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PREPARATION AND CHARACTERIZATION OF MAGNETIC NANOPARTICLES LOADED WITH ANTIMICROBIAL AGENT

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ABSTRACT

Background: Bacterial infections are an important cause of serious health issues worldwide. Various antibacterial drugs have been developed but they have numerous side effects. Development of drug loaded magnetic nanoparticles will help to achieve targeted drug delivery while sustaining the release of drug. It will also enhance its antibacterial activity by using iron oxide. **Method:** Drug loaded iron oxide nanoparticles were developed to sustain and enhance the antibacterial activity of drug. Chitosan was used as a polymer. The method adopted to prepare magnetic nanoparticles was co-precipitation. Formulated magnetic nanoparticles were tested for drug release, surface morphology, antibacterial activity and FTIR. **Results:** It was observed from the findings that both formulations were effectively loaded with drugs. It was also found that the release of drug levofloxacin was sustained over a period of 48 hrs. The SEM results showed the semi spherical nanoparticles effectively loaded with drug. FTIR spectrum revealed characteristic peaks related to functional groups. Raman spectroscopy showed characteristic bands of both drugs and drug loaded iron oxide nanoparticles. Antibacterial assay results showed an enhanced antibacterial activity by using iron oxide nanoparticles **Conclusion:** It was concluded from the study that magnetic nanoparticles could be one of the best approaches to load antibacterial drugs while sustaining and enhancing their antibacterial activity.

Keywords: Magnetic nanoparticles, Chitosan, Levofloxacin, Sustained release, Antibacterial activity.

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INTRODUCTION

Novel drug delivery systems employ the use of specific carriers that get bind with the drug molecules enabling their delivery at specific sites of body. Revolutionary benefits attained with the use of novel drug delivery systems owe to the specific characteristics of such carriers. Carriers in novel drug delivery systems include microparticles, polymeric micelles, liposomes, niosomes, dendrimers, quantum dots, hydrogels, nanogels, nanoparticles and magnetic nanoparticles [**1**].

Recent advancement in nanotechnology and molecular biology has enabled the evolution of specific functionalized nanoparticles that overcome the limitations of conventional diagnostic and therapeutic agents [**2**]. They have variety of uses but because of unique physical properties, they are used in targeted drug delivery systems. The magnetic nanoparticles are considered as "clever particles" which has a magnetic core which directs particles to their target, a layer for the attachment of receptors, and a drug loaded inside the inner cavities of particles [**3**].

Super paramagnetic iron oxide nanoparticles are extensively used for applications such as magnetic resonance imaging, immunoassay, detoxification of biological fluids, drug delivery, tissue repair, hyperthermia and cell separation.

Hyperthermia provides a striking method for cancer treatment, it is linked with less harmful effects in contrast to radiotherapy and chemotherapy [**4**]. Upon locating a malignancy or lesion, external magnetic fields control the direct particle assembling to provide therapeutic effects. To make the therapeutic treatments effective, transition metals (e.g. Fe, Co, Ni) or metal oxides (e.g. Fe_3O_4, g-Fe_2O_3) are used to achieve magnetization. Small iron oxide NP's are used for diagnosis in vitro, over more than 50 years. Nanoparticle surfaces must be modified to improve biocompatibility and reduce aggregation [**5**].

Antibacterial activity is linked to compounds which in the vicinity kill bacteria and decrease their growth. The increase of resistance of bacteria to antibacterial drugs is a usual occurrence, and is a main problem. The infectious diseases are the extreme healthcare challenges all over the world because bacteria have become resistant against various antibacterial agents. Nano materials have appeared as novel, antimicrobial agents. Numerous classes of the antimicrobial NPs and nano carriers for the delivery of antibiotics have proved their efficacy, for handling infectious diseases which include the ones resistant to antibiotics [**6**].

The magnetic core and surface coating of magnetic nanoparticles determines their prospective in drug delivery systems. The coatings are known to eradicate or diminish its aggregation under bodily conditions. Chitosan, as a derivative of chitin, is a striking natural biopolymer from renewable resources because of the presence of functional groups of amino and hydroxyl within its structure. Because of the enormous surface to volume ratio, compared to bulk form of chitosan, chitosan nanoparticles have exceptional, biological, physico chemical and antimicrobial properties. These exclusive properties make chitosan NPs a favorable biopolymer for use of DDSs [**7**].

