

Preparation of chitin nanogels containing nickel nanoparticles



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ABSTRACT

In this work, we developed 120–150 nm sized nickel nanoparticles loaded chitin nanogels (Ni-Chitin NGs) by regeneration chemistry approach to investigate and determine its cytocompatibility and antibacterial activity against *Staphylococcus aureus*. The nickel nanoparticles were prepared by hydrothermal method. The prepared Ni-Chitin NGs were well characterized by SEM, FTIR, TG/DTA/DTG and XRD and the *in vitro* cytocompatibility was tested on A549 and L929 cells which showed that they are completely non-toxic. Ni-Chitin NGs showed better toxicity to the bacterial strains when compared to previous study with other nanoparticles using serial dilution method. The rhodamine labeled-Ni-Chitin NGs showed cellular localization on both L929 and A549 cells without perturbing their cellular constituents. These studies showed that the Ni-Chitin NGs could be used for various applications in biomedical filed.

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1. Introduction

Nanogels are of great importance in biomedical field including drug delivery and tissue engineering. The characteristics of “nanogels” can be derived from their parent “Hydrogels” (Chacko, Ventura, Zhuang, & Thayumanavan, 2012). The ideal features of a nanogels include their optimum size (ranging from 10 to 200 nm), tunable degradation, high functionality, biocompatibility, excellent drug loading capacity, good release characteristics, high aqueous dispersion, prolonged blood circulation and immunocompatibility (Rejinold et al., 2012). Nanogels can be obtained through different methods including, chemical cross-linking of polymers (Soleimani et al., 2013), physical assembly of polymers, ionic and covalent based synthesis (Ryu et al., 2010), photo polymerization and radical polymerization cross linking (Sanson & Rieger, 2010). Recently, a number of reports which point out the use of drug loaded nanogels (Farag & Mohamed, 2012) for enhanced antimicrobial effects. The chelate of nickel with chitin shows the highest binding constant value among a number of transition metal ions as reported and it is amenable to a homogeneous dispersion of metal particles when treated with NaBH₄ (Muzzarelli, 2011).

Nano sized nickel has immense potential in electronics, medical diagnostics, and biotechnology. The size and shape of nickel

nanoparticles can be tuned to obtain high metallic and catalytic activity (Tayel et al., 2011). Recent reports have shown that the positively charged nickel nanoparticles can bind with negatively charged membranes of bacteria, thereby disrupting the membrane, inducing its antibacterial effects (Díaz-Visurraga, Gutierrez, Von Plessing, & García, 2011; Stoimenov, Klinger, Marchin, & Klabunde, 2002). The major limitations of metallic nanoparticles are toxicity (Li, Xu, Chen, & Chen, 2011) when a high dose is administered for the required anti-bacterial activity. This can be overcome by entrapping metallic nanoparticles inside a nanogel. Highly stable, swell able and cytocompatible chitin nanogels have been recently developed from our research group (Rejinold et al., 2012). Since chitin has antibacterial activity, it could have an adequate effect even after combining with nickel nanoparticles (Aranaz et al., 2009; Benhabiles et al., 2012).

Thus the aim of this work is to develop cytocompatible nickel nanoparticles loaded chitin nanogels for better anti-bacterial activity and to investigate cytocompatibility of the same on cell lines *in vitro*. The work mainly focuses on the material characterization, swelling and cellular localization in detail.

2. Materials and methods

2.1. Materials

Chitin (degree of acetylation 72.4%, molecular weight 150 kDa) was purchased from Koyo Chemical Co., Ltd., Japan. Calcium chloride and methanol were purchased from Qualigens, India.

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Staphylococcus aureus strain SA113 ATCC 35556, obtained from Amrita Institute of Medical Sciences, India. Luria Bertani and Agar-Agar, Type-I purchased from Hi-Media, India. TEA (triethanolamine), NiCl_2 , NaOH and all the reagents used for the preparation of nickel were purchased from Nice Chemicals, India. Elisa plate reader, BioTek Power Wave XS, USA. The L929 and A549 cell lines were purchased from NCCS Pune, India and anti-actin (rhodamine-B conjugated phalloidin, Texas red), DAPI were purchased from Sigma Aldrich, Bangalore, India.

