A STUDY OF WOUND HEALING AND ANTIBACTERIAL ZINC OXIDE NANOPARTICLE - POLY (N-ISOPROPYL ACRYLAMIDE)

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ABSTRACT

In current studies, zinc oxide nanoparticle poly-(N-isopropylacrylamide)-conjugate (ZnO-NIPAM) has been synthesized with the assessment of its wound repair and bacterial inhibition properties.The developed conjugates were aimed to determine its antibacterial activity with enhanced stability.Combination of nanomaterials with organic molecules is done to improve their activity and stability..Topographic analysis of the nanoparticle was investigated usingScanning electron microscopy of field emission –FESEM, antibacterial activity was evaluated using standard well diffusion method and wound-heal assay was determined using in-vitro scratch assay. The results proved significant antibacterial properties along with potential wound healing characteristics.UV-Vis and FESEM analysis showed the particle characteristics in support to literature survey. The obtained results shall be applicable for different pharmaceutical and biomedical studies in future.

Keywords: Zinc oxides, nano-particle, poly (N-isopropylacrylamide), N-isopropylacrylamide, Bacterial inhibition.

INTRODUCTION

In the current scenario, hygiene products are highly essential for the prevention of dissemination of bacterial pathogens. Several works were conducted world-wide to meet this objectives till date. Among them, nanoparticles play a crucial role to combat with infectious agents. Particularly, metal nanoparticles have significant characteristics to provide hygiene environment and products to consumers. Yasuyuki et al., 2010; Turner, (2017) reported that a number of metals (Al, Ag, Co, Cd, As, Zn, Cu, Fe, Ga, Hg, Mn, Mo, Ni, Sb, Pb, Te, Cr) have significant antimicrobial properties. Maret (2011) specifically highlighted the use of metal oxides as nanoparticles for more promising results in terms of antimicrobial properties. Zinc is easily available and it's well known oxide forms were reported to be highly potent against several types of bacteria and other microbes.

Different applications of zinc oxide nanoparticles (ZnO NPs) have been reported in the field of medicine, pharma and engineering. Beek et al., (2004) notified the application of ZnO NPs in solar cells, gas sensors (LPG sensors). Vaseem et al., (2010) and Baruwati et al., (2006) reported its application as chemical sensors and biosensors which were widely applicable in engineering sciences. ZnO nanoparticles have several advantages like, ability to accelerate wound healing (Mishra et al., 2017), the cytostatic activity against cancer cells (Mishchenko et al., 2019), anti-inflammatory activity (Agarwal and Shanmugam, 2020), antimicrobial and fungicidal activities (Dadi et al., 2019),

However, organism has adverse reaction over ZnONps to protect them using defense mechanism which is named as hormesis; it stimulates two level of defense like genomic and transcriptional. DNA polymerases have poor validation activity which leads to add abnormal bases in the strands. This in turn caused spontaneous mutations and genome plasticity (Tkachenko, 2018). Genome plasticity increases the resistance of microbes to metals and metal oxide nanoparticles (Graves et al., 2015).

To reduce the defence reactions of microbes against nanoparticles, significance of conjugates were highlighted recently. Many research articles were published with regards to conjugates and its applications. Noor Akbar et al., (2021) highlighted that ZnO NPs was conjugated with ampicillin, quercetin, ceftriaxone, and amphotericin-B. ZnO NPs were conjugated with bovine serum albumin for different applications (Pawan Kumar et al., 2013).Won et al. (2008) tested a different conjugation in which the particles were coupled to linoleic acid and gamma-linolenic acid..KandasamySaravanakumar et al., (2020) recently studied the anti-biofilm activity of zinc oxide nanoparticles + β-D-glucan conjugates

Ability of conjugated mediated zinc oxide nanoparticles was reported to provide different mechanism of actions as reported; damage to the cell membrane (Sirelkhatim et al., 2015), binding to DNA and proteins(Dutta et al., 2012), hindering in the processes of DNA amplification, and expression of genes (Xie et al., 2011).

Conforming to the benefits of nanoparticle medicated conjugates, a zinc oxide nanoparticle poly-(N-isopropylacrylamide)-conjugate (ZnO-NIPAM) was produced with the intention of looking at its wound repairing and bacterial inhibition powers in current research.Topographic analysis of the nanoparticle was investigated using Field emission scanning electron microscopy and its biocompatibility was investigated using MTT assay.

