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RESEARCH PAPER

Antimicrobial Drugs Encapsulated in Fibrin Nanoparticles for Treating Microbial Infested Wounds

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ABSTRACT

Purpose *In vitro* evaluation of antibacterial and antifungal drugs encapsulated fibrin nanoparticles to prove their potential prospect of using these nanocomponent for effective treatment of microbial infested wounds.

Methods Surfactant-free oil-in-water emulsification-diffusion method was adopted to encapsulate 1 mg/ml each of antimicrobial drugs (Ciprofloxacin and Fluconazole) in 4 ml of aqueous fibrinogen suspension and subsequent thrombin mediated cross linking to synthesize drug loaded fibrin nanoparticles.

Results Ciprofloxacin loaded fibrin nanoparticles (CFNPs) showed size range of 253 ± 6 nm whereas that of Fluconazole loaded fibrin nanoparticles (FFNPs) was 260 ± 10 nm. Physico chemical characterizations revealed the firm integration of antimicrobial drugs within fibrin nanoparticles. Drug release studies performed at physiological pH 7.4 showed a release of 16% ciprofloxacin and 8% of fluconazole while as the release of ciprofloxacin at alkaline pH 8.5, was 48% and that of fluconazole was 37%. The antimicrobial activity evaluations of both drug loaded systems independently showed good antibacterial activity against *Escherichia coli* (*E.coli*), *Staphylococcus aureus* (*S. aureus*) and antifungal activity against *Candida albicans* (*C. albicans*). The *in vitro* toxicity of the prepared drug loaded nanoparticles were further analyzed using Human dermal fibroblast cells (HDF) and showed adequate cell viability.

Conclusion The efficacies of both CFNPs and FFNPs for sustained delivery of encapsulated anti microbial drugs were evaluated *in vitro* suggesting its potential use for treating microbial infested wounds (diabetic foot ulcer).

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INTRODUCTION

The use of nanoparticles as drug delivery vehicles for wound healing has great potential to revolutionize the future of diabetes therapy. Nanoparticles have drawn increasing interest for their ability to deliver drugs in the optimum dose range resulting in effective therapeutic efficacy of the drug and weakened side effects (1,2). Depending on the chemical composition of the nanoparticles, they can hold wide variety of compounds making them as an effective drug delivery system (3). The reason why nanoparticles have drawn interest in medical purpose is based on their important and unique features, as their surface to mass ratio is much larger, their properties and their ability to absorb and carry encapsulated compounds such as drugs, probes and proteins. Especially in the area of drug delivery, nanoparticles of size ranging (size >100 nm) should be needed for loading sufficient amount of drug into the particles (4). Pharmaceutical industry has been using nanoparticles to reduce toxicity and side effects of the drugs. For formulations used in drug delivery the main focus is on reducing the toxicity of the free drug, where as the toxicity of the nanoparticle carrier is not significantly considered. Therefore natural biodegradable nanoparticles are to be chosen for drug delivery.

Fibrin is a coagulation protein derived from its precursor blood plasma protein, fibrinogen that plays an integral role in the wound healing process (5). Because of its biocompatible nature, fibrin is utilized as a effective in drug delivery agent and also as a major matrix protein for tissue engineering applications (6). Fibrin is an appealing drug delivery vehicle because it can be

injected where it gels *in situ*, it is degraded naturally and it stimulates the body's own wound healing response (7–9). To initiate wound healing, it is often desirable to locally deliver tissue-specific growth factors in a controlled manner. It also serves as an ideal substrate for cell attachment, proliferation and also as an extra cellular matrix for tissue regeneration (10–14). Fibrin supplemented with antibiotics has been used to treat experimental osteomyelitis, facilitate wound healing (15,16).

Control of microbial wound infestations is of prime importance in the treatment a non-healing wounds like that in case of diabetic foot ulcers. Bacterial infestations occurring on wound site exudates will affect the healing process by reducing the oxygen tension, degrading extracellular matrix proteins and thus delaying re-epithelialization and wound closure (17,18). A study in dermatological wound showed that 10–40% of normal population is colonized with *S. aureus* while as, almost 71% of the patients with chronic wound were colonized with *S. aureus* (19). An increased bacterial load on the wound surface amplifies pro inflammatory environment. Therefore, in order to improve wound healing it is necessary to create an environment unfavorable to micro-organisms and favorable for host repair mechanism (20). Therefore antimicrobial drugs are effective against a broad range of micro-organisms such as yeast, mold, and bacteria including MRSA when it is provided at an appropriate concentration (21).

Ciprofloxacin (4-fluoroquinolone) was chosen for this study because it has an intrinsic activity against most pathogens in bone and soft tissue infections. Ciprofloxacin has broad spectrum antimicrobial activity against gram negative and gram positive organism (22).

Fluconazole, a synthetic broad spectrum antifungal agent that selectively inhibit triazole of the fungal enzymes involved in ergosterol synthesis (23). Fluconazole is FDA approved for the treatment of systemic *Candida* infections (24–26).

In this study, we used fibrin, a natural bio-polymer as the drug delivery carrier of antibacterial and antifungal drugs at the site where antimicrobial activity is required in a sustained way without loss of bioactivity. Recently it was shown that a polyethylene glycol-based fibrin gel (PEGylated fibrin gel) induces vasculogenesis both *in vitro* and *in vivo* (27). To exploit the inherent ability of fibrin as a substitute for a three-dimensional matrix and as a carrier to control the release of both the drugs, we encapsulated Fluconazole and Ciprofloxacin into the fibrin nanoparticles. This drug loaded systems are expected to primarily control infestations and may further aid in vascularization and wound healing process when applied to the microbial infested wound sites.

MATERIALS AND METHODS

Materials

Fibrinogen was purchased from Himedia, India. Thrombin and potassium bromide (KBr) was purchased from Merck, India. *S. aureus* (ATCC 25923) and *E.coli* (ATCC 25922) and *C. albicans* (ATCC 10231) strains were kindly provided by Microbiology lab of Amrita Institute of Medical Sciences, Kochi, India. Alamar Blue® reagent and live dead assay kit was purchased from Invitrogen. $DiOC_2$ and Triton X-100 were purchased from Sigma Aldrich. Human dermal fibroblast cells (HDF) and growth medium were purchased from PromoCell, Germany. Luria-Bertani broth, Sabouraud Chloramphenicol Agar and Agar-Agar were purchased from Himedia, India. Ciprofloxacin was purchased from Micro Labs, India and Fluconazole was purchased from Sigma Aldrich. All chemicals were used without further purification.

Synthesis of Drug-Encapsulated Fibrin Nanoparticles

The drug encapsulated fibrin nanoparticles were synthesized by a surfactant-free water-in-oil emulsification-diffusion method. 1 ml. aqueous suspension of each drugs (Ciprofloxacin and Fluconazole) were mixed with 4 ml. aqueous suspension of fibrinogen and thrombin were taken separately in individual applicators and administrated simultaneously into a pre-heated vegetable oil system that can induce thrombin-FXIIIa mediated cross linking of fibrin molecules (Fig. 1). In brief, 5 ml aqueous suspensions of both fibrinogen-FXIIIa cryoprecipitate and thrombin were instantaneously injected into 40 ml of purified vegetable oil. This was kept under constant magnetic stirring at 400 rpm and maintained at a temperature of 70–80°C to crosslink and emulsify in the oil phase. The stirring was continued for 6–8 h for the complete cross linking of fibrin moieties and its uniform dispersion in oil phase to occur. For synthesizing antimicrobial drugs loaded fibrin nanoparticles, 1 mg/ml each of both antimicrobial drugs (Ciprofloxacin and Fluconazole) were independently cross-linked with 4 ml of aqueous fibrinogen suspension during the synthesis procedure. The nano constructs thus formed in the emulsion were then centrifuged at 10 000 rpm for 15 min. This resulted in the formation of a density gradient layer of nano constructs at the oil–water interface, which was pooled up and harvested from the emulsion. The resultant constructs were further redispersed in water and probe sonicated for 10 min. to attain a uniform nano size distribution and preserved after lyophilizing for 48 h (1).