2.2. Synthesis of nickel nanoparticles

In a typical procedure 0.2 M TEA was added drop wise into 40 mL aqueous solution of 0.02 M NiCl_2 under stirring. Then 1.0 mL hydrated hydrazine (80%) and 0.02 g NaOH was added (Ni, Zhao, Zhang, Yang, & Zheng, 2005). The whole mixture was stirred for another 1 h to obtain a homogeneous green solution and subsequently transferred into a 50 mL autoclave, sealed and maintained at 110 °C for 12 h. After the hydrothermal treatment, the fluffy particles floating on the solution was collected, washed with distilled water and dried at room temperature (Motlagh, Youzbashi, & Sabaghzadeh, 2011).

2.3. Synthesis of Ni-Chitin NGs

5 mg of nickel nanoparticles was added to 10 mL of 0.5% chitin solution and sonicated with amplitude of 75% for 20 min to make a uniform distribution of nickel nanoparticles within the chitin solution. This step was followed by the addition of equal volume of methanol so as to regenerate chitin nanogels loaded nickel nanoparticles. Further washing steps were used to remove the excess methanol to develop monodispersed Ni-Chitin NGs (Tamura, Furuike, Nair, & Jayakumar, 2011).

2.4. Rhodamine-labeled-Ni-Chitin NGs

The rhodamine-123 was labeled with Ni-Chitin NGs to differentiate Ni-Chitin NGs with the other stains during cellular localization studies. Briefly, 20 μL rhodamine-123 was added to the final suspension of Ni-Chitin NGs (2 mL) and incubated for 4 h, centrifuged at 20,000 rpm for 10 min, and resuspended in water for further studies.

2.5. Characterization of Ni-Chitin NGs

2.5.1. Surface morphology

The prepared Ni-Chitin NGs were characterized by scanning electron microscope (JEOL-JSM-6490LA) for size determination and also for surface topography of Ni-Chitin NGs. For the analysis, Ni-Chitin NGs were diluted and redispersed in water, from this dilution a few drops have been added and sputtered to make it more conductive for SEM imaging.

2.5.2. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis was conducted to identify the structure of the nanogels to determine the presence of bonding and also the chemical structure of the unknown formed nanogels. FTIR spectra were recorded on Perkin-Elmer spectrum RXI by mixing potassium bromide powder (1%, w/w) to lyophilized sample and pelletized. Pelletized sample were analyzed with the rate of 100 scans per sample. FTIR was analyzed with corresponding wave number and bond vibrations compared with the control FTIR data for the further confirmation of the sample (Kumirska et al., 2010).

2.5.3. X-ray diffraction (XRD) studies

X-ray diffraction analysis was done by PAN analytical X'PERT PRO X-ray diffractometer for confirming the presence of nickel. In this study we determine the conformational change of nickel loaded chitin nanogel structure. This can be clearly compared with XRD of nickel nanoparticles as well as chitin nanogels. This is one of the important parameter to know the presence of nickel in nanogels.

2.5.4. TG/DTA

In preparation for TGA, Ni-Chitin NGs were lyophilized and ground to powder form. Thermal degradation studies were done using S II TG/DTA 6200 EXSTAR, where it was used to determine the thermo responsive properties of modified composite nanogels with varying temperature from room temperature to 500 °C. Experiments were conducted in the presence of nitrogen gas in order to maintain lifeless nanogels. All measurements were studied with 2 mg samples in the aluminum pan.

