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

Recently, there has been an augmenting demand for chitosan-metal nanocomposites in biomedicine as drug delivery and diagnostic agent due to their notable efficacy and desired biocompatibility. In this present study, chitosan-selenium nanocomposite (CS-SeNC) prepared by *in vitro*, economical green chemistry principle was evaluated for quorum quenching activity against quorum sensing virulence genes expression in the human pathogenic multidrug-resistant bacterial pathogen *Pseudomonas aeruginosa*. Biocompatibility of the synthesised CS-SeNC was evaluated on the zebrafish model by determination of expression pattern of oxidative stress enzymes genes – catalase (CAT), superoxide dismutase (SOD1), apoptosis genes p53 and Bax genes in liver tissue. Histopathological analysis of the liver was also conducted. The silencing effect of the synthesised nanocomposite on major virulence factors encoding genes Las B and RhlI was investigated by determining relative gene expression rate adopting quantitative real-time reverse transcriptase-polymerase chain reaction. This is followed by studies that measure relative gene expression of respective quorum sensing genes, the effect of CS-SeNC on the quorum sensing genes mediated virulence factors like biofilm formation, pyocyanin production, swarming motility and elastase activity were demonstrated. 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 of *P.aeruginosa*. The absence of marked expression of oxidative stress enzymes genes apoptosis genes in the CS-SeNC administrated zebrafish model reveals the best biocompatibility. Liver histology has not shown any marked effect, confirming the best biocompatibility. The present study implies that CS-SeNC prepared by *in situ* green science principles is the promising antibacterial agent against multiple drug-resistant bacterial strains, which exhibit an antagonistic effect by inhibiting the molecular mechanism of pathogenesis with noteworthy biocompatibility.

1. Introduction

There is an increasing demand for well-designed nanostructures developed from diverse nanomaterials with a potential application, mainly in the biomedicine field [1]. Various nanoformulations, including organic and inorganic nanocarriers, porphyrin-laden nanoparticles, photosensitisers based amphiphilic molecules like porphyrin, supramolecular polymers, and porphyrin-based block copolymers, have been developed to deliver porphyrins, which protect the pre-mature release of photosensitiser and provide tumour-selective and site-specific targeting [2]. DNA based and stimuli-responsive smart nanocarrier systems can efficiently deliver encapsulated cargos in response to various driving thresholds [3].

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 [4]. 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 [5, 6]. In recent years, nanocomposite-based products in the industry, medicine, and day-to-day life activities for notable applications have been increased [7].

Eco-friendly methods for preparing multifunctional nanocomposites utilising acceptable low cost or green methodology are now gaining more attention [8]. 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 [9], Zinc oxide doped polyketones hybrid nanocomposites [10], polyvinyl alcohol-graphene oxide-silver nanocomposite [11] raspberry-like PGMA@AgNPs composite [12], silicon nanocomposites [13] plasma polymer silver nanocomposite [14], NiO nanoparticles doped PVA-MF polymer nanocomposite based potential antimicrobial efficacy of polymeric nanocomposites have recently reported [15].

In this study, chitosan- selenium bionanocomposite was prepared using green science principles to study the molecular mechanism of inhibiting major virulence genes of the human pathogenic bacterial strain *Pseudomonas aeruginosa*. This study utilises chitosan for selenium based nanocomposite preparation. Chitosan is extensively utilised among the various polymers used for nanocomposite preparation [16]. 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 [17]. 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 [18]. Selenium NPs (SeNPs) attracted widespread attention because of their exceptional physicochemical properties, including biocompatibility, chemical stability, minimum toxicity associated with highly selective toxicity [19, 20]. Recent studies reveal the notable biological activities of selenium nanoparticles like antimicrobial, anticancer activity, and antioxidant activity suggests the possible use in biomedicine [21, 22]. 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 in minimum concentration [23]. 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 [24–27].

Antimicrobial activity of diverse selenium based nanostructures as selenium nanocomposites have been reported. Potential antibacterial activity of surface modified nano selenium with bovine serum albumin, ascorbic acid, glucose, chitosan reveals notable antimicrobial activity against *Staphylococcus aureus* (ATCC 6538), *Enterococcus faecalis* (ATCC 29212), *Bacillus subtilis* (ATCC 6633), and *Kocuria rhizophila* (ATCC 9341), as well as *Escherichia coli* (ATCC 8739), *Salmonella abony* (NCTC 6017), *Klebsiella pneumoniae* (NCIMB 9111) and *Pseudomonas aeruginosa* (ATCC 9027), and the standard yeast strain *Candida albicans* (ATCC 10231) [28]. Graphite selenium zinc oxide nanocomposite prepared by Sonde et al. [29] reveals marked antibacterial activity against water-borne bacterial strains associated with

photodegradation of methylene blue. Selenium doped ZnO nanostructure synthesised via green science method by Suresh Kumar and Nesaraj [30] shows antibacterial activity against human pathogenic bacterial strains. The antimicrobial activity of nanocomposite composed of vanadium pentoxide-selenium- multiwalled carbon nanotubes have recently been studied by Francklin et al. [31].

With this objective, the current study is undertaken to evaluate the molecular mechanism inhibition of virulence genes produced by *Pseudomonas aeruginosa*. *P. aeruginosa* major Gram-negative, aero bacilli are known to cause a wide range of severe infections with an elevated rate of mortality and morbidity [32]. *P. aeruginosa* infection is often refractory to antibiotic therapy due to multidrug resistance, making it a World Health Organization and U.S. Centers for Disease Control priority pathogen for therapeutic development [33]. *P. aeruginosa* produces various virulence factors in a coordinated system that enables host colonisation and adaptation [34–36]. These virulence factors comprise biofilm production, pyocyanin, elastase, and rhamnolipid and are under the control of a cell density-dependent signalling regulation known as quorum sensing (QS) [37–39]. Quorum sensing (QS) – is a bacterial cell-to-cell communication mechanism via a distinct communication network that deals with the binding of diffusible signals to their cognate receptor proteins, thereby regulating gene expression in response to bacterial cell density [40]. It is well documented that QS regulates sundry bacterial physiological processes, including bioluminescence, antibiotic biosynthesis, virulence determinants, swarming, *Agrobacterium* plasmid conjugal transfer and biofilm differentiation [41].

