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Nanoencapsulated Copper Pthalocyanine as Photodynamic Antimicrobial Chemotherapy

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By

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ABSTRACT

The growing resistance developed by microbes towards antimicrobial agents has driven our focus on developing alternative treatment modalities such as Photodynamic antimicrobial chemotherapy (PACT). It would be difficult for the microbes to develop resistance towards singlet oxygen generated during the PACT process. Photosensitizers are the vehicles of the transfer and translation of light energy in to a type II chemical reaction (singlet oxygen generation) in photodynamic antimicrobial chemotherapy. PACT is proposed as a potential, low cost approach to treatment of locally occurring infection.

Photodynamic inactivation (PDI) of Escherichia coli and Staphylococcus aureus has been studied in cultures treated with photoactivated copper pthalocyanine. Phototoxic Copper pthalocyanine, a structural analog of porphyrin, is tested for its antibacterial activity using visible light. In the presence of the visible light, the generally nontoxic copper pthalocyanine is photoactivated, causing cell death. The main limitation of this technique would be the uptake kinetics of photosensitizers in to the microorganism. Nanoencapsulated photosensitizer drug carrier system is believed to penetrate the polymicrobial species with in the biofilms. The generation of the singlet oxygen had been confirmed using the singlet oxygen sensor green studies.

INTRODUCTION

Photodynamic therapy (PDT) is the clinical application of photodynamic inactivation (PDI). PDT is a non-invasive treatment modality that is used in certain superficial malignancies[2]. It employs visible light to activate photosensitive compound, called photosensitizer. The activated photosensitizer can directly react with molecules in its direct environment by electron or hydrogen transfer, which leads to the production of free radicals (Type I reaction) or by energy transfer to oxygen, which generates a highly reactive singlet oxygen (Type II reaction). Both pathways can lead to cell death.

Over the past few years, Photodynamic therapy has been clinically applied for various cancers of the lung, gastrointestinal tract, the head and neck region, bladder, prostate, nonmelanoma skin cancers and actinic keratosis[1]. Photodynamic therapy has produced a complete response in a very high percentage of patients, and the frequency of follow up treatments for recurrences is no greater than found with other treatment modalities. Successful treatment of atherosclerosis or plaque with PDT has also been reported. PDT is also employed in the treatment of noncancerous conditions such as psoriasis and age related macular degeneration (ARMD).

Like PDT, PACT makes use of a photosensitizer, visible or ultra-violet light in order to give a phototoxic response, normally via singlet oxygen[3]. Currently, the major application of PACT is in the disinfection of blood products, particularly for viral inactivation. PACT is clinically applied in the treatment of oral infections. This technique has been effective in vitro against bacteria (including drug resistant strains), yeasts, viruses and parasites. A wide range of photosensitizers, both natural and synthetic, are available with differing physiochemical make up and light absorption properties.

The science of photodynamic antimicrobial chemotherapy (PACT) is still in its infancy, but follows similar principles to that of PDT. Indeed, while PDT is currently used only in the more accessible tumors, the use of PACT may also be limited to localized infection due to the problems of systemic light delivery. However, with the advent of optical fibre technology, deep-seated infection should become amenable to the photodynamic approach.

A very wide selection of light sources is available, ranging from state-of-the-art laser technology to basic tungsten-filament lamps. Indeed, the assumption that a laser is essential for the photodynamic therapy of malignant disease has hindered the growth and acceptance of this discipline considerably. What is important, both in PDT and PACT, is the ability to excite the photosensitizer at its target site with minimal photoeffect on the surrounding tissue. For example, the disinfection of virally contaminated blood currently carried out in parts of Europe utilizes light boxes containing fluorescent tubes. It should be remembered that PACT, like PDT, uses low-power light rather than the lasers used in ablative therapy. Microbial photo-killing is attained with milliwatts rather than tens (or hundreds) of watts.

An ideal photosensitizing agent with potentially optimal properties for the treatment of microbial infections should be endowed with specific features in addition to the expected photophysical characteristics, such as a high quantum yield for the generation of both the long-lived triplet state and the cytotoxic singlet oxygen species[4]. Such features include a large affinity for microbial cells, a broad spectrum of action in order to efficiently act on infections involving a heterogeneous flora of pathogens, a mechanism of cell inactivation minimizing the risk of inducing the selection of resistant strains or promoting the development of mutagenic processes, and the possibility to identify a therapeutic window which allows the extensive killing of the disease-inducing microbial cells with minimal damage to the host tissue in the area of infection and the

prevention of the regrowth of the pathogens after the treatment.

