Size Attenuated Copper Doped Zirconia Nanoparticles Enhances In Vitro Antimicrobial Properties

S. Nishakavya1 · Agnishwar Girigoswami1 · A. Gopikrishna1 · R. Deepa1 · A. Divya1 · S. Ajith1 · Koyeli Girigoswami1

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Abstract
Biofilm formation hinders the activity of antimicrobial drugs at the site of infections and any agent that can act on both Gram-positive and Gram-negative bacteria by inhibiting the bacterial growth and rupturing the biofilm is needed to manage infection. In the present study, we have synthesized zirconia nanoparticles (ZrO2 NPs) and copper doped zirconia nanoparticles (Cu-ZrO2 NPs) and characterized them using dynamic light scattering, X-ray diffractometry, and scanning electron microscopy (SEM). The size of the Cu-ZrO2 NPs drastically reduced compared to ZrO2 NPs, and the antimicrobial activity was studied against Gram-positive bacteria (Lactobacillus sp.) and Gram-negative bacteria (Pseudomonas aeruginosa), respectively. The synthesized Cu-ZrO2 NPs showed superior inhibitory action against Lactobacillus sp. compared to ZrO2 NPs, due to the negatively charged cell wall of Lactobacillus sp., which could attract readily the positively charged Cu-ZrO2 NPs, thereby inhibiting its activity. The biocompatibility was tested using XTT assay in FL cells, and the results demonstrated that Cu-ZrO2 NPs were nontoxic to mammalian cells. Hence, it could be proposed that the synthesized Cu-ZrO2 NPs possess possible biomedical applications and can be used as antibacterial agents without causing toxicity in mammalian cells.

Keywords Zirconia · Copper doped zirconia · Antimicrobial properties · Biofilm inhibition · Nanoparticles

Introduction
The nanoparticles (NPs) engineered from different metal oxides are immensely used in sunscreens, coating with self-cleaning properties, cosmetics, water treatments, paints, polymeric coatings, etc. [1, 2]. The metal oxide NPs have characteristic chemical and physical properties as they have an ultrasmall size and a huge density of surfaces and...
edges, enhancing their surface area. Some researchers have demonstrated that metal oxide NPs are also toxic to zebrafishes as well as some organisms [3, 4]. Since the metal oxide NPs exist in a wide range of shape, size, chemical composition, and functionalization of surface, they pose different risks in the organisms. The material’s electronic properties affect the size effect of metal oxides [5]. The semiconducting nature of the metal oxide NPs is due to the bandgap of these NPs which consists of orbitals indicating the electronic structure of a semiconductor. As the NP size decreases, the number of atoms present on the surface and interface increases, generating strain or stress resulting in structural perturbation [6].

Zirconium dioxide (ZrO₂) is a refractory and chemically inert metal oxide that exists in several crystalline phases depending on temperature. ZrO₂ has a thermodynamically stable crystalline phase of monoclinic (1170°C), tetragonal (1170–2370°C), and cubic phase (2370–2680°C) under atmospheric pressure [7]. Although the monoclinic phase of ZrO₂ is only thermodynamically stable at room temperature, tetragonal and cubic zirconia can also exist at metastable phases at this temperature. Moreover, because of the low surface energy, the zirconia NPs (ZrO₂ NPs) are preferentially stabilized with the tetragonal structure [8]. The tetragonal phase of ZrO₂ is more valuable for applications in technology compared to the monoclinic phase, due to its vital role in transformation toughening [9]. ZrO₂ exhibits a unique set of properties such as high corrosion resistance, chemical inertness, ionic conductivity, excellent thermal stability, higher isolectric point, lack of toxicity, low thermal conductivity, mechanical strength, and good biocompatibility [10–12]. Although the tetragonal structure is very unstable, the most common stabilization method in the tetragonal phase is the doping of zirconia lattice by bi- or trivalent ions [13]. Zirconia ceramics are becoming an appropriate material for biomedical applications such as hip joint replacement material and dental implants because of white color and their excellent biocompatibility [14].

ZrO₂ NPs are doped for increasing the stability using metal oxides like Y₂O₃, MgO, CaO, Cr₂O₃, Fe₂O₃, NiO, and CuO, yielding cubic and tetragonal phases [15]. Doping with copper to the ZrO₂ NPs can improve its antimicrobial activity, yielding a superior bionanomaterial compared to only ZrO₂ NP. When ZrO₂ NPs are doped with Cu, the process allows stabilizing the tetragonal state, makes the surface of the NPs more active to sintering, and reduces the probability of martensitic tetragonal-monoclinic transition during cooling powders from higher temperatures (higher than 1100°C). The durability of ceramic materials can be increased by varying the type or amount of dopants [13]. So far, there has been no report on the use of copper doped ZrO₂ NPs (Cu-ZrO₂) as an antimicrobial nanoparticle for its use in biomedicine. Copper NPs are capable of influencing the cell membrane by making structural changes, along with bactericidal properties due to the ability of copper to donate and accept electrons continuously.