There are four different interconnected QS signalling networks in *P. aeruginosa*, viz. LasI/LasR, RhII/RhIR, the *Pseudomonas* quinolone signal (PQS)/MvfR and IQS (integrated QS). All these pathways are well regulated hierarchically. The two QS pathways, Las and Rhl, produce AHL signalling molecules such as 3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) and butyryl-L-homoserine lactone (C4-HSL) by the LasI and RhII synthase enzymes [42]. Quorum quenching (QQ) refers to the process of interrupting QS. It has been regarded as a promising innovative methodology to attenuate bacterial pathogens. Interfering with this QS system by applying QS inhibitors (QSI) is a novel therapeutic target that effectually reduces virulence in opportunistic pathogens [43]. There are various ways by which quorum quenching can inhibit quorum sensing, either by degradation of quorum sensing signalling molecules or antibodies mediated blocking [44]. It is anticipated that the signalling molecules produced by pathogenic bacterial strains could be inhibited or inactivated, which prevents the manifestation of pathogenesis utilising green science quorum quenching principles. Recently, various nanoparticles (NPs) have become popular to treat bacterial infections as an alternative to antibiotics. Since NPs follow a different mechanism of action to target the bacteria and do not need to penetrate the bacterial cell, new pathways emerged [45]. The potential application of nanocomposites for the molecular mechanism inhibition of pathogenic strains has gained attention recently in biomedicine due to its high efficacy, selective target toxicity and biocompatibility. Anti-quorum sensing activity of chitosan- gold nanocomposite [28] Kaempferol loaded chitosan nanoparticles [46], chitosan-zinc oxide nanocomposite [47, 48] zinc oxide nanoparticles [49, 50] would suggest the possible utilisation of nanomaterials as effective inhibitors of quorum sensing.

This study synthesised biogenic selenium nanoparticles using a yeast consortium, adopting an eco-friendly in situ method. Synthesised nanoparticles were fabricated with chitosan as a chitosan- selenium nanocomposite (CS-SeNC) using an ionic gelation method. Suitable analytical methods like UV-visible spectroscopy, Fourier transform infrared spectroscopy (FTIR), electron microscopy, and x-ray diffraction (XRD) were used to determine the synthesised nanocomposite's structural and functional properties. The synthesised nanocomposite was checked for quorum quenching activity using quantitative real-time PCR to measure *Pseudomonas aeruginosa* Las B expression Rho I genes. Following this, the effect of nanocomposite on the major virulence factors production was also investigated.

Noteworthy biocompatibility of synthesised nanocomposite was studied using an adult zebrafish model. Those NPs should enter the body and contact tissues and cells directly for biomedical applications. Thus, it is necessary to explore their biocompatibility [51, 52]. Biocompatibility or biological response of composite material, including biocomposites, is complex, complicated and challenging [53]. The biocompatibility of the diverse nanocomposites using various models has been reported. Ti3C2Tx (MXene) nanocomposite using zebrafish embryo model [54] silver polyvinyl hydrogel nanocomposite using melanoma cell lines [55] polyacrylate/ZnO nanocomposite [56] using activated sludge, passion fruit like nanoarchitecture using zebrafish mode [57] chitosan nanocomposite on zebrafish model [58]. In this present investigation, the biocompatibility of CS-SeNC was checked using the zebrafish model by determining oxidative stress enzymes, apoptosis genes expression pattern, and liver histopathological analysis. The respective test group of animals was administered with lethal concentration 50 (LC₅₀) of nanocomposite followed by the determination of oxidative stress enzymes and apoptosis genes. qRT-PCR was carried out to study the expression level of oxidative stress enzymes like catalase (CAT), superoxide dismutase SOD 1 and apoptosis genes, p53 and Bax. Histopathology of the tested group was also studied to assess the nanocomposite induced effect.

2. Materials And Methods

2.1. Reagents, chemicals and culture medium

Analytical grade chitosan of medium molecular weight (65 kDa) with 8A0% deacetylation 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 (Muller Hinton (MH) Agar, Muller Hinton Broth), antibiotics discs were purchased from Hi-Media, India. All the reagents, including enzyme-substrate used for antioxidative 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 (20ng) 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 using a yeast consortium constructed with *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Torulopsis* species. Respective yeast strain was isolated from grapes adopting a culture-dependent method. Grape homogenate derived from healthy grapes was serially diluted in sterile saline followed by plating Sabouraud Dextrose Agar with chloramphenicol to prevent bacterial contamination. Inoculated plates were incubated at 28°C for 48–72 h. After the incubation period, the yeast colonies that formed were purified. The pure culture of the respective isolate was identified by suitable selected identification methods [59, 60]. For yeast consortium preparation, pure culture of the respective yeast strains was inoculated into 100 mL of Sabouraud Dextrose Broth separately incubated at 30°C for 24 h

2.2.2. Yeast consortium mediated selenium nanoparticles synthesis (SeNps)

Known volume (1mL) 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. The inoculated flask was kept for incubation at $30 \pm 1^\circ\text{C}$ (Ambient condition). The flask was observed regularly for colour change. The colour change from pale yellow to reddish-orange indicates the synthesis of selenium nanoparticles. After the primary confirmation of SeNps, the reaction mixture was centrifuged at $10,000 \times g$ for 10 min. The collected pellet was lyophilised and subjected to various instrumental methods for characterisation.

2.2.3. Characterisation

Various characterisation techniques were used to determine synthesised nanoparticles' structural and functional properties. Standard methods did the processing of the sample for the analysis. UV visible spectroscopy was initially carried out to assess surface plasmon absorption maxima (SPAM) using double beam UV visible spectrophotometer (Shimadzu-1800 spectrophotometer) with 200–800 nm scanning range Fourier transform infrared spectroscopy (FTIR) analysis was done with the sample, which was pelletised with potassium bromide in a suitable ratio followed by recording spectra at $400\text{--}4000\text{ cm}^{-2}$ (Bruker Optic GmbH Tensor 27). Scanning electron microscopy (SEM) and atomic force microscopy (AFM) analysed the particle's morphology and topography. SEM micrograph was taken using SUPRA 55-CARL ZEISS (Germany) at 19 kV acceleration voltage (magnification range 35–10,000, resolution 200 Å). In the case of 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 30kV voltage 100mA current with Cu K beta radiations with a running scan speed/duration (Rigaku Smart lab).

2.3. Preparation of chitosan- selenium nanocomposite (CS-SeNC)

Simple green science principles were adopted to prepare chitosan-selenium nanocomposite (CS-SeNC). In this method, a known volume of selenium nanoparticles suspension (1 mL) was suspended in 100 mL of deionised water- an acetic acid mixture containing 0.1% of chitosan (Analytical grade chitosan of medium molecular weight (65 kDa) with 80% diacylation Brookfield viscosity > 200 000 cps) was purchased from Sigma- Aldrich (USA) followed by dropwise addition of 0.1 M sodium salt of tripolyphosphate (ionic gelation method). 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.