When an aromatic molecule absorbs light of certain energy, it may undergo an electronic transition to the singlet excited state (electron spins paired). Depending on its molecular structure and environment, the molecule may then lose its energy by electronic or physical processes, thus returning to the ground state, or it may undergo a transition to the triplet excited state (electron spins unpaired). At this stage the molecule may again undergo electronic decay back to the ground state, it may undergo redox reactions with its environment, or its excitational energy may be transferred to molecular oxygen (also a triplet-state molecule) leading to the formation of the labile singlet oxygen.

The ability of a molecule to instigate redox reactions and/or to form singlet oxygen depends on the production of a sufficient population of triplet state molecules. This in turn depends on the decay rates of both the triplet and initially-formed singlet states. Thus, for example, a highly fluorescent molecule which undergoes significant electronic decay from the excited singlet state would not be expected to form a high proportion of the triplet excited state (Figure 1). Photosensitizers are usually aromatic molecules, which are efficient in the formation of long-lived triplet excited states. In terms of the energy absorbed by the aromatic-system, this again depends on the molecular structure involved: furocoumarin photosensitizers (psoralens) absorb relatively high energy ultraviolet (UV) light (300–350 nm), whereas macrocyclic, heteroaromatic molecules such as the phthalocyanines absorb lower energy, near-infrared light (around 700 nm).

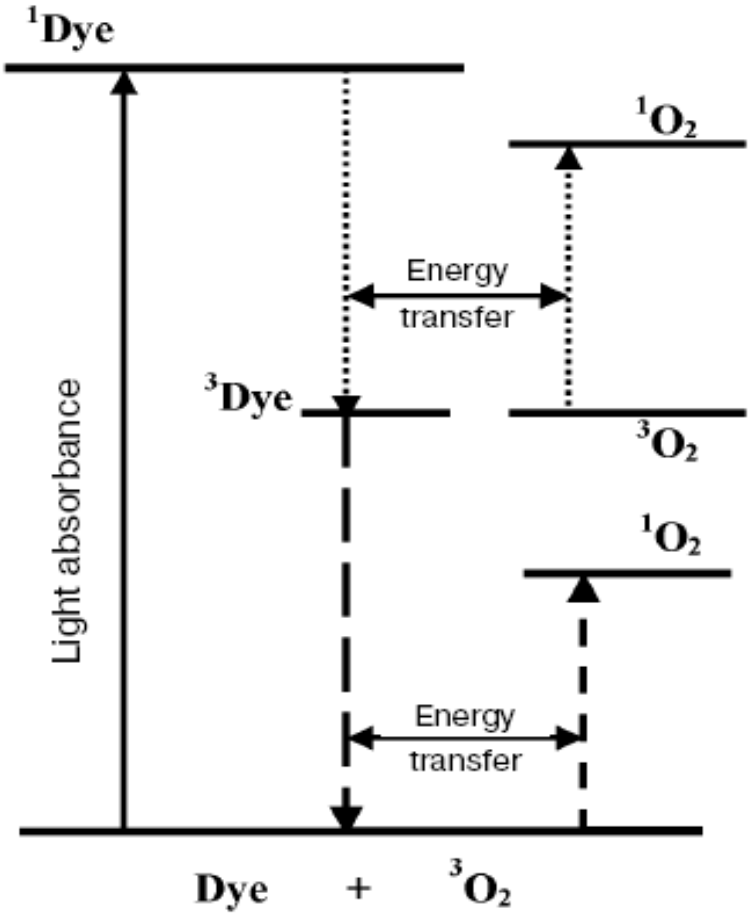

Figure 1 : Excited state diagram.

Microbial cells are characterized by large differences in their cellular structure and organization, which has obvious effects in modulating the interaction of exogenously added photosensitizing agents with cell constituents, hence in affecting the efficiency and the mechanism of the photoinactivation processes. Gram positive and Gram negative bacteria have profound differences in their three-dimensional architecture. Both groups of bacteria present an outer cell wall. In particular, in Gram positive bacteria the outer wall (15-80nmthick) contains up to 100 peptidoglycan layers, which are intimately associated with lipoteichoic and negatively charged teichuronic acids. [6]This wall displays a relatively high degree of porosity, since various macromolecules, such as glycopeptides and polysaccharides with a molecular weight in the 30,000–

60,000 range, were found to readily diffuse to the inner plasma membrane. Thus, in this class of bacteria, the outer wall does not act as a permeability barrier for the most commonly used photosensitizers, whose molecular weight does not generally exceed 1,500–1,800 Da.

