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Der Pharmacia Lettre, 2015, 7 (2):100-111
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Biocompatible polymer chitosan stabilized silver nanoparticles-Azithromycin (CS-AgNp-AZ) and levofloxacin (CS-AgNp-LF) drug nano conjugate fabricated wound dressing for the improved anti bacterial activity against human pyogenic bacteria

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ABSTRACT

In the present study, an attempt has been made to study the improved anti bacterial property of biocompatible polymer chitosan stabilized silver nanoparticles-azithromycin (CS-AgNp-AZ) and levofloxacin (LF) nano drug conjugate fabricated wound dressing against human pathogenic bacteria. Nano drug conjugate was prepared by chemical reduction of metal salt precursor with reducing agent and further stabilization with chitosan by ionic gelation method. Synthesized nano drug conjugate characterized by Ultra Violet Visible Spectroscopy and Scanning Electron Microscopy. Synthesized nano drug conjugate was coated on the wound dressing by pad dry cure method and the fabricated dressing was further characterized by scanning electron microscopy which revealed complete dispersion of the particles on the fibre surface. The coated fabrics were examined in the range of basic physical-mechanical parameters such as the tensile strength, the bursting strength and air permeability. Distinct changes was not observed in the all the tested physical-mechanical properties of the nano drug conjugate coated dressing material. Anti bacterial activity revealed all the tested bacteria were highly susceptible to the both nano drug conjugate fabricated wound dressing. The present study would suggest the possible utilization of nano drug conjugate fabricated wound dressing against pyogenic bacteria and thus further development of biocompatible chitosan stabilized nano drug conjugate mediated anti microbial dressing.

Key words: biocompatible polymer, chitosan, nano drug conjugate, pyogenic bacteria, wound dressing

INTRODUCTION

The discovery of antibiotics in the 20th Century marked a watershed in the treatment of infections. The ability to treat the serious infections of the pre-antibiotic era stimulated advances in medical fields and enlarged the scope of medical care. However, while a drastic change has taken place in the causes of fatal infections, they are still a major cause of death the world over [1]. While demographic changes and drug access issues are important reasons in the developed and developing worlds, respectively, “relentless and Dizzying Rise of Antimicrobial Resistance” has contributed in a large measure to the persistence of infections as a major cause of morbidity and mortality [2]. The rapid emergence of resistance to antibiotics amongst pathogens generates visions of the ‘potential post-antibiotic era threatening present and future medical advances [3,4]. The use of antimicrobial agents for textiles has also become indispensable to avoid cross-infection by pathogenic microorganisms, to control the infestation by microbes, and arrest metabolism in microbes [5]. Recently, an awareness of general sanitation, contact disease transmission, and personal protection has led to the development of antibacterial fibers to protect wearers against the spread of bacteria and diseases rather than to protect the quality and durability of the textiles [6]. Development of anti microbial wound dressing against human pyogenic microorganism is an important area in health care to fight against human pathogenic microorganism. Because of high efficacy and compatibility, nanoparticles coated wound dressing is quite popular in various part of the world. Nanotechnology is significant on account of its pre-eminence upon the

comprehension, use, and control of matter at magnitudes of a minute scale, akin to approaching atomic levels, with which to manufacture new substances, instruments, and frameworks [7,8,9]. silver nanoparticles are in great use in the medicinal, pharmaceutical, agricultural industry and in water purification [10,11]. Since noble metal nanoparticles such as silver, gold nanoparticles are widely applied to human contacting areas. Silver nanoparticles have been reported to have antimicrobial activity against a wide range of microorganism [12]. Taken together, this compound as a highly safe compound may be considered for combination therapy against pathogenic microorganism due to its potential synergistic effect with important antibiotics [13]. These nanoparticles exhibit tolerable monodispersity and in the case of particles synthesized extracellularly, exhibit excellent long term stability. Silver nanoparticles have been reported to have antimicrobial activity against a wide range of microorganisms [14]. An important factor is the protection provided by the encapsulation of the drug- nanoparticles in the biological milieu, decreasing toxicity and allowing the drug to reach the specific site. Drug delivery nano carriers systems, such as liposomes [15] and polymer-based carriers [16] have also arisen as appealing methods with a great potential in the treatment of infections, due to several factors especially good biocompatibility and ample range and extent of drugs that they can carry. Another important factor is the protection provided by the encapsulation of the drug in the biological milieu, decreasing toxicity and allowing the drug to reach the specific site [17]. Chitosan is the natural polymer has been reported as a polymer-based protective agent to stabilize the metal nanoparticles. Because of the biocompatibility, biodegradability, nontoxicity and adsorption properties of chitosan, it was used as a stabilizing agent to prepare Ag, Au and Pt nanoparticles [18]. These chitosan- protected nanoparticles can be easily integrated into systems relevant for pharmaceutical, biomedical, and biosensor applications. Therefore, it has attracted considerable interest due to its medicinal properties, such as antifungal, antibacterial, antiprotozoal, anticancer, antiplaque, antitartar, hemostatic, wound healing and potentiates anti-inflammatory response, inhibits the growth of cariogenic bacteria, immunopotential, antihypertensive, serum cholesterol lowering, immune enhancer, increases salivary secretion (anti-xerostomial) and helps in the formation of bone substitute materials [19]. In the present study, an attempt has been made to study the improved anti bacterial property of biocompatible polymer chitosan stabilized silver nanoparticles-azithromycin (CS-AgNp-AZ) and levofloxacin (LF) nano drug conjugate fabricated wound dressing against human pathogenic bacteria.