2.6. Swelling studies of Ni-Chitin NGs

Swelling study of the Ni-Chitin NGs was done according to our previous protocols. Degree of swelling is the swelling ratio of nanogels where, W_w (wet weight) and W_d (dry weight) of Ni-Chitin NGs were calculated respectively (Rejinold et al., 2012).

$$\text{Swelling ratio} = \left[\frac{(W_w - W_d)}{W_d} \right] \times 100$$

2.7. In vitro Ni-Chitin NGs antibacterial study

Antibacterial activities of Ni-Chitin NGs were tested against *S. aureus* strain SA113 (Lordanescu & Surdeanu, 1976). Briefly, SA113 cells were cultured overnight in pre-sterilized LB broth at 37 °C with 160 rpm shaking. 5 mg/mL of Ni-Chitin NGs was added to the 1 mL overnight culture of SA113 and incubated further for 24 h. The bacterial cultures were serially diluted and plated in the LB agar plates for the determination of bacterial viability by colony counting (Maya et al., 2012).

2.8. Cell lines and culture procedure

Normal mouse fibroblast (L929) and non-small cell lung carcinoma cell (A549) lines (NCCS, Pune, India) were maintained in MEM supplemented with 10% fetal bovine serum and 0.5% antibiotics. The cells were incubated at 37 °C with 5% CO_2 . After reaching confluence, the cells were trypsinized and sub cultured in the growth medium for further studies.

2.9. Cellular localization and cytocompatibility studies

L929 and A549 cells were seeded in a density of 5×10^4 cells onto acid etched cover slips placed in 24-well plate. The experiments were done with 0.8 mg/mL concentration of Ni-Chitin NGs according to the existing protocols. Cytocompatibility studies were performed using MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium] assay. 10^4 cells were added to 96 well plates for overnight attachment and after reaching 90% confluency, the cells were incubated for 24 h with different concentrations of Ni-Chitin NGs diluted with media. Cells in the media without the nanogels were acted as negative control and Triton X-100 as a positive control. The optical density of the solution was measured at a wavelength of 570 nm using Elisa plate reader.

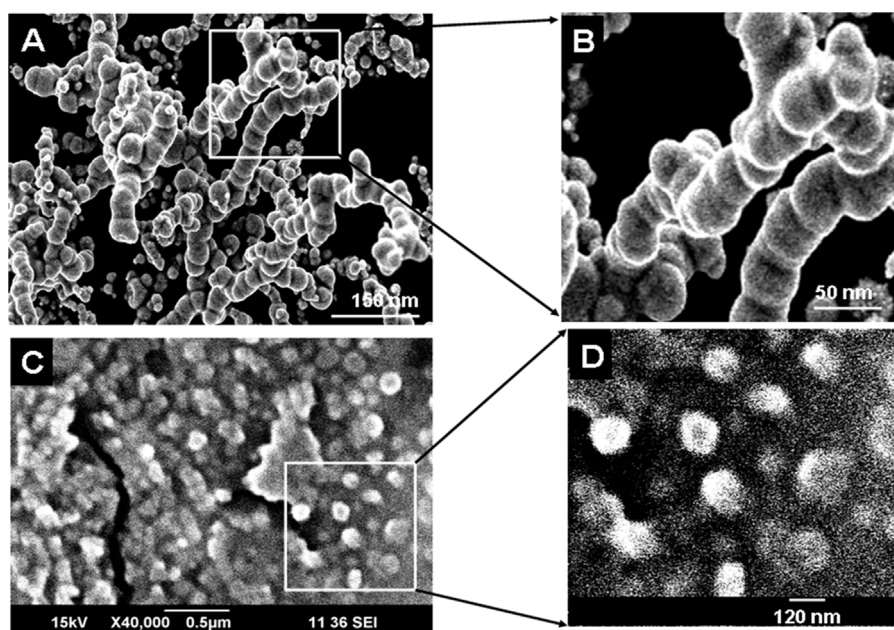
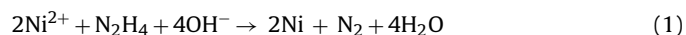


Fig. 1. (A) Size and topography analysis of the nickel nanoparticles by SEM and (B) higher magnified version of the nickel nanoparticles; (C) morphology of Ni-Chitin NGs and (D) higher magnification of Ni-Chitin NGs.

