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Synthesis and characterization of targeted bar encapsulated polylactic-co-glycolic acid nanoparticles to inhibit porphyromonas gingivalis biofilm formation.

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SYNTHESIS AND CHARACTERIZATION OF
TARGETED BAR ENCAPSULATED POLYLACTIC-CO-GLYCOLIC ACID
NANOPARTICLES
TO INHIBIT *PORPHYROMONAS GINGIVALIS* BIOFILM FORMATION

By
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Thesis approved on

April 13, 2017

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DEDICATION

To my beloved family

ACKNOWLEDGEMENTS

I would like to sincerely thank every individual who has helped me throughout my research and thesis writing. It's their love, support and encouragement that has helped me to successfully complete this thesis.

Firstly, I would like to express my gratitude to my mentors Dr. Donald R. Demuth and Dr. Jill M. Steinbach-Rankins, you both have been tremendous mentors for me. I would like to thank you for encouraging my research and for allowing me to grow as a research scientist. Your motivation and advice on both research as well as on my career have been priceless. I would also like to thank Dr. Richard J. Lamont for serving as my committee member and giving his valuable suggestions. I would especially like to thank Dr. Douglas Darling, Program Director of Masters in Oral Biology for his constant support and encouragement.

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ABSTRACT

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Periodontal disease is one of the most prevalent infectious diseases worldwide. Between 30-50% of the global adult population suffers from periodontal disease. Some form of periodontitis is present in 46% of American adults, corresponding to annual expenditures in excess of 14 billion dollars for treatment and prevention. Current treatments for periodontal diseases involve mechanical removal of plaque, correction of risk factors, gingival surgery and/or antibiotic therapy. To our knowledge there is no effective therapeutic approach that aims to limit pathogen colonization of the oral biofilm or re-colonization after treatment. Interaction of the pathogen *Porphyromonas gingivalis* with oral streptococci is critical in the pathogenesis of periodontal diseases. Our previous studies identified a peptide (BAR) that potently inhibited this interaction and reduced *P. gingivalis* virulence *in vivo*. However, BAR required higher concentrations and prolonged exposure in limiting *P. gingivalis* in a pre-established biofilm. This study is aimed at developing targeted BAR encapsulated poly(lactic-co-glycolic acid; PLGA) nanoparticles (NPs) to increase the efficacy of BAR to inhibit *P. gingivalis* colonization in a dual species biofilm. NPs encapsulating BAR were synthesized using double emulsion solvent evaporation technique. We generated NPs with particle size of 207±19 nm and zeta potential of -15mV.

Loading and controlled release properties of NPs were determined using a fluorescently labeled BAR. We showed that BAR-NPs had a total payload of 14.12±0.39 µg/mL of BAR per mg of NP, resulting in the sustained-release of BAR for up to 4 hours. Furthermore, NPs encapsulating BAR peptide potently inhibited pre-formed *P.gingivalis/ S. gordonii* biofilm. These results suggest that enhanced inhibition of *P. gingivalis* was be obtained with BAR-NPs, identifying a novel therapeutic approach to effectively targeting *P. gingivalis* colonization of the oral cavity.

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CHAPTER 1

INTRODUCTION

Periodontitis is defined as an inflammatory disease of the supporting tissues of the teeth caused by specific microorganisms or groups of microorganisms, resulting in progressive destruction of the periodontal ligament and alveolar bone with increased probing depth formation, gingival recession, or both (1). Periodontal disease is one of the most prevalent infectious diseases worldwide affecting between 30-50% of the global adult population suffers from periodontal disease (2-4). In America 46% of the adults suffer from some form of periodontal disease; corresponding to annual expenditures in excess of 14 billion dollars for treatment and prevention. An estimated 18% of the US population has moderate periodontitis and 7% is afflicted by severe periodontitis (5). Studies have shown that periodontal disease occurs more often in patients with systemic disease like diabetes mellitus, AIDS, leukemia and Down's syndrome. Periodontal diseases are also proven to be risk factors for various systemic diseases such as infective endocarditis, cardiovascular disease, prosthetic device infection, diabetes mellitus, respiratory diseases, rheumatoid arthritis and adverse pregnancy outcomes (6-10).

