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Antibacterial and antifungal activity of chitosan coated iron oxide nanoparticles

P Nehra[a], RP Chauhan[b], N Garg[c] and K Verma[c]

[a]School of Biomedical Engineering, National Institute of Technology, Kurukshetra, India; [b]Department of Physics, National Institute of Technology, Kurukshetra, India; [c]Department of Microbiology, Kurukshetra University, Kurukshetra, India

ABSTRACT

Background and aim: The emergence of resistance against antimicrobial agents has led to the development of more efficient agents and new techniques for treatment of various microbial infections. The aim of the present study is to determine the antibacterial and antifungal activity of bare and chitosan coated Fe_3O_4 nanoparticles (NPs) against five organisms, *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*), *Candida albicans* (*C. albicans*), *Aspergillus niger* (*A. niger*) and *Fusarium solani* (*F. solani*).

Methods: Fe_3O_4 NPs were synthesised by coprecipitation and surface coating was done by chitosan polymer to avoid agglomeration. The antimicrobial property of NPs was tested by agar well diffusion and analysed by measuring the diameter of the inhibition zone.

Results: Average particle size of Fe_3O_4 and chitosan coated Fe_3O_4 NPs was 10.4 ± 4.9 and 11.4 ± 5.2 nm, respectively. Mean diameter of inhibition zone of synthesised chitosan coated Fe_3O_4 NPs was in the range 14.5 to 18.5 mm. The effect of chitosan coated Iron oxide nanoparticles was *F. solani/A. niger* < *C. albicans* < *E. coli/B. subtilis* ($p < 0.001$).

Conclusions: Chitosan coated Fe_3O_4 NPs are effective antimicrobial agents and so may be developed as a microbial resistant coating for biomedical devices.

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KEYWORDS
Iron oxide nanoparticles; chitosan; antimicrobial activity; agar well diffusion technique

Introduction

Nanotechnology has emerged as one of the most versatile fields in recent years. Nanoparticles (NPs) are drawing increasing attention due to their distinctive characteristics and minimal harmful aftereffects. Magnetic NPs are becoming popular in bioengineering and biomedical applications due to their capability to act at the cellular and molecular level when exerted to *in vitro* and *in vivo* applications. Among NPs, iron oxide (Fe_3O_4) NPs are popular due to features such as superparamagnetic, biocompatibility, crystalline structure, nontoxicity, monodispersity, water soluble and cost effective [1,2]. The crystalline morphology of Fe_3O_4 NPs consists of a high number of edges, corners and potentially reactive sites which create an interest as potential antibacterial agents [3]. NPs with the size of less than 100 nm have consistent physical and chemical properties. To make them suitable for bioengineering and biomedical applications they need to be coated to assure their nontoxicity and stability in the biological medium. The surface coating agents may be enzymes, antibodies, proteins, drugs and polyelectrolytes. This is done to achieve better interactive properties on the surface of the NP [4].

The preferred process for the synthesis of Fe_3O_4 NPs is coprecipitation, because of its simplicity, and that it is carried out under the mild condition without using any hazardous solvents. It has the potential for large-scale production, is cost effective and water soluble, all the necessary requirements for biomedical applications [5]. The crucial challenge in synthesis is to attain the optimum size and shape of the NPs, which is achieved by controlling pH, temperature, the nature of the salts, ionic strength and the insertion of the surface coating agents. NPs usually aggregate due to high surface energy and magnetisation. To avoid such aggregation, NPs are coated with a surface coating agent [6].

Chitosan, a biopolymer which is obtained from deacetylation of chitin, is a biocompatible, biodegradable, linear polysaccharide and consisting of three types of functional groups (amino, and primary and secondary hydroxyl groups). These serve as an anchor for combining therapeutics, imaging agents and targeting ligands [5,7]. Chitosan has antimicrobial activity against various microbes, but negligible toxicity towards human cells [8].

