Green Synthesis of Chitosan–Selenium Bionanocomposite with High Biocompatibility and Its Marked Impact on Las B and RhII Genes Expression in Pseudomonas aeruginosa

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Abstract

In this present study, quorum quenching activity (QQ) of chitosan–selenium bionanocomposite with high biocompatibility was investigated. QQ activity was studied in Pseudomonas aeruginosa by measuring the expression level of Las B and RhII genes. Impact of gene expression on the major virulence factors production was also evaluated. Relative gene expression of both the genes was significantly reduced in all the dosages of CS–SeNC treatment, with subsequent reduction in the production of all the tested virulence factors. Biocompatibility of the nanocomposite was checked in zebrafish model which reveals that the administrated nanocomposite was not shown any undesirable effect on apoptosis, oxidative stress gene expression and liver histology. The present study implies that CS–SeNC prepared by in situ green science principles is a promising antibacterial agent against multiple drug-resistant bacterial strains, which exhibit an antagonistic effect by inhibiting the molecular mechanism of pathogenesis with noteworthy biocompatibility.

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1 Introduction

Nanomaterials based on nanocomposites are being extensively utilised in the various disciplines of science, engineering and technology due to noteworthy physical, chemical and biocompatibility properties [1]. The incorporation of metal nanoparticles in a polymer matrix generates new nanocomposites materials. Combining the different properties of these components (polymer and nanoparticles) can render material with improved optical, electronic, mechanical and antimicrobial properties [2, 3]. In recent years, nanocomposite-based products in the industry, medicine, and day-to-day life activities for notable applications have been increased [4].

Eco-friendly methods for preparing multifunctional nanocomposites utilising acceptable low cost or green methodology are now gaining more attention [5]. Green synthesised nanocomposites have documented their potential role in biomedicine, particularly as an antimicrobial agent. Antibacterial activity of cationic polymer–silver nanocomposite against Gram-negative and Gram-positive bacterial strains [6], Zinc oxide doped polyketones hybrid nanocomposites [7], polyvinyl alcohol–graphene oxide–silver nanocomposite [8] raspberry-like PGMA@AgNPs composite [9], silicon nanocomposites [10] plasma polymer silver nanocomposite [11], NiO nanoparticles doped PVA-MF polymer nanocomposite based potential antimicrobial efficacy of polymeric nanocomposites have been recently reported [12].

In this study, chitosan is selected for nanocomposite preparation. Among the polymers, Chitosan is extensively utilized for nanocomposite preparation [13]. Because of specific characteristics of chitosan like biocompatibility, biodegradability, nontoxicity and adsorption properties, it can be used as a stabilising agent to prepare various bioactive nanomaterials with notable biological activities, and these chitosan-protected nanoparticles can be easily integrated into systems for diverse pharmaceutical, biomedical, and biosensor applications [14]. This study has selected selenium nanoparticles to prepare antibacterial active nanocomposite. Selenium is an important micronutrient that plays many functions in the biological
system and protects the cells from oxidative stresses [15]. Selenium NPs (SeNPs) attracted widespread attention because of their exceptional physicochemical properties, including biocompatibility, chemical stability, minimum toxicity associated with highly selective toxicity [16]. Recent studies reveal the notable biological activities of selenium nanoparticles like antimicrobial, anticancer activity, and antioxidant activity suggesting the possible use in biomedicine [17]. A combination of SeNPs with different pharmacologically active drugs for the potential pharmacological activity has been reported, which is used to minimise the risk of antibiotic resistance as the drug is required at a minimum concentration [18]. Recent reports reveal the potential antibacterial activity of selenium nanoparticles against human pathogenic bacteria. Different studies have proposed the antibiotic activity of selenium nanoparticles synthesised via various routes under various conditions against human pathogenic bacterial strains [19]. With this background, the present study is undertaken to evaluate molecular mechanism inhibition of chitosan–selenium nanocomposite (CS–SeNC) in Pseudomonas aeruginosa by measuring the expression rate of Las B and Rho genes. The biocompatibility of the synthesised nanocomposite was also investigated with the zebrafish model.

