Mycosynthesis of highly fluorescent selenium nanoparticles from *Fusarium oxysporum*, their antifungal activity against black fungus *Aspergillus niger*, and in-vivo biodistribution studies

Sk Najrul Islam1 · Syed Mohd Adnan Naqvi1 · Azam Raza1 · Amit Jaiswal2 · Akhilesh K. Singh2 · Manish Dixit2 · Atul Barnwal2 · Sanjay Gambhir2 · Absar Ahmad1

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Abstract
In the past few years, photo-luminescent inorganic materials have been studied extensively as fluorescent sensors, and diagnostic and bioimaging tools. The assessment of photoluminescence (PL) properties of selenium nanoparticles (Se NPs), especially mycosynthesized Se NPs, is still in its infancy. Herein, we have biosynthesized highly dispersed fluorescent Se NPs (42 nm) using endophytic fungus *Fusarium oxysporum*, and fully characterized them using sophisticated instruments like TEM, XRD, UV–Vis spectrophotometer, FTIR, and PL spectrometer. To determine the therapeutic efficacy and side effect profiles, these crystalline Se NPs were radiolabeled with technetium-99m (99mTc) and their biodistribution and renal clearance times were investigated in the normal Wister rat. The results showed that these Se NPs may be useful for targeting the lungs and liver dysfunction as significant accumulation of these NPs was observed in the liver (approx. 19.47 ± 4%) and lungs (at 6 ± 1%) after 10 min of post-injection. Quick circulation and the presence of Se NPs in kidney (3.8 ± 2%) also suggested the easy excretion of these NPs from the body through urinary tract. Furthermore, the antioxidant activity of Se NPs (IC50, 159.5 μg/mL) has been investigated using DPPH free radical scavenging assay with scavenging efficacy of 80.4% whereas ascorbic acid (IC50, 5.6 μg/mL) was used as a positive control. Additionally, the microscopic study of the inhibition zone encircled around Se NPs confirmed their strong antifungal and antisporeulant activity against the black fungus *Aspergillus niger*.

Keywords Antifungal · *Aspergillus niger* · Biodistribution · Fluorescent · *Fusarium oxysporum* · Selenium nanoparticles

Introduction
Fluorescent nanoparticles and their surface functionalization have been receiving great scientific acclaim in the field of biomedical science as the biological traits and effects of nanoparticles can be easily changed by straightforward surface modification (He et al. 2019; Sanità et al. 2020; Jung and Neuman 2021). However, this modification typically involves the use of harmful chemicals for capping, which often limits their applications in healthcare (Sukhanova et al. 2018). Thus, the biological approaches for nanoparticle synthesis are being adopted to overcome these toxicity issues as the biosynthesized nanoparticles are highly stable, water dispersible, and capped by nontoxic phytochemicals or natural protein molecules (Mukherjee et al. 2001; Shankar et al. 2003; Sudhasree et al. 2014; Zhang et al. 2021). Our group has done pioneering work in the biosynthesis of biomedical important nanomaterials using fungi, bacteria, and plant extract (Ahmad et al. 2003, 2004, 2007; Syed and Ahmad 2012; Islam et al. 2021a). The fungi are referred to as a "biofactory" for producing NPs, because this process is highly scalable, controllable, and inexpensive. The fungi-based bio-inspired method is better than the conventional biosynthesis approaches in terms of cost and reproducibility, since plant-based biosynthesis is geographically and seasonally variable, while bacterial biosynthesis requires expensive, sophisticated equipment for NPs’ separation and
purification. Nowadays, biosynthesized Se NPs become a promising material in healthcare, indicating significant effectiveness as an anticancer (Cruz et al. 2019; Vahidi et al. 2020), antimicrobial, and antioxidizing agent (Korde et al. 2021). Although several studies on the biosynthesis of Se NPs utilizing bacteria (Wadhwani et al. 2016; Ashengroh and Hosseini 2021; Bulgarini et al. 2021), fungi (Wadhwani et al. 2016; Abu-Elghait et al. 2021), and algae (Tehrani and Hosseini 2021; Bulgarini et al. 2021) have been published, little is known about their photoluminescence properties, especially for mycosynthesized Se NPs. In all the previous studies, the capability of saprophytic fungi has been investigated for the bio-reduction of selenium into Se NPs. Herein, we have biosynthesized Se NPs from the salt selenium (IV) chloride using the endophytic fungus Fusarium oxysporum, a well-known, important source of different enzymes that have various applications in biotechnology and nanomaterial synthesis (Ibrahim et al. 2021).