2.3.1. Characterisation

Specific interaction of selenium nanoparticles with chitosan to transform as functionalised chitosan-selenium nanocomposite was studied by suitable analytical techniques described earlier. Changes in UV visible absorption spectra, a characteristic shift of absorption peaks due to interaction of functional groups with the nanoparticles and chitosan, crystalline phase, particles morphology, and distribution, including topography changes, were analysed using UV- visible spectroscopy, FTIR, XRD, SEM, AFM and particle size analysis. Processing and analysis of nanocomposite were carried out as described earlier.

2.4. Antibacterial activity

2.4.1. Multidrug-resistant tested bacterial strain - *Pseudomonas aeruginosa*

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 method such as molecular characterisation technique using 16S rRNA was also done to confirm the isolated bacterial strain. Genomic DNA of the bacterial strain was isolated using standard DNA isolation procedure; the isolated pure DNA was amplified with 16S rRNA primers. Nucleotide sequences of the 16S rRNA have been evaluated using NCBI's blast analysis.

The isolated bacterial strain studied multiple drug resistance (MDR) by determining antibiotic susceptibility or resistance patterns. Respective tested antibiotics disc was gently placed on Muller Hinton (MH) Agar Media (Hi-media, India) that was seeded with *Paeruginosa* inoculum (prepared in Muller Hinton Broth). The zone of inhibition was calculated 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

Antibacterial activity was primarily confirmed by agar cup diffusion assay. In contrast, a liquid medium microdilution assay was used to determine minimum inhibition concentration (MIC). Inoculum for these assays was prepared in sterile Muller Hinton Broth (MH). The inoculated medium was incubated at 37°C under shaking conditions. Viable inoculum thus obtained was used for antibacterial assays. In agar cup diffusion assay, sterile MH agar plates were swapped with the bacterial inoculum thus prepared, followed by the addition of 25, 50, 75 and 100 µg) 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 [61]. 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 24 h. A lower concentration of the nanocomposite that inhibited bacterial growth was considered MIC.

2.5. Evaluation of Nanocomposite induced virulence genes silencing studies

Nanocomposite based effect on the gene silencing of major virulence factors encoding genes Las B and RhII was investigated by determination of relative gene expression rate (%). A quantitative real-time reverse transcriptase-polymerase chain reaction was used to measure 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' GGAATGAACGAGGCGTTCTC 3') and (Reverse – 5' GGTCCAGTAGTAGCGGTTGG 3') genes were designed. RhII primers Forward-5'-GTAGCGGGTTTGCGGATG-3 'and reverse- 5' CGGCATCAGGTCTTCATCG-3' were also designed based on nucleotide sequence alignments.

2.5.2. qRT-PCR

Pseudomonas aeruginosa strain was grown in MH broth supplemented with 25, 50 and 100 µg 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 period of incubation, the culture broth was centrifuged, and the collected cell pellets were used for further studies. Total RNA of the respective treatment group, including control, was isolated by RNA isolation kit (Thermofisher Scientific) adopting manufacture instructions. Purity of the isolated RNA was done by spectrophotometric measurement of the samples at 260 and 280 nm, revealing that the 1.8-2 range was considered an acceptable purity range of extracted RNA. cDNA synthesis was carried out with the synthesised pure RNA thus obtained (20ng) using Prime Script 1st strand cDNA Synthesis Kit (TAKARA) following the manufacturer's instruction. Analysis of the synthesised cDNA was done by 1.2% agarose gel electrophoresis.

The mRNA expression levels of specific target genes were quantified by qPCR using SYBR green dye (Roche). Analysis of gene expression was done by the comparative threshold $\Delta\Delta C_t$ method. Pro C was used as an internal control (housekeeping gene). The reaction was processed in a real-time PCR machine, LightCycler® Nano, Roche. No template control (NTC) was incorporated as a negative control. The real-time PCR conditions are according to the manufacturer instruction as follows: initial denaturation at 95°C for 10 min, continued by 45 cycles of 3 step amplification consisting of denaturation at 95°C for 15s, annealing 60°C for 15s and extension at 72°C for 30 s. The melting curve was analysed from 60°C 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 studied. 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 *P. aeruginosa*; the inoculated broth was incubated under shaking conditions at 35°C for 24 h.

2.5.3.1 Biofilm assay

The effect of CS-SeNC on biofilm development was investigated by the microtitre plate crystal violet assay described elsewhere. In this method, the bacterial inoculum thus prepared was transferred to the 96 well plate under aseptic condition, followed by aliquots of CS-SeNC (50, 75 and 100 µg). To compare the efficacy, free selenium nanoparticles (F-SeNps) and chitosan CS) were also tested. 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 washed with phosphate-buffered saline (PBS). Washed microtitre plate was stained with 100 µL of crystal violet (final concentration of 0.1%) at ambient condition for 39 min. After the incubation period, destaining was done with ethanol after washing the stained microtitre plate. Ethanol solubilised biofilm was measured spectrophotometrically at 570 nm after the destaining. 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.

2.5.3.2. Pyocyanin production

CS-SeNC impact on pyocyanin production was studied by the modified method of Essar et al. [62]. The pyocyanin was quantified by extracting pyocyanin from the culture broth of respective treatment groups. Culture supernatant was obtained from the respective treatment group, followed by extraction with chloroform. The absorbance of the extracted layer carried out by spectrophotometric measurement revealed the amount of pyocyanin.

2.5.3.3. Swarming motility

CS-Se NC induced effect on the swarming motility was also studied to determine quorum quenching activity. In this assay, the bacterial inoculum thus prepared was spot inoculated on 0.3% agar concentration of MH 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.

2.5.3.4. Elastase activity

The effect of the CS-SeNC on the elastase activity also reveals 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 (1mL) was mixed with substrate elastin-Congo red suspended in 100mM Tris-HCl, 1 mM CaCl₂ 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.

2.6. Biocompatibility assessment using Zebrafish model

2.6.1. Zebrafish Collection and Maintenance

Adult zebrafish that are healthy and devoid of malformations or infections were chosen for the study. Before priming the experiment, fishes were acclimatised to lab conditions according to OECD guidelines (OECD, 203). Fishes were grouped and separated into various tanks for the treatment with the given sample.