Development of dental plaque biofilms

Periodontal disease starts with a microbial biofilm known as dental plaque. A milder form of periodontal disease which just exhibits inflammation of the gingiva is known as gingivitis. Maturation of biofilm within subgingival pocket together with host-systemic and lifestyle factors cause attachment loss and lead to periodontitis.
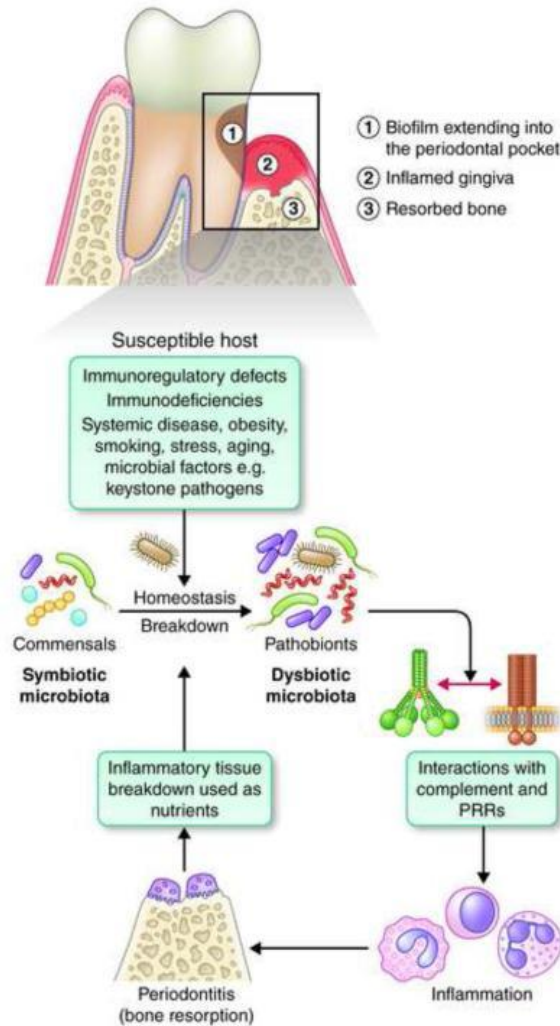
Fig 1. Pathogenesis of periodontal disease. (Hajishengallis, G. (2014). Immunomicrobial pathogenesis of periodontitis: keystones, pathobionts, and host response. *Trends in immunology*, *35*(1), 3-11.)

A biofilm is a structured community of microbial cells attached to a surface and is enclosed in a polymeric matrix. In the oral cavity these surfaces can be teeth or restorative materials. During the initial stages of biofilm formation there is adsorption of salivary macromolecules on oral tissue surfaces which results in formation of the salivary pellicle. Bacteria can attach to this pellicle and actively grow (12). Bacterial cells participate in a communication process known as quorum sensing, that is an important pathway for biofilm maturation. Quorum sensing is a cell-cell communication process that involves signaling molecules called auto inducers (13) that allow bacteria to regulate gene expression in

response to changes in cell-population density. Distinct stages in biofilm formation are: acquired pellicle formation, initial adhesion, maturation and dispersion (14).

The first step of biofilm formation is the attachment of an acquired pellicle. An acquired pellicle is a thin protein-containing film derived from salivary glycoproteins on the tooth surface. Interactions between various glycoproteins, other salivary components, and the tooth surface are involved in the formation of acquired pellicle. After formation the pellicle is ready for bacterial adhesion.

Bacterial adhesion to the pellicle is the second step of biofilm formation. Some of the planktonic bacteria recognize binding proteins like α-amylase and proline-rich glycoproteins/proteins and bind to the pellicle. However, in this stage the attachment is reversible and those initially attached bacteria can detach from the pellicle easily. Early attachments are primarily based on electrostatic or physical attachments, but later, chemical forces become predominant (14). As the bacteria attach to the pellicle, they begin to secrete extracellular polymeric substances which helps the bacteria stay bound together and attached to the pellicle. Surface attachment structures including fimbriae and fibrils are the primary means through which bacteria attach to the pellicle *Actinomyces* spp, *Streptococcus* spp, *Haemophilus* spp, *Capnocytophaga* spp, *Veillonella* spp, and *Neisseria* are the main pioneer bacterial genera attaching to the tooth surface (15). Early colonization help these species occupy space and seize advantage in later competition with other species.

Later colonizing bacteria recognize polysaccharide or protein receptors on the pioneer bacterial cell surface and attach to them. Bacteria coaggregate, forming the typical corn cob forms, bristle brush forms, or other forms in mature oral biofilm. Later colonizing

bacterial species include *Fusobacterium nucleatum*, Treponema spp, *Tannerella forsythensis*, *P. gingivalis*, *Aggregatibacter actinomycetemcomitans* (14). The microbial structure of a mature biofilm is significantly different from the initial biofilm, including a populational shift that occurs during biofilm development (17). Mature biofilms typically contain many porous layers and water channels through the biofilm, providing the bacteria essential nutrients (18). Moreover, cell aggregation is the basis of bacterial interactions in biofilm formation. Generally, one bacterial species can coaggregate with many other bacterial species by cell-to-cell recognition, but this aggregation is specific because one bacterial strain cannot aggregate with any random bacterial strains. The fundamental mechanism promoting this aggregation is via polysaccharide or protein recognition between bacteria (19).