2 Materials and Methods

2.1 Reagents, Chemicals and Culture Medium

Analytical grade chitosan of medium molecular weight (65 kDa) with 8A0% deacylation and Crosslinker-Sodium Tripolyphosphate (STPP) purchased from Sigma-Aldrich (USA). Sodium selenite anhydrous-reagent grade highly water-soluble with 99.9% purity was obtained from Hi-Media, India. The medium used for yeast inoculum preparation (Sabouraud dextrose agar), antibacterial assays (Mueller Hinton (MH) Agar, Muller Hinton Broth), antibiotics discs were purchased from Hi-Media, India. All the reagents, including enzyme-substrate used for antioxidant enzymes assays purchased from Sigma-Aldrich (USA), were used as received without further purification. For biofilm inhibition studies with high purity analytical grade, crystal violet and ethanol were purchased from RAMKEM (India). CDNA synthesis was carried out with the synthesised pure RNA thus obtained (20 ng) using Prime Script 1st strand cDNA Synthesis Kit (TAKARA).

2.2 Synthesis of Selenium Nanoparticles

2.2.1 Construction of Yeast Consortium

Selenium nanoparticles were synthesised by yeast consortium composed of Saccharomyces boulardii, Saccharomyces cerevisiae, Torulopsis species. Yeast strains were isolated from ripen grapes samples adopting culture-dependent method. Grape homogenate derived from healthy grapes was serially diluted in sterile saline, followed by plating on sabouraud dextrose agar. Identification of yeast strain was done by suitable selected identification methods [20, 21]. For yeast consortium preparation, the pure culture of the respective yeast strain was inoculated into 100 mL of sabouraud dextrose broth (SDB), incubated under shaking condition at 30 °C for 24 h.

2.2.2 Yeast Consortium Mediated Selenium Nanoparticles Synthesis (SeNps)

A known volume (1 mL) of yeast inoculum from the yeast consortium was inoculated into 100 mL of SDB medium followed by aseptic addition of 0.1 molar final concentration of sodium selenite, incubated at 30 ± 1 °C. The colour change of the broth from pale yellow to reddish-orange indicates selenium nanoparticles synthesis. After the primary confirmation of SeNps synthesis, the reaction mixture was centrifuged at 10,000xg for 10 min. The collected pellet was lyophilised and used for further studies.

2.2.3 Characterisation

Various analytical techniques were used to characterize synthesised nanoparticles UV visible spectroscopy analysis was initially carried out to determine the surface plasmon absorption maxima (SPAM) using a double beam UV visible spectrophotometer (Shimadzu-1800 spectrophotometer) with 200–800 nm scanning range Fourier transform infrared spectroscopy (FTIR) analysis was done by recording spectra at 400–4000 cm⁻² (Bruker Optic GmbH Tensor 27). Particle Morphology and surface topography were confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM micrograph was taken using SUPRA 55-CARL ZEISS (Germany) at 19 kV acceleration voltage (magnification range 35–10,000, resolution 200 Å) equipped with energy dispersive atomic X ray spectroscopy (EDAS). For AFM analysis, the processed sample was analysed using Ntegra Prima at contact mode. The crystalline phase of the nanoparticles was demonstrated with X-ray diffraction (XRD) under 30 kV voltage 100 mA current with Cu K-alpha radiations with a scan speed of 10°/min (Rigaku Smart lab).

2.3 Preparation and Characterization of Chitosan–Selenium Nanocomposite (CS–SeNC)

In a typical procedure of CS–SeNC preparation, known volume of selenium nanoparticles suspension (1 mL) was suspended in 100 mL of deionised water–acetic acid mixture containing 0.1% of chitosan (Analytical grade chitosan,
Sigma-Aldrich USA, medium molecular weight – 65 kDa with 8A0% diacylation Brookfield viscosity >200,000 cps) followed by dropwise addition of 0.1 M sodium salt of tripolyphosphate. The reaction mixture was kept at ambient temperature (30 °C) for 3 h under stirring. After stirring, the reaction mixture was centrifuged at 10,000× g for 10 min; the collected pellet was washed thrice with deionised water. The washed pellet was lyophilised and used for further studies. Characterization of the prepared nanocomposite was done by UV–visible spectroscopy, FTIR, XRD, SEM, AFM and particle size analysis as described above.

2.4 Antibacterial Activity

2.4.1 Bacterial Strain

_Pseudomonas aeruginosa_ used in this study was obtained from non-HIV UTI patients adopting standard microbiological methods. The strain was identified based on distinct morphological, cultural, and biochemical characteristics. A non-culture dependent process such as the molecular characterisation technique using 16 S rRNA was also done to confirm the isolated bacterial strain. Genomic DNA of the bacterial strain was isolated using a standard DNA isolation procedure; the isolated pure DNA was amplified with 16 S rRNA primers. Nucleotide sequences of the 16 S rRNA have been evaluated using NCBI’s blast analysis.