In the present study, we have synthesized ZrO₂ NPs and Cu-ZrO₂ NPs by sol-gel method and characterized those using different photophysical techniques like dynamic light scattering (DLS), X-ray diffractometry (XRD), and scanning electron microscopy (SEM). The antimicrobial activity and anti-biofilm activity of ZrO₂ NPs and Cu-ZrO₂ NPs were assessed for *Pseudomonas aeruginosa* and *Lactobacillus* sp., using turbidity assay and standard crystal violet staining method, respectively. The biocompatibility of the synthesized NPs was also evaluated by XTT assay using FL cells (HeLa derivative) and live-dead cell assay using A549 cells. The purpose of this study was to isolate a superior nanoparticle compared to ZrO₂ NPs with high bacteria-resistant properties.
Materials and Methods

Materials

Zirconium alkoxide, copper nitrate, ethanol, nitric acid, DMEM, antibiotics solution (penicillin and streptomycin), trypsin-EDTA, XTT assay kit, acridine orange, and ethidium bromide were procured from HiMedia, India. Fetal bovine serum was purchased from Gibco, South America. FL and A549 cells were procured from NCCS, Pune.

Sol-Gel Synthesis of Zirconia and Copper Doped Zirconia Nanoparticles

The synthesis of ZrO₂ NPs and Cu-ZrO₂ NPs were done according to Asadi et al. [15], with slight modifications. Zirconium alkoxide (Zr(OCH₂CH₂CH₃)₄) was used as a precursor for the synthesis process. To synthesize pure ZrO₂, 4.25 ml zirconium alkoxide was added to 18.75 ml ethanol and then stirred well. Ethanol was used as a medium to complete the polymerization uniformly, and nitric acid (HNO₃) was used to adjust the pH (4.0). Further, a mixture of 1.485 ml of distilled water and 6.25 ml ethanol was added slowly to the prior mixture of zirconium alkoxide and ethanol. Then, it was allowed to stir for additional 2 h to complete the polymerization and aging process.

To synthesize the Cu-ZrO₂ NPs (5 % doping by copper), 3.705 ml zirconium alkoxide was added to 0.151 g of copper nitrate (Cu(NO₃)₂.3H₂O), and further the mixture was stirred in 18.75 ml of ethanol and the pH was adjusted to 4.0 with nitric acid (HNO₃). A mixture of (1.485 ml water and 6.25 ml ethanol) was added slowly to the prior mixture of zirconium alkoxide and ethanol. Then it was allowed to stir for 2 h further to complete the polymerization and aging processing. The gel obtained after aging for both the synthesized ZrO₂ and Cu-ZrO₂ NPs was dried and calcinated at 300 °C and crushed in mortar pestle to obtain a fine powder. The synthesized ZrO₂ NPs and Cu-ZrO₂ NPs were characterized using UV-visible spectroscopy, DLS, XRD, and SEM according to Girigoswami et al. [3, 16]. For UV-visible spectroscopy, DLS, and zeta potential, we have dissolved the powdered nanoparticles in double distilled water and sonicated two times in bath sonicator for 15 min each with a 10-min interval for cooling. The sonicated nanoparticle samples were suspended in distilled water and taken in a quartz cuvette for procuring the measurements. Powdered XRD was done at SAIF, IIT, Madras, where CuKα was the source in the diffractometer with 1.54-Å wavelength, and the particle size was calculated according to the Debye-Scherrer equation.

\[
D = 0.9 \frac{\lambda}{\beta \cos \theta}
\]

where

\(D\) is the crystal size
\(\lambda\) is the wavelength of X-ray
\(\beta\) is the full width at half maximum (in radians)
\(\theta\) is the angle of diffraction (in radians)

The samples were prepared for SEM analysis by spreading a thin film of nanoparticle suspension over clean and dust-free aluminum foil and dried under dust-free atmosphere.
Antimicrobial Activity Assay

The synthesized ZrO$_2$ NPs and Cu-ZrO$_2$ NPs were evaluated for inhibition of bacterial growth against both Gram-positive and Gram-negative bacterial strains such as *P. aeruginosa* (Gram-negative) and *Lactobacillus* sp. (Gram-positive) according to Mftah et al. [17]. UV-visible spectrophotometer (Shimadzu (Japan) UV-1800) was used for this study. The bacterial cultures were maintained on LB agar. These cultures were incubated overnight in 5 ml LB broth at 37 °C with a shaker speed of 150 rpm until the culture reached an absorbance of 1.0 at 600 nm, corresponding to $10^8$ colony-forming units per ml.

