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Research Article

Surface Modified Implant - Release of Antibiotic in the Presence of Aerobic Microorganisms

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ABSTRACT

Aerobic microorganisms are responsible for majority of infections. By means of formation of biofilms, resistance against various drug delivery systems are been reported. Most of the aerobic microorganisms are catalase and superoxide dismutase positive. The main objective of this study is to create antibiotic loaded implants, which releases antibiotics during the presence of aerobic microorganisms, thereby preventing the formation of biofilms and improving the treatment efficacy. Stainless steel plates were coated by dip coating method. The solution was prepared by using Polyhydroxybutyrate-co-hydroxyvalerate in Dichloromethane and followed by addition of ofloxacin. By spray coating technique, sodium formate was coated over the polymer film. Parameters such as In vitro drug release, zone of inhibition were studied. Coated plates with uniform thickness of 9µm were obtained. In all bacterial strains, Staphylococcus *aureus*, Clostridium *sporogens*, Pseudomonas *aeruginosa*, the zone of inhibition was noticed with a inhibited zone area of 64mm for S.*aureus* and 19mm for C.*sporogenes*. The Invitro drug release study showed that the total encapsulated drug was released over a period of 6 hours. Antibiotic coated plate's offers a new perspective for treating implant related infections and also overcoming formation of biofilms. The study can be applied for creating biosensors to detect microorganisms.

INTRODUCTION

Infection are defined as "invasion of the body by various agents -including bacteria, fungi, protozoans, viruses, and worms - and its reaction to our system can lead to tissue damage and disease" [1]. The main goal of treating the various types of wound infections should be to reduce the bacterial load in the wound to a level at which wound healing processes can take place. Over the last few centuries, an infection tends to possess a great challenge in terms of preventing and treating it to our nature. Antimicrobial resistance is an issue great significance for public health at the global level [2,3]. Considered as sweet drugs, antibiotics are often prescribed inappropriately and inadequately and have thus become one of the highly abused agents [4]. Bacterial pathogens causing acute infections are increasingly exhibiting resistance to the commonly used antibiotics and have great threat to public health [5].

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To overcome these challenges, various research works are been carried out by using drug delivery systems. Combining various polymers with different drugs, a huge variety of delivery systems are created targeting infections [6]. The main drawback of these systems is their inability to prevent the formation of biofilms and also they tend to release the drug even during the absence of micro organisms at the target site of action [6,7].

The main objective of this study is to create antibiotic loaded implants, which releases antibiotics during the presence of aerobic microorganisms, thereby preventing the formation of biofilms and thus increasing the treatment efficiency.

MATERIALS AND METHODS

Materials

Ofloxacin was obtained as a free gift sample from Caside pharmaceuticals, India. Poly hydroxybutyrate-co-hydroxyvalerate (PHBV) 12 % was purchased from Goodfellow Chemicals,

USA. Steel plates were obtained as gift sample from KRR private limited, which were cut down into small plates with equal dimensions (1 inch × 1 inch). Other chemicals such as Dichoromethane, Sodium formate were purchased from Sigma Aldrich, India.

Polymer Coating

The steel plates were coated by dip coating method. Prior to coating, the plates were undergone surface treatment by using hydrogen peroxide and sodium hydroxide [8]. The polymer solution was prepared by using 15 % of PHBV (w/v) in Dichloromethane and to which the drug was added at a ratio of 5:1 (Polymer: Drug) respectively. Implants were kept immersed in the solution for a period of 60 min. Later they were withdrawn manually and were kept outside at room temperature for the solvent to get vaporized.

After 24 hours, by following spray coating protocol, 3 % of sodium formate solution was prepared and coated over the primary polymer coating. The second coating was done three times for each implant.

Surface Characterization

The surface morphology of coated implants was investigated by using scanning electron microscope (SEM). The implants were cut down into small fragments and were mounted onto metal stubs using double-sided adhesive tape, sputter-coated with a thin platinum layer using an Auto-sputter fine coater (JFC 1600, JEOL, Japan) and directly analyzed by cold field emission SEM (JEOL, JSM-6701F, Japan).