MATERIALS AND METHODS

Synthesis of Zinc oxide nanoparticles (Yadav et al., 2006)

The common method reported by Yadav et al., was utilized for synthesizing zinc oxide nanoparticles. (2006). In general, a wet chemical technique was selected with zinc nitrate and sodium hydroxide as precursors chemicals whereas soluble starch is used as a stabilizing agent.In 500 ml of distilled water with zinc nitrate, different quantities of soluble starch (0.1%, 0.5%, and 1.0%) were dissolved. (14.874g - 0.1M). To dissolve the zinc nitrate wholly, the mixture was continuously agitated. For 2 hours, 0.2 M of a sodium hydroxide solution was added drop wise. The supernatant was disposed of after allowing the solution to settle overnight. The resulting solution was centrifuged at 10,000X g for 10 minutes to obtain nanoparticles. (NPs). NPs were washed and dried overnight at 80°C to convert zinc hydroxide to zinc oxide.

Developing Zinc oxide nanoparticle poly-(N-isopropylacrylamide)-conjugate (ZnO-NIPAM) (Ruben and Wolfgang, 2007)

Zinc oxide nanoparticle poly-(N-isopropylacrylamide)-conjugate (ZnO-NIPAM)adopting the technique established by Ruben and Wolfgang (2007.The method was described in our previous publication (Basil Baby and) (fig-9)

Bacterial inhibition of the ZnONps and ZnO-NIPAMconjugate

The bacterial inhibition efficiency of ZnONps and ZnO-NIPAM conjugates were determined against test bacteria (*Staphylococcus aureus*, *Pseudomonas aeruginosa*,*Enterococcusfaecium* ,*Proteus* spand*Klebsiella pneumonia*) using a standard well diffusion method. Mueller-Hintn agar (MHA) plates were prepared using dehydrated media (Hi Media Laboratories, Mumbai, India). Over the prepared media surface, 0.1ml of each test bacteria was uniformly swabbed evenly using 12h cultures. In both of the sides of solid media plates, two 6mm well was made using a sterile cork-borer/well cutter. About 20µl of the samples (ZnONps and ZnO-NIPAM conjugates) were loaded under sterile conditions. All the plates were incubated at 37°C for 24h. The inhibitory zones around the well were measured in millimeter and recorded separately.

Zn nanoparticle chemical characterization

Scanning electron microscopy ofField emission (FESEM)

The morphology and size of the prepared zinc oxide nanoparticles analysis was performed using scanning electron microscopy of field emission (JSM-7500F, JEOL, Japan). A drop of nanoparticle powder on a copper grid that had been coated with carbon before being transported to the microscope. High-resolution photographs of zinc oxide nanoparticles were taken, and the shape and morphology of ZnNPs has been studied further.

UV-Vis spectrophotometer examination

The UV-Vis spectrophotometer (Elico–BL 198) was employed to determine the wavelength at regular intervals, a wavelength between 300 and 800nm was used to examine the generation of zinc oxide nanoparticles. When the sample became overly concentrated, dilutions were made. The UV-visible reading has been taken and then analysed with Origin Pro or Microsoft Excel.

ZnO-NIPAM nanocomposite covering wound healing materials

Wound healing materials were coated with the developed conjugate using the typical two dip-coating process described by Gollwitzer et al. (2003). The method was well explained in our previous publication (Basil Baby and). After coating the materials, all samples were subjected to FESEM analysis and wound healing studies.

Antibacterial activity of ZnO-NIPAM coated wound healing materials

Antibacterial activity of ZnO-NIPAM coated wound healing materialswas studied using standard well diffusion method against wound causing organisms. The method was well explained in our previous publication (Basil Baby and). All experiments were run three times to get standard deviation data using the SPSS - 9 (Windows 7.0) software.

In vitro wound scratch test

Wound healing ability of developed conjugate on L_{929} mouse fibroblast cell lines were studied using a standard in vitro wound scratch model. The steps and procedure was well explained in our previous publication (Basil Baby and).

RESULTS AND DISCUSSION

Bacterial inhibition of ZnONps and ZnO-NIPAM conjugates

Bacterial inhibition analysis of ZnONps and ZnO-NIPAM conjugates was determined according to the Microbiological standards (well diffusion method). The ability of the samples to inhibit the growth of test bacteria was measured in millimeter and recorded in Table-1. Among the test bacteria, *Enterococcus faecium*showed 19mm and 14mm for ZnO-NIPAM and ZnONps. *Staphylococcus aureus* exhibited 17mm and 12mm for ZnO-NIPAM and ZnONps. About 18mm, 17mm and 18mm of zones were obtained for ZnO-NIPAM conjugates and 13mm, 11mm and 13mm were observed for ZnONps against *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus* sp respectively (Fig. 1, Fig.7).