Using standard microbiology methods [9,10], we tested the antimicrobial properties of synthesised NPs on two species of bacteria (*Escherichia coli* [*E. coli*] *and Bacillus subtilis* [*B. subtilis*]) and three species of fungus

CONTACT P Nehra poonamnehra007@gmail.com

(*Candida albicans* [*C. albicans*], *Aspergillus niger* [*A. niger*] *and Fusarium solani* [*F. solani*]). *C. albicans* is responsible for infection Candidiasis, and forms a biofilm on the surface of the implantable biomedical devices [11]. *A. niger* is a common cause of fungal ear infection (otomycosis), *F. solani* is responsible for corneal infection (keratitis), and *E. coli* is responsible for 80% of Urinary tract infections (UTIs) [12–14]. Our objective was to synthesise chitosan coated Fe_3O_4 NPs and evaluate their antimicrobial activity, thus determine whether these NPs may be used as microbial resistant coating and for anti-infective application in biomedical devices.

Materials and methods

Chemicals for the synthesis of nanoparticles, Iron (II) chloride tetrahydrate ($FeCl_2{\cdot}4H_2O$), Iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), Acetic acid (CH_3COOH), Sodium hydroxide (NaOH), Chitosan (75% deacetylated) were purchased from LobaChemie Pvt Ltd, India and growth medium for the micro-organisms were purchased from HiMedia, India. The test organisms *E. coli* (MTCC 736), *B. subtilis* (MTCC 443), *C. albicans* (MTCC 183), *A. niger* (MTCC 281) and *F. Solani* (MTCC 350) were purchased from Institute of Microbial Technology (IMTECH), Chandigarh, India. All glass wares were purchased from Borosil, India.

Iron (II, III) oxide nanoparticles were synthesised by coprecipitation method reported by Bellova et al. [15] with some modification. 2.534 g of iron (II) chloride tetrahydrate and 6.488 g of iron (III) chloride hexahydrate were dissolved in a 200 ml of deionised (DI) water using a magnetic stirrer until a homogenous solution was formed. The solution was heated at 65 °C in a water bath for 15 to 20 min and followed by the addition of 28 ml of 25% of sodium hydroxide. After completion of the reaction, the colour changes to black due to the formation of precipitate. The solution was centrifuged at 6000 rpm for 20 min. The supernatant (solution) was discarded and the pellet was washed three times with DI water. After washing, the pellet was dried at 70 °C to obtain the powder form of Fe_3O_4 NPs.

The coating of chitosan on iron (II, III) oxide NPs was done by the method reported by Samani et al. [16]. 40 mg of chitosan was mixed in 200 ml of DI water containing 11.60 ml of acetic acid. In the above solution, 0.14 g of synthesised iron (II, III) oxide nanoparticles were added, and kept on a magnetic stirrer for approximately 15–20 h. During the process, the colour changes from black to brown. The suspension was centrifuged at 8000 rpm for 40 min. The supernatant (solution) was discarded and the precipitate was washed two times with DI water. After washing, the precipitate was dried at 65 °C to obtain the powder form of iron oxide nanoparticles.

Crystal structure of synthesised nanoparticles was analysed by X-ray diffraction (XRD). Fourier Transform-Infrared Spectroscopy (FTIR) technique was used to identify the chemical groups and chemical interactions involved in the synthesis of nanoparticles. The morphological features of synthesised nanoparticles were studied using scanning electron microscope (SEM).

Five microbes were used in the study of the antimicrobial test. Initially, all the microbes were inoculated in their desired growth medium viz. *E. coli* (Tryptic soy agar), *B. subtilis* (Nutrient agar), *C. albicans* (Yeast extract peptone dextrose-YEPD), *A. niger* (Czapek yeast extract-CYA) and *F. Solani* (Potato sucrose agar). The Well diffusion technique was used to test the antimicrobial property of NPs. Lawns of each organism were prepared on agar, four wells cut, and filled with Fe_3O_4 NPs (40 µg/1 ml of distilled water), chitosan coated Fe_3O_4 NPs (30 µg/1 ml of distilled water), chitosan (20 µg/1 ml of distilled water) and an antibiotic: Cefixime (20 µg/1 ml of distilled water) for bacteria and Amphotericin B (20 µg/1 ml of distilled water) for fungi. Agar plates were incubated for 48 h at 30 °C to observe the results. The diameter of inhibition zones of various controls are expressed as the mean ± standard deviation. Differences were sought by analysis of variance followed by Tukey's *post hoc* test. All experiments were performed in triplicate and repeated three times ($n = 9$).

