In the similar study, a cationic surfactant alkyltrimethyl ammonium bromides with water soluble initiator was used by Xu et al. [14]. W. Ming et al. synthesised nanoparticles of different monomers with high polymer ratio and smaller NP size using modified micro – emulsion technique [15]. The effect of temperature, surfactant concentration, nature of initiator on polymeric NPs also studied by many researchers [16,17].

This work mainly focuses on developing a novel synthesis method for PSNPs using micro-emulsion polymerization technique. These PSNPs will be used to develop a simple and robust biosensor against the highly infectious viral disease of chickens, named *infectious bursal disease virus* (*IBDV*).

II. EXPERIMENTAL

1. Reagent & Material Used

For polystyrene nanoparticle synthesis styrene monomer (from Acros), sodium dodecyl sulfate (from Himedia), amyl alcohol and methanol (from Merck); potassium persulphate, magnesium sulphate and sodium hydroxide (from Fisher) were used.

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Department of Veterinary Biochemistry, G B Pant University of Agriculture & Technology, Pantnagar has provided the purified polyclonal antibodies specific to *Infectious Bursal Disease Virus* (*IBDV*).

For developing a specific quartz crystal surface, synthesised (PSNPs) and bovine serum albumin (BSA) (from Acros) were used. for developing quartz crystal surface. Methanol and phosphate-buffered saline (PBS), pH 7.4 were used for washing the crystal surface in subsequent steps.

2. Sensing Device Used

Stanford Research System (SRS) (Model: QCM200) quartz crystal microbalance analog controller (USA) was used for measurement. This QCM system contains a sensor unit and sensor chip *i.e.* an AT-cut piezoelectric quartz crystal of fundamental frequency of 5 MHz having gold coated electrodes on both sides of crystal was used as transducer.

III. SYNTHESIS OF POLYSTYRENE NANOPARTICLES *(***PSNPs***)*

1. Removal of Inhibitor from Styrene Monomer

This is done by shaking twice with aa aqueous 10% NaOH solution. This biphasic system was separated and the monomer styrene was washed three times with double distilled water to remove traces of NaOH. Magnesium sulfate *(*$MgSO_4$*)* was then added to washed styrene to get rid of water and stored at – 20 °C.

2. Polymerisation of Styrene Monomer to form PSNPs

Micro – emulsion polymerization method was used for synthesis of PSNPs. Micro – emulsion was prepared by stirring continuously

double distilled water *(*90 mL*)* with SDS *(*10 mg*)* and amyl alcohol *(*1.24 mL*)*. this polymerizing mixture was continuously pursed with nitrogen *(*60 bubbles per minute*)* to ensure complete absence of oxygen. To this oil – in – water type emulsion, destabilized styrene monomer *(*2.7 mL*)* followed by an aqueous solution of initiator (0.06 gm of potassium persulphate, KPS in 2 mL water) was added with constant stirring. This solution was continuously stirred at 65 – 70 °C for ~4 hours in order to ensure maximum conversion of styrene to PSNPs. The mixture turned milky white indicating the formation of PSNPs. The micro – emulsion having PSNPs were precipitated out in methanol *(*PSNPs to methanol ratio 1:20 – 100*)* for further study.

3. Characterization of PSNPs

Characterisation of PSNPs was done with TEM (make JEOL) and FTIR (Model NICOLET 6700, Thermo). A thin film of PS nanoparticle was deposited on a carbon coated copper grid for size measurement using TEM microscope (JEM 1011) operating at 80 kV.

4. Process of Biosensor Development

Quartz crystal microbalance gives response to any mass deposited on its surface. Its surface must be modified so that it responds to a specific entity particularly for biosensor development. In present study, the biosensor development includes the modification of gold surface with PSNPs followed by immobilization of IBDV antibody. The free sites left on modified crystal surface were masked with bovine serum albumin *(* 1% solution in PBS*)*. This modified crystal was then tested in final step for detection with specific and non-specific antigens. Each and every step during the process of biosensor development is accompanied with incubation and washing with appropriate solvent/buffer solution. Incubation is required in each step as the process of attachment is very slow.

a. ***Developing a layer of PSNPs:*** A layer of PSNPs was deposited on gold surface by incubating crystal for ~18 h with 100 μL of PSNPs in methanol. For the characterization of deposited monolayer, scanning electron microscope was used.