In the mature biofilm, bacterial cells detach from the biofilm by single cell detachment or a group of cells detaching. Bacterial detachment is attributed to two reasons. The first is due to limited nutrients present at the original site, prompting the bacteria to identify and move to a new site with more nutrients for growth. This relocation can occur either by active dispersion or passive dispersion. Active dispersion is induced by the bacteria themselves, while passive dispersion is caused by other bacterial species which compete for the nutrient (20). The second reason for cell detachment is due to host defense processes, such as the shear force of saliva, which serves to limit biofilm growth (21).
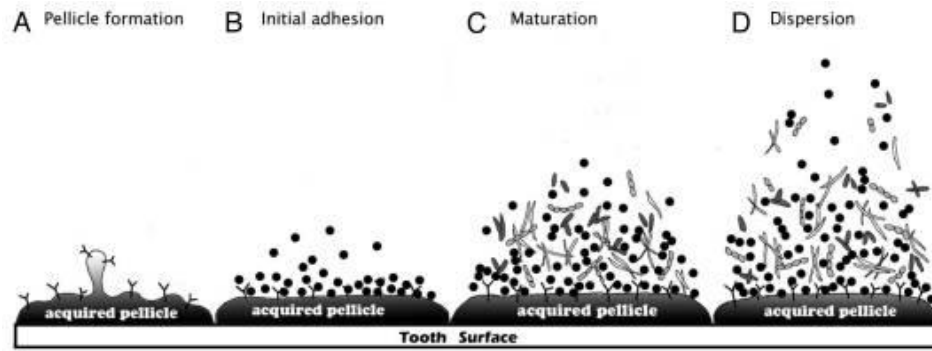
Fig 2. Stages of biofilm development. (Huang, R., Li, M., & Gregory, R. L. (2011). Bacterial interactions in dental biofilm. *Virulence*, *2*(5), 435-444.)

Role of bacteria in Periodontitis

There are two main hypotheses regarding the role of bacteria in periodontal disease. The non-specific plaque hypothesis suggests that no one specific bacterial species is any more significant in causing periodontal disease, relative to another. According to this hypothesis, the accumulation of plaque results in oral diseases - not the individual bacteria or its virulence (22). The conclusion from this hypothesis indicates that since any plaque has the ability to cause disease, mechanical removal of plaque is an important step in disease prevention. In light of this theory all plaque was viewed as the cause and plaque control became the focus of periodontal therapy. The relative merits of this hypothesis is arguable since plaque associated diseases are polymicrobial infections, but in which only certain pathogens predominate. In contrast to this theory, the Specific plaque hypothesis proposes that only a few specific species are actively involved in disease pathogenesis (23). This hypothesis suggests that the primary concern is the specific bacteria that causes disease. As such, procedures are needed to remove only those species and not all the other species in the biofilm. However, this hypothesis could not explain the absence of pathogens in certain disease sites or the presence of pathogens in healthy sites. A more recent

ecological plaque hypothesis combines the merits of both specific and non-specific plaque hypothesis. According to ecological plaque hypothesis, disease is a result of enrichment of some pathogens caused by ecological stress and an imbalance in the total microflora (22).

Dental biofilms are a complex community that may consist of more than 700 different bacterial species (24). Primary colonizing organisms that initiate the formation of the biofilm consists of Gram positive organisms such as streptococci and *Actinomyces* spp. Over the time there is a shift in microflora from Gram positive to Gram negative organisms and from facultative anaerobes to strictly anaerobic species (12). Populational shifts in the microflora can result in dysbiosis and are believed to cause periodontal disease. In subjects with periodontal disease, red complex are directly associated with periodontal disease and includes *Tannerella forsythia*, *Porphyromonas gingivalis* and *Treponema denticola* (25).

Role of *Porphyromonas gingivalis* in Adult Periodontitis

P. gingivalis is a non-motile, asacchrolytic, rod shaped, Gram negative anaerobic bacterium which belongs to phylum Bacteriodetes. *P. gingivalis* was found in 87.75% of sub-gingival plaque samples obtained from patients with chronic periodontitis (26). *P. gingivalis* produces a multitude of virulence factors which results in destruction of periodontal tissues by direct or indirect modulation of host inflammatory response (27). Hajishengallis et al, 2012 identified *P. gingivalis* as a keystone pathogen in the development of periodontal diseases in mice. Keystone pathogens are specific pathogens that when present even at low abundance can cause disease by altering a normal microbiota leading to disruption of normal host-microbe homeostasis (i.e., dysbiosis) (28).
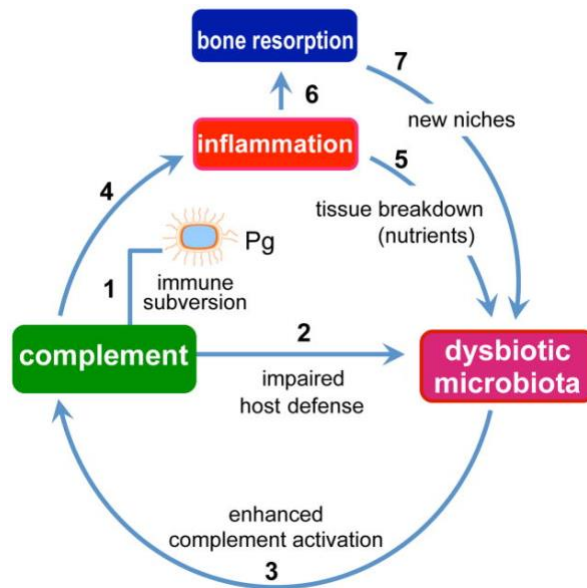
Fig 3. *P. gingivalis* induced dysbiosis. (Hajishengallis, G., Darveau, R. P., & Curtis, M. A. (2012). The keystone-pathogen hypothesis. *Nature Reviews Microbiology*, *10*(10), 717-725.)