Antibiogram of the tested bacterial strain was determined by disc diffusion assay as described elsewhere. Respective tested antibiotic disc was gently placed on Muller Hinton (MH) Agar Media (Hi-media, India) that was seeded with _P.aeruginosa_ inoculum (prepared in Muller Hinton Broth). The zone of inhibition was measured after the incubation period. The measured zone of inhibition was compared with the standard antibiogram which reveals the antibiotic resistance pattern.

2.4.2 Antibacterial Susceptibility Testing

Agar cup diffusion assay was initially carried out to determine anti-bacterial activity of nanocomposite. Sterile MH agar plates were swapped with the mid log phase culture of bacterial strain followed by the addition of 25, 50, 75 and 100µg of nanocomposite into the agar cups (8 mm) that were made on the MH agar plates using sterile gel puncture. Three replicates and control were maintained. The zone of inhibition was measured with the zone reader after the incubation period.

Clinical and laboratory standard Institute (CLS) protocol was adopted to determine the MIC of the Nanocomposite against tested bacterial strain using double dilution 96 well microtitre plate assay [22]. The known volume of bacterial culture (0.1 mL) was inoculated into each well, followed by the addition of aliquots under aseptic conditions, incubated at 37 °C for 24h. A lower concentration of the nanocomposite that inhibited bacterial growth was considered as MIC.

2.5 Evaluation of Nanocomposite Induced Virulence Genes Silencing Studies

Nanocomposite induced effect on Las B and RhII was investigated by determination of relative gene expression rate (%). A quantitative real-time reverse transcriptase-polymerase chain reaction was carried out to record the relative gene expression rate.

2.5.1 Primers Selection

Nucleotide sequence alignments of _Pseudomonas_ specific region of the LasB gene encoding elastase were obtained through databases. Based on these alignments, LasB primers (Forward-5′GGAATGAACGAGCAGTTCTC3′) and (Reverse-5′GGTCCAGTAGAGCGGTG3′) genes were designed. RhII primers Forward-5′-GTAAGCGGT TTGCGGATG-3′ and reverse-5′CGGCATCAGGTCTTC ATCG-3′ were also designed based on nucleotide sequence alignments.

2.5.2 qRT-PCR

For qRT-PCR study, the bacterial strain was cultured in MH broth supplemented with 25, 50 and 100µg of free selenium nanoparticles, chitosan and nanocomposite separately. The respective treatment group was incubated under shaking conditions at 37 °C for 24 h. After the incubation period, the culture broth was centrifuged, and the collected cell pellets was used for total RNA extraction. Total RNA of the respective treatment group including control group was isolated by RNA isolation kit (Takara). Analysis of gene expression was done by the comparative threshold ΔΔCt method. According to the manufacturer’s instructions, real time PCR was done (initial denaturation at 95 °C for 10 min, continued by 45 cycles ,annealing at 60 °C for 15 s and extension at 72 °C for 30 s). The melting curve was analysed from 60 to 95 °C for 20 s.

2.5.3 Evaluation of Virulence Factors Production Potential

The influence of nanocomposite on major virulence factors like biofilm formation, pyocyanin production, swarming motility, and elastase production was also investigated. The inoculum required for these bioassays was prepared in sterile MH broth. The known volume of MH broth (100 mL) was
inoculated with a pure culture of \textit{P. aeruginosa}; the inoculated broth was incubated under shaking conditions at 35 °C for 24 h.

\subsection*{2.5.3.1 Biofilm Assay}

Microtitre plate crystal violet assay was adopted to determine the anti biofilm effect. Known volume of bacterial inoculum (0.1 mL) was transferred to the 96 well plate under aseptic conditions. Aliquots of nanocomposite, free selenium nanoparticles and chitosan was added separately. Inoculated microtitre plate was incubated at 35 °C for 72 h under static conditions. After the incubation period, the content of the microtitre plate was removed completely, washed with phosphate-buffered saline (PBS). Washed microtitre plate was stained with 100 μL of crystal violet (final concentration of 0.1%) at ambient conditions for 30 min followed by adding ethanol. Ethanol solubilised biofilm was measured spectrophotometrically at 570 nm. The rate of biofilm formation was determined by measuring the differences in the optical density of the control and the respective treatment groups. Structural changes of the biofilm collected and processed with the standard condition were examined by scanning electron microscopy (SEM). Biofilm derived from the respective treatment group was analysed by Carl–Zeiss Supra 55.

\subsection*{2.5.3.2 Pyocyanin Production}

CS–SeNC’s impact on pyocyanin production was studied by the modified method of Essar et al. [23]. Pyocyanin was extracted from the culture broth of respective treatment groups with chloroform. The absorbance of the extracted layer revealed the amount of pyocyanin.