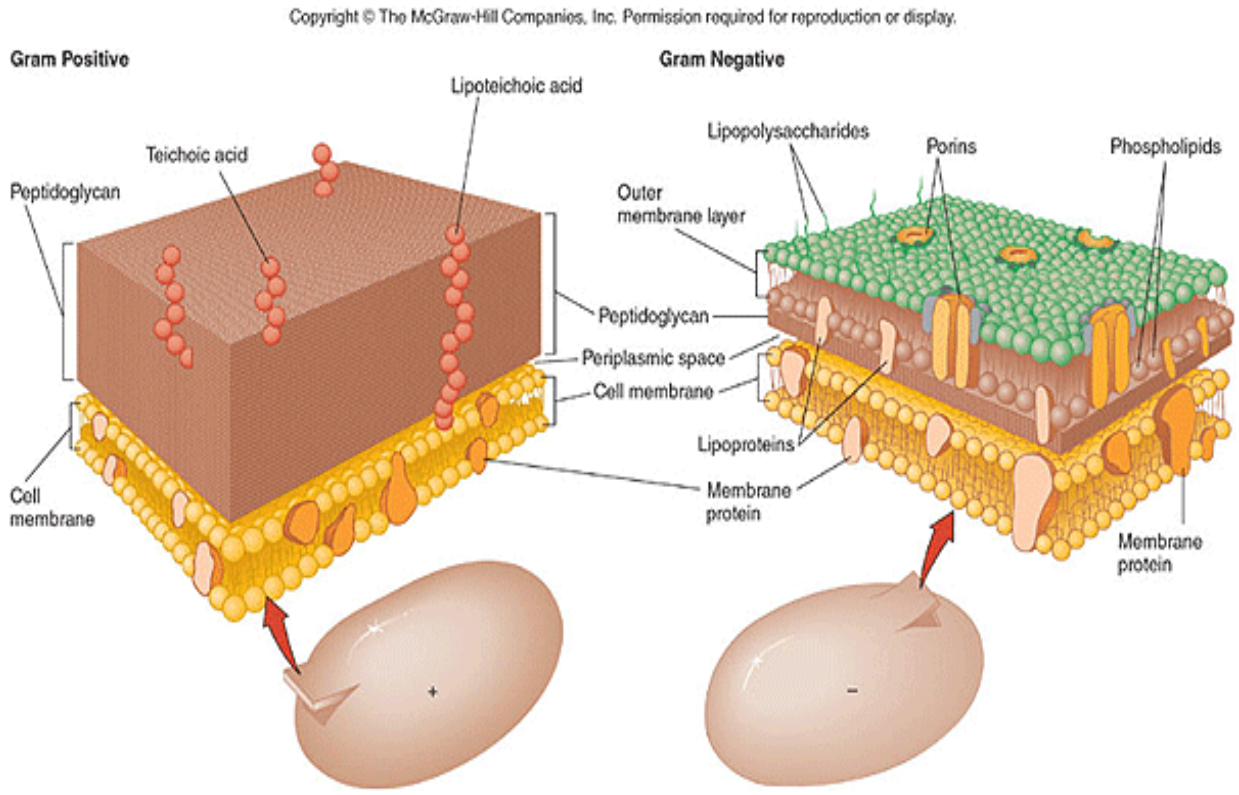

Figure 2 : Composition of gram +ve and gram –ve bacterial outer mambrane.

On the contrary, the outer wall of Gram negative bacteria possesses an additional 10–15 nm thick structural element, which is external to the peptidoglycan network and has a very heterogeneous composition, including proteins with porin function, lipopolysaccharide trimers and lipoproteins giving the outer surface a quasi-continuum of densely packed negative charges. Such a highly organized system inhibits the penetration of host cellular and humoral defense factors and triggers mechanisms of resistance against several antibiotic drugs. Only relatively hydrophilic

compounds with a molecular weight lower than 600–700Da can diffuse through the porin channels. It is thus necessary to devise suitable strategies, which enhance the permeability of the outer wall in order to make Gram negative bacteria sensitive to the action of photodynamic processes[5,7].

Copper pthalocyanine is an intensly blue-green coloured aromatic macrocyclic compound.

