Nanoencapsulated Drug-Carrying System for Photodynamic Antimicrobial Chemotherapy (PACT)

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ABSTRACT

The purpose of this study is to develop nanoparticulate drug-carrying systems that are capable of directly delivering photodynamic antimicrobial agents to treat patients with chronic wounds. In the United States, the expense of treating chronic wounds constitutes over half of the total cost for all skin diseases. Pathogenic biofilms are the primary hindrance to the wound healing. Owing to bacterial species within biofilms being exceptionally resistant to many traditional therapies, Photodynamic Antimicrobial Chemotherapy (PACT) can provide an effective alternative treatment for chronic wounds. The main advantage of PACT would be the unlikelihood of bacteria developing resistance to reactive oxygen species. However, the major limitation of this technique would be the uptake kinetics of the photosensitizers into microorganisms. To overcome the problem, we have successfully formulated an oil-in-water nanoemulsion to deliver the chemotherapeutic agents.

Keywords: drug-delivery, photodynamic therapy, biofilm, nanoemulsion, hydrophobic drug

1 INTRODUCTION

In 2010, more than 7 million people suffered from chronic wounds and increases by 10% annually.[1] Approximately 80,000 people undergo amputation each year due to wounds that do not heal.[2] Chronic wounds are a pandemic problem that can greatly affect a person's quality of life. The management and treatment cost billions of dollars a year globally. Most health professionals consider a wound to be chronic when it has not healed within 4 to 6 weeks. Surgical textbooks identify chronic wounds are those that do not heal in 3 months.[3] Regardless the length of the time, wounds that do not heal through an orderly progression, halt in a stage dominated by inflammatory processes are considered chronic wounds.

The most common chronic wounds are venous, diabetic, and pressure ulcers. Venous ulcers usually occur in the legs, accounting for about 70% to 90% of chronic wounds. The elderly are affected the most by these ulcers which penetrate deep into the skin and become infected easily. Occasionally, if a venous ulcer persists for a long time, skin cancer could develop at the edge.[4-6] In the United States, 8.3% of the population is diabetic and each year about 65,700 non-traumatic lower-limb amputations are performed in people with diabetic ulcers. In addition, there are about 2.5 million Americans currently hospitalized who are suffering from pressure ulcers (bed-sores).[6,7] The statistical data shows a great need for the treatment of chronic wounds. 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.

Biofilms are highly structured groups of pathogenic microbes living within a protective extracellular matrix that they produce. The extracellular polymeric substance provides physical protection to the microorganisms from its environment. Biofilms in chronic wounds are difficult to detect and highly resistant to the host immune system and antibiotic elimination. Presently, the most common management of biofilm infections is physical removal of the biofilm called surgical debridement.[11] This procedure involves aggressive removal of massive amount of necrotic tissues. Theoretically it would be the preferred method, however, due to the invasive nature it is not always the best option. The life threatening dangers to the patient are tremendous such as anesthesia risk, bleeding, sepsis, and bacteremia.[11.12]

In order to overcome problems associated with the treatment of chronic wounds, non-invasive Photodynamic Antimicrobial Chemotherapy (PACT) can provide an alternative method to heal chronic wound. 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. After the inactive, nontoxic drug is applied topically or injected, it can only be activated by irradiation with a certain wavelength of light. Once the drugs are switched on by light, they can produce highly reactive intermediates to destroy the targeted cells without damaging the surrounding healthy tissues. Once the irradiation is removed, the photosensitive drug will return to its stable, non-harmful state.

The main limitation of this technique would be the uptake kinetics of the photosensitizer in microorganisms. In

general, neutral, anionic, and cationic photosensitizers can efficiently eliminate Gram-positive bacteria.[13] Only hydrophilic cationic photosensitizers can kill Gram-negative bacteria. The porous cell wall of Gram-positive bacteria allows most photosensitizers to cross. However, the cell envelope (outer membrane) of Gram-negative bacteria forms an effective permeability barrier between the cells and its environment.[14] This has led to intensive research on particulate delivery systems to overcome this situation. Studies have shown that using a nanoemulsion as a carrier for biomedical applications can improve efficacy in solubilizing, protecting, and targeting drugs for specified delivery.[15-17] Therefore one can anticipate such an approach to greatly advance current chronic wound diagnostics and treatments.