Results

Crystal structure of synthesised nanoparticles was analysed by XRD technique. The Figure 1 shows the XRD pattern of both bare and chitosan coated Fe_3O_4 nanoparticles. It exhibits six diffraction peaks at 2θ values. The major peak at 35° and minor peaks at 30°, 43°, 53°, 57° and 62° which correspond to the (3 1 1), (2 2 0), (4 0 0), (4 2 2), (5 1 1) and (4 4 0) crystallographic planes of the Fe_3O_4 NPs. This confirms the spinal structure of Fe_3O_4 NPs and reveals that the synthesised NPs contain magnetite- Fe_3O_4 and maghemite -γ- Fe_2O_3 and does not contain any other form of iron oxide NPs such as haematite, goethite, or any other iron hydroxide [17]. The lattice parameter of synthesised NP is 8.3840 Å, which is more close to the lattice parameter of magnetite (8.3960 Å) then maghemite (8.3515 Å) [18]. This confirmed that the synthesised NPs were predominantly magnetite than the maghemite. The 2θ value of the peak (3 1 1) of magnetite is at 35.423°, maghemite is at 35.631° [19]. The 2θ value of the peak (3 1 1) of synthesised NPs is at 35.57° which further confirmed the presence of magnetite. The coating of chitosan on Fe_3O_4 NPs does not affect the crystal structure of Fe_3O_4 NPs, since the peaks are at the same diffracting angle, correspond to 8.3840 Å lattice. All the obtained experimental peaks were compared with JCPDS reference code-75-0033.The size of the nanoparticles was determined by Debye–Scherrer equation: Particle size = $0.9\lambda/\beta \cos\theta$, where λ is the wavelength of X-ray (0.154 nm), β is the full width at half maximum (FWHM) in radians, θ is the Braggs angle and particle size is in nanometres (nm). The average particle size

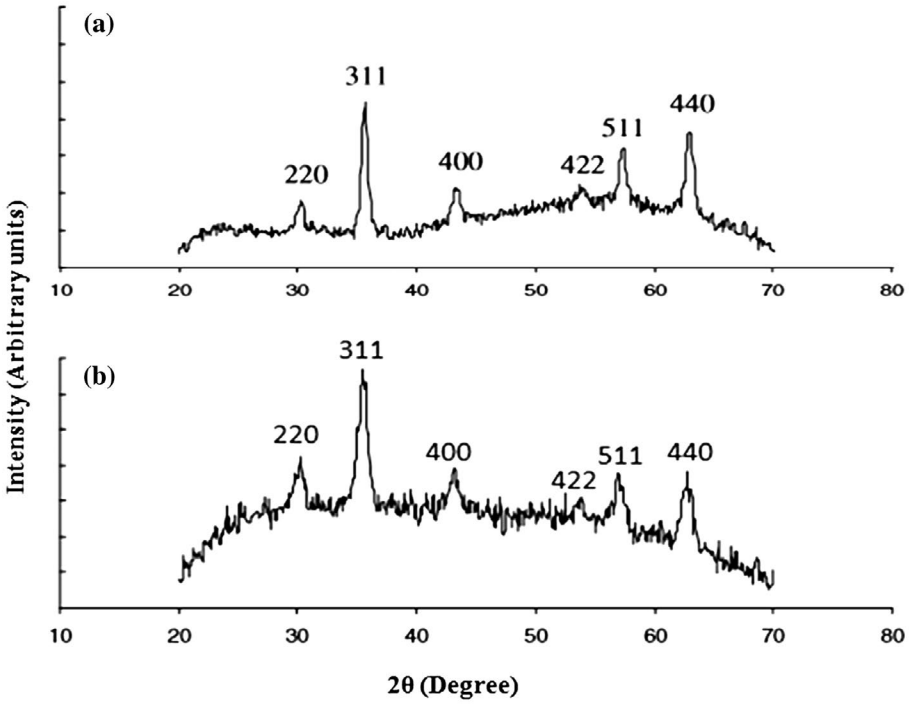

Figure 1. XRD pattern (a) Fe_3O_4 nanoparticles and (b) chitosan coated Fe_3O_4 nanoparticles.