\subsection*{2.5.3.3 Swarming Motility}

Bacterial inoculum was spot inoculated on MH medium with 0.3% agar supplemented separately with SeNps, chitosan and SeNC. Inoculated plates were incubated at 35 °C for 24 h. After the incubation period, the swarming motility was determined by measuring the turbid zone as a swarm around the spot inoculation. A reduction percentage represented results.

\subsection*{2.5.3.4 Elastase Activity}

The effect of the CS–SeNC on the elastase activity also indicates the noteworthy impact of Las B gene expression. Culture supernatant derived from the protease production medium was used as an enzyme source. A known volume of culture supernatant (1 mL) was mixed with substrate elastin-Congo red suspended in 100 mM Tris-HCl, 1 mM CaCl\textsubscript{2} reaction buffer, incubated for 3 h at 37 °C with shaking at 180×g. Enzyme activity was determined from the absorbance of the reaction mixture at 495 nm.

\subsection*{2.6 Biocompatibility Assessment Using the Zebrafish Model}

\subsection*{2.6.1 Nanocomposite Treatment}

Heathy, infection or malformation free adult zebrafishes were chosen for the study. Before the initialisation of the experiment, fishes were acclimatised to lab conditions according to OECD guidelines (OECD, 203). Acclimated fishes were exposed to 3 µg, 6 and 9 µg of nanocomposite separately. Control group was maintained without nanocomposite treatment. At the ending of the experiment, fishes were euthanised by decapitation with proper anaesthesia. Liver tissue was used as the target for biocompatibility assessment study. Biocompatibility was assessed by measuring changes in liver histopathology, relative expression rate of oxidative stress and apoptosis genes of nanocomposite treatment groups.

\subsection*{2.6.2 Liver Histopathology}

Adopting standard methods, the liver tissue was aseptically dissected out from the respective treatment group and used for further studies. Dissected tissue was fixed in a suitable fixative. Histopathological analysis was carried out by the method of Lendrum et al. [24]. In brief, the tissues were detached from fixative, washed in running tap water and processed for dehydration in an augmenting percentage of ethyl alcohol followed by methyl benzoate washing. Washed tissues were embedded in paraffin wax. Paraffin blocks were cut with a thickness of 5µ and stained with hematoxylin and eosin.

\subsection*{2.7 qRT-PCR Analysis of Oxidative Stress Enzymes Genes and Apoptosis Genes}

Total RNA was isolated from the liver tissue of respective treatment group using RNA isolation kit (Thermofisher scientific). Pure RNA thus obtained was converted into cDNA using cDNA synthesis kit (TAKARA). qRT-PCR was done to determine the gene expression of oxidative stress enzymes—catalase (CAT), superoxide dismutase (SOD 1) and apoptosis genes using 2−ΔΔCt method. Beta-actin was used as an internal control. qRT-PCR was carried out with reaction mixture consist of respective gene forward, reverse primer and synthesized cDNA. According to the manufactures instruction, amplification was carried out at desired conditions [denaturation (95 °C for 15 s)] annealing ( 60 °C for 15 s followed by extension at 72 °C for the 30 s ). The melting curve was analysed from 60 to 95 °C for the 20 s. The expression of the
respective target gene has its own \((ct)\) amplification value at its threshold level.

3 Results and Discussion

3.1 Synthesis and Characterisation of Selenium Nanoparticles (SeNps)

Colour change of the culture broth from pale yellow to redish-orange is the primary confirmation of selenium nanoparticles synthesis which was observed at 72 h. UV–Vis spectroscopy analysis of the reaction mixture containing synthesized selenium nanoparticles shows broad surface plasmon absorption maxima at 370 and 650 nm (Fig. 1). Further characterization was done by FTIR analysis. FTIR is an important analytical technique used to determine the functional groups. FTIR spectra that depicted in Fig. 2 shows absorption peaks at 3905.00, 3854.51, 3840.47, 3822.08, 3802.56, 3446.90, 2362.00, 2344.83, 2078.36, 1643.75, 1052.35 and 681.16 cm\(^{-1}\). The wideband recorded at 3822 and 3802 cm\(^{-1}\) could be assigned to stretching vibrations of water OH groups. The sharp band observed at 3446 cm\(^{-1}\) could be assigned to alcohol OH stretch. The bands noted at 2362 cm\(^{-1}\) assigned to C≡C stretch, and the bands at 1643 cm\(^{-1}\) can be acknowledged as amide I and amide II due to the carbonyl and NH stretching vibrations in the proteins’ amide linkages. The band at 1052 cm\(^{-1}\) shows the C–OH stretch. These findings confirm the synthesis and stabilisation of nanoparticles by the proteins present in the yeast.