Significant inhibitory zones were observed for Zinc oxide nanoparticles. Thus, our results well correlate with the previous studies of the other authors. In order to increase the stability of the nanoparticles N-isopropylacrylamide (NIPAM) were conjugated with the zinc and silver nanoparticles. The inhibitory zones were found to be higher for the conjugates i.e the NIPAM also exerts bacterial inhibition. The nanoparticles when combined with NIPAM shows synergistic mode of action. Conjugation of metals with NIPAM has a lots of advantages like good stability, excellent bio-compatibility, large surface-to-volume ratio, and controllable particle size, extensive adsorption of proteins (Ya Sun *et al.*, 2019).

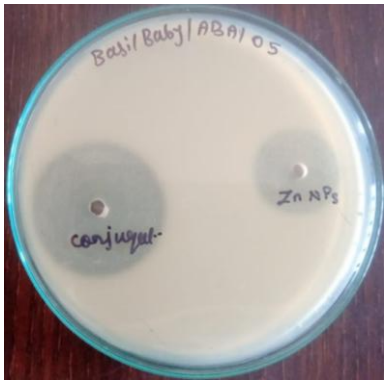
Table-1: Bacterial inhibition of ZnONps and ZnO-NIPAM conjugates

S. No	Test Organisms	Inhibitory zones (mm)	
		ZnO-NIPAM	ZnONps
1	*Enterococcus faecium*	19	14
2	*Staphylococcus aureus*	17	12
3	*Klebsiellapneumoniae*	18	13
4	*Pseudomonas aeruginosa*	17	11
5	*Proteus* sp	18	13

Fig. 1: Bacterial inhibition of ZnONps and ZnO-NIPAM conjugates

Enterococcus faecium *Staphylococcus aureus*

Klebsiella pneumonia *Pseudomonas aeruginosa*



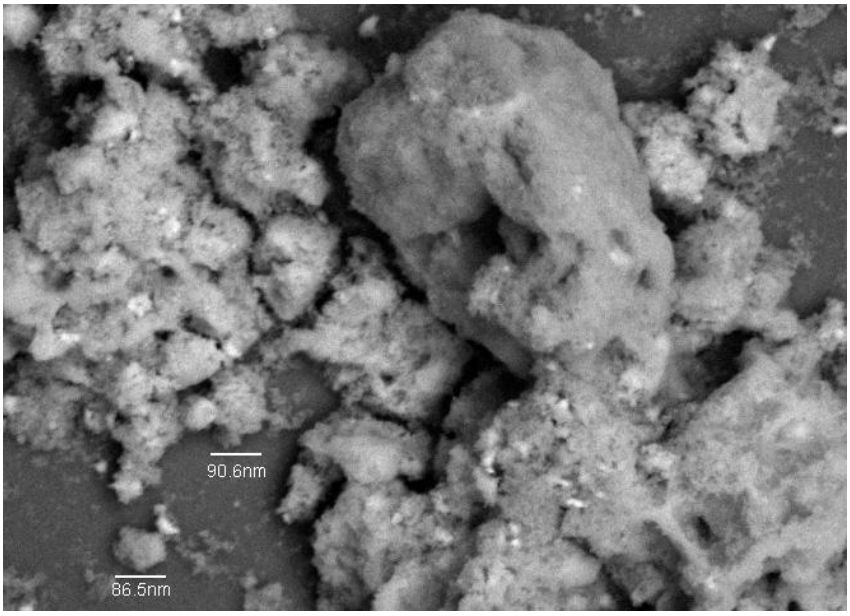
Proteus sp

Scanning electron microscopy of Field-emission -ZnO NPs

Size, shape, general morphology and particle distribution of obtained nanoparticles were topographically observed using scanning electron microscope offield-emission (FESEM).

In Fig. 2, nanoparticles were found as coalesced ornano-clusters. This may be due to indirect heating the reaction mixtures of the synthesis medium for 48hours; and hence the nanoparticles were found tend to be aggregated.

