EFFICACY OF CIPROFLOXACIN CONTAINING EUDRAGIT NANOPARTICLES AGAINST URINARY CATHETER ASSOCIATED *STAPHYLOCOCCUS AUREUS* BIOFILMS

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Abstract: The formation of biofilms by *Staphylococcus aureus* are associated with drastically enhanced resistance against most antimicrobial agents, contributing to the persistence of the bacteria despite antibacterial therapy. The main aim of the present study was the synthesis and characterization of Ciprofloxacin containing Eudragit RL-100 nanoparticle to combat biofilm associated infection. In the present investigation *Staphylococcus aureus* biofilms was formed on urinary catheter surface and Ciprofloxacin containing nanoparticle was added. Ciprofloxacin applied directly and antibiotic Gentamycin was used as positive control. Biofilm quantification assay showed 74.18% reduction in biofilms after 24 h in response to Ciprofloxacin containing nanoparticle while Ciprofloxacin added directly showed 60.25 % reduction. This result was further confirmed through scanning electron microscopy and colony forming unit (cfu) assay. It is concluded from the study that the potential of drug nanoparticles against biofilms associated infection and reveals enhanced potential of Ciprofloxacin containing nanoparticle with respect to drug added directly because of better penetration of nanoparticle through biofilms matrix and better availability of drug to microbial vicinity. The substantial antibacterial activity of drug encapsulated nanobiosystem could of great interest for the biomedical field, opening new system against infectious biofilm.

Keywords: Biofilms, Ciprofloxacin, Eudragid RL-100, Urinary Catheter.

Introduction: Microbial biofilm is a characteristic feature of most bacteria having an inherent ability to form structured communities of bacterial cell adherent on living surface [1]. The opportunistic pathogen *Staphylococcus aureus* can form biofilm on many host tissue and implanted medical devices often causing chronic infections [2]-[5]. In response to certain environmental cues, bacteria living in the biofilm are capable of using active mechanism to leave biofilm and return to the planktonic state in which sensitivity to antimicrobials is regained [6]-[8]. The increasing occurrence of multidrug resistant, extensive drug resistant and pandrug resistant microbial strains has gradually rendered traditional antimicrobial treatment ineffective [9] and estimates suggest that as many as 80% of chronic bacterial infections are biofilm associated [10]. In this context, finding and testing new preventive/therapeutic strategies for biomaterial associated infections have become a top priority at the international level. Nanotechnology is expected to open some new ways to fight and prevent diseases using atomic scale tailoring of materials [11]. Conventionally drugs are directly spread over the biofilm, which may cause harmful effect on the cell or tissue or organ. Conventional drug delivery needs high doses to make up the bio availability [12]-[13]. Drug encapsulated particle has unique physicochemical properties such as small and controllable size, large surface area to mass ratio, high reactivity, and functionalizable structure. They can increase the bioavailability, solubility of much potent drug. The purpose of this study is to combine the unique properties of nanoparticles with the antimicrobial activity of the Ciprofloxacin in order to obtain a drug encapsulated nanobiosystem that could be used to minimize the biofilm growth on urinary catheter.

Materials And Methods

Microorganism and Culture conditions: A standard *Staphylococcus aureus* strain MTCC 96 was used in the present study. The strain was cultured in nutrient agar media (Himedia) and incubated for 24 h at 37°C in shaking incubator.

Chemicals: Ciprofloxacin and Gentamycin were purchased from Sigma Aldrich; India and Eudragid RL-100 polymer was obtained from Evonik Degussa India Pvt. Ltd, Mumbai, India. Antibacterial agent, agar and CV used for the biofilm growth and quantification were purchased from Sigma chemicals. Urinary catheter was purchased from Alpha medicare and devices Pvt. Ltd. Dichloromethane (DCM) and hydrophilic surfactant polyvinyl alcohol (PVA) was obtained from Merk India Pvt. Ltd. All other chemicals and materials were of analytical grade and were used as procured.

Determination of minimum inhibitory concentration (MIC) of Ciprofloxacin: MIC of drug was determined by agar dilution assay [14]. The agar plates were prepared in triplicate by adding nutrient agar containing different concentrations of Ciprofloxacin (0.03–5% v/v). The plates were inoculated with 10^3 cfu, using inocula of the strain prepared as above, and incubated for 48 h at 37°C. Plates with Tween-20, but without any drug were used as control. Numbers of colonies were counted after 48 h of incubation. The lowest concentration of Ciprofloxacin required to completely inhibit the growth of *Staphylococcus aureus* was designated as

the MIC.