3. Results and discussion

3.1. Preparation and characterization of nickel nanoparticles and Ni-Chitin NGs

The formation of metallic nickel is attributed to the reduction of nickel chloride by hydrazine hydrate as follows:



TEA could coordinate with Ni^{2+} forming a very stable complex, which could decrease the concentration of nickel ions, in favor of nickel particles to grow at a relative slow rate to the form of worm like structures (Wang, Sun, Yu, Qiu, & Meng, 2009). In addition to the role of ligands, TEA also acted as a “directing agent”, linking the spherical nickel crystals together (Couto et al., 2007).

Ni-Chitin NGs were prepared via regeneration chemistry. The aggregated form of nickel nanoparticles was completely redispersed in chitin solution with high amplitude (75%) probing. These nickel nanoparticles were encapsulated into the regenerated chitin nanogels by adding equal volumes of methanol and calcium chloride. The obtained gels were then probe sonicated for several times followed by centrifugation at 20,000 rpm for 15 min.

SEM image of worm like nickel nanoparticles revealed with a size around 50 nm (Fig. 1A and B). SEM revealed spherical shaped Ni-Chitin NGs in the range of 120–150 nm (Fig. 1C and D). This is in the optimum size range for having a better penetration through bacterial membrane. FTIR spectroscopy was taken for the confirmation of nanogels. The broad absorption band centered at around $3400\text{--}3500\text{ cm}^{-1}$ is due to the —OH stretching vibration. It was clear that the Ni-Chitin NGs gave only the split transmittance peaks of chitin (Kumirska et al., 2010) at 1660 and 1638 cm^{-1} corresponding to the amide-I region and transmittance peak at 1560 cm^{-1} corresponding to the amide II region. This revealed that the nickel was covered by the chitin nanogels (Fig. 2A–C).

Fig. 3A shows XRD peaks corresponding to pure nickel phases (JCPDS-number-01-87-07120). Fig. 3B shows the intensity of the nickel peaks were not much predominant in the Ni-Chitin NGs which explain the encapsulation of nickel into chitin nanogels.

Major peaks of chitin were also present in the chitin nanogels at 10 and 20° .

Thermogravimetric analysis was conducted to determine the effect of arrangement and condition on the thermal decomposition of nanogels. In Ni-Chitin NGs, a three step decomposition occurred, the first step degradation was due to the release of H_2O at the temperature of $60\text{--}80^\circ\text{C}$, Second degradation occurred at the range at 270°C which is having at a range of 40% loss and third degradation with a loss in mass due to the release of short chains N-acetyl glucosamine. The Ni-Chitin NGs showed enhanced thermal stability and slow degradation compared to the control chitin and chitin nanogels. This decrease in the degradation and thermal stability of Ni-Chitin NGs could be due to the entrapment of nickel nanoparticles within the chitin nanogels (Fig. 4A–C).