X-ray diffraction (XRD) is one of the most important tools to analyse crystalline phase of the materials. XRD pattern of the synthesized selenium nanoparticles that depicts in the Fig. 3 shows well resolved peaks indicating crystalline nature. The position of diffraction peaks at 20 values of 23.48°, 29.63°, 45.66°, 56.63° and 76.84° corresponds to miller indices (100), (101), (111), (112) and (212) of selenium nanoparticles respectively (Fig. 3a). All the diffraction peaks readily match to hexagonal phase with lattice constants \(a = 4.357\) Å and \(c = 4.945\) Å and have good agreement with the standard JCPDS file no. 06-0362 (Fig. 3b).

SEM micrograph shows aggregated, dense rough spherical particles with a size range of 39-43 nm (Fig. 4a). Characteristic morphology of the nanoparticles was due to the presence of capping agents—bioreducers produced by yeast strains that capped heavily, which may increase the roughness and particles morphology. SEM equipped with EDAS analysis used to determine elemental composition in the

**Fig. 1** Synthesized selenium nanoparticles (SeNps), UV–Visible absorption spectra of SeNps
sample which confirms the presence of selenium. The samples exhibited strong absorption peaks at corresponding to selenium (Fig. 4b). Noted additional peaks of carbon and oxygen can be attributed to the protein molecules, which are probably involved in the capping of the produced nanoparticles, as already stated above. Atomic force microscopy (AFM) analysis reveals the presence of well-agglomerated particles with nano dimensions (Fig. 4c, d).

### 3.2 Preparation of CS–SeNC

Various analytical techniques are used to characterize chitosan–selenium nanocomposite. UV visible absorption spectra that depicts in Fig. 5a indicates surface plasmon absorption maxima at 339 and 650 nm which corresponds to chitosan and selenium nanoparticles respectively. Characteristic interaction of chitosan with selenium nanoparticles which was then transformed into chitosan–selenium nanocomposite can easily inferred from this data. FTIR analysis was used to confirm the structural modification. FTIR spectra that presented in Fig. 5b reveal chitosan’s interaction with the selenium nanoparticles. These peaks showed notable absorption bands corresponding to bio reducers or capping agents (protein of yeast consortium) of SeNps that were recorded at 3500 cm\(^{-1}\), 1649 and 1438 cm\(^{-1}\). The vibration of OH groups of chitosan appear around 3500 cm\(^{-1}\), at 1649 cm\(^{-1}\), where usually the vibration of the amide I group of chitosan occurs.

The XRD analysis was carried out to investigate the amorphous or crystalline nature of Chitosan–selenium nanocomposite (CS–SeNC) and shown in Fig. 6. Figure 6c revealed the broad peak at 19.3\(^{\circ}\) corresponding to miller indices of (110) attributed to the presence of chitosan [JCPDS no. 39-1894]. The slight shift in the peak position was due to the different sources of chitosan [25]. The broad diffraction band was observed because of amorphous nature of biopolymer [26, 27]. From the Fig. 6c, it was also noticed that the diffraction peak at 29.35\(^{\circ}\) (101) corresponds to selenium nanoparticles and presence of diffraction peaks of both chitosan and selenium nanoparticles confirms the successful formation of composite. Further, well resolved diffraction peaks with corresponding miller indices (100), (101), (111), (112) and (212) revealed that of the selenium nanoparticles (Fig. 6b). The diffraction peaks were in correlation with hexagonal phase of selenium (Se) with lattice constants a = 4.357 Å and c = 4.945 Å and are in good agreement with the standard JCPDS data showed in Fig. 6a (JCPDS No. 06-0362). Furthermore, the Chitosan–selenium nanocomposite XRD exhibited amorphous peaks which is the characteristic of composite material and presence of more bioactive compounds as the capping agents may be responsible for the existence of noise [28].
no impurity peaks were observed, suggesting that high purity materials was formed.

