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# Using Photoactive Vitamin Nanoparticles as Photodynamic Antimicrobial Chemotherapeutic (PACT) Agents to Treat Chronic Wounds

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**By**

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**Abstract**

The objective of the proposed study is to develop new materials and methods to manage microbial growth in chronic wounds using phototherapy. Chronic wounds are considered as a worldwide health problem. The most common chronic wounds can be classified into three categories: venous ulcers, diabetic ulcers, and pressure ulcers. Venous ulcers, which usually occur in the legs, account for about 70% to 90% of chronic wounds and can become infected easily. Venous ulcers are sores that develop after veins in the legs have been damaged. These ulcers can penetrate deeply into the skin. Occasionally, if a venous ulcer persists for a long time, skin cancer might develop at the edge. It has been shown that several bacteria species can colonize chronic wounds as highly persistent biofilm communities. Chronic wound pathogenic biofilms are host-pathogen environments that colonize and exist as a cohabitation of many bacterial species that cooperate to promote their own survival and the chronic nature of the infection. Bacterial biofilms are highly organised microbial communities living within a protective extracellular matrix. They are difficult to detect and highly resistant to immune or antibiotic elimination. There are specific major populations of bacteria that are evident in the biofilms of all chronic wound types. Species including *Staphylococcus, Pseudomonas, Peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia*, and *Serratia* spp are found to be common chronic wound pathogens.

Our preliminary studies have shown that non-toxic vitamins such as riboflavin (vitamin B2) and cobalamin (vitamin B12) can produce singlet oxygen and free radicals upon irradiation. Furthermore, our data has shown, upon irradiation with low energy visible light, riboflavin and cobalamin both can cleave and damage native double-stranded DNA. It is very important to use low energy visible light in the process owing to it penetrates human tissue much deeper then the high energy UV light. Also, visible light will not damage normal human cells like UV light. The delivery of visible light is a localized process normally using laser light transported by optical fibers. We have the knowledge and technology to discover and develop the drugs as well as design a portable laser system for PACT that can be patented and eventually commercialized.

## Introduction

The objective of this invention is to use photoactive vitamin nanoparticles as new photodynamic antimicrobial chemotherapeutic agents to heal chronic wounds. Chronic wounds are considered as a worldwide health problem due to the treatment being often inadequate. Incorrect diagnosis, overuse of systemic antibiotics and inadequate use of compression therapy frequently aggravate the complications.

It has been shown that several bacteria species can colonize chronic wounds as highly persistent biofilm communities. Chronic wound pathogenic biofilms are host-pathogen co-exist environments that many bacterial species cohabitate to promote their own survival. Many people have the misconception that systemic antibiotics alone can treat these infections. However, the truth is that use of antibiotics alone is often harmful, because they often breed bacteria that are resistant to the antibiotic being used. For that reason, photodynamic antimicrobial chemotherapy (PACT) has the potential to represent an alternative antibacterial, antifungal, and antiviral treatment for drug-resistant microorganisms. It is very unlikely for any organisms to develop resistance to cytotoxic reactive molecules such as reactive oxygen species (ROS) generated by the photosensitizers. Our studies have shown that non-toxic vitamins such as riboflavin (vitamin B6), phylloquinone (vitamin K1), and menaquinone (vitamin K2) are excellent photosensitizes that can produce singlet oxygen and free radicals upon irradiation. We have also developed a unique nano-emulsion to increase the solubility of these otherwise hard to dissolve, hydrophobic vitamins for faster, more effective delivery to the target cells. This invention will provide a novel photodynamic chemotherapeutic regime for the treatment of chronic wound ulcers caused by microbial biofilms.

Photodynamic therapy (PDT) is a technique that uses the combination of light and nontoxic drugs to destroy specific targeted cells.[1-11] After the inactive and nontoxic drug is applied topically or injected, it localizes in the selected tissue and can only be activated by irradiation with certain wavelengths of light. When these photosensitive drugs are activated, they can produce highly reactive intermediates and ultimately lead to the selective death of targeted cells without affecting normal tissue. Currently, PDT is being applied mostly in the treatment of cancer.[2,4,6,8,9] However, several studies have

shown that PDT can also be utilized in antimicrobial management.[1,4,5,7] Research interest in antimicrobial PDT diminished for many years due to the availability of antibiotics. However, in recent years, the emergence of antibiotic-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis,* has renewed interest in alternative treatments.