2.6.2. LC₅₀ determination

Zebrafishes of interest were weighed and separated in diverse tanks for the study. The dosages were fixed as 3 µg, 6 µg and 9 µg. Also, control fishes were maintained in a separate tank, and the fishes were maintained under treatment for 5 days.

2.6.3. Dissection of Fishes from Liver Tissues:

Each fish was slowly anesthetised by adding ice chips into the fish water until the temperature reached 12°C. Those were taken for dissection when the fish remained still to any external stimuli. The liver tissues were removed carefully and stored.

2.6.4. Tissue fixation

The dissected liver tissue was stored in formalin for histopathology analysis, stored in PBS for Lipid Peroxidation assay and stored in RNA later for RNA isolation.

2.6.5. Histopathological analysis

Histological processing was done by adopting the procedure described by Lendrum et al. [63]. In brief, the tissues were detached from fixative, washed in running tap water and processed for dehydration in an augmenting percentage of ethyl alcohol. After this, the tissues were cleaned in methyl benzoate, and the

cleaned tissues were embedded in paraffin wax. Paraffin blocks were cut with a Leica manual rotary microtome at a thickness of 5 μ . They were stained with Hematoxylin and counterstained with eosin.

2.6.6. Lipid Peroxidation Assay

The degree of lipid peroxidation was evaluated by measuring the 2-thiobarbituric acid reactive substances (TBARS). The reaction mixture (0.1 mL) was treated with 0.2 mL of 8.1% (w/v) SDS, 1.5 mL of 0.8% of 2-thiobarbituric acid, 1.5 mL of 20% acetic acid, 0.7 mL of H₂O and then boiled for 60 min. After cooling, the sample was centrifuged (11,000 $\times$ g for 3 min), and the absorbance of the supernatant solution was measured at 532 nm. Malondialdehyde bis (dimethyl acetal) yields malondialdehyde by acid treatment.

2.7. qRT-PCR analysis of oxidative stress enzymes genes and apoptosis genes

2.7.1. RNA isolation

RNA isolation was done using TAKARA RNA iso plus reagent as per the manufacturer protocol. The isolated RNA was analysed on a 0.8% agarose gel containing 1 mg/mL ethidium bromide, 6 μ L of the PCR products with 4 μ L of 6X gel loading dye were loaded into the wells and electrophoresis was performed in 1 X TAE buffer at 50 V for 2 h. The gel was visualised under UV and photographed.

2.7.2. cDNA synthesis

The first-strand cDNA was synthesised from 20 ng of total RNA by reverse transcription method using Prime Script 1st strand cDNA Synthesis Kit from TAKARA following the manufacturer's instruction.

2.7.3. qPCR Analysis

Analysis of gene expression of oxidative stress enzymes – catalase (CAT), superoxide dismutase (SOD 1) was done by the 2 – $\Delta\Delta$ Ct method to confirm noteworthy biocompatibility. Beta-actin was used as an internal control. Per 5 μ L of the reaction mix, 10 picomolar each of forward and reverse primer (like CAT, SOD1 and p53- 50.1°C respectively, then for Bax – 50.9°C and Beta-actin – 52.1°C), 200 ng cDNA, 1x reaction mix were used. The reaction was conducted in a real-time PCR machine, LightCycler® Nano, Roche. No template control (NTC) was incorporated as a negative control. The real-time PCR conditions are according to the manufacture instruction as follows: initial denaturation at 95°C for 10 min, continued by 45 cycles of 3 step amplification consisting of denaturation at 95°C for 15s, annealing 60°C for 15s and extension at 72°C for the 30s. The melting curve was analysed from 60°C to 95°C for the 20s. The expression of the respective target gene has its own (ct) amplification value at its threshold level.

3. Results And Discussion

Current studies reveal that many pathogenic bacteria carry out cell-cell communication systems interceded by the production of signalling molecules in a density-dependent method to express certain

phenotypic traits in a density-dependent manner. The signalling principle is called quorum-sensing (QS). A novel strategy is to inhibit bacterial infections without preventing the growth by effectively suppressing bacterial cell communication – quorum sensing using certain chemical modulators [64]. Inhibiting quorum sensing or bacterial cell communication via green science principles plays a major role in drug development against life-threatening infections. In this study, chitosan-selenium nanocomposite was prepared through green science eco-friendly principles. The synthesised nanocomposite was evaluated against quorum sensing mediated virulence genes and production dependent virulence factors.

3.1. Synthesis and Characterisation of selenium nanoparticles (SeNps)

Green technology principles were adopted to synthesise selenium nanoparticles (SeNps) using biological synthesis mechanisms. In this method, a yeast consortium consisting of *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Torulopsis* species cultured in SDB medium was used to reduce selenite salt sodium optimum condition. Bio reduction of selenite salt into selenium nanoparticles was confirmed by converting the broth into a bright reddish-orange. The colour change started to appear at 48 h of incubation, and the colour change was retained up to 72 h. During the biogenic synthesis of nanoparticles, the microorganisms interact with target ions from their environment. The metal ions get converted into the elemental metal through enzymes generated by the microbial cell activities [65].

The formation of selenium nanoparticles in the culture broth was inspected visually and by a UV-visible spectrophotometer. The colour of the culture broth supplemented with sodium selenite changed from pale yellow to reddish-orange, thus delivering evidence for reducing selenite to elemental selenium (Fig. 1). The characteristic red colour was due to the surface plasmon resonance of selenium nanoparticles. The time-dependent synthesis of nanoparticles was monitored with UV-vis at different time intervals (24h, 48h and 72h). At 24h, the culture broth showed a broad absorption band between 370 to 570 nm. At 48h, the absorption band was found to be shifted between 230 to 670 nm. At 72h, the absorption band was found to be shifted between 360 to 650 nm (Fig. 2). Harikrishnan et al. [66] reported the formation of SeNps after 24 hours during their studies with *Saccharomyces cerevisiae* using selenite as the precursor. Dungan et al. [67] reported a similar finding, who obtained SeNps from *Stenotrophomonas maltophilia*.