b. ***Study of Different Concentrations of PSNPs:*** For this study, 1 mL of PSNPs synthesized by micro – emulsion polymerization technique was precipitated out in different amount of methanol (20, 50, and 100 ml of methanol). For complete precipitation, these mixtures were incubated overnight. These resulting solutions were used as such to develop monolayer on gold surface of quartz crystal.

c. ***Immobilization of Antibody on PSNPs Modified Crystal Surface:*** The quartz crystal modified with PSNPs was subjected to 100 μL of *IBDV* antibody solution in PBS buffer *(*5μg*/*mL*)* and frequency was recorded for ~1 h. Unattached antibodies was removed by rinsing crystal with PBS buffer after ~24 h incubation.

d. ***Blocking of Unmodified Crystal Surface:*** The unmodified crystal surface was checked by applying BSA solution *(*1 *%* in PBS*)* for ~ 1h to avoid false and non – specific sensing. This crystal was washed many times with PBS to remove unbound material.

e. ***Study of Antibody – Antigen Interaction:*** Detection of antigen specific for IBDV is laid on the specific interaction between the corresponding antibody (Ab) and antigen (Ag), resulting Ab – Ag complex formation. The change in frequency of the quartz crystal directly indicates the reaction during the formation of the Ab – Ag complex

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VI. RESULT & DISCUSSION

1. Characterisation of PSNPs Synthesized by FTIR

The Fourier Transform IR spectroscopy was used to study the chemical structure of synthesized PSNPs (Figure – 1). The phenyl ring in styrene was characterized by specific peaks 3025.2, 1492.7, and 755.7 cm^{-1} were assigned to the stretching vibration of C – H bond , the C = C stretching vibration and rocking vibration of C – H bond of phenyl ring respectively [18]. The other three peaks at 1597.2, 1492.7 and 1452.1cm^{-1} were more characteristics peaks for the aromatic compounds. An absorption band in region 3100–3000 cm^{-1} assigned to C–H stretching of aromatic ring whereas absorption just below 3000 cm^{-1} in region 2900 – 2820 cm^{-1} assigned to C – H stretching for CH_2 group present in polymeric chain.

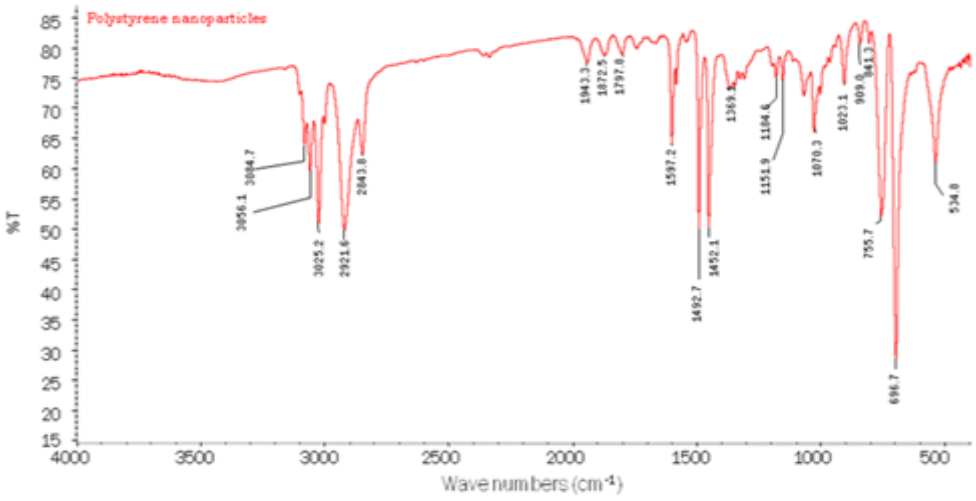

Figure 1: FTIR spectra of PSNPs synthesized

The absorption peaks at 696.7 and 755.7 cm^{-1} in the range of 690 – 860 cm^{-1} confirmed that the product contains a monosubstituted benzene ring. In addition, the absence of absorption peak for stretching vibration of conjugated C = C bond with aromatic ring in range 1680 – 1620 cm^{-1} confirm the polymerization of styrene to polystyrene**.**

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2. Transmission Electron Microscopy study of PSNPs

The particle size and shape were measured directly from transmission electron micrograph. The spherically shaped NPs have average size of 75 nm. In order to study the effect of preservation and sonication on particle size, TEM micrographs were recorded at different time intervals after sonication. It was observed that sonication only dispersed the NPs and it had no effect to the particle size.

