Targeted Methylene Blue-Containing Polymeric Nanoparticle Formulations for Oral Antimicrobial Photodynamic Therapy

Master’s Thesis Dissertation Presented

By

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to

The Bouve’ Graduate School of Health Sciences in Partial Fulfillment of the Requirement for the Degree of Master of Science in Pharmaceutical Science with Specialization in Pharmaceutics

Northeastern University
Boston, Massachusetts

July 2009
ABSTRACT

Periodontitis is a chronic inflammatory disease of the periodontal tissues, which can lead to tooth loss and significant sub-gingival tissue deformities. The inflammatory process is initiated by chronic infection resulting from the presence of multi-species bacterial biofilms (dental plaques). Mechanical removal of the periodontal biofilms with or without antimicrobial agents is the current method of treatment. Antiseptics and antibiotics are also used. However, development of resistance in the target organisms is a problem associated with the use of such agents. On the other hand, Porphyromonas gingivalis is a microorganism present in dental plaque and a key player in the development of periodontitis. Selective targeting of P. gingivalis in dental plaque may lead to the development of a strategy for prevention of periodontal diseases.

In the present study, we explored a) the in vitro effects of poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with the photosensitizer methylene blue (MB) and light against Enterococcus faecalis, and b) the preparation of a conjugate between a monoclonal antibody PF18 against Porphyromonas gingivalis and MB-loaded polymeric nanoparticles for selective photosensitization of the microbe. MB-encapsulated nanoparticles of poly(lactic-co-glycolic acid) (PLGA) with negative and positive surface charge were prepared and characterized. The uptake and distribution of cationic and anionic nanoparticles in E. faecalis was investigated by transmission electron microscopy (TEM) after incubation with PLGA complexed with colloidal gold particles for 5 min. A large fraction of nanoparticles were found to be concentrated onto the cell walls of microorganisms. The synergism of light and cationic MB-loaded nanoparticles led to approximately \( >1 \log_{10} \) reduction of colony-forming units in planktonic phase. On the other hand, the selectivity of an PF18-modified gold nanoparticle conjugate was demonstrated by TEM using P. gingivalis and E. faecalis (negative control).
Our results indicate that a) PLGA nanoparticles encapsulated with photoactive drugs may be an effective adjunct in antimicrobial periodontal treatment, and b) a targeting approach may be possible for selective suppression of key pathogenic bacteria in dental plaque.
DEPARTMENTAL APPROVAL RECORD

NORTHEASTERN UNIVERSITY
Graduate School of Bouvé College of Health Sciences

Thesis Title: Targeted Methylene Blue-Containing Polymeric Nanoparticle Formulations for Oral Antimicrobial Photodynamic Therapy

Author: Niraj Patel
Department: Pharmaceutical Sciences

Approved for Thesis Requirements of the Master of Science Degree in Pharmaceutical Science

Dissertation Committee

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Director of Graduate Programs
GRADUATE SCHOOL APPROVAL RECORD

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I have waited eagerly to write this section and always thought that it would be the easiest part of this thesis on the contrary it is proving to be quiet difficult. I cannot find all the right words to express my heartfelt gratitude to the people instrumental in the completion of this thesis.

First and foremost, I would like to express my deepest thanks and appreciation to two great personalities of my life, whose confident and support always being a strong base of my life as a researcher, My advisors, Dr. Mansoor Amiji and Dr. Nikos Soukos. They were like pillars for my research carrier. Their ideas, constant support, guidance, motivation and patience, none of this would have been possible.

I would like to thank my dissertation committee members Dr. Robert Campbell and Dr. Toshi Kawai for their time, support, suggestions and advice through the course of my thesis work. I would also like to thank Dr. Robert Campbell for giving me permission to use various analytical instruments in his laboratory.

My deepest thanks to my seniors Srinivas Ganta, Harikrishna Devalapally, Arun Iyer for their valuable suggestions, advice, and help, my colleagues and friends Chinmay Bakshi, Deepti Deshpande for their encouragement and company. My closest friends Hardik Patel, Tushar Patel Piyush Patel, Ashwin Patel and Sachin Thakkar for being inspirations to live life for, and providing much needed fun distractions and just being there for me.

And lastly, to my lovely wife Nirali Patel and my parents – for giving me the chance to challenge hard life every movement and being my source of inspiration and strength - and to my family for making my dreams come true. To them I dedicate this thesis.
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1. INTRODUCTION

1.1. Periodontitis

Periodontitis results from the accumulation of bacterial biofilms (plaques) that colonize tooth surfaces and epithelial cells lining the periodontal pocket/gingival sulcus (subgingival dental plaques). Bacteria growing in biofilms adhere to a solid surface where they multiply and form microcolonies embedded in an extracellular polymeric matrix, which includes water and nutrient channels (Costerton JW, 1999). These biofilms are among the most complex biofilms that exist in nature comprising more than 700 bacterial species or phylotypes (Kroes et al., 1999; Kumar et al., 2005; Sakamoto et al., 2005). Periodontitis involves progressive loss of the alveolar bone around the teeth and may eventually lead to the loosening and subsequent loss of teeth if left untreated. Mechanical removal of the biofilms is the current method of treatment. Antimicrobial agents are also used, but biofilm species exhibit several resistance mechanisms (Anderson GG, 2008; Del Pozo JL, 2007; Fux CA, 2005). In addition, disruption of the oral microflora and the difficulty of maintaining therapeutic concentrations of antimicrobials in the oral cavity are also problems associated with the use of these agents (Wilson M., 2004).