MATERIALS AND METHODS

Synthesis and characterization of silver nanoparticles

Silver nanoparticles (AgNPs) were synthesized by chemical reduction of 1 mM silver nitrate with with 0.1M sodium borohydride as reducing agent. Synthesis of silver nanoparticles was confirmed by the conversion of the reaction mixture into brown colour and further characterization of the synthesized silver nanoparticles was carried out with determination of Plasmon absorption maxima with UV-Vis spectroscopy and particle morphology with scanning electron microscopy (SEM) and Fourier Transform Infrared Spectroscopy (FT-IR)

Preparation of chitosan stabilized silver nanoparticles –azithromycin (CS-AgNp-AZ) and levofloxacin (CS-AgNp-LF) nanoconjugate

Chitosan was obtained from Rolex chemical industries, Mumbai and refined twice by dissolving it in dilute HOAc solution. The solution was filtered, the chitosan was precipitated with aqueous sodium hydroxide, and the precipitate was dried in vacuum at room temperature [20]. Chitosan stabilized azithromycin and levofloxacin -silver nanoparticles conjugate was prepared with three ml aqueous solution of respective antibiotics with the final concentration of 0.1mg/ml, five /ml of synthesised nanoparticles and 5ml of 0.2% of chitosan solution. The mixture was stirred under magnetic stirrer for 3hrs to get the complete homogenous mixture. The slurry thus obtained was freeze dried and used for further studies. Characterisation of nanoparticles loaded antibiotic was carried out with FT-IR and Scanning electron microscope (SEM)

Evaluation of minimum inhibitory concentration (MIC)

The minimum inhibition concentration (MIC) was determined by a turbidometric method. In this method, a series of tubes each containing 2ml broth medium was prepared. The nanoparticles were accurately quantified and dispersed into the broth medium. Briefly, 2mL of nanoparticles added into the tubes separately. After mixing, 2 ml of mixture from these tubes were transferred to the next tubes, and a similar procedure was repeated for the subsequent tubes. Hence, each tube contained a test sample solution with half of the concentration of the previous one. The addition of the nanoparticles did not affect the pH of the medium. The bacteria suspension was then added to the tubes to achieve a final bacterial concentration of 10^5 cells/ml. The bacterial concentration was estimated from the optical density of the suspension based on standard calibration with the assumption that the optical density of 1.0 at 600nm for *Pseudomonas aeruginosa*, *Staphylococcus aureus* suspension is equivalent to approximately 10^9 cells/ml. All the tubes were incubated in an orbital shaker with a shaking speed of 200 rpm/min at 37°C for 20 h. The MIC was determined as the minimum concentration at which there is no visible change in the turbidity of the medium.

Coating of biocompatible polymer stabilized nano drug conjugate

Nano drug conjugate was coated on the wound dressing using pad-dry-cure method [21]. Sterile wound dressing obtained from the health care centre was cut to the size of 10×10 cm, immersed in minimal inhibitory concentration (MIC) of respective nano drug conjugate suspension with acrylic binder (1%) for 5 minutes then it was passed through a padding mangle, which was running at a speed of 15 m/min with a pressure of 15 kgf/cm² to remove excess solution. A 100% wet pick-up was maintained for all of the treatments. After padding, the fabric was air-dried and then cured for 3 min at 140°C. The fabric was then immersed for 5 min in 2 g/l of sodium lauryl sulfate to remove unbound nanoparticles. Then the fabric was rinsed at least 10 times to completely take out all the soap solution. The fabric thus washed was air-dried and dried pieces were used for further studies.

Characterization of nano drug conjugate coated wound dressing

The surface topography of nano drug conjugate coated dressing was characterized by scanning electron microscopy and FTIR. In a SEM setup, the nanoparticulate sample, coated to be conductive (e.g. gold, platinum), is scanned in a high vacuum chamber with a focused electron beam. FTIR analysis helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanomaterials. The samples were analyzed for their variations in chemical groups using FTIR spectroscopy. The wavelength of light absorbed is characteristic of the chemical bond and can be seen in this annotated spectrum. By interpreting the infrared absorption spectrum, the chemical bonds in a molecule can be determined. Effect of nano drug conjugate coating on mechanical properties was also studied. Respective nano drug conjugate coated wound dressing were conditioned at 65 ± 2% relative humidity and 27 ± 2°C before analysis. The coated fabrics were examined in the range of basic physical-mechanical parameters such as the tensile strength, the bursting strength, air permeability, friction measurements and UV blocking test followed by its functional properties by adapting standard methods.

Anti bacterial activity

The antibacterial activity of coated dressing was tested against pathogenic bacteria adopting agar diffusion assay against clinical isolate of *Staphylococcus aureus* and *Pseudomonas aeruginosa* isolated from pyogenic patients. The strains were maintained on brain heart infusion agar slants. A single colony of the test strains were grown overnight in Mueller Hinton broth on a rotary shaker (200 rpm) at 35°C. The inoculums were prepared by diluting the overnight cultures with 0.9% NaCl to a 0.5 McFarland standard. A lawn of the test organism was made on the Muller Hinton agar (Hi media, Mumbai, India) and the seeded plates were incubated at 37°C for 24 hours and the plates were observed for zone of inhibition. After the incubation period the diameter of the zone was recorded.