The current study involves the development and characterization of Levofloxacin loaded chitosan- magnetic nanoparticles. Drug loaded magnetic nanoparticles were produced by co precipitation method. Magnetic nanoparticles like ferrous oxide Fe3O4 also known as magnetite and ferric oxide also known as maghemite are compatible biologically. They are used and inspected for treatment of various cancers, sorting of stem cells, targeted drug delivery, gene therapy, analysis of DNA and MRI. Magnetic nanoparticle consists of following parts: 1) A magnetic core 2) Protective coating 3) Organic linker 4) Active molecule. There are various routes for the synthesis of magnetic nanoparticles. The most used ones are microemulsion, sol-gel method, hydrothermal synthesis, thermal decomposition and co-precipitation. There are three methods of preparation of magnetic nanoparticles: 1. Physical 2. Chemical 3. Biological

Table 1: Comparison of methods of preparation.

Technique	**Method**	**Product Morphology**	**Advantages**	**Disadvantages**
Physical	Gas/Aerosol phase methods (Spray pyrolysis and Laser pyrolysis)	Spheres and irregular spheres	Easy to execute	Problematic in controlling the size of particle
	Electron beam lithography	Spheres and rods	Well controlled inter particle spacing	Requires expensive and highly complex machines
Chemical	Co-precipitation	Spheres	Simple and effective	Nanoparticles are of broad size distribution. Coating can be difficult.
	Thermal Decomposition	Spheres, cubes	Narrow size distribution	Control of particle size is difficult.
	Microemulsion method	Spheres	Little solvent involved. Narrow size distribution Good shape control	Low yield
Biological	Microbial Incubation	Small platelets, spherical or rod-like spheres, irregular spheres	Good reproducibility and scalability, high yield, and low cost	Slow and laborious

MATERIAL AND METHOD

$FeCl_3.6H_2O$ ferric chloride hexahydrate and $FeCl_3.4H_2O$ ferric chloride tetrahydrate purchased from Universal Chemicals Lahore. Sodium Hydroxide NaOH was purchased from Panreac Quimica SLU Barcelona. Chitosan was gifted by Saffron Pharmaceuticals. Glacial acetic acid ethanol was purchased from Sigma-Aldrich UK. Phosphate Buffer Saline (PBS) was self-prepared using potassium monobasic phosphate and NaOH. Fresh deionized and distilled water bottles were purchased from Batteries Care Enterprises Lahore and Super Scientific Store Faisalabad.

Preparation of Iron Oxide Nanoparticles

Firstly, a solution of Ferric chloride hexahydrate ($FeCl_3.6H_2O$) 4g and ferric chloride tetrahydrate ($FeCl_2.4H_2O$) 4g was prepared in 50 mL of water. The above solution of ferric and ferrous ions was added gradually into a solution of 2M NaOH with constant stirring, having a room temperature pH of less than 10. At room temperature, solution was sonicated for 60 minutes. Finally, the filtration of particles, and three times washing with deionized water was performed. The resultant particles were dried on hot plate at 70-90 ºC for 3-4 hours [**8**].

Preparation of Chitosan-Coated Magnetic Nanoparticles

2g of chitosan was added in a 1% acetic acid solution and dissolved (Vieira, Arias et al. 2019). Acetic acid solution 1% was prepared by adding 1ml acetic acid to 99 ml water. The dried Fe_3O_4 were mixed with chitosan solution in a beaker, and constantly stirred for 18 hours. The separation of MNP's coated with chitosan was done by placing a permanent magnet under the beaker, the MNP's settle down while the remaining solution is decanted. The separated MNP's were dried on a hot plate at a temperature of 70°C for 2 hrs [**9**].