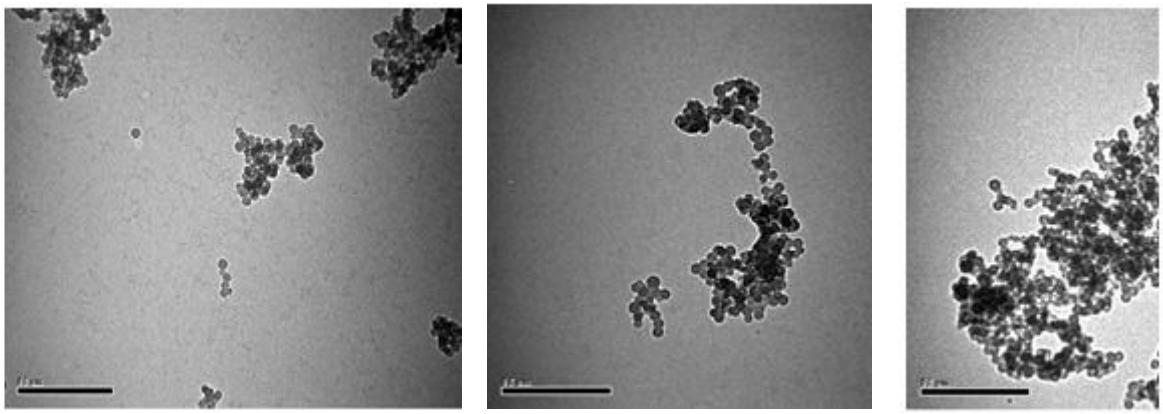

Figure 2: TEM images of PSNPs showing their size & shape.

3. Study of PSNPs Immobilized Quartz Crystal by Scanning Electron Microscopy

The surface morphology of modified AT – cut quartz crystal with PSNPs was studied with Hitachi S-5500 in-lens Field Emission SEM. The specimen area was about 60 nm wide with up to 0.4 nm resolution. The image clearly shown the homogenously distributed NPs on crystal surface. This NPs decorated surface has larger possibility to hold larger number of Ab as compared to bare crystal surface (Figure – 3). The SEM image supports the research laid on assumption that NP beared surface can attach large number of Antibody.

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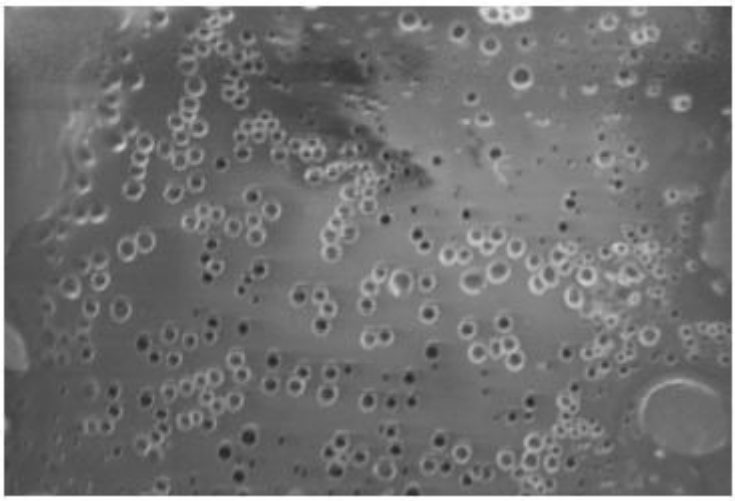

Figure 3: SEM image of polystyrene nanoparticles immobilization on gold surface

4. Explanation of Biosensor Development Process

The first step in development of a specific biosensor for *IBDV* was the crystal surface modification with PSNPs to provide larger surface area for the immobilization of antibodies specific to *IBDV*. The average fundamental vibrating frequency for untreated crystal surface about 4996575.28 Hz. The average frequency observed for base line development with 100 µL methanol was 4996040.55 Hz. A 100 µL methanol supension containing the PSNPs was the added to crystal. As some mass deposited on the quartz surface, the fundamental frequency decreased and acquire a minimum frequency of 4995957.01 Hz after a total decrease of 17.84 Hz in ~1 ½ h (figure-4). After that, crystal was placed for incubation.