RESULTS AND DISCUSSION

Synthesis and characterization of chitosan stabilized nano drug conjugate

Silver nanoparticles (AgNPs) synthesis adopting chemical reduction was primarily confirmed by colour change of the reaction mixture from pale yellow to brown clearly indicating the formation of silver nanoparticles. The characteristic brown colour due to the excitation of Plasmon vibrations in the nanoparticles provides a convenient signature of their formation. Brown colour formation was observed after the drop wise addition of sodium borohydride to the silver nitrate and tri sodium citrate solution. Synthesized silver nanoparticles characterized by UV-Vis spectroscope which reveals a strong broad surface Plasmon peak located at 420nm (Figure 1). Particle morphology size and shape with transmission electron microscopy reveals spherical particles with the size of 20-40nm (Fig-2). Chitosan stabilized azithromycin and levofloxacin silver nano drug conjugate was primarily confirmed by surface plasma absorption maxima (SPAM) by UV-vis absorption spectroscopy, scanning electron microscopy (SEM) analysis with and FT-IR. Distinct shift in the surface plasma absorption maxima was observed after the stabilization of chitosan with the respective drug nano conjugate (Figure 3,4). The scanning electron microscopy study reveals chitosan stabilized azithromycin and levofloxacin -silver nanoparticles conjugate as spherical rough particles with the size range of 60 to 70nm and electron scarce thin chitosan coating. (Figure 5 a,b) The confirmation of the coating was also confirmed using EDX which shows the peak for all the elemental composition in the respective nano drug conjugate (Figure 5 c). Further characterization was carried out with FT-IR which helps to detect the functional groups, structure of a compound and purity of the sample in a given environment in terms of frequencies of radiation present in the nanoparticles. When the FTIR spectrum of respective nano drug conjugate were compared with free silver nanoparticles and free antibiotics, it was found that almost all the absorbed peaks were modified upon coating with chitosan (Figure 6,7,8).

Anti bacterial activity

Anti bacterial activity was studied by MIC determination adopting broth dilution method. All the tested bacteria were susceptible to the tested concentration of the nano drug conjugate (Table 1). In the case of CS-AgNp-AZ treatment, *Pseudomonas aeruginosa* (0.5 mg/ml) showed the highest sensitivity followed by *Staphylococcus aureus* and *E.coli*

(0.9 and 1.4 mg/ml). Similar finding was also recorded in CS-AgNp-LF treatment which revealed 0.6,1.1 and 1.6 mg/ml of MIC against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E.coli*. But the MIC was found to be high in the both free antibiotics treatment against all the tested strains.3.2,2.9 and 2.3,4.1,3.4 and 2.2 mg/ml of MIC was recorded in free azithromycin and levofloxacin treatment against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E.coli*.

Fabrication of wound dressing

Synthesized nano drug conjugate was coated on the wound dressing by pad dry cure method using binder with minimum inhibitory concentration of respective nano drug conjugate. Surface topography of wound dressing with SEM reveals complete dispersion of nano conjugate on the fiber surface and the size of the embedded particles was 60-70 nm (Figure 9,10). The particle size plays a primary role in determining their adhesion to the fabrics. It is reasonable to expect that the largest particles agglomerates will be easily removed from the fiber surface, while the small particles will penetrate deeper and adhere strongly into fabric matrix. The effect of coating on fabric strength was evaluated for tensile strength and bursting strength addition to air permeability. Table 2 shows the effect of coating on the mechanical properties of coated material. Nano coating did not cause any effect on the mechanical properties except friction. Nano coated wound dressing due to its nano-size and uniform distribution, friction was found to be significantly lower. Air permeability is an important factor in the performance of textile materials used to provide an indication of the breathability of coated material. The uniform and very thin distribution of nano drug conjugate increased the air permeability and hence its breathability. In case of bursting strength, no significant variation was observed due to coating. The coefficient of friction is the parameter most frequently used to evaluate degrees of smoothness or roughness, so it is important to match this quantity with the tactile feel of the fabrics studied. Anti bacterial copper nanoparticles were coated on the wound dressing and the effect of coating on mechanical properties has been studied by Karthick Raja Namasivayam *et al* [22]. Their study revealed distinct effect was not observed in the nano coated material except air permeability.

Anti bacterial activity

Anti bacterial activity of respective nano drug conjugate fabricated wound dressing was studied by solid plate agar diffusion assay (Table 3). All the tested bacterial strains were susceptible to the both the chitosan stabilized nano drug conjugate treatment. Minimum bactericidal effect was observed in free silver nanoparticles and chitosan against all the tested strains. Anti bacterial activity of both nano drug conjugate was found to be higher against all the tested strains than the free antibiotics by the increase in zone of inhibition with minimum 7 to maximum 8mm. In the case of CS-AgNp-AZ fabricated wound dressing, the zone of inhibition against *P.aeruginosa*, *Staph.aureus* and *E.coli* was found to be 20.0,18.5 and 17.0mm (Figure 11,12). Free azithromycin revealed 12.0,10.0 and 10.0mm of zone of inhibition against the respective bacterial strains. Enhanced anti bacterial activity was also recorded in CS-AgNp-LF fabricated wound dressing which revealed 19.0,18.0 and 16.0mm of zone of inhibition against the respective tested strains. Antibacterial activity of cotton fabrics impregnated with biogenic silver and copper nanoparticles has been reported [23,22,24]. Improved anti bacterial activity of biogenic silver nanoparticles-antibiotics (AgNp-abt) conjugate coated wound dressing against human pyogenic bacteria has studied.

