Spring 2012

Using Photoactive Nanoparticles as Photodynamic Antimicrobial Chemotherapeutic (PACT) Agent to Treat Chronic Wounds

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MASTER'S PROJECT

Submitted in partial fulfillment of the requirement

For the Degree of Master of Science,

With a Major in Analytical Chemistry

Governors State University

University Park, IL 60484

2012

Abstract:

The purpose of this study is to use photoactive vitamin nanoparticles as photodynamic antimicrobial chemotherapeutic agent to treat patients with chronic wounds. Chronic wounds are considered as a pandemic health problem which affects millions of people and increases ~10 % annually. According to the American Academy of Dermatology, the expense of treating chronic wounds constitutes over half of the total cost for all skin diseases. The main cause of chronic wounds is due to the existence of biofilms. Biofilms are complex microbial communities containing and are reported to be major factor contributing to multiple chronic inflammatory diseases. Owing to bacterial species with in biofilms being exceptionally resisted to many traditional therapies, Photodynamic Antimicrobial Chemotherapy (PACT) can provide an effective alternative antimicrobial, antifungal, and antiviral treatment for drug-resistant microorganism. PDT uses both light and nontoxic vitamin photosensitizers to destroy specific targeted cells. The main advantage of PACT would be that it is very unlikely for bacteria to develop resistance to reactive oxygen species. However, the main limitation of this technique would be the uptake kinetics of the photo sensitizers in microorganisms. We have developed a unique nano-emulsion to increase the solubility of these otherwise hard to dissolve, hydrophobic vitamins for faster, more effective delivery to the targeted cells. This provides a photodynamic chemotherapeutic regime for the treatment of chronic wound ulcers caused by microbial biofilms.

Introduction:

Chronic wounds are the major public health problem affecting millions of individuals. The term "chronic wound" is generally accepted, but yet no simple definition has been agreed upon.[1] A mechanistic definition such as "those not following normal wound healing trajectory" have proposed but the most common definition have been "ulcers (wounds) older than 3 months of age". No matter the length of time, a wound which does not heal in an orderly progression but halts in an inflammatory process is considered a chronic wound. [1-2] The treatment of chronic wounds is often inadequate, extremely painful, time consuming, and costly. Approximately $25 billion is spent annually on the treatment of chronic wounds.

Chronic wounds, includes diabetic food ulcers, pressure ulcers, and venous leg ulcers. The elderly are affected the most by these ulcers which penetrate deep into the skin and become infected easily.[3] It has been estimated that 15% of individuals with diabetes mellitus will develop lower extremity ulcers and 14-24% of diabetic patients with foot ulcers will eventually undergo amputation. Approximately 100,000 lower limb amputations are performed in diabetic patients each year in the United State. [4-6] Pressure ulcers are common and expensive problem in acute care, rehabilitation unit, nursing home, and home care populations. Venous leg ulcers are often painful inflictions that have been estimated to affect 1% of the world's population. The statistical data shows a great need for the treatment of chronic wounds. [6-7] There are biological and physiological reasons for wounds that are not healing. A primary barrier to healing is the continuing influx of poly-morphonuclear leukocytes (PMNs, a category of white blood cells) from the host blood circulatory system to the open wounds. Activated PMNs release cytotoxic enzymes and inflammatory mediators that can damage host tissues. [8-10] Owing to this continuous influx of PMNs, the healing and the destructive processes within the chronic wounds are

imbalanced; and the main reason for this distress is the presence of biofilms. Biofilm-related diseases are typically persistent infections that develop slowly, seem to be rarely resolved by immune defenses, and respond transiently to antimicrobial therapy.

Biofilm are defined as a group of microorganisms that exist in a self-synthesized protective matrix and are encased within a three dimensional matrix of extracellular polymeric substances. Extracellular polymeric substance provides physical protection to the microorganisms from the environment. They are extremely difficult to detect in chronic wounds and exceptionally resistant to the host immune system as well as antibiotic elimination. Currently, the management of infections due to biofilms is physical removal by a method called surgical debridement.[11] The procedure requires aggressive abstraction of necrotic tissue until healthy tissue is visible. Hypothetically, this is the preferred method, but due to its aggressive nature it's not always the optimum choice. Risks can be life threatening to patients such as anesthesia, bleeding, sepsis, and bacteremia. [11,12]