The antimicrobial activities of the synthesized ZrO$_2$ NPs and Cu-ZrO$_2$ NPs were evaluated against the abovementioned microorganisms using a turbidity test. For both Gram-positive and Gram-negative bacteria, 4.5 ml of LB broth was taken in test tube and 0.5 ml of the overnight microbial suspension was added in each test tube. Different concentrations (50 μg/ml, 100 μg/ml, and 150 μg/ml) of ZrO$_2$ and Cu-ZrO$_2$ NPs, only antibiotics (ampicillin for Gram-positive and streptomycin for Gram-negative) which served as a positive control, were added to the test tubes containing cultures. After incubation of a further 24 h at 37 °C, the O.D. at 600 nm was measured. The experiment was performed in triplicate, and the antimicrobial activity was compared between the ZrO$_2$ NP and Cu-ZrO$_2$ NP treated bacteria cultures taking the antibiotic-treated O.D. as 100 %.

Biofilm Formation Assay

Growing a Biofilm

The wild-type *P. aeruginosa* and *Lactobacillus* sp. culture was grown overnight in an LB medium, respectively, and used for biofilm formation assay according to O’Toole GA [18]. The overnight culture was diluted to 1:100 into fresh medium for biofilm assays. A standard biofilm assay medium for *P. aeruginosa* as well as *Lactobacillus* sp. was used; i.e., M63 minimal medium was used which was supplemented with magnesium sulfate, casamino acids, and glucose. In 96-well plates, 100 μl diluted bacterial culture was added and was incubated for 72 h at 37 °C for the formation of biofilm. We typically used 3 replicate wells for each treatment.

Staining the Biofilm

Post incubation, the cells were discarded by turning the plate upside down and shaking away the liquid and then submerging the plate in a tray of water. This process was repeated two times to remove the unattached cells and the media components that may get stained, which significantly lowered the background staining. In each well, 125 μl of 0.1 % crystal violet (CV) solution was added and kept at room temperature for 10–15 min. Further, it was rinsed 3–4 times with water by submerging in a tray of water and blotted vigorously on paper towel stacks to remove the excess cells and dye from the plate. The plate was dried overnight.

Quantifying the Biofilm

The quantification of the biofilm was done by adding 125 μl of 30 % acetic acid in water into each well of the microtiter plate stained the previous day, to solubilize the CV. The
microtiter plate was further incubated at room temperature for 10–15 min. A total of 125 μl of the CV, which got solubilized, was transferred to a new flat-bottomed microtiter dish. Absorbance was measured in a Robonik ELISA plate reader at 550 nm, taking 30 % acetic acid as the blank.

**Biocompatibility Study Using XTT Assay**

As done earlier, FL cells were maintained in DMEM supplemented with 10 % FBS and 1 % antibiotics at 37 °C in a 5 % CO₂ humidified atmosphere [16]. The XTT assay was done following the manufacturer’s protocol (HiMedia). A total of 10⁴ cells were seeded in 96-well plate and allowed to adhere for 24 h. After 24 h, the cells were treated with different concentrations (30 μg/ml, 50 μg/ml, 100 μg/ml, and 150 μg/ml) of ZrO₂ and Cu-ZrO₂ NPs. After 48 h of treatment, the XTT dye was added to the cells under sterile conditions and allowed to incubate at 37 °C for 4 h. The metabolically active cells turned the solution orange in color and its O.D. was measured using a Robonik ELISA plate reader at 450 nm. The percentage of viability was calculated as done earlier [16].