The in-vivo biodistribution study of different NPs has attracted a lot of attention in preclinical research to understand the therapeutic effectiveness, toxicity, and interactions between organs with nanoparticles (Khan et al. 2014; Nabanichang 2019; Yuan et al. 2019). Huang et al. reported chirality-dependent biodistribution of glutathion@Se NPs with the preferential accumulation of L-glutathion@Se NPs in the liver, spleen, and pancreas (Huang et al. 2020). Similar findings were made in another investigation, which discovered that Se NPs accumulated in the liver and kidney after being administered orally, with no detrimental effects on biochemical and hematologic indicators (Chandramohan et al. 2021). Radiolabel vitamin C-coated Se NPs were also reported to have poor physiochemical stability (Korany et al. 2020). As the NPs with different compositions and morphologies show distinct affinities toward various organs, we explored the biodistribution study of fluorescent Se NPs (mycosynthesized) capped with natural proteins, in normal Wister rat to figure out the effect of capping proteins in the biodistribution of Se NPs (Li and Huang 2008; Wei et al. 2018). When the biomass of the fungus Fusarium oxysporum is suspended in sterilized double distilled water, it releases various enzymes and proteins into the water. Our group previously reported that the enzyme with a molecular weight of 44-kilodalton was responsible for the enzymatic reduction of precursor ions (Kumar et al. 2007) while low molecular weight protein (13- kilodalton) capped the synthesized nanoparticles (Khan and Ahmad 2014). In this study, we have also confirmed the presence of capping protein using UV–visible and FTIR spectrometer, while residual phytochemicals were washed out using centrifugation. Parallelly, we have investigated the antioxidant activity of protein–capped fluorescent Se NPs. In the present COVID-19 pandemic situation, Aspergillus infection caused by the black fungus Aspergillus niger has become life-threatening to immune compromised SARS-CoV-2-infected patients in intensive care units. A recent study exposed that one-third of the ventilated COVID-19 patients also had Aspergillus infections and roughly 50% of them died. Thus, different antifungal drugs have been used as a preventive measure for seriously ill COVID-19 patients even if they have not tested positive for fungal infection. The excessive use of these drugs increases antifungal drug resistance and is no longer effective for fungal inhibition. In view of the public health and current pandemic situation, our group has biosynthesized Se NPs and unveiled their antifungal and antispore activity against the black fungus Aspergillus niger.

Materials and methods

Chemicals

The analytical reagent-grade chemicals and reagents were commercially accessible and utilized as delivered without further refining. Selenium tetrachloride (SeCl$_4$) that was used for the biosynthesis of fluorescent Se NPs was procured from Sigma-Aldrich, while fungal media components were obtained from Hi-media (Malt extract, Yeast extract, Peptone) and Qualigens (D-glucose). Technetium-99m was obtained as pertechnetate elute from $^{99m}$Tc generator which was acquired from the SDS Life Sciences Pvt Ltd. Whatman paper No.1 sheets were obtained from Merck (Darmstadt, Germany). Equipment ANaI (Tl) γ-ray scintillation counter (Scaler-Ratemeter SR7 model, UK) was utilized to detect the radioactivity. A high-powered sonicator was employed for the encapsulation process. Additionally, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ascorbic acid were purchased from Alfa Aesar and SD-fine, respectively, for free radical scavenging assay.