Determination Of Ofloxacin Drug Loading

A small amount of free drug was dissolved in 0.1M HCl. The ofloxacin content was assayed by means of UV spectrophotometer (Lambda 25, Perkin Elmer, USA).The standard concentration profiles were done and the drug loading efficiency was calculated.

In Vitro Drug Release

In vitro release patterns were studied using conventional dialysis technique. Ofloxacin coated stainless steel implants were placed in a dialysis bag and dialyzed against 200 mL of phosphate saline buffer (PBS).The PBS was prepared initially and the pH was adjusted to 7.4 with sodium hydroxide. Followed by 0.1M of HCl was added at the ratio of 9:1 (v/v) of PBS and HCl respectively. pH of the final solvent was 6.2. During the dissolution process the temperature was stabilized at 37±1 °C, and the sink conditions were maintained through out the course of study. The In vitro release was aided by continuous stirring using a magnetic stirrer.

Thickness of Film

The thickness of the coated film was done by screw gauge apparatus. Prior to any coating, the thickness of implant is measured and after the coating again, the thickness of the plate is measured. The difference between these values indicates the thickness of film.

Zone of Inhibition

Zone of inhibition was determined by the Kirby-Bauer antibiotic testing method. Known quantities of bacteria are grown on agar plates in the presence of coated implants. If the bacteria are susceptible to a particular antibiotic, an area of clearing surrounds the wafer where bacteria are not capable of growing (called a zone of inhibition).The size of the zone and the rate of antibiotic diffusion are used to estimate the bacteria's sensitivity to that particular antibiotic. In general, larger zones correlate with smaller minimum inhibitory concentration (MIC) of antibiotic for those bacteria. This information can be used to choose appropriate antibiotics to combat a particular infection.

The bacterial strains that were used are Staphylococcus aureus (ATC 6538) and Clostridium sporogenes (ATC 11437). All the bacteria were cultures in Nutrient Agar. The mediums that were used for inoculating the S.aureus and C.sporogenes were Muller Hinton agar and Brucella blood agar respectively. Under aseptic conditions, first the plates were cultured with standardized inoculums of each bacterial strain respectively using sterile cotton swabs. Followed by which the antibiotic coated implants were carefully placed on the plate. The plates were incubated under their respective environments for a period of 24 hours and following which the zone of inhibition was observed.

RESULTS

Nature of Films

Based upon various factors, the film properties got varied. In case of plates which were undergone surface treatment initially prior to dip coating process, had a uniform coating of the antibiotic over it, with a fine thickness of few microns were obtained [Fig 1]. Also the adhesive property of these films were found to be good,

where only under external pressure they get peeled away from the surfaces of the plates, otherwise they remain intact with the surface of the plates. In case of stainless steel plates which were not undergone any surface treatment initially, had poor adhesive properties and the thickness were not uniform in nature and were larger in thickness.

Thickness of Film

The thickness of coated film was around 9 µm. The particles were distributed uniformly over the plate surface. In the case of plates, which did not undergo any surface treatment, the thickness of film was larger in size.

Zone of Inhibition

In all bacterial strains, S.*aureus*, C.*sporogens*, P.*aeruginosa*, the zone of inhibition was noticed. In S.*aureus*, the diameter of the inhibited area was 64mm. In the case of C.*sporogenes*, the bacterial strain had good viability and the area of inhibition was 19 mm [Fig 2]. Also in P. *aeruginosa* the zone of inhibition was noted and was of significant distance. Rest while in the control groups, there was no growth of any contaminants.

Scanning Electron Microscopy

The scanning electron microscopy image of the antibiotic coated implants shows that the antibiotic particles are incorporated uniformly over the film [Fig 3]. The drug – ofloxacin – particle size was of few microns.

***Invitro* Drug Release**

The maximum absorbance peak of ofloxacin content was obtained at 294 nm by spectrometry. 98.5 % of encapsulation efficiency was achieved. The In vitro release of the ofloxacin coated plates [Fig 4]. Initially for the first one hour, faster release was achieved, were 16 % of the encapsulated drug got released into the medium. Followed by which the drug is released at a constant rate where the total amount of drug gets released into the medium over a period of 6 hours.