**Live‑Dead Cell Assay Using Acridine Orange and Ethidium Bromide**

The study of live and dead assay (apoptosis) was done using the fluorescent dyes acridine orange (AO) and ethidium bromide (EtBr) according to Liu et al. [19] with slight modifications. A549 lung fibroblasts were used for this study. A549 cells were cultured in DMEM, supplemented with 10 % FBS and 1% antibiotic solution at 37 °C, humidified atmosphere inside a CO₂ incubator (5% CO₂). The cells were maintained in exponential phase by subculturing them two times a week. In a six-well plate, 10⁵ cells were inoculated and after 24h, treated with ZrO₂ and Cu-ZrO₂ at 100 μg/ml dose each. The dose was selected based on the XTT assay results, and two six-well plates were taken with each having duplicate wells for each treatment. Untreated cells were taken as control. The treatment was done for further 48 h, and 40 μl of filtered (0.22-μm filter) AO/EtBr dye (100 μg/ml) was added to each well and incubated for 3 h. After 3 h of incubation, the medium from all the wells of 6-well plate was aspirated (dead cells) to the 2 ml Eppendorf tube, respectively. The inverted microscope images were taken after the medium removal from 6-well plate, for imaging the live cells. Later, the 6-well plate was trypsinized, and the cells were suspended with DMEM and added to the same respective tubes where the dead cells were collected previously. Thus, each tube has both live cell and dead cells stained with AO/EtBr. It is known that live cells have an intact cell membrane and retain the AO dye, whereas the dead cells get stained with EtBr. To remove the excess dye, the cells were washed by centrifugation (1500 rpm for 5 min) and resuspended in 1 ml DMEM. From the cell suspension, 25 μl of cells was added on the grease-free glass slides and observed under Lumia in vivo live animal imaging system for detecting live cells (AO staining) and dead cells (EtBr staining) using proper filters for excitation and emission of the dyes. The excitation wavelengths for AO and EtBr were 500 nm and 430 nm, respectively, and the emission was recorded using DsRed filter available in the instrument. The images of the slides were captured in the system, and to quantitate the amount of fluorescence emitted for AO and EtBr, the ROI tool was used, and the photon flux was recorded as done earlier [20]. A graph was plotted taking the type of cell treatment along abscissa and photon flux along ordinate in logarithmic scale.
Statistical Analysis

All the experiments were done in triplicates and the mean and standard deviations were calculated and plotted in the graphs. Student’s *t*-test was used to calculate the significance level of the data.

Results

Characterization of the Synthesized Nanoparticles

The synthesized ZrO$_2$ and Cu-ZrO$_2$ NPs were characterized photophysically and photochemically for the measurement of hydrodynamic diameter and particle stability using zeta sizer, absorbance using UV-visible spectrophotometry, crystal structure using X-ray diffractometry, and surface morphology using scanning electron microscopy. Figure 1a and b showed the hydrodynamic diameter of the synthesized NPs with a major scattering peak at 321 nm for ZrO$_2$ and 240.5 nm for Cu-ZrO$_2$, respectively. The crystal size of the ZrO$_2$ and Cu-ZrO$_2$ were determined using the data obtained by X-ray diffractometry (XRD) according to Meenakshi et al. [3]. The Debye-Scherrer equation was used for calculating the crystal size. The crystal size was found to be 84 nm for ZrO$_2$ NPs (Fig. 1e) and 108 nm in Cu-ZrO$_2$ NPs (Fig. 1f), respectively. The nanoparticle stability can be assessed by using zeta potential values of the NPs when they are suspended in an aqueous solution. We have suspended the ZrO$_2$ and Cu-ZrO$_2$ NPs in water with very high dilutions, ultrasonicated for 20 min two times after a cooling time of 10 min. The particles were taken in plastic cuvettes and inserted with an electrode to measure the zeta potential using Malvern Zetasizer. The zeta potential of synthesized ZrO$_2$ and Cu-ZrO$_2$ NPs was found to be −6.93 mV and + 28.9 mV, respectively, as shown in Fig. 1(c and d). The increase in zeta potential values after copper doping shows the high stability of the NPs even after the decrease in their size.

The surface morphology of the synthesized ZrO$_2$ NPs was visualized using scanning electron microscopy. The NPs are perfectly spherical in shape as seen in Fig. 2a. The size of the synthesized zirconia NPs ranged between 175 and 200 nm, having an average diameter of nearly 190 nm. The SEM image of copper doped ZrO$_2$ NPs is shown in Fig. 2b. The shape of the NPs is also spherical, and the average diameter is 30 nm. The SEM images clearly indicated that after doping with copper, the size of ZrO$_2$ NPs has reduced nearly 6 times. The absorption spectra of synthesized ZrO$_2$ are shown in Fig. 2c, with a broad absorption range in the UV region. The maximum absorbance was observed at 335 nm, which is attributed to its UV light absorption properties. The energy-dispersive X-ray spectroscopy (EDS, EDX, EDXS, or XEDS) can be used for the chemical characterization of a sample. To determine whether the synthesis of ZrO$_2$ NPs was successful without any impurity, the EDX analysis was done. The data obtained from EDX analysis has spectral peaks corresponding to the different kinds of elements that the sample contains. The ordinate corresponds to the number of counts, whereas the abscissa corresponds to the energy of X-rays. Figure 2d shows the EDX analysis of the synthesized ZrO$_2$ NPs that contain 45.7 wt % oxygen (O) and 54.3 % zirconium (Zr). The result indicates that the synthesis was done successfully without any impurities.
Antimicrobial Activity