The use of non-invasive Photodynamic Antimicrobial Chemotherapy (PACT) can overcome problems which are associated with the current treatment of chronic wounds. The principle of PACT is the same as that of traditional photodynamic therapy. It is a non-intrusive technique that uses a combination of light and nontoxic drugs (photosensitizers) to destroy targeted cells. Once the inactive, nontoxic drug is put on either topically or injected it can only be activated by irradiation at a certain wavelength After the drug is irradiated it can then produce extremely reactive oxygen species to destroy those cells which were targeted without causing damage to any healthy tissue. Once the irradiation is removed, the photosensitive drug will return to its stable, non-harmful state. The main limitation of PACT is the uptake kinetics of the nontoxic drug in microorganisms. Generally, neutral, anionic, and cationic photosensitizers can effectively

eradicate Gram-positive bacteria.[13] The only photosensitizer that can kill Gram-negative bacteria is hydrophilic cationic. Gram-positive bacteria have a porous cell wall which lets most photosensitizers to cross. On the other hand, the cell envelop, outer membrane, of Gram-negative bacteria forms an effective permeability obstruction between the cell and environment.[14] Therefore, intensive research has been done on particulate delivery systems to overcome this situation. Nanoemulsion studies have been found to be an efficient carrier for biomedical applications that improves efficacy in solubilizing, protecting, and targeting drugs for specified delivery.[15-17] Hence this approach can be used to improve current chronic wound diagnostics and treatments.

Experiment:

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.

Bacteria Study and Cell count:

Each emulsion formulation will be tested for its ability to deliver the PACT drugs into bacteria biofilm of E coli.

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. 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 grown biofilm coupons are removed and drop each emulsion formulations on the top of the coupons. Half of the coupons remain in the dark and the other half are incubated for 30 minutes at 35˚C and then irradiated to light for 30 minutes.

Then the both the coupons that remains in dark and irradiate to light are removed and diluted 9x times individually in tryptic soy broth, each in single test tube.

We have set four test tubes, each with 9 mL of sterile tryptic soy broth, and label from 1to4. Then 1 mL sample is taken from the bacterial suspension 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. Mix well. The same dilutions are followed for all the 4 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:

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 remains on dark is 92 cells and for the Petri plates that irradiate to light are 0 cells. Thus the percentage of bacteria that is killed is calculated as follows:

Number of colonies on the plate times the reciprocal of dilution factor gives the percentage of killing the microorganisms.

Thus the % kill microorganisms is 100%

Formulation 2:

We have seen that for formulation 2 (Riboflavin with polysorbate80 as surfactant) the bacterial cell count for the Petri plates that remains on dark is 147 cells and for the Petri plates that irradiate to light are 0 cells. Thus the percentage of bacteria that is killed is calculated as follows:

Number of colonies on the plate times the reciprocal of dilution factor gives the percentage of killing the microorganisms.

Thus the % kill microorganisms is 100%

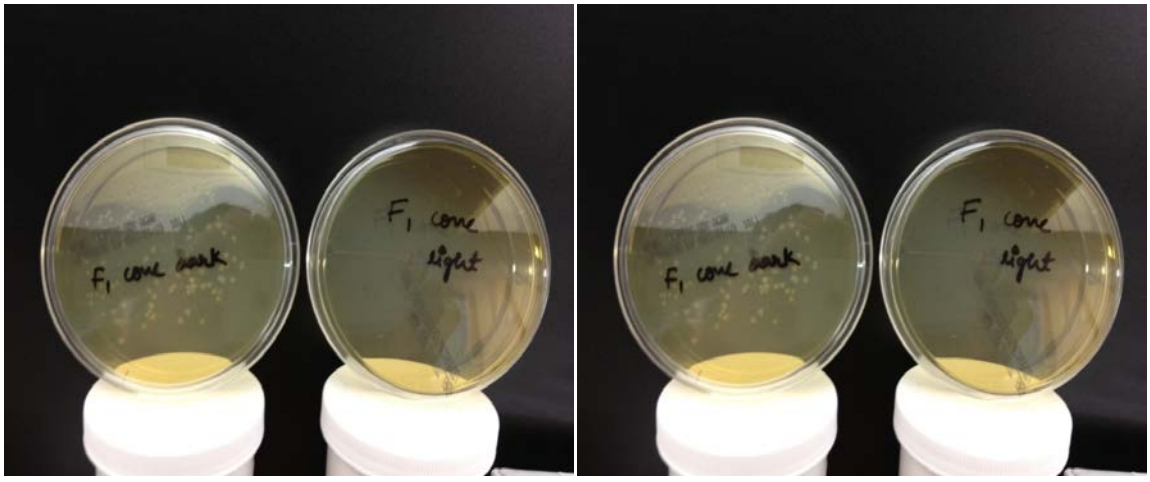
Formulation 3:

We have seen that for formulation 3 (riboflavin with poloxamer-407 as surfactant) the bacterial cell count for the Petri plates that remains on dark is 897 cells and for the Petri plates that irradiate to light are 142cells. Thus the percentage of bacteria that is killed is calculated as follows:

Number of colonies on the plate times the reciprocal of dilution factor gives the percentage of killing the microorganisms.

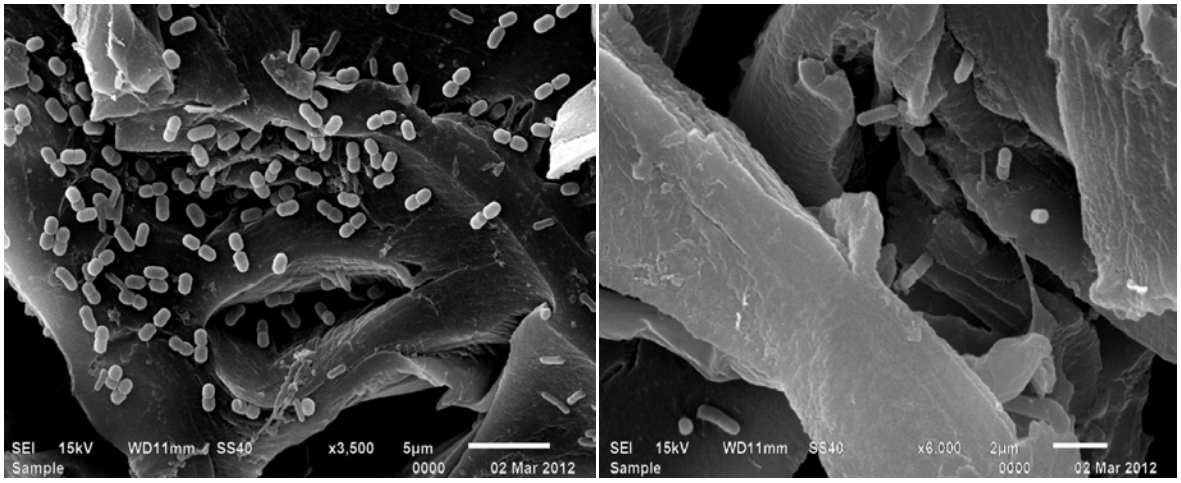
Thus the % kill microorganisms is 84.2%

Figure1



The above figures are the Petri plates which are after incubation to 35˚C.

Figure 2



The above figure is the biofilm of E coli which are identified by scanning through electron microscopy.

Bacteria Study:

The bacterial study of Copper Pthalocyanin and Riboflavin was conducted using biofilm of E. coli. Each formulation will be tested for its ability to deliver the PACT drugs into bacteria. E coli (Gram negative) are cultured in Petri dishes with nutrient broth culture media. A concentration 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/cm^2 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 is due to the less penetration of the drug with poloxamer-407 as a surfactant. Our result indicates our nanoemulsion can easily pass through the bacterial membrane and release the photosensitizes

	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:

We have shown that photodynamic antimicrobial chemotherapy (PACT) has the potential to represent an alternative antibacterial, antifungal, and antiviral treatment for drug-resistant organisms. PDT uses both visible light (>395nm in wavelength) and a non-toxic vitamin photosensitizer to destroy specific targeted cells. Nanoemulsion also has proven to be an effective way for drug delivery. We have developed a unique nanoemulsion to increase the solubility of these otherwise hard to dissolve, hydrophobic vitamins for the faster, more effective delivery to the targeted cells. Thus the technique provides a photodynamic chemotherapeutic regime for the treatment of chronic wound ulcers by microbial biofilm.

Acknowledgment:

I would like to express my sincere thanks to my advisor Dr. Patty K-L. Fu for her guidance and support throughout my research work as well as writing of this thesis. I also thank the rest of my thesis committee: Prof Dr.Walter Henne and Dr.Stephen D.Kent for their encouragement. This Research was supported by the College of Arts and Science, Governors State University.

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