DISCUSSION

The hypothesis of this study is that the encapsulated drug gets released during the presence of aerobic organisms in the environment. Niekus *et al* demonstrated the formation of hydrogen peroxide from superoxide dismutase in Campylobacter sputorum subspecies bubulus [9]. They demonstrated the ability to produce superoxide anion radicals (O_2^-) during oxidation of formate and lactate. Furthermore they showed that C.sputorum was found to produce H_2O_2 while oxidizing formate. Based upon this principle, the work was designed so that, it could be applied for clinical applications especially in combating infections.

Mechanism of Release

The principle of the study depends upon the production of enzymes, *super oxide dismutase*, and *catalase,* by the microorganisms respectively. Thereby the superior formate coating gets degraded and the antibiotic is released to the target site.

According to our hypothesis, in the presence of oxygen in the medium, the formate gets oxidized thereby leading to production of super oxide anions. Since these radicals are lethal to the microorganisms, they tend to stimulate to release superoxide dismutase such a way to breakdown the radicals into hydrogen peroxide (H_2O_2). By nature as such hydrogen peroxide has bactericidal effects thereby they can also inhibit the growth of microorganisms. Hence this is lead to release of enzyme *catalase*, to breakdown the hydrogen peroxide to water and oxygen [Fig 5]. Thereby the drug comes in contact with the medium and hence it gets released.

To confirm this overall mechanism, the drug release was tested with *C.sporogenes*, S.*aureus and P.aeruginosa*. C. sporogenes are anaerobic organisms, which are *catalase* and superoxide dismutase negative, while the later organisms are aerobic organism, are *catalase* and superoxide dismutase positive. Prior to procedure it was found that all organisms are sensitive to ofloxacin. When the antibiotic loaded plates were kept, the area of the inhibited zone was more in the *S.aureus and P.aeruginosa* than *C.sporogenes*. Being a *catalase* and superoxide dismutase negative, there should not be any zone of inhibition. But in our study, an inhibited zone area of 19 mm was found. This might be due to high concentration of loaded drug in the implant or due to presence of any free drug without proper coating of formate. Comparing to *C.sporogenes, S.aureus* had a significant area of inhibition even though both are sensitive to the drug.

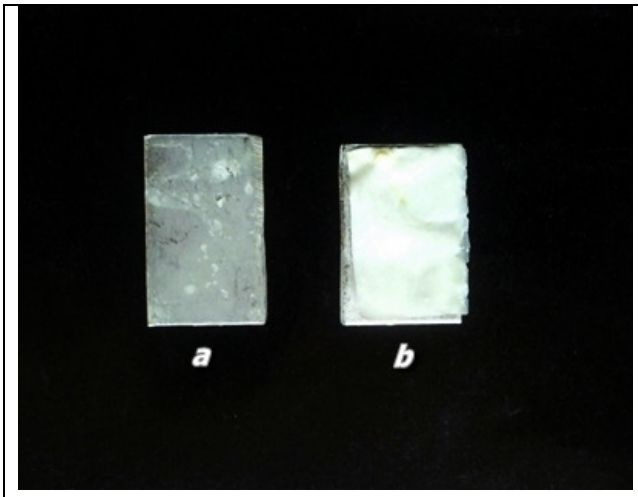

Figure 1: Antibiotic coated plates. (a) Plates undergone surface treatment prior to coating and (b) plate not undergone any surface treatment

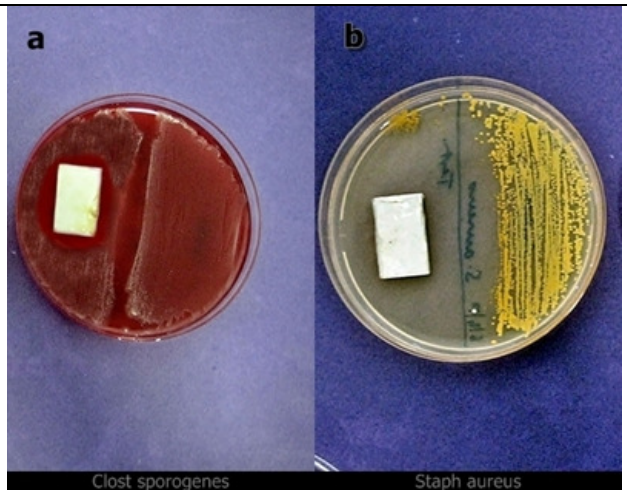

Figure 2: Ofloxacin coated plates exhibiting antibiotic sensitivity against (a) Clostridium sporogenes; (b) Staphylococcus aureus