FESEM analysis conducted to study the morphological characteristics reveals distinct changes in size and shape. SEM micrograph shows well distributed, less aggregated, spherical particles with a size range of 142–160 nm (Fig. 7a), indicating an effective coating or noteworthy interaction of chitosan with the selenium nanoparticles, which transformed into SeNC, brought about well-distributed or well-aggregated core particles with the particle size bigger than free selenium nanoparticles. EDAS analysis was used to determine the elemental composition which demonstrates the presence of selenium with carbon, oxygen and sulphur (Fig. 7b). Sulphur identified might have been derived from Sulphur-containing amino acids, methionine, observed in the EDAS spectrum. Surface topology of the nanocomposite was analysed by atomic force microscopy (AFM). AFM was used as the prime technique to screen the dissolution and aggregation pattern. AFM micrograph indicates well-distributed particles with thickness higher than free selenium nanoparticles (Fig. 7c, d). Chitosan interaction with selenium nanoparticles followed by transformation into functionalised CS–SeNC has brought about notable changes in the surface topology. This can also be seen from the well-dispersed particles without pores or other defects. Nano dimensional spherical, well-dispersed spots confirm the homogenous status of the synthesised SeNC.

### 3.3 Antibacterial Activity

Anti-bacterial activity of the synthesized nanocomposite was tested against *P. aeruginosa*. All the characterization methods revealed the bacterial strain belong to *Pseudomonas aeruginosa*. Antibiogram results indicate that the tested bacterial strain exhibited notable resistance to most antibiotics tested.

Antibacterial activity was studied with free selenium nanoparticles (F–SeNps), chitosan (CS) and the Nanocomposite (CS–SeNC) by well diffusion assay. The turbidometric micro broth dilution method was carried out to determine minimum inhibition concentration (MIC). Results indicate that CS–SeNC inhibited *P. aeruginosa* significantly (*P > 0.05%*). In the well diffusion assay, all the tested dosages of CS–SeNC brought about marked antibacterial activity by recording a higher zone of inhibition than F–SeNps and chitosan. Results further reveal that the tested bacterial strain was not inhibited at the lower dosages of F–SeNps and chitosan. Hence, no zone of inhibition was recorded in this treatment group (Table 1).

The merit of using SeNps as an antibacterial agent is that they have a high surface area to volume ratio, are target-specific, highly biocompatible, and highly reactive. The interaction of nanoparticles with cellular components like the ribosome, DNA, and RNA by permeation through the cell membrane via endocytosis, then the cytosol, damages the genetic components of the bacterial cells, which causes alteration in the metabolic processes to lead to bactericidal action [29, 30].

It can be seen that the unique surface membrane chemistry of chitosan brought about sustained or controlled release of SeNps. SeNps was reported to show marked antimicrobial activity against the diverse pathogenic microorganism [31]. MIC was determined by broth micro-dilution assay, which indicates that even the least tested dosage of CS–SeNC inhibited the growth of the tested bacterial strain. Data obtained from the analysis showed that within various concentrations tested against *P. aeruginosa*, 25 μg of CS–SeNC inhibited the bacterial growth effectively, which shows that the MIC value in this study was estimated to be 25 μg (Table 2). In F–SeNps and chitosan, the MIC value was 75 μg and 100 μg, respectively.
Fig. 4 Characterization of F-SeNps a SEM micrograph, b EDAS spectra, and c AFM micrograph—2D image & 3D image
Fig. 5  a UV–Visible absorption spectra of CS–SeNC, b FTIR spextra of CS–SeNC
3.4 Virulence Gene Silencing Studies

Recent studies reveal that quorum sensing or bacterial cell communication can be targeted to prevent pathogenesis via quorum quenching (QQ) principles [27]. In *P. aeruginosa*, various genes are responsible for bacterial signalling, triggering different virulence factors production. Las B and Rhl are the two major quorum-sensing systems in *P. aeruginosa* that produce diverse virulence factors mediating pathogenesis. Nanotechnology principles are being extensively utilised to inhibit the Las B and Rhl expression systems, interrupting bacterial pathogenesis [2]. In this present study, CS–SeNC synthesised via green science principles was evaluated against Las B and Rhl gene expression of *P. aeruginosa*, followed by studying changes in the virulence factors production.

CS–SeNC induced effect on the expression of Las B and Rhl was studied by quantitative real-time PCR. cDNA bands that recorded in the respective treatment clearly reveals the gene expression (Fig. 8a, b). The relative gene expression profile of both genes (Las B and Rhl) was analysed quantitatively to determine CS–SeNC induced effect. Quantification studies suggest that all the dosages of CS–SeNC significantly reduced the relative gene expression of Las B and Rhl (P > 0.05%). Interestingly, F–SeNps reduced the expression level of both Las B and Rhl only at high concentrations (Fig. 8c, d). However, the expression level was found to be lower than CS–SeNC. Notable changes were not observed in all the dosages of chitosan treatment.