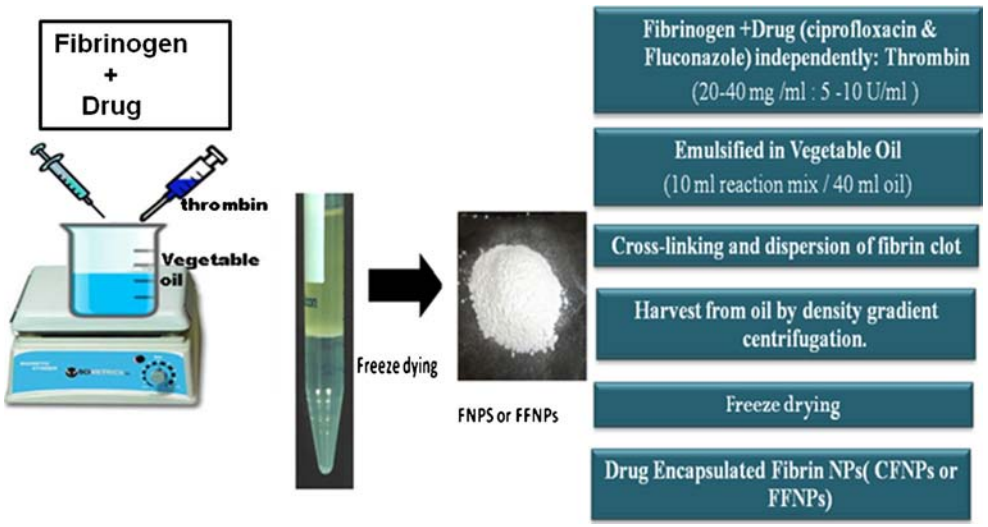

Fig. 1 Method of drug loaded fibrin nano particle synthesis.

Characterization of Antibacterial and Antifungal Drug Encapsulated in Fibrin Nanoparticles

Dynamic Light Scattering Analysis

The hydrodynamic particle size distribution was analyzed using the technique dynamic light scattering (DLS) measurements (Malvern Zeta Sizer).

SEM Analysis

The size as well as the surface morphology of drug loaded fibrin nanoparticles were analyzed by scanning electron microscopy (SEM). For SEM analysis, freeze-dried nano constructs were redispersed in MilliQ water were dropped on aluminum stub and sputter coated with platinum using an automatic fine platinum coater (JEOL JFC-1600) at 10 mA for 120 s before imaging under SEM (JEOL-JSM-6490LA).

FT-IR Spectroscopy

The interaction between fibrin and drugs were accessed using Fourier transform infrared spectroscopy (FT-IR spectroscopy, Perkin-Elmer Co., Model SPECTRUM RXI, FT-IR). The scan range was from 4,000 cm^{-1} to 400 cm^{-1}. Briefly 175 mg KBr and 2 mg sample was mixed thoroughly and pelletized using a pelletizer (Hydraulic pellet press, Kimaya Engineers).

Determination of Encapsulation Efficiency of Antibacterial and Antifungal Drug Loaded Nanoparticles

For checking the encapsulation efficiency of both drug loaded nanoparticles systems, 1 mg each of drug loaded lyophilized nanoparticles were mixed with 5 ml of water and sonicated for 10 min. The suspension was filter centrifuged through a Centricon ® plus filter device of MW cutoff 300 kDa (Millipore) for 8,000 rpm for 10 min. The resultant supernatant was again collected and quantified using an UV absorption spectrophotometer (UV-1700 Pharmaspec, Shimadzu) at 318 nm for Ciprofloxacin and 260 nm for Fluconazole.

$$\text{Efficiency}(\%) = \frac{\text{Amount of drug in the lyophilized powder Encapsulation}}{\text{Initial amount of drug}} \times 100$$

In Vitro Drug Release Studies

In vitro drug release profile from antibacterial and antifungal drug loaded nanoparticles system was determined under alkaline pH of 8.5 at 37°C. 1 mg each of drug loaded lyophilized nanoparticles were redispersed in 5 mL Tris buffer (8.5) and PBS (7.4), separate falcon tubes were taken for each time interval. The falcons were incubated at 37°C under gentle shaking. At definite time intervals 3 mL of the release solution was collected and stored. The sample solutions were analyzed using an UV absorption spectrophotometer (UV-1700 Pharmaspec, Shimadzu) at 318 nm for Ciprofloxacin and 260 nm for Fluconazole. The release percent can be determined by the following equation:

$$\text{Drug Release}(\%) = \frac{\text{Released drug at a definite time}}{\text{Amount of drug entrapped within nanoparticles system}} \times 100$$

Kinetic Modeling of In Vitro Drug Release in Phosphate Buffer pH 8.5

To investigate the mechanism of drug release from the antibacterial and antifungal drug loaded fibrin nanoparticles, the obtained drug release data were analyzed and plotted into various kinetic models, namely zero order rate kinetics, first order rate kinetics, Higuchi model, Korsmeyer Peppas model and Hixson-Crowell cube-root model (28). The *in vitro* drug release data were fitted into the above mentioned kinetic

models and the linearity of each plot was determined from the value of the regression coefficient.

In Vitro Antimicrobial Activity Evaluation

We have evaluated the antimicrobial potential of the prepared antibacterial and antifungal drug loaded nanoparticles against bacteria as well as fungus. The culturing medium with the microbes were taken as control for the experiments.

In Vitro Antibacterial Studies

S. aureus (gram positive) and *E. coli* (gram negative) were used for evaluating the antibacterial activity of the prepared antibacterial drug loaded nanoparticles. LB broth and LB agar were used as culturing media for bacteria. The bacteria were inoculated in sterilized LB broth and then incubated overnight at 37°C in a shaking incubator. The concentration of bacteria was 1×10^6 colony forming units per milliliter (CFU/mL). The concentration of bacteria was taken in comparison to the McFarland standard (McFarland standards are used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria will be within a given range).

The antibacterial drug loaded nanoparticles were then added to the medium and were incubated at 37°C for 24 h. After the incubation, the optical density of the bacterial culture with the raw drug and drug loaded fibrin nanoparticles were noted.

In Vitro Antifungal Studies

C. albicans was used to assess the antifungal activity and SC agar (Sabouraud Chloramphenicol) was used as the culturing medium. *C.albicans* were inoculated in sterilized SD broth (Sabouraud Dextrose) and then incubated overnight at 37°C in a shaking incubator. The concentration of fungus was 1×10^6 colony forming units per milliliter (CFU/mL). The antifungal drug loaded nanoparticles were then added to the medium and were incubated at 37°C for 24 h. After the incubation, the optical density of the culture with the raw drug and drug loaded fibrin nanoparticles were noted.

Microbial Uptake of Antibacterial and Antifungal Drug Encapsulated Fibrin Nanoparticles

The nanoparticles was tagged with umbelliferone dye and incubated for 2 h. The dye exhibits fluorescence, which was measured at excitation/emission maxima 360/449 nm. Bacterial smear was prepared and heat fixed. The umbelliferone tagged nanoparticles were allowed to interact with the bacteria and fungus. Fluorescence image was taken.

Membrane Potential of Bacteria

S. aureus and *E.coli* was grown in Luria Bertani broth at 37°C with 160 rpm shaking. In order to determine the effect of antibacterial drug loaded nanoparticles on the bacterial cell membrane potential, cells from the mid-exponential phase cultures centrifuged and pellet was resuspended in PBS, pH 7.4 at room temperature at an optical density of 0.3–0.5 at 610 nm. To the 1 ml of the *S. aureus* and *E.coli* suspension, antibacterial drug loaded nanoparticles were added and incubated for 1 h in shaking rotar, followed by the addition of 10 µl of 3 mM $DiOC_2$ (Diethyloxacarbocyanine) and 2 µl of Triton X-100. The samples were incubated at room temperature for 5 min and the relative green fluorescence was measured at Excitation/Emission 480 nm/520 nm and red fluorescence was measured at 480 nm/620 nm. Cells with $DiOC_2$ alone served as a negative control while cells with both $DiOC_2$ and Triton X-100 were used as a positive control.