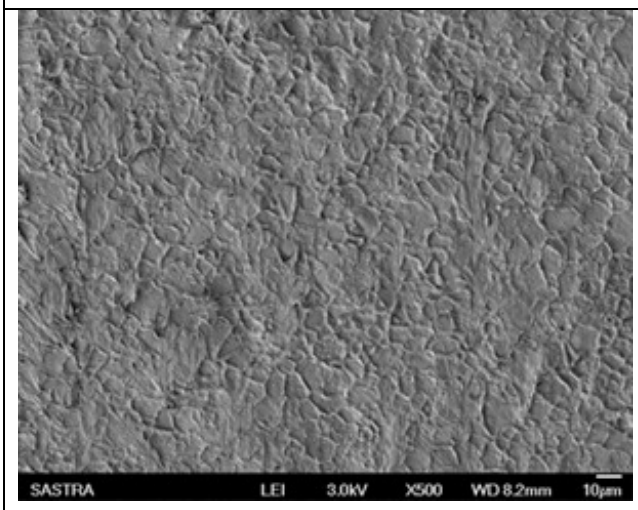

Figure 3: SEM image of ofloxacin coated film

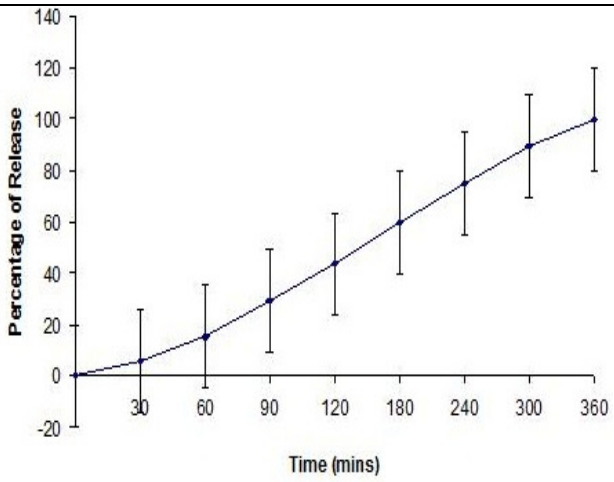

Figure 4: Drug release pattern of antibiotic loaded plates

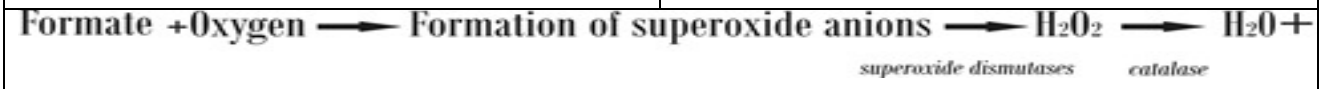

Figure 5: Proposed mechanism of drug release

To confirm this overall mechanism, the drug release was tested with *C.sporogenes*, S.aureus *and P.aeruginosa*. C. sporogenes are anaerobic organisms, which are *catalase* and superoxide dismutase negative, while the later organisms are aerobic organism, are *catalase* and superoxide dismutase positive. Prior to procedure it was found that all organisms are sensitive to ofloxacin. When the antibiotic loaded plates were kept, the area of the inhibited zone was more in the *S.aureus and P.aeruginosa* than *C.sporogenes*. Being a *catalase* and superoxide dismutase negative, there should not be any zone of inhibition. But in our study, an inhibited zone area of 19mm was found. This might be due to high concentration of loaded drug in the implant and due to presence of any free drug without proper coating of formate. Comparing to *C.sporogenes, S.aureus* had a significant area of inhibition even though both are sensitive to the drug.