Preparation of Drug-Chitosan Magnetic Nanoparticles

Drug loading in chitosan- MNP's was performed by adding the drug solution (0.58g drug in 50 ml deionized water) drop wise with constant stirring into the aqueous dispersion of MNP's. The aqueous dispersion was prepared by suspending formed MNP's in 50 ml deionized water. The drug solution containing the mixture of CS-MNP was stirred magnetically. The stirring was carried out at room temperature for duration of 18 hours, to assist uptake of drug. Separation of the final product which is drug coated with CS-MNP, was achieved by placing permanent magnet under the beaker. After decanting the top layer drug loaded MNP's were obtained and dried on a hot plate for 30 mins to one hour.

For levofloxacin the solvent used was deionized water because it is a water soluble drug. In this way levofloxacin and MNP's were prepared (**Fig. 1**). In the whole procedure, all solutions were prepared using deionized water.

Figure 1: Conceptual image of drug loaded MNP's.

Physical Appearance of Magnetic Nanoparticles

The drug loaded, chitosan coated magnetic nanoparticles were evaluated with naked eye to check the appearance.

Magnetic Properties of Magnetic Nanoparticles

The magnetic properties of MNP's were analyzed by dispersing the MNP's in water inside a glass container. Then that glass container was placed in front of the magnetic field produced by a permanent magnet, to see how the magnetic nanoparticles behave [**10**].

In vitro Release of Levofloxacin from the Magnetic Nanoparticles

The *in vitro* release profile of levofloxacin from nanoparticles was performed on the LFX MNP's formulation. Nanoparticles equivalent to 10 mg of levofloxacin were re dispersed in 10 ml of pH 7.4 phosphate buffer solution and 1ml was placed in a dialysis membrane bag with a molecular cutoff of 5 kDa which act as a donor compartment, tied and placed in a beaker containing 100 ml of pH 7.4 phosphate buffer solution which acts as a receptor compartment. The entire system was kept at 37°C in a shaking water bath for 48hrs. At appropriate time intervals (0.5, 1, 3, 5, 7, 10, 12, 24, 48h), 2 ml of the release medium was removed and 2 ml fresh pH 7.4 phosphate buffer solution was added into the system. The amount of levofloxacin in the release medium was evaluated by UV-visible spectrophotometer at 288.5 nm [**11**].

Fourier Transform Infrared Spectroscopy (FTIR)

An (FT-IR) spectroscopy study was carried out with the help of nicolet 6700 fourier transform infrared spectroscope. The transmission mode was used in the wavelength range of 4000-650 cm^{-1}. The gas used was nitrogen. The numbers of scans performed were 128 having a resolution of 8 cm^{-1}. The spectra obtained from the spectroscopic study are shown in the results.

Scanning Electron Microscopy (SEM)

SEM of magnetic nanoparticles was performed using TESCAN Vega 3 LMU – Variable pressure Scanning Electron Microscope. This scanning electron microscope is capable of imaging and elemental characterization of metals, ceramics, polymers and biological specimens. Detectors used in this microscope include secondary electron, back scattered electron and energy dispersive detectors (EDX).

Particle Size Analysis using Zeta Sizer

Particle size was analyzed by using Zeta sizer (Malvern Instruments, UK). Suspension of particles (to be analyzed) was prepared by using water that was filtered through a filter of 0.45 µm. Then this aqueous suspension was placed in clear disposable zeta cell. Evaluation was done by dynamic light scattering method. Particle size was measured by dynamic light scattering method and recorded [**12**].

Raman Spectroscopy

Molecular vibration, crystal structure, chemical composition of the prepared magnetic nanoparticles was determined by Raman spectroscopy with model in Via Raman Microscope by Renishaw UK [**13**].

Antibacterial Assay

Firstly, Muller Hilton agar was prepared by adding agar 23g in 750ml distilled water. Then the prepared agar media was poured in conical flask. All the apparatus including petri plates, test tubes and prepared agar media were placed in autoclave for sterilization at 121ºC for 90 mins. After 90 mins, temperature was reduced to 50 ºC. All the apparatus was taken out. All petri plates were placed under UV lamp. Then 20-30 ml agar media was poured in each petri plate and allowed to solidify for 20 mins. Bacterial strains were refreshed by adding them in 5-10 ml sterilized water and allowed to stand for 30 mins. Streaking of refreshed bacterial strains was performed by dipping the cotton bud in refreshed bacterial culture. Then it was streaked out in both directions on petri plates containing solidified agar media. Using a cork borer, a small hole was created in the agar media. Then our samples solutions were poured inside the wells. After 24 hrs of incubation at 37ºC, the zones of inhibition were checked and measured.