2 EXPERIMENTAL METHODS

2.1 Materials

Riboflavin , Phylloquinone, Vitamin F and Trizma acetate buffer (Sigma Life Sciences), Tween 20 (Sigma-Aldrich), Ethidium bromide (MP Biomedicals, LLC.) were used without further purificatio. Fischer-Biotech Electrophoresis FP-SB-710, Thermoscientific NAPCO series 8000WJ CO_2 incubator, ESCO class II biological safety cabinet.

2.2 Nanoemulsion

The riboflavin drug (Active Pharmaceutical Ingredient) used in the study is hydrophobic and less permeable to cross the cell barrier. To intervene this, a nanoemulsion of drug is being prepared for optimal drug delivery. Emulsion formation depends upon the hydrophilic and lipophilic nature of the drug, solvent, and emulsifier. The surfactant being used has a hydrophilic-lipophilic balance (HLB) value in the required HLB range of the drug to form the oil-in-water (O/W) emulsion. Tween 20 is the emulsifier used in the preparation to lower the surface tension of the drug in the water. Riboflavin is dissolved in Vitamin F(oil phase) in the preparation.

Primary Emulsion

Vitamin F with vitamin	4 Parts
Water	2 Parts
Tween 20 (Emulsifier)	1 Parts

Riboflavin is dissolved in Vitamin F by vortexing in a vial (oil phase). One part of Tween 20 surfactant is added to the 2 parts of water and vortexed to form a homogenous solution (water phase). The oil to surfactant ratio is maintained to be greater than 1:2. The oil phase is added to the water phase in a drop-wise fashion while vortexing to disperse the oil droplets uniformly into the water phase. The emulsion is formed by sonicating for 1.0 h in the sonicator. The shear forces of vortexing and sonic waves during sonication favor the formation of the nanoemulsion. A 1:10 dilution of the primary emulsion is made with water to form a clear emulsion.

2.3 Singlet Oxygen Study

Singlet oxygen determination has been carried out by UV-Vis spectroscopy (Ocean Optics) with loggerpro-3.6.0 Vernier software. The 9,10-anthracene dipropionic acid (ADPA) photo-bleaching method is used to confirm singlet oxygen generation. ADPA is easily converted to a photo inactive endoperoxide by singlet oxygen. An ADPA solution in deuterium oxide (D_2O) is used as the singlet oxygen acceptor. Mixed solutions of the photosensitizer and ADPA are irradiated with visible light. The reaction progress is monitored by recording the decrease of the 400 nm absorption peak of ADPA verses irradiation time. The intensity of ADPA absorption decreased as the irradiation time increased, which indicates the generation of singlet oxygen.

2.4 DNA Study

Drug formulation at different concentrations, pUC19 Plasmid (120 μM), NaCl (5 mM) are added to transparent eppendorf micro tubes. A uniform volume of 20 μL is maintained in all tubes. The tubes are incubated in the dark for 0.5 h with subsequent irradiation of the light samples under visible light in the photoreactor (2.8×10^{-3} W/cm^2 ; 5.0 J/cm^2) for 0.5 h.The dark and light samples are loaded into the gel with gel loading solution (4 μL) and gel electrophoresis (90 V; 1.5 h) is carried out using agarose (2.0%) stained with ethidium bromide in 1X TAE running buffer. The gel is imaged on Gel Doc 200 transilluminator (Bio-Rad, Hercules, CA) with the supercoiled DNA at position 2 and the nicked DNA at position 1. The degree of photocleavage is estimated by the integration of the image intensity at position 1 and position 2 by Quantity One Analysis system software (Bio-Rad, Hercules,CA).