Preparation of Ciprofloxacin Loaded Eudragit RL-100 Nanoparticles: Ciprofloxacin loaded Eudragit RL-100 Nanoparticles were prepared by High Pressure Homogenization Emulsification (HPHE) - Solvent Evaporation method [15]-[16] and with some modifications. In brief, Eudragit RL-100 and Ciprofloxacin were mixed in 1:1 w/v ratio and dissolved in 20 ml of dichloromethane (DCM) and then they were mixed by using laboratory magnetic stirring at 500 rpm for 2 min, homogeneous mixture was formed. Above formulated homogeneous mixture containing polymer and drug was then dispersed in 100 ml of Poly vinyl alcohol (PVA) solution using a high-speed homogenizer (Omni-TH International) for 15 min at 35,000 rpm for getting preemulsion. The resulting pre-emulsion was immediately passed through a high-pressure homogenizer (HPH) at 500 bar press for five cycles. The nanosuspension obtained was collected in a glass beaker and kept on a magnetic stirrer at 500 rpm for 3 hours to evaporate DCM completely from the formulated nano formulation. The formed nanoparticles were recovered by centrifugation at 10,000 rpm for 20 min, followed by the dried nanoparticles collected at 48 hr after freeze drying. The effect of several variables on the characteristics of the formulated Ciprofloxacin loaded Eudragit RL-100 nanoparticle was evaluated.

Biofilm formation and quantification:

Biofilm were developed using *Staphylococcus aureus* on urinary catheter and quantified [17]-[18]. Briefly, 1 ml of 1.5 x 10^8 cfu/ml of *Staphylococcus aureus* was added in eppendorf tubes having 0.5x0.5cm token of urinary catheter [19]-[20] for 90 min of adhesion phase. After 90 min the catheter pieces were washed with sterilized PBS to remove loosely adhered cells. The catheter pieces were then transferred to new eppendorf tubes and 5% (v/v) concentration of Ciprofloxacin was added along with the same concentration of drug equivalent nanoparticle (amount of nanoparticle synthesized by 5% Ciprofloxacin) separately taking Gentamycin (10 X MIC value) as positive control and incubated at 37°C for 24 h, 48h and 72 h. Quantification of biofilm was performed using CV assay at 570nm [21].

Scanning Electron Microscopy (SEM): *Staphylococcus aureus* biofilm formed on urinary catheter tokens were fixed with 2.5% (v/v) glutaraldehyde in PBS for 2 h at room temperature. They were then treated with 1% (w/v) uranyl acetate for 1 h, and washed with distilled water. The samples were dehydrated with ethanol series (30%, 50%, 70%, 90% and 100%). All samples were dried to critical point by Polaron critical point drier, coated with gold and viewed under SEM.

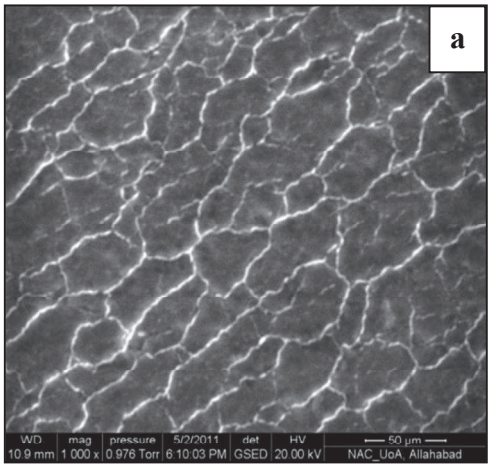
Statistical analysis: All experiments were performed in triplicate and results were expressed as means ± standard deviations. Statistical analyses of the differences between mean values obtained for experimental groups were performed using Student's t-test. P-values of 0.05 or less were considered significant.

Results:

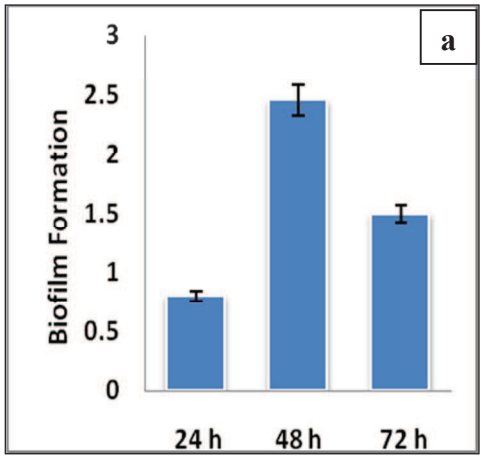
Assessment of antibacterial effect and determination of MIC

Antibacterial effect of Ciprofloxacin, Gentamycin and Eudragit RL-100 was screened by Zone of inhibition assay and Ciprofloxacin and Gentamycin showed antibacterial property but no zone of inhibition was observed in case with Eudragid RL-100. MICs value for Gentamycin and Ciprofloxacin were determined for *Staphylococcus aureus*. The result showed that Gentamycin 4 µg/ml was able to completely inhibit the growth of *Staphylococcus aureus* while Ciprofloxacin showed complete inhibition at 1.0% concentration.