RESULTS AND DISCUSSION

Physical Appearance of Magnetic Nanoparticles

The fabricated MNP's were physically evaluated with naked eye. They were small particles being black in color due to the presence of ferric chloride.

Magnetic Properties of Magnetic Nanoparticles

The magnetic properties of drug loaded iron oxide nanoparticles were determined by placing

their solution in front of a permanent magnet. It was seen that all the magnetic nanoparticles stick to the walls of the glass container facing the permanent magnet.

Fourier Transform Infrared Spectroscopy (FTIR)

To evaluate structural properties, confirm presence of specific functional groups and assure chemical cross-linking within network structure, polymer (chitosan), active drug (levofloxacin), blank magnetic nanoparticles and drug-loaded magnetic nanoparticles of levofloxacin, physical mixture of all these were characterized by fourier transform infrared spectroscopy by scanning them in range of 4000 to 500 cm^{-1} to obtain their respective spectra. The FTIR spectrum was used to evaluate the functional groups existing in the chitosan. The spectrum of pure chitosan shows several characteristic peaks at 3280, 2865, 1616, 1541 and 1374 cm^{-1}. A peak at 3280 cm^{-1} corresponds to N-H and O-H stretching. The absorption band at 2865 cm^{-1} is attributed to C-H stretching vibrations (Nehra, Chauhan et al. 2018). A band at 1616 cm^{-1} is due to the N-H bending vibrations. 1374 cm^{-1} is due to C-N stretching vibrations and 1004 cm^{-1} is due to C-O-C stretching vibrations. FT-IR of drug LFX as shown in **Fig. 2** showed the following characteristic peaks: peak at 3257.79 cm^{-1} due to carboxylic group, 2801.82 cm due to alkanes group stretching, 1715.53 cm^{-1} due to stretching of carbonyl group, 1288.07 cm^{-1} due to stretching of amines, in between 1100 to 1400 cm^{-1} due to the presence of halogen group. FTIR spectrum of synthesized iron oxide nanoparticles show various well defined peaks at 3724, 3261, 1617 and 1540 cm^{-1}. The peak at 3724 cm^{-1} is present in all spectras of magnetic nanoparticles which shows that this peak is specific to MNP's. The peak positioned at 3261 cm^{-1} is due to bending vibration of OH group. Two peaks at 1617 and 1540 cm^{-1} are due to chitosan coating of iron oxide nanoparticles. Few smaller peaks between 500 to 600 cm^{-1} are due to the presence of Fe-O, which confirms the synthesized iron oxide nanoparticles. The peak carboxylic group appears as it is and on the same wavenumber 3257 cm^{-1} but the alkane group is absent [**14**]. The peak of C=O shifted to a slightly higher wave number from 1715.53 cm^{-1} to 1767.82 cm^{-1} which shows an increase in vibration of the bond. The wave number corresponding to C-N group is increased greatly from 1288.07 cm^{-1} to 1337.91^{-1}. The halogen group is present as it is. These functional groups confirm the presence of drug LFX in MNP's (**Table 2**).

Scanning electron microscopy (SEM)

Surface properties of iron oxide MNP's and drug loaded chitosan magnetic nanoparticles were evaluated by scanning electron microscope. For this purpose, photographs of high resolution were taken at magnifications of 50kx, 25kx, 10kx and 5kx as depicted in **Fig. 3.**

The images of iron oxide nanoparticles reveal rough semi-spherical morphology. The particles are found to be scattered without aggregation which confirm the presence of high- purity magnetite nanoparticles. The images show effective coating of magnetic nanoparticles with chitosan [**15**].

The images reveal effective drug loading of levofloxacin in MNP's. The empty spaces are filled with drug as indicated by the white spots. Some particles are rough surfaced and some are spherical in shape.