Antimicrobial activity was assessed using turbidity test at different concentrations of ZrO$_2$ and Cu-ZrO$_2$ NPs (50μg/ml, 100 μg/ml, 150 μg/ml). The activity was checked against...
Fig. 2 The SEM image of a ZrO$_2$ nanoparticles and b Cu-ZrO$_2$ nanoparticles. The absorption spectra (c) and EDAX (d) of ZrO$_2$ nanoparticles
Lactobacillus sp. and P. aeruginosa (Fig. 3), and the inhibitions were observed in both Gram-negative and Gram-positive bacteria. Compared to ZrO2 NPs, the Cu-ZrO2 NPs had higher antimicrobial activity against both Lactobacillus sp. and P. aeruginosa, respectively.

Biofilm Formation Assay

The concentration of ZrO2 and Cu-ZrO2 NPs for anti-biofilm activity was monitored at 50 μg/ml, 100 μg/ml, and 150 μg/ml (Fig. 4). The biofilm formation was observed in both bacteria types, Lactobacillus sp. and P. aeruginosa. The inhibition of biofilm was significantly higher for Cu-ZrO2 NPs in Lactobacillus sp. compared to only ZrO2 NPs, whereas the effect on inhibition of biofilm in the case of P. aeruginosa was similar for both ZrO2 and Cu-ZrO2 NPs at all the doses exploited (no significant difference). The schematic representation of biofilm inhibition is shown in Fig. 5.

Biocompatibility Assay

The exposure of FL cells to the different concentrations of ZrO2 and Cu-ZrO2 NPs (30 μg/ml, 50 μg/ml, 100 μg/ml, and 150 μg/ml) is shown in Fig. 6. The data shows that the ZrO2 NPs were toxic to the cells at a low dose, but the toxicity was reduced as the dose increased. On the other hand, Cu-ZrO2 NPs were not toxic to the FL cells at the different doses used. The decrease in toxicity of the ZrO2 NPs at a high dose may be attributed to the aggregation of the NPs, which did not enter the cells to exert their toxicity. Thus, it was safe to use Cu-ZrO2 NPs for their insignificant toxicity.

Live-Dead Assay

The population of A549 cells after exposure to 100 μg/ml was visualized under inverted microscope after removing the supernatant (dead cells) as shown in Figure S1. The amount of fluorescence emitted for AO and EtBr from the A549 cells 48-h post-treatment with ZrO2 and Cu-ZrO2 is shown in Fig. 7. Figure 7a, c, and e shows the AO fluorescence for control, ZrO2, and Cu-ZrO2 treated cells, respectively. The EtBr fluorescence emitted from A549 cells after control, ZrO2, and Cu-ZrO2 nanoparticle treatment is shown in Fig. 7b, d, and f, respectively. The photon flux emitted for A549 cells for AO and EtBr quantitated using the ROI tool is plotted in Fig. 7g.

Discussion

Nanotechnology has shown its profound application in biomedical science for its antimicrobial activity, biofilm inhibition, and also to control the proliferation of cancer cells [21–23]. Among the widespread oral diseases, dental caries is the most common disease [24], and nowadays treatments in the dental field using biomaterials can change the mechanical, chemical, and physiological conditions inside the oral cavity. The extent of change is dependent on the size as well as the quality of the biomaterial inserted for restoration, which further can affect the oral cavity microbiology [25]. ZrO2 is known to be doped with various stabilizers for the achievement of high strength along with fracture toughness [26]. A high propagation rate of osteoblasts was seen, showing an exceptional tissue
Fig. 3 Antimicrobial activity of ZrO$_2$ and Cu-ZrO$_2$ nanoparticles at different doses (50, 100, and 150 μg/ml) against *Lactobacillus* sp. and *P. aeruginosa* (* represents $p<0.001$)
response apart from the new formation of cortical bone in good density (around 97.5%) of the zirconia-based implants following the osseointegration period [27]. Dental Medicine Nanosystems (DMN) with innovative applications, dental materials for restorative systems, adhesives and bonding systems, cement and sealant systems, and tissue regenerative systems have been established [28]. Limited studies exist that have explored the antimicrobial effects of NPs counter to cariogenic and periodontal disease causing bacteria grown in the simulated oral condition [29].