Maintenance and growth condition of fungal strain

The strain of endophytic fungus Fusarium oxysporum was maintained on potato dextrose agar (PDA) slants by monthly sub-culturing and stored in the B.O.D. incubator at 25 °C. The preserved strain of the fungus Fusarium oxysporum was then transferred into 100 mL aqueous MGYP medium which was prepared in 500 mL Erlenmeyer flask by adding malt extract (0.3%), glucose (1%), yeast extract (0.3%), and peptone (0.1%). Then, the mycelia-containing flasks were kept on a rotary shaker (200 rpm) at 25 °C. The fungal strains started to grow at pH 7–9, and after 96 h of continuous shaking, the biomass was separated from the culture medium by centrifugation (7000 rpm, 15 min) at 15 °C. Then, the separated biomass was washed thrice with sterile distilled water for further use.
Biosynthesis of fluorescent Se NPs

Biosynthesis of Se NPs was carried out by adding approximately 20 gm of biomass into 100 mL of freshly prepared aqueous SeCl₄ solution (2 mM) in a 500 mL sterilized Erlenmeyer flask. The reaction mixture was then placed on the rotary shaker (200 rpm) at room temperature. After 72 h of reaction completion, the mycelia were separated from the respective medium by a simple filtration method. The obtained red filtrate containing highly dispersed fluorescent Se NPs was lyophilized and stored as a powder for further investigation.

Techniques used for characterization of Se NPs

The UV–Vis–NIR spectrophotometer (Cary 5000) was used in the wavelength range of 200–800 nm to investigate the absorbance of mycosynthesized Se NPs. To examine the characteristic bond vibration frequencies, the Fourier transform infrared (FTIR) spectrometry (PerkinElmer) was performed in the wavelength range of 400–4000 cm⁻¹. Using photoluminescence (PL) spectroscopy, fluorescence behavior of Se NPs was explored. The shape, size, and average particle-size distribution of Se NPs were surveyed using Transmission Electron Microscopy (TEM, JEM-JEOL, JAPAN). To obtain Transmission Electron Micrographs (TEM images), the Se NPs were dropcasted on carbon-coated copper grid and show under the TEM operating at a 100,000× magnification. Finally, X-ray powder diffraction (XRD) technique (Rigaku X-ray Diffractometer) and selected area electron diffraction (SAED) were used to confirm the crystallinity and unit lattice parameters of Se NPs. XRD diffractogram of lyophilized Se nanopowder was measured in the 2θ range of 20°–80° with a step size of 0.04° and a time of 5 s per step at 40 kV voltage and a current of 30 mA. For SAED analysis, same Se NPs-loaded carbon-coated copper grid was used for the recording of SAED pattern at an angular magnification of 0.001 nm per degree.

Radiochemical yield of ⁹⁹mTc-Se NPs

The radiochemical yield percentage of ⁹⁹mTc-Se NPs was determined by instant thin-layer chromatography (ITLC, 12 cm long and 1 cm wide), marked at a distance of 2 cm from the lower end and lined into sections 1 cm each up to 10 cm. A few drops from the mixture were spotted using a hypodermic syringe, and then, the strip was developed in an ascending manner in a jar using only acetone as a developing solvent. After complete development, the strips were dried and cut into fragments 1 cm each, and then, the sections were counted by NaI (TI) γ-ray scintillation counter for measuring radioactivity. The percentage of radiochemical yield was calculated by division of the radioactivity of radiolabeled nanoparticles (⁹⁹mTc-Se NPs) by the total activity multiplied by 100. The radiolabeling efficiency was in the range of > 95% and stability was measured at different points of time, at both solution conditions and physiological states.

In-vitro stability study

The in-vitro stability of ⁹⁹mTc-Se NPs formulation was estimated to investigate the radiochemical tolerance in physiological conditions. Exactly 0.1 mL of the final preparation of ⁹⁹mTc-Se NPs (1 μCi/500 μL) was incubated with 0.9 mL of Human Serum albumin (pH = 7.4) for different time intervals (0, 100, 200, 350, 1440 min) at 37 °C. The radiochemical yields were determined by the above-described ITLC method at different time intervals.