2.5 Cell and Bacteria Study

Human skin fibroblasts (Hs-27) from the American Type Culture Collection, cell line CRL-1634 (Manassas, VA) are used in the cell study. The cells were cultured in Dulbecco's Modified Eagle Media (10% fetal bovine serum, 50 μg/mL gentamicin, 4.5 mg/mL glucose, and 4 *mM* L-glutamine). The cells are cultured in BD-Biocoat (60 mm) culture dishes (at 5.0% CO_2 at 37^{o}C in the incubator). The media is washed away from the culture plates using 1X phosphate buffer for the assesment of cytotoxicity and photocytotoxicity of the nanoparticles. Uniform volume of nanoemulsion (test), 1X PBS (blank), and placebo (control) are added to the culture dishes. The culture dishes are incubated for 1.0 h in the dark and in visible light seperately for photocytotoxicity. The supernant from the culture dishes are decanted and washed with 1X PBS. The cells are lysed using cell lysis solution (20 μM sodium laurylsarcosine solution) for approximately 1.0 h on

a shaker. The protein content from the lysed culture dishes are quantified using BCA Protein Assay (Thermopierce). The culture dishes are incubated for an hour and the absorbance intensity of the purple color complex formed is observed at 570 nm in Biotek ELx800 micro plate reader for the dark and light samples.

E coli (Gram negative) and *S. aureus* (Gram positive) are cultured in petri dishes with nutrient broth culture media. The nanoemulsion is added to the bacterial culture and incubated for an hour. Half of the culture remains in the dark and the other half is irradiated for 0.5 h. The absorbance readings of both the irradiated and dark samples are observed on Biotek Epoche Absorbance Plate reader.

3 RESULTS

3.1 Nanoemulsion

An oil-in-water preparation is used to form the nanoemulsion of riboflavin shown in Figure 1. Riboflavin is slightly soluble in water, but by using a nanoemulsion the riboflavin solubility can be increased by 10-fold. The same results can be achieved with phylloquinone. In Figure 2, the encapsulation of phylloquinone can be seen when the concentration is increased 3-fold. The TEM displays that the phylloquinone is in the oil phase then encapsulated in the water phase.

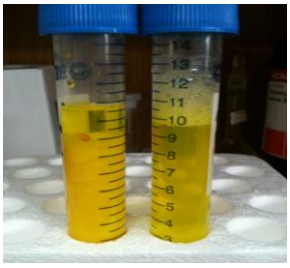

Figure 1. Left, percipatation formed with 250 µM riboflavin in water. Right, 2.0 mM riboflavin nanoemulsion.

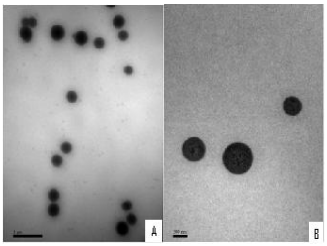

Figure 2. TEM of phylloquinone encapsulated nanoparticles. (A) Indicated Magnification is X25000 and the Total Magnification is X28800. (B) Indicated Magnification is X60000 and the Total Magnification is X69900.

3.2 Singlet Oxygen Study

Singlet oxygen study was also preformed on riboflavin to ascertain its generation over time. Figure 3 shows that after only 0.25 h of irradiation, riboflavin is able to produce a significant amount of singlet oxygen. The sensor used in the singlet oxygen study is 9,10-anthracene dipropionic acid (ADPA). Our result has shown that riboflavin is an excellent source of producing reactive oxygen species when irradiated with low energy visible light.

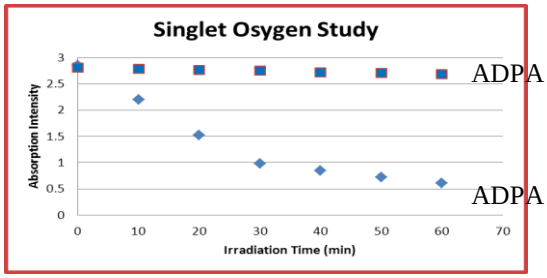

Figure 3. The absorption intensity of ADPA (at 400 nm, in the presence of riboflavin) decreased as the irradiation time increased indicates ADPA was converted to an endoperoxide derivative by singlet oxygen.

3.3 DNA Study

The image of the EtBr-stained gel in Figure 4 shows the DNA photocleavage of 120 μM pUC19 bacterial plasmid by riboflavin. The control lanes show that irradiation of the plasmid (pUC19) alone with visible light for 5.0 J/cm^2 does not result in DNA damage (Lanes 1 and 2). In addition, along with the supercoiled plasmid (Form I), a small amount of circular (nicked, Form II) is found in the plasmid. As shown in Figure 4, the addition of 25 μM riboflavin (lane 3 and 4) to 120 μM pUC19 plasmid does not result in cleavage in the dark (Lane 3), however, irradiation of the mixture for 5.0 J/cm^2 with visible light (λirr= 400 – 700 nm, 0.5 h) results in the formation of nicked ds-DNA (Lane 4, Form II).