Secondary caries is reported to be one of the important reasons for teeth re-restoration, irrespective of the used restorative material. Streptococcus mutans (S. mutans) and

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**Fig. 4** The percentage inhibition of biofilm formation by a ZrO$_2$ and b Cu-ZrO$_2$ nanoparticles at different concentrations (50, 100, and 150 μg/ml) for Lactobacillus sp. and P. aeruginosa (* represents $p<0.001$; ** represents $p<0.005$)

**Fig. 5** Scheme showing the destruction of bacterial biofilm by ZrO$_2$ nanoparticles and Cu-ZrO$_2$ nanoparticles
Lactobacillus have been used to study in vitro secondary caries for a long duration because S. mutants and Lactobacillus are capable of producing a series of acid. They can also survive at low pH for an extended period, directing toward teeth demineralization and caries lesion [30]. The Gram-negative bacteria responsible for dental caries is P. aeruginosa [31]. In general, the altered restorative materials or the secondary caries prevention involves two strategies: first to reduce the demineralization and/or enhance the remineralization of the hard tooth tissues and secondly to hinder the metabolism of bacteria related to caries and/or to reduce the bacterial count/inhibit the growth of bacteria present in the dental plaque or/and dentin with caries under restorations. Researchers have documented that such modified restorative materials can release doped ions such as zinc, Ag-Cu alloy, aluminum, calcium, and fluoride that can inhibit bacterial growth and/or reduce the colonization as well as acidogenicity of oral plaque, thereby contributing as an antibacterial agent and reduce the replacement rate of restoration [32].

In this study, we have successfully synthesized ZrO₂ NPs and characterized them using different photophysical tools. To incorporate antimicrobial activity to the synthesized ZrO₂ NPs along with its other properties like increased microhardness, its strong interaction with active phase, high thermal stability, and chemical inertness, we have doped the ZrO₂ NPs with a small amount of Cu (5 %) and wanted to study their antimicrobial activity and biocompatibility. The synthesis of ZrO₂ NPs and Cu-ZrO₂ NPs was done using the sol-gel method and was characterized using UV-visible spectroscopy, FTIR, DLS, zeta potential, SEM imaging, and EDX analysis. The XRD data showed the crystal size of ZrO₂ NPs was 84 nm and for Cu-ZrO₂ NPs 108 nm. The noise obtained in the XRD pattern shows the partial amorphous nature of the ZrO₂ NPs and Cu-ZrO₂ NPs (Fig. 1e and f). This may be attributed to the low calcination temperature that we have used (200 °C) during synthesis. The typical peaks obtained for ZrO₂ NPs at 20 value of 30 ° and 51 ° corroborates with the JCPDS pattern of ZrO₂ (cubic ZrO₂-27-0997 and tetragonal ZrO₂-80-0965). The two pictures showed that after doping with Cu to the ZrO₂ NPs, the crystal size has increased compared to ZrO₂. Dopants affect grain growth, and it is due to the strain in the lattice of compounds the size increases. In some studies, it has been found that the size of the crystallites of alkali-treated zirconia which was calculated from data obtained by XRD was around the

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**Fig. 6** The cell viability assessed using XTT assay for FL cells after treatment with ZrO₂ and Cu-ZrO₂ nanoparticles at different doses
Fig. 7 The fluorescence images captured using Lumia in vivo live animal imaging system. The dose of nanoparticles treated were 100 μg/ml for both ZrO$_2$ and Cu-ZrO$_2$. The fluorescence emitted by acridine orange from A549 cells without treatment (a) and 48h after treatment with ZrO$_2$ (c) and Cu-ZrO$_2$ (e) is depicted, respectively. The ethidium bromide fluorescence emitted from A549 cells without treatment (b) and 48h after treatment with ZrO$_2$ (d) and Cu-ZrO$_2$ (f) is shown, respectively. g Fluorescence intensity emitted from acridine orange and ethidium bromide as quantitated by photon flux is plotted for different treated groups in a semi logarithmic scale.
A similar XRD pattern was found in previous studies also, which showed a combination of both tetragonal and monoclinic structures, similar to our finding [33]. The absorbance spectrum of ZrO$_2$ NPs showed a broad peak in the UV region with a $\lambda_{\text{max}}$ of 335 nm (Fig. 2c). Previous studies have also demonstrated that the absorption spectrum of microwave synthesized ZrO$_2$ NPs have a broad absorption peak in the UV region [34]. Thus, the characteristic peak of ZrO$_2$ NPs was obtained supporting its synthesis. The hydrodynamic diameter of both ZrO$_2$ NPs and Cu-ZrO$_2$ NPs was monitored to assess their size. The hydrodynamic diameter of ZrO$_2$ NPs and Cu-ZrO$_2$ NPs was 321 nm and 240 nm, respectively, as obtained by DLS studies (Fig. 1a and b). The findings of DLS studies showed that the size of NPs reduced after doping with Cu. The incorporation of Cu may strain the lattice of the ZrO$_2$ NPs and reduce the size having a higher affinity for oxygen.