Particle Size Analysis using Zeta Sizer

Particle size of prepared magnetic nanoparticles were evaluated by dynamic light scattering method and recorded. The particle size of optimum formulation of magnetic nanoparticles is shown in **Fig. 4**.

Table 2: Interpretation of FTIR spectrum of LFX and LFX MNP's.

Functional groups	-COOH cm^{-1}	-CH_3 cm^{-1}	C=O cm^{-1}	C-N cm^{-1}	F (halogen group) cm^{-1}
LFX	3257.79	2801.82	1715.53	1288.07	1085.95
LFX MNP's	3257.83	Absent	1767.82	1337.91	1090.12
Physical mixture of LFX-CS-blank MNP's	3259.25	Absent	1724.52	1289.25	1086.60

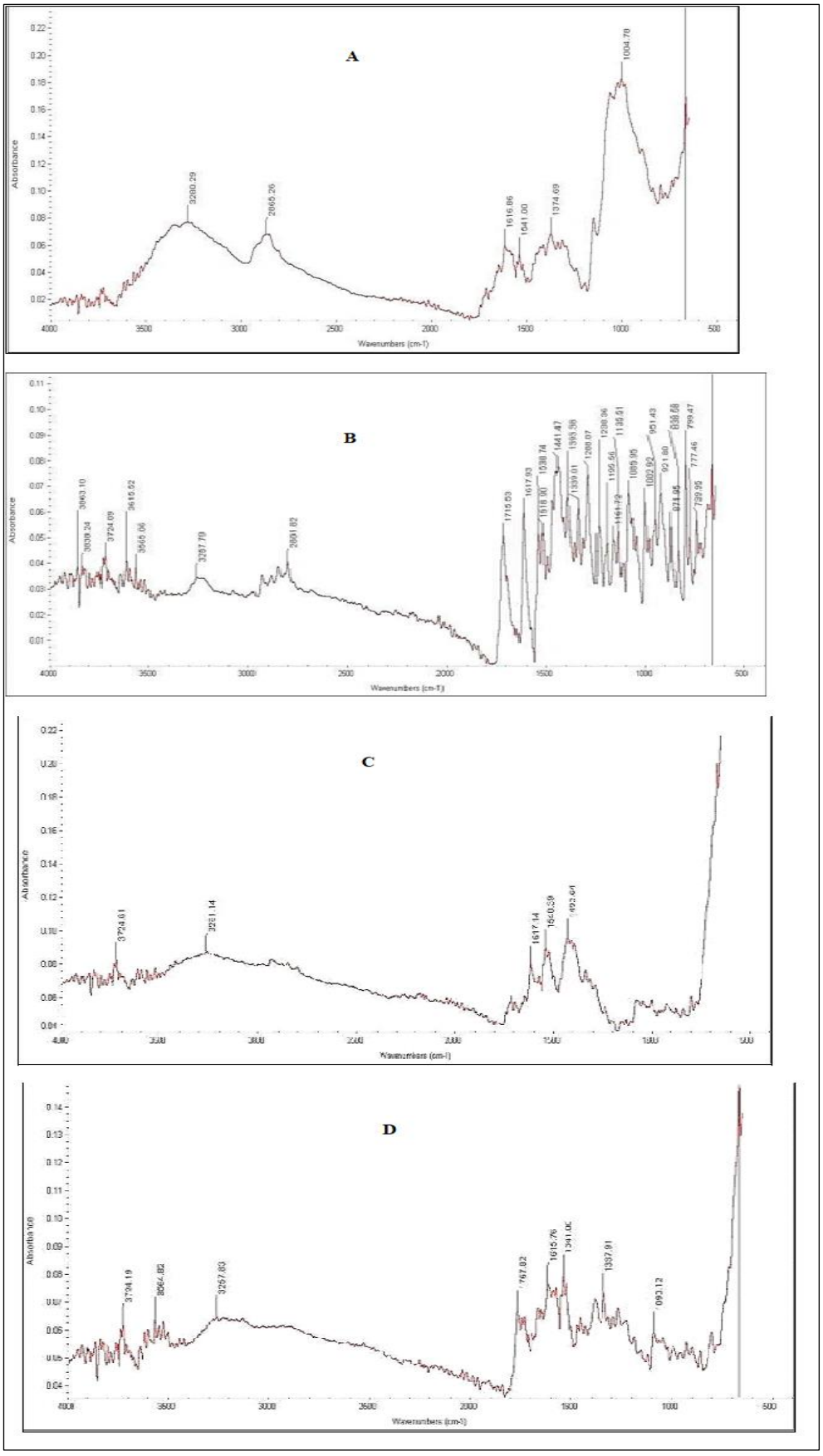

Figure 2: FTIR spectrum of physical mixture of drug LFX, chitosan and blank MNP's.

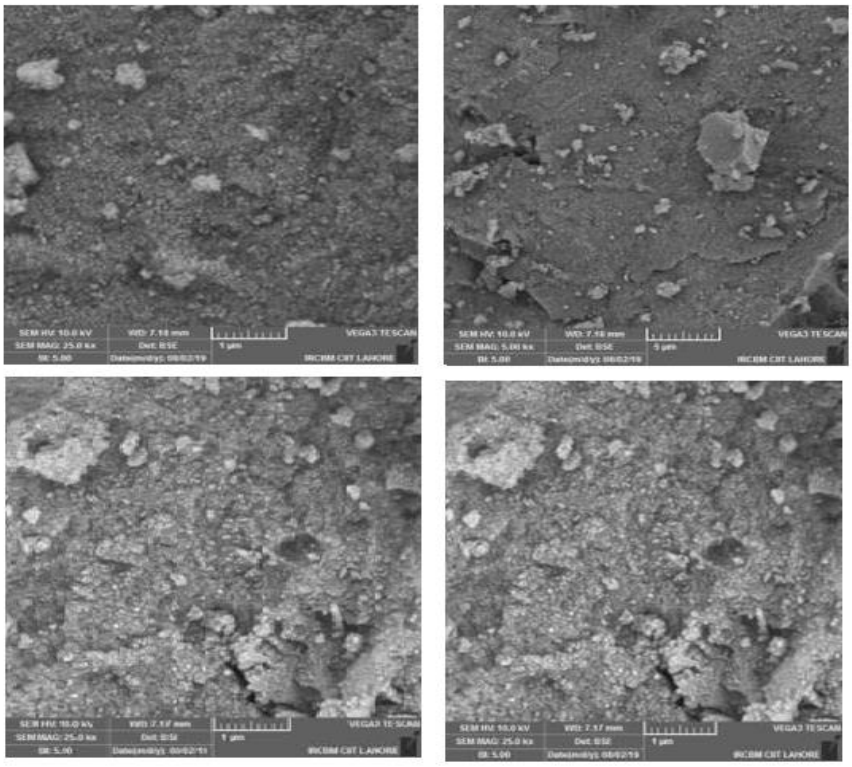

Figure 3: Scanning electron microscopy photographs of iron oxide NP's at different magnifications.