Fig. 2: Field-emission scanning electron microscopy analysis of ZnO NPs

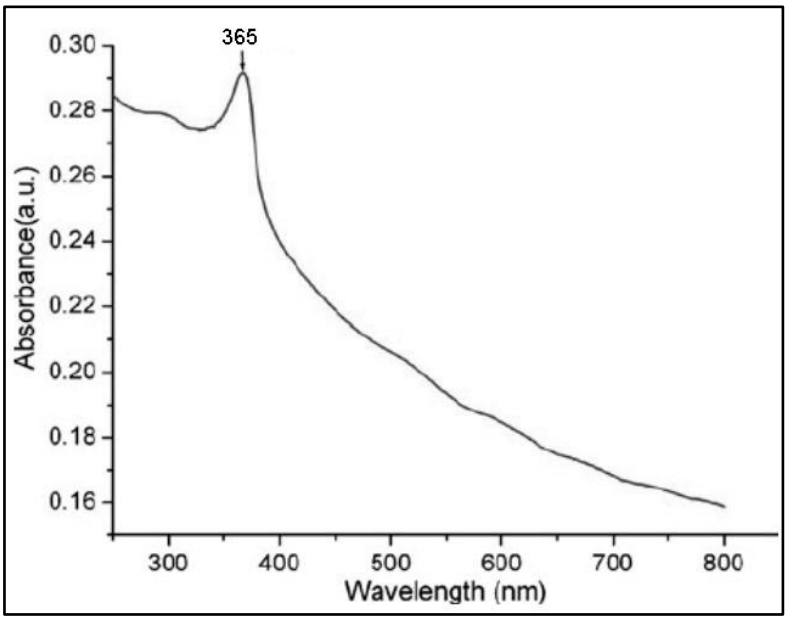


Coalesced or nano-clusters of nanoparticles (varied size of 86.5nm to 90.6nm)

UV-Vis spectrophotometer analysis - ZnO NPs

To prove the formation of ZnO as nanoparticles, the method was studied at 365nm of wavelength. In Fig. 3, the absorption spectrum of synthesized ZnO NPs was presented.At the measured wavelength, an intrinsic band-gap of Zn-O absorption was observed.A similar result was achieved for the absorption band that represents ZnO NPs between the wavelength of 350nm to 380nm. Akhil et al., (2017) and Zak et al., (2011) obtained absorption peak at 370nm respectively which were found very close to the absorption peak (365nm) obtained in our present study. Bian et al., (2011) also obtained a close peak at 371nm followed by Lavand et al., (2015) at 375nm and Talam et al., (2012) at 355nm.

Fig. 3: UV-Vis spectrophotometer studies of ZnO NPs



ZnO-NIPAM antibacterial properties-coated wound healing materials

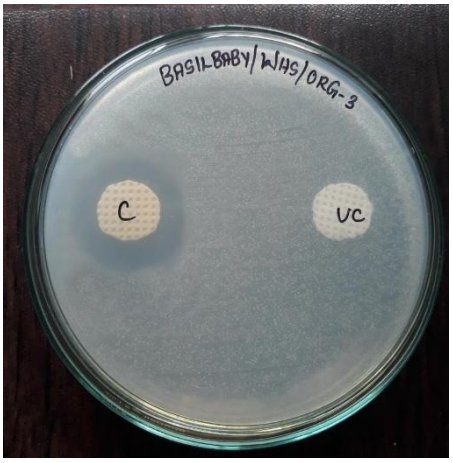
Antibacterial activity of ZnO-NIPAM coated wound healing materials when tested against wound pathogens showed good inhibitory zones around the sample. Maximum inhibitory zone of 33±1.05mm was obtained against *Staphylococcus aureus.Enterococcus faecium*expressed 32±0.57mm, *Pseudomonas aeruginosa*revealed 29±1.25mm, *Klebsiella pneumonia* and *Proteus* sp. exhibited 29±1.25mm and 29±1.05mm of inhibitory zones respectively (Table-2, Fig-4 and, Fig-6). Antibacterial mechanism of zinc oxide nanoparticles depends on its concentration and size. Khwaja*et al.,* (2018) revealed that concentration between 20mg/l to 100 mg/l with average particle size of 480nm have potent antibacterial mode of action. (Fig-8) The specified sized particles permeated in to cell or plasma membrane of bacteria targeting to damage lipids, proteins, carbohydrates and DNA.