To further exploit the stability of the NPs, the zeta potential of synthesized ZrO$_2$ NPs and Cu-ZrO$_2$ NPs was observed. Zeta potential is taken as a measure to evaluate the stability of NPs [3]. The zeta potential of ZrO$_2$ NPs and Cu-ZrO$_2$ NPs was $-6.9$ mV and $+28.9$ mV, respectively (Fig. 1c and d). The results showed that even though the size of the NPs has reduced, it has increased the stability. This shows that Cu-ZrO$_2$ can be a superior nanoparticle compared to only ZrO$_2$, based on its small size and high stability. The surface morphology of ZrO$_2$ NPs and Cu-ZrO$_2$ NPs and the chemical composition of ZrO$_2$ NPs were monitored using SEM analysis (Fig. 2a and b) and EDX analysis (Fig. 2d), respectively.
The shape of both ZrO\textsubscript{2} NPs and Cu-ZrO\textsubscript{2} NPs was spherical, but the diameter was nearly 178 nm for ZrO\textsubscript{2} NPs and 30 nm for Cu-ZrO\textsubscript{2} NPs. The SEM results corroborated with our findings of DLS studies, where we found that the hydrodynamic diameter of Cu-ZrO\textsubscript{2} NPs was reduced compared to only ZrO\textsubscript{2} NPs. The chemical composition of ZrO\textsubscript{2} NPs was analyzed using EDX. The data showed that the peaks obtained by the EDX correspond to Zr and O. Thus, there was minimum impurity present in the synthesized ZrO\textsubscript{2} NPs, and our synthesis was successful.

In our study, antimicrobial activity using turbidity test of ZrO\textsubscript{2} NPs and Cu-ZrO\textsubscript{2} NPs showed the inhibitory action for both Gram-positive (\textit{Lactobacillus} sp.) and Gram-negative bacteria (\textit{P. aeruginosa}) (Fig. 3). The physicochemical characteristics of surfaces of specific materials are reported to influence the process of bacterial adhesion significantly [35]. Our results with Cu-ZrO\textsubscript{2} NPs also showed significant antimicrobial activity against \textit{P. aeruginosa} and \textit{Lactobacillus} sp., and we can propose that this nanoparticle can be used as an effective biomaterial with antimicrobial property. Zn and Cu have been reported to have a potential anti-biofilm capability, and this property was useful to prevent biofilm formation synergistically against \textit{S. mutans} and \textit{Streptococcus sanguinis} [36]. Our results for the assessment of anti-biofilm formation showed that ZrO\textsubscript{2} NPs and Cu-ZrO\textsubscript{2} NPs showed inhibition of biofilm formation against both bacteria \textit{Lactobacillus} sp. and \textit{P. aeruginosa}, respectively (Fig. 4). The inhibition was significantly higher for Cu-ZrO\textsubscript{2} NPs against \textit{Lactobacillus} sp. than only ZrO\textsubscript{2} NPs and is schematically illustrated in Fig. 5. Moreover, the biocompatibility of these synthesized NPs was needed to be assessed, because any agent which will come in contact with biological fluids needs to be biocompatible. The in vitro cell culture study showed that Cu-ZrO\textsubscript{2} NPs did not show any significant toxicity against FL cells when treated with low as well as high doses (Fig. 6), although the ZrO\textsubscript{2} NPs were found to be toxic at low doses (50 μg/ml), but nontoxic at high doses. The toxicity at a low dose may be attributed to its ultrasmall size, which could enter the cells and exhibit its toxicity. At high doses, there are possibilities of aggregation of the ZrO\textsubscript{2} NPs, and they may not enter the cells to elicit the toxic response.