1.2. Periodontal diseases and Porphyromonas gingivalis

In health, dental plaque remains stable for prolonged periods of time due to a dynamic balance among the resident members of its microbial community [Marsh, 2003]. This stability (termed microbial homeostasis) stems from a balance of dynamic microbial interactions, including synergism and antagonism (Alexander, 1971; Marsh, 1989). It has been argued that plaque-mediated diseases (gingivitis, periodontitis) are a result of the breakdown of this homeostasis, in which enrichment of pathogens occurs within the microbial community due to a major disturbance to the local habitat [Marsh, 1994; Marsh & Bradshaw, 1997]. In periodontal disease there is a shift in the composition of subgingival plaque’s microflora that colonizes tooth surfaces and epithelial cells in the periodontal pocket to a more proteolytic Gram-negative anaerobic community [Moore & Moore, 1994; Socransky et al., 1998]. Gram-negative anaerobes, such as Porphyromonas gingivalis, have been implicated as pathogens associated with the initiation and progression of periodontitis (Dahlén GG, 1993; Ximenez-Fyvie et al., 2000). P. gingivalis has also been associated with a variety of diseases outside of the oral cavity including pneumonia, low birth weight and abscesses (Wilson, 2004). Additionally, P. gingivalis has been implicated in human heart disease and stroke (Meyer & Fives-Taylor, 1998). If subgingival colonization and multiplication of distinct bacterial species cause periodontal disease, then non-specific removal of bacterial plaque is not the only possibility for prevention but also control and therapy. Specific suppression of key pathogens becomes a valid alternative. If microorganisms, such as P. gingivalis are associated with the development and progress of periodontal disease, then their elimination should be a primary objective of therapy.

1.3. Antimicrobial photodynamic therapy

The use of photodynamic therapy (PDT) for inactivation of microorganisms was first demonstrated more than 100 years ago, when Oscar Raab reported the lethal effect of acridine hydrochloride and visible light on Paramecia caudatum (Raab, 1900). Photodynamic therapy for human infections is based on the concept that a photosensitizing agent, known as photosensitizer, can be preferentially taken up by bacteria and subsequently activated by light of the appropriate wavelength in the presence of oxygen to generate singlet oxygen and free radicals that are cytotoxic to microorganisms (Figure 1). It is unlikely that bacteria would develop resistance to the cytotoxic action of singlet oxygen free radicals. Photodynamic therapy has emerged as an alternative to antimicrobial regimes and mechanical means in eliminating dental plaque species due to the pioneering work of Professor Michael Wilson and colleagues at the Eastman Dental Institute, University College London, UK (Wilson, 1993). Several studies
have shown that oral bacteria are susceptible to PDT in planktonic cultures (Wilson et al., 1993; Soukos et al., 1998; Williams et al., 2003) and plaque scrapings (Sarkar & Wilson, 1993). However, incomplete suppression of oral bacteria was reported after their sensitization with methylene blue and exposure to red light in mono- (Soukos et al., 2000; Soukos et al., 2003) and multispecies oral biofilms (Fontana et al., 2009). The mechanism responsible for the reduced susceptibility of these oral biofilms to PDT may be related to failure of drug penetration (Stewart et al., 1998). Water channels can carry solutes into or out of the depths of a biofilm, but they do not guarantee access to the interior of the cell clusters (Stewart, 2003), whose diameter may range from 20 to 600 µm (Rani et al., 2005).

**FIGURE 1:** Mechanism of photodynamic therapy

### 1.4. Nanoparticle-based antimicrobial PDT

Nanoscale systems that have been proposed for application in PDT are ceramic-based nanoparticles (Roy et al., 2003), semiconductor quantum dots (Samia et al., 2003), polymeric nanoparticles for the encapsulation of PS (Moreno et al., 2003), gold nanoparticles as PS delivery vehicles (Wang et al., 2004), and most recently iron oxide nanoparticles for combined hyperthermia and PDT (Gu et al., 2005).

**Why nanoparticles?** Nanoparticles containing photosensitizing agents have several advantages over photosensitizing molecules not encapsulated in nanoparticles. Reasons include (Koo et al., 2007): 1) A larger “critical mass” for the production of reactive oxygen species, which destroy cells. 2) Limit cell’s ability to pump the drug molecule back out and reduce possibility of multiple-drug-resistance. 3) Selectivity of treatment by localized delivery agents, which can be achieved by either passive targeting or by active targeting via the charged surface of the nanoparticle. 4) The nanoparticle matrix can reduce immunogenicity and other side effects. Studies in PDT have also focused on the use of polymer-based nanoparticles for PS delivery and release systems, in particular those with biocompatible and biodegradable polymers. These systems are able to target different organs and control the release of the PS molecules by the incorporation of site-specific moieties, e.g. the modification of the particles’ surface with poly(ethylene oxide) to improve the carrier’s biocompatibility and biodistribution (Devalapally et al., 2007; Shenoy and Amiji, 2005; Shenoy et al., 2005). Engineered biodegradable polymeric nanoparticles, made of poly(lactic-co-glycolic acid) (PLGA) (Langer, 1998), were used as a drug delivery system for various PS (Vargas et al., 2004; Gomes et al.,
The nanoparticle matrix PLGA is polyester co-polymer of PLA and PGA that has received approval by FDA due to its biocompatibility and its ability to degrade in the body through natural pathways (Panyam et al., 2002). Once encapsulated within PLGA, the excited state of the PS is quenched, which results in loss of phototoxicity (McCarthy et al., 2005). When the nanoparticles were incubated with cells, they showed a time-dependent release of the PS, which then regained its phototoxicity and resulted in an activatable PDT-nanoagent (McCarthy et al., 2005). Although PLGA nanoparticles loaded with various compounds (e.g. antibiotics) have been used for bacterial targeting (Kim et al., 1999; Pandey et al., 2003; Lecároz et al., 2006; Esmaeili et al., 2007; Jeong et al., 2008; Al-Ahmad et al., 2008), the use of PLGA nanoparticles as carriers of PS has not been previously explored in antimicrobial PDT.

Methylene blue has been previously encapsulated into polyacrylamide, sol-gelica silica and organically modified silicate nanoparticles for phototargeting tumor cells in vitro (Tang et al., 2005). Recently, MB-containing silica-coated magnetic nanoparticles were proposed as potential carriers for PDT (Tada et al., 2007).

1.5. Antibody-Targeted Antibacterial Approaches using Photodynamic Therapy

Antibodies conjugated with photosensitizers have been used for bacterial targeting: IgG to target Staphylococcus aureus (Gross et al., 1997) and Methicillin-resistant Staphylococcus aureus (MRSA) (Embleton et al., 2002), monoclonal antibodies to target P. gingivalis (Bhatti et al., 2000) and Pseudomonas aeruginosa in a mouse infection mode (Griffiths et al., 1997). Embleton et al. (2002) have shown that MRSA can be selectively killed by an IgG-tin chlorine e6 conjugate directed toward protein A in the S. aureus cell wall, exploiting the fact that IgG binds via its Fc region to protein A. In further work by Embleton et al. (2004), a polyclonal antibody was raised against MRSA capsular polysaccharides and was designed to bind to multiple MRSA strains. This antibody proved to be very effective when used as a targeting molecule in the PDT of multiple MRSA strains. A related approach involves physical damage to the target bacterium by the combination of pulsed laser energy and absorbing nanoparticles selectively attached to the bacterium (Zharov et al., 2006). Gold nanoparticles are the most promising candidates for application as photothermal sensitizers and can easily be conjugated to antibodies. Recently, Zharov et al. (2006) targeted the surface of S. aureus using gold nanoparticles conjugated with anti-protein A antibodies.

1.6. Significance of the thesis research

This thesis brings together researchers from different fields (oral biology and microbiology, periodontology, photochemistry, photophysics, pharmaceutics and nanomedicine) for the development of two photochemotherapeutic systems for the prevention and/or treatment of periodontal disease based on recent advances in photomedicine and nanotechnology. In this thesis, we proposed:

a) The use of light as a bactericidal agent is proposed as a method of eliminating oral bacteria in vitro after their sensitization with a photosensitizing drug (MB) loaded in a biocompatible and biodegradable polymeric nanoparticle, whose matrix is made of poly(lactic acid) and poly(glycolic acid). We envision the application of PDT in clinical practice as follows. The nanoparticles will be applied in the dental pocket for a short time (5-15 minutes). It is possible that nanoparticles with a positive charge will strongly bind to the negatively charged matrix of plaque-grown periodontopathogens as well as to the negatively charged bacterial species due
to their charge and conformation. Then the dental plaque will be exposed to light for 5 minutes, which will lead to complete killing of dental plaque bacteria.

b) The selective suppression of P. gingivalis in the dental plaque environment can be achieved using photodynamic therapy mediated by a conjugate between a MAb against P. gingivalis and MB-loaded polymeric nanoparticles as above. In this thesis we were able to prepare and characterize this conjugate. Testing of the conjugate on dental plaque bacteria in planktonic and biofilm phase will be performed in future studies.

We envision the application of PDT in clinical practice as follows. The nanoparticles or the conjugate will be applied in the dental pocket for a short time (5-15 minutes). Then the dental plaque will be exposed to light for 5 minutes. This will lead to complete elimination of species or selective suppression of P. gingivalis when nanoparticles or the conjugate is used, respectively.

In a clinical setting PDT would be applied in the dental pocket using a fiber optic. This technique would offer the following advantages in the hypothetical case of its in vivo application: (a) Rapid application of the drug in the dental pocket (antiseptics and antibiotics are difficult to maintain at high concentrations within the lesion), (b) Rapid bacterial killing after a short time of exposure of the dental pockets to light, (c) As the killing is mediated by reactive oxygen species, the development of resistance would be unlikely, (d) Killing could easily be confined to the disease lesion by restricting irradiation to this region, so that microflora at other sites would remain intact, and (e) Light would be delivered topically, rapidly and non-invasively.
2. OBJECTIVES AND SPECIFIC AIMS

2.1. Statement of the Problems

**Problem 1:** Several studies have shown that oral bacteria are susceptible to PDT in planktonic cultures (Wilson et al., 1993; Soukos et al., 1998; Williams et al., 2003) and plaque scrapings (Sarkar & Wilson, 1993). However, incomplete supression of oral bacteria was reported after their sensitization with methylene blue and exposure to red light in mono- (Soukos et al., 2000; Soukos et al., 2003) and multispecies oral biofilms (Abernethy et al., 2005; Fontana et al., 2007). The mechanism responsible for the reduced susceptibility of these oral biofilms to PDT may be related to failure of drug penetration (Stewart et al., 1998). Water channels can carry solutes into or out of the depths of a biofilm, but they do not guarantee access to the interior of the cell clusters (Stewart, 2003), whose diameter may range from 20 to 600 µm (Rani et al., 2005). The problem of limited penetration is even greater in a clinical setting, where application of both the PS and light is practical only for short time periods (<15 minutes).

**Problem 2:** According to the current opinion, there are distinct bacterial species, which have been implicated as pathogens associated with the initiation and progression of periodontitis. If subgingival colonization and multiplication of distinct bacterial species cause periodontal disease, then non-specific removal of bacterial plaque may not be the only possibility for prevention but also control and therapy. **Specific suppression of key pathogens** becomes a valid alternative. *Porphyromonas gingivalis*, the most important of these key periodontal pathogens, has also been associated with a variety of diseases outside of the oral cavity including pneumonia, low birth weight and abscesses. Additionally, *P. gingivalis* has been implicated in human heart disease and stroke. Therefore, its elimination or suppression should be a primary objective of therapy.

2.2. Objectives and Hypotheses

**Objective 1:** The long-term goal is to develop a photochemotherapeutic system for prevention, control and/or treatment of periodontitis based on recent advances in photomedicine and nanotechnology.

The hypothesis to be tested was: Complete photodestruction of *Enterococcus faecalis*, an oral bacterium, in planktonic phase may be possible after their sensitization with methylene blue (MB)-loaded polymeric nanoparticles followed by exposure to red light of 665 nm.

**Objective 2:** The long-term goal is to develop a photochemotherapeutic system for prevention of periodontitis by selectively suppressing key pathogens in dental plaque.

The hypothesis to be tested was: The conjugation between a monoclonal antibody against *Porphyromonas gingivalis* and MB-loaded polymeric nanoparticles is possible.

2.3. Specific Aims

**Specific Aim 1.** To prepare and characterize MB-loaded biocompatible and biodegradable polymeric nanoparticles

a) MB-encapsulated nanoparticles of poly(lactic-co-glycolic acid) (PLGA) with negative and positive surface charge will be prepared;
b) The particle size, surface charge, and morphology of each type of MB-loaded spherical nanoparticles will be evaluated by ZetaPALS instrument and scanning electron microscopy (SEM);
c) The capacity and efficiency of MB encapsulation will be verified by UV-visible spectroscopy; and
d) The in vitro release of MB in simulated environment will be investigated to insure that sufficient agent is available for effective PDT.

**Specific Aim 2. To investigate the bacterial destruction mediated by MB-loaded nanoparticles in vitro**

The photodynamic effects of cationic and anionic MB-loaded PLGA nanoparticles will be investigated using cultures of *Enterococcus faecalis* in planktonic phase. These effects will be evaluated by total viable counts.

**Specific Aim 3. To prepare and characterize a conjugate between the MAb PF18 against P. gingivalis and MB-loaded PLGA nanoparticles**

a) The specificity of PF18 to P. gingivalis was characterized using gold nanoparticles;
b) The end group activated polymer was synthesized for active attachment of the MAb;
c) MB-loaded PLGA nanoparticles were prepared and characterized using activated Pluronic F108;
d) The surface of MB-loaded nanoparticles was modified using the MAb PF18; and
e) The surface modification of the conjugate was characterized.
3. MATERIALS AND METHODS

3.1. Preparation, Characterization and Optimization of MB-Loaded Surface Modified PLGA Nanoparticles

a) Preparation of Methylene Blue Oleate salt. Since MB is a very hydrophilic photosensitizer, it was found that pure drug could not be efficiently encapsulated in hydrophobic PLGA nanoparticles. In order to enhance the loading efficiency, we have prepared MB-oleate salt by reaction MB with sodium oleate (Figure 2). In a typical process, 50 mg of MB was dissolved in 5 ml of dehydrated ethanol and 100 mg of sodium oleate was dissolved in 100 ml of deionized distilled water by stirring with magnetic stirrer at room temperature. MB in ethanol was added drop-wise to the aqueous sodium oleate solution and stirred to insure complete mixing. The mixture was left at room temperature overnight to allow the ethanol to evaporate. MB oleate salt formed is insoluble in water and thus precipitates out. The MB oleate salt was purified from the remaining sodium oleate by chloroform extraction. MB oleate salt was then confirmed by measuring visible absorbance where the fatty acid salt has a characteristic red shift as compared to the absorbance of free MB.

![Figure 2](image.png)

**Figure 2.** The chemical structures of methylene blue and sodium oleate used for preparing methylene blue oleate salt.

b) Preparation of MB-loaded PEO-PLGA Nanoparticles. PEO-modified PLGA nanoparticles were prepared by using the solvent displacement method, in which PLGA was dissolved with Pluronic® F108 (an ABA triblock copolymer of poly(ethylene oxide)/poly(propylene oxide)/poly(ethylene oxide)) in acetone in 85-to-15% weight ratio. For MB-loaded PEO-PLGA nanoparticles, MB oleate was added at 10% (w/w) of the polymer in the organic phase. The organic phase consisting of PLGA, Pluronic® F108 and MB oleate in acetone was added drop-wise to pre-cooled deionized distilled water under moderate stirring condition. The stirring speed, acetone-to-water volume ratio, addition speed, and temperature of the dispersion were optimized to generate particles of approximately 200 nm in diameter. The dispersion was then left stirring at room temperature overnight until all of the acetone had evaporated. The aqueous dispersion of MB-loaded PEO-PLGA nanoparticles was centrifuged, washed with deionized distilled water, and lyophilized for approximately 48 hours resulting in free-flowing dry powder.

c) Characterization of Control and MB-Loaded PEO-PLGA nanoparticles. Blank control and MB-loaded PEO-PLGA nanoparticles were characterized for particle size and size distribution, surface charge, and were observed with scanning electron microscopy (SEM).

**Particle Size Analysis.** Blank and drug-loaded PEO-PLGA nanoparticles were dispersed in deionized distilled water and subjected to particle size analysis using the Brookhaven Instrument’s Zeta PALS (Holtsville, NY) light scattering instrument at 90° scattering angle and 25°C. The count rate was adjusted in the range of 50 - 500 kcps by diluting the samples.

**Surface Charge Analysis.** Surface charge (zeta potential) measurements were also performed with the Brookhaven Instrument’s Zeta PALS (Holtsville, NY) instrument. The nanoparticle samples were dispersed in deionized water and the zeta potential values were measured at the
default parameters of dielectric constant, refractive index, and viscosity of water. The average zeta potential values were determined based on the electrophoretic mobility using the Smoluchowski-Helmholtz equation.

**Scanning Electron Microscopy.** SEM analysis was carried to confirm the size and determine the nanoparticle shape and surface morphology. Freeze-dried samples of PEO-PLGA nanoparticles, with and without the encapsulated MB, were mounted on an aluminum sample mount and sputter coated with gold-palladium to minimize surface charging. The coated samples were observed with a Hitachi S-4800 ((Pleasanton, CA) field emission scanning electron microscope at the accelerating voltage of 3.0 kV and a working distance of 3 mm. PLGA-Pluronic F 108 nanoparticles were evaluated for shape and surface morphology by SEM.

**Determination of MB Loading in PEO-PLGA Nanoparticles.** In order to determine the MB loading capacity and efficiency in PEO-PLGA nanoparticles, a standard curve of MB was prepared by dissolving the oleate salt at different concentrations ranging from 10 µg/mL to 250 µg/mL in acetone. The absorbance of solution was measured at 640 nm with a Shimadzu UV/VIS spectrophotometer (Columbia, MD).

The MB loading in PEO-PLGA nanoparticles was measured by dissolving a known amount of the nanoparticle sample in acetone. The absorbance of this solution at 640 nm was measured and the amount of incorporated MB was determined using the standard curve. At 10% (w/w) MB loading, the maximum efficiency was observed to be 45-55%.

**In Vitro MB Release Studies from PEO-PLGA Nanoparticles and CTAB-PLGA Nanoparticles.** Release of MB oleate from PLGA nanoparticles was evaluated and compared against two different types of PLGA nanoparticles. One formulation was modified with Pluronic® F-108 and had a net negative surface charge, while the other with the CTAB had a positive surface charge. The MB oleate release was observed in PBS at 37°C and was plotted as cumulative percentage releases over 24 hour time-points.

3.2. Bacterial photodestruction mediated by cationic or anionic MB-loaded nanoparticles *in vitro*

The photodynamic effects of MB-loaded nanoparticles with positive or negative charge were investigated on: a) *E. faecalis*, and b) dental plaque microorganisms in samples obtained from natural human plaque.

**E. faecalis culture.** *Enterococcus faecalis* (ATCC 29212) was used in this study. Cultures were maintained by weekly subculture in plates comprised of trypticase soy agar (Becton, Dickinson, and Co., Sparks, MD) with 5% sheep blood (Northeast Laboratories, Waterville, ME). For experimental purposes, the microorganism was grown in the presence of 80% N₂, 10% H₂, 10% CO₂ at 35°C in an anaerobic chamber for 72 hr; harvested from plates; and re-suspended in brain heart infusion (BHI) broth. Cells were dispersed by vortexing and repeated passage through Pasteur pipettes. Cell numbers were measured in a spectrophotometer (wavelength, 600 nm; 0.1 optical density unit equals approximately 10⁸ cells/ml) in 1-ml cuvettes.

**Photosensitizer.** Methylene blue (Sigma, St Louis, MO) was dissolved in BHI broth to give solutions at concentrations of 12.5 (for *E. faecalis* cultures) and 50 µg/ml (for dental plaque species) before use. The ultraviolet-visible absorption spectra of MB in BHI broth were recorded from 300 to 700 nm using quartz cuvettes with 1 cm path length on a diode array spectrophotometer (model 335907P-000, ThermoSpectronic, Rochester, NY), and were characterized by a long-wavelength maximum at 665 nm. Both *E. faecalis* and dental plaque
microorganisms were exposed to cationic and anionic MB-loaded nanoparticles. The concentration was 12.5 µg/ml equivalent to MB for E. faecalis and 50 µg/ml equivalent to MB for dental plaque species as above.

**Photodynamic treatment of bacterial suspensions.** For the photodynamic treatment of microorganisms, aliquots of bacterial suspensions (10⁸/ml) were placed in sterile microcentrifuge tubes and were centrifuged (7000 rpm for 4 minutes). The supernatants were aspirated and 1 ml of phosphate buffered saline (PBS) with MB-loaded PLGA nanoparticles or free MB was then added. Cultures in triplicate were resuspended with the nanoparticles or free MB and placed in the wells of 24-well plates for 5 min before they were exposed to light. All wells were irradiated with red light from a diode laser (BWTEK Inc., Newark, DE) with an output power of 1 Watt and a central wavelength of 665 nm for 10 min in the dark at room temperature. The system was coupled to an optical fiber 1 mm in diameter that delivered light into a lens. This formed a uniform circular spot on the base of the 24-well plate, 2 cm in diameter. The laser possessed a spectral stability of ± 2 nm with an output power stability of 10 mW. Power measurements were quantified with a power meter (Ophir Optronics LTD, Danvers, MA). Distance adjustments between the lens and the illuminated plates created fields of irradiation with appropriate dimensions and power densities. The light exposure was from above with an irradiance of 100 mW/cm² and an energy fluence of 30 J/cm². All plates were kept covered during the illumination in order to maintain the purity of the culture. After illumination of the appropriate wells, bacterial suspensions underwent serial dilutions in BHI broth and 100 µl aliquots were plated on blood agar plates for anaerobic incubation for 7 days. The following experimental groups were used for E. faecalis experiments: 1) No light/No MB-nanoparticles (control), 2) Treated only with light, 3) Treated only with anionic MB-loaded nanoparticles, 4) Treated only with cationic nanoparticles, 5) Treated only with free MB, 6) Treated with anionic MB-loaded nanoparticles and light, 7) Treated with cationic MB-loaded nanoparticles and light, and 8) Treated with free MB and light.

The primary endpoint for evaluation was the mean number of colony-forming units (CFU) per group.

**Bacterial uptake of nanoparticles by E. faecalis.** The uptake and distribution in E. faecalis was investigated by transmission electron microscopy (TEM) using PLGA complexed with colloidal gold particles. Colloidal gold nanoparticles (10-15 nm) were prepared by reduction of chloroaureic acid with sodium citrate. To a flask containing 85 mL of boiling HPLC water, Chloroaureic acid (HAuCl₄) solution (10 mL, 5 mM) was added, and the solution was allowed to return to a boil. A freshly prepared solution of sodium citrate (5 mL, 0.03 M) was then added to the flask. After a few minutes, the solution turned from colorless to a deep wine-red color. Heating was stopped at this point and the resulting sol was left to cool overnight. The nanoparticles were centrifuged at 30000 rpm for 10 min and the supernatant was discarded. The remaining pellet was re-dispersed in deionized distilled water for further use. Bacteria (10⁸/ml) were incubated with PLGA-Au-Pluronic® nanoparticle suspension (100 µg/ml) for 2.5, 5 and 10 min, centrifuged and washed twice with phosphate buffered saline (PBS). Then, microorganisms were fixed in 2.5% glutaraldehyde solution at room temperature for 1 hour, washed with distilled water, and postfixed in 1% osmium tetroxide and uranyl acetate. The cells were dehydrated with ethanol and embedded in Epon®. Thin-sectioned samples were prepared and examined using a transmission electron microscope (Brand Inc., City, ST).
3.3. Preparation and Characterization of the conjugate between the MAb PF18 and MB-loaded PLGA nanoparticles for selective targeting of *P. gingivalis*

**Characterization of the conjugate specificity**

To evaluate the specificity of the MAb (PF18) against *P. gingivalis* (ATCC 33277) in culture, we formulated gold nanoparticles and made them surface active using Traut’s reagent to attach the antibody. Using transmission electron microscopy (TEM, Brand Inc., City, ST), we compared the selectivity of these conjugates between two different bacterial species, *P. gingivalis* and *E. faecalis*.

For these studies, the bacterial cells were cultured in BHI broth and isolated and washed with PBS. After washing, the cells were suspended in PBS and added to a parafilm substrate and coated on a formvar-coated nickel TEM grid. The bacterial cells on TEM grid were fixed with 2% paraformaldehyde for 5 minutes and non-specific binding was blocked with 1% bovine serum albumin in PBS. The antibody-modified gold nanoparticles were incubated with the bacterial cells for 30 minutes and then it was washed with PBS to remove unbound conjugate. The samples were then air-dried and negatively stained with 2% uranyl acetate and observed with a TEM.

**Synthesis of end group activated Pluronic F108 polymer for active attachment of antibody**

Thiol-modified Pluronic® F108 copolymer was synthesized by a two-step reaction in order to attach the antibody to the nanoparticle surface by disulfide linkages (Thun Li 1996). Briefly, 2 gm of Pluronic® F108 was dissolved in benzene and was slowly added to the stirred solution of 4-nitrophenyl chloroformate in benzene (6 mL). The product was precipitated with diethyl ether after overnight stirring. It was recovered by filtration and purified by re-dissolving in benzene and re-precipitating with diethyl ether (Product 1). To synthesize Pluronic® F 108-2-pyridyl disulfide, 1.13 g of mercaptoethylamine hydrochloride was dissolved in methanol acetic acid mixture (1.2:0.8) and was added drop-wise to a stirred solution of 6.74 gm of 2,2'-dithiopyridine in 30 mL of methanol. The yellow mixture obtained was stirred for 30 minutes at room temperature and was precipitated with diethyl ether. It was separated by filtration and purified by again re-dissolving in methanol and re-precipitating with diethyl ether until crystals appeared white. The product was dried under vacuum. Following this step, 0.6 g of the above product (2-(2-pyridylthio) ethylamine hydrochloride) was reacted with 300 mg of triethylamine (TEA) in 3 mL methanol to neutralize the hydrochloride salt and the yellow reaction mixture is being kept stirring for 15 hours. To remove excess TEA, 0.2 to 0.25 mL of 10 M HCl was added and subjected to dialysis (with 6,000 mol. wt. cut-off membrane) in a beaker containing 4.0 L of de-ionized distilled water with several washings over a period of 48 hours. Finally the product was lyophilized to yield white free-flowing powder (Product 2). Thiol-modified Pluronic® F108 was characterized by Elman’s reagent and the degree of thiolation was determined as mmol of SH groups per mg of polymer.

**Preparation and Characterization of MB loaded PLGA Nanoparticles using activated Pluronic F108**

Thiol-modified Pluronic® F108 was incorporated in PLGA nanoparticles. These nanoparticles were prepared by the solvent dispersion method as described earlier. In this case, Pluronic® F108 (15 mg), PLGA (70 mg), thiol-modified Pluronic F108 (5 mg) and MB Oleate (10 mg) were dissolved in 2.5 ml of acetone and this solution was added drop-wise in pre-cooled de-ionized distilled water with continuous stirring at room temperature to form the nanoparticles. The suspension was allowed to continue stirring overnight for removal of the entire organic solvent. Stirring speed, addition rate of organic phase, aqueous to organic phase volume ratio
Thiol-modified PEO-PLGA nanoparticles and p-nitrophenyl chloroformate activated PEO-PLGA nanoparticles were characterized by measuring particle size and surface charge analysis.

a) Particle Size and Size Distribution: The hydrodynamic diameter of both type PEO-PLGA nanoparticles in aqueous suspension was measured by dispersing them in the deionized distilled water. The count rate was adjusted between 50-500 kcps. Mean hydrodynamic diameter was measured using Brookhaven Instrument’s ZetaPALS® (Holtsville, NY) light scattering instrument at 90° scattering angle and 25°C.

b) Surface Charge (Zeta Potential) Measurements: Both types of nanoparticles were measured using Brookhaven Instrument’s Zeta PALS (Holtsville, NY) instrument. The parameters were fixed by default (dielectric constant, refractive index, and viscosity of water) and mean zeta potential was measured using Smoluchowski-Helmholtz equation.

Surface Modification of MB-loaded nanoparticles using Monoclonal antibody (Mab) specific for *P.gingivalis*
To attach MAb or Fab fragment to the nanoparticles, we used thiol modified pluronic F108 as a linker for disulfide (-S-S-). Preparation of MB loaded thiol modified Pluronic F108 – PLGA was prepared by phase dispersion method. To attach the MAb or Fab fragment a cross linking between –SH of the end group of Pluronic F108 present in MB loaded Thiol modified Pluronic F 108 –PLGA nanoparticles was made and thiol group of amino acid (Cysteine) in the presence of reducing agent EDTA in PBS with overnight shaking at 4°C.

Characterization of the surface modification of MB-loaded nanoparticles with MAb
BCA Assay for protein analysis: To estimate the amount of the bound antibody to the nanoparticles, we used BCA assay. Briefly, a standard curve was prepared using Albumin standard protein solution in PBS in dilution ranging from 5 µg/ml to 250 µg/ml. Free (Unbound) antibody present in PBS was removed by centrifugation and supernatant was measured for Free antibody concentration. Relative bound antibody was calculated for different MAbs and Fab (Fragment) which was shown in the Table.
4. RESULTS AND DISCUSSION

4.1. Characterization of MB-Loaded Surface Modified PLGA Nanoparticles

The diameter of PEO-PLGA nanoparticles ranged from 190 to 250 nm (Table 1). The particle size remained the same with inclusion of MB. The surface charge of the nanocarriers, in the absence and presence of encapsulated payload, was determined by zeta potential measurements and was found to be \(-17.5\) and approximately \(-38\) (anionic nanoparticles) to \(+44\) mV (cationic nanoparticles), respectively (Table 1).

Table 1. Particle Size and Surface charge of Blank, MB loaded Cationic and MB loaded anionic nanoparticles.

<table>
<thead>
<tr>
<th></th>
<th>Blank nanoparticles</th>
<th>Anionic (PEO-PLGA) nanoparticles</th>
<th>Cationic (CTAB-PLGA) nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Hydrodynamic Diameter (nm)</td>
<td>189.2 ± 2.2</td>
<td>218 ± 3.1</td>
<td>205 ± 1.3</td>
</tr>
<tr>
<td>Mean Zeta Potential (MV)</td>
<td>-17.5 ± 0.74</td>
<td>-38.30 ± 0.17</td>
<td>43.5 ± 1.83</td>
</tr>
</tbody>
</table>

After freeze-drying, the surface morphology of the nanocarriers was visualized by SEM. PLGA nanoparticles were spherical in shape and had a smooth surface (Figure 3). UV-visible spectroscopy verified the capacity and efficiency of MB encapsulation.

![Figure 3. Scanning electron micrograph (SEM) of the blank PEO-PLGA nanoparticles. The inset shows higher magnification SEM image showing individual spherical nanoparticles of 190-250 nm in diameter.](image)

To determine the MB loading capacity and efficiency in PEO-PLGA nanoparticles, a standard curve of MB was prepared by dissolving the oleate salt at different concentrations ranging from 10 µg/mL to 250 µg/mL in acetone. The standard curve was shown to be linear over this concentration range (Figure 4).

![Figure 4. Standard curve of methylene blue oleate in acetone by UV/VIS spectroscopy at 640 nm.](image)
Release of MB oleate from PLGA nanoparticles was evaluated and compared against two different types of PLGA nanoparticles. One formulation was modified with Pluronic® F-108 and had a net negative surface charge, while the other with the CTAB had a positive surface charge (Figure 5).

![Figure 5](image.png)

Figure 5. Cationic nanoparticles released MB oleate much faster than anionic nanoparticles. After 12 hours, over 80% of encapsulated MB oleate was released in PBS at 37°C from the cationic nanoparticles and approximately 28% from anionic PEO-PLGA nanoparticles.

In the future, in order to optimize the PLGA nanocarrier formulations for bacterial targeting with PDT, we have to consider several challenges. In the first instance, we have to formulate the nanocarrier systems in order to efficiently encapsulate MB at 10% (w/w), to have the desired physical properties of size (~150 nm in diameter), surface charge, and stability. Our studies show that PLGA nanocarriers can efficiently encapsulate >55% MB for anionic and approximately 20% for cationic nanoparticles. The surface morphology was smooth and round for both types of nanoparticulate formulations to achieve desired particle size and pass through the small water channels present in the biofilms. Also, slow release for prolonged period of time was achieved in anionic nanoparticles compared with cationic nanoparticles. Another challenge is to insure that the nanocarriers are able to diffuse in the water-channels of the biofilm and efficiently release the encapsulated drug in the active form. Release and drug stability studies are critical in order to address this requirement. The third challenge is to insure that the sufficient concentrations of MB are released in order to have a positive effect in eradication of the biofilm organisms. PLGA nanoparticle size, surface charge, and the efficient encapsulation of MB will be critical in order to achieve the desired pharmacological benefit of PDT. Optimization of the formulation at each step will be important in order to achieve the desired success.

4.2. Antimicrobial Photodynamic Therapy

In the present study, we explored a new approach for antimicrobial therapy against the microorganism *E. faecalis* with light activation of targeted MB-loaded PLGA nanoparticles. *E. faecalis* is a gram-positive commensal bacterium inhabiting the human gastrointestinal tracts (Macovei L et al 2006), a leading cause of nosocomial infections due to its resistance to multiple antibiotics (Richards MJ et al 2000), and a microorganism associated with failures of endodontic treatment (Peculiene V et al 2000). The nanoparticle matrix PLGA is polyester co-polymer of PLA and PGA that has received approval by FDA due to its biocompatibility and its ability to degrade in the body through natural pathways (Panyam, J et al 2002). Once encapsulated within PLGA, the excited state of the PS is quenched, which results in loss of phototoxicity. When the nanoparticles were incubated with cells, they showed a time-dependent release of the PS, which then regained its phototoxicity and resulted in an activatable PDT-nanoagent (McCarthy JR et al 2005). This nanoagent has several favorable properties for use as a PS,
including nontoxicity in extracellular spaces and time-dependent intracellular release of the PS. On the other hand, MB is a well-established PS and has been used in PDT for targeting various gram-positive and gram-negative oral bacteria (Harris F et al 2005). Intravenous administration of MB is FDA approved for methemoglobinemia. Its high quantum yield of $^1{\text{O}}_2$ generation ($\Phi_{\Delta} \sim 0.5$) (Redmond RW et al 1999) in the therapeutic window of 600-900 nm, coupled with its low toxicity, has led to the testing of MB as a promising candidate for PDT of cancer (DeRosa MC et al 2002). Methylene blue has been previously encapsulated into polyacrylamide, sol-gelica silica and organically modified silicate nanoparticles for phototargeting tumor cells *in vitro* (Tang W et al 2005). Recently, MB-containing silica-coated magnetic nanoparticles were proposed as potential carriers for PDT (Tada DB et al 2007).

**Why cationic PLGA nanoparticles?** There is strong evidence that the positive charge of a PS enhances its uptake and phototoxicity on bacterial species (Minnock et al., 1996; Merchat et al., 1996; Soukos et al., 1998; Hamblin et al., 2002), and therefore cationic PLGA nanoparticles are likely to serve as better alternatives to neutrally charged nanoparticles. Recently, cationic biodegradable PLGA nanoparticles composed of chitosan have been studied as gene carriers in the nasal mucosa of mice *in vivo* (Kumar et al., 2004). The results of this study showed that PLGA nanoparticles facilitated gene delivery and subsequent expression with increased efficiency and without causing inflammation.

In our thesis studies, the cationic MB-loaded PLGA nanoparticles showed superior photodynamic effect on *E. facealis* species over anionic nanoparticles and free MB (*Figure 6*).

![Cationic MB-loaded PLGA nanoparticles exhibited superior photodynamic effect on *E. facealis* species over anionic nanoparticles and free MB](image)

**Figure 6:** Incubation of *E. facealis* bacteria with cationic MB-loaded nanoparticles (12.5 µg/ml equivalent to MB) for 5 min followed by exposure to red light for 5 min (100 mW/cm², 30 J/cm²) led to >1 log bacterial killing. Anionic MB-loaded nanoparticles (12.5 µg/ml equivalent to MB) and free MB (12.5 µg/ml) led to 73% and 13% killing, respectively.(p value for L+MB+, L+neg+, L+cat+ is < 0.05 v/s no light no drug( control)).

Cationic MB-loaded PLGA nanoparticles exhibited significant dark toxicity on *E. faecalis* species (*Figure 7*).

![Cationic nanoparticles loaded with MB reduced bacterial survival by 94% in the absence of light, whereas anionic nanoparticles by 21%.](image)
TEM also demonstrated that nanoparticles were not internalized by microorganisms, but they were mainly concentrated onto their cell walls (Figure 8). This may have rendered the cell wall permeable to MB released by the nanoparticles. In this case, the intracellular localization and the local surroundings of MB influence the phototoxicity. Sensitization of MB with light leads to the production of singlet oxygen (1\(^1\)O\(_2\)) that can migrate about 0.02 µm after its formation targeting important intracellular components. There is also another scenario, according to which photodestruction takes place within the cell wall. In this case the intracellular content may have leaked out. However, the fact that MB-loaded nanoparticles alone exhibited a toxicity ranging from 21% to 94% suggests that MB penetrated the bacterial cell wall.

**Figure 8:** The uptake and distribution of cationic (b) and anionic (c) nanoparticles in *E. faecalis* (a) was investigated by transmission electron microscopy (TEM) after incubation with PLGA complexed with colloidal gold particles for 5 min. A large fraction of nanoparticles were found to be concentrated onto the cell walls.

A more thorough evaluation of the photodynamic effects of MB-loaded nanoparticles would also require knowledge of various parameters, which would lead to a maximum photodynamic effect on microbes, such as: the amount of MB encapsulated in nanoparticles; the incubation time of MB-loaded nanoparticles with microorganisms; the power density (mW/cm\(^2\)) and energy fluence (J/cm\(^2\)) of light; the In addition, the therapeutic window where microorganisms would be killed by MB-loaded nanoparticles while leaving normal cell intact as well as the role of nanoparticle charge should also be explored. Currently, we are investigating the effects of cationic and anionic MB-loaded PLGA nanoparticles as well those of free Mb on dental plaque bacteria obtained from human natural dental plaque.

### 4.3. Preparation and Characterization of the conjugate between the MAb PF18 and MB-loaded PLGA nanoparticles for selective targeting of *P. gingivalis*

Our results confirm specific binding of antibody-modified gold nanoparticles to *P. gingivalis* (Figure 9, left image). On the other hand, the antibody-modified gold nanoparticles did not bind to the negative control *E. faecalis* (Figure 9, right image).
**Figure 9:** TEM analysis of *P. gingivalis* (left image) specific monoclonal antibody binding to the bacterial cell surface in culture. In these studies, *E. faecalis* (right image) was used as a negative control.

The two products obtained by the Synthesis of end group activated Pluronic F108 polymer for active attachment of antibody are shown in **Figure 10**.

**Figure 10:** p-nitrophenyl chloroformate activated Pluronic F 108 intermediate also used as an amide linker for proteins (product 1, upper). Thiol activated Pluronic F 108 linker for thiol conjugation with proteins (product 2, lower).

<table>
<thead>
<tr>
<th>Nanoparticles type</th>
<th>Mean Hydrodynamic Diameter (nm)</th>
<th>Mean Surface Charge (mv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thiol activated Pluronic F 108</td>
<td>$195 \pm 3.8$</td>
<td>$-8.4 \pm 1.2$</td>
</tr>
<tr>
<td>P-nitrophenyl chloroformate activated Pluronic F 108</td>
<td>$209.4 \pm 6.4$</td>
<td>$-4.94 \pm 0.96$</td>
</tr>
</tbody>
</table>

**TABLE 2** Particle size and Surface charge of MB loaded PLGA nanoparticles surface modified with activated Pluronic F 108 for MAb conjugation.
**Figure 11:** Standard curve prepared using Bovine Serum Albumin to characterize MAb Conjugated nanoparticles for attachment of Different types of Mabs.

**TABLE 3.** Amount of different antibodies (based on different antigen affinity) actively attached to the surface of MB loaded nanoparticles measured using BCA assay.

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Percent Antibody Bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF 9</td>
<td>68</td>
</tr>
<tr>
<td>PF 18</td>
<td>48</td>
</tr>
<tr>
<td>BSO 2</td>
<td>65</td>
</tr>
</tbody>
</table>
5. CONCLUSIONS

From the experimental data of our pilot study we conclude that in future optimized formulations of MB loaded PLGA nanoparticles can be an adjunct to the standard Periodontal Therapy to complete eradication of the Diseases. Also by selective targeting, we may able to suppress pathogenic microorganisms which are the key triggers to the advance stages of Periodontitis and prevent Diseases in healthy human beings. Our research will provide the base for future development of the antibody conjugated nanoformulations for selective targeting of pathogens and also for the adjuvant therapy against chronic periodontitis to get rid of all the microorganisms present in human plaque.
6. REFERENCES