Table 1. Minimum inhibitory concentration (MIC) of nano drug conjugate against pathogenic bacteria

S.No.	Treatment	MIC (mg/ml)		
		<i>P.aeruginosa</i>	<i>Staph.aureus</i>	<i>E.coli</i>
1	Free AZ	3.2	2.9	2.3
2	Free LF	4.1	3.4	2.2
3	CS-AgNp-AZ	0.5	0.9	1.4
4	CS-AgNp-LF	19.0	18.0	1.6

Table 2. Effect of chitosan stabilized nano drug conjugate treatment on mechanical properties of wound dressing

Treatment on fabrics	Tensile strength				Air permeability [m/(kPa.s)]	Bursting strength (kg/cm ²)
	Av. breaking load (kg)		Strain (%)			
	Warp	Weft	Warp	Weft		
Control	21.07	18.79	17.05	13.07	6.70	4□38
CS-AgNp-AZ	17.21	21.01	15.73	16.12	9.65	5□56
CS-AgNp-LT	18.01	24.21	14.01	17.31	10.11	61.0

Table 3.Zone of inhibition (mm) of chitosan stabilized nano drug conjugate against human pathogenic bacteria

S.No.	Treatment	Zone of inhibition(mm)		
		<i>P.aeruginosa</i>	<i>Staph.aureus</i>	<i>E.coli</i>
1	Free AZ	12.0	10.0	10.0
2	Free LF	11.5	10.4	9.2
3	CS-AgNp-AZ	20.0	18.5	17.0
4	CS-AgNp-LF	19.0	18.0	16.0

Figure 1. UV absorption spectra of silver nanoparticles

Overlay Spectrum Graph Report

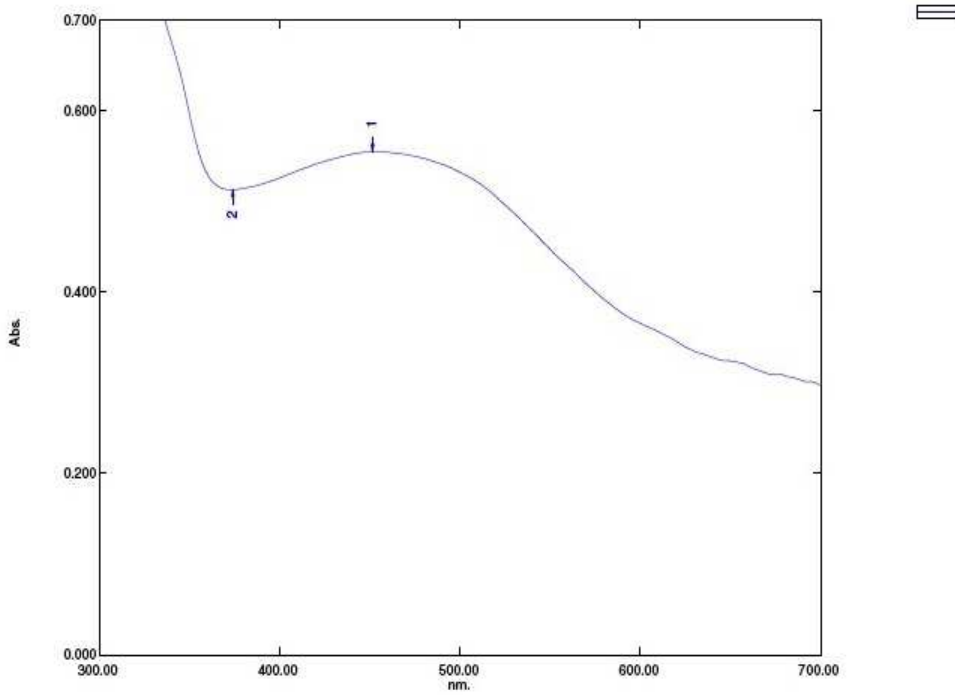


Figure 2.Scanning .electron micrograph of silver nanoparticles

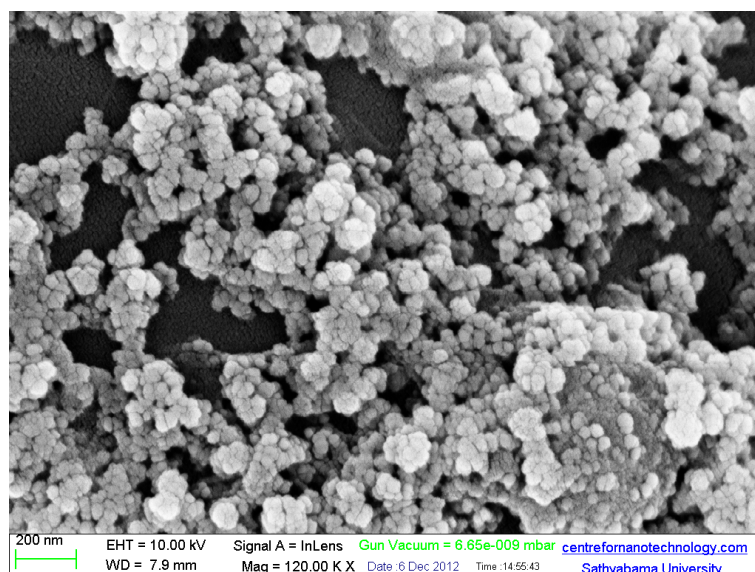


Figure 3. UV –vis absorption spectra of chitosan stabilized silver-azithromycin drug nano conjugat