Table-2: Antibacterial activity of the ZnONps and ZnO-NIPAM conjugates

S. No	Test Organisms	Inhibitory zones (mm)	
		ZnO-NIPAM Coated material	**Uncoated material**
1	*Enterococcus faecium*	32±0.57	0
2	*Staphylococcus aureus*	33±1.05	0
3	*Klebsiellapneumoniae*	29±1.25	0
4	*Pseudomonas aeruginosa*	30±0.75	0
5	*Proteus* sp	29±1.05	0

Fig. 4: Antibacterial activity of the ZnONps and ZnO-NIPAM conjugates

Enterococcus faecium *Staphylococcus aureus*



Klebsiella pneumonia *Pseudomonas aeruginosa*

Proteus sp

In-vitro woundscratch assay

Wound closure, cell migration and cell proliferation was investigated under in-vitro condition. Developed conjugate, ZnO-NIPAM showed promising results in terms of wound healing when tested under in-vitro conditions. Following observations were recorded in the present study.

An evident scratch was made on L_{929}fibroblast cell-lines. From 0^{th} hour to 24^{th} hour, healing was measured. During this period, cell migration was found evident due to significant number of cell proliferation. This facilitated the wound closure and healing within the specified incubation period. In Fig. 5, step wise cell migration, cell proliferation and wound healing was recorded and presented. At 0^{th} hour, no cell migration was observed. After 12^{th} hour, cell-migrationwas found evident. After incubating for 24hours, cell-proliferation facilitating towards wound closure was significantly observed.

The obtained results were found supportive to the research work as per literature survey. *Peltophorumpterocarpum* leaf extracts where subjected to scratch assay by Annamalai et al., (2019). The researchers found promising wound closure properties during the assay. *Beta vulgaris* extracts where used by the researchers, Udayakumar et al., (2020) to investigate the wound closing ability.The researchers also compared different Psidiumguajava plant extracts. When compared to Psidiumguajava, Beta vulgaris had better

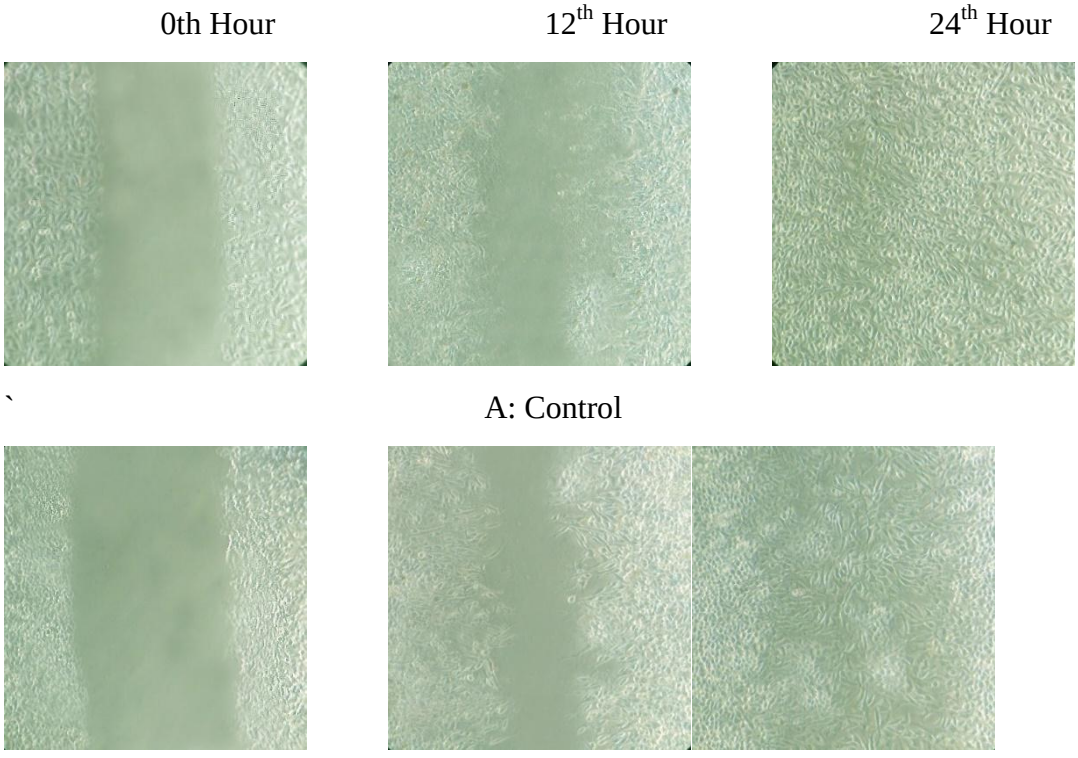
cell-epithelialization. Srinivasa Rao Bolla et al. employed Aristolochiasaccata leaf extract to determine wound healing properties under invitro environments in yet another study. (2019). Microscopic studies revealed positive and promising wound closing abilities of the leaf extracts with 48 hours. Maximum of about 99.05% wound closure was found evident. These results were found supportive for the developedZnO-NIPAM conjugate interms of wound closing ability.