In-vivo biological studies

The in-vivo preliminary evaluation was evaluated in groups of normal Wistar rat at different point intervals. Exactly, 75 μL having 11–14 MBq of the radiolabeled NPs was intravenous (I.V.) injected in the tail vein. For each group, the rats were anesthetized and then weighed at 10, 60, 120 min and 6 h post-injection (p.i.). All organs were extracted, flushed with saline, and weighed, and their radioactivity was estimated using a well-type NaI gamma counter where the background was excluded. The percentage injected activity per gm of tissues (%ID/g) was estimated using the formula given below and the tissue uptakes were evaluated.

\[
\% \text{ ID/g} = \frac{\text{Counts per gramin}}{\text{Counts dose given}} \times 100
\]

Antifungal, antisporulant, and antioxidant activity

The antifungal and antisporulant activity of Se NPs has been investigated against the black fungus *Aspergillus niger* by
the disc diffusion method. Initially, the stoke solution of Se NPs having a concentration of 8 mg/mL was prepared and subsequently diluted to 4, 2, 1, 0.5, and 0.25 mg/mL by adding distilled water. 30 μL from each prepared solution (containing 240, 120, 60, 30, 15, and 7.5 μg) were loaded onto sterile discs (A, B, C, D, E, and F, respectively) and placed on PDA petri plates having the spores of Aspergillus niger. Fluconazole 10 μg (mcg) antifungal disc (H) was used as positive control, while 30 μL water (G) was considered a negative control. To determine the anti-sporulation activity of Se NPs, the hyphal growth of fungus Aspergillus niger was closely monitored using an optical microscope as microscopic visualization is considered the best method to assess sporulation.

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out for the assessment of the antioxidant activity of fluorescent Se NPs. The absorbance of Se NPs aliquots (1000, 500, 250, 125, 62.50, and 31.25 μL/mL) was measured at 517 nm. The different concentrations of these aliquots were prepared by the addition of 1 mL Se NPs solution into 3 mL of methanolic DPPH solution (0.02 mM). The radical scavenging capacity of Se NPs in reference with standard ascorbic acid (100, 50, 25, 12.5, 6.25, and 3.125 μL/mL) and methanol (negative control) was evaluated followed by the IC50 value determination. The percentage (%) of inhibition for Se NPs and ascorbic acid were calculated by applying the following formula:

\[
\text{% Inhibition} = \frac{\text{Abs}(C) - \text{Abs}(T)}{\text{Abs}(C)} \times 100
\]

where Abs(C) and Abs(T) represent absorbance of the control sample and test sample, respectively.

Results and discussion

Nanoparticles characterization

The initial confirmation of Se NPs formation was obtained through visual observation that showed a color change from yellowish white to clear red during the reaction. After completion of the reaction, the red filtrate was analyzed by UV–visible spectroscopy. The strong absorption peak observed at 265 nm was due to surface plasmon resonance (SPR) of protein-capped Se NPs (Fig. 1a), while the absorption maxima were red-shifted at 276 nm (Fig. 1b) when mycosynthesized Se NPs were calcined at 200 °C (Kokila et al. 2017; Mellinas et al. 2019). The shifting of absorption maxima to a higher wavelength was attributed to the degradation of protein molecules from the surface of Se NPs.

The visualization of intense sky-blue fluorescence and the PL spectra confirm the photo-luminescent behavior of biogenic Se NPs (Fig. 1c). The highly intense emission peak that appeared at 360 nm (excited at 250 nm) was characterized by natural protein-capped biogenic Se NPs, which was not detected for chemogenic nano Se (Prasanth and Sudarsanakumar 2017; Piacenza et al. 2020). It was also observed that when biogenic Se NPs’ solution was excited at 280 nm, the wide red-shifted emission peak appeared in the wavelength range of 390–420 nm. The redshift of excitation-dependent emission spectra can be attributed to the non-homogeneous size distribution as the PL properties of NPs are highly dependent on NPs size and capping agent (Piacenza et al. 2020).