After aspirating the medium containing the floating cells (dead) in an Eppendorf tube, the remaining cells were captured for imaging under an inverted microscope (Figure S1). Typical snapshots showed that the control cells and Cu-ZrO\textsubscript{2} treated cells had a similar number of cells attached to the plate, whereas the number of adhered cells in ZrO\textsubscript{2} treated cells was less than control and Cu-ZrO\textsubscript{2} treated plates. This showed that Cu-ZrO\textsubscript{2} nanoparticles could cause less damage leading to lower cell killing. Further to quantify the amount of apoptotic cells and live cells, AO/EtBr staining was done, and amount of fluorescence was recorded in Lumia in vivo live animal imaging system using appropriate filters. Equal amounts of cells were loaded to capture the fluorescence for all three groups, namely, control, ZrO\textsubscript{2}, and Cu-ZrO\textsubscript{2}. Figure 7a, c, and e show the AO fluorescence emitted by control, ZrO\textsubscript{2}, and Cu-ZrO\textsubscript{2} nanoparticles treated cells, respectively. EtBr fluorescence was shown in Fig. 7b, d, and f for control, ZrO\textsubscript{2}, and Cu-ZrO\textsubscript{2} nanoparticles treated cells, respectively. This emitted fluorescence was quantified by measuring the photon flux and plotted in Fig. 7g. The results show that the AO fluorescence depicting live cells had photon flux of $8.44 \times 10^9$ and $3.989 \times 10^9$ for control and Cu-ZrO\textsubscript{2} nanoparticles treated cells, respectively, whereas the value was $1.107 \times 10^9$ for ZrO\textsubscript{2} treated cells. This shows that minimum viability was observed for the ZrO\textsubscript{2} group compared to control and Cu-ZrO\textsubscript{2} group, and there were three times more live cells present in Cu-ZrO\textsubscript{2} treated cells than ZrO\textsubscript{2} treated cells. The results of dead cells represented by EtBr staining showed a photon flux of $6.56 \times 10^7$, $8.43 \times 10^8$, and $2.078 \times 10^8$ for control, ZrO\textsubscript{2}, and Cu-ZrO\textsubscript{2} treated cells, respectively. The number of dead cells was highest for ZrO\textsubscript{2} treated cells compared to control and
Cu-ZrO$_2$ treated group, and ZrO$_2$ treatment showed four times higher killing than Cu-ZrO$_2$ treatment. A similar result was observed for the inverted microscope image also, where we observed less adhered (live) cells for ZrO$_2$ group compared to the control and Cu-ZrO$_2$ group.

Thus, from our results, it can be concluded that doping of ZrO$_2$ NPs with Cu yielded a superior ZrO$_2$ NPs, which has unique properties of killing both Gram-positive and Gram-negative bacterial cells and could also inhibit the biofilm formation against *Lactobacillus* sp. Further studies are required to be done with other strains of bacteria and explore their efficacy in killing these bacteria, inhibiting biofilm formation.

**Conclusion**

We have shown that ZrO$_2$ NPs and Cu-ZrO$_2$ NPs were synthesized by the sol-gel method and characterized with various analytical techniques. The XRD study of pure ZrO$_2$ NPs and Cu-ZrO$_2$ NPs shows that both the synthesized particles are amorphous in nature, although the crystal size increase due to copper doping ensures that the doping is done successfully. The stability also increased after doping as revealed by the increase in zeta potential. Antimicrobial studies revealed that ZrO$_2$ NPs and Cu-ZrO$_2$ NPs both inhibited microbial growths as tested against the Gram-positive and Gram-negative bacteria, Cu-ZrO$_2$ being more effective at the doses used. The biofilm formation was also found to be inhibited by both the NPs without showing any significant difference in inhibition. The study showed that Cu doping in ZrO$_2$ NPs could enhance the protection against bacteria and thereby can be proposed as a superior antibacterial agent compared to only ZrO$_2$ NPs. The toxicity study of ZrO$_2$ NPs and Cu-ZrO$_2$ NPs in FL cells as well as A549 cells showed that Cu-ZrO$_2$ NPs were much more biocompatible to mammalian cells and can be safely used as a component for biomedical implants inside our body.

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**Data Availability** All the authors declare that all data and materials support their published claims and comply with field standards.

**Code Availability** Not applicable

**Author Contribution** Nishakavya S, Divya A, Deepa R, Ajith S, and Gopikrishna A have executed the experiments. Agnishwar Girigoswami and Koyeli Girigoswami have given the concept and prepared the final manuscript.

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**Declarations**

**Consent to Participate** Not applicable

**Conflicts of Interest** The authors declare no conflict of interest.
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