The lyophilised nanoparticles' IR spectrum was recorded to identify the functional groups of the biomolecules involved in reducing and capping the synthesised nanoparticles. The major absorbance bands present in the spectrum of nanoparticles were at 3784, 3708, 3433, 2352, 1598, 1355, 1159, 1046 and 585 cm^{-1} (Fig. 3a). The wideband noted at 3784 and 3708 cm^{-1} could be assigned to stretching vibrations of water OH groups. The sharp band found at 3433 cm^{-1} could be assigned to alcohol OH stretch. The bands observed at 2352 cm^{-1} assigned to $\text{C} \equiv \text{C}$ stretch and the bands at 1598 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 found in 1159 and 1046 cm^{-1} shows the C-OH stretch. These findings

corroborate the synthesis and stabilisation of nanoparticles by the proteins present in the yeast. XRD confirmed the crystal structure of the produced nanoparticles. The XRD spectrum showed slightly sharper respective peaks of diffraction 23.22° (100), 29.52° (101), 41.14° (110), 43.60° (102), 45.50° (111), 51.48° (201), 55.50° (202), 61.40° (112), 64.88° (210), and 71.40° (113) (Fig. 3b). This pattern has good agreement with the standard JCPDS data (JCPDS No. 06-0362). The presence of more bioactive compounds as the capping agents may be responsible for the existence of noise.

SEM analysis of the synthesised nanoparticles was done to determine morphological characteristics (size & shape). 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. EDAX analysis used to determine elemental composition demonstrates the presence of selenium. The samples exhibited strong absorption peaks at 1.47keV, corresponding to Se's characteristic absorption (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 used to study surface topography of the synthesised nanoparticles reveals the presence of well-agglomerated particles with nano dimensions (Fig. 4c, d). Surface topology also indicates the absence of pores or notable defects.

3.2. Preparation of CS-SeNC

The chitosan-based nanocomposite is now extensively utilised in various fields, mainly biomedicine, due to its high efficacy and biocompatibility [28]. Chitosan is the most commonly employed polymer among the various polymers utilised in the metal-polymer nanocomposites preparation with potential antimicrobials and drug delivery agents. In this study, chitosan-based selenium nanocomposite (CS-SeNC) was prepared using in situ green science principles, then determined structural-functional properties and adopted suitable analytical techniques. Primary confirmation of selenium nanocomposite was done by UV visible absorption spectroscopic analysis reveals specific interaction of chitosan with the selenium nanoparticles to transform it into functionalised CS-SeNC by recording notable shift of surface plasmon resonance (Fig. 5a).

FTIR analysis was also used to confirm structural modification investigation. FTIR spectra depicted in Fig. 5b reveal chitosan's interaction with the selenium nanoparticles. Characteristic changes in the absorption spectra were due to the interaction of chitosan functional groups with the nanoparticles. Along with these peaks, notable absorption bands corresponding to bio reducers or capping agents of SeNps synthesis were recorded at 3500 cm^{-1} , 1649 and 1438 cm^{-1} . This was due to the interaction of bioreducers proteins of the yeast consortium. The vibration of OH groups of chitosan appears around 3500 cm^{-1} , at 1649 cm^{-1} , where usually the vibration of the amide I group of chitosan occurs.

The crystalline phase of the synthesised CS-SeNC was analysed by XRD, which shows the diffraction peaks only at 29.52° (101), 41.14° (110). The diffraction peak at 41.14° (110) reveals the chitosan interaction with selenium (diffraction peak at 101) which in turn is structurally modified as CS-SeNC

(Fig. 5c). The absence of remaining diffraction peaks with retention of selenium peak at 101 confirms chitosan interaction and transformation into a less crystalline structure. 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. 6a), 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. EDAX analysis was used to analyse the elemental composition demonstrates selenium with Carbon, Oxygen and Sulphur (Fig. 6b). Sulphur identified might have been derived from Sulphur-containing amino acids, methionine, observed in the EDAX spectrum. Atomic Force Microscopy (AFM) studied the surface topology of the synthesised SeNC was studied by Atomic Force Microscopy (AFM). AFM was used as the prime technique to screen the dissolution and agglomeration pattern. AFM micrograph indicates well-distributed particles with thickness higher than free selenium nanoparticles (Fig. 6c, 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

Antibacterial activity was evaluated against multidrug-resistant human pathogenic bacterial strain *Pseudomonas aeruginosa*. The clinical isolate of *Paeruginosa* used in this study was identified by culture-dependent methods (cultural, biochemical characteristics) followed by 16S rRNA analysis. Selected culture-dependent methods reveal Gram-negative, aerobic bacilli, oxidase, citrate positive non-Enterobacteriaceae members. Molecular methods based on 16S rRNA analysis confirm the isolated bacterial strain reveal 99.0% similarity with *Paeruginosa* (Fig. 7). The agar diffusion assay's antibiogram determination was studied using a multidrug-resistant pattern of the tested strain (Fig. 8). 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 determined minimum inhibition concentration (MIC). Results indicate that CS-SeNC inhibited *Paeruginosa* 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 (Fig. 9). 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, and damage the genetic components of the bacterial cells, which causes alteration in the metabolic processes that leads to bactericidal action [68, 69].

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

Treatment	MIC (µg)
F-SeNp	75.0 ^a
CS	100.0 ^a
CS-SeNC	25.0 ^a
^a Column carries alphabet is statistically significant (P < 0.5%)	

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 [70]. Released nanoparticles from the membrane chemistry principles of chitosan, which interact continuously with the bacterial cell components. MIC was determined by broth microdilution assay, which indicates that even the least tested dosage of CS-SeNC inhibited the growth of 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 found to be 75 µg and 100 µg, respectively.

3.4. Virulence genes silencing studies

Recent studies reveal that quorum sensing or bacterial cell communication can be targeted to prevent pathogenesis via quorum quenching (QQ) principles [43]. In *P.aeruginosa*, various genes are responsible for bacterial signalling, triggering various 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 [67]. 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. *P.aeruginosa* was treated with three different dosages of F-SeNps, chitosan, and CS-SeNC, followed by total RNA extraction. cDNA was synthesised from extracted total RNA as the template. The expression pattern of cDNA was analysed in agarose gel electrophoresis. The Agarose gel electrophorogram shown in Fig. 10,11 reveals characteristic changes in the cDNA expression pattern. CS-SeNC treatment group shows very thin, light DNA bands, unlike thick or sharp bands observed in the control, chitosan, F-SeNps treatment groups. The relative gene expression profile of both genes (Las B and Rhl) was analysed quantitatively to determine CS-SeNC induced effect. The relative gene expression level shown in Fig. 12,13 indicates the characteristic changes. Quantification studies indicate 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. 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 *Paeruginosa* 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 factors. 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. Considerable 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 (Fig. 14). 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 RhlI 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 *Paeruginosa*. SEM micrograph depicted in Fig. 15 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 on 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 of the biofilm. However, the level of biofilm damage was found to be lower than that of CS-SeNC.