Most importantly, TEM images (Fig. 2a and b) of biofabricated Se NPs were captured to confirm the shape and size of NPs. The captured electron micrograph demonstrated that the synthesized NPs are spherical with an
average particle size of 42 nm, whereas the SAED pattern (Fig. 2c) confirmed the crystalline phase of Se NPs.

Further powder X-ray diffractometer (XRD) was performed to identify the crystallographic structure of myco-synthesized Se NPs. Figure 3a shows the diffraction pattern of as-synthesized Se NPs. The sharp and intense peaks appearing at 2θ angles of 27.31°, 30.74°, 39.35°, 49.05°, 57.45°, 65.47°, and 72.78° were indexed as (101), (011), (101), (110), (021), (012), and (120) planes conforming the hexagonal phase of Se NPs with space group P3121 (81.6%) and R3-MH (18.4%) (Ref. Pattern: 98-007-8796, 98-009-2700) (Islam et. al. 2022).

Fourier transform infrared spectroscopy (FTIR) was executed to investigate the bond vibration frequencies associated with protein-capped Se NPs. The peaks that appeared at 3415, 2947, 1630, 1520, 1395, and 1070 cm⁻¹ in the FTIR spectrum (Fig. 3b) are the characteristic peaks of proteins, whereas the peak observed at 530 cm⁻¹ is due to the Se–Se bond vibration (Khiralla and El-Deeb 2015; Qian et al. 2017). The intense peaks at 3415 and 2947 cm⁻¹ are identified for stretching vibration of O–H (alcohol) and N–H (amines). The sharp peaks obtained at 1630 and 1520 cm⁻¹ correspond to amide I (N–C=O-stretching mode) and amide II (N–H bending mode), which are the main two characteristic peaks of proteins (Islam et al. 2021b), while the remaining two absorption band appeared at 1395 and 1070 cm⁻¹ may be assigned to C–O-stretching mode (Khiralla and El-Deeb 2015).

Figure 4a and b shows the stability of the radiolabeled nanoparticles at room temperature while keeping the radioactivity at physiological saline and human serum albumin solution, respectively, and assessing its stability using ITLC method at different time points. The result showed that the Se NPs was chelated excellently with the metallic radionuclide (⁹⁹mTc) at physiological pH range or at human serum albumin environment.

The randomly dotted ring in SAED pattern indicating the polycrystalline nature of Se NPs, while particle-size distribution histogram exploring the average particle diameter of 42 nm

The NPs synthesized in this study are within the size range for biodistribution and no external capping agents were incorporated during synthesis.

Data in Fig. 5a and b showed that radiolabeled nanoparticle is well distributed as the maximum percentage injected dose per gm organ (% ID/g) of ⁹⁹mTc-Se NPs complex in the liver was 19.47 ± 4% at 10 min post-injection (p.i.) with merely no significant accumulation in spleen, but the expected value in stomach and lungs is about 9.4 ± 2 and 6 ± 1% at 10 min p.i. The data suggested that these radiolabeled nanoparticles are well above the size of the hydrodynamic diameters (HDs) of renal clearance threshold (<6 to 8 nm). The activity at the kidney is around 3.8 ± 2 after 6 h p.i. suggested the excretion of nanoparticles through the urinary tract. The experiment at post 6 h showed the accumulation mainly in the liver (approx. 2.2% ID/g) and lungs (at 3.4% ID/g) and this is suggestive of the particle size of the nanoparticles. These nanoparticles may be useful for lung or liver dysfunction. The Se NPs has the advantages of enjoying a quick circulation in the blood and allow for passive targeting through the enhanced permeability and retention (EPR) effect; in addition, the nanoparticle can actively target lungs and liver through molecular interaction or affinity which will be beneficial in assessing the functionality of these organs.