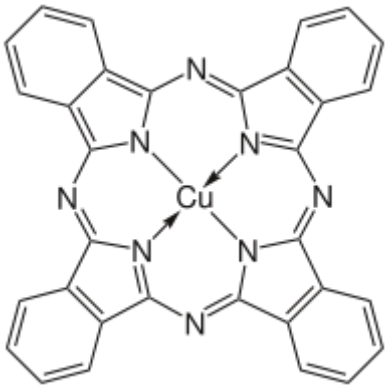

Figure 3: Structure of copper pthalocyanine.

Molecular Formula : C32H16CuN8

Molecular Weight : 576.08 g mol−1

State : Solid

Water Solubility : Not soluble

UV-VIS Absorption : 678nm.

Solubility of copper pthalocyanine dye is checked with a range of solvents like ethylacetate , TFA (tri-fluoro acetic acid), haxane , toulene , acetic acid, acetonitrile , mathanol and chloroform. Though copper pthalocyanine was partially soluble in Ethylacetate, it was found to be the ideal solvent.

EXPERIMENTAL METHODS AND PROCEDURE

PREPARATION OF NANOPARTICLE

- 5 mg of copper Phthalocyanine (CuPc) and 2.0 mL of surfynol- 465(to increase the solubility) was added to 20mL of ethyl acetate (organic phase) over low heat with constant stirring.
- 2gms of high molecular weight PEG (Mr > 2000 g/mol) was dissolved in 20 mL of water (water phase).
- Organic phase was added to the water phase drop by drop with vigorous stirring until all the ethyl acetate has evaporated. Sonicatd for 30 minutes.

Preparation of tryptic soy broth:

- 15 gms of agar has been weighed and added to 500 ml of distilled water over low heat with constant stirring.
- Autoclaved for 30 minuttes and allowed to cool.

BACTERIAL STUDY:

Two sets of test tubes were taken for the bacterial study of gram positive and gram negative respectively. Each set containing six test tubes of which three labelled as dark and other three labelled as light.

In all the test tubes 3 ml of tryptic soy broth, 0.5 ml of bacteria and the prepared nanoemulsion with different concentrations for both the sets of gram positive and gram negative

bacteria were taken (three test tubes with concentration of100µl, 50 µl, 25 µl as dark and other three test tubes with 100µl, 50 µl, 25 µl as light).

All the test tubes are incubated at 35˚C , after 30 minuttes of incubation the light labelled test tubes are irradiated to light for 45 minuttes and further incubated for 24 hours. Then all the test tubes are diluted 9x times individually in tryptic soy broth, each in single test tube.

For the 9x dilution, we have set four test tubes labelled from 1 to 4, each with 9 mL of sterile tryptic soy broth. 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 as 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, tube 3 is 1:1000 and tube 4 is 1:10000 respectively and mixed well.

Then 1mLof dilution broth suspension is taken from tube 4 and added it to the surface of sterile nutrient medium in a Petri dish. Spreaded evenly and plates are incubated and allowed the bacteria to multiply for 24 to 48 hours at 37˚C

RESULTS

U.V STUDY:

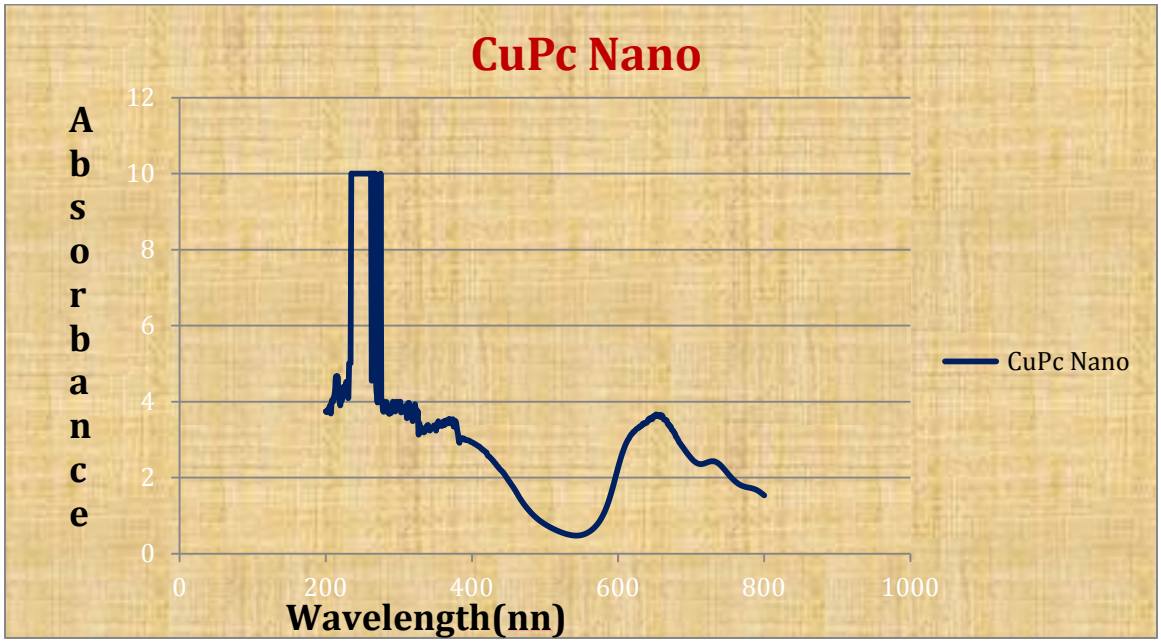

Figure 4 : U.V absorbance of copper pthalocyanine nanoparticle.

In the above figure the blue line represents the typical UV-VIS spectrum of aqueous copper pthalocyanine drug.

By observing the graph, the peak is seen in between 600 to 700 nm which coincides with the optimum wavelength of copper pthalocyanine (678nm).

FLUORESCENCE STUDY:

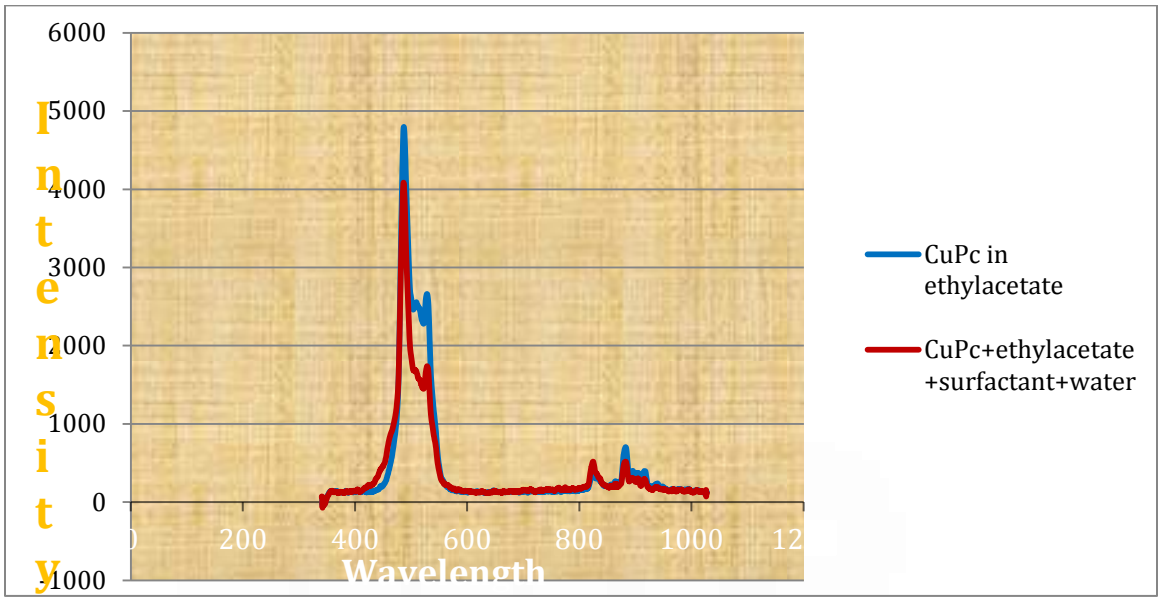

Figure 5 : Fluoresecence of encapsulated nanoparticle

The red line from the above graph represents the fluorescence of nanoencapsulated drug, shows the intensity at 4000. The blue line from the above graph representing the CuPc in solvent ethylacetate shows the intensity at 4800.

The hypochromic effect of red line compared to that of blue line, indiacates the increase of hydrophilicity. It is evident that the copper pthalocyanine is encapsulated inside the nanoparticle.