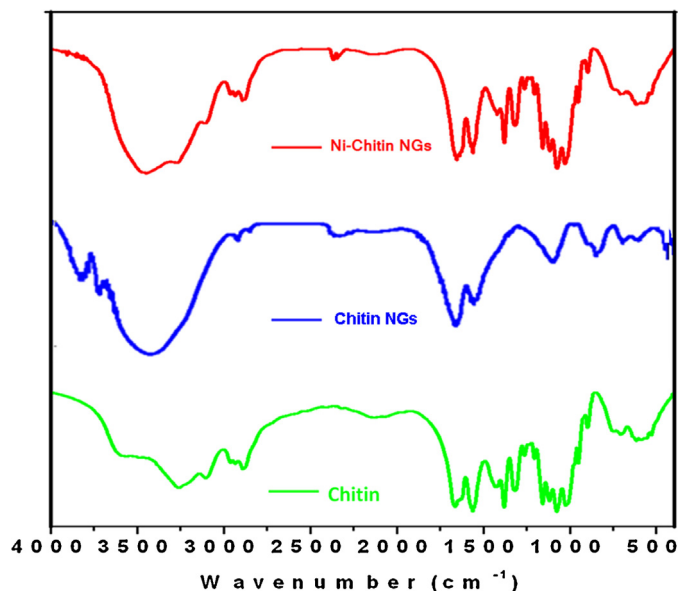


Fig. 2. FTIR analysis of (A) Ni-Chitin NGs; (B) control chitin nanogels and (C) control chitin.

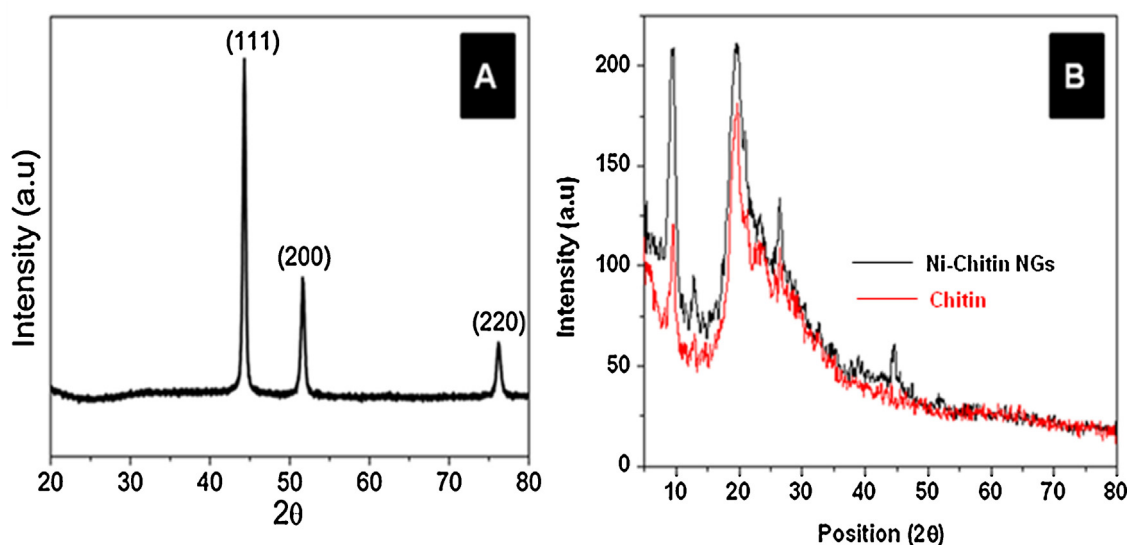


Fig. 3. (A) XRD of control nickel nanoparticles, (B) XRD analysis of chitin (control), Ni-Chitin NGs.

Swelling behavior of Ni-Chitin NGs was performed to quantify the amount of water uptake by nanogels when subjected to different pH of solutions (Fig. 5A). This determines the relationship between the swellings of polymer in a solvent. The higher swelling in acidic pH of the Ni-Chitin NGs would be due to the ionization of the pendant groups such as -NHCOCH_3 and -OH/NH_2 . Since the pH of bacteria is acidic in condition where these nanogels could easily swell and release of nickel nanoparticles could kill the bacteria in the acidic pH conditions.