**Antifungal, antisporulant, and antioxidant study**

It is previously mentioned that the different antifungal drugs have been used as a preventive measure for seriously ill COVID-19 patients even if they have not tested positive for fungal infection, because 50% of one-third ventilated COVID-19 patients with *Aspergillus* infections had died in intensive care unit. The excessive use of these drugs increases antifungal drug resistance and is no longer effective for fungal inhibition. In view of the public health...
and current pandemic situation, our group has biosynthesized Se NPs and unveiled their antifungal and antispore-lant activity against the black fungus *Aspergillus niger*. The antifungal activity of Se NPs was evaluated after 24, 36, and 48 h of incubation at different concentrations of 240, 120, 60, 30, 15, and 7.5 μg/30 μL (from discs A–F) against the black fungus *Aspergillus niger*. The strong antifungal activity of mycosynthesized Se NPs was ascertained by the zones of inhibition which were encircled across the sterile discs containing Se NPs (Fig. 6a). It was also observed that the zone of inhibition increases with increasing NPs concentration. The microscopic study (Fig. 6b) showed that the aerial hyphae which contain structures (conidiophores, vesicle, metule, phialide, and conidia) for spores’ production were suppressed without affecting the growth of vegetative mycelia (anti-sporulation) near inhibition boundary, while both the aerial mycelia and vegetative growth of *Aspergillus niger* were completely inhibited in the zone of inhibition (antifungal) by Se NPs. The antifungal efficiency of Se NPs was compared with standard antifungal compound (10 mcg
fluconazole, disc H) and the evidence indicates the potential of Se NPs to act as an effective antifungal measure against *Aspergillus niger*. In-vitro antioxidant activity of mycosynthesized Se NPs has been determined by DPPH assay. The gradual decolorization of the purple color and decrease in the absorption maxima (517 nm) of DPPH with an increase in concentration of Se NPs confirmed its antioxidant potency (Fig. 7a) which was compared with the standard antioxidant ascorbic acid (Fig. 7b). The free radical scavenging efficiency of Se NPs (1 mg/mL) was estimated (80.4%) with IC50 value of 159.5 μg/mL (Fig. 7c), while IC50 value for ascorbic acid was found to be 5.6 μg/mL (Fig. 7d). The significant antioxidant activity of mycosynthesized Se NPs discloses its efficacy to serve as an effective antioxidant agent for various industrial and biomedical applications.

**Conclusion**

The present study demonstrated the potency of endophytic fungus *Fusarium oxysporum* to allow mycosynthesis of highly fluorescent, protein-capped, water dispersible Se
Fig. 6  a Antifungal activity of Se NPs against Aspergillus niger at different incubation periods of 24, 36, and 48 h. Sterile discs from A to F contain 240, 120, 60, 30, 15, and 7.5 µg of selenium nanoparticles, while disc G is filled with double-distilled water. 10 mcg fluconazole disc (H) is used as positive control. b Microscopic study suggesting antifungal and antisporulant activity of Se NPs against Aspergillus niger. The zone of inhibition and microscopic investigation clearly illustrate the antifungal and antisporulant efficacy of Se NPs.
NPs without the use of external capping agents. These spherical, crystalline Se NPs with average particle diameter of 42 nm have been fully characterized and their biodistribution, antiradical, antifungal, and antisporeulant activity were elucidated. The biodistribution studies unfolded the influence of capping protein which determines the preferential localization of Se NPs in liver and lungs. Our myco-synthesized Se NPs also showed excellent antifungal and antisporeulant activity against black fungus *Aspergillus niger* which has become life-threatening to SARS-CoV-2 patients during pandemic. At a time when drug resistance is rising to high levels, our mycogenic Se NPs exhibiting effective antifungal, antisporeulant, and antioxidant activity will find major use in the development of a novel and improved drug. Since our kidneys can filter up to 50 nm in size, our mycosynthesized Se NPs can treat lung and liver disorders and seamlessly pass through the kidneys and get expelled out of the body via urine. This will prevent selenium retention in the body, which might lead to selenium toxicity.

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**Declarations**

**Conflict of interest** The authors have no conflicts of interest in the publication.
Ethical approval The ethical standards are followed throughout the animal study and animal care standards set out by the institutional animal ethics committee (IEAEA/2018/174). They were given free access to food and drink while staying in groups of six. They were kept with a 12-h light/dim cycle at a constant room temperature.

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