Conclusion

ZnONps and ZnO-NIPAM composites underwent bacterial inhibition research in accordance with the Microbiological standards. (well diffusion method). The samples' capacity to stop the development of test bacteria was quantified in millimetres and listed in Table 1. Enterococcus faecium demonstrated 19mm and 14mm for ZnO-NIPAM and ZnONps, respectively, among the test microorganisms. For ZnO-NIPAM and ZnONps, Staphylococcus aureus displayed 17mm and 12mm, respectively. Zones of about 18mm, 17mm, and 18mm were obtained for ZnO-NIPAM composites, while ZnONps against Klebsiella pneumonia, Pseudomonas aeruginosa, and Proteus sp. were noted at 13mm, 11mm, and 13mm, respectively. (Fig. 1).

Zinc oxide nanoparticles were found to have significant inhibiting zones. Our findings therefore closely align with those of the other authors' earlier research. The zinc and silver nanoparticles were conjugated with N-isopropylacrylamide (NIPAM) to improve the stability of the nanoparticles. The conjugates, i.e., the NIPAM, which also causes bacterial inhibition, were found to have higher inhibitory zones. When combined with NIPAM, the nanoparticles exhibit a synergistic mechanism of action. Excellent biocompatibility, decent stability, extensive protein adsorption, a high surface-to-volume ratio, and controllable particle size are just a few benefits of metals and NIPAM conjugation. (Ya Sun et al., 2019).

Fig. 5: Test for self-healing scratches: *In vitro* Wound Scratch Assay



B: ZnO-NIPAM Conjugate (100µg)