SINGLET OXYGEN STUDY:

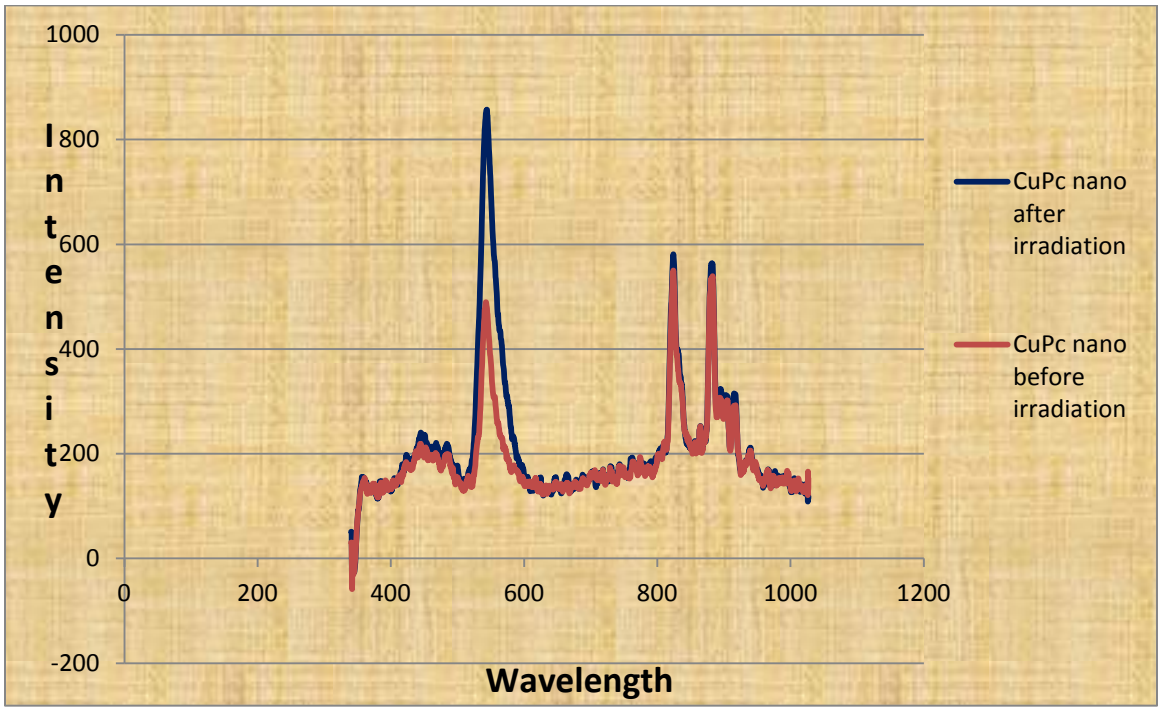

Figure 6 : Fluoresence of copper pthalocyanine nanoparticle before and after irradiation

Singlet oxygen study was also performed to ascertain the generation of singlet oxygen(type II reaction) in photodynamic antimicrobial chemotherapy. The sensor used in the singlet oxygen study is singlet oxygen sensor green.

Fluorescence study of prepared CuPc nanoemulsion is done before the irradiation and after the irradiation. CuPc nano before irradiation shows less intensity when compared with the intensity of CuPc nano after irradiation. Our result shows that the prepared nanoencapsulated drug produces sufficient amount of singlet oxygen.

BACTERIAL STUDY:

Figure 7 : Comparison of growth of gram –ve bacteria (E.coli) in dark and light.

After incubating the bacterial streaked petri plates for 48 – 72 hours, the plates were observed for the bacterial growth in dark and visible light. Bacterial colonies were seen in the petri plates incubated in the dark, whereas in the petri plates which were irradiated to visible light for 45 minutes, were devoid of bacterial growth. From the above results, we can confirm nanoencapsulated copper pthalocyanine's potential as photodynamic antimicrobial chemotherapy agent.

CONCLUSION

Our results shows that the nanoencapsulation was successful. PDT uses light, nanoencapsulated photosensitizer and the singlet oxygen to destroy the specific target cells. The singlet oxygen studies and the bacterial cell culture studies have confirmed the photodynamic inactivation of bacteria by nanoencapsulated CuPC. We have developed a unique nanoparticle to increase the permeability to cross the cell barrier of bacteria. We have proven that the nanoemulsion to be an effective way for drug delivery and are efficient in treating the infections caused by broad spectrum of bacteria.

In vivo studies of antibacterial activity using photodynamic antimicrobial chemotherpy should be done. Photodynamic antimicrobial chemotherapy of yeast, viruses and parasites using the encapsulated drug still needs to be investigated further.

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