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Development and In vitro Characterization of Erythrosine Nanoparticles for Chronic Sinusitis using Photodynamic Therapy

by

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Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Pharmaceutical Sciences

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An Abstract of Development and In vitro Characterization of Erythrosine Nanoparticles for Chronic Sinusitis using Photodynamic Therapy

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Antimicrobial therapy for sinusitis has been shown to reduce or eliminate pathologic bacteria associated with rhinosinusitis and improve the symptoms associated with the disease. However, the continuing rise in antibiotic resistance, the ongoing problem with patient compliance, and the intrinsic difficulty in eradication of biofilms complicates antibiotic therapy. The introduction of photodynamic antimicrobial therapy (PAT) using erythrosine, a photosensitizer, could eliminate the bacteria without inducing antibiotic resistance or even requiring daily dosing. In the present study, erythrosine nanoparticles were prepared using poly-lactic-co-glycolic acid (PLGA) and evaluated for their potential in PAT against Staphylococcus aureus biofilms. PLGA nanoparticles of erythrosine were prepared by nanoprecipitation technique. Erythrosine nanoparticles were characterized for size, zeta potential, morphology and in vitro release. Qualitative and quantitative uptake studies of erythrosine nanoparticles were carried out in S. aureus biofilms.

Photodynamic inactivation of S. aureus biofilms in the presence of erythrosine nanoparticles was investigated by colony forming unit assay. Nanoprecipitation technique resulted in nanoparticles with a mean diameter of 385nm and zeta potential of -9.36 mV.
Erythrosine was slowly released from nanoparticles over a period of 120 h. The qualitative study using flow cytometry showed the ability of *S. aureus* cells to internalize erythrosine nanoparticles. Moreover, erythrosine nanoparticles exhibited a significantly higher uptake and antimicrobial efficacy compared to pure drug in *S. aureus* biofilms. In conclusion, erythrosine-loaded PLGA nanoparticles can be a potential long term drug delivery system for PAT and are useful for the eradication of *S.aureus* biofilms.
Dedicated to my parents
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Chapter 1

Introduction

The field of antimicrobial chemotherapy has always been a constant challenge, particularly in view of the resistance developed towards various antimicrobial agents and a wide variety of pathogens encountered. A variety of new antimicrobial regimens have therefore been developed and refined in order to combat the resistance associated with microbial infections. However, development of resistance towards the antimicrobials by various mechanisms, including thickening of the outer wall, encoding of new proteins that prevent the penetration of drugs, onset of mutants deficient in those porin channels that allow the influx of foreign chemicals, have progressively compromised the treatment results [1]. Hence, an alternative approach that eliminates the development of antimicrobial resistance needs to be cultivated. Photodynamic antimicrobial therapy (PAT) is an alternative strategy to alleviate resistance associated with microbial infections. The term ‘photodynamic action’ is used to distinguish photosensitized reactions from the physicochemical processes occurring in emulsions of photographic films [2].
Photodynamic therapy was originally developed for treating cancers and has now achieved regulatory approval in several countries [3, 4]. Photodynamic therapy is based on the utilization of a non-toxic photosensitizer (PS) localized in certain cells, which could be activated by low doses of visible light at an appropriate wavelength. Activation of the PS generates a singlet oxygen and free radicals that are cytotoxic to the target cells [5]. The development of bacterial resistance towards singlet oxygen and free radicals is highly unlikely. The singlet oxygen has a short half-life and the diffusion of singlet oxygen is limited to a few nanometers; hence, the cytotoxicity is confined to the immediate cells. However, the delivery of PSs to the vicinity of target cells is challenging. In this review, we provide comprehensive and updated information on the PAT, with special emphasis on the use of polymeric nanoparticles in delivering PSs to the target cells.

1.1 History and development of photodynamic therapy

Light has been used for the treatment of various diseases in ancient Egypt, Greece, and Indian civilizations [6]. In Greece, it was known as ‘heliotherapy’ and it was recommended for treating diseases like vitiligo, psoriasis, and skin cancer [7]. Historical records before 3000 years ago reported the use of vegetables and plant substances to produce photoreactions in the skin. Later it was ignored for centuries until the early nineteenth century, when it started to flourish again. In the nineteenth century, researchers found that the combination of light with certain chemicals could induce cell death. A German medical student, Oscar Raab reported that Paramecia caudatum
survived when treated with Acridine for 15 h, but died within 2 h when exposed to both acridine and light at the same time [8]. This study postulated that light converts acridine to an active chemical energy, which formed the basis for PDT (Fig.1-1). In the same year, a French neurologist found that epilepsy patients treated with eosin developed dermatitis in sun-exposed areas [9]. In 1903, Von Tappeiner and Jesionek proposed the use of eosin in topical skin diseases and reported their experiences with topical 5% eosin as a photosensitizer with artificial light to successfully treat lupus vulgaris, non-melanoma, condylomatalata and skin cancers[8]. Since then, PDT has received increased attention and has been tested for use against several types of cancers.

During the period 1908-1913, the photodynamic properties of hematoporphyrins were studied on paramecia, mice, guinea pigs, and humans after exposure to the sunlight (Fig. 1-2). Hausman et al., showed that hematoporphyrin activated by light could photosensitize in mice and guinea pigs [10]. The effect of hematoporphyrins in humans was first studied by Friedrich Meyer Betz, a German doctor, in 1912 [11]. In 1942, Auler and Banzer reported the accumulation of injected porphyrins in tumor tissues.
The modern era of PDT was initiated by the development of hematoporphyrin derivatives, which is a giant step in the evolution of PDT. Inspired by the tumor localizing effect of porphyrins, Samuel Schwartz started developing components with better tumor localizing properties than the pure hematoporphyrin and these are known as ‘hematoporphyrin derivatives’ (HpD) [12]. These investigations firmly established the use of PDT in cancer cells. Later conducted *in vivo* studies reported a delay in the growth of gliomas in the presence of PDT with porphyrins [9]. In 1978, Doughtery and coworkers developed a krypton ion laser system that can excite porphyrins in endoscopy. They also used the red light from a xenon arc lamp for activating HpD in treating early lung cancers in an animal model [13]. Since then, many studies have confirmed the clinical effectiveness of PDT in treating cancers. This led to the first regulatory approval

### 1.2 Photodynamic therapy for bacterial diseases

Since the early days of PDT, it was well known that microorganisms can be killed by using a combination of a PS and harmless visible light. The prerequisite for photosensitization of a microbial cell is the binding/ internalization of the PS into the cytoplasmic membrane [14]. In the 1990’s, researchers reported the differences in susceptibility of Gram positive bacteria and Gram negative bacteria towards PDT. The difference in susceptibility is majorly due to the variation in the structural anatomy and physiology of their cell membranes (Fig. 1-3).
Anionic or neutral PS molecules were found to be internalized efficiently by the Gram positive bacteria, whereas the Gram negative bacteria were relatively resistant [15, 16]. The outer wall of the Gram positive bacteria consists of a peptidoglycan layer linked to teichuronic acid groups, which exhibit high level of porosity [17]. This allows large molecules (molecular weight: 30-57 kDa) to readily pass through the cytoplasm [18]. Therefore, the Gram positive bacteria doesn’t act as a permeability barrier for PS molecules with molecular weight less than 1800 Da [19]. But the outer wall of the Gram negative bacteria has a heterogeneous composition consisting of peptidoglycan, lipoproteins, and lipopolysaccharides, which are densely packed and give the wall a negative charge [17]. Only very low molecular weight PS molecules can pass through the
cell membrane. Studies have shown that PS molecules pass through the Gram negative bacterial membrane when a cationic agent such as polymixin is administered [14, 20]. Some researchers have achieved a better penetration by using the cationic porphyrins or phthalocyanines [21, 22]. Recent studies have shown that anionic or neutral PS molecules can penetrate through the cell membrane when linked to an oligomer (such as polylysine), which exists as a cation at the physiological pH [23]. Photosensitivity also depends on the physiological state of the bacteria. Cells in logarithmic phase are found to be more susceptible compared to the cells in stationary phase [19]. The differences between the Gram positive and Gram negative bacteria are listed in Table 1.1.