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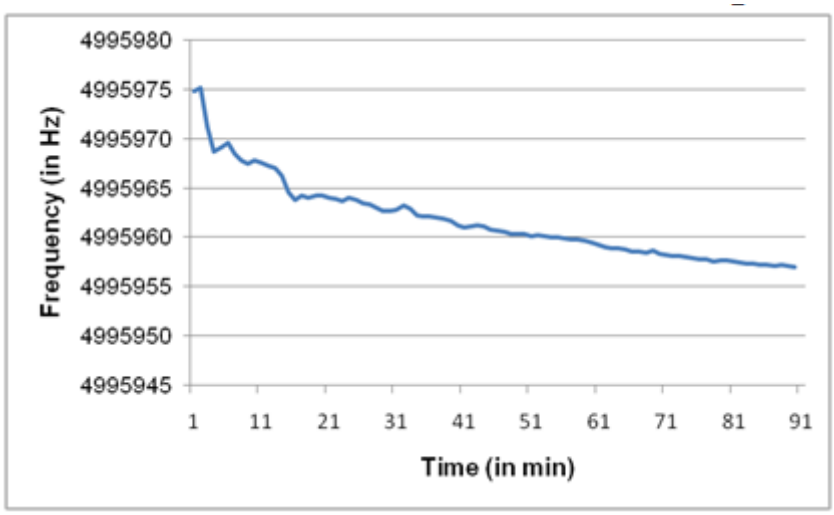

Figure 4: Immobilization of PSNPs on quartz crystal. Frequency shift accompanying the PSNPs immobilization in methanol on bare crystal monitored by QCM

This modified quartz crystal with PSNPs was then used for immobilization of *IBDV* antibody. The average vibrating frequency recorded for this modified surface specific to *IBDV* was 4995636.871Hz. After blocking the free surface with 1% BSA solution crystal was exposed to non-specific RK_2 antigen. This antigen is specific to Cd–*Rhizobacteria*. The observed frequency confirmed no interaction between modified surface specific to *IBDV* and RK_2–Antigen which is specific to Cd – *Rhizobacteria*. Now, this crystal was washed with PBS buffer in order to taken away RK_2 antigen from crystal. After washing with PBS buffer solution to remove unbound material, this modified quartz crystal was exposed to *IBDV* Antigen. The real time monitoring of vibrating frequency of modified quartz crystal with NPS during immobilisation of *IBDV* antibodies, exposer of crystal to non – specific RK_2 antigen and to a specific *IBDV* Antigen shown in figure – 5.

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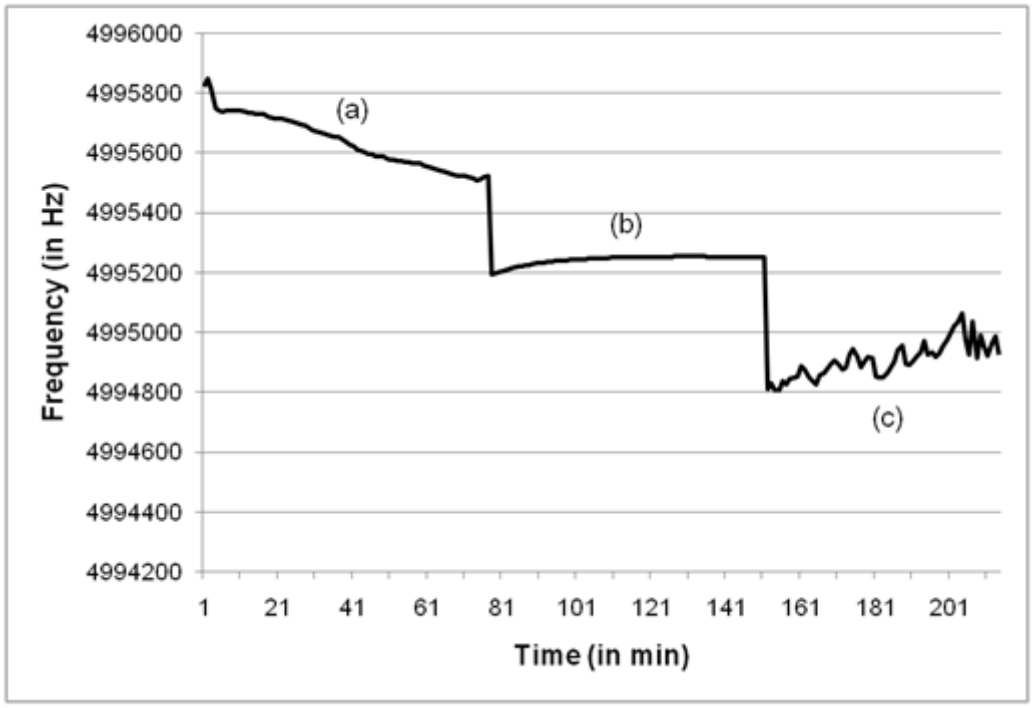