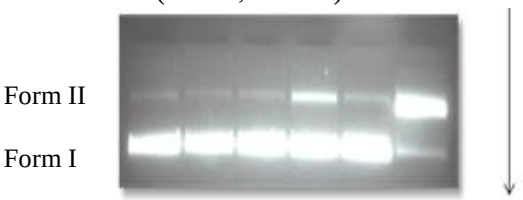

Figure 4. Ethidium Bromide stained agarose gel showing the photocleavage of 120 µM bacterial plasmid DNA. From left: Lane 1: DNA only, dark. Lane 2: DNA only, irradiated. Lane 3: DNA + 25 µM Riboflavin, dark. Lane 4: DNA + 25 µM Riboflavin, irradiated. Lane 5: DNA + 50 µM Riboflavin, dark. Lane 6: DNA + 50 µM Riboflavin, irradiated (18.8 mg/Kg).

	Human Skin		E. Coli		S. Aureus		Acute Oral Toxicity
	Dark	Irradiated	Dark	Irradiated	Dark	Irradiated	
Riboflavin	No Effect	2,170 ± 110	197 ± 9.85	85 ± 4.20	275 ± 13.8	117 ± 5.85	>10,000
Phylloquinone	No Effect	18,700 ± 938	1,100 ± 55	423 ± 21.1	3,270	475 ± 23.8	25,000

Table 1. LD50 (lethal dose 50 in mg/Kg) of Riboflavin and Phylloquinone in both dark and irradiated conditions . Acute oral systematic toxicity of both was cited from the official MSDS.

3.4 Cytotoxicity and Photocytotoxicity

The cytotoxicity and photocytotoxicity study was executed using human fibroblast skin cells. Table 1 shows that both riboflavin and phylloquinone have no toxic effect on human skin cells in the dark. There is a slight adverse effect when the riboflavin and phylloquinone is irradiated with light, but these numbers are much lower compared to the acute oral toxicity obtained from the MSDS. Furthermore, the reactive oxygen species that are formed when irradiated with visible light have such a short lifetime and can not travel far, so only those cells which are irradiated with light will be effected in the end.

3.5 Bacteria Study

The bacterial study of riboflavin and phylloquinone was conducted using *E. coli* and *S. aureus*. A concentration as low as 85 mg/Kg of riboflavin can cause 50% fatality of bacteria after an hour of incubation followed by 0.5 h of irradiation with low intensity light, ~5.0 J/cm^2. These results were compared with the acute oral toxicity of riboflavin and phylloquinone obtained from the official MSDS. Table 1 confirms that at a low concentration, riboflavin and phylloquinone are able to eradicate bacteria upon irradiation. *S. Aureus* is a gram-positive bacteria that can be easily penetrated by photosensitizers due its porous cell wall. However, *E. Coli* is a gram-negative bacteria which has a outer membrane that is hard to penetrate. Our results indicate our nanoemulsion can easily pass through the bacterial membrane and release the photosensitizers.

4 DISCUSSION

We have shown that photodynamic antimicrobial chemotherapy (PACT) has the potential to represent an alternative antibacterial, antifungal, and antiviral treatment for drug-resistant organisms. Non-toxic vitamins such as riboflavin and phylloquinone can produce singlet oxygen and free radicals upon irradiation. Our data has shown that riboflavin not only has a great DNA binding constant ($K_b > 10^4$ M^{-1}), it can also cleave bacterial DNA under the irradiation of the visible light ($\lambda > 395$ nm). Nanoemulsion also has proven to be an effective way for drug delivery. Their photobiological properties and phototoxicity towards prokaryotic cells still needs to be investigated further. In addition, spin-off applications of this work would offer opportunities for other environmental friendly uses such as bioremediating hazardous waste sites, biofiltering industrial water, and forming biobarriers to protect soil and groundwater from contamination.

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