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Nano Encapsulated Drug Delivery for Biofilms

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By

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Abstract

Biofilms are now considered ubiquitous in the natural world. Bacterial biofilms have been observed to be extremely heterogeneous, both structurally and with regard to the physiology of the bacterial cells within them. The prevailing conceptual model depicts bacterial biofilms as being made up of microcolonies, which serve as the basic unit of the greater biofilm structure. A major concern with this approach is the frequently observed development of resistance to antimicrobial compounds. A number of elements in the process of biofilm formation have been studied as targets for novel drug delivery technologies. The present study aimed to penetrate biofilm by gram positive and gram negative bacteria by in-vitro culture technique, with developed nano emulsion containing photodynamic agents. The results of this study are encouraged and significantly prevent the formation of microcolonies, building bocks of biofilms.

Introduction

Bio-films are defined as collection of organisms (microcolonies) which are attached to a surface by a matrix. This matrix is called as Extracellular Polymeric Substances (EPS). It has good protection against macrophages and antibiotics which allows surviving even in hostile environment. Due to their resistant phenotype it is difficult to eradicate. Attaching to the wet surface is the natural tendency of microbes and after attaching they manufacture a protective carbohydrate matrix which allows to attach to each other and to host wound surface[3]. Bio films are found in Chronic wounds[9]. There are number of mechanisms by which various microbial species are able to attach to a surface and promote cell-cell interactions and grow as a complex structures called biofilms.

Formation of Biofilm occurs in a sequence of steps . They are Attachment of bacteria to a solid surface, Proliferation and accumulation in multilayer cell cluster, Formation of bacterial community enclosed in a self-produced polymeric matrix[5]. Attachment of microorganisms to surface and biofilm development are very complex processes and affected by several variables. Generally attachment occurs on surfaces that are rough , more hydrophobic and coated surface conditioning film. Cell surface property, particularly the presence of extra cellular appendages , the interaction involved in cell-cell communication and EPS production are important for the formation and development of biofilm[7]. Hydrophobicity of cell surface is important in adhesion as hydrophobic interaction tends to increase with an increasing non-polar nature of one or both surfaces involved.

Extracellular Polymeric Substances is responsible for binding cells and other particulate material together and to the surface. EPS composes of polysaccharides , proteins , nucleic acids , lipids , phospholipids and humic substances. Proteins and phospholipids are the major components of EPS . EPS acts as barrier and tolerate high amount of biocides and also delays or prevents antimicrobials reaching target microorganisms by diffusion limitation[6]. Lipids and nucleic acids influence the rheological properties and the stability of the biofilm significantly. Extracellular DNA required for initial establishment of biofilm. Cell-Cell communication is a driving force for self-organization and co-operation among the cells. These signals governors cell attachment and detachment , facilitates their adaption to change in environment . They respond to external environment and modulate their gene expression accordingly. Bacteria produce diffusible organic signals called auto inducer (AI) molecules which accumulate in surrounding environment. Their functions are extracellular enzymes biosynthesis, biofilm development, antibiotic biosynthesis, bio surfactant production and EPS synthesis.

There are different methods in treating biofilm on wound , they are differentiated in to mechanical debridement and chemical debridement. Mechanical Debriement is to completely removing all necrotic tissues and biofilm in the wound bed while leaving tissues untouched. Hydro surgery[7] can be used in which debridement of wound tissue is done by both cutting and aspirating the necrotic soft tissue. Wound dressing can also be done to remove the moisture content in wound. Commercial antimicrobial and topical enzymatic agents used in wound care cannot penetrate into biofilm and cannot eliminate critical wound bacterial colonization and stimulate wound healing. Chemical Debridement is using of agents like silver and bleach containing compounds and antibiotic can provide bactericidal effect with biofilm. Silver coating

cam also be done to prevent biofilm formation on industrial equipment. Ionic silver has a effect in wound care due to interference with the transport system of biofilm.

But the above mentioned techniques helps in treatment of biofilms only to an extent and they cannot prevent the reoccurrence of the bacterial biofilms . The use of non-invasive Photodynamic Antimicrobial Chemotherapy (PACT) can overcome most of the problems which are associated with the biofilms. The principle of PACT is the same as the traditional photodynamic therapy . this is a non-intrusive technique that uses a combination of light and non-toxic drug (photosensitizer) to destroy targeted cells. The inactive drug is activated by irradiation at certain wave length producing reactive oxygen species , which destroys the targeted cells without causing much damage to healthy cells. Once the irradiation is stopped the photosensitive drug gets back into its intial inactive form. The main limitation of photodynamic therapy is the uptake kinetics of the inactive drug by the micro organisms. Neutral , cationic and anionic photosensitizers are effective on gram positive bacteria[19] and where as hydrophilic cationic photosensitizers are effective on gram negative bacteria. Gram negative bacteria are more resistant because of their cell envelope and the outer membrane which provides very good protection against environment and antibiotics. Nano emulsion studies are found to be efficient in biological applications which have good efficacy in solubilization and targeted drug delivery[15-17]. In the present study we formulated different formulations of nano emulsions and we have grown biofilm in invitro conditions and successfully tested the formulations on the biofilms.