Porphyromonas gingivalis- Streptococcus gordonii interaction

The primary niche of *P. gingivalis* in the oral cavity is the sub-gingival pocket. Before *P. gingivalis* colonizes the sub-gingival pocket it often adheres to primary colonizers of the oral cavity like *Streptococcus gordonii* (29). Previous studies have shown that adherence of *P. gingivalis* to oral streptococci is driven by a protein-protein interaction that occurs between the minor fimbrial antigen (Mfa) of *P. gingivalis* and the streptococcal antigen I/II (30). A region encompassing amino acids 1167 to 1250 of the SspB polypeptide of *S.gordonii* antigen I/II was shown to be essential for adherence of *P. gingivalis* (31-33). Subsequently, This region of SspB was compared with the corresponding region of the *Streptococcus mutans* antigen I/II and demonstrated that a protein determinant comprised amino acids 1167 to 1193, designated BAR (Ssp<u>B</u> <u>A</u>dherence <u>R</u>egion), was sufficient to promote *P. gingivalis* adherence (34-36). A synthetic peptide encompassing this region of SspB functions as a potent inhibitor of *P. gingivalis* adherence to *S. gordonii* and its

subsequent formation of biofilms *in vitro* (IC_{50}= 1.3μM). BAR also inhibited *P. gingivalis* virulence and colonization of the oral cavity *in vivo* (37). These data suggest that the initial interaction between *P. gingivalis* and *S. gordonii* provides a potential target to prevent the colonization of *P. gingivalis* in the oral cavity and one can contemplate formulating BAR in an oral rinser or varnish to limit *P. gingivalis* colonization of the oral cavity. However, a possible limitation of this approach is that BAR can only be delivered transiently. Moreover, BAR-mediated disruption of pre-formed two-species biofilms or more complex three-species biofilms *in vitro* required a higher concentration of BAR, as well as a prolonged exposure. To address these challenges, the goal of this project was to develop methods to deliver higher localized concentration of BAR for a prolonged duration of time.
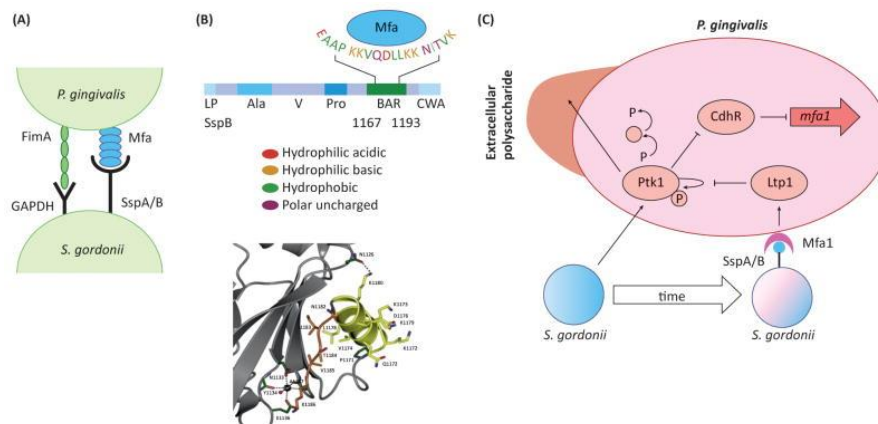
Fig 4. Mechanism of *P. gingivalis* – *S. gordonii* co-aggregation. (Lamont, R. J., & Hajishengallis, G. (2015). Polymicrobial synergy and dysbiosis in inflammatory disease. *Trends in molecular medicine*, *21*(3), 172-183.)

<u>Nanoparticles in oral diseases</u>

The use of nanotechnology in drug delivery has gained a lot momentum in the recent years (38). Nanoparticles are appealing vehicles to use for drug delivery because of their characteristic features like higher surface-to-volume ratio, their ability to encapsulate

various hydrophobic and hydrophilic compounds. A variety of materials including dextran, gelatin, liposomes, fullerenes, metals, silica and polymers have been used to formulate nanoparticles. For drug delivery, it is often desirable for NPs to release the drug upon reaching the appropriate sites and hence, biodegradable formulations can aid in this endeavor. Poly(lactic acid ,PLA), poly(lactic-co-glycolic acid, PLGA), polycaprolactone (PLCL) are all different biodegradable formulations that have been used for nanaoparticle synthesis (39).

In the last few decades PLGA has been most widely accepted and studied for drug delivery and tissue engineering applications. PLGA is a family of FDA-approved biodegradable polymers that are highly biocompatible and have been extensively used as controlled delivery vehicles for drugs, proteins, and various other macromolecules such as DNA and RNA (40-42). Favorable degradation characteristics, sustained-release properties, and surface modification has made PLGA a popular candidate for NP formulations. In addition, mechanical strength and ability to undergo hydrolysis can be altered by controlling parameters like molecular weight and the monomer ratio of ratio of lactic to glycolic acid (43).