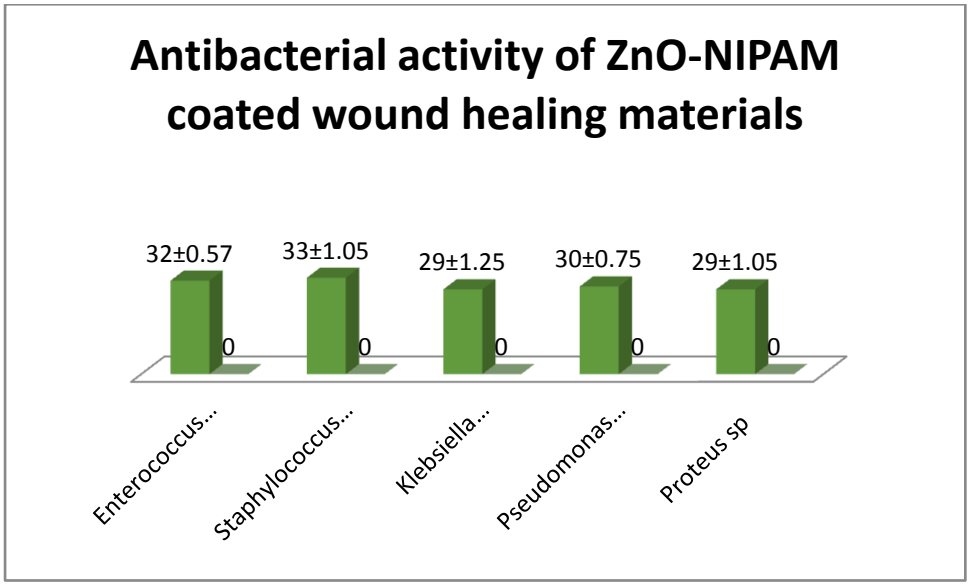


Fig. 6Antibacterial activity of ZnO-NIPAM coated wound healing materials

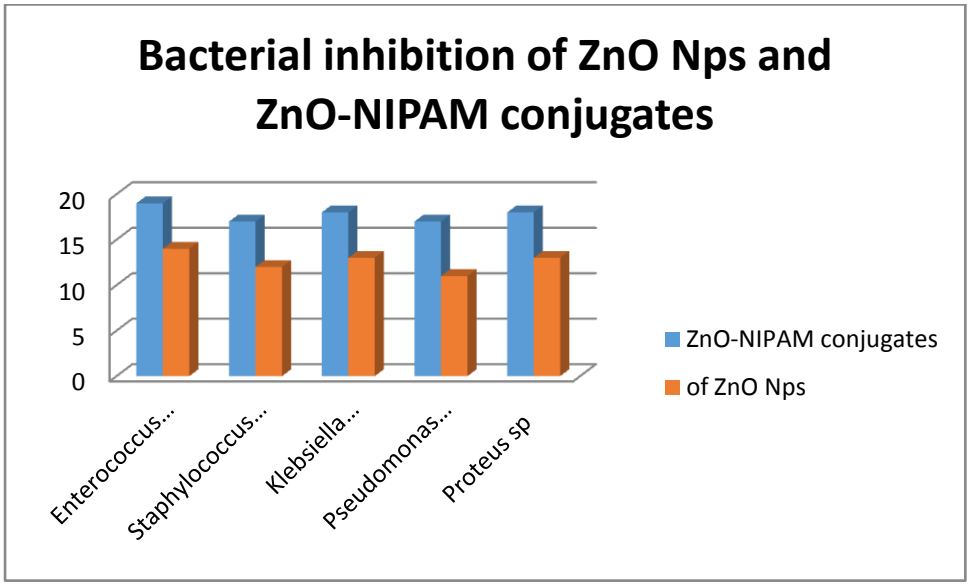


Fig. 7Bacterial inhibition of ZnONps and ZnO-NIPAM conjugates

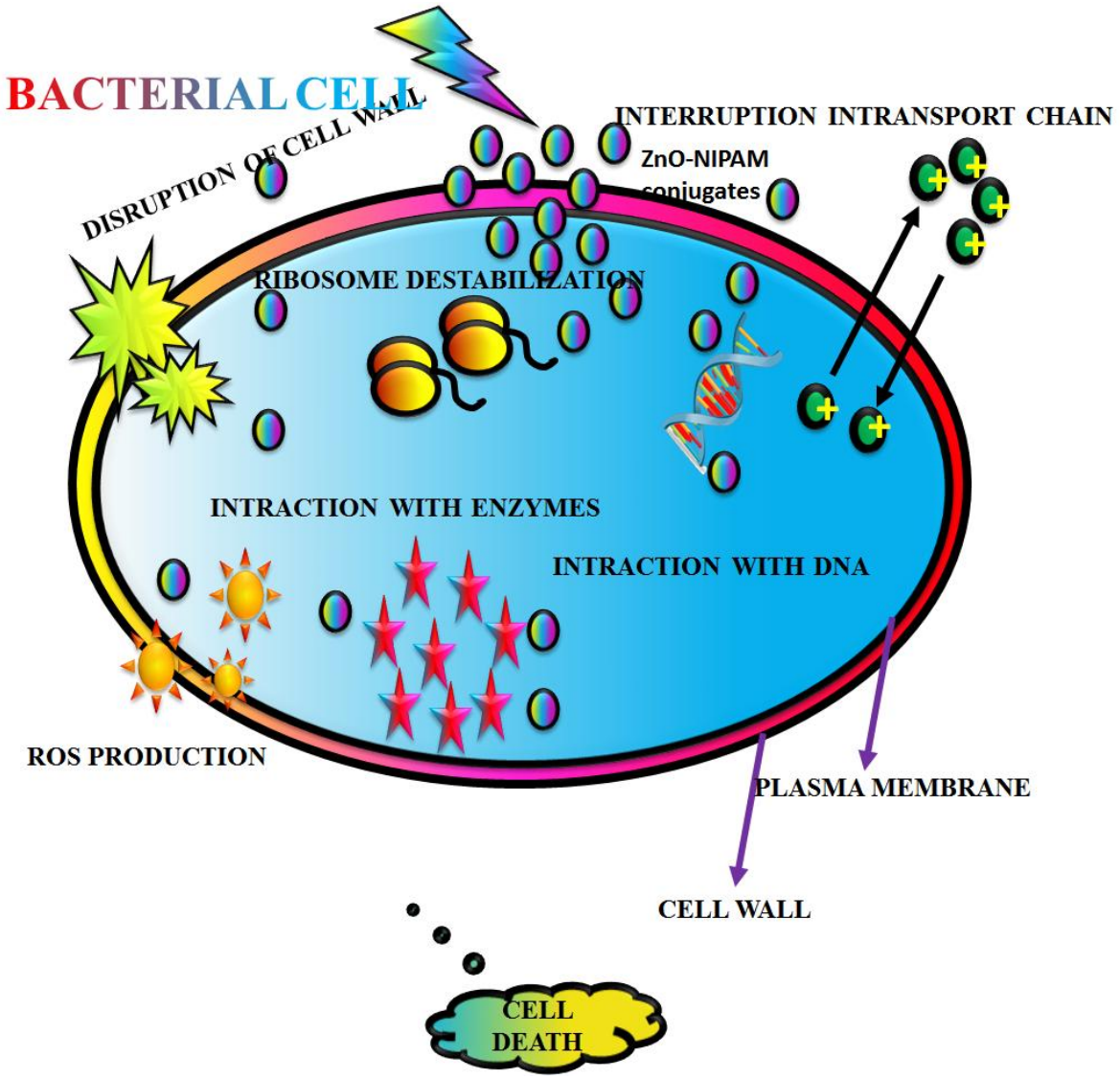


Fig. 8Antibacterial mechanism ofZnO-NIPAM coated wound healing materials