Experimental Methods and Procedures

The biofilm of E coli (Gram negative) are successfully grown using CDC Bio-Reactor.

- Bio-reactor is a one liter vessel with an effluent spout at approximately 400 ml.
- A 5 gallons of diluted broth is made to run through the bio reactor continuously for 24 to 72 hrs till the formation of bio film.
- Continuous mixing of the reactor's bulk fluid is provided by a baffled stir bar that is magnetically driven.
- An UHMW polyethylene top supports eight independent rods. Each rod houses three removable coupons (biofilm growth surfaces) for a total of 24 sampling opportunities.
- The bioreactor operates as a continuous flow stirred tank reactor, as such nutrients are continuously pumped into and flow out of the reactor, leaving only biofilm.
- The bioreactor provides opportunity for checking the samples periodically . The bio film growth can be checked from 24 hrs from the starting time and for every hour .
- The samples also can collected periodically depending on the experimental conditions .
- The used broth can also be collected and disposed off safely without any kind of contamination.

Preparation of Nanoemulsion Formulations

The nanoemulsion drug is being prepared for optimal drug delivery. We have chosen hydrophobic photosensitizers which are less permeable to cross the cell barrier. Many studies have shown that using nanoemulsions as carriers for biomedical applications can improve efficacy in solubilizing, protecting, and targeting microorganisms for specified delivery. Therefore one can anticipate that our approach can greatly advance current chronic wound treatment.

In this study we have chosen copper Phthalocyanine (CuPc) and riboflavin (vitamin B2) as hydrophobic photosensitizers. The main reason for using this as a PACT agent is owing to the certainty of its non-toxic nature towards human tissue. In order to promote the drug delivery oil-in-water nanoemulsion formulations have been developed.

Preparation of oil in water (o/w) nanoemulsion

Formulation 1

In this formulation we use Copper phthalocyanine (CuPc) which will have a final oil phase.

- Dissolve 5.0 mg of copper phthalocyanine (CuPc) and 2.0 mL of surfynol-465 (surfactant, wetting agent) in 20 mL of ethyl acetate (organic phase) over low heat with constant stirring.
- 2 gm of poly ethylene glycol (PEG200) is dissolved in 20 mL of water (water phase).
- Add the organic phase into water phase drop by drop with vigorously stirring over low flame until all the ethyl acetate has evaporated.
- Sonicate for 20 minutes.

Preparation of water in oil in water (w/o/w) nanoemulsion

- A water-oil-water (w/o/w) double emulsion method is developed to entrap hydrophilic vitamin riboflavin inside the double coated nanoparticles.

Formulation 2

- In this formulation we use riboflavin (vitamin B2), as photosensitizer.
- First 30 mg of riboflavin and 2.0g poly ethylene glycol (PEG 200) is dissolved in 20 mL of water (water phase).
- Dissolve 20 mL of castor oil and 2.0mL of surfynol-465 (organic phase) over low heat with constant stirring.
- The water phase is added drop by drop into the organic phase with constant stirring, reverse micelles are formed in this step (w/o emulsion).
- The final water phase is prepared by dissolving 2.0mL of polysorbate80 (surfactant) in 20 mL of water.
- Finally, the w/o emulsion from above is added drop by drop into the final water phase with constant stirring.
- Keep stirring until all the water is evaporated, a double emulsion is formed in this step.
- Surfynol-PEG is good pair of hydrophobic-hydrophilic double emulsion.

Formulation 3

- In this formulation we use riboflavin (vitamin B2), nanoparticles.
- First 30 mg of riboflavin and 2.0g poly ethylene glycol (PEG 200) is dissolved in 20 mL of water (water phase).
- Dissolve 20 mL of castor oil and 2.0mL of surfynol-465 (organic phase) over low heat with constant stirring.
- The water phase is added drop by drop into the organic phase with constant stirring, reverse micelles are formed in this step (w/o emulsion).
- The final water phase is prepared by dissolving 2.0mL of poloxamer-407 in 20 mL of water.
- Finally, the w/o emulsion from above is added drop by drop into the final water phase with constant stirring.
- Keep stirring until all the water is evaporated, a double emulsion is formed in this step.
- Surfynol-PEG and Surfynol-Poloxamer-407 are all good pairs for a hydrophobic-hydrophilic double emulsion.

The grown biofilm coupons are removed and drop each emulsion formulations on the top of the coupon and each coupon is placed in a test tube containing TSB.

Half of the test tubes are kept in dark (that means covering the test tube with aluminum foil to prevent exposure to light) and the other half are incubated for 30 minutes at 35˚C and then irradiated to light for 30 minutes. The test tubes that are kept in dark and irradiated to light are incubated for 24 hours.