3.5 Virulence Factors Status

The effect of CS–SeNC on the QS mediated virulence factors production potential of *P. aeruginosa* was confirmed by studying changes or modulation in biofilm formation, pyocyanin production, swarming motility pattern and elastase activity. Results indicate that the synthesised nanocomposite brought about a drastic reduction of all the tested virulence factor. Among the treatment groups, all the virulence factors were inhibited by the CS–SeNC significantly (P > 0.05%). No marked effect on the virulence factors status was observed in chitosan treatment. In contrast, F–SeNps inhibited the virulence factor’s production in a concentration-dependent manner. Biofilm formation is a vital QS mediated virulence factor. The development or progression depends on the signalling molecules expressed via Las and Rhl expression systems. Inhibition or alteration of these expression systems prevents disturbance of signalling molecules production, affecting biofilm development. In this study, expression of Las B and Rhl decreased drastically in CS–SeNC treatment compared to F–SeNps and Chitosan treatment. Notable reduction in the respective gene expression pattern studied in this present investigation indicates the antibiofilm effect. Scanning electron microscopy (SEM) studies were further studied to demonstrate the CS–SeNC mediated effect on the structural framework of biofilm derived from *P. aeruginosa*. SEM micrograph depicted in Fig. 9 shows the characteristic changes in the structural framework of biofilm. The Control group shows compact, rough dense cell aggregates bounded by a thick matrix into a unique structural framework. When the biofilm was exposed to CS–SeNC treatment, characteristic changes were observed. A complete alteration in the structural framework of biofilm was easily inferred from the SEM micrograph that shows the weakened biofilm framework with segregated cell masses. Fragmentation of the dense cell masses followed by changes in the biofilm matrix was observed in the CS–SeNC treatment groups. Degenerated biofilm framework with detached cells clearly shows the antibiofilm effect of CS–SeNC. However, no marked impact on the biofilm framework was observed in chitosan treatment groups. F–SeNps treatment groups also show antibiofilm effect by recording structural changes weakening the biofilm. However, the level of biofilm damage was found to be lower than that of CS–SeNC. Considerable

![Fig. 6 XRD pattern](image-url)
reduction in biofilm development or formation was noted. CS–SeNC treatment reduced biofilm formation to 10–50% at 50–100 μg dosage, whereas 60–80% and 80–95% of biofilm formation were observed in F–SeNps and chitosan treatment, respectively.

The effect of CS–SeNC on the pyocyanin pigment production reduction was studied by spectrophotometric measurement of extracted pigment from the culture-free supernatant. The data collected was used to evaluate the reduction in pigment production (Fig. 10a). Results show that CS–SeNC with all the dosages reduced pyocyanin production significantly (P > 0.05%). No marked effect on the pyocyanin production was recorded in chitosan and F–SeNps treatment.

Studies on Swarming motility—an important QS mediated virulence factor of *P. aeruginosa*, were carried out by measuring changes in swarm diameter (Fig. 10b). CS–SeNC with all the dosages recorded a significant reduction in swarm diameter. As in the control group, no changes in swarm diameter were observed in chitosan (all the tested dosages). However, F–SeNps with high dosages show a noted effect on swarming. As in other virulence factors, elastase production was also affected by CS–SeNC treatment. A high rate of elastase production

Table 1 Zone of inhibition (mm) against *P. aeruginosa*

| Concentration (µg) | F–SeNp | CS | CS–SeNC |
|-------------------|--------|----|---------|
| 25                | 0.0    | 0.0| 17.0a   |
| 50                | 8.0    | 0.0| 22.0a   |
| 75                | 10.0   | 8.0| 27.0a   |
| 100               | 12.0   | 8.0| 34.0a   |

*aColumn carrying the alphabet is statistically significant (P< 0.5%)

Table 2 Minimum inhibition concentration (µg) of *P. aeruginosa*

| Treatment    | MIC (µg) |
|--------------|----------|
| F–SeNp       | 75.0a    |
| CS           | 100.0a   |
| CS–SeNC      | 25.0a    |

*aColumn carrying the alphabet is statistically significant (P<0.5%)*
3.6 Biocompatibility Studies

Among the various model systems, Zebrafish (Danio rerio) has been used as a popular vertebrate model to determine toxicity profiles due to genomic, physiological homology with humans. Notable characteristics of embryos or developmental stages like transparency rapid developmental period enable zebrafish to be a promising candidate for determining the toxicity profile of nanomaterials [32]. Biocompatibility of synthesised nanocomposite was studied using an adult zebrafish model and liver tissue was used for determining gene expression profiles of oxidative stress enzymes genes CAT, SOD and apoptosis genes p53, Bax genes. Beta-actin was used as a housekeeping gene. The CS–SeNC induced effect on liver histopathological parameters were also investigated to confirm noteworthy biocompatibility.