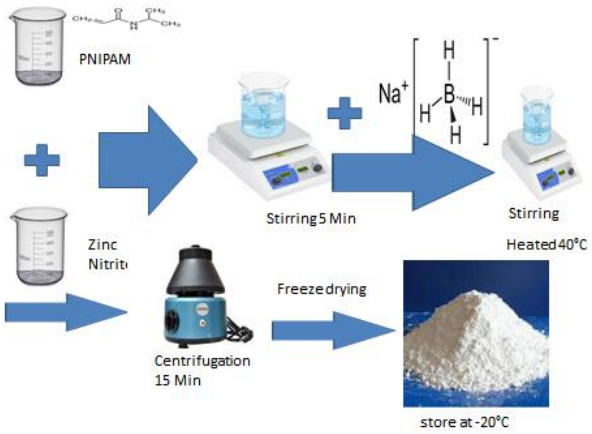
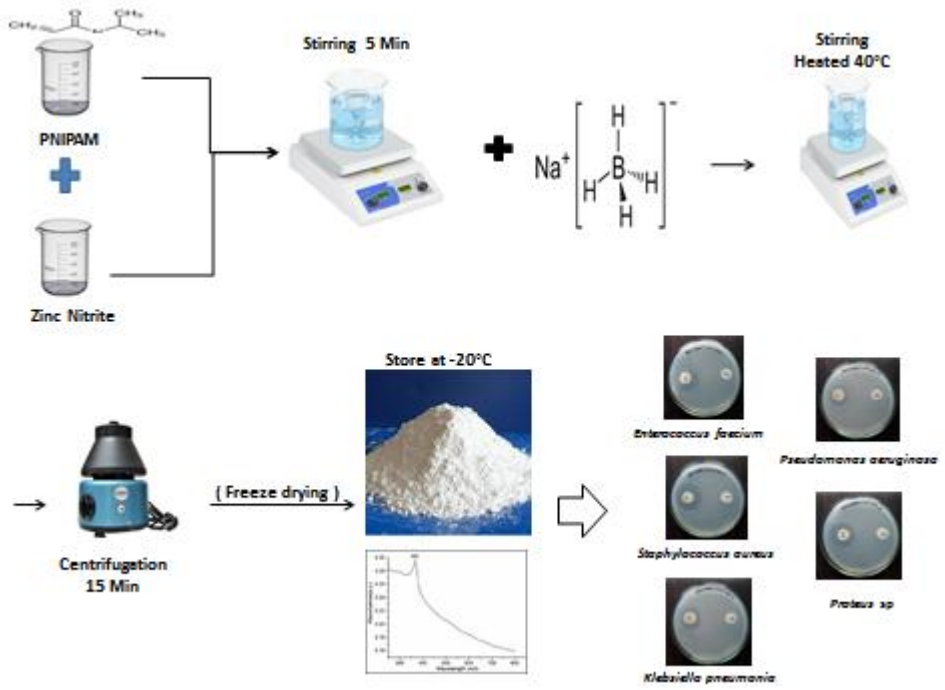


Fig. 9 Preparation of ZnO-NIPAM



ABSTACT DIAGRAM

Conflict of Purpose

In this study, the authors state that they have no conflicts of interest.

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