Serial dilution is made 9x times with each test tube for counting of the bacteria .Four test tubes, each with 9 mL of sterile tryptic soy broth, and labelled from 1to4. Then 1 mL sample is taken from the bacterial suspension(test tube with coupon) that we wish to count and add it to the first tube. Mix well, this is 1:10 dilution ratio because we have added 1 mL to 10mL total. Then 1mLof dilution broth suspension is removed from tube 1 and added to tube 2. Mix well, this dilution and each following mix increasingly will be diluted by a factor of 10. Thus, tube 2 is 1:100 dilutions. Then 1mLof dilution broth suspension is removed from tube 2 and added to tube 3 and mixed well. The same dilutions are followed for all the test tubes. Then 1mLof dilution broth suspension is taken from tube 4 and added it to the surface of sterile nutrient medium in a Petri dish. Spread evenly and incubate the plates upsides down allow the bacteria to multiply for 24 to 48 hours at 37˚C.

Results & Discussion

The Petri plate after incubating to 24 to 48 hours, the bacterial colonies that grew on the plates are counted.

Formulation 1

We have seen that for formulation 1(copper phalocyanin) the bacterial cell count for the Petri plates that has sample from test tubes kept in dark are 92 cells and for the Petri plates that have samples from test tubes kept in light have no growth i.e 0 cells. Thus the percentage of bacteria that are killed is calculated as the number of colonies on the plate times the reciprocal of dilution factor gives the percentage of killing the microorganisms.

Therefore the formulation 1 has killed all the bacteria , almost 100%.

Formulation 2

We have seen that for formulation 2 (Riboflavin with polysorbate80 as surfactant) the bacterial cell count for the Petri plates that have sample from test tubes kept in dark have 147 cells and for the Petri plates that have samples from test tubes kept in light have 0 cells. Therefore we can say that the formulation 2 also have prevented the growth of bacteria upto 100%

Formulation 3

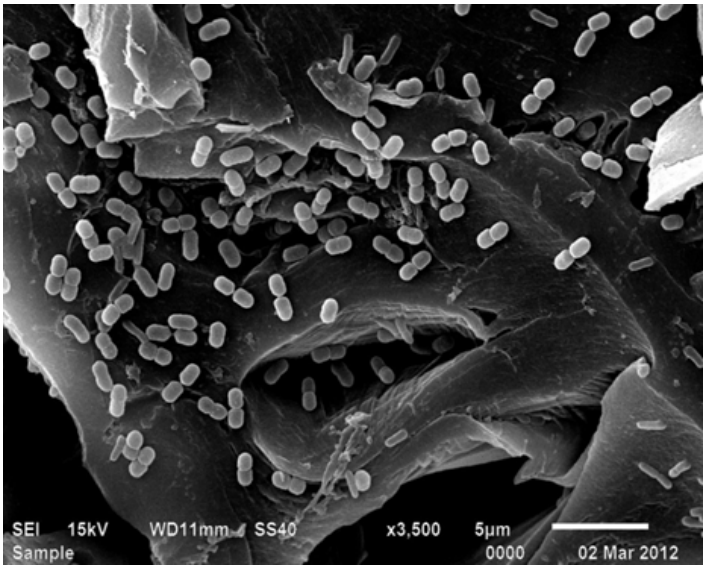
We have seen that for formulation 3 (riboflavin with poloxamer-407 as surfactant) the bacterial cell count for the Petri plates that have saples from test tubes kept in dark have 897 cells and for the Petri plates that have samples from test tubes kept in light have 142cells. The

percentage of bacteria that is killed is calculated and the percentage of the prevention of growing of bacteria is 84.2% .

Figure1

The following figures are the Petri plates which are after incubation to 37˚C.

Figure 2 : SEM Images of biofilm



The results shows that concentration of 5.0 mg of copper pthalocyanin can kill 100% of bacteria after incubation followed by 0.5 h of irradiation with low intensity light, ~5.0 J/cm2 and a concentration of 30 mg Riboflavin with polysorbate 80 as a surfactant can kill 100% of bacteria, where as the concentration of 30mg Riboflavin with poloxamer-407 as a surfactant can kill only 84.2% . This condition in using riboflavin is due to the less penetration of the drug with poloxamer-407 as a surfactant. Hereby we can say that the nanoemulsions formulated can easily pass through the bacterial membrane releasing the photosensitizers which when activated producing reactive oxygen species killing the bacteria inside the biofilm.

Table 1 : Results of formulations used on biofilm coupons kept in light and dark

	Formulation 1 (copper pthalocyanin)	**Formulation 2 (Riboflavin with polysorbate80)**	**Formulation3 (Riboflavin with poloxamer-407)**
Concentration	0.001	0.03	0.03
Dark	92 cells	147 cells	142 cells
Light	0 cells	0 cells	897 cells
% Kills	100%	100%	84.2%

Conclusion

From the above results we can assume that photodynamic antimicrobial chemotherapy (PACT) has the potential to represent an alternative antibacterial treatment for drug-resistant organisms. PDT uses normal visible light and nontoxic vitamin photosensitizer to destroy specific targeted cells. Nano emulsions, that we formulated are very much effective in drug delivery across the biofilms. We have developed a nanoemulsion in such a way to increase the solubility of these drugs which are otherwise hard to dissolve hydrophobic vitamins for the faster and more effective delivery to the targeted cells. Therefore the experimental procedure and results provides a photodynamic chemotherapeutic regime for the treatment of chronic wound ulcers by microbial biofilm.

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