PLGA degradation is a collective process of bulk diffusion, surface diffusion, bulk erosion and surface erosion. The initial burst of active agent during the release from nanoparticles is dependent on the drug and polymer hydrophobicity. In the second phase, the drug is gradually released as water hydrolyzes the polymer into oligomeric and monomeric products (43-44).
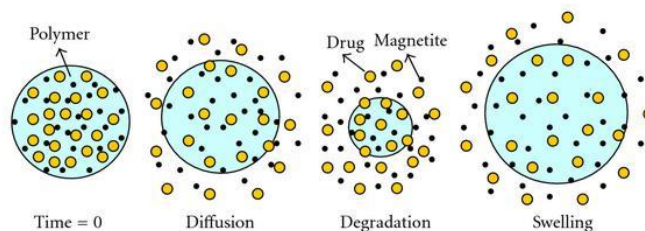
Fig 5. Schematic showing PLGA NP degradation. (Asmatulu, R., Fakhari, A., Wamocha, H. L., Chu, H. Y., Chen, Y. Y., Eltabey, M. M., ... & Ho, J. C. (2009). Drug-carrying magnetic nanocomposite particles for potential drug delivery systems. *Journal of Nanotechnology*, *2009*.)
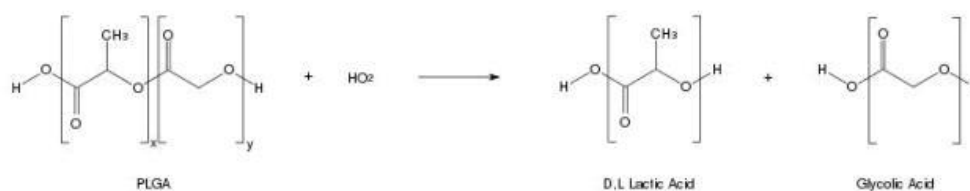
Fig 6. Hydrolysis of PLGA. (Makadia, H. K., & Siegel, S. J. (2011). Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers*, *3*(3), 1377-1397.)

Nanofibers

In the recent years polymer nanofibers (NFs) have been used in drug delivery due to their ease in synthesis, primarily through an electrospinning process. Various drugs, proteins, RNA, DNA and growth factors have been incorporated into electrospun fibers (45-47). PLGA nanofibers have been shown to increase the efficacy of various drugs. Combined with the above mentioned properties and the ability to use it under a periodontal pack after a surgery makes nanofibers a suitable candidate for treatment of periodontal diseases (48).

CafA adhesin and targeting the nanoparticles

Targeting of NPs are has been widely used in drug delivery to improve the local concentration of the drug. One method of achieving targeting is to surface modify NPs with proteins or drugs to achieve specific targeting or binding properties. In addition to

specifically targeting delivery to particular niches (here in the oral cavity), surface modification may decrease the initial release of encapsulant from the NPs, aiding in prolonged release (49-51). Basic rationale here is to use a bacterial surface protein that can aid in targeting these NPs to the areas in oral cavity where *P. gingivalis* is present. To achieve this, we envision the use of CafA adhesin. CafA or coaggregation factor A is one of the 14 cell surface proteins of *Actinomyces oris* that possess the LPXTG anchoring motif that is recognized by sortase. CafA adhesin allows for *Actinomyces*/*Streptococcus* coaggregation (52). Surface modification of NPs with CafA represents an approach to target NPs or fibers to streptococcal cells, a primary initial niche of *P. gingivalis* in the oral cavity.
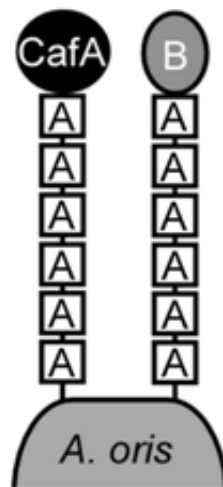
Fig 7. Schematic representation of fimbrial structures of *Actinomyces oris*. (Reardon-Robinson, M. E., Wu, C., Mishra, A., Chang, C., Bier, N., Das, A., & Ton-That, H. (2014). Pilus hijacking by a bacterial coaggregation factor critical for oral biofilm development. *Proceedings of the National Academy of Sciences*, *111*(10), 3835-3840.)

BAR-modified PLGA Nanoparticles

Previous studies in our lab has shown that BAR-modified NPs potently inhibit *P. gingivalis* adherence to *S. gordonii*. When administered simultaneously with *P. gingivalis*, BAR-modified NPs exhibited an IC$_{50}$ to 0.3μM which is significantly lower when

compared to IC_{50} of 1.3μM for free BAR peptide. However, in the oral cavity there is presence of well-established and complex biofilms. Hence it is important to know if BAR-modified NPs are more potent in such conditions. Parallel to this study that seek to evaluate the efficacy of CafA modified NPs, we also intend to test the efficacy of BAR-modified NPs in an established *P. gingivalis/ S. gordonii* biofilm and compare them with free BAR.

CHAPTER 2

HYPOTHESIS AND SPECIFIC AIMS

Research Hypothesis

We hypothesize that the delivery of BAR by sustained release PLGA (poly lactic-co-glycolic acid) nanoparticles (NPs) that are targeted to specific niches in oral cavity will increase the effectiveness of BAR against the colonization of *P. gingivalis* in the oral cavity.