In vitro drug release:

The pattern of drug release varies with each technique. The release kinetics can be adjusted by the choice of the coating technique, the film thickness and polymer composition [10]. In our study, by following dip coating method, the drugs are incorporated in the surface of the PHBV film. In our study, we observed that the total encapsulated drug gets eliminated into the medium over a time period of 6 hours. The thickness of film is 9 µm. Also the pH of the solvent can play major role, since ofloxacin is highly soluble in acidic solution than basic. In our study, the final pH of the releasing medium was 6.1. Since the medium is too acidic, it would have enhanced the ofloxacin that are coated in the film to get released at a faster rate. Sree Harsha *et al* showed that by varying the pH of the releasing the medium, the releasing pattern of the ofloxacin loaded drug delivery systems gets altered [11]. They showed that when the pH of the medium is too acidic the drug gets easily dissolved in the medium and gets released at a faster rate comparing with the other case, where the pH of the releasing medium is basic.

In drug-polymer matrix system, the drug is dispersed or dissolved in the polymer, and the release rate depends upon various factors [12]. The diffusion of a drug molecule through the polymer matrix is dependent upon the solubility of the drug in the polymer matrix and the surrounding medium, the diffusion coefficient of the drug molecule, the molecular weight of the drug, its concentration throughout the polymer matrix, and the distance necessary for diffusion [13]. The faster release of drug from the coated drug may be due to the presence of higher concentration of the drug in the film. The obtained release pattern may be due to combination of both surface and bulk erosion mechanism and also the pH of the releasing medium. It can be presumed that when the antibiotic coated plates are exposed to the release media, the drug- polymer coated thin film becomes hydrated in presence of aqueous media, leads to generate more porosity throughout the film, which allows more drug to release from coating. Also the thickness of the film plays a vital role on the drug elution. As the thickness of the film is very less the entire surface degradation process proceeds rapidly [14].

It is well known that the products of oxygen reduction—hydrogen peroxide, superoxide radical, and hydroxyl radical—are highly toxic for cells and bring about damage to cell macromolecules [15]. Also hydrogen peroxide plays a major role in overcoming the formation of biofilm and promotes wound healing [16-18]. In our study, for the release of the drug from the medium it can be noted that, formation of hydrogen peroxide is essential. The formed hydrogen peroxide can itself act as an anti - bactericidal agent other than the loaded drug and thereby improving the treatment efficacy.

FURTHER APPLICATIONS

The rate of enzyme getting released and its properties get varied between organisms. The breakdown of hydrogen peroxide to water and oxygen by *catalase* is associated with a release of energy – exothermic reaction. Based upon this, temperature based biosensors can be made to detect the organism in the given sample. Sensors are been devised to detect the micro organism based upon microbial redox reactions. The approach to the detection of biological activity described in this study is based on the recording of microbial redox reactions. In two laboratory applications, measurement of microbial activity in a biological wastewater treatment plant and in a biofilm, two sensor systems were investigated which monitor the microbiological activity online and in real time and thus its used for monitoring and control. The findings obtained showed considerable potential for optimizing biological processes on the basis of microbial activity [19].

LIMITATION OF THE STUDY

(i) At random concentrations of the organisms, the zone of inhibition studies were performed.
(ii) The minimum concentrations of organism for stimulating the drug release from the plate should be evaluated.
(iii) In studying the In vitro drug release; pH of the releasing medium was 6.2.
(iv) In vivo release from the coated plates should be evaluated.

CONCLUSION

The present study, demonstrates the potential of ofloxacin coated plates for treating infections caused by aerobic micro organisms. Plates were coated with antibiotic by dip coating method, followed by spray coating of sodium formate. Antibacterial effects were studied against S.*aureus* and C.*sporogenes*. Comparing with S.*aureus*, a significant zone of inhibition was formed in the case of C.*sporogenes*. In drug release study, the total amount of encapsulated drug was released over a period of 6 hours. Also the hydrogen peroxide produced during the course of mechanism could play a major role in preventing formation of biofilms. Hence these results indicates that these smart drug delivery systems releases drug during the presence the aerobic micro organisms and thereby improving the treating efficacy.

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