Overlay Spectrum Graph Report

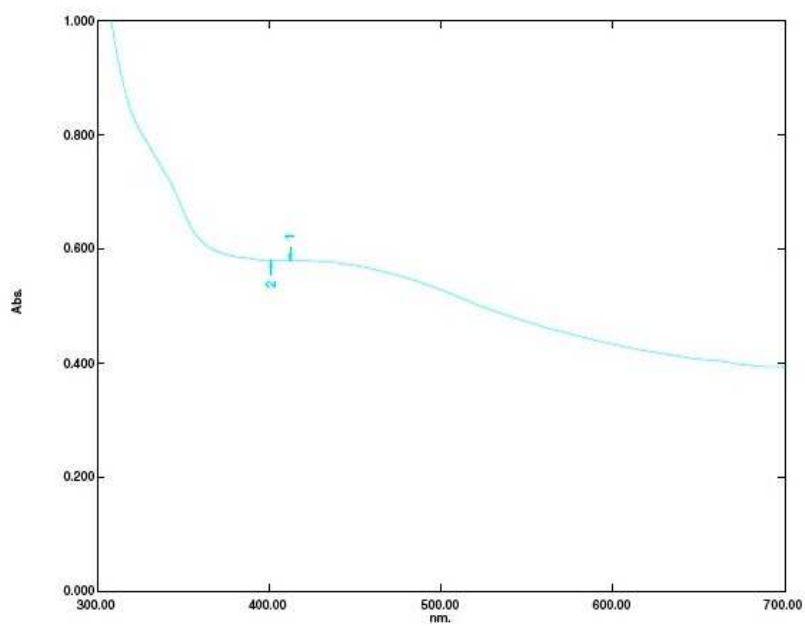


Figure 4. . UV –vis absorption spectra of chitosan stabilized silver- levofloxacin drug nano conjugate

Overlay Spectrum Graph Report

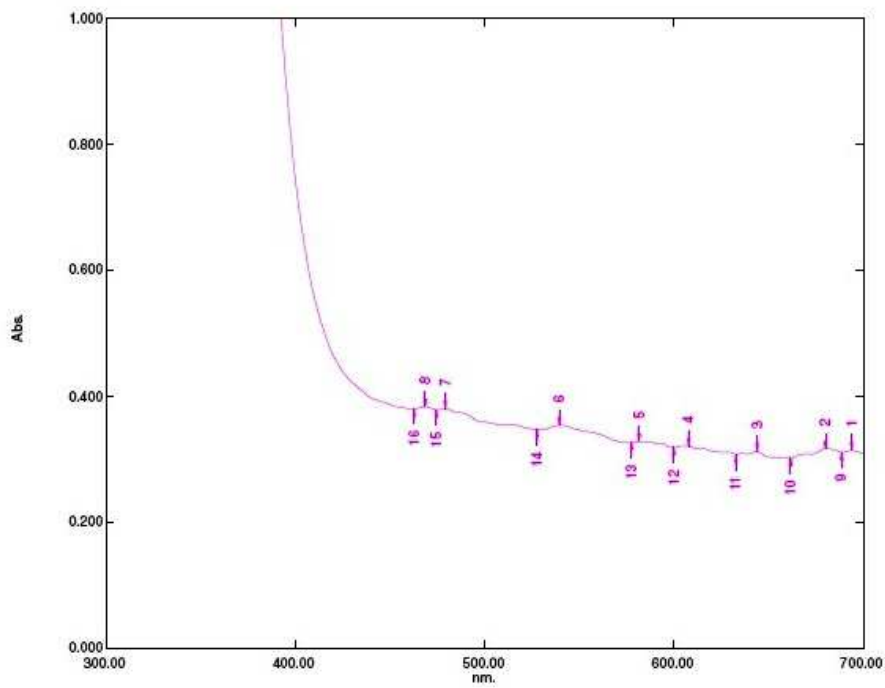


Figure 5a. Scanning electron micrograph of chitosan stabilized silver-levofloxacin nano drug conjugate

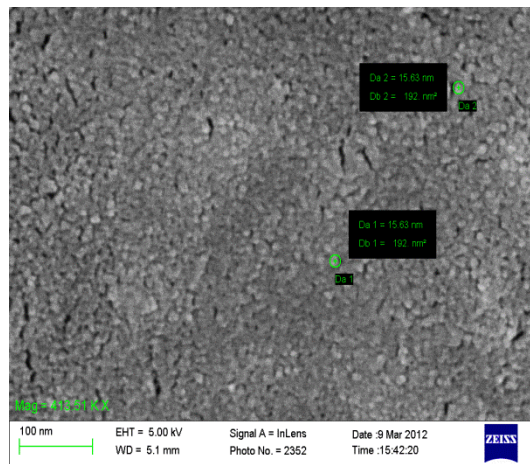


Figure 5b. Scanning electron micrograph of chitosan stabilized silver-levofloxacin nano drug conjugate