Specific Aims

1. Synthesize and characterize PLGA NPs that encapsulate BAR to provide prolonged-release of the peptide.

2. Target the BAR-NPs to specific niches to the oral cavity using CafA adhesin.

3. Determine the functional efficacy of NPs on an established biofilm.

CHAPTER 3

METHODS

Peptide Synthesis

The BAR peptide used in this study, is shown in Table 1. The peptide is comprised of residues 1167 to 1193 of the SspB (Antigen I/II) protein sequence of *S. gordonii*. The peptide was synthesized by BioSynthesis, Inc. (Lewisville, TX) and was obtained at more than 90% purity.

Table 1. Sequence of BAR peptide.

Peptide	Peptide Sequence
BAR	NH2-LEAAPK<u>K</u>VQDLLKKANITVKGAFQLFS-OH

To assess the amount of BAR encapsulated within the NPs, a peptide designated as F-BAR was synthesized (BioSynthesis, Inc.) and 6-carboxyfluorescein was covalently attached to the epsilon amine of the lysine residue (underlined in Table 1).

Peptide containing a covalently attached biotin at its N-terminus was also synthesized and obtained at more than 85% purity.

Growth of Bacterial Strains

P. gingivalis strain ATCC 33277 was grown in Trypticase soy broth media (TSBY media) (Difco) supplemented with 0.5% (w/v) yeast extract, 1 µg/ml (final concentration) menadione, and 5 µg/ml (final concentration) hemin. TSBY media was reduced for 24 hours under anaerobic conditions consisting of 80% N_2 10% CO_2 10% H_2. Next, *P. gingivalis* was inoculated into the medium and grown anaerobically for 48 hours at 37°C. *S. gordonii* DL-1 was cultured aerobically without shaking in brain-heart infusion (BHI) broth supplemented with 1% yeast extract for 16 hours at 37°C. *A. viscosus* (ATCC 43146) was grown anaerobically in Trypticase soy broth supplemented with 5% defibrinated sheep blood. Media was reduced for 24 hours under anaerobic conditions consisting of 80% N_2 10% CO_2 10% H_2. *A. viscosus* was inoculated to the reduced media and grown anaerobically for 24 hours at 37º C. Bacterial plasmid was extracted using Wizard® Genomic DNA Purification Kit (Promega). *E. coli* strains DH5α and XL1 were grown in Luria-Bertani broth (LB) at 37ºC overnight. The pQE-60 vector DNA was harvested from DH5α cells using QIAprep® Spin Miniprep Kit (QIAGEN). Electrocompetent XL1 cells were harvested from the overnight culture and stored at -80ºC in 50 µL aliquots.

Nanoparticle Synthesis

Nanoparticles encapsulating BAR or F-BAR were synthesized using a double emulsion solvent evaporation technique. One hundred milligrams of 50:50 PLGA crystals were dissolved in 2mL dichloromethane (DCM) overnight. The following day the PLGA/DCM mixture was vortexed while adding 200µL of prepared mixture of BAR (4.3µg/mg of PLGA) in TE buffer. PLGA/DCM/peptide solution was sonicated to ensure

homeogeneity. To create the second emulsion, PLGA/DCM/peptide solution was added drop wise to 2 mL 5% PVA while vortexing. This mixture was then sonicated and added to 50mL 0.3% PVA in DI water. The resulting solution was stirred for 3 hours on a stir plate to allow for excess DCM evaporation. After 3 hours the solution was transferred to tubes and centrifuged at 13,000 rpm for 10 minutes. After each centrifugation, the supernatant was discarded and the pellets were re-suspended in 20 mL distilled water and centrifuged. After 2 washes, the NPs were resuspended in 5mL distilled water and transferred to a cryotube. NPs were subsequently frozen at -80˚C for 2 hours and lyophilized for 48 hours. All NPs were stored at -20˚C. F-BAR-NPs were synthesized in the similarly but were protected from light during the entire procedure to ensure minimal loss of fluorescence.
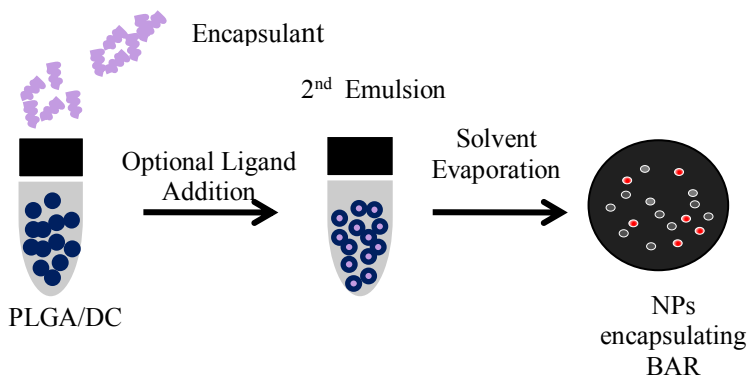
Fig 8. Overview of the double emulsion solvent evaporation technique for NP synthesis.

Nanofiber synthesis

In a scintillation vial, 120 mg of 50:50 PLGA was dissolved in hexafluoroisopropanol (HFIP) overnight on a tabletop shaker. The day of synthesis BAR (4.3μg/mg of PLGA) in 200μL of TE was added to the PLGA/HFIP mixture. PLGA/HFIP/BAR solution was then transferred into a syringe which was then placed in the syringe driver. This emulsion was electrospun at a voltage of 20mV at a flowrate of

0.8mL per hour. The voltage clip was attached to the syringe needle. The fine mist of solution was collected on a mandrel and allowed to dry for 15 minutes. After 15 minutes the fiber was removed from the mandrel using a razor blade and placed in a petri dish in a dessicator for 24 hours. After dessication, fibers were stored at 4˚C.
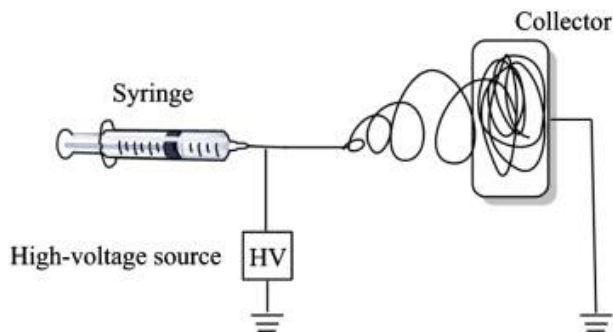
Fig 9. Schematic showing nanofiber synthesis. (Hu, X., Liu, S., Zhou, G., Huang, Y., Xie, Z., & Jing, X. (2014). Electrospinning of polymeric nanofibers for drug delivery applications. Journal of Controlled Release, 185, 12-21.)

BAR-Modified NPs

To synthesize BAR-modified PLGA NPs, the NP surfaces were first modified with avidin-palmitate to attach biotinylated BAR. Avidin-palmitate was synthesized as previously described (50). Briefly, 40 mg/4.8 mL solution of avidin was made in 2% (w/v) sodium deoxycholate (NaDC) in PBS and warmed to 37ºC. Four milligrams per four milliliter solution of palmitic acid-NHS (PA-NHS, Sigma) was prepared in 2% (w/v) NaDC and sonicated until well-mixed. To the reaction vial containing avidin 3.2 mL of the above made PA-NHS solution was added dropwise, and reacted overnight at 37ºC. The following day, the reaction was dialyzed in 1200 mL of 0.15% (w/v) NaDC in PBS heated to 37ºC using a 3500 molecular weight cut off (MWCO) dialysis tubing to remove free

PA-NHS. After overnight dialysis at 37ºC, complexed avidin-palmitate was transferred to a storage vial from the dialysis cassette and stored at 4ºC.

NPs were synthesized using an oil-in-water (o/w) single emulsion technique. 100 mg PLGA crystals were dissolved in 2 ml of DCM overnight. The next day, 2 ml of 5% (w/v) polyvinyl alcohol (PVA) solution was mixed with 2 ml of 5mg/ml avidin-palmitate to obtain a well-mixed solution. To create the single emulsion, 2 mL PLGA/DCM solution was added dropwise to 4 ml PVA/avidin-palmitate while vortexing. The solution was then sonicated to ensure homogeneity. This emulsion was then added to 50 mL of 0.3% PVA for 3 hours. to allow for the evaporation of excess DCM. The solution was then transferred to tubes and centrifuged at 13,000 rpm at 4°C. Supernatant was discarded and the pellets were resuspended in 9 ml distilled water. The resuspended NPs were incubated with biotinylated BAR for 30 minutes on a benchtop at a molar ratio of 3:1 BAR: avidin. NPs were washed and lyophilized as previously mentioned in the double emulsion technique and stored at -20°C.

Physical Characterization of Nanoparticle/ Nanofiber

Nanoparticle size and morphology were determined using scanning electron microscopy (SEM). NPs were mounted on a mounting pin with carbon tape and sputter coated with gold under vacuum. Average particle diameter and size distribution were determined from SEM images of at least 400 particles per batch using image analysis software (ImageJ, National Institutes of Health). Zeta potential was measured with a Zetasizer Nano ZS (Malvern) in diH_2O to determine particle charge. Nanoparticles with a

negative charge has less chances of aggregation. Nanofiber size, morphology and zeta potential were determined similarly.

Functional Characterization of Nanoparticles

To determine the total payload of the NPs, 2-3 mg of NPs were dissolved in 500μL of DCM. To this 500μL of TE buffer was added and vortexed for 1 min to extract the peptide into the aqueous solution. The solution was then centrifuged at 13,000 rpm for 5 minutes at 4˚C and the aqueous phase is carefully removed without disturbing the organic phase. Five hundred microliters of TE buffer was added to the remaining organic phase and vortexed and centrifuged again to remove any remaining peptide. The amount of F-BAR encapsulated was then determined by comparing fluorescence to a known standard of F-BAR in TE. The standard was plotted by serially diluting a 1mg/mL solution of F-BAR in TE. The standard and the NP samples were measured for fluorescence at 488/515 nm (excitation/emission).

To determine the sustained-release characteristics of BAR from the NPs, F-BAR NPs were added to 1x PBS and incubated at 37˚C for 1 hour. After 1 hour the solution was centrifuged and supernatant was removed. To the remaining NPs fresh PBS was added and incubated until the next time point. This procedure was repeated and supernatants were obtained at 1, 2, 4, 8, 24, 48 and 72 hour time points. The amount of F-BAR released at each time point was measured by comparing to a known standard of F-BAR in PBS. The standard was plotted by serially diluting a 1mg/mL solution of F-BAR in PBS. The standard and the NP samples were measured for fluorescence at 488/515 nm.

Functional Characterization of Nanofibers

To determine the total payload of NFs, 2-3 mgs of NFs were dissolved in DMSO and compared to a known standard of F-BAR in DMSO. The NF samples and standards were measured for fluorescence at 488/515 nm.

The sustained-release of BAR from NFs were determined similar to the sustained release of NPs as mentioned above.

Dual Species Biofilm

Formation of *P. gingivalis*/ *S. gordonii* biofilms were carried out as previously described (32). *S. gordonii* cells were harvested by centrifuging a 15 ml culture of *S. gordonii* at 5600 rpm for 5 min. The supernatant was discarded and the cell pellet was resuspended in 1 ml of 1x PBS. *S. gordonii* cells were labeled with 20 μl of 10mM hexidium iodide for 15 min at room temperature on a rocker platform protected from light. After incubation the labeled samples were centrifuged at 5600 rpm for 5 min, the supernatant was discarded, and the cells were resuspended in 1ml of 1x PBS. Following this, the optical density (O.D) was measured at 600 nm to determine cell count. For all experiments, the optical density of *S. gordonii* cells was adjusted to 0.8 for uniformity of the *S. gordonii* cell amounts in each well. After adjusting the optical density, 1ml of *S. gordonii* cells were added to a 12 well culture plate containing a sterilized micro-coverslip in each well. The 12 well cell culture plate was wrapped in aluminum foil to protect the labeled cells from light and placed on a rocker platform in the anaerobic chamber at 37ºC for 24 hours. *P. gingivalis* cultures used for biofilm formation were optimized using a similar approach. 15 ml of *P. gingivalis* cells were centrifuged for 5 min at 5600 rpm. The

supernatant was discarded and the cell pellet was resuspended in 1 ml of prereduced 1x PBS. *P. gingivalis* cells were labeled with 15 µl of carboxyfluorescein–succinylester (4 mg/ml). Cells were incubated with the fluorescent dye for 30 min on a rocker platform protected from light. Following incubation, cells were centrifuged at 5600 rpm for 1 min and the supernatant was discarded. The pelleted cells were resuspended in 1ml of pre-reduced 1x PBS. Optical density of *P. gingivalis* cells were adjusted to 0.4. After removal of unbound *S. gordonii* cells and washing, labeled *P. gingivalis* (1 ml) cells were then added to each well of the microtiter plate. The 12 well cell culture plate was covered with aluminum foil and incubated at 37ºC for 24 hours in an anaerobic chamber. Following incubation, the supernatant was removed from the wells of the 12 well cell culture plate and the treatment solutions containing free BAR or BAR-NPs in PBS were added to the appropriate wells and incubated for 1-3 hours. Following incubation the supernatant was removed and the wells were washed with pre-reduced 1x PBS to remove non-adherent bacterial cells. The cells were subsequently fixed with 4% (w/v) paraformaldehyde, excess paraformaldehyde was removed, and the cells were washed with pre-reduced 1x PBS. The coverslip was then mounted on to a glass slide using Prolong Gold anti-fade reagent and viewed using confocal laser scanning microscopy.

Confocal Microscopy

    P. gingivalis- S. gordonii biofilms were visualized using a Leica TCS SP8 confocal microscope (Leica Microsystems). The slides were viewed using an argon laser for visualization of FITC-labeled *P. gingivalis* and the HeNe-G laser to visualize hexidium iodide- labeled streptococci. *P. gingivalis* binding was determined from randomly chosen

frames using LAS X software. Z-stack images of the biofilms were obtained using a z-step size of 0.7 μm.

Image Analysis

The images obtained from confocal microscopy were processed and reconstructed into 3D images using the Volocity software. Images were imported into Volocity as .tiff files. Uniform filters were used to remove noise from the images and were further analyzed to quantify the extent of *P. gingivalis* binding. The image brightness and contrast was adjusted equally for all frames, and a snapshot of the image was captured. Next, the ratio of green to red fluorescence was determined. Each sample was analyzed in triplicate and 3 independent frames were measured for each well. Statistical significance between samples was determined using ANOVA. The variation was considered statistically significant when $P<0.05$.

Recombinant CafA expression in *E. coli*

Forward and reverse primers used for amplification of *cafA* gene are shown in Table 2.

<p align="center">**Table 2.** Primers used for amplification of *cafA* gene.</p>

Forward Primer	5'- CAC CAT GGT GAT CGA CTG GAT CGA CTG -3'
Reverse Primer	5'- CAG ATC TGA TCT TGC CCA GCG C -3'

The *cafA* gene of *A. viscosus* (lacking the LPXTG anchoring motif) was amplified using the primers shown in Table 2 by PCR using Radiant™ HiFi Ultra Polymerase. PCR protocol and cycling conditions used were as described in Table 3.

Table 3.1. PCR protocol used for amplification of the *cafA* gene.

Component	50µl Reaction	Final Concentration/Notes
RadiantTM 5x