3.2. Antibacterial activity of Ni-Chitin NGs

The antimicrobial activity of the Ni-Chitin NGs against SA113 was determined by using serial dilution method and by calculating the number of colony forming units (CFU). Incubation of SA113 overnight culture with 5 mg/mL of Ni-Chitin NGs leads to decrease in ~ 3.2 CFU/mL of SA113 growth (Fig. 5B and C). The antibacterial activities exerted by Ni-Chitin NGs against SA113 were due to its efficacy in damaging the membrane structure and generation of

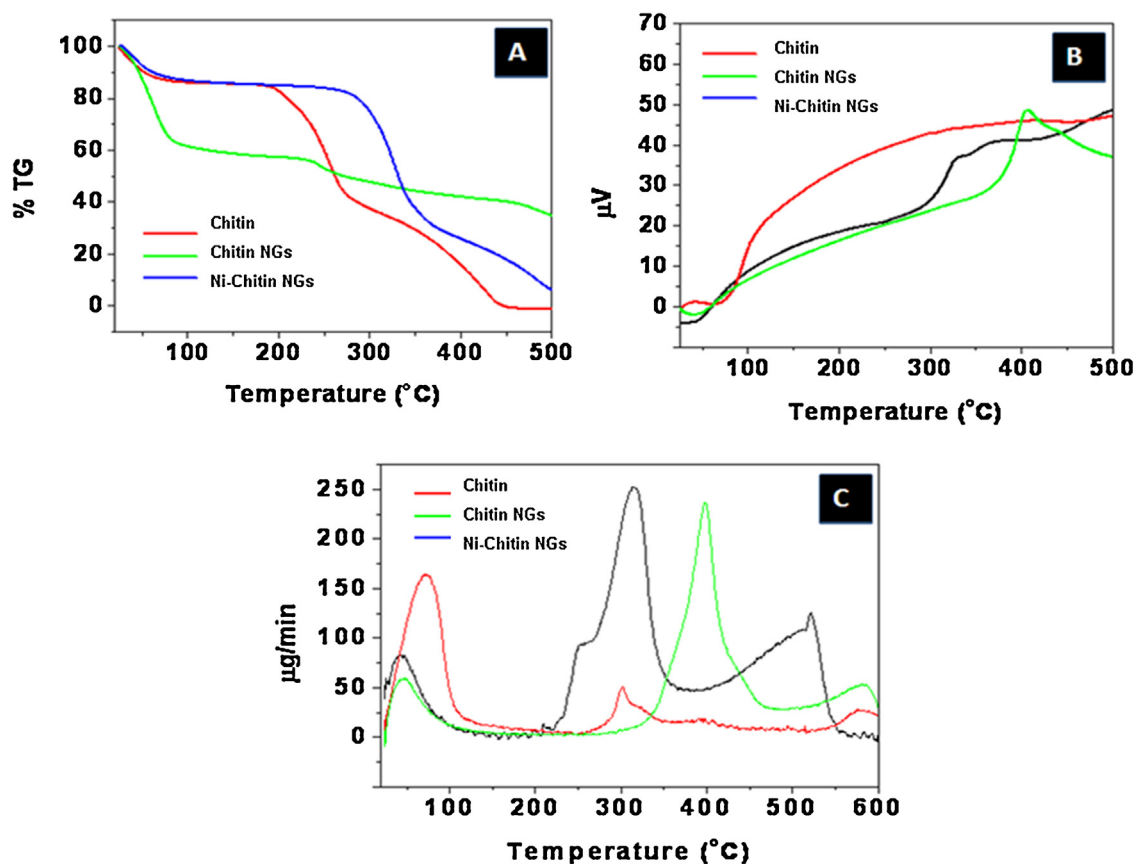


Fig. 4. (A) TGA, (B) DTA and (C) DTG analysis of chitin (control), chitin nanogels (control) and Ni-Chitin NGs.