It has been established that there are specific major populations of bacteria that are habited in all chronic wound biofilms. Species including *Staphylococcus, Pseudomonas, Peptoniphilus, Enterobacter, Stenotrophomonas, Finegoldia*, and *Serratia* spp are found to be common chronic wound pathogens.[12] Bacterial biofilms are highly organized microbial communities living within a protective extracellular matrix. They are difficult to detect and highly resistant to immune or antibiotic elimination. It has been suggested that biofilm presence may contribute to the intractable inflammatory processes seen in chronic wounds. Several clinical studies showed there was a statistically significant ($p$<0.001) that chronic wound patients had developed biofilms (> 60%) verses that of acute wound patients (6%). Molecular analyses of chronic wound specimens have revealed the diverse polymicrobial communities and the presence of bacterial colonization.[13-15]

Various metallic compounds have been investigated for their utility as PDT agents, most notably cisplatin in the treatment of cancer.[16] The primary problems with cisplatin are its selective utility against various forms of cancer, its toxicity to human cells, and the resistance that tumor cells may develop against it.[16] Other metallic compounds have been thoroughly investigated for their photoreactive properties and their utility as PDT agents and, more specifically, PACT agents.[17-25] While many suffer from the same shortcomings as cisplatin, some do not have the same side effects; however, all the metallic compounds suffer from problems related to synthesis. That is, they have complex syntheses and may be costly to manufacture. Naturally occurring compounds such as riboflavin and phylloquinone do not have the drawbacks associated with synthesis. Naturally occurring compounds have the added benefit that they will not harm normal tissue. That is, they remain inert until photoactivated, thus, they are great candidates for PACT agents.

Our research would eventually solve many of the major problems associated with post-wound infections by developing a new, fully characterized treatment that is based on well-known, inexpensive, and innocuous aqueous vitamin nanoparticles in combination with light therapy.

## Methods

### DNA Photocleavage

The DNA photocleavage experiments were carried out on a 20 μL total sample volume in 0.5 mL transparent Eppendorf microtubes containing 120 *μM* pUC19 plasmid and 30 *μM* of DAP in 10 *mM* phosphate buffer, 5 *mM* NaCl, pH = 7.5. All samples were irradiated for 30 min after 30 minutes of incubation following addition of DAP. After irradiation, the samples were loaded into the gel following the addition of 4 μL of DNA gel loading solution to each reaction mixture. In showing the pH dependence of photocleavage, the pH of each reaction mixture was varied using phosphate buffer before adding DAP and irradiation. All the photolysis experiments were conducted in air at room temperature. The electrophoresis was carried out using 2% agarose gel stained with 0.5 mg/L ethidium bromide in 1X TAE running buffer (TAE = tris-acetate EDTA) at 90 V for 1.5 hours, and the gels were imaged on a GelDoc 2000 transilluminator (Bio-Rad, Hercules, CA). The extent of photocleavage was calculated from the ratio of nicked (circular) to supercoiled ds-DNA determined from the integration of image intensity available in the QuantityOne Analysis System software (Bio-Rad, Hercules, CA). In these measurements, no correction was made for the difference in EtBr emission from the different forms of DNA.

### Cytotoxicity and Photocytotoxicity Assays.

Human skin fibroblasts (Hs-27) were obtained from the American Type Culture Collection, cell line CRL-1634 (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle Media, containing 10% fetal bovine serum, 50 μg/mL gentamicin, 4.5

mg/mL glucose, and 4 *mM* L-glutamine. The cell cultures were incubated in a humidified atmosphere containing 5% $CO_2$ at 37 $^{o}$C.

For assessing the cytotoxicity and photocytotoxicity of nanoparticles, >80% confluent monolayers of Hs-27 in 60 mm culture dishes were used. The monolayers were first washed twice with phosphate-buffered saline (PBS) to ensure that the culture dishes were free of any culture medium. Fresh PBS (3 mL) containing various concentrations of DAP was then added to cover the fibroblasts. The cells were irradiated through the PBS buffer, which does not absorb light in the visible region, immediately without an incubation period. The spectral output of the visible light was ~2.8 x $10^{-3}$ W/$cm^2$, and all samples were irradiated no longer than 30 min (~5 J/$cm^2$) at room temperature. Following irradiation, the cells were removed from the dishes by trypsinization, reseeded into 24-well culture dishes, and incubated for 48 hours. N-lauroyl sarcosine (200 μL, 40 *mM*) was then added to each well and the cells were allowed to lyse for at least 30 min. Quantitative determination of the protein content in each well was undertaken using Peterson's modification of the micro-Lowry method. The lysate was treated with 200 μL Lowry reagent for 30 min and then with 100 μL Folin-Ciocalteau phenol reagent for 30 min. A portion (200 μL) of the contents of each well was transferred to a 96-well plate for absorbance determination using a 96 well MRX plate reader (Dynatech Laboratory, DynPort, VA). The absorbance measured at 630 nm is proportional to the total protein content and the number of cells in each well.

**Nanoemulsion:**

Due to most photoactive vitamins in nature being either hydrophobic or low in solubility, we have developed a biocompatible nano-emulsion in order to deliver lipophilic vitamins to target cells more effectively. We used high a HLB (hydrophilic-lipophilic balance) emulsifier to prepare our oil in water nanoparticles. Tween 20 has a HLB of 16.7 which serves as the emulsifier. We use vitamin F (mixture of linoleic acid and alpha linoleic acid) as our primary fatty acid which has a HLB of 14-15. It meets the criteria of HLB for solubilizing oil in water to make clear nanoemulsion. HLB value needs to be in between13-18.

**Making Emulsion:**

| | |
|---|---|
| Oil | 16% |
| Emulsifier | 4% |
| Water | 80% |

**Primary Emulsion:**

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

Hydrophobic vitamin was dissolved in fatty acid (vitamin F) by vortexing in a centrifuge tube. Then one part of Tween 20 and 2 parts (v/v) of water were added to it and vortexed for 5 min. This primary emulsion was sonicated for 1 hour in Brason 2500 sonifier. This primary emulsion was diluted 1:10 to avoid aggregation and Tween 20 was added drop by drop to make clear emulsion. It was again sonicated for additional 30 minutes to reduce the particle size and get a clear emulsion.

**Results**

Our data has shown that riboflavin not only has an excellent DNA binding constant ($K_b > 10^4\ M^{-1}$). Shown in Figure 1, it can also cleave DNA under irradiation with visible light ($\lambda > 395$ nm). A singlet oxygen study using 9,10-anthracene dipropinoic acid (ADPA) as the singlet oxygen sensor has shown that riboflavin is capable of producing a large quantity of singlet oxygen after only 15 minutes of irradiation (Figure2).

**Figure1:**


Ethidium bromide stained agarose gel showing the photocleavage of 120 μM plasmid DNA from Form I – supercoiled to Form II – nicked by riboflavin after 30 min incubation and 30 min of irradiation. From the left: Lane1: 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).

**Figure 2:**

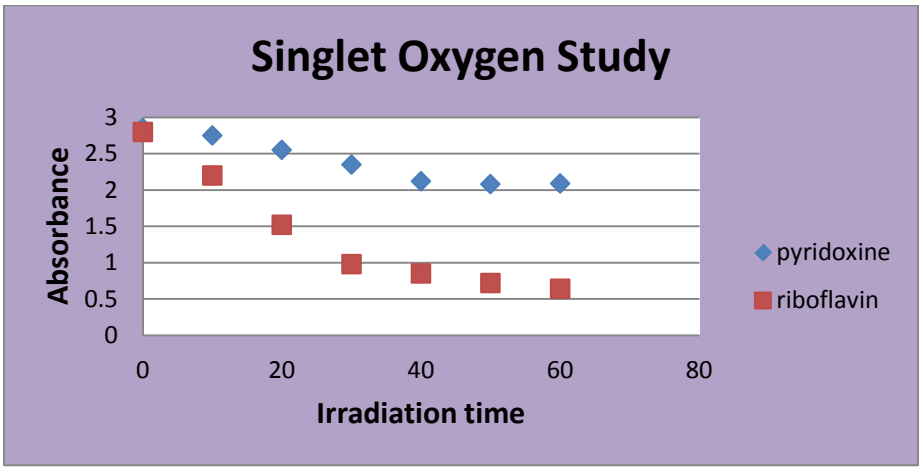
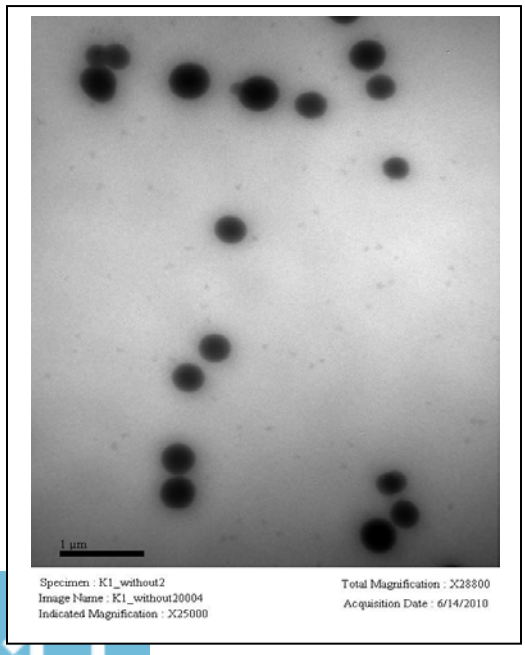
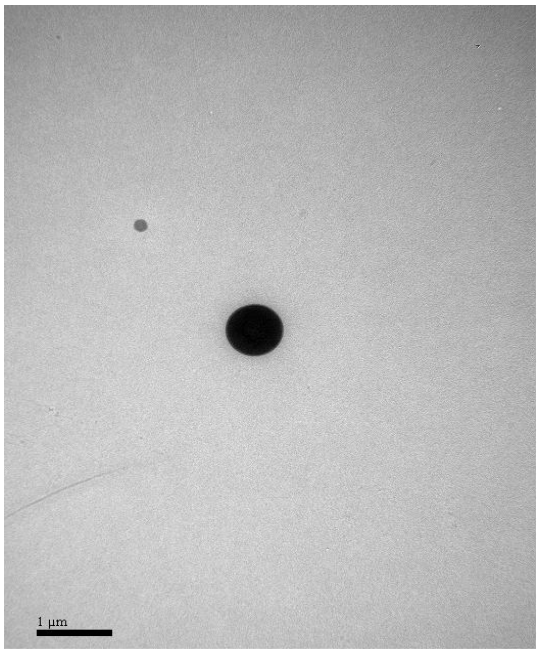



**Figure 3**: TEM image of vitamin K encapsulated nanoparticles.



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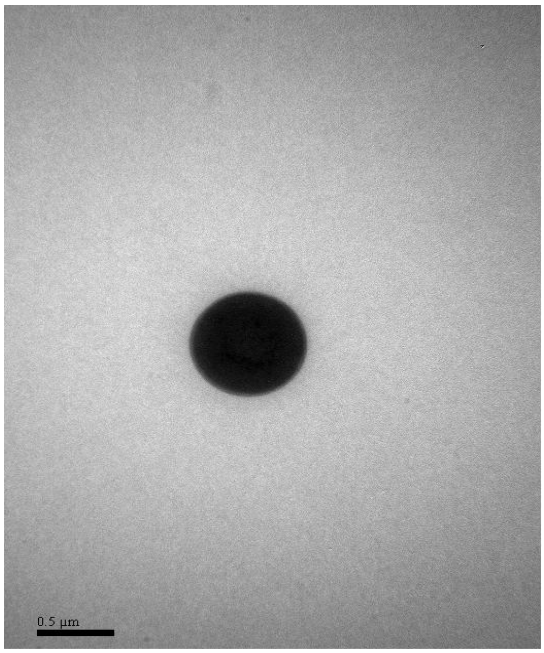


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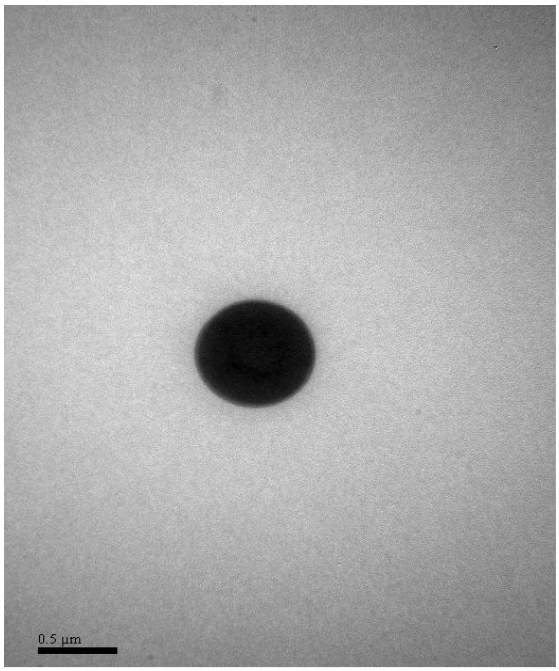


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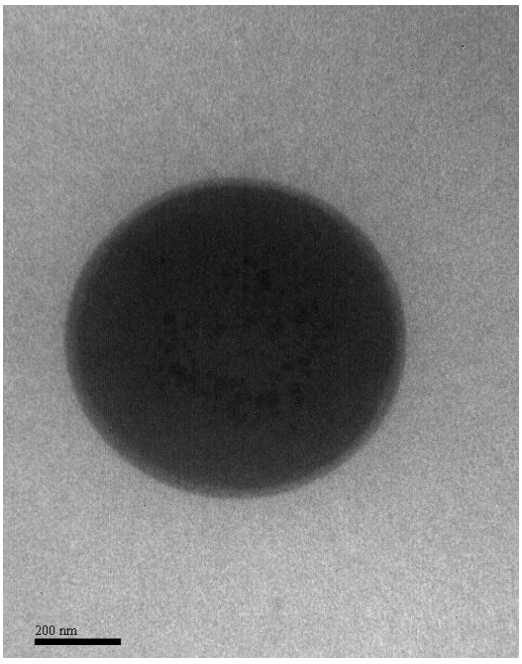


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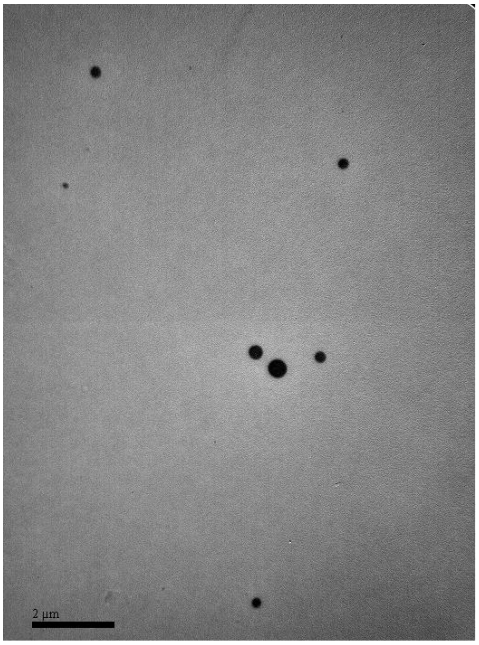


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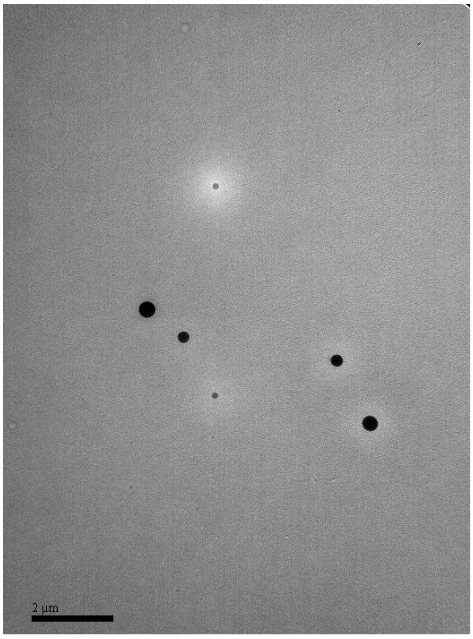


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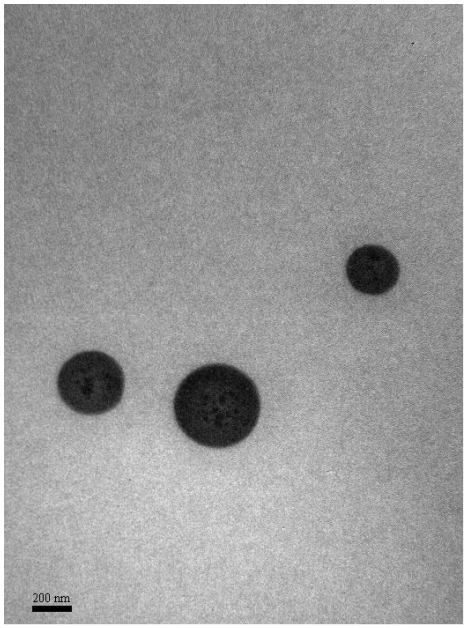


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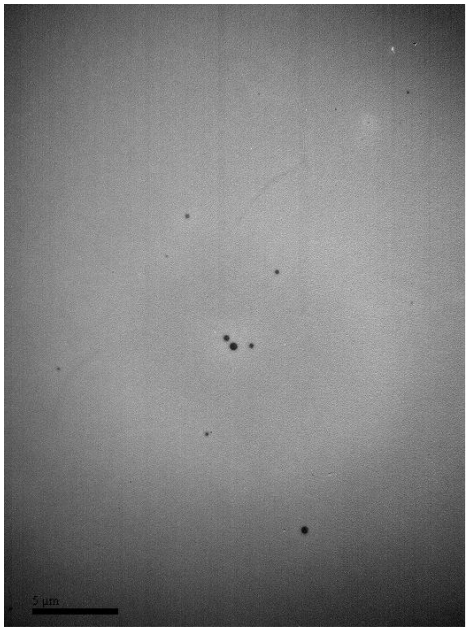


D

**Figure4:** Riboflavin solubility increased more than 3 fold by using nanoemulsion. Picture on the left shows riboflavin precipitation in water at 250 μM, picture on the right shows clear solution at 750 μM.

Antibacterial study has shown that after one hour incubation and 30 minutes irradiation with low intensity light (about 5J/cm$^2$), a concentration of riboflavin as low as 85 mg/Kg can cause 50% bacteria fatality. Compare with the acute oral toxicity recorded on the official MSDS of LD50 > 10g/Kg. Therefore, needless to say, the concentration used for eradicating bacteria would have no effect to the normal human cells. The same result was also obtained in the phylloquinone study. Table 1 shows the complete summary of our bacterial study with LD50 values in mg/Kg.

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

| | **Human Skin** | | ***E coli*** | | **Acute Oral Toxicity** |
|---|---|---|---|---|---|
| | **Dark** | **Irradiated** | **Dark** | **Irradiated** | |
| **Riboflavin** | **No Effect** | **2,100** | **197** | **85** | **> 10,000** |
| **Phylloquinone** | **No Effect** | **18,779** | **1,100** | **423** | **25,000** |

## Conclusion

Our research will provide significant improvement to heal chronic wounds by using non-expensive, non-toxic photoactive vitamin nanoparticles as new photodynamic antimicrobial chemotherapeutic agents. The expense in lost manpower, hospitalizations, debilitation, and even death is costly globally. Conventional methods of killing bacteria (such as antibiotics and disinfection) are often ineffective due to increasing rates of multidrug resistance and can cause additional damage to surrounding tissue, thus inhibiting recovery and overall successful surgical outcomes. The huge doses of antimicrobials required to treat patients who are already immunosuppressed are medically undesirable, and are increasingly ineffective. As a result, new strategies are urgently needed for immediate use and PACT has proven to be the solution. 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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I would like to dedicate this thesis to my family. The extent and quality of research work I did in Dr. Fu's lab here in Governors State University needed full dedication which could not have been possible without unconditional support from my family. I thank to my parents, elder brother Abhijit and sister-in-law Priyanka for believing in me and supporting me throughout my two years journey in Governors State University. Thank you Mom for letting me come to USA and live all these days without me. Thank you dear brother Abhijit for being there with family all the time, without you it was impossible to fly all the way to USA away from family. I thank my sister-in-law Priyanka for being with my brother all the time.

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Life here at GSU was a learning and memorable experience. Here I made many good friends in and outside the school. Living with completely strange students and then gradually making bonds with them sharing light and tough moments with them is unforgettable, especially time spent with my roommates. There has been time when we were together as family during our peaks and valleys of professional and personal life. I thank to my friends for being with me and help me make through tough times. I had lot of fun with my friends here in GSU and that really helped me in refreshing mind and getting it ready for studies, exams and extensive research work in lab.

Finally I would like to thank my research advisor Dr. Patty Fu for guiding me throughout the research work in her lab. I am very glad that I got the mentor like her who is very patient and kind to her students. I have attended American Chemical Society's regional meetings and other conferences with Dr. Fu. Because of Dr. Fu's efforts I was able to attend ACS National meeting at Boston in 2010. All of this was a great learning experience for me. I feel I am very fortunate to meet her in GSU.

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