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. 16a). 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 *Paeruginosa*, was carried out by measuring changes in swarm diameter (Fig. 16). 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 shows 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 reduction was observed in CS-SeNC (Fig. 16c). Chitosan treatment groups revealed no impact on elastase production as in control groups. In *Paeruginosa*, the production of diverse virulence factors that mediate pathogenesis is controlled by Las and Rhl QS system. Expressed products of these genes like Las B and RhlI synthases are essential for producing QS mediating signal molecules, triggering various virulence factors like pyocyanin production, elastase, protease production, biofilm formation [17]. Interruption of the gene expression by CS-SeNC proposed in this study correlates with the characteristic reduction in the virulence mentioned above factors production.

3.6. Biocompatibility studies

Biocompatibility of the synthesised Nanocomposite was tested with 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 lipid peroxidase (LPO) and histopathological analysis of liver tissue were also investigated to confirm noteworthy biocompatibility. Among the various model system, Zebrafish (*Danio rerio*) has been used as a popular vertebrate model to determine toxicity profile due to genomic, physiological homology with humans. Notable characteristics of embryos or developmental stages like transparency rapid developmental period enable zebrafish as the promising candidate for determining the toxicity profile of nanomaterials [48].

The study group was composed of control, CS-SeNC treatment with 3,6 and 9 µg. Adult zebrafishes were exposed to three different dosages – 3, 6 and 9 µg as LC_{50} was found to be 6 µg whereas 3 µg –below LC_{50} , 9 µg above LC_{50} dosage. Primary confirmation of biocompatibility was carried out by evaluating lipid peroxidase (LPO) activity. All the tested dosages of CS-SeNC did not induce any sign of undesirable stress, which was easily inferred from no marked effect in the LPO level (Fig. 17). No significant changes in LPO activity were recorded in CS-SeNC treatment ($P < 0.05\%$) which confirms the best biocompatibility of the nanocomposite.

Likewise, reactive oxygen species such as Hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) are generated by reactions that, in turn, generate lipid peroxidation (LOOH). The absence of impact on LPO level in CS-SeNC exposed treatment group clearly shows the administered nanocomposite was not inducing any sign of oxidative stress, which further confirms the best biocompatibility. Assessment of antioxidative enzymes is a noteworthy parameter for the biocompatibility evaluation. qRT-PCR evaluated relative mRNA expression of oxidative stress and apoptosis genes markers

Further confirmation of biocompatibility was carried out by quantitative assessment of marker genes in the liver tissue. The quantitative PCR was used to analyse the expression of oxidative stress genes (SOD, CAT) and apoptosis genes (p53 and BAX) in a nanocomposite treatment group zebrafish liver sample. Total RNA from the CS-SeNC treatment group was extracted and electrophoretically separated using a standard RNA isolation procedure explained previously. Agarose electrophorogram reveals the presence

of 28s and 18s RNA (Fig. 18). First-strand cDNA was synthesised from 20 ng of total RNA, thus isolated by reverse transcription method using PrimeScript 1st strand cDNA Synthesis Kit from TAKARA following the manufacturer instruction, followed by respective gene primer designing, qPCR using SYBR green dye (Roche), and the $2^{-\Delta\Delta Ct}$ method. These gene expression analyses indicate that the relative mRNA expression of all the tested oxidative stress genes and apoptosis genes confirmed the absence of significant variation (Fig. 19–22). No significant changes in gene expression level in all the treatment groups exposed to CS-SeNC for ten days. 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. 23). 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 be 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 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. Conclusion

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 RhII 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 of 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.

Declarations

Conflicts of interest

We declare that no conflicts of interest

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References

1. G. De Crozals, R. Bonnet, C. Farre, C. Chaix, Nano Today **11**, 435 (2016)
2. M. Qindeel, S. Sargazi, S.M. Hosseinikhah, A. Rahdar, M. Barani, V.K. Thakur, S. Pandey, R. Mirsafaei, ChemistrySelect **6**, 14082 (2021)
3. F. Sabir, M. Zeeshan, U. Laraib, M. Barani, A. Rahdar, M. Cucchiarini, S. Pandey, Cancers (Basel). **13**, (2021)
4. S. Kumar, F. Ye, S. Dobretsov, J. Dutta, Appl. Sci. **9**, (2019)

5. S. Zhang, L. Wang, X. Liang, J. Vorstius, R. Keatch, G. Corner, G. Nabi, F. Davidson, G.M. Gadd, Q. Zhao, *ACS Biomater. Sci. Eng.* (2019)
6. A.M. Rabel, S.K.R. Namasivayam, M. Prasanna, R.S.A. Bharani, *Int. J. Biol. Macromol.* **138**, 1109 (2019)
7. R. Rosman, B. Saifullah, S. Maniam, D. Dorniani, M.Z. Hussein, S. Fakurazi, *Nanomaterials* **8**, (2018)
8. A. Regiel-Futyra, M. Kus-Liśkiewicz, V. Sebastian, S. Irusta, M. Arruebo, A. Kyzioł, G. Stochel, *RSC Adv.* **7**, 52398 (2017)
9. L. Mei, Z. Lu, X. Zhang, C. Li, Y. Jia, *ACS Appl. Mater. Interfaces* **6**, 15813 (2014)
10. S.J. Almeahmadi, K.A. Alamry, M.A. Elfaky, S. Alqarni, J.A. Samah, M.A. Hussein, *Mater. Res. Express* **7**, (2020)
11. M. Cobos, I. De-La-Pinta, G. Quindós, M.J. Fernández, M.D. Fernández, *Polymers (Basel)*. **12**, (2020)
12. R.J.B. Pinto, S. Daina, P. Sadocco, C.P. Neto, T. Trindade, *Biomed Res. Int.* **2013**, (2013)
13. R. Nigmatullin, F. Gao, V. Konovalova, *J. Mater. Sci.* **43**, 5728 (2008)
14. S. Wiesenmueller, P. Cierniak, M. Juebner, E. Koerner, D. Hegemann, K. Mercer-Chalmers, Bender, J. *Biomater. Appl.* **33**, 327 (2018)
15. S. Ahmad Bhat, F. Zafar, A. Ullah Mirza, A. Hossain Mondal, A. Kareem, Q. Mohd. Rizwanul Haq, and N. Nishat, *Arab. J. Chem.* **13**, 5724 (2020)
16. G. Mendoza, A. Regiel-Futyra, V. Andreu, V. Sebastián, A. Kyzioł, G. Stochel, M. Arruebo, *ACS Appl. Mater. Interfaces* **9**, 17693 (2017)
17. A. Khurana, S. Tekula, M.A. Saifi, P. Venkatesh, C. Godugu, *Biomed. Pharmacother* **111**, 802 (2019)
18. S.A. Wadhwani, U.U. Shedbalkar, R. Singh, B.A. Chopade, *Appl. Microbiol. Biotechnol.* **100**, 2555 (2016)
19. S. Zheng, X. Li, Y. Zhang, Q. Xie, Y.S. Wong, W. Zheng, T. Chen, *Int. J. Nanomedicine* **7**, 3939 (2012)
20. L.B. Truong, D. Medina-Cruz, E. Mostafavi, N. Rabiee, *Molecules* **26**, (2021)
21. J.X. de Miranda, F. de O. Andrade, A. de Conti, M.L.Z. Dagli, F.S. Moreno, T.P. Ong, *J. Trace Elem. Med. Biol.* **28**, 486 (2014)
22. K.A. Amin, K.S. Hashem, F.S. Alshehri, S.T. Awad, M.S. Hassan, *Biol. Trace Elem. Res.* **175**, 136 (2017)
23. Y. Zhang, X. Li, Z. Huang, W. Zheng, C. Fan, T. Chen, *Nanomed. Nanotechnol. Biol Med* **9**, 74 (2013)
24. M. Vahdati, T. Tohidi Moghadam, *Sci. Rep.* **10**, (2020)
25. G. Liao, J. Tang, D. Wang, H. Zuo, Q. Zhang, Y. Liu, H. Xiong, *World J. Surg. Oncol.* **18**, (2020)
26. D. Chudobova, K. Cihalova, S. Dostalova, B. Ruttkay-Nedecky, M.A. Merlos Rodrigo, K. Tmejova, P. Kopel, L. Nejd, J. Kudr, J. Gumulec, S. Krizkova, J. Kynicky, R. Kizek, V. Adam, *FEMS Microbiol. Lett.* **351**, 195 (2014)
27. Awanish Kumar and Kumar Suranjit Prasad, *Asian J. Pharm. Clin. Res.* **4** (2019)
28. N. Filipović, D. Ušjak, M.T. Milenković, K. Zheng, L. Liverani, A.R. Boccaccini, M.M. Stevanović, *Front. Bioeng. Biotechnol.* **8**, (2021)