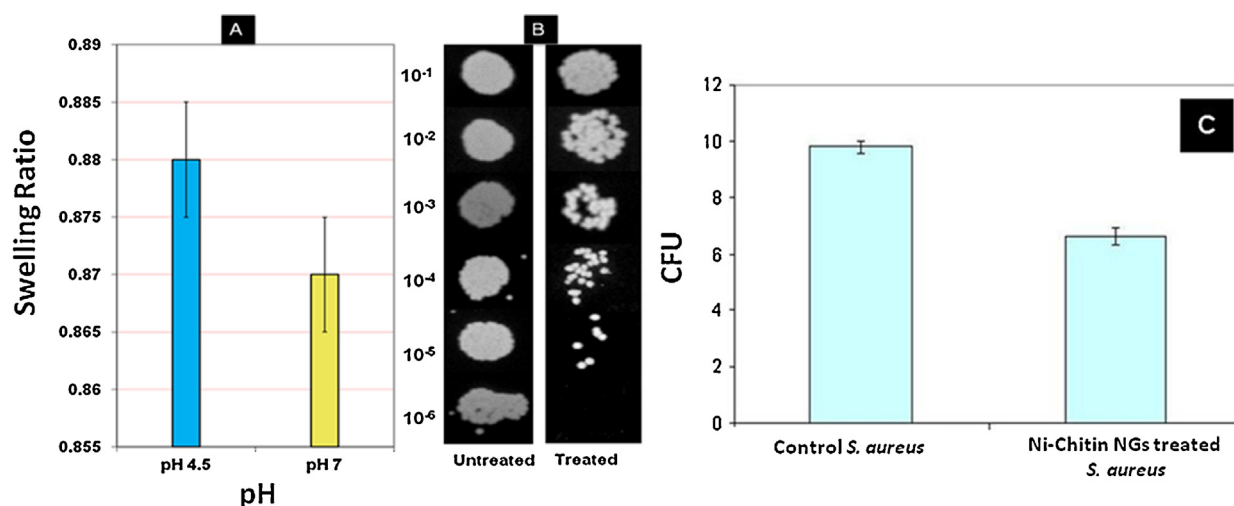


Fig. 5. (A) Swelling studies of Ni-Chitin NGs at pH 7 and 4.5, (B) antibacterial study with *S. aureus* by serial dilution method and (C) bacterial viability of CFU/mL.

reactive oxidative species (ROS) (Díaz-Visurraga et al., 2011). Nickel nanoparticles and chitin nanogels have positive charge and can easily enter deeply into the bacteria where, phosphorus and sulphur compounds of the DNA and proteins, respectively will interact with Ni-Chitin NGs which would kill the bacteria effectively (Fuhrmann & Rothstein, 1968; Kumar, Rani, & Salar, 2010).

3.3. Cellular localization and cytocompatibility of Ni-Chitin NGs

The rhodamine-123 labeled Ni-Chitin NGs (0.8 mg/mL) showed green fluorescence inside L929 and A549 cells. To have a clear idea of the localization, the cells were stained with DAPI (nuclear stain) and anti-actin (cytoskeleton stain), so that the uptake can be