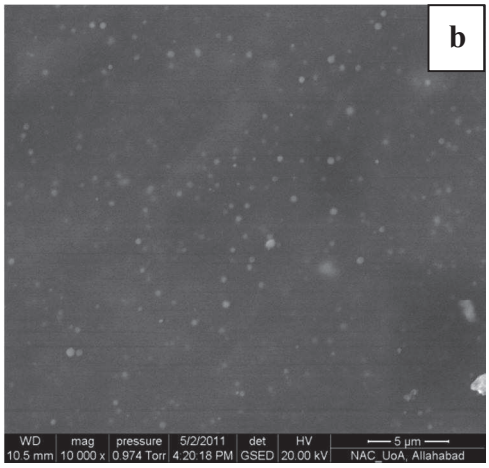
Synthesis of Nanoparticles: The nanoparticle was synthesized as described above. Shape and surface characteristics of the nanoparticles were investigated using scanning electron microscope (SEM), the study revealed nanoparticle synthesized was with rounded shape and size was around 150 nm (Fig. 1b). Fig. 1a shows the surface characteristics of Urinary catheter.



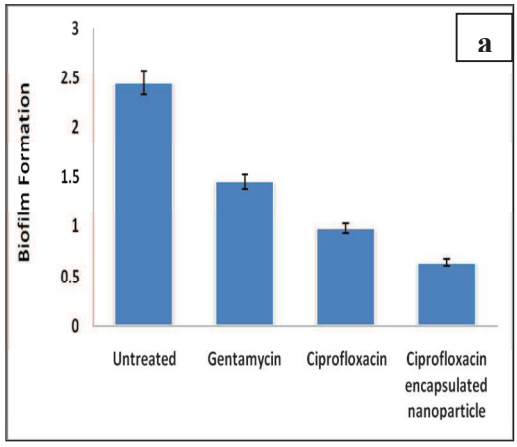
(a) Urinary Catheter Inner surface



(a).Biofilm formation by *Staphylococcus aureus* on urinary catheter surface after different time



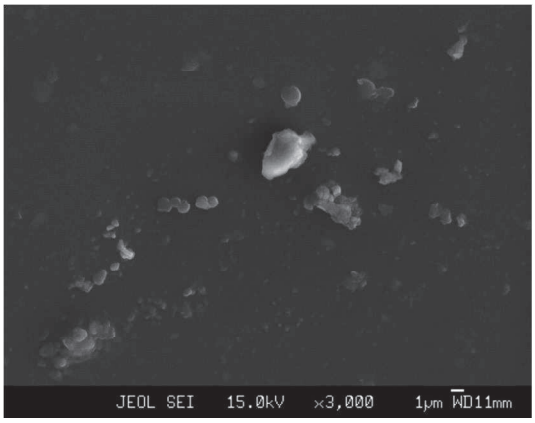
(b) Synthesized encapsulated Eudragid RL-100 nanoparticles at 5KX



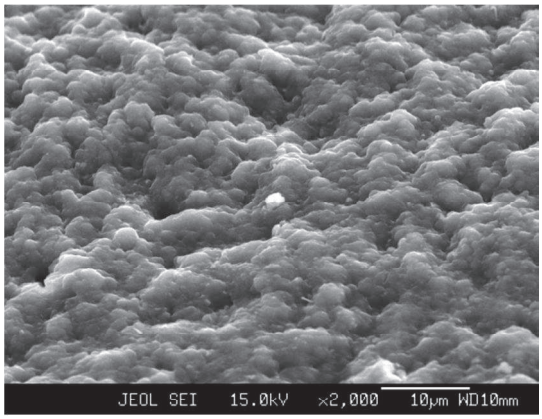
(b) Staphylococcus aureus biofilm reduction in response to Gentamycin, Ciprofloxacin and Ciprofloxacin encapsulated nanoparticle.

Nanoparticle mediated Biofilm inhibition
Biofilm quantification assay showed maximum biofilm formation on urinary catheter after 48 h (Fig. 2.a). The quantification assay showed that biofilm reduction was 41.00% with Gentamycin while 60.25 % and 74.18% was reduced by the application of 5% w/v Ciprofloxacin and 5% w/v Ciprofloxacin equivalent encapsulated nanoparticle respectively (Fig. 2.b).
SEM Analysis To evaluate the relevance of reduction assay SEM was employed. Scanning electron microscopic analysis of control biofilms and those treated with Ciprofloxacin and Ciprofloxacin encapsulated nanoparticle is shown in (Fig. 3). Visualization of the ultrastructure in general revealed that most reduction to biofilm constituents was caused by Ciprofloxacin encapsulated nanoparticle followed by Ciprofloxacin (Fig. 3c, d) as compared to the untreated biofilm and Gentamycin control (Fig. 3a, b). Ciprofloxacin and Ciprofloxacin encapsulated nanoparticle treated cells demonstrated reduction in adhering cells and biofilm development. This suggests that despite the relative minimal diffusion, Ciprofloxacin and Ciprofloxacin encapsulated nanoparticle may be exerting a metabolic interference in biofilm.