29. O.I. Sonde, M. Gbenga Peleyeju, F.O. Oladoyinbo, A.R. Oloyede, T.A. Afolabi, H.A. Adesokan, O. Ademola, Arotiba, E.O. Dare, J. Nanoanalysis **4**, 41 (2017)
30. P.S. Kumar, A.S. Nesaraj, Int. J. Pharm. Sci. Res. **8**, 2395 (2018)
31. M. Francklin Philips, J. Thangarathinam, J. Princy, C.A. Crispin Tina, C.A. Crispin, Tina, A. Kasthuri, J. Nanosci. Nanotechnol **21**, 5673 (2021)
32. S. Karthick Raja Namasivayam, G. Venkatachalam, R.S. Arvind, Bharani, Sustain. Chem. Pharm. **17**, (2020)
33. A.F. Pinto, M. Ebrahimi, M. Saleeb, Å Forsberg, M. Elofsson, H. Schöler, J. Biomol. Screen. **21**, 590 (2016)
34. M. Saleeb, C. Sundin, Ö Aglar, A.F. Pinto, M. Ebrahimi, Å Forsberg, H. Schöler, M. Elofsson, Eur. J. Med. Chem. **143**, 568 (2018)
35. A.E. Marsden, J.M. King, M.A. Spies, O.K. Kim, T.L. Yahr, Antimicrob. Agents Chemother. **60**, 766 (2016)
36. A.A. Ahmed, F.A. Salih, Iran. J Basic Med Sci **22**, 568 (2019)
37. S.A.K.S. Ahmed, M. Rudden, T.J. Smyth, J.S.G. Dooley, R. Marchant, I.M. Banat, Appl. Microbiol. Biotechnol. (2019)
38. T. Sawa, J. Intensive Care **2**, (2014)
39. A.R. Kroken, C.K. Chen, D.J. Evans, T.L. Yahr, and S. M. J. Fleiszig, MBio **9**, (2018)
40. A. Arnoldo, J. Curak, S. Kittanakom, I. Chevelev, V.T. Lee, M. Sahebol-Amri, B. Kosciuk, L. Ljuma, P.J. Roy, A. Bedalov, G. Giaever, C. Nislow, R.A. Merrill, S. Lory, I. Stagljar, PLoS Genet. **4**, (2008)
41. A. Kaminski, K.H. Gupta, J.W. Goldufsky, H.W. Lee, V. Gupta, S.H. Shafikhani, Sci. Rep. **8**, (2018)
42. J. Béatrice, P. Maud, A. Stéphane, C. Franç Cois, G. Frédéric, G. Benoit, M.O. Husson, FEMS Microbiol. Lett. **243**, 271 (2005)
43. D.E. Fateme Moradi, A.A. Imani, Foladi, A. Bahador, Pharm. Regul. Aff. Open Access **4**, (2015)
44. S.G. Ali, M.A. Ansari, Q.M. Sajid Jamal, H.M. Khan, M. Jalal, H. Ahmad, A.A. Mahdi, Silico Pharmacol. **5**, (2017)
45. M. Ahumado, J.C. Drosos, R. Vivas-Reyes, Mol. Biosyst **10**, 1162 (2014)
46. S. Ilk, N. Saglam, M. Özgen, Artif. Cells Nanomedicine Biotechnol. **45**, 907 (2017)
47. M.S.E.M. Badawy, O.K.M. Riad, F.A. Taher, S.A. Zaki, Int. J. Biol. Macromol. **149**, 1109 (2020)
48. F. Hemmati, R. Salehi, R. Ghotaslou, H.S. Kafil, A. Hasani, P. Gholizadeh, M.A. Rezaee, Int. J. Biol. Macromol. **163**, 2248 (2020)
49. F.M. Husain, Q. Zia, M.F. Khan, E. Ahmad, A. Jamal, M. Alaidarous, S. Banawas, M.M. Alam, B.A. Alshehri, M. Jameel, P. Alam, M.I. Ahamed, A.H. Ansari, I. Ahmad, ACS Omega **5**, 32203 (2020)
50. M. Pourmbarak Mahnaie, H. Mahmoudi, Eur. J. Med. Res. **25**, (2020)
51. L. Gunti, R.S. Dass, N.K. Kalagatur, Front. Microbiol. **10**, (2019)