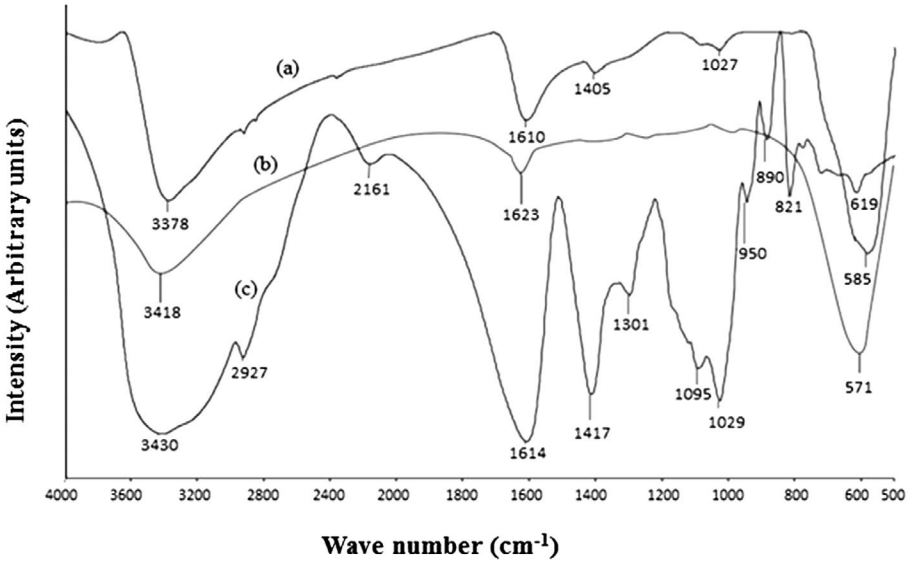

Figure 2. FTIR analysis (a) Chitosan coated Fe_3O_4 nanoparticles, (b) Fe_3O_4 nanoparticles and (c) Chitosan polymer.

calculated from this equation is 10.4 ± 4.9 nm of Fe_3O_4 NPs and 11.4 ± 5.2 nm of Chitosan coated Fe_3O_4 NPs.

The presence of chitosan on the surface of Fe_3O_4 NPs was confirmed by the FTIR analysis. Figure 2 shows the characteristic peaks of bare and chitosan coated Fe_3O_4 NPs. The presence of absorption band at 571 cm^{-1} in the spectrum of Fe_3O_4 NPs and at 585 cm^{-1} in the chitosan coated Fe_3O_4 NPs, correspond to the FeO bond. The presence of a peak at 619 cm^{-1} in the spectrum of chitosan polymer and at 585 cm^{-1} in the chitosan coated Fe_3O_4 NPs correspond to the primary amine group. It indicates that the Fe_3O_4 NPs are successfully coated by chitosan polymer [5]. The peak at 3430 cm^{-1} in the chitosan spectrum is due to OH and NH stretching vibrations, 2927 cm^{-1} is assigned to the OH stretching vibrations and the 1417 cm^{-1} and bands around 1095 cm^{-1} are due to CO stretching vibrations.

The images obtained using SEM indicate that the Fe_3O_4 NPs and chitosan coated Fe_3O_4 NPs are both spherical in shape. The diameters of both NPs range from 8 to 20 nm. The results obtained from XRD and SEM indicates that the coating of chitosan on Fe_3O_4 NPs does not have any effect on their size [20].

Fe_3O_4 was tested on *E. coli* and *B. subtilis* alone, giving in both cases a mean (SD) diameter of inhibition of 14.0 (0.5) mm. The effects of chitosan coated Fe_3O_4 NP, chitosan polymer and an antibiotic on bacterial or fungal growth are shown in Table 1 and Figure 3. Overall, there

Table 1. Inhibition diameter (in mm) of bacterial or fungal growth.