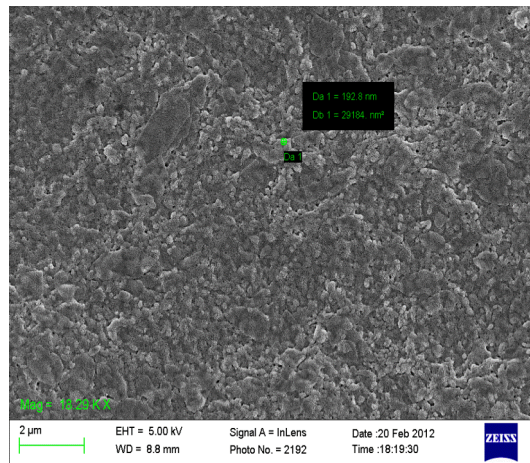
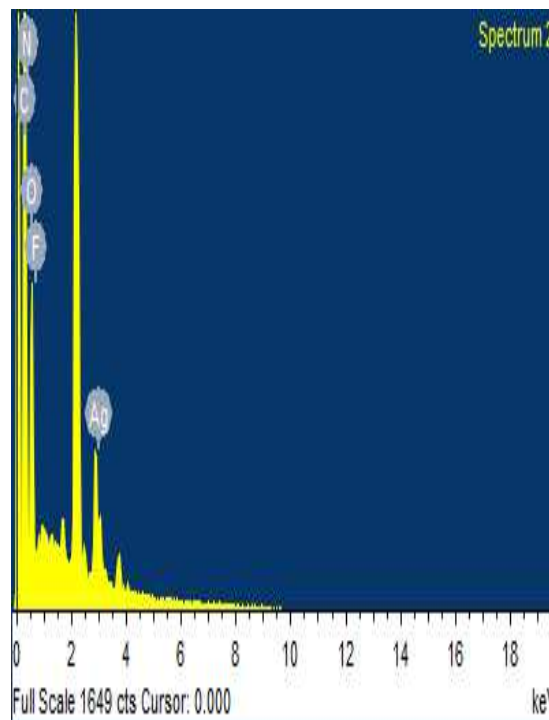


Figure 5c. EDX spectra of chitosan stabilized silver-Azithromycin nano drug conjugate