52. S. Karthick Raja Namasivayam, R.S. Arvind, Bharani, K. Karunamoorthy, Int. J. Biol. Macromol. **120**, 921 (2018)
53. X. Li, L. Wang, Y. Fan, Q. Feng, F.Z. Cui, J. Nanomater. 2012, (2012)
54. G.K. Nasrallah, M. Al-Asmakh, K. Rasool, K.A. Mahmoud, Environ. Sci. Nano **5**, 1002 (2018)
55. S. Kumaraswamy, S.L. Patil, S.H. Mallaiah, J. Bioact Compat. Polym. **35**, 435 (2020)
56. C. Zhu, H. Wang, Z. Mahmood, Q. Wang, H. Ma, PLoS One 13, (2018)
57. M. d'Amora, D. Cassano, S. Pocoví-Martínez, S. Giordani, V. Voliani, Nanotoxicology **12**, 914 (2018)
58. M. Liu, D. Xu, K. Wang, F. Deng, Q. Wan, G. Zeng, Q. Huang, X. Zhang, Y. Wei, RSC Adv. **5**, 96983 (2015)
59. H. Weber, J. Basic. Microbiol. **25**, 520 (1985)
60. J. Barnett, Arthur, *Roger William Payne, David Yarrow* (Cambridge University Press (CUP), 1990), p. 1012
61. L. Qi, Z. Xu, X. Jiang, C. Hu, X. Zou, Carbohydr. Res. **339**, 2693 (2004)
62. D.W. Essar, L. Eberly, A. Hadero, I.P. Crawford, J. Bacteriol. **172**, 884 (1990)
63. A.C. Lendrum, J. Clin. Pathol. **16**, 609 (1963)
64. A.B. Aliyu, N.A. Koorbanally, B. Moodley, P. Singh, H.Y. Chenia, Phytochemistry **126**, 23 (2016)
65. M. Shah, D. Fawcett, S. Sharma, S.K. Tripathy, and G. E. J. Poinern, Materials (Basel). **8**, 7278 (2015)
66. H. Hariharan, N.A. Al-Harbi, P. Karuppiyah, S.K. Rajaram, Chalcogenide Lett. **9**, 509 (2012)
67. R.S. Dungan, S.R. Yates, W.T. Frankenberger, Environ. Microbiol. **5**, 287 (2003)
68. W. Ahmad, S. Shams, A. Ahmad, Y. Wei, Q. Yuan, A.U. Khan, M.S. Khan, A. Ur, Rahman, M. Iqbal, Appl. Nanosci. **10**, 1191 (2020)
69. P. Verma, World J. Pharm. Pharm. Sci. **4**, 652 (2015)
70. W. Jiang, Y. Fu, F. Yang, Y. Yang, T. Liu, W. Zheng, L. Zeng, T. Chen, ACS Appl. Mater. Interfaces **6**, 13738 (2014)

Figures

Figure 1

Synthesized Selenium nanoparticles

Figure 2

UV-Visible absorption spectra of synthesized SeNps

Figure 3

Characterization of F-SeNps (a) FTIR spectra (b) XRD pattern

Figure 4

Characterization of F-SeNps (a) SEM micrograph (b) EDAX spectra (c) AFM micrograph- 3D image (d) AFM micrograph – 2D image

Figure 5

Characterization of CS-SeNC (a) UV-vis spectra (b) FTIR spectra (c) XRD pattern

Figure 6

Characterization of CS-SeNC (a) SEM micrograph, (b) EDAX spectra, (c) AFM micrograph- 2D image, (d) AFM micrograph – 3D image

Figure 7

Figure 8

Antibiogram of *P. aeruginosa*: (a) Penicillins: ampicillin (10 mcg), amoxycillin (20 mcg), ticarcillin (75 mcg), piperacillin (100 mcg); (b) gentamicin (10 mcg), tobramycin (10 mcg), amikacin (30 mcg), netilmicin (30 mcg); ciprofloxacin; (c) cephalixin (30 mcg), cefuroxime (30mcg), cefazolin (30mcg), cefotaxime (30mcg), ceftriaxone (30mcg); (d) cephalixin (30 mcg), cefuroxime (30mcg), cefazolin (30mcg), cefotaxime (30mcg), ceftriaxone (30mcg); (e) cefpodoxime 10 mcg), cefdinir (5 mcg), cefepime (30 mcg); imipenem (10mcg), meropenem (10 mcg); (f) piperacillin + tazobactam (100/10 mcg), ticarcillin + clavulinic acid (75/10 mcg), cefoperazone + sulbactam (75/10 mcg), cefotaxime + sulbactam (30/10 mcg), ceftriaxone + sulbactam (30/10 mcg).

Figure 9

Anti bacterial activity against *Paeruginosa* 1 - 100 µg, 2 - 75 µg, 3 - 50 µg, 4 - 25 µg

Figure 10

cDNA expression pattern of Las B. lane 1 – control 2 - Chitosan-100 µg 3 - 75 µg 4 - 50 µg 5 -F-SeNps 100 µg 6 -75 µg 7 - 50 µg 8 - CS-SeNC 100 µg 9 -75 µg 10 - 50 µg

Figure 11

cDNA expression pattern of RhII 1 - control 2 - Chitosan-100 µg 3 - 75 µg 4 - 50 µg 5 -F-SeNps 100 µg 6 -75 µg 7 - 50 µg 8 - CS-SeNC 100 µg 9 -75 µg 10 - 50 µg

Figure 12

Relative expression of Las B

Figure 13

Relative expression of RhII

Figure 14

Biofilm formation of *Paeruginosa*

Figure 15

SEM micrograph of biofilm (a) Control (b) F-SeNps (c) Chitosan (d) CS-SeNC

Figure 16

Virulence factors status of *P. aeruginosa* (a) Pyocyanin production (b) Swarming motility- swarm reduction (c) Elastase enzyme activity- activity reduction

Figure 17

LPO activity of CS-SeNC administrated Zebrafish

Figure 18

Extracted RNA from the CS-SeNC administrated Zebrafish

Figure 19

Relative mRNA expression of CAT gene in CS-SeNC administrated Zebrafish

Figure 20

Relative mRNA expression of SOD1 gene in CS-SeNC administrated Zebrafish

Figure 21

Relative mRNA expression of p53 gene in CS-SeNC administrated Zebrafish

Figure 22

Relative mRNA expression of p53 gene in CS-SeNC administrated Zebrafish

Figure 23

Histopathological analysis of liver tissue in CS-SeNC administrated Zebrafish. (a) Control, (b) 3 μ g, (c) 6 μ g, (d) 9 μ g

Supplementary Files

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