Microbe	Chitosan coated Fe_3O_4 NP	Chitosan polymer	Antibiotic*
E. coli	16.0 (0.5)	23.0 (0.5)	26.1 (0.5)
B. subtilis	18.0 (0.5)	25.0 (1.1)	27.0 (2.1)
F. solani	15.0 (0.1)	25.0 (1.1)	25.0 (0.1)
C. albicans	16.0 (1.1)	20.0 (0.5)	24.0 (1.1)
A. niger	15.0 (0.5)	21.0 (0.5)	24.0 (1.1)
Mean	16.0 (1.3)	23.2 (2.4)	25.2 (1.7)

Note: Data are mean (SD) diameters of inhibition. NP = nanoparticle.
*Cefixime for bacteria, amphotericin B for fungi.

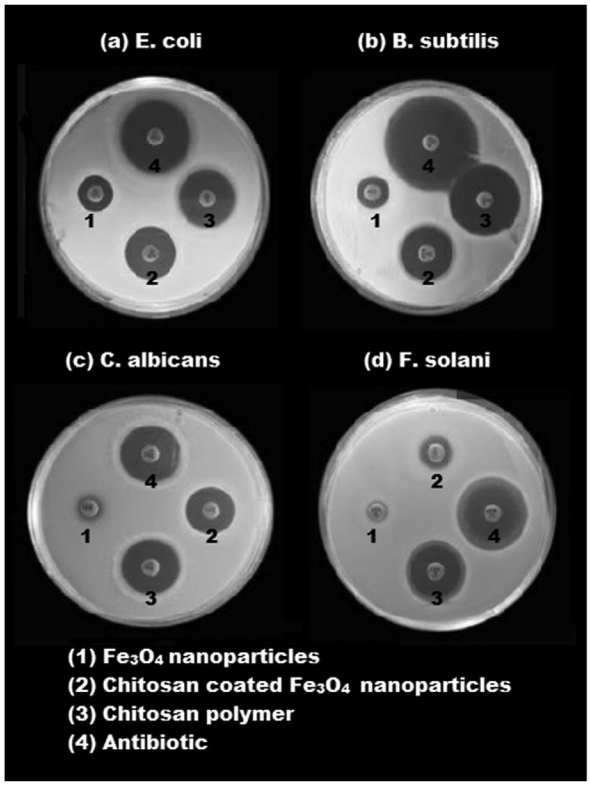

Figure 3. Antimicrobial activity (inhibition zone) of (1) Fe_3O_4 nanoparticles, (2) Chitosan coated Fe_3O_4 nanoparticles, (3) Chitosan polymer, (4) Antibiotic against (a) *E. coli*, (b) *B. subtilis*, (c) *C. albicans* and (d) *F. solani*.

were marked effects of all three agents on microbial growth, in the order (least effective - most effective) chitosan coated Fe_3O_4 NP < chitosan polymer < antibiotic (ANOVA $p < 0.001$, $n = 45$/group), with significant differences between each agent (Tukey $p < 0.01$). This pattern was reproduced for each microbe ($p < 0.001$, $p < 0.05$ between groups, $n = 9$/group), except the differing effects of chitosan polymer and antibiotics on *F. solani*, which was not significant. There were also marked differences between the resistance/sensitivity profiles of each agent on the different microbes. The effect of chitosan coated Fe_3O_4 NP was *F. solani/A. niger* < *C. albicans/E. coli* < *B. subtilis*. The effect of chitosan polymer was *C. albicans/A. niger* < *F. solani/E. coli/B. subtilis*. The effect of antibiotics was *A. niger/C. albicans* < *F. solani* < *E. coli* < *B. subtilis* (all differences $p < 0.001$, $p < 0.05$ between groups). *E. Coli* shows more antimicrobial activity than *B. Subtilis* because the charge on the surface of gram negative bacteria is more negative than gram positive bacteria [21]. The positively charged surface of metal oxide NPs may bind to cell membrane which is negatively charged through electrostatic interactions which disrupt bacterial functions [22].