The quantitative PCR was used to analyse the expression of oxidative stress genes (SOD, CAT) and apoptosis genes (p53 and BAX). These gene expression analyses indicate that the nanocomposite was not inducing any undesirable effect on the oxidative stress and apoptosis genes (Fig. 11). The relative expression of these enzymes’ genes shows no significant difference between the control and other treated groups. These results suggest that the expression of oxidative stress and apoptosis genes is similar to the control group revealing the non-toxic nature of the CS–SeNC.

Histopathological examination of Zebrafish liver tissue dissected from the CS–SeNC treatment group was also studied to determine the biocompatibility. Histopathological studies indicate no altered liver architecture when treated with all the tested dosages of CS–SeNC. Livers from the control and treated group exhibited normal histology, with hexagonal hepatocytes and nuclei localised in the centres of cells (Fig. 12). The absence of infiltration of inflammatory cells further confirms the biocompatibility of CS–SeNC. Though a lower dosage of nanocomposite did not show any toxicity and oxidative damage to the liver of zebrafish, the experiment needs to have proceeded for long-term exposure. However, these results reveal the high degree of safety or notable biocompatibility of CS–SeNC, which can be used for possible biomedical applications.

Chitosan–selenium nanocomposite proposed in this study shows specific unique characteristics. This study synthesised selenium nanoparticles using a simple, economical green route using yeast consortium and transformed them into highly stable chitosan–selenium bionanocomposite. Prepared bionanocomposite reveals marked antibacterial activity against human pathogenic strain Pseudomonas aeruginosa by inhibiting Las B and Rho I genes that mediate bacterial cell communication termed quorum sensing. Followed by gene expression impact, the nanocomposite drastically reduced virulence factors production. The biocompatibility of the nanocomposite using the zebrafish model also
indicates its uniqueness. The prepared nanocomposite was not inducing any effect on the expression of antioxidative enzymes and apoptotic genes. These findings demonstrate the possible utilisation of chitosan–selenium bionanocomposite as an effective antibacterial agent with high efficacy and biocompatibility.

The novelty of the chitosan–selenium bionanocomposite can be seen from the below-mentioned features. Inhibition of the molecular mechanism of pathogenesis is an essential criterion for drug development. The present study proposed the significant inhibition of the major quorum sensing genes Las B and RhII of Pseudomonas aeruginosa responsible for virulence factors. This study synthesised selenium nanoparticles using a simple, economical green route using yeast consortium and transformed them into highly stable chitosan–selenium bionanocomposite. Prepared bionanocomposite reveals marked antibacterial activity against human pathogenic strain Pseudomonas aeruginosa by inhibiting Las B and Rho I genes that mediate bacterial cell communication termed quorum sensing. Followed by gene expression...
impact, the nanocomposite drastically reduced virulence factors production. Biocompatibility of the nanocomposite using the zebrafish model also indicates its non-toxic and high biocompatible nature. The prepared nanocomposite was not inducing any effect on the expression of antioxidative enzymes and apoptotic genes. These findings demonstrate the possible utilisation of chitosan–selenium bionanocomposite as an effective antibacterial agent with high efficacy and biocompatibility.

4 Conclusions

Chitosan-based polymer-based metal nanocomposites have gained recent attention in biomedicine due to their high efficacy and biocompatibility rather than other diversified fields of science and technology. Chitosan–selenium nanocomposite (CS–SeNC) synthesised in this study adopting green route principles was evaluated for its antagonist action at the molecular level against the genes that trigger the pathogenesis of multiple drug-resistant human pathogenic bacteria *Pseudomonas aeruginosa*. The highly stable CS–SeNC thus synthesised down-regulated the expression of Las B and RhlII quorum sensing virulence genes, which reduced the production of virulence factors like biofilm formation, pyocyanin production, swarming motility and elastase activity. The absence of oxidative stress and apoptosis gene markers induction confirmed the biocompatibility of the zebrafish model. Alteration or tissue morphology changes in the liver also reveal noteworthy biocompatibility. Further studies using in vivo infection model will be helpful to utilise CS–SeNC as an effective antimicrobial agent against drug-resistant pathogenic microorganisms.
Fig. 11  Relative mRNA expression of oxidative stress and apoptotic genes in CS–SeNC administrated Zebrafish a CAT, b SOD, c p53, and d Bax
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Declarations

Conflict of interest  We declare that no conflict of interest.

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