Figure 5: Monitoring of vibrational frequency of quartz crystal modified with NPs during biosensor study for various steps**:** (a) for immobilization of *IBDV* antibody. (b) the specificity checking of biosensor with non-specific RK_2 – Antigen (c) interaction with specific *IBDV* Antigen.

This study was based on the fact that the vibration frequency of a quartz crystal will decrease if some mass is deposited on the surface of the crystal. The observations were consistent with those expected before the study, except for the step of interaction between the antibody immobilized quartz surface and the specific IBDV antigen. When the modified quartz crystal exposed to specific IBDV antigen, the vibrational frequency started to increase and speed up after ~15 min of exposer to antigen. This clearly infer that the interaction of Antigen with immobilised antibody is stronger than the forces holding the antibodies on PSNPs modified surface.

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Effect of Different Dilution on Biosensor Development:

Effect of dilution on surface modification and consequent steps was also studied by developing the surface with 100 µl PSNPs solution from 20, 50 and 100 ml methanol PSNPs suspensions (Figure 6). It was observed that the pattern of the attachment is similar in each case except the amount of PSNPs attached to the surface. It is clear from the figure that as the dilution increases the attachment becomes fast and systematic as there is limited interaction between the particles.

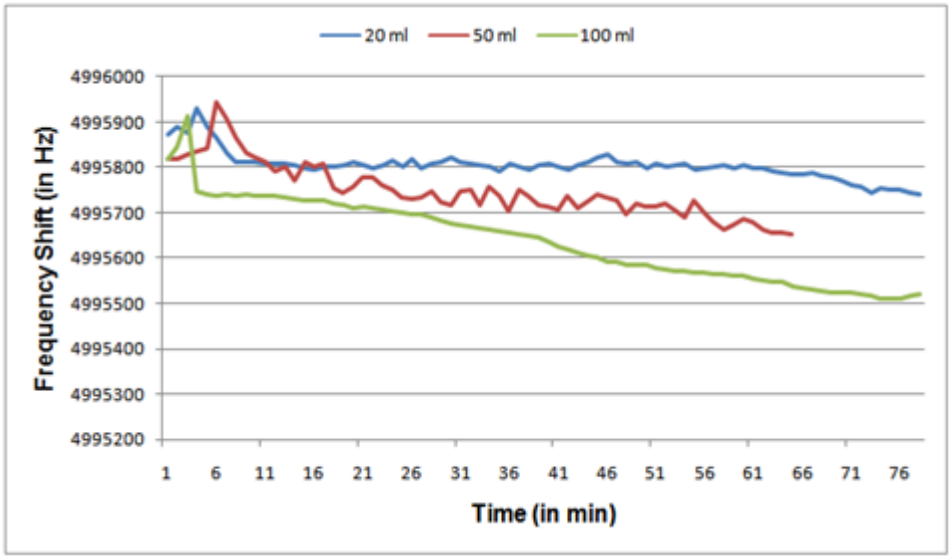

Figure 6: Development of PSNPs surface using different concentrations in methanol

Table 1 is the summary of the frequency of the quartz crystal after each step of modification. The data from table 1 confirmed that a large quantity of PSNPs attached to the crystal surface in the case of 20 ml methanol suspension and the least in the case of 100 ml methanol suspension. It means the amount of PSNPs attached directly proportional to concentration.

Process	20 ml	50 ml	100 ml
Bare crystal	4996577.53	4996581.749	4996593.415
Methanol Base line	4996047.833	4996052.325	4996031.195
PSNPs immobilization	4992595.137	4994743.485	4995499.01
Antibody immobilization	4992227.594	4994262.605	4995204.54
After detachment	4994216.621	4995080.376	4996000.974

Table 1: Average frequency of crystal associated with different steps during biosensor development. This average frequency was calculated after the incubation followed by washing with solvent/buffer and air drying.