Discussion

The emergence of resistance against antimicrobial agents has led to search for of more efficient antimicrobial agents. This is a major topic of concern since 1965 when for the first time reduced activity of penicillin towards the Streptococcus pneumoniae was reported [23]. The antibacterial effect of antibiotics may be enhanced if combined with natural products such as Punica granatum (pomegranate) [24].

Various researchers have investigated the mechanism behind the antimicrobial activity of iron oxide NPs. Some have demonstrated that iron oxide NPs have dose-dependent antibacterial activity against *E. coli*, whilst others showed that iron oxide NPs have no antibacterial activity [25,26]. We have extended this work by showing the antifungal activity of Fe_3O_4 NPs. We synthesised iron (II, III) oxide NPs with negative surface potential and coated them with chitosan polymer to change the surface potential and functional group. Due to the presence of free hydroxyl group in the chitosan polymer, its gets strongly bonded to the iron (II, III) oxide NPs via hydrogen bonding [27].

There are several explanations for the increased antimicrobial activity of chitosan coated Fe_3O_4 NPs compared to bare Fe_3O_4 NPs. Cationically charged amino group of chitosan combines with the anionic component on the cell membrane of microbes such as sialic acid, neuraminic acid and N-acetylmuramic acid. Due to this, chitosan may suppress the microbial growth by chelating various transitional metal ions, inhibiting enzymes and by impairing the exchange with the medium [6]. The increase in the antimicrobial activity is due to the greater stability of chitosan coated Fe_3O_4 NPs in aqueous medium as compared to the bare Fe_3O_4 NPs because chitosan protects them from aggregation. Various studies have associated the antimicrobial activity with pH, they reported that the rise in pH from 6 to 7 of the reaction medium declined the antibacterial activity of chitosan towards *E. coli* [28,29].

The antibacterial drugs and antibiotics show bactericidal properties due to the oxidative stress generated by reactive oxygen species (ROS) [30]. Recent studies also demonstrated that ROS also play a role in cell signalling, including gene expression, apoptosis and the activation of cell signalling cascades [31]. Silver NPs also show antibacterial property due to ROS generation [32]. In our case, ROS can be generated by Fe_3O_4 NPs which lead to the inhibition of microbial growth. Keenan et al.

described a procedure in which iron (Fe^{2+}) reacted with oxygen to create hydrogen peroxide [33]. This hydrogen peroxide further reacted with ferrous ions and produced hydroxyl radicals which damage the biological macromolecules.

Several studies concluded that the small sizes of NPs are responsible for the bactericidal effects because they easily penetrate the cell membrane and then react with intracellular oxygen which causes the disruption due to the generation of oxidative stress [34]. Some studies also concluded that the concentration of nanoparticles was a considerable factor for promoting antimicrobial activity [35–38]. One study also concluded that the appropriate external magnetic field may be directed to iron oxide NPs to kill bacteria inside the body [39]. Magnetic NPs whose size is less than 30 nm have remanence and coercivity of almost zero, and such NPs show superparamagnetic behaviour which is the desired property of biomedical applications [5]. These NPs can easily be isolated from the medium through an external magnetic field during waste disposal to prevent the environment from contamination.

Limitations of our study include testing the effects of NPs on only five microbes, and that only one concentration of each antimicrobial agent was used. Nevertheless, this work represents an advance in biomedical science because it shows that NPs have antimicrobial activity which makes them suitable for the microbial resistant coating and anti-infective application for biomedical devices.

Summary table

What is known about this subject:
- Fe_3O_4 nanoparticles show good antibacterial properties.
- Chitosan polymer shows excellent antibacterial and antifungal properties.
- Chitosan coated Fe_3O_4 nanoparticles can be synthesised in the range of 10–20 nm.

What this study adds:
- Surface modification of Fe_3O_4 nanoparticles by Chitosan has improved its antibacterial properties.
- Chitosan coated Fe_3O_4 nanoparticles also show good antifungal properties.
- Chitosan coated Fe_3O_4 nanoparticles can be synthesised by coprecipitation method with size less than ~15 nm.

Disclosure statement

No potential conflict of interest was reported by the authors.

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