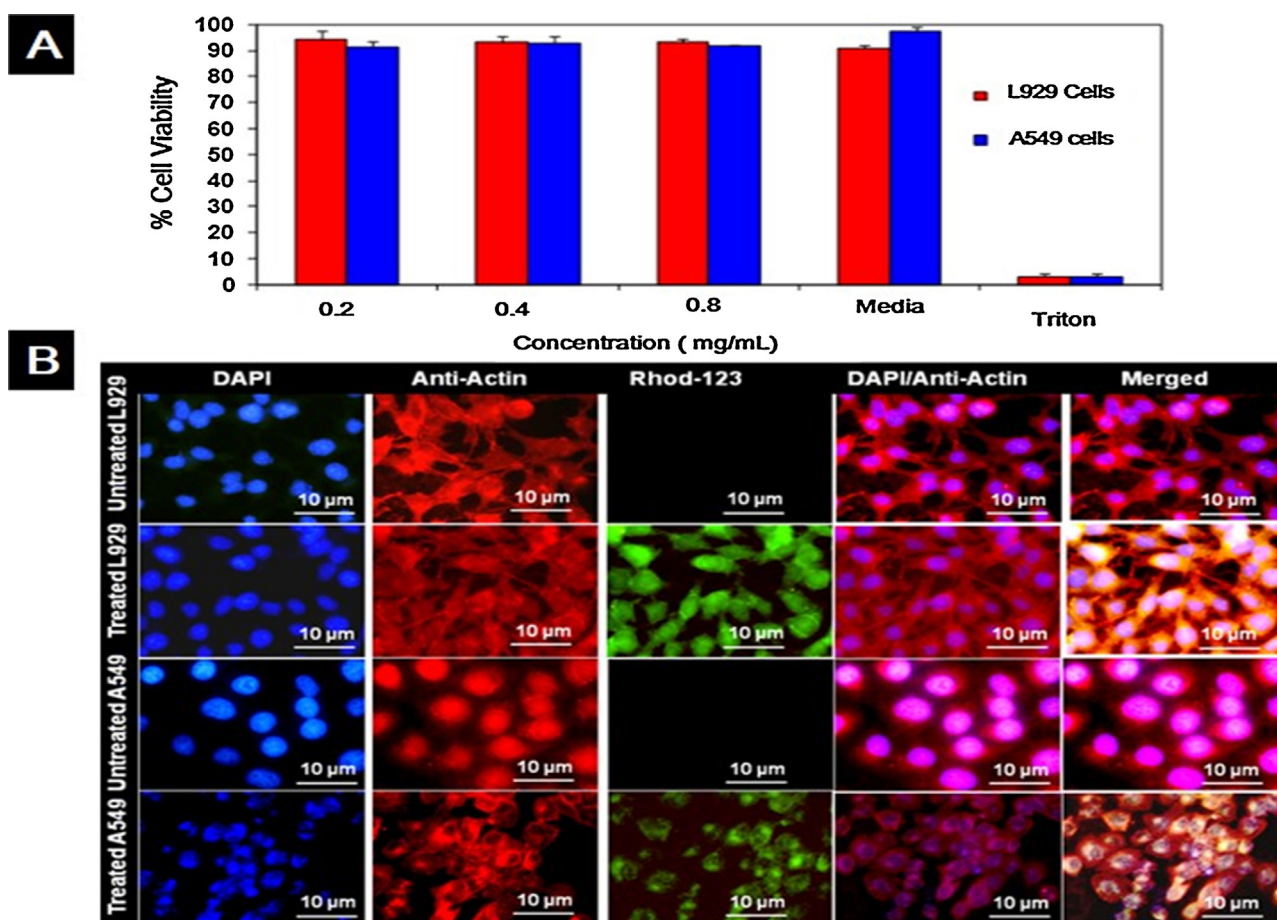


Fig. 6. (A) Cytocompatibility of Ni-Chitin NGs on L929 and A549 cells (24 h study) and (B) cellular localization of the rhodamine-123 labeled-Ni-Chitin NGs after 24 h exposure on L929 and A549 cells.

recognized critically. Fig. 6 visualizes most of the particles are inside the cytoplasm, especially in T47D cells, whereas Ni-Chitin NGs were located peripherally on L929 cells.

4. Conclusions

In summary, we have prepared and characterized nickel loaded chitin nanogels. At the optimized level, SEM image showed 120–150 nm sized Ni-Chitin NGs which were prepared by regeneration chemistry. They showed an enhanced anti-bacterial activity against *S. aureus*. The rhodamine-123-labeled-Ni-Chitin NGs were well localized in A549 cells and L929 cells. Furthermore, Ni-Chitin NGs are cytocompatible toward L929 and A549 cells up to a concentration range of 800 µg/mL. So the prepared Ni-Chitin NGs can be used for biomedical applications. However, current results support the cytocompatibility and antibacterial activity of Ni-Chitin NGs at lower concentration (800 µg/mL).

It should be kept in mind that in many countries the presence of trace nickel in cosmeceuticals is prohibited, because nickel can trigger allergic reactions. Clinical evidence of damages to soft and bone tissues in the oral cavity has been reported after the application of nickel piercing devices. Furthermore, a thorough *in vivo* toxicological study of the Ni-Chitin NGs has to be carried out before using this system in medical field.

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