Live/Dead Staining of Bacterial Cells

Live/dead staining was performed to assess the viable bacterial cells on the antibacterial and antifungal drug loaded nanoparticles. The bacteria were inoculated in sterilized LB broth and then incubated overnight at 37°C in a shaking incubator. The antibacterial drug loaded nanoparticles were then added to the medium and were incubated at 37°C for 24 h. After the incubation smear was prepared and allowed to dry and then the Acridine orange/Ethdium bromide (Himedia, India) was added to the smear and was incubated for 20 mins. Excess dye was washed away. After 20 min of incubation, glass slides were viewed under fluorescence microscope (Olympus BX51).

Evaluation of Cell Viability of the Antibacterial and Antifungal Drug Loaded Nanoparticles

Cell viability of the antibacterial and antifungal drug loaded nanoparticles was evaluated by Alamar Blue® assay. The cell viability of antibacterial and antifungal drug loaded nanoparticles with different concentration of drug was evaluated on HDF cells. The antibacterial and antifungal drug loaded nanoparticles were sterilized by ethylene oxide gas. HDF cells were cultured in fibroblast growth medium. Sterile antibacterial and antifungal drug loaded nanoparticles were added in 12 well plates containing 5×10^4 cells. The cells with the materials were then incubated up to 48 h and Alamar Blue® assay was performed. HDF cells alone are taken as positive control. The optical density was measured at 570 nm with 620 nm set as the reference wavelength using a microplate spectrophotometer (Biotek PowerWave XS, USA).

Live/Dead Staining on HDF Cells

Live/dead staining was performed to assess the viable HDF cells on the antibacterial and antifungal drug loaded nanoparticles. This fluorescence two-color cell viability assay simultaneously detects the viable and non-viable cells based on two parameters, intracellular esterase activity and intact plasma membrane integrity. In the live cells, non-fluorescent calcein-acetoxymethyl is converted to bright green fluorescent calcein by the intracellular esterases (EX/EM - 495/515 nm). On the other hand, (EthD-1) enters cells with damaged membranes and produces a bright red fluorescence upon binding to the nucleic acids of dead cells (EX/EM - 495/635 nm). Ethylene oxide sterilized drug loaded nanoparticles were placed in a 24 well plate and HDF cells were seeded at a density of 3×10^4 cells/well and incubated at 37°C for 12 and 48 h respectively. After 4 h of incubation, the cells were fed with additional growth medium. At the pre-determined time intervals, the nanoparticles were removed from the wells and the cells were washed with PBS and stained with PBS containing 2 µmol/l calcein-AM and 4 µmol/l EthD-1 for 20 min at 37°C. After 20 min of incubation, the cells were washed with PBS and visualized using a fluorescence microscope.

Statistical Analysis

All the experiments were triplicated and the obtained data was analyzed using student *t*-test to find the statistical significance (p). A value of $p<0.05$ was considered as statistically significant.

RESULTS

Preparation and Characterization

The green emulsification method was adopted for the preparation of antibacterial (Ciprofloxacin) and antifungal (Fluconazole) drug loaded fibrin nanoparticles. In brief, 1 mg/ml drugs were added independently to 4 ml of fibrinogen suspension and cross linked with 100 units of Thrombin. For the preparation of fibrin nanoparticles, coconut oil was used as the oil phase. Here no additional surfactants were used to stabilize the emulsion, making it a completely bio friendly synthesis route.

Size Analysis Using DLS

The hydrodynamic particle size distribution of both antibacterial and antifungal drug loaded fibrin nanoparticles was found to be in the range of about 254 ± 6 nm for ciprofloxacin loaded fibrin nanoparticles (CFNPs) whereas that of fluconazole loaded fibrin nanoparticles (FFNPs) was 260 ± 10 nm.

SEM Analysis

Figure 2a represents SEM images of both CFNPs and FFNPs. The obtained images revealed the spherical morphology of drug loaded nanoparticles. The particle size of both CFNPs and FFNPs was in the range of 200-300 nm.

FT-IR Spectroscopy

FT-IR spectroscopy analysis (Fig. 2b) was performed to confirm the incorporation of drugs into the fibrin nanoparticles. The FT-IR spectrum of nanofibrin showed the characteristic peaks at 1,391 and 1,544 cm^{-1}. Spectrum of Fluconazole control displayed a broad peak at 3,487 cm^{-1} due to O-H stretching vibrations, aromatic C=N stretching vibrations at 1,620 cm^{-1} and aromatic C-F stretching vibrations at 1,210 and 1,220 cm^{-1}. Spectrum of Ciprofloxacin control showed a peak at 3,500 cm^{-1} due to O-H stretching vibrations and a peak at 1,300 cm^{-1} due to OH bending vibration.

Spectrum of FFNPs showed a peak broadening at 3, 487 cm^{-1} due to the intermolecular hydrogen bonding between Fluconazole and fibrin. Spectrum of CFNPs showed a sharp peak at 3,500 cm^{-1} which was due to the intramolecular H-bonding. The FT-IR spectra showed that both the drugs Fluconazole and Ciprofloxacin are well integrated within the fibrin moieties.

Encapsulation Efficiency and *In Vitro* Drug release Studies of Antibacterial and Antifungal Drug Loaded Nanoparticles

The encapsulation efficiency of both CFNPs and FFNPs was found to be 66% and 56% respectively. The higher percentage of encapsulation obtained in CFNPs can be attributed to the higher hydrophilic interaction of the drug Ciprofloxacin with fibrin where as Fluconazole, being a hydrophobic drug showed lesser encapsulation.

In vitro drug release studies were carried out for different time intervals. Samples were withdrawn and cumulative percentage drug release was calculated. Drug release studies were done via the direct dispersion method at pH 8.5 and at physiological pH 7.4 and the release pattern is shown in Fig. 3i. The drug release at pH 8.5 of CFNPs was found to be 48% and for FFNPs was found to be 37%. The drug release at pH 7.4 of CFNPs was 16% and for FFNPs was found to be 8%. The release was higher in alkaline pH. This was due to the presence of more salt content that precipitates out fibrin protein and therefore drug is released. The release pattern obtained in both the nanosystems was slow and sustained that makes the system suitable for prolonged applications of chemokines.

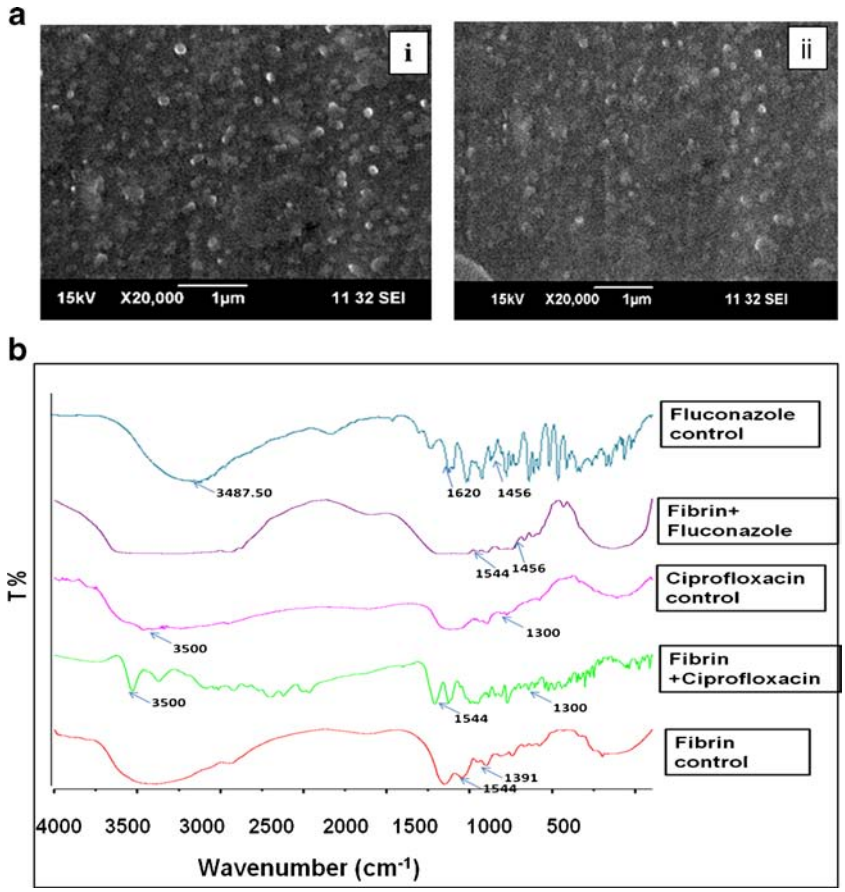

Fig. 2 (**a**) SEM images of (***i***) Ciprofloxacin loaded fibrin nanoparticles (CFNPs) (***ii***) Fluconazole loaded fibrin nanoparticles (FFNPs). (**b**) FT-IR spectra of control drugs and drug loaded fibrin nanoparticles.