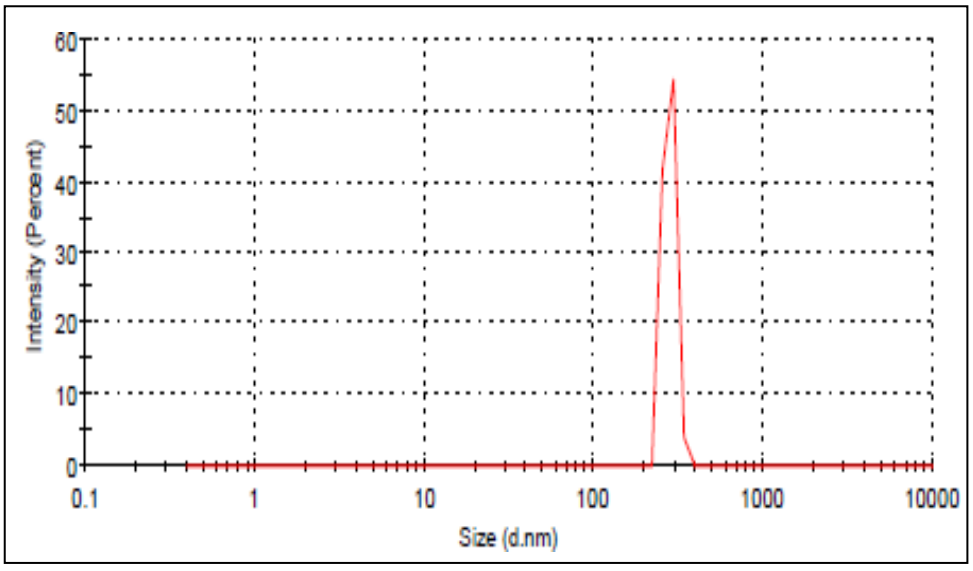

Figure 4: Particle size of magnetic nanoparticles.

Average hydrodynamic diameter of magnetic nanoparticles was evaluated using Zetasizer (Malvern Instruments, UK) and size found to be 200 nm that is within the range of nanoparticle size (0- 200 nm). In vitro drug release study of levofloxacin loaded MNP's. The release profile of levofloxacin loaded, chitosan coated iron oxide nanoparticles was studied. It was found out that cumulative release % of levofloxacin increase with time. For upto 12 hrs initial burst release phase was observed after which a slow

and sustained release was observed for upto 48hrs. After 48hrs, 91 % of the drug was released from the Fe3O4/CS/nanoparticles [**16**]. Hydrophilic nature of the polymer chitosan allows the penetration of release medium, which causes rapid dissolution of the drug in close contact to the surface of the nanoparticles. These all factors contribute to initial quick release of the drug from the nanoparticles. Also the size of the drug molecule is smaller than the MNP's, so drug levofloxacin easily diffuses through nanoparticles surface in a short time. The slower release is then caused by changing release mechanism due to drug diffusion through matrix of chitosan.

Antibacterial Assay of Levofloxacin Magnetic Nanoparticles

Antibacterial assay of drug loaded MNP's and blank drug was performed by agar well diffusion method. Their antimicrobial activity was determined against two types of bacteria: *Staphylococcus aureus* and *Escherichia coli*. The drug solutions were used in four different concentrations i.e. 500 µg/mL, 250 µg/mL, 125 µg/mL, 75 µg/mL. The diameter of the zones of inhibition was measured and results were interpreted.

The results show that both levofloxacin has good antibacterial activity against Staphylococcus aureus and E-coli. Their zones of inhibition are shown in **Fig. 5**. Levofloxacin zone of inhibition measured against *S. aureus* was 17mm and *E. coli* was 18mm. The zone of inhibition was increased in case of LFX MNP's to 21mm against *S. aureus* and 22 mm against *E. coli*. This shows that LFX MNP's having iron oxide has improved antibacterial effects in comparison to drug LFX alone [**17**].

This improved antibacterial activity of levofloxacin was due to the presence of chitosan and iron oxide in the nanoparticles. Both chitosan and iron oxide possess antibacterial activities of their own, thus enhancing the activity of drug levofloxacin. So we can say that LFX MNP's possess better antibacterial activities than the drugs alone due to chitosan. It was also found out that in case of *E. coli* the positively charged surface of metal oxide NPs may bind to cell membrane which is negatively charged through electrostatic interactions which disrupt bacterial functions thus enhancing the antibacterial effects of iron oxide nanoparticles. Similar results were found by.

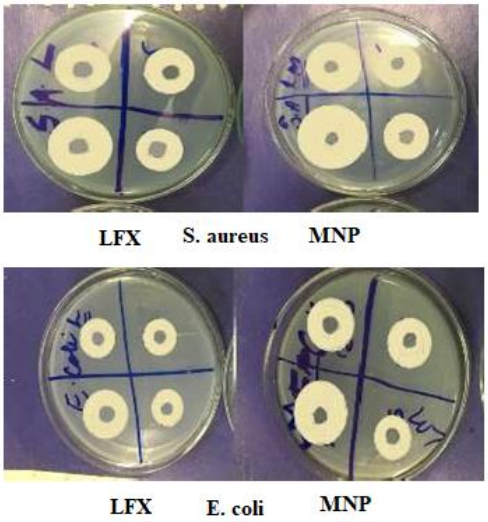

Figure 5: Antibacterial activity of **(a)** drug LFX **(b)** LFX MNP's showing zone of inhibitions against E-coli bacteria by agar well diffusion method.