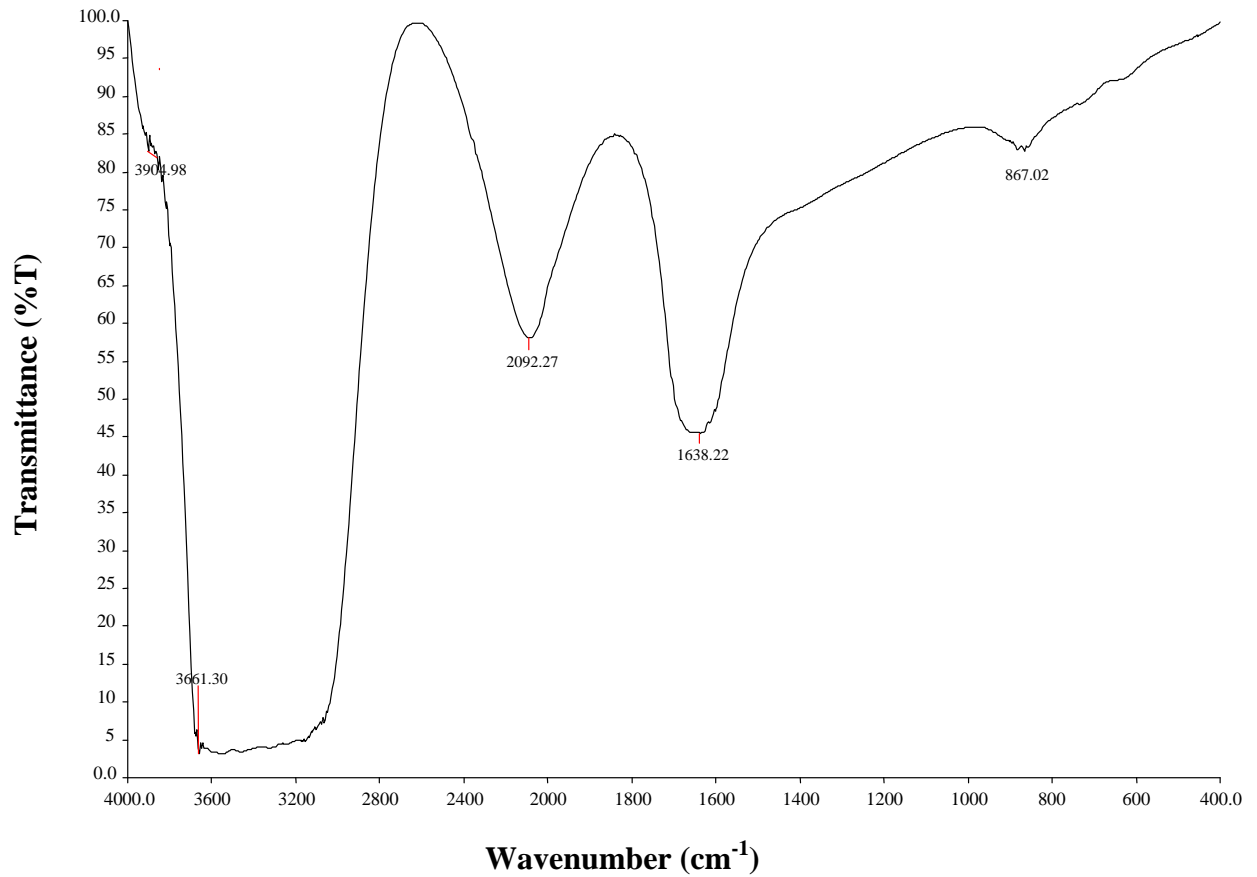


Figure 6. FTIR spectra of free silver nanoparticles

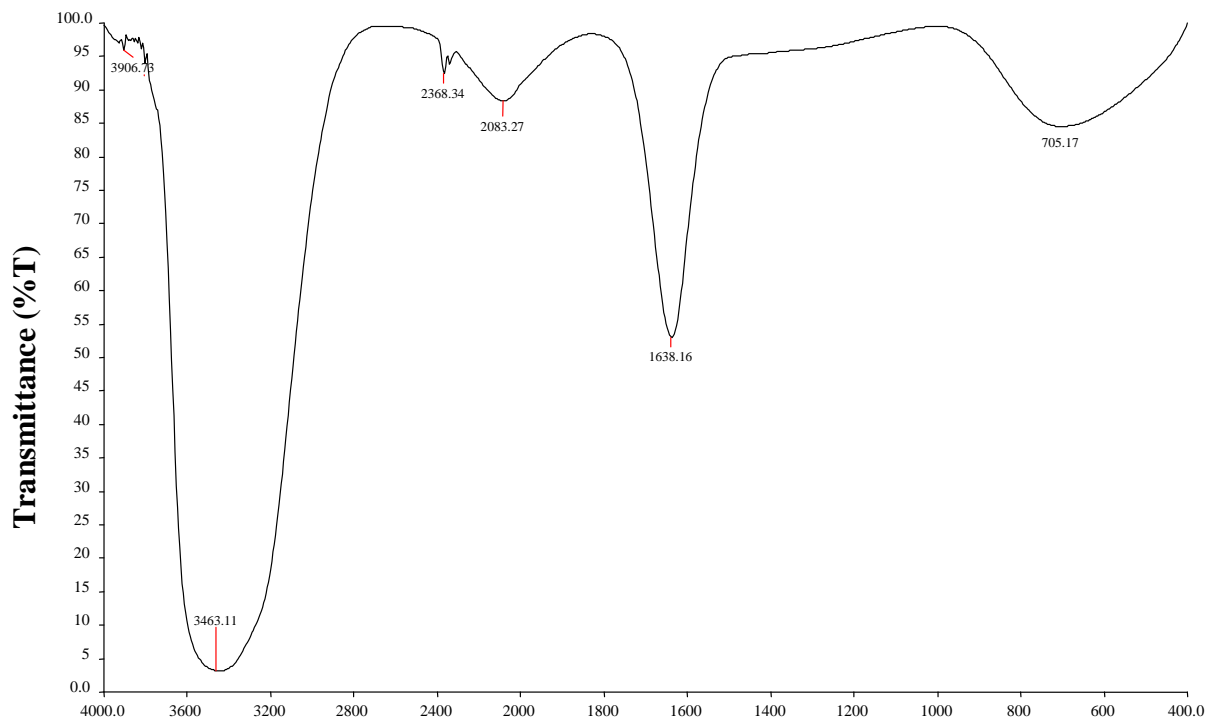


Figure 7. FTIR spectra of chitosan stabilized silver-levofloxacin nano drug conjugate