From the first step data, it was expected that the surface contains the larger amount of PSNPs will provide the maximum surface for immobilization during the exposure of the crystal to *IBDV* immobilization. But the observation was not as expected. Maximum antibodies were immobilized in the case of 50 ml methanol suspension. It means that this provide a smooth & larger surface area to antibodies. The minimum amount of antibody was immobilized in the case (iii) as was expected. The reason for the attachment of lesser amount of antibody in case (i) than case (ii) may be hazy deposition of PSNPs.

In the case of 100 ml methanol almost all the PSNPs immobilized on crystal get detached from the crystal surface during the antibody-antigen interaction leaving behind almost bare crystal. The system acquired a frequency of 4996000.974 Hz as compared to fundamental frequency of 4996593.415 Hz and quite below the base frequency of 4996031.195 Hz. Similarly, in the case of 50 ml methanol, a greater amount of material is removed from surface as compared to first one. The detachment of antibodies with polystyrene nanoparticles may due to the greater force exhort during the course of

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complex forming in between antibody and antigen. As there is no force that can hold the nanoparticles on surface due to this the antibodies can take them away to solution from crystal surface. Consequently, it can be concluded that antibody attachment to PSNPs modified surface more firmly than the attachment of PSNPs to the gold surface.

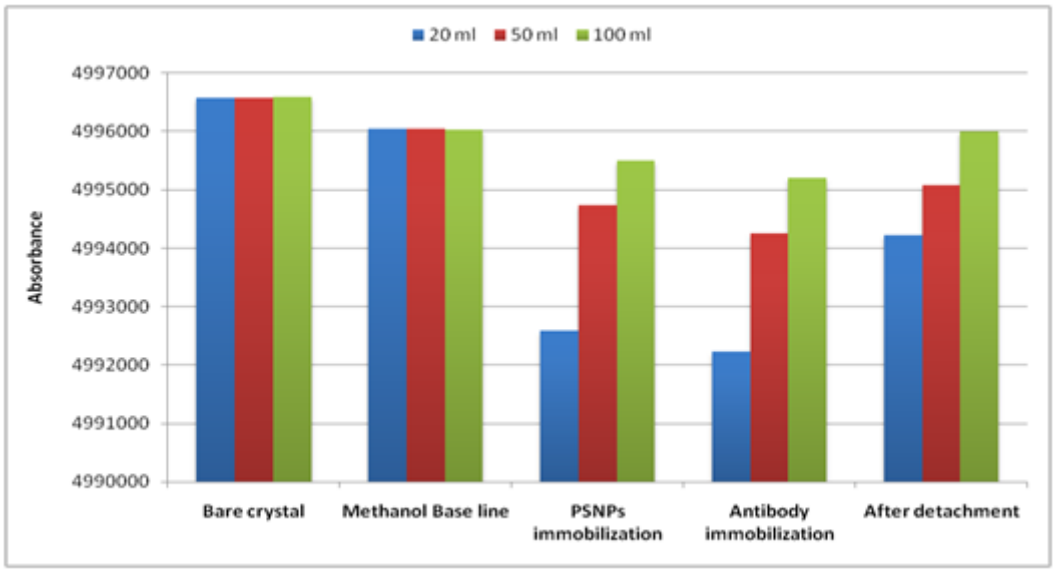

Figure 7: Graphical representation for the average vibrational frequencies **(**Hz**)** associated with different steps monitored by QCM**.**

V. CONCLUSION

In the field of nanotechnology, polymeric nanoparticles are playing an important role and likely to replace metal NPs. Polystyrene is already being used in drug and pharmaceutical industries, sensor technology, etc. The present study involved the synthesis of spherical nano–sized polystyrene particles by Micro-emulsion polymerization technique. The synthesized nanoparticles were characterised by FTIR and TEM images. The monolayered distribution of these polystyrene nanoparticles on quartz crystal surface was identified by SEM scanning. This modified surface was then used for antigen recognition through Antibody – Antigen complex formation. Our future study focuses on the surface modification of PSNPs so that these can bind

themselves and the antibody firmly to the crystal surface.

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