Raman Spectroscopy

The drug levofloxacin, iron oxide nanoparticles and levofloxacin nanoparticles were characterized by raman spectroscopy and various peaks were obtained by scanning them in the range of 200-4000 cm^{-1}. **Fig. 6** shows the nature of the iron oxide core determined by raman spectroscopy. The irradiation of energy on the sample produces molecular effects which lead to Raman effect. The raman spectrum peaks of magnetite were investigated where the peak at 707 cm^{-1} was identified as band characteristic and is related to Eg phonon modes whereas the peak at 339 cm^{-1} is related to A1g phonon mode [**18**].

Raman spectra of levofloxacin showed characteristic bands. The band at 1774.7 cm^{-1} shows C=O stretching vibration and -OH in-plane bending vibration. The band at 1614 cm^{-1} corresponds to C-C stretching vibration in aromatic ring. The band at 1432 cm^{-1} is due to CH_2 bending vibration. In the Raman spectra of levofloxacin magnetic nanoparticles, the band at 1432 cm^{-1} was shifted to a slightly higher wave number of 1485 cm^{-1} due to increase in bending vibrations of CH_2. The characteristic band of magnetite moved to 714 cm^{-1} due to addition of drug levofloxacin, but at the same time confirming the presence of drug in iron oxide nanoparticles.

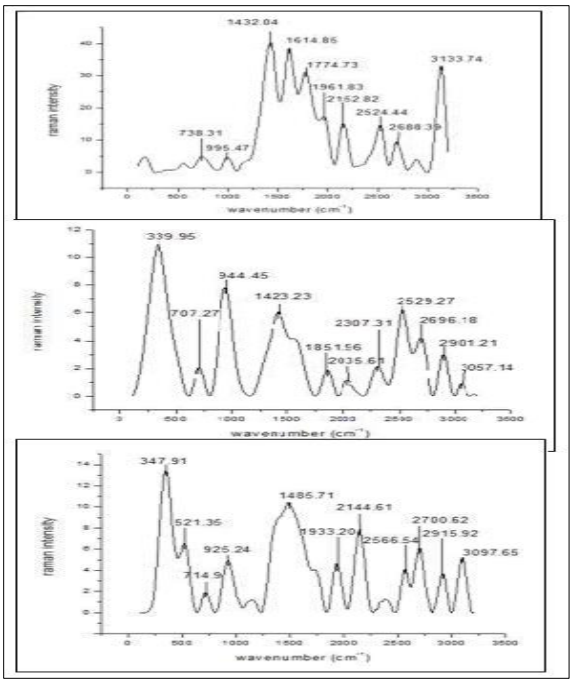

Figure 6: Raman spectra of **(a)** drug levofloxacin **(b)** iron oxide nanoparticles **(c)** levofloxacin MNP's.

CONCLUSION

Magnetic iron oxide nanoparticles have shown the excellent capacity for association of drug levofloxacin using the polymer chitosan. The objective of this research was to formulate magnetic iron oxide nanoparticles by co-precipitaion method. Two drugs were used for this research work levofloxacin. Both are antimicrobial drugs having activity agains wide range of bacteria for example *S. aureus* and *E. coli*. They are used for upper respiratory tract infections, urinary tract infections e.t.c. The prepared magnetic nanoparticles were checked for different parameters such as magnetic properties, particle size, FTIR, SEM, raman spectroscopy, in- vitro release and antibacterial activity. Results of all parameters were satisfactory and confirmed that prepared formulation can be effectively used for loading of levofloxacin. The study showed that iron oxide nanoparticles can be used to enhance the antibacterial activity of both drugs. The formulation also provided sustained release of drug levofloxacin over a period of 48 hrs.

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