Kinetic Modeling of *In Vitro* Drug Release in Phosphate Buffer pH 8.5

From the kinetic models (Fig. 3ii), we conclude that the best fit model for the FFNPs is zero order (regression coefficient of the zero order is close to unity). It describes the system FFNPs where the drug release rate is independent of its concentration of the dissolved substance. The best fit model for the CFNPs is Hixson crowell model (Table I). The 'n' value of the Korsemeyer-Pappas model predicts that the mechanism of diffusion is Non-Fickian.

In Vitro Antimicrobial Activity Evaluation

In Vitro Antibacterial Activity Evaluation

We have evaluated the antibacterial activity of the prepared CFNPs. In the Figs. 4 and 5 it was clear that the numbers of bacterial colonies were decreased in presence of (CFNPs). Three different dosage of the antibacterial drug ciprofloxacin (33 μg/ml, 66 μg/ml, 99 μg/ml) of fibrin (w/w) was taken. The number of viable bacterial colonies was found to be decreasing with increased dosage of the drug. The reduction in bacterial colonies was same in both *S.aureus* and *E.coli* (Figs. 4 and 5). The antibacterial activity was compared with the same concentration of raw drugs as well.

In Vitro Antifungal Activity Evaluation

Antifungal activity evaluation was done against *C. albicans* which proved the antifungal activity of the (FFNPs) to fungus (Fig. 6i). Three different dosage of the antifungal drug was taken 28 μg/ml, 66 μg/ml and 84 μg/ml. The number of viable fungal colonies was found to be decreasing with increased dosage of FFNPs (Fig. 6ii). The antifungal activity was compared with the same concentration of raw drugs as well.

Microbial Uptake of Antibacterial and Antifungal Drug Encapsulated Fibrin Nanoparticles

The nanoparticle was tagged with umbelliferone dye and incubated for 2 h. *E.coli*, *S. aureus* and *C. albicans* microbial smear were prepared on sterile glass slides and heat fixed. The umbelliferone tagged CFNPs and FFNPs were allowed to interact with the bacteria and fungus respectively. Excess dye was washed off. Fluorescence images were taken after interaction of umbelliferone tagged CFNPs with *S. aureus*, *E.* coli and *C. albicans* (Figs. 7, 8 and 9). The obtained images showed bright fluorescence of umbelliferone on the bacterial

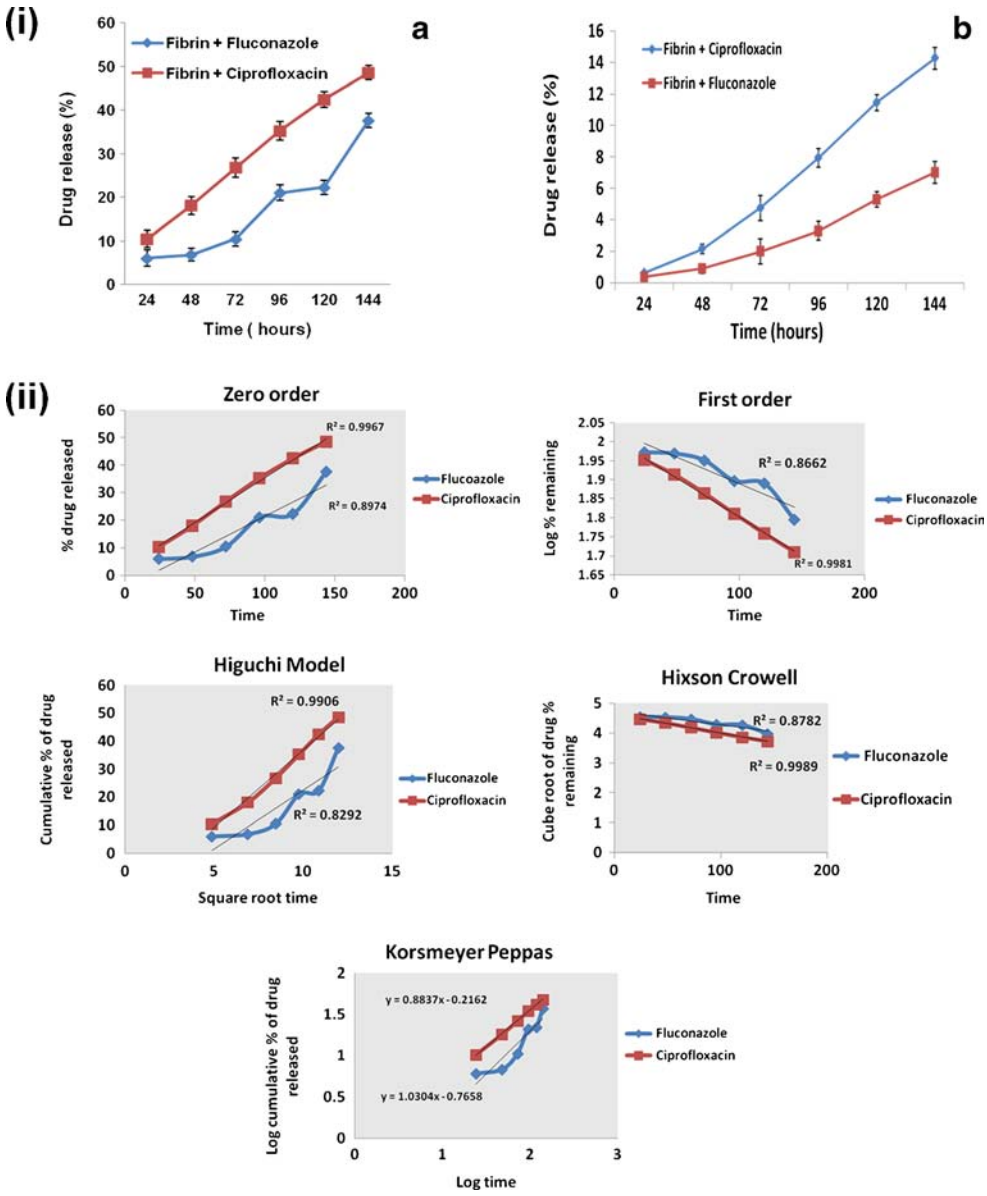

Fig. 3 (**i**) *In vitro* drug release profiles of CFNPs and FFNPs : (**a**) Drug release at pH 8.5 (**b**) Drug release at pH 7.4. (**ii**) *In vitro* drug release data fitted into various kinetic models.

and fungal cells. This proved the uptake of CFNPs and FFNPs into the bacterial and fungal cells.

Membrane Potential Evaluation of Bacteria

$DiOC_2$ assay was used to study the variation in the membrane potential. Diethyloxacarbocyanine ($DiOC_2$) exhibits green fluorescence in all bacterial cells, but the fluorescence shifts toward red emission as the dye molecules self associate at higher concentrations caused by larger membrane potentials. Triton could destroy membrane potential by eliminating the proton gradient, thus it was used as a positive control in the study. Control live bacterial cells are taken as negative control. In this study we, Fig. 10i show that CFNPs could change the membrane potential that caused depolarization, possibly leading to the cell disruption. Therefore, this suggests that the membrane depolarization could be one of the mechanisms of antibacterial activity of drug loaded fibrin nanoparticles.

Table I Best Fit Kinetic Model

NPs	Zero order R^2	First order R^2	Higuchi model R^2	Korsmeyer- peppas model n	Hixson crowell model R^2	Best fit model
FFNPs	0.897	0.866	0.8292	0.8837	0.878	Zero order
CFNPs	0.996	0.998	0.990	1.030	0.9989	Hixson crowell model

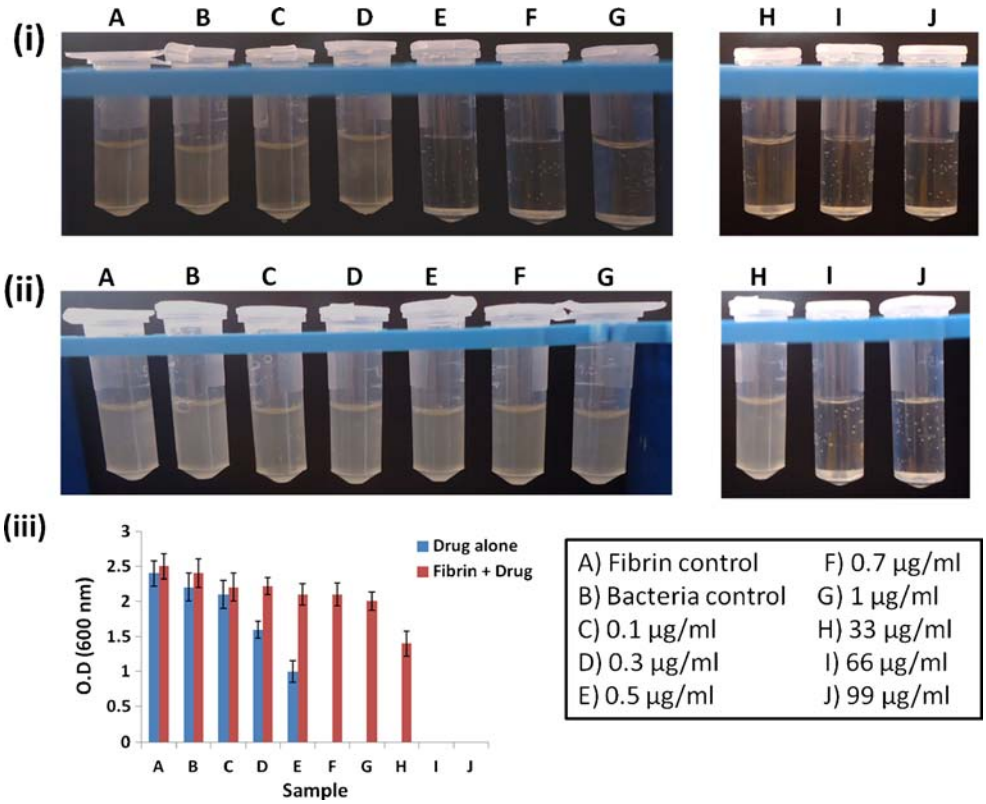

Fig. 4 (**i**) Antibacterial activity study of ciprofloxacin raw drug against *S. aureus* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml (***ii***) Antibacterial activity study of ciprofloxacin drug loaded fibrin nanoparticles against *S. aureus* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml drug loaded fibrin nanoparticles. (***iii***) Optical density measurement data of antibacterial activity study against *S. aureus* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml.

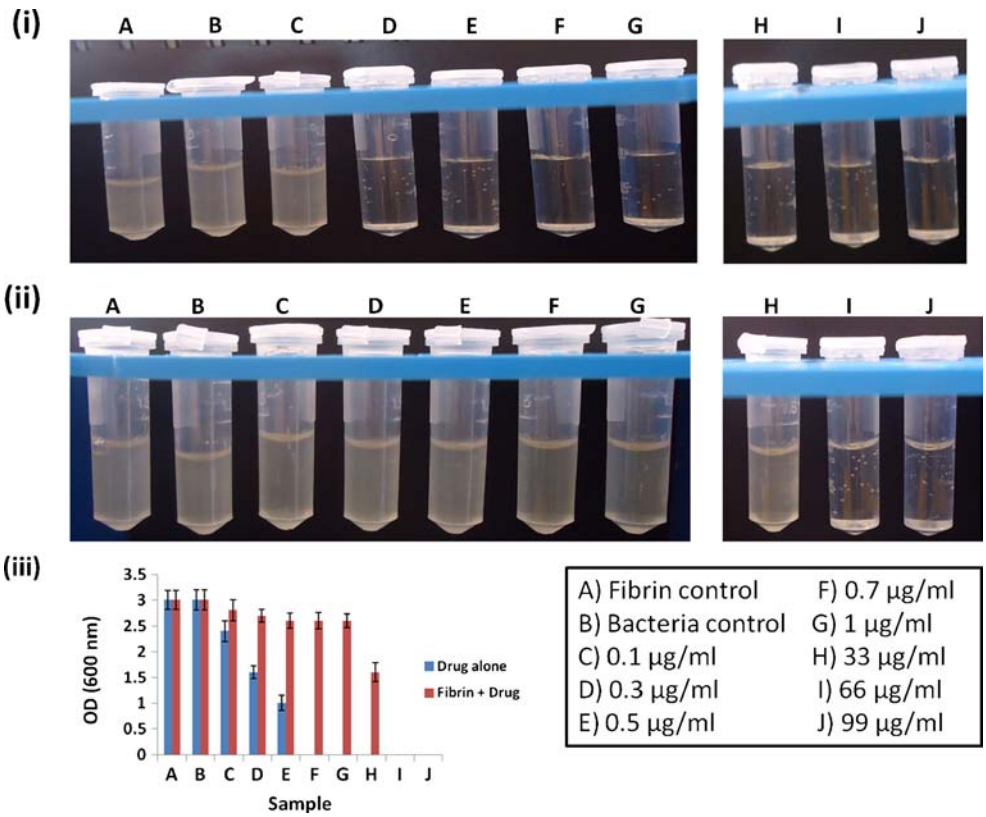

Fig. 5 (***i***) Antibacterial activity study of ciprofloxacin raw drug against *E. coli* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml (***ii***) Antibacterial activity study of ciprofloxacin drug loaded fibrin nanoparticles against *E. coli* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml drug loaded fibrin nanoparticles. (***iii***) Optical density measurement data of antibacterial activity study against *E. coli* (**a**) Fibrin control (**b**) Bacteria control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 33 (**i**) 66 (**j**) 99 μg/ml.

Antimicrobial Activity Evaluation by Live Dead Assay

Figure 10ii and 10iii evaluates the antibacterial activity of CFNPs by live dead assay. Three different dosage of the antibacterial drug ciprofloxacin were taken. The viability of bacterial cells was found to be decreasing with increased dosage of the drug. The reduction in bacterial viability was same in both *S. aureus* and *E. coli*. Acridine orange is a green-fluorescent nucleic acid dye that stains both live and dead bacteria with intact and damaged cell membranes. Acridine orange fluoresce red for dead bacteria but with less intensity when compared to EtBr. EtBr is a red-fluorescent nucleic acid dye that stains only dead bacteria with damaged cell membranes.

Evaluation of Cell Viability of the Antibacterial and Antifungal Drug Loaded Nanoparticles

Figure 11 depicts the cytocompatability of the prepared antibacterial and antifungal drug loaded nanoparticles assessed using Alamar Blue® assay. Alamar Blue® is the cell viability marker which uses the reducing power of cells as an indicator of metabolic activity. Resazurin, the active ingredient of Alamar Blue® reagent, is a non-toxic, cell permeable compound that is blue in color and virtually non-fluorescent. When upon entering the cells, resazurin is reduced to resorufin, a compound that is red in color and is highly fluorescent. Viable cells continuously convert resazurin to resorufin, increasing the overall fluorescence and color of the media surrounding cells. Three different dosages of both the antibacterial and antifungal drug loaded nanoparticles were taken Fig. 11ii and iv. With increasing dosage of drug, cell viability reduced when compared to fibrin control (no drug) and positive control (cells incubated in media alone). However, when the drug concentration increased the cell viability reduced which shows that at higher dosage drug was found to be toxic to the cells when compared to lower dosage of drug which had above 70% cell viability for both antibacterial and antifungal drug loaded nanoparticles.

Live Dead Staining of HDF Cells

Figure 11v–vi depicts live dead staining of CFNPs and FFNPs. Live/dead staining was performed to evaluate the HDF cell viability of the antibacterial and antifungal

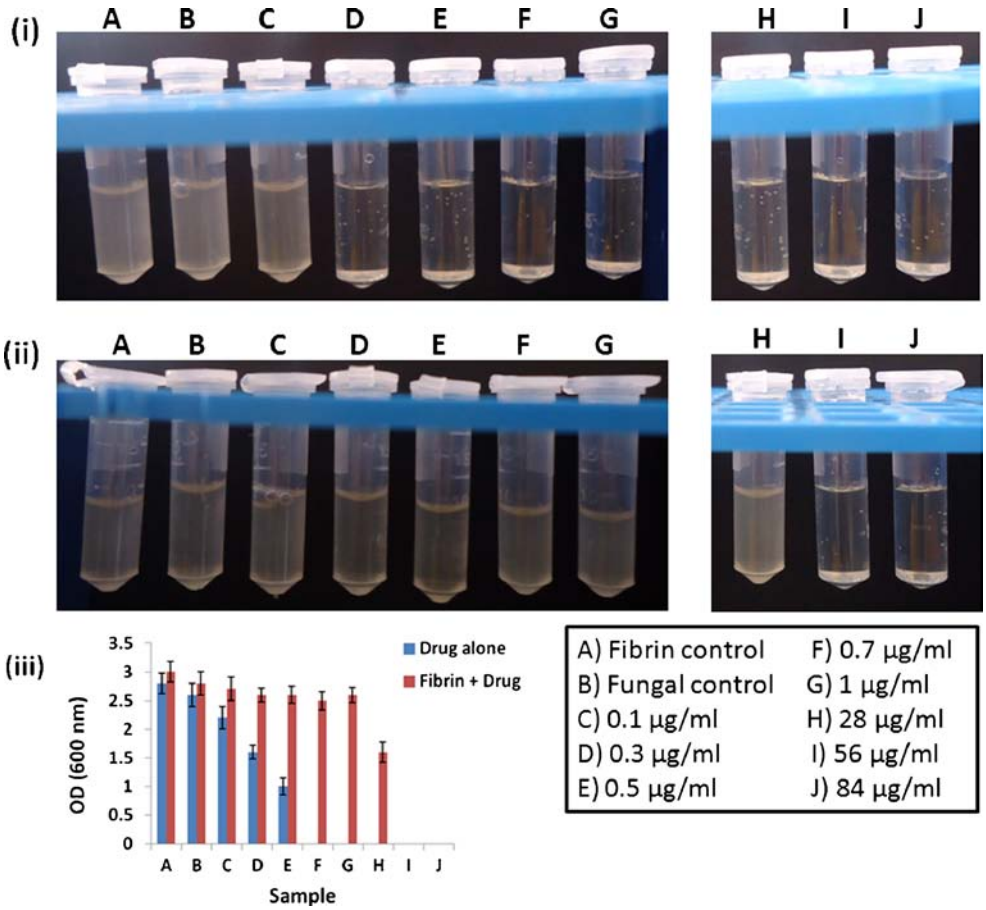

Fig. 6 (***i***) Antifungal activity study of fluconazole raw drug against *C. albicans* (**a**) Fibrin control (**b**) Fungus control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 28 (**i**) 56 (**j**) 84 μg/ml (***ii***) Antifungal activity study of fluconazole drug loaded fibrin nanoparticles against C. albicans (**a**) Fibrin control (**b**) Fungus control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 28 (**i**) 56 (**j**) 84 μg/ml drug loaded fibrin nanoparticles. (***iii***) Optical density measurement data of antifungal activity study against *C. albicans* (**a**) Fibrin control (**b**) Fungus control (**c**) 0.1 (**d**) 0.3 (**e**) 0.5 (**f**) 0.7 (**g**) 1 (**h**) 28 (**i**) 56 (**j**) 84 μg/ml.

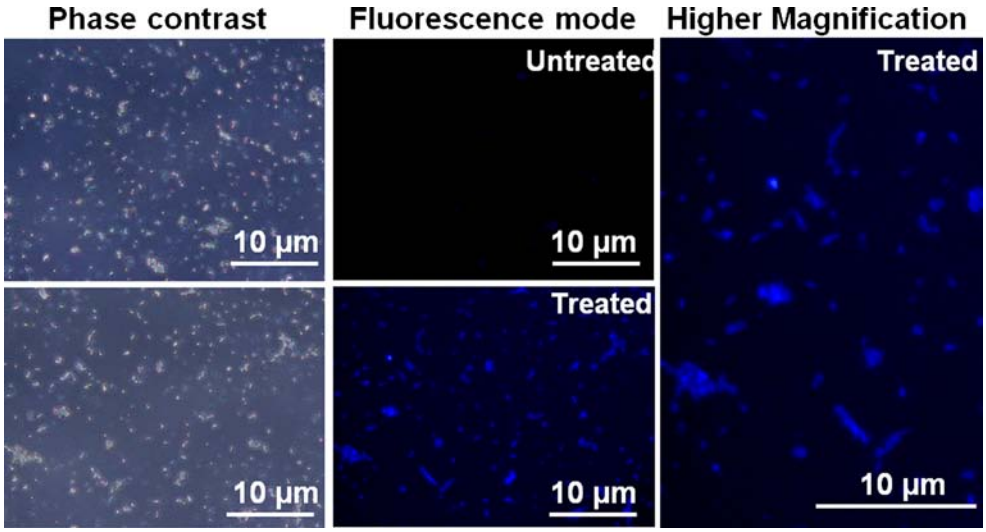

Fig. 7 Bacterial uptake of Ciprofloxacin loaded fibrin nanoparticles against *E. coli*.

drug loaded nanoparticles. When the drug concentration increased, the cell viability reduced compared to lower dosage of drug. Dead cells have damaged membranes; the EthD-1 enters damaged cells and is fluorescent when bound to nucleic acids. EthD-1 produces a bright red fluorescence in damaged or dead cells. Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non fluorescent cell-permeant calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells.

DISCUSSION

The CFNPs and FFNPs were prepared using completely bio friendly synthesis route. The SEM images revealed the spherical morphology of drug loaded nanoparticles and the particle size of both CFNPs and FFNPs was in the range of 200–300 nm. The size range of the drug loaded nanoparticles was further confirmed using DLS and size was found to be 254 ± 6 nm and 260 ± 10 nm for CFNPs and FFNPs respectively. FT-IR spectroscopy analysis was performed to confirm the integration of drugs into the fibrin nanoparticles. Spectrum of FFNPs showed a peak broadening at 3, 487 cm^{-1} due to the intermolecular hydrogen bonding between Fluconazole and fibrin. Spectrum of CFNPs showed a sharp peak at 3,500 cm^{-1} which was due to the intramolecular H-bonding. The FT-IR spectra showed that both the drugs Fluconazole and Ciprofloxacin are well integrated within the fibrin moieties.

The encapsulation efficiency of CFNPs was higher when compared to FFNPs this higher percentage of encapsulation obtained in CFNPs can be attributed to the higher hydrophilic interaction of the drug Ciprofloxacin with fibrin where as Fluconazole, being a hydrophobic drug showed lesser

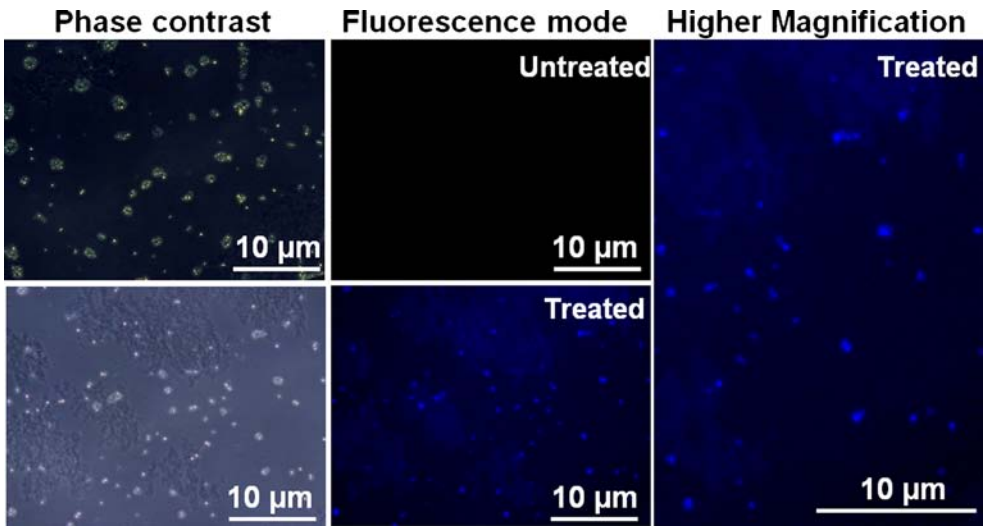

Fig. 8 Bacterial uptake of Ciprofloxacin loaded fibrin nanoparticles against *S. aureus*.

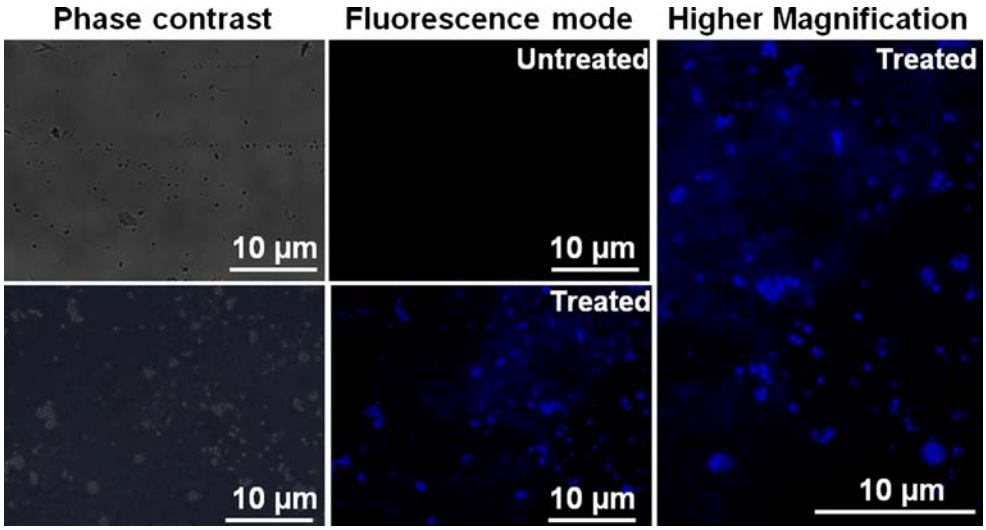

Fig. 9 Fungal uptake of fluconazole loaded fibrin nanoparticles against *C. albicans*.

encapsulation. The drug release at pH 8.5 was higher when compared to the drug release at pH 7.4, the release was higher in alkaline pH was due to the presence of more salt content that precipitates out fibrin protein and therefore drug is released. The best fit model for the FFNPs is zero order and it describes the system FFNPs where the drug release rate is independent of its concentration of the dissolved substance. The best fit model for the CFNPs is Hixson crowell model. It describes the drug releases by dissolution and with the changes in surface area and the diameter of the particles.

The numbers of viable bacterial colonies were found to be decreasing with increased dosage of the drug. The mechanism behind antibacterial activity is, a subunit of the essential bacterial enzyme DNA gyrase is the target action of the drug ciprofloxacin. The inhibition of purified DNA gyrase is correlated with antibacterial activity (29). The antibacterial activity was due to the interaction of ciprofloxacin released from the CFNPs with bacterial DNA gyrase enzymes which is necessary to separate bacterial DNA, thereby inhibiting cell division.

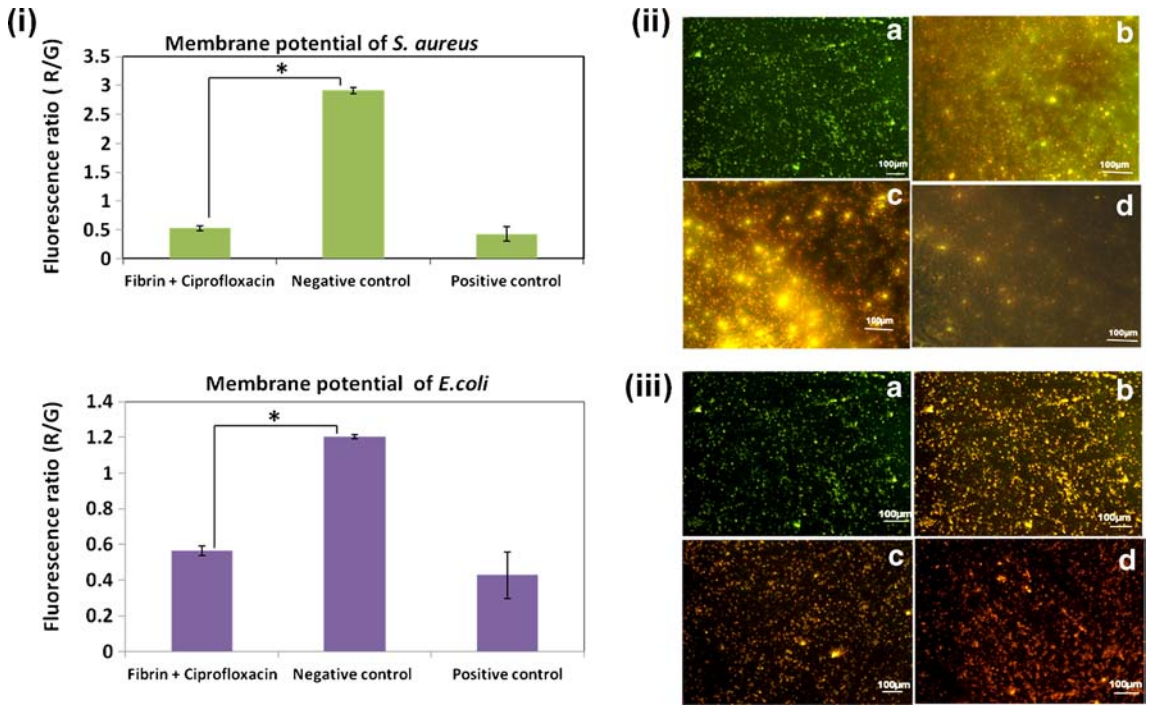

Fig. 10 (***i***) Membrane potential evaluation of the CFNPs against *S. aureus* and *E. coli*. (***ii***) Live Dead assay of *E. coli*: (**a**) *E. coli* control (**b**) fibrin + ciprofloxacin (33 μg) (**c**) fibrin + ciprofloxacin (66 μg) (**d**) Fibrin + ciprofloxacin (99 μg). (***iii***) Live Dead assay of *S. aureus*: (**a**) *S. aureus* control, (**b**) fibrin + ciprofloxacin (33 μg), (**c**) fibrin + ciprofloxacin (66 μg), (**d**) fibrin + ciprofloxacin (99 μg).

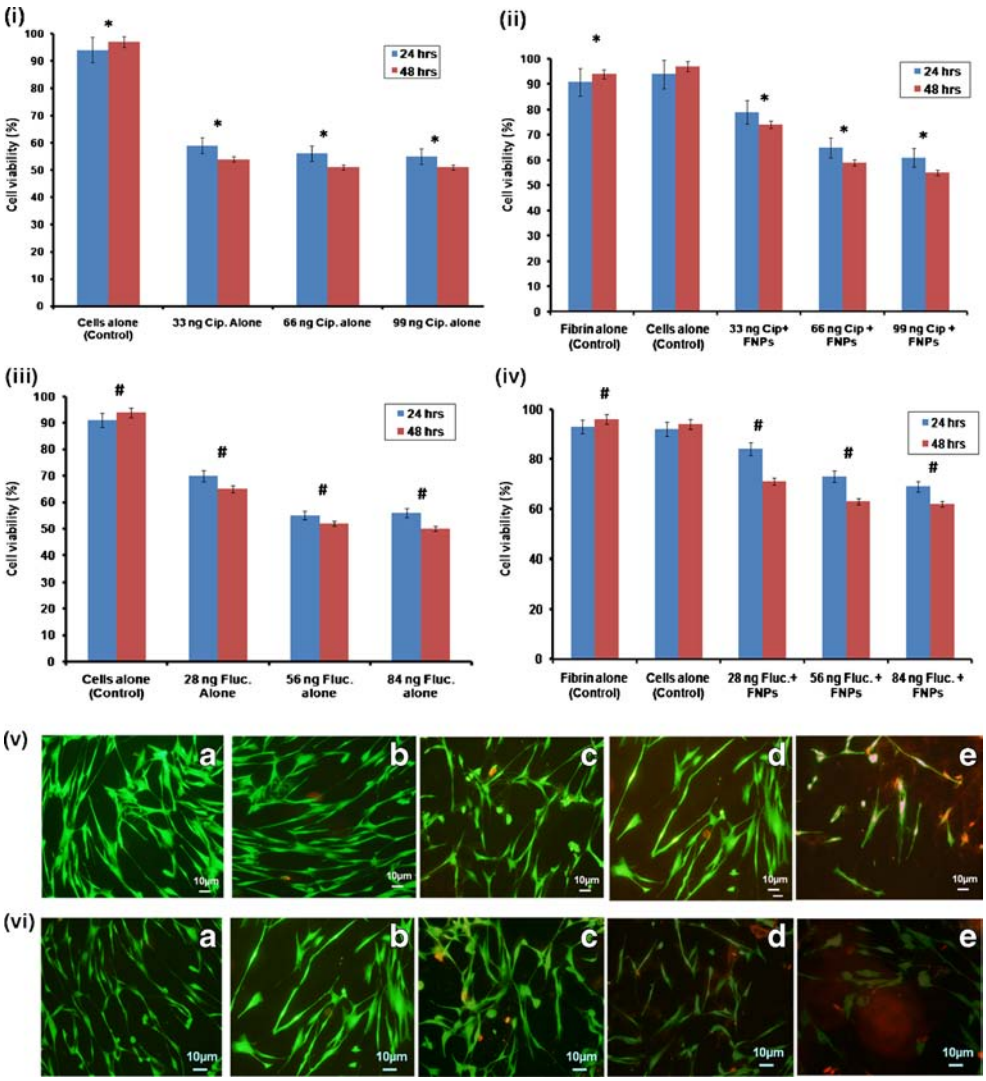

Fig. 11 Cell viability studies of the antibacterial and antifungal drug loaded nanoparticles plotted as percentage of viability in HDF Cell lines (***i***, ***ii***) Three different concentrations of bare and ciprofloxacin loaded fibrin nanoparticle systems. * ($p < 0.05$) statistical significance compared to controls (***iii***, ***iv***) Three different concentrations of bare and fluconazole loaded fibrin nanoparticle systems Positive control. # ($p < 0.05$) statistical significance compared to controls (***v***) Live dead staining of Ciprofloxacin loaded fibrin nanoparticles: (**a**) control HDF cells (**b**) Fibrin control (**c**) Fibrin + 33 μg Ciprofloxacin, (**d**) Fibrin + 66 μg Ciprofloxacin, (**e**) Fibrin + 99 μg Ciprofloxacin. (***vi***) Live dead staining of Fluconazole loaded fibrin nanoparticles: (**a**) control HDF cells (**b**) Fibrin control (**c**) Fibrin + 28 μg Fluconazole, (**d**) Fibrin + 56 μg Fluconazole, (**e**) Fibrin + 86 μg Fluconazole.

The number of viable fungal colonies were found to be decreasing with increased dosage of FFNPs. Ergosterol serves as a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells. The antifungal drug Fluconazole inhibits the fungal enzymes involved in ergosterol synthesis thereby disrupting the fungal cell wall. The failure of ergosterol synthesis then leads to the death of fungi (30). Bacterial uptake of antibacterial and antifungal drug loaded fibrin nanoparticles proved the uptake of CFNPs and FFNPs into the bacterial and fungal cells. Membrane potential evaluation of bacteria showed that CFNPs could change the membrane potential, possibly leading to the cell disruption. Therefore, this suggests that the membrane depolarization could be one of the mechanisms of antibacterial activity of drug loaded fibrin nanoparticles. Antimicrobial activity evaluation was carried out by live dead assay, the viability of microbial cells was found to be decreasing with increased dosage of the drug proving that drug showed toxicity to bacterial and fungal cells.

Overall, the results indicate that fibrin nanoparticles loaded antibacterial drug or antifungal drug required higher concentration to induce anti-microbial activity than the corresponding free drug. This may give an impression that the nanoparticle reduces the therapeutic efficacy. However, it is not because the fibrin nanoparticle did anything to undermine the potency of the drug, but the pattern of release from nanoparticles is slow and sustained. Thus, the bandage with drug loaded nanoparticles would have a long-term local action compared to free drug and could be kept for 3–5 days before removal, cleaning and re-placement. Also, it is worth to mention that the drug concentrations used in this study is much less than the routine systemic dosage given.

Evaluation of cell viability of the antibacterial and antifungal drug loaded nanoparticles was done using Alamar Blue® assay and it showed that when the drug concentration increased the cell viability reduced which shows that at higher dosage drug was found to be toxic to the cells when compared to lower dosage of drug. The toxicity of ciprofloxacin to HDF

cells was due to matrix degrading activity of ciprofloxacin as well as decreased matrix synthesis (31). This was further confirmed by Live/dead staining of HDF cells, when the drug concentration increased, the cell viability reduced compared to lower dosage of drug.

CONCLUSION

The green emulsification method was adopted for the preparation of antibacterial and antifungal drug loaded fibrin nanoparticles. To exploit the inherent ability of fibrin as a substitute for a 3D matrix and as a carrier to control the release two antimicrobial drugs, we encapsulated both the drugs Fluconazole and Ciprofloxacin independently into the fibrin nanoparticles. CFNPs was synthesized by adding 1 mg/ml drugs independently to 4 ml of fibrinogen suspension and cross linked with 100 units of Thrombin. FFNPs same above explained methodology was followed to get nanoparticles of size range of 254 ± 6 nm for Ciprofloxacin loaded fibrin nanoparticles (CFNPs) whereas that of Fluconazole loaded fibrin nanoparticles (FFNPs) was 260 ± 10 nm. Physico chemical characterization was performed and revealed that antimicrobial drugs were firmly integrated within the fibrin matrix. The *in vitro* drug release kinetics at pH 8.5 was studied using this drug loaded nano formulations proved that 48% and 37% of antibacterial and antifungal drugs were released from the fibrin matrix in a sustained manner. The best fit kinetic model for the FFNPs is zero order and for CFNPs is Hixson crowell model. Antibacterial and antifungal evaluation was carried out by serial dilution method and thus addition of Ciprofloxacin and Fluconazole drugs showed decrease in bacterial count and hence provided good antibacterial and antifungal activity against *E. coli*, *S. aureus* and *C.albicans* respectively. The *in vitro* toxicity of the prepared drug loaded nanoparticles was further analyzed in Human dermal fibroblast cell lines (HDF) and showed adequate cell viability thus drug loaded nano formulations proved to be non-toxic. This drug loaded fibrin nanoparticles is expected to primarily control infection when applied to an infected wound. Therefore the antibacterial and antifungal drugs encapsulated fibrin nanoparticles were proved to be excellent drug delivery system. The above results prove the efficacy of antimicrobial drug loaded systems as an effective candidate for incorporating within the wound dressing bandages for treating microbial infested wounds.

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