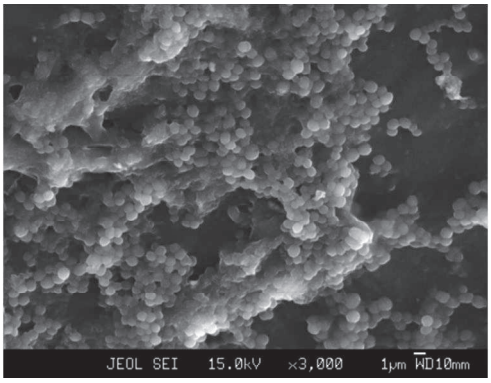
Discussion: Biofilm are 100 to thousand times [22] resistant than their planktonic counter parts. The reason behind the resistant phenotype includes presence of thick extracellular polymer matrix armour. Most of the antibiotic part entrapped into the EPS or become inactive during chemical reactions. The factors like numbers of microorganism agents that can inhibit the growth of biofilm-associated microorganisms are greatly needed and would enhance the number of effective therapeutic alternatives [26] Taking into account this, the present study was carried out to assess the antibacterial properties of drug Ciprofloxacin and Ciprofloxacin encapsulated nanoparticle on biofilm



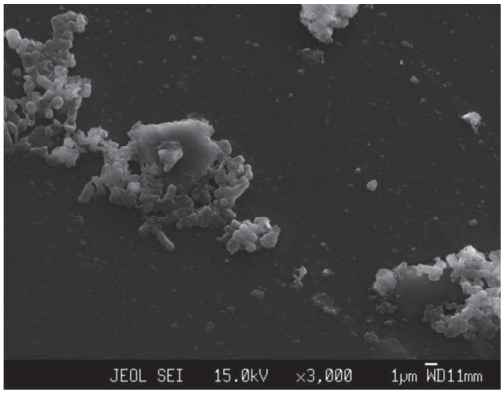
(a) Biofilm growth after 48h



(b) Effect of Gentamycin on Biofilm growth



(c) Effect of Ciprofloxacin on Biofilm growth.



(d) Effect of *Ciprofloxacin* encapsulated nanoparticle on Biofilm growth.

[23] and their protection by self produce exopolysaccharide matrix which affect the antibiotic to penetrate and kill the organism in biofilm. Use of long term urinary catheter is frequently associated with microbial adhesion that leaves to microbial colonization and biofilm development. However, the process of differential microbial adhesion to the urinary catheter surface may include many of probable reasons. Electrostatic forces generated by net charge distribution on urinary catheter surface and bacterial wall leaves to generation of electric potential which affect microbial adhesion [24]. Vander Waal forces, acid base interaction may also affect microbial colonization [25].n this context, new growth. Biofilm pattern was observed after 24 h, 48 h and 72 h at 37°C. Quantification of biofilm was performed using CV assay at 570nm [21]. Biofilm quantification assay showed maximum biofilm formation on urinary catheter after 48 h (Figure 2. a). Gentamycin, Ciprofloxacin, Ciprofloxacin equivalent encapsulated nanoparticle gave 41%, 60.25% and 74.18% reduction in *Staphylococcus aureus* biofilm (Figure 2.b). Antimicrobial screening of Ciprofloxacin, Gentamycin and Eudragid RL-100 was measured using zone of inhibition assay and no zone of inhibition was observed in case with Eudragit RL-100. The data showed that Eudragid RL-100 do not have any antimicrobial activity in its own and the

biofilm inhibition observed in case with Ciprofloxacin encapsulated nanoparticle was due to the presence of Ciprofloxacin. Earlier, the antimicrobial activity of Ciprofloxacin has been proved against the biofilm system [20]. Current study clearly demonstrated that efficacy of Ciprofloxacin encapsulated nanoparticle was increased by 13.93% in contrast with when the Ciprofloxacin was used without encapsulation inside the nano-particles. It can be predicted by the data shown in the study that the availability of Ciprofloxacin enhanced to the biofilm residing microorganisms which may be due to more efficient penetration to microbial vicinity and ability to overcome from biofilm associated barriers.

Conclusion: In summary, the research presented in this article conclusively demonstrates the anti-biofilm potential of Ciprofloxacin and Ciprofloxacin equivalent encapsulated nanoparticle. Ciprofloxacin encapsulated nanoparticle shows maximum reduction of *Staphylococcus aureus* biofilm, may find use in future therapeutic strategies.

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