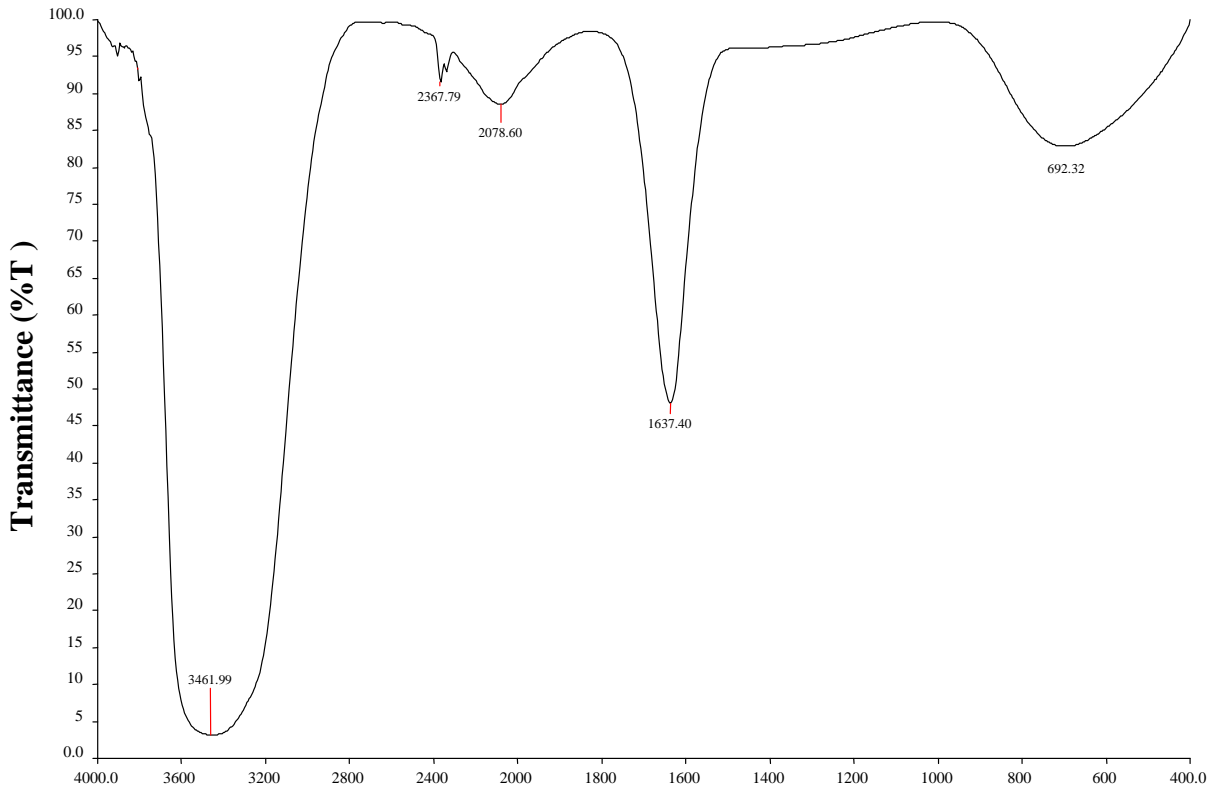


Figure 8. FTIR spectra of chitosan stabilized silver- azithromycin nano drug conjugate

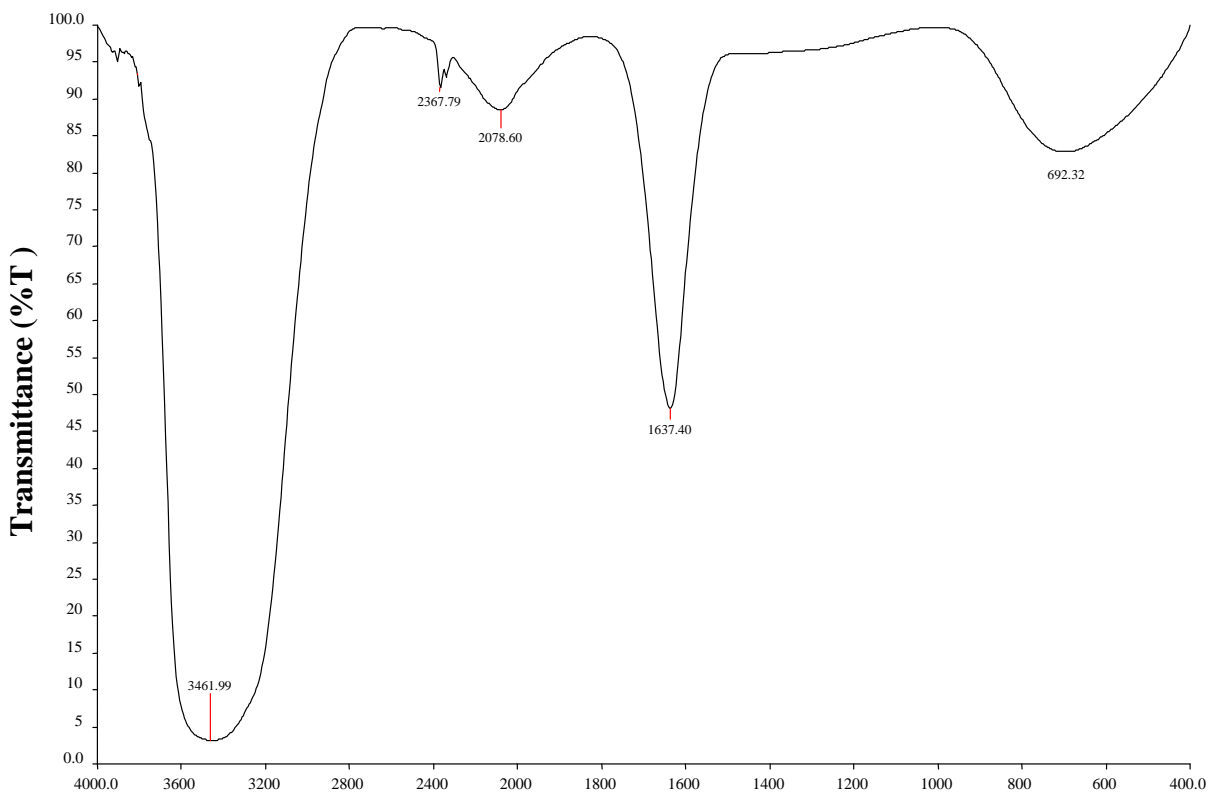


Figure 11. Zone of inhibition (mm) of chitosan stabilized Silver –levofloxacin nano drug conjugate against pathogenic bacteria

a. *Pseudomonas aeruginosa*



b. *Staphylococcus aureus*



C.E.coli



Figure 12. Zone of inhibition (mm) of chitosan stabilized Silver –Azithromycin nano drug conjugate against pathogenic bacteria

a.P.aeruginosa*b.Staph.aureus**c.E.coli*

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