**Table 1.1**: Comparative characteristics of the Gram positive and Gram negative bacteria

<table>
<thead>
<tr>
<th>Character</th>
<th>Gram positive bacteria</th>
<th>Gram negative bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nature of cell wall</td>
<td>Rigid</td>
<td>Flexible</td>
</tr>
<tr>
<td>Gram stain</td>
<td>Purple</td>
<td>Pink/Red</td>
</tr>
<tr>
<td>Peptidoglycan layer</td>
<td>Multi-layered (thick)</td>
<td>Single-layered (thin)</td>
</tr>
<tr>
<td>Teichoic acids</td>
<td>Present</td>
<td>Absent</td>
</tr>
<tr>
<td>Periplasmic space</td>
<td>Absent</td>
<td>Present</td>
</tr>
<tr>
<td>Lipo-polysaccharide content</td>
<td>High</td>
<td>Virtually none</td>
</tr>
<tr>
<td>Resistance to physical disruption</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Inhibition by basic dyes</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Antibiotic resistance</td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>Susceptibility to anionic detergents</td>
<td>Low</td>
<td>High</td>
</tr>
</tbody>
</table>

Due to the above listed differences in the cell structure, PS molecules should undergo some modifications based on the type of bacteria involved; in order achieve sufficient penetration into the bacterial cytoplasm.
1.3 Mechanisms of photodynamic therapy

There are three basic principles of photodynamic therapy:

1) The Grotthus-Draper Law (also called as Principle of Photochemical Activation) states that for a PS to be activated, the light used must be of an appropriate wavelength so that it can be absorbed by the system to bring about a photochemical change.

2) The Stark Einstein Law (also called as Photoequivalence law) states that each mole of PS that is involved in the photochemical reaction absorbs one quantum of the light emitted.

3) The Bunsen-Roscoe Law states that a photochemical response is proportional to the product of light intensity and exposure.

When a photosensitizer is illuminated by a light of an appropriate wavelength, the chromophore gets transformed from its stable, quiescent ground state to an excited single state. Here, the molecule might undergo an intersystem crossing into a long-lived high energy triplet state or it might emit fluorescence to come back to its original stable ground state. The triplet state is also unstable and can undergo a nonradiative transition giving rise to phosphorescence or it might transfer energy to another molecule [24]. However, the triplet-singlet state transitions are forbidden as the change in electron spins are required [25]. In the body, where oxygen levels are significant, the triplet state readily transfers energy to the surrounding oxygen or substrate molecules which absorbs energy and converts into a singlet state oxygen or substrate radicals, which are cytotoxic [26].
Photosensitization reactions induced by the activation of PSs are known to cause damage to the biological membranes through the oxidation of proteins, nucleic acids, and lipids. This damage is triggered by both type I and type II reactions.
1.3.1 Types of reactions

1.3.1.1 Type I reactions: Type I reaction mechanisms involve electron transfer between the excited photosensitizer and substrate yielding free radicals such as superoxide and hydroxyl radicals [27]. In anoxic environments, the energy from the excited photosensitizer is transferred to a substrate (such as a cell membrane or an organic substrate), which eventually form substrate radicals by the transfer of a proton or an electron. These substrate radicals cause cell death by the destruction of the cell membrane. In hypoxic environments, the substrate radicals may further react with oxygen to produce reactive oxygen species (cytotoxic). These reactions depend on the concentration of the substrate.

\[
\text{hv} + \text{PS} \rightarrow \text{PS}^1 \rightarrow \text{PS}^3
\]

\[
\text{PS}^3 + \text{S} \rightarrow \text{S}^{o+} + \text{PS}^{o3-}
\]

\[
\text{PS}^{o3-} + \text{O}_2 \rightarrow \text{PS} + \text{O}^{o2-} \rightarrow \text{HO}^o; \text{H}_2\text{O}_2
\]

In type I photoreaction, the excited photosensitizer in the triplet state (PS\(^3\)) captures an electron from the substrate and forms two radical: substrate (S\(^{o+}\)) and photosensitizer (PS\(^{o3-}\)) radicals. In hypoxia environments (where O\(_2\) is present), the photosensitizer radical transfers an electron to the molecular oxygen forming a superoxide radical (O\(^{o2-}\)).

1.3.1.2 Type II reactions: In a type II reaction, the energy is directly transferred to the molecular oxygen to form an excited state singlet oxygen. The singlet state oxygen is a very strong oxidizing agent and damages the biological membrane through oxidation. It
has a very short half-life (nanoseconds) and the diffusion is limited only to a small
distance (about 100nm). Hence, the cytotoxicity is limited to the immediate cells [5].

\[ h\nu + PS \rightarrow PS^1 \rightarrow PS^3 \]
\[ PS^3 + O_2 \rightarrow PS + ^1O_2 \]
\[ ^1O_2 + S \rightarrow S(O) \]

In type II reactions, the photosensitizer transfers its excitation energy to the molecular
oxygen in the ground state (O_2) resulting in the singlet state oxygen (^1O_2), which oxidizes
substrates such as proteins and nucleic acids.

Both type I and type II reactions may occur simultaneously and the ratio between these
processes depends on the type of photosensitizer used, the concentrations of substrate and
oxygen, and the binding capacity of the photosensitizer to the substrate [28, 29]. It is
generally believed that the single state oxygen (^1O_2) generated from type II reactions is
largely responsible for the PDT effect [30, 31]. However, recent studies have reported an
amplified PDT response with the radicals generated from type I reactions, especially
under low O_2 environments [32, 33].

1.3.1.3 Type III reactions: Few researchers have suggested that there might be a
mechanism that occurs parallel with type I and type II reactions [34]. These reactions
occur between the photosensitizer in the triplet state (PS^3) and the free radicals present in
the system. This reaction is also known as a triplet-doublet process or a modified type I
reaction [35, 36]. Very less is known about these reactions.
1.4 Photosensitizer

Photodynamic antimicrobial therapy (PAT) is highly dependent on the photosensitizer used. Photosensitizers are chemical compounds that are capable of absorbing light of an appropriate wavelength and transforms it to useful energy [37]. More than 400 natural and synthetic photosensitizers are known and developed in the past few years which include dyes, drugs, cosmetics, and chemicals [38]. In PAT, an ideal photosensitizer should possess the following properties [37, 39].

- Have a minimum dark toxicity and be cytotoxic only in the presence of light.
- Minimal tendency to aggregate.
- Capable of generating high triplet states (PS\textsuperscript{3}) with a long half-life and also be able to generate a singlet oxygen and other reactive oxygen species (ROS).
- Have a strong absorbance at longer wavelengths (600-800nm), where the tissue penetration of light is the maximum.
- Should be characterized by a rapid clearance from the body and low skin phototoxicity.
- Should be stable, chemically pure, and easily synthetized.
- Should be retained preferentially by the target tissue.
- Preferably water soluble, for use in aqueous media

1.4.1 Photosensitizers: Pharmacokinetics and distribution

The time interval between the injection of a photosensitizer and light treatment is a very important parameter to determine the efficacy of PAT [40]. Therefore, understanding the pharmacokinetics and distribution of photosensitizer is important. The pharmacokinetics...
and distribution of a photosensitizer depends on its delivery vehicle and the mode of administration [41]. The nature of delivery vehicle affects the relative transfer of the drug to different serum proteins [42, 43]. Hence, comparisons can only be made by similar modes of delivery. The physical characteristics of PSs (such as lipophilicity, solubility) also determine the pharmacokinetics and distribution into a particular organ [41]. The organ distribution data of photosensitizers revealed higher concentrations in the liver due to its large blood supply [44, 45]. Accumulation of the photosensitizer in the liver was found to be inversely proportional to its lipophilicity [46]. PS also accumulate in the lungs, spleen, and kidneys in large amounts. Even the distribution of PS in these organs is proportional to its charge and hydrophobicity of the molecule. The digestive system takes about moderate amounts of PS molecules [47]. The lowest concentrations are found in heart, skeletal muscle, and brain [48].

1.4.2 Classification of photosensitizers

Photosensitizers used in PAT can be classified in various ways with certain limitations. Depending on when photosensitizers were generated, they are categorized under three generations. This is useful for doctors and scientists working in clinics. Photosensitizers can also be classified based on the chemical structure. This classification is widely accepted by chemists, but has limited utility in clinics.

1.4.2.1 Different generations of photosensitizers: Photosensitizers employed in PDT can be divided into three generations.
1.4.2.1.1 *First generation photosensitizers:* These photosensitizers are developed in the 1970’s and early 1980’s. The photosensitizers in this generation are mostly porphyrin based or hematoporphyrin derivatives (HpD). Though hematoporphyrin was demonstrated as a powerful photosensitizer back in 1910, its chromatography showed that it has many components [49]. Hence, hematoporphyrin was treated with 5% sulfuric acid and acetic acid for purification. These are known as hematoporphyrin derivatives (HpD) [39]. Despite the presence of several components than its pure form, HpD had better localizing properties. These derivatives were further purified to Photofrin. These photosensitizers had a few disadvantages which include: the difficulty in purifying derivatives, prolonged photosensitivity due to poor clearance, and poor accumulation of photosensitizers in the target tissue. These sensitizers retained in cutaneous tissues for 2-3 months during which patients must avoid bright light [39].

1.4.2.1.2 *Second generation photosensitizers:* They were developed in the late 1980’s to overcome the disadvantages of the first generation photosensitizers. These are chemically pure substances and have a high absorbance at longer wavelength regions (675-800 nm). Light with these wavelengths can penetrate to a depth of 2-3 cm [39]. They also have high triplet state yields with photosensitivity lasting for a short time [49]. The second generation photosensitizers include expanded porphyrins (benzoporphyrin derivative, boronated porphyrin), chlorophyll derivatives (chlorins, purpurins), dyes (xanthenes, phthalocyanines), and intrinsic photosensitizers such as 5-aminolevulinic acid. 5-amino levulinic acid is converted to a photosensitizer, proto-porphyrin IX *in situ* [50]. The first
photosensitizers to be clinically approved are dyes [51]. Though the second generation photosensitizers are 100 times more active when compared with the previous generation, they cause significant pain during the therapy due to severe skin photosensitivity [50].

1.4.2.1.3 Third generation photosensitizers: First and second generation photosensitizers have been conjugated to various modifiers such as antibodies, liposomes, nanoparticles, in order to increase the selectivity of the PS to a particular tissue [51]. Compared to the first and second generation photosensitizers, they bind specifically to the target cells and sensitize their killing without affecting the normal cells.

1.4.2.2 Classification based on chemical structure: Chemically, photosensitizers can be divided into four categories.

1.4.2.2.1 Porphyrin related structures: Photofrin is a commercially available porphyrin photosensitizer and has the longest clinical history among all photosensitizers (Fig. 1-5) [52]. Photofrin and porphyrin related structures have a weak absorption at longer wavelengths and have a long lasting photosensitivity[53]. Hence, derivatives such as tetraaryl porphyrins with a stronger absorption at longer wavelengths along with high triple state yields were developed. These derivatives had a low solubility, which was overcome by the introduction of hydrophilic sulfonate groups. Recently, dendritic porphyrin derivatives have been developed for targeting approaches.
1.4.2.2 Phthalocyanine related structures: Phthalocyanine, a tetapyrrole derivative, is used in several areas of photonics such as optical memory, optical power limiting, and photomedicine (Fig. 1-6) [54]. They resemble porphyrins in their structure, but have maximum absorption peaks at longer wavelengths (650-700 nm) [55]. Their uptake and efficacy is dependent on the number of hydrophilic groups [56]. Hence, the uptake kinetics and cell retention are different for hydrophobic and hydrophilic phthalocyanines. Various metallic complexes of phthalocyanine such as zinc phthalocyanine show photobiological activity, which is two times more efficient than other commonly used photosensitizers such as methylene blue. Additionally, these complexes have a low cytotoxicity profile and high capacity to penetrate through the cellular membrane [57]. Amphiphilic phthalocyanines are obtained by the direct sulfonation of parent system and
the activity is related to the degree of sulfonation. Disulfonic acid of aluminium phthalocyanine is more effective compared to tetrasulfonic acid derivatives [58].

One limitation of using phthalocyanine photosensitizers is that they show aggregation in solution, that leads to low photosensitization efficiency due to low quantum yields of excited states [57].

\[ \text{Figure 1-6: General structure of phthalocyanine derivatives} \]

1.4.2.2.3 Chlorins and bacteriochlorins: Chlorin core is obtained by the reduction of pyrrole double bond and further reduction of a second pyrrole double bond gives the bacteriochlorins (Fig. 1-7). They maximum absorption peaks at longer wavelengths (650-670 nm for chlorins and 730-800 nm for bacteriochlorins). As these are reduced forms, they are prone to oxidation. To prevent from oxidation, large substituents are placed next to the reduced pyrrole ring [59]. Metalated chlorins and bacteriochlorins have efficient
photosensitizing activity as metallation strengthens the absorption of red light and moves their red absorption bands to longer wavelengths. These also have a high quantum yield of excited states [59]. But, these (SnET2) impart long-term skin photosensitization, which limit their utility [37, 53]. Synthetic derivatives have also been developed and evaluated for use as photosensitizers.

![General representation of chlorins](image)

**Figure 1-7**: General representation of chlorins

1.4.2.2.4 Phenothiazine derivatives: These are an important class of photosensitizers which are majorly used in antimicrobial therapy (Fig. 1-8). Methylene blue is currently being used for decontamination of freshly frozen plasma units [60]. Most photosensitizers in this class bring about the antimicrobial effect by their association with the nucleic
acids inside the cell. Improved phototoxicity is observed against the Gram positive and Gram negative bacteria by modifying the structure. The addition of methyl or ethyl groups or nitro groups to methylene blue resulted in enhanced phototoxicity as these derivatives are more resistant to reduction compared to methylene blue. Reduction of methylene blue results in the formation of colorless neutral species which do not show phototoxicity[61]. Toluidine blue is another phenothiazine dye that predominantly shows phototoxicity against oral bacteria[61].

![Figure 1-8: General representation of phenothiazines](image.png)

1.5 Clinically approved photosensitizers

Though many substances exhibit photosensitizing properties, very few have made it to clinical trials and are commercially available [62].
Table 1.2: A list of clinically approved photosensitizers

<table>
<thead>
<tr>
<th>Trade Name</th>
<th>Photosensitizer</th>
<th>Clinical application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Photofrin</td>
<td>HpD</td>
<td>Oesophageal cancer, cervical cancer, gastric cancer</td>
</tr>
<tr>
<td>Foscan</td>
<td>Temoporfin</td>
<td>Head and neck cancer</td>
</tr>
<tr>
<td>Levulan</td>
<td>Amino Levulinic acid (ALA)</td>
<td>Actinic keratoses</td>
</tr>
<tr>
<td>Metvix</td>
<td>m-ALA</td>
<td>Actinic keratosis, basal cell carcinoma</td>
</tr>
<tr>
<td>Visudyne</td>
<td>Vertiporfin</td>
<td>Wet-age related macular degeneration</td>
</tr>
<tr>
<td>Photoheme</td>
<td>HpD</td>
<td>Skin, breast and gastrointestinal cancer</td>
</tr>
<tr>
<td>Photochlor</td>
<td>2-(1-Hexyloxyethyl)-2-devinyl pyropheophorbide-a (HPPH)</td>
<td>Oesophageal cancer</td>
</tr>
</tbody>
</table>

1.6 Light sources

The clinical approach of PDT depends on the selection of an appropriate light source and a light delivery system [63]. Light should penetrate into the tissues in order to be absorbed by the photosensitizer. Generally, light with a shorter wavelength has less tissue penetration and are mostly absorbed, resulting in skin photosensitivity. Hence, a photosensitizer with maximum absorption at longer wavelengths is more suitable. The ‘optical window’ of a living tissue ranges between 600 nm – 1300 nm; however, wavelengths greater than 850 nm hardly generate singlet state oxygen [64]. Studies have postulated that the wavelength of light ranging between 600-700 nm has 50-200% more penetration compared to the light of shorter wavelengths [65, 66].

The penetration of light into a tissue also depends on the optimal characteristics of the tissue, which can be defined by its optimal penetration depth (OPD) [67, 68]. OPD is defined as the depth at which the intensity of the propagating light is attenuated to 37% of
its initial value. At 635 nm, the OPD of brain is 800 µm, whereas for bladder it is 4mm [69]. Hence, the choice of the light source must be determined by considering the location and depth of the lesion, action spectrum of the photosensitizer (relative effectiveness at different wavelengths for generating a desired response), optimal wavelength to generate high yields of singlet state oxygen, fluence rate of light (high fluency of light causes oxygen depletion), cost and size of the light source [49]. Most light sources used for PDT were developed to have an optimized output near the wavelengths between 600-700nm. Based on the source, light sources can be divided into laser and non-laser sources.

1.6.1 Laser sources

Laser is an ideal light source for PDT due to its monochromaticity and high intensity coherent light. Since, coherence is lost after penetration into the tissue; this property would be of no importance in PDT. The monochromaticity allows the delivery of light of a precise wavelength where the photosensitizer has its maximum absorption. Two laser systems are popularly used for photodynamic inactivation of bacteria: Helium-Neon laser and diode laser [70]. Helium-Neon laser is a gas laser developed by Javan and coworkers in 1961. It operates at wavelengths in the red part of the visible spectrum. The best and most widely used Helium-Neon laser operates at 632.8 nm. The power of the laser beam is between 0.5-50 mW [70]. These have been used extensively for PDT of *Staphylococcus aureus*, *Pseudomonas aeruginosa* [71], *Streptococcus mutans* [72], and *Fusobacterium nucleatum* [73].
Diode lasers represent a major breakthrough in the clinical use of PDT [63]. These are made up of semiconductor substances and were easily portable due to their light weight [74]. They have an air cooling system utilizing 120 V power and with an ability to deliver both continuous and pulsed light [63]. The limitation of using these lasers is that they offer only a single output wavelength of light [63]. They have been used for the photodynamic treatment of *Staphylococcus epidermidis* [75], *Actinobacillus actinomycetemcomitans*, *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, and *Streptococcus sanguis* [73].

### 1.6.2 Non-laser sources

Non-laser sources used in PAT majorly include lamps and light emitting diodes (LED’s). Two types of lamps are available, lamps that emit a continuous spectrum (incandescent lamps) and lamps with spectrum in bands (metallic vapor lamps) [70]. The maintenance of lamps is easier and cheaper. Filters are employed in order to narrow the spectrum to an appropriate wavelength [70]. Incandescent lamps consist of a glass bulb with a tungsten filament inside the lamp. The glass bulb can be filled with vacuum or an inert gas. Light is produced by heating the filament using electric current. These lamps can deliver a power up to 250 mW/cm² over a wide range of spectrum [63]. Incandescent lamps have been used for the photodynamic inactivation of Gram positive bacteria such as *Staphylococcus aureus* and Gram negative bacteria such as *Pseudomonas aeruginosa* [15].
Gaseous discharge lamps or Xenon lamps are based on the electric discharge between two electrodes in a vapor or gas atmosphere. The emitted radiation depends on the gas. Xenon and mercury vapor lamps are the most commonly used lamps [70]. In xenon lamps, the cathode is a metallic tube containing zirconium dioxide and the anode is a metallic plate. They emit a broad spectrum light ranging from 300nm-1200nm and are characterized by large output and high fluence rates. Xenon lamps have been employed for photodynamic inactivation of *Staphylococcus aureus*, *Escherichia coli* [76], *Staphylococcus epidermidis* [77].

Light emitting diodes (LEDs) have been recently used in the photodynamic antimicrobial therapy because of their low cost and versatility. They consist of an array of semiconducting LED chips and are used to emit low powered broad spectrum light [78]. The choice of the emission wavelength ranges from ultra violet (350 nm) to near infrared (1100 nm) [63]. LEDs have been used in inactivating bacteria such as *Staphylococcus aureus*, *Porphyromonas gingivalis* [79], *Enterococcus faecalis* [80].

1.7 Factors controlling the effectiveness of photodynamic therapy

The efficiency of photodynamic therapy depends on various factors such as the efficiency of the photosensitizer to produce a singlet state of oxygen, the delivery system, the light fluency rate, dose of the light, and the localization of photosensitizer in the target tissue. Some of the factors that contribute to the success of the treatment are described below.
1.7.1 Optimum tissue penetration depth of photoactivating light

The depth of penetration of the light determines the efficiency of PDT. Currently, PDT is limited to topical, subcutaneous, and endoscopic applications because of the limited tissue depth at which the singlet state oxygen can be generated [81]. The light typically used to activate the currently approved photosensitizers cannot approach 5-10 cm in depth. Tissues have a high penetration in a phototherapeutic window ranging between 780 nm-950 nm [82]. At shorter wavelengths, the light is attenuated by the hemoglobin and at longer wavelengths, the light is absorbed by water. But, there are no photosensitizing agents that have an absorption maximum in the phototherapeutic window other than Tookad® ($\lambda=763$nm) [81]. Hence, some researches have now focused on synthesizing novel photosensitizing agent with simultaneous two-photon absorption for improved penetration depth [82, 83].

1.7.2 Localization of photosensitizer in target tissue

Photodynamic therapy depends on the retention of the photosensitizer in the target cell. Normal cells can also accumulate photosensitizers just like the bacterial cells. This can lead to undesired toxic effects. Hence, the photosensitizer needs to be accumulated selectively in the bacterial cells. Higher localization of photosensitizer in the bacterial cells, leads to improved therapeutic efficacy at a reduced dose [84]. Initially researchers have attempted to develop new photosensitizers by changing the physicochemical properties such as logP value in order to improve their specificity [85]. Hence, second generation photosensitizers had a better efficacy compared to first generation photosensitizers. Recently, researchers have developed third generation photosensitizers
where the PS has been conjugated to carriers such as liposomes, micelles, nanoparticles in order to target them to specific cells [86].

1.7.3 Administration of photosensitizer and light

The interval between the administration of photosensitizer and light exposure is critical for the efficiency of the treatment. In case of topical administration, there is no delay in light exposure after the administration of photosensitizer [84]. With other modes of administration, the PS needs to accumulate in the bacterial cell before light exposure. When systemically administered, the delay in light exposure should not be more than the half-life of the PS. The PS-light interval of currently approved photosensitizers is pretty long. Photofrin has an interval in the range of 40-50 h and Foscan has an interval between 90-110 h [84].

1.8 Nanoparticles for PDT

As mentioned earlier, the delivery of PS is a critical parameter that determines the efficiency of PDT. The selective accumulation of PS in diseased cells is required to avoid damage to the healthy cells. Therefore, PS molecules should be delivered using an appropriate delivery system. The delivery system can be selected on the basis of the barrier properties of the target site, physicochemical properties of PS, the dose of PS to be delivered, and patient acceptability. Several drug delivery systems such as liposomes, polymeric micelles, and nanoparticles have been developed and evaluated for the delivery of photosensitizers. Nanoparticles have been increasingly used to controlled the release of PSs at the target site [87].
1.8.1 The use of nanoparticles in photodynamic therapy

Nanoparticles are colloidal systems with a diameter less than 1 µm [88]. Nanoparticles offer several advantages over conventional dosage forms. They a) prevent the drug from premature degradation, b) enhance the absorption of drugs due to improved intracellular penetration, c) target drugs to selected cells/tissues, d) prevent the drug efflux by multidrug resistance pumps, and d) control the release of drugs from a few days to a few weeks [89, 90]. There is a vast literature on the application of nanoparticles in PDT for cancer [91-93]. But, the research related to the application of nanoparticles in PAT is limited. The use of nanoparticles in the PAT helps in improving the antimicrobial effect by increasing the absorption of photosensitizer into bacterial cells.

![Diagram of nanoparticles as a delivery system in photodynamic antimicrobial therapy](image)

**Figure 1-9:** Nanoparticles as a delivery system in photodynamic antimicrobial therapy

Nanoparticles have been classified as active and passive nanoparticles based on their functional role in PAT [87]. Passive nanoparticles can be subdivided into biodegradable
and non-biodegradable nanoparticles based on the type of polymer used in their preparation.

1.8.2 Passive nanoparticles

Many photosensitizers suffer from low bioavailability and poor bio-distribution. Nanoparticles can serve as a platform for improving the cellular uptake, bioavailability, distribution, and pharmacokinetics of photosensitizers. They offer several advantages when compared to free PS molecules. Nanoparticles increase the concentration of photosensitizer reaching the target cell and thereby increase the yield of singlet state oxygen inside the cell. Nanoparticles reduce the ability of the target cell to efflux PS molecules and thus modulate the drug resistance. They also prevent the photosensitizer to self-aggregate and form ineffective dimers or trimers. They are classified based on the material composition into biodegradable and non-biodegradable nanoparticles.

1.8.2.1 Biodegradable nanoparticles

Biodegradable nanoparticles are made of polymers that can degrade in the biological environment and release the photosensitizer. These polymers degrade by simple hydrolytic or enzymatic hydrolytic processes. The chemical composition of these polymers can be designed in such a way that, they can accommodate photosensitizer molecules with varied physicochemical properties such as molecular weight, solubility, and charge. The degradation of the polymer and drug release kinetics can be optimized by changing the composition of the polymer. Incorporating site specific moieties on the
surface of these nanoparticles allows active targeting into specific cells [94]. The advantages of these nanoparticles include high drug loading and sustained drug release.

The use of biodegradable nanoparticles in PDT began in 1990 in order to control the release of PS [95]. Till now, researchers have exploited various PS encapsulated in nanoparticles for the photodynamic treatment in cancer [96-98]. However, the use of nanoparticles in PAT has just been explored. Previously, biodegradable nanoparticles loaded with antibiotics have been used to target bacterial cells [99-101]. Among the biodegradable polymers, poly-lactic-co-glycolic acid (PLGA) is used as a drug delivery carrier due to its favorable properties such as biocompatibility, biodegradability, bioresorbability, and mechanical strength [94]. PLGA has been used to prepare methylene blue loaded nanoparticles for photosensitization of *Enterococcus faecalis*, which led to 99% bacterial killing. The synergism of PLGA nanoparticles and light contributed to a 10-fold killing of *E.faecalis* in the infected root canals of human teeth [102]. Indocyanine green loaded nanoparticles showed good antimicrobial photodynamic effect on *P.gingivalis* [103]. Tsai *et al.*, showed that biodegradable nanoparticles reduced the dose of the photosensitizer to induce photodynamic inactivation and improved the overall antimicrobial efficacy[104].

1.8.2.2 *Non-biodegradable nanoparticles*

These nanoparticles are not traditionally used in PDT because of their inability to degrade in the biological environment. PSs are not toxic to target cells by themselves rather they act like catalysts, which upon radiation convert the dissolved oxygen to toxic products.
Like catalysts, they are not destroyed during the process and hence can be used repeatedly, if properly activated. In such conditions, non-biodegradable polymers can be used as carriers to avoid the usage of free PS[105]. The following are the advantages of non-biodegradable nanoparticles over biodegradable nanoparticles: [94]

1) The size, shape, and porosity of non-biodegradable nanoparticles can be easily controlled.

2) The inert materials used in the preparation of non-biodegradable nanoparticles make them stable to environmental fluctuations.

3) They are not affected by the microbial attack.

4) The pore size of these nanoparticles allows oxygen diffusion, but doesn’t allow the efflux of drug out of the nanoparticles.

Most of the non-biodegradable nanoparticles are ceramic based, but a few of them are made from organic polymeric nanoparticles. Ceramic nanoparticles offer several advantages over organic polymeric nanoparticles such as stability and lower particle size. The particle size, shape, and porosity of ceramic nanoparticles can be better controlled over polymeric nanoparticles [106]. Polyacrylamide polymers are used to synthesize non-biodegradable nanoparticles and the PS molecules are encapsulated in the non-porous core of polyacrylamide [94]. Silver [107], and copper [108] nanoparticles have also received a lot of attention. Moreover, it is hypothesized that the bacteria are far less likely to acquire resistance against these nanoparticles [109].

Epple et al., concluded that methylene blue loaded calcium phosphate nanoparticles showed significant toxicity against P. aeruginosa and S. aureus after 30 min of incubation time prior to irradiation [110]. Zhang et al., studied the effect of PS loaded
silica nanoparticles when compared to free PS. They reported that the association of PS with nanoparticles made the PS more resistant to photo bleaching. They also showed the superior killing efficiency of PS loaded silica nanoparticles in comparison to the free PS in Methicillin resistant S.aureus and S.epidermidis [77]. Wiesner et al., also showed that hydroxylated and polymer coated fullerenes are better generators of singlet state oxygen when compared to titanium dioxide nanoparticles [111].

1.8.3 Interaction between photosensitizer and nanoparticles: Three different types of interactions between nanoparticles and PS have been reported [112].

1.8.3.1 Photosensitizer encapsulated within the nanoparticles: The photosensitizer is physically entrapped inside the nanoparticles. This kind of interaction results in improved penetration of the photosensitizer into the bacterial cell. Methylene blue entrapped within polyacrylamide has shown to inactivate both planktonic bacteria and biofilms upon irradiation [113].

1.8.3.2 Photosensitizer bound to the surface of nanoparticles: PS is covalently bound to the surface of the nanoparticles functionalized with amino groups, carboxyl groups on the surface so as to link it with the photosensitizer [112]. The bacteria can easily bind to nanoparticles which is then killed by exposure to the singlet oxygen produced by the photosensitizer [112]. Angeli et al., and coworkers reported high photodynamic action of dyes such as methylene blue, rose bengal, and eosin on E.coli when covalently immobilized on polystyrene beads [114]. Bezman et al., and coworkers demonstrated that
the photodynamic inactivation of *E. coli* in the presence of light and rosebengal covalently bound to polystyrene beads is not due to the internalization of rosebengal into the cell [115]. This study concluded that the bacteria binds to the nanoparticles and is photodynamically inactivated by the $^{1}\text{O}_2$ produced by the PS.

1.8.3.3 *Photosensitizer alongside nanoparticles:* The nanoparticles are too big to penetrate the bacterial cell and the mechanism of action is due to the physical/chemical interactions of the PS with nanoparticles in the microbial environment [112]. Narband et al., demonstrated the enhanced antimicrobial photodynamic action against *S. aureus* when toluidine blue was conjugated to gold nanoparticles [116].

1.8.4 Active nanoparticles: They act as catalysts to generate singlet state oxygen from the dissolved oxygen available in the biological environment. These also can serve as light sources for activating the PS. To serve as a light source, nanoparticles should have an emission spectrum similar to the photosensitizer and the light they emit should be of high luminescence. Moreover, nanoparticles must be non-toxic, stable, and soluble in the biological environment [94]. Active targeting nanoparticles can be sub-classified by the mechanism of activation.

1.8.4.1 *Photosensitizing nanoparticles:* These include quantum dots and fullerenes. Quantum dots are nanoparticulate imaging probes characterized by high quantum yields and photo stability. Their fluorescence emission can be modified by changing their size [94]. They can be targeted to specific tissues. These transfer their energy to the
surrounding oxygen generating singlet oxygen molecules. Fullerenes transfer energy from incident radiation to the surrounding oxygen. They produce different reactive oxygen species based on the solvents. With polar solvents, they produce free radicals (such as superoxide and hydroxyl radical) and with non-polar solvents, they produce singlet oxygen molecules [105].

1.8.4.2 Self-lightning nanoparticles: Here luminescent nanoparticles are attached to the photosensitizer. Upon irradiation with an ionizing radiation (such as X-rays), luminescence is generated from the nanoparticles which excites the PS [105]. This results in the generation of singlet oxygen molecule that kills the target cell.

1.8.4.3 Up conversion nanoparticles: They are modified composites which convert low energy light to high energy light which inturn activates the PS. They provide advantages such as deep tissue penetration of light and enable delivery of PS to specific tissues [105].
Chapter 2

Significance of the Research

Billions are spent on over 11.4 million visits for sinusitis to physicians each year and is the fifth most common disease that is treated with antibiotics affecting over 32 million people of all ages each year [117, 118]. A variety of antibiotic regimens have therefore been developed and refined in an effort to reduce the morbidity associated with chronic sinusitis. However, the increase in antibiotic resistance by many bacterial strains has progressively compromised the treatment results of chronic sinusitis. The introduction of a non-resistance inducing antimicrobial treatment that could reduce the prevalence of sinusitis would lay the groundwork for a dramatic change in the treatment of sinusitis and result in substantial cost savings to the health care system.

The central hypothesis of this research is that PDT can be used locally to treat chronic sinusitis by destroying the biofilm in situ. Erythrosine was used as a photosensitizer. Here, we developed poly-lactic-co-glycolide (PLGA) nanoparticles containing erythrosine (PS) that can be delivered to the bacteria infecting the sinus mucosa and compared it with the free drug (PS). The potential of PLGA nanoparticles in controlling and disrupting the formation of biofilms within the nasal cavity has not been identified. We hypothesize that erythrosine loaded PLGA nanoparticles may have better
antimicrobial properties compared to the free drug and provide a sustained therapeutic action. We propose that erythrosine nanoparticles are taken up into the bacterial cell by an endocytotic process and provide a sustained release of erythrosine to reduce the sinus biofilm over time which can then be stimulated without reapplication. The absence of resistance makes the transition of this application from bench side to bed side commercially viable.
Chapter 3

Preliminary Study

Cell culture
For microscopic observations and uptake studies, Methicillin resistant strain of 
*Staphylococcus aureus Rosenbach* (ATCC: BAA 1692, MRSA) was procured. For liquid 
cultures, *S. aureus* cells were grown in TrypticSoyabean Broth (TSB), a minimum 
essential medium. Cells growing in log phase were transferred to a 6-well plate with a 
medium containing TSB with 1% glucose and grown in an incubator maintained at 37°C 
until cells reached 50-70% confluency.

Determination of irradiation time for photodynamic inactivation using live/dead assay
*S.aureus* cells were seeded in a 96-well plate at a density of 5 X 10^7 cells and allowed to 
grow for 24 hours. Unbound bacteria were eliminated by washing the biofilm with 
phosphate buffered saline (PBS). Each well was spread out so as to minimize light 
contamination from adjacent wells during treatment within the clear well plate. LED was 
used as a light source with a power density of 16.55mW/cm^2. The control group (C) had 
only biofilm suspended in 200µl of PBS. The light only group (L) had biofilm suspended 
in 200µl PBS and received 15 min of light irradiation. The erythrosine group was 
incubated with 200µl of erythrosine (10ug/ml) and then treated with light for different
irradiation time intervals (30 sec, 1, 5, 10, and 15 min). Prior to light treatments, erythrosine was extracted and the well was rinsed with PBS. Following the treatment, the wells were incubated with 100µl of live/dead assay for 15 min. The fluorescence was measured using a microplate reader (Perkin Elmer Victor Multilabel Counter). The results (Fig 3-1) indicated a decrease in live/dead ratio between the control, light, and erythrosine groups. There was no change in the live/dead ratio between the groups treated with different intervals of light irradiation. Based on these results, it was concluded that a minimum of 30 second light irradiation would be sufficient to eliminate the bacteria. But, the power density of light could be increased to 20mW/cm² for better antimicrobial effect.

**Figure 3-1:** Live/Dead Assay results of PDT treatment after one day biofilm growth. T30- 30 sec of light irradiation; T1- 1 min of light irradiation; T5- 5 min of light irradiation; T10- 10 min of light irradiation; T15- 15 minutes of light irradiation
Chapter 4
Development and Invitro Characterization of Erythrosine nanoparticles for Chronic Sinusitis using Photodynamic Therapy (PDT)

4.1 Introduction

Sinusitis is an infection or inflammation of the paranasal sinuses associated with obstruction of the sinus ostia and retention of secretions. Chronic sinusitis is the fifth most common disease, affecting over 32 million people in the United States each year [121, 122]. A variety of antibiotic regimens have therefore been developed and refined in an effort to reduce the morbidity associated with chronic sinusitis. However, the increase in antibiotic resistance by many bacterial strains has progressively compromised the treatment results for chronic sinusitis [123]. The introduction of a non-resistance-inducing antimicrobial treatment that could reduce the prevalence of sinusitis would lay the groundwork for a dramatic change in the treatment of sinusitis and result in substantial cost savings for the health care system.

Photodynamic antimicrobial therapy (PAT) is a promising alternative therapy to combat antibacterial resistance [5]. PAT has been proven effective against many types of bacteria [124, 125] that are found in dental plaque [126] and other biofilm-producing pathogens [72]. Erythrosine, an FDA-approved food color and an oral agent, is commonly used to
disclose dental biofilms or plaque. Erythrosine possesses antibacterial properties to Gram-positive and Gram-negative organisms [127-129] and oral bacteria [130], as well as anti-fungal properties [131]. When compared to other photosensitizers such as methylene blue or photofrin, erythrosine was found to be more effective [130]. The formation of singlet oxygen when exposed to light of an appropriate wavelength is the mechanism responsible for its antimicrobial activity in photodynamic therapy [132]. To achieve cell death, erythrosine needs to penetrate the bacterial cell wall and enter cytoplasm, where the photodynamic action can be exerted.

Delivery systems such as liposomes, polymeric micelles, and nanoparticles are widely used to deliver photosensitizers. Nanoparticles are colloidal systems with a diameter of less than 1 µm [133]. Nanoparticles offer several advantages over conventional dosage forms: They a) prevent the drug from premature degradation, b) enhance the absorption of drugs due to improved intracellular penetration, c) target drugs to selected cells/tissues, d) prevent the drug efflux by multi-drug resistance pumps, and d) control the release of drugs from a few days to a few weeks [90, 134]. Synthetic biodegradable polymers are widely used in the preparation of nanoparticles. Polylactic-co-glycolic-acid (PLGA) is one such biodegradable copolymer, approved by the FDA for use as a suture material and as a polymer matrix in drug delivery systems. PLGA degrades into lactic and glycolic acid by hydrolysis of the ester bonds, both of which are metabolized into carbon dioxide and water through Kreb’s cycle. The degradation rate of the polymer can be modified by changing the polymer composition and molecular weight [133].
The potential of PLGA nanoparticles in controlling and disrupting the formation of biofilms within the sinus cavities has not been identified. We hypothesize that erythrosine-loaded PLGA nanoparticles might have a variety of potential antimicrobial properties on bacterial biofilms and provide sustained therapeutic action following intra-nasal administration. Therefore, we propose that erythrosine encapsulated in PLGA nanoparticles may be effectively taken up by the bacterial cells and provide sustained release of erythrosine to reduce the pathologic sinus biofilm formation over time, which can then be repeatedly stimulated photodynamically without reapplication. The aim of the present study was to develop and characterize erythrosine-loaded PLGA nanoparticles for PAT.

4.2 Materials and Methods

**Materials**

Erythrosine (Lot SLBC7873V), PLGA 65/35 (Lot SLBB3262V, M.W. 40-75 kDa), PLGA 75/25 (Lot 071M1473V, M.W. 66-107 kDa), and PLGA 85/15 (Lot MKBJ9550V, M.W. 50-75 kDa) were purchased from Sigma Life Sciences (St. Louis, MO). Polyvinyl alcohol (Lot A011215403, M.W. 88,000 Da) was purchased from ACROS Organics (NJ, USA). Tryptic soy broth (Soyabean Casein Digest medium-Bacto™, Lot 2030828) and Mueller-Hinton broth (Lot 3240477) were purchased from Fisher Scientific (Pittsburgh, PA). Potassium chloride (Lot 083966), hydrochloric acid (Lot 091256), and HPLC grade solvents including acetone (Lot 121151) and methanol (Lot 113904) were purchased from Fisher Scientific (Pittsburgh, PA).
**Preparation of erythrosine-loaded PLGA nanoparticles**

Nanoparticles were prepared by nanoprecipitation method using three different grades of PLGA (65/35; 75/25; 85/15). Erythrosine (5 mg) and PLGA (50 mg) were dissolved in 5 ml acetone. In a separate beaker, 0.5g of polyvinyl alcohol (PVA) was dissolved in HCl-KCl buffer solution, pH 2.45. The acetone solution was then added drop-wise to the PVA solution under constant stirring using a magnetic stirring bar. The resultant mixture was stirred for 2 h, followed by rotary evaporation in order to completely eliminate acetone. The resulting suspension was centrifuged using a Beckman Coulter Allegra 64R ultracentrifuge at 20,000 g for 20 min. The nanoparticles were washed in order to eliminate unentrapped erythrosine and freeze-dried for 24 h in the presence of 2.5% trehalose. Blank particles were prepared similarly without the addition of erythrosine.

**Determination of drug content**

The amount of erythrosine in nanoparticles was determined by UV-spectrophometer (Agilent 680 UV-Visible Spectrophotometer). Briefly, a specific amount of lyophilized nanoparticles was dissolved in 5 ml of acetone. The mixture was then vortexed for 5 min. The absorbance of the final solution was measured at 530 nm. Empty PLGA nanoparticles dissolved in acetone were used as blank. Drug entrapment efficiency was expressed as a percentage of the drug in the lyophilized nanoparticles with respect to the initial amount of nanoparticles used for preparation.

\[
\text{Entrapment efficiency (\%)} = \frac{\text{Amount of erythrosine in nanoparticles (mg)}}{\text{Initial amount of erythrosine taken (mg)}} \times 100
\]
**Determination of particle size and surface charge**

The size of nanoparticles was determined by dynamic light scattering technique using Nicomp 380 ZLS (Particle sizing systems, CA) at room temperature. A dilute suspension of nanoparticles was placed in a Durex® borosilicate glass culture tube and positioned in the path of a He-Ne laser ($\lambda = 658$ nm). The scattered light was collected at a 90° scattering angle and detected using a photodiode array detector. The neutral density filter was tuned until the scattered intensity fluctuated around 300kHz. Baseline and channel width were automatically adjusted using the Nicomp software. A Chi-squared value of 3 was used as a criterion to verify the presence of a single particle population. Nicomp distribution analysis was considered when the Chi-squared value was higher than 3 [135]. Zeta potential was also determined using the same instrument. Samples were placed in standard 1 cm square glass cuvettes, and the electrode assembly fit into the cuvette. Readings were made by collecting the scattered laser light at an angle of 14.1°C at room temperature. All measurements were taken in triplicate.

**Morphology of nanoparticles**

The surface morphology of nanoparticles was determined by using Hitachi S-4800 High Resolution Scanning Electron Microscope (Hitachi High-Technologies Corp., Tokyo, Japan). Erythrosine-loaded PLGA 85/15 nanoparticles were attached to double-sided tape, spray-coated with gold at 0.6 kV and viewed with a scanning electron microscope (SEM) at an accelerating voltage ranging between 1-10 kV.
In vitro drug release

Five milligrams of erythrosine-loaded PLGA 85/15 nanoparticles were dispersed in 1 ml release medium (phosphate buffer solution, pH 7.4) and enclosed in a regenerated cellulose dialysis membrane (Fisher Brand, MWCO = 12-14 kDa). The bag was immersed in 44 ml of release medium contained in a centrifuge tube. The centrifuge tubes were placed in a shaker bath at 37±0.5 °C and 60 oscillations/min. Samples were withdrawn at regular time intervals and replaced with an equal volume of fresh buffer. The samples were analyzed using a microplate fluorometer (Fluoroskan Ascent™, Thermo Scientific Labs, Waltham, MA) at an excitation wavelength of 465 nm and emission wavelength of 530 nm.

Fourier-Transform Infrared Spectroscopy (FT-IR)

FT-IR was performed to investigate the interactions between PLGA and erythrosine. The FT-IR spectra was obtained in the micro attenuated total reflection mode (ATR) with a Varian Excalibur Series FTIR spectrometer equipped with a microscope UMA 600 and a Germanium crystal. For each spectrum, a 256-scan interferogram was collected with a 4 cm\(^{-1}\) resolution from 4000 cm\(^{-1}\) to 400 cm\(^{-1}\).

Flow cytometric assessment of erythrosine nanoparticle uptake in S. aureus biofilms

A frozen 1 ml culture of S.aureus (ATCC: BAA-1692) was removed from the -80°C freezer and thawed. The culture was inoculated into a 100 ml flask of tryptic soy broth (TSB) and after 24 hours, 1 ml of the above medium was transferred to 100 ml of TSB
with 1% glucose and grown aerobically in a water shaker at 37°C. *S.aureus* cells suspended in TSB were seeded in a 6-well plate (Corning, Rochester, NY) at a density of ~8 X 10⁸ cells per well. Cells were allowed to grow for 72 h at 37°C. Uptake study was carried out in phosphate buffered saline (PBS), pH-7.4, under aseptic conditions. Unbound bacteria were eliminated by washing the biofilm in each well with PBS under aseptic conditions [136]. The growth medium was replaced with 1 ml of erythrosine nanoparticulate suspension (125 µg/ml). Nanoparticulate suspension and cells incubated with an equal amount of PBS were used as a control. After incubation for 1 h, cells were washed with PBS thrice. Cells were then scraped and resuspended in PBS. The fluorescence intensity of each sample was analyzed by the fluorescence-activated cell sorter flow cytometry (FACSCalibur, *BD Biosciences*, San Jose, CA) using an FL2 detector. A minimum of 10,000 events/sample were analyzed using the CellQuest software.

**Quantitative uptake in *S.aureus* biofilms**

*S.aureus* cells were suspended in TSB and seeded in a 96-well plate (Corning, Rochester, NY, USA) at a density of ~5 X 10⁷ cells per well and were allowed to grow for 24 h. Unbound bacteria were eliminated by washing biofilm with PBS, and uptake was initiated by the addition of 100 µl of erythrosine (125 µg/ml) or the nanoparticulate suspension containing an equivalent amount of erythrosine. The cells were incubated for 1 h at 37°C under aseptic conditions. Cells incubated with same amounts of PBS or blank PLGA nanoparticles were used as controls. After incubation, drug solution was removed
and the cell monolayer was washed three times with 100 µl of ice-cold stop solution to stop the uptake. The cells were lysed by adding 50 µl of 0.5% TritonX-100 in 0.2N NaOH. The cell lysate was analyzed using a microplate reader, and cell uptake was expressed as percent uptake of erythrosine per mg of protein.

**Photodynamic inactivation of S.aureus cells**

A stock solution of 10µM of erythrosine and an equivalent amount of erythrosine-loaded nanoparticles was prepared aseptically in a hood. *S.aureus* cells were suspended in sterile phosphate buffer at a concentration of $10^8$ colony-forming units/ml. One milliliter of cell suspension was placed in each well of a 24-well plate (Corning, Rochester, NY). Cells were incubated in the dark with erythrosine or erythrosine-loaded nanoparticles (1 ml) for different time periods and irradiated with light. Cells incubated with the same amounts of PBS only and light irradiation only were used as controls. Cells were irradiated after 0, 0.5, 2, 8, 16, and 24 h with an LED (DC4100 - 4-Channel LED Driver, Thorlabs, Newton, NJ) with a central wavelength of 530 nm and a fluence rate of 20.38 mW/cm$^2$. The system was coupled to an optical fiber (1 mm in diameter) that was placed 1 cm above the well so as to provide uniform distribution of light. The output power was measured by a power meter (LaxMax-TOP, Coherent, Santa Clara, CA) connected to a OP-2 VIS sensor. Cells were irradiated for 30 seconds with a total light dose of 0.6084 J/cm$^2$. Care has been taken to prevent the settling of bacteria during exposure and sampling. The number of live bacteria in samples was measured by the standard agar plate method. Samples were diluted with sterile phosphate buffer until the bacterial cells were diluted enough to count precisely. Fifty microliters of the sample or diluted sample
solution was taken and spread on the agar plate using glass beads and incubated for 16 hours at 37°C. The number of live bacteria was expressed as colony-forming units (CFU) per ml.

Statistics

Two-tailed Student’s $t$ test was used to assess the differences between two means, and a value of $p < 0.05$ was considered significant.

4.3 Results and Discussion

Erythrosine undergoes photochemical reactions in the presence of proper light frequency to generate singlet oxygen, which is highly reactive and kills bacterial pathogens [137]. Erythrosine has shown beneficial antibacterial properties against Gram-positive and Gram-negative organisms [127-129] as well as anti-fungal properties [131]. Since most of the oral bacteria are found in chronic sinusitis, it is hypothesized that erythrosine is an excellent candidate for use as a photosensitizer in the treatment of chronic sinusitis using PAT. In this study, we prepared erythrosine nanoparticles and evaluated their potential in PAT against *Staphylococcus aureus* biofilms. Erythrosine, a 2,4,5,7-tetraiodofluorescein disodium salt, has polycyclic groups that are responsible for its lipophilic nature, and the presence of sodium groups imparts hydrophilic character to this molecule (Fig. 4-1). At acidic pH, erythrosine exists in unionized form and could be extracted into the organic phase. Hence, erythrosine nanoparticles were prepared by nanoprecipitation method. The nanoprecipitation technique resulted in uniform size particles with low Chi-squared and polydispersity values. The migration of erythrosine from the organic phase (acetone) to
the aqueous phase (2.5% w/v PVA solution) was prevented by manipulating the pH of the external environment and stirring rate. Optimum entrapment of efficiency was obtained when the pH of the aqueous phase was maintained at 2.45. A further reduction in pH only resulted in aggregation of nanoparticles (data not shown). Hence, the pH of the outer phase (or aqueous phase) was maintained at 2.45. An increase in the rate of agitation decreased the size of nanoparticles (data not shown). Hence, the mixture of acetone and PVA solution was stirred at 800 rpm in order to obtain the optimum particle size.

![Structure of erythrosine](image)

**Figure 4-1:** Structure of erythrosine

The effect of the lactide/glycolide ratio on entrapment efficiency and particle size was also investigated. Nanoparticles were prepared using three grades of PLGA polymer (PLGA 65/35, PLGA 75/25, and PLGA 85/15). The particle size and entrapment efficiency values were summarized in Table 1. A slight increase in the entrapment efficiency was observed at higher lactide/glycolide ratios. The rank-order in terms of entrapment efficiency was PLGA 85/15> PLGA 75/25> PLGA 65/35, from highest to lowest concentration. The increase in the entrapment efficiency with higher lactide/glycolide ratios might be due to the better interaction/packing of erythrosine
inside the PLGA polymer. The mean particle sizes of nanoparticles prepared using PLGA 65/35, PLGA 75/25, and PLGA 85/15 were found to be 410.3 nm, 433.8 nm, and 385 nm, respectively (Table 4.1, Fig. 4-2).

**Table 4.1:** Particle size and entrapment efficiency of erythrosine nanoparticles

<table>
<thead>
<tr>
<th>Polymer grade</th>
<th>Particle Size (nm)</th>
<th>Chi-squared value</th>
<th>Polydispersity index</th>
<th>Entrapment Efficiency (%) (mean±S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA (65:35)</td>
<td>410.3</td>
<td>5.812</td>
<td>0.193</td>
<td>33.8±2.3</td>
</tr>
<tr>
<td>PLGA (75:25)</td>
<td>433.8</td>
<td>2.888</td>
<td>0.180</td>
<td>35.3±1.3</td>
</tr>
<tr>
<td>PLGA (85:15)</td>
<td>385.0</td>
<td>2.350</td>
<td>0.218</td>
<td>53.4±3.3</td>
</tr>
</tbody>
</table>

The Chi-squared values of PLGA 75/25 and PLGA 85/15 nanoparticles were less than 3, indicating a uniform particle size distribution. As the Chi-squared value of PLGA 65/35 nanoparticles was higher than 3, the data was analysed using Nicomp distribution. Two particle populations were resolved using the Nicomp distribution analysis (Fig. 2B). Peak 1 was observed at 32.5 nm, while peak 2 was observed at 390.5 nm. Polydispersity index values in all cases were less than 0.3, indicating stable solutions and homogeneous nanoparticle size distribution [133]. Based on the size and entrapment efficiency values, erythrosine nanoparticles prepared by PLGA 85/15 were further characterized and used in the uptake and photodynamic inactivation studies.
Figure 4-2: Particle size distribution of erythrosine-loaded nanoparticles (A) PLGA 65/35-intensity weighting Gaussian distribution, (B) PLGA 65/35-intensity weighting Nicomp distribution, (C) PLGA 75/25-intensity weighting Gaussian distribution, (D) PLGA 85/15-intensity weighting Gaussian distribution.

The zeta potential of erythrosine-loaded PLGA 85/15 nanoparticles was found to be -9.36 mV (Fig. 4-3). The overall surface charge of nanoparticles depends on the drug used, type of polymer, and choice of stabilizing agent. The nanoparticles were found to have a low negatively charged surface. Generally, PLGA nanoparticles exhibit a negative zeta potential due to the presence of carboxylic acid groups on the surface [138]. Negative charges on the surface of nanoparticles will facilitate the uptake and transport of nanoparticles across the cell membrane [139].
Figure 4-3: Zeta potential of erythrosine loaded PLGA (85/15) nanoparticles

The morphology of nanoparticles was studied by scanning electron microscopy (SEM). SEM images revealed the spherical shape and smooth surface of erythrosine nanoparticles (Fig. 4-4). SEM images also confirmed the size of nanoparticles with no signs of aggregation.
**Figure 4-4:** Scanning electron microscopy image of erythrosine loaded in PLGA 85/15 nanoparticles, inset: high-resolution image of nanoparticles.

Erythrosine nanoparticles exhibited a biphasic release pattern (Fig. 4-5). Approximately 20% of erythrosine was released from nanoparticles within 2 h, followed by a sustained release. Erythrosine was completely released from nanoparticles in 120 h. The initial burst release could be due to the presence of erythrosine on the surface of nanoparticles or due to the release of surface adsorbed erythrosine. Though erythrosine is water soluble and exists in ionized form at pH 7.4, a prolonged release was observed for up to 120 h. This could be attributed to the slow diffusion of erythrosine through the PLGA 85/15 matrix.
FTIR spectra of pure drug, PLGA 85/15, and erythrosine-loaded PLGA 85/15 nanoparticles were compared to investigate the presence of drug-polymer interactions (Fig. 4-6). The FTIR spectra of erythrosine showed characteristic peaks at 1233 cm\(^{-1}\) (C-OH stretch) and a broad band from 3200 cm\(^{-1}\) to 3500 cm\(^{-1}\) corresponding to O-H stretch. The medium intensity bands between 1600 cm\(^{-1}\) - 1500 cm\(^{-1}\) indicate vibrations of the aromatic ring. In the case of PLGA 85/15, a characteristic peak at 1748 cm\(^{-1}\) corresponds to C=O stretching, and a split peak between 2900 cm\(^{-1}\) - 3000 cm\(^{-1}\) corresponds to C-H
stretch. The spectrum of erythrosine-loaded PLGA 85/15 nanoparticles showed characteristic peaks of PLGA 85/15 and erythrosine with a lower intensity. No polymer-drug interaction or shifting of characteristic peaks was evident from the spectrum. This concludes the absence of chemical interactions between the drug and the polymer.

Figure 4-6: FTIR spectra of pure erythrosine, PLGA 85/15 polymer, and erythrosine-loaded PLGA (85/15) nanoparticles
The uptake of nanoparticles in *S.aureus* cells was studied by the light scatter analysis of the bacterial suspensions using a flow cytometer. Side-scatter (SSC) intensity and forward-scatter (FSC) intensity of *S.aureus* cells incubated with erythrosine nanoparticles were compared with the control (*S.aureus* cells incubated with PBS). Both SSC and FSC intensities of *S.aureus* cells increased in the presence of erythrosine nanoparticles (Fig. 4-7). SSC intensity increases with the intracellular density or the granularity of the cells [140]. When nanoparticles are ingested by the bacterial cell, the cell density increases and thereby an increase in the SSC intensity is observed [141]. The flow cytometric histogram of nanoparticles eliminates the possibility of aggregation of nanoparticles resulting in the increase in SSC intensity. Cells incubated with nanoparticles have also shown an increase in FSC intensity when compared to the control. The uptake of nanoparticles can also be studied by fluorescence intensity emitted by the bacterial cells. Flow cytometry results show the number of bacteria emitting fluorescence upon ingestion of nanoparticles (Fig. 4-8). The increase in the fluorescence intensity might be attributed to the uptake erythrosine loaded particles by the bacterial cells. From the results, the difference between the fluorescence intensity of the control bacteria and that of the bacteria incubated with the drug-loaded nanoparticles is clearly evident. Fluorescence in the control is due to autofluorescence of the cells. Approximately 87.48% of the bacterial cells have taken up by the erythrosine-loaded nanoparticles. This study indicates the internalization of nanoparticles by the bacterial cells.
**Figure 4-7:** Qualitative uptake of erythrosine-loaded PLGA nanoparticles by flow cytometric (FCM) light scatter, (A) FCM histogram of PBS, (B) FCM histogram of nanoparticles, (C) FCM histogram of blank bacteria, (D) FCM histogram of bacteria incubated with erythrosine nanoparticles for 1 h. The x-axis indicates the forward scatter, and the y-axis indicates the side scatter.
Figure 4-8: Fluorescence intensity measured by a flow cytometer, (A) fluorescence intensity of bacteria incubated with phosphate buffer saline, (B) fluorescence intensity of bacteria incubated with erythrosine nanoparticles for 1 h.
The uptake of erythrosine from neat-drug and drug-load nanoparticles was quantitatively estimated in *S.aureus* cells. After 1 h of incubation, the uptake of erythrosine was \( \sim 25 \) times higher in the presence of erythrosine nanoparticles compared to the free drug (Fig. 4-9). The uptake of erythrosine in *S.aureus* cells from nanoparticles and pure drug was approximately \( 14.83 \pm 0.15 \) and \( 0.60 \pm 0.19 \) µg per mg of protein, respectively. This indicates that the bacteria cells can internalize erythrosine nanoparticles easily when compared to the free drug. As the bactericidal activity depends on the internalization of erythrosine within the bacterial cells, it can be speculated that erythrosine-loaded nanoparticles could be a better alternative in photodynamic therapy when compared to free erythrosine.

**Figure 4-9:** Quantitative uptake of erythrosine in *S.aureus* biofilms, \((n=3)\). Error bars represent the standard deviation (S.D.), ** \( p < 0.05 \)
Illumination of \textit{S.aureus} cells after incubation with free erythrosine and erythrosine nanoparticles at different time intervals decreased the cell viability. The survival bacteria after photodynamic inactivation were represented as logarithmic values of colony-forming units (CFU) per milliliter (Fig. 4-10). No significant loss of cell viability was observed in the control groups (both \textit{S.aureus} only and light irradiation only) until 24 h. \textit{S.aureus} cells treated with free erythrosine and erythrosine-loaded nanoparticles produced similar effects after 0.5 and 2 h compared to the control groups, with both showing a significant reduction in the viability of \textit{S.aureus} cells. At 8, 16 and 24 h, the photodynamic inactivation of erythrosine nanoparticles was significantly higher compared to pure erythrosine. No significant decrease in viability was observed after 8, 16, and 24 h in the cells treated with erythrosine. With time, erythrosine nanoparticles were found to be more effective in killing all bacteria cells. After 16 hours incubation, free erythrosine resulted in a 5-fold reduction in the mean $\log_{10}$ CFU/ml. However, \textit{S.aureus} cells treated with erythrosine nanoparticles resulted in a complete loss of viability after 16 hours. Erythrosine nanoparticles were found to be highly effective in killing \textit{S.aureus} cells. This could be attributed to the sustained released of erythrosine from PLGA nanoparticles. Hence, nanoparticles could be selectively targeted to bacterial cells so that they could be in contact with the bacterial cells for about 16 h. The delivery of photosensitizers using nanoparticulate carriers is relatively new, and the extent to which nanoparticles affect the photochemical properties of photosensitizers remains unknown. Recent studies indicate that the photodynamic efficiency of photosensitizers loaded in nanoparticles depends on its release rate from the nanoparticles [142]. Moreover, there could be a decrease in the reactive oxygen species when free erythrosine
is used, due to self-quenching of erythrosine in aqueous solution [143]. This indicates that photodynamic therapy using erythrosine nanoparticles had a better bactericidal effect when compared to neat erythrosine.

**Figure 4-10**: Comparison of phototoxicity of free erythrosine (pure drug) and erythrosine nanoparticles after photodynamic treatment, (n=3). Error bars represent the standard deviation (S.D.)

From a clinical perspective, erythrosine nanoparticles can be delivered to the infected sinus mucosa via powder insufflation. Oftentimes, traditional PDT agents result in
insufficient drug accumulation for a variety of reasons, including the efflux of photosensitizer by the multi-drug resistance pumps in bacteria. Our study indicated that erythrosine nanoparticles could be easily taken up by the bacterial biofilms and provide sustained release of erythrosine to reduce the pathologic sinus biofilm formation over time which can then be stimulated repeatedly without reapplication. The clinical advantages of the application of the photosensitizing agent every 2-3 days coupled with the ease of simple, light exposure for the treatment make the transition from bench to bedside straightforward.

4.4 Conclusion

In summary, we have successfully prepared and characterized erythrosine nanoparticles for PAT. PLGA 85/15 nanoparticles were found to be superior in terms of particle size and entrapment efficiency. Nanoparticles sustained the release of erythrosine over a period of 120 hours. The flow cytometry study revealed the ability of *S. aureus* biofilms to ingest PLGA nanoparticles. Nanoparticles exhibited a significantly higher uptake of erythrosine compared to the pure drug. Photodynamic inactivation study also revealed the enhanced and sustained bactericidal effect erythrosine nanoparticles. The use of PLGA 85/15, an FDA approved biodegradable polymer, in the preparation of erythrosine nanoparticles makes this formulation clinically attractive. Photosensitizing agents entrapped in nanoparticles would lay the groundwork for a dramatic change in the treatment of sinusitis and result in substantial cost savings to the health care system. Further *in vivo* studies are warranted to evaluate the safety and efficacy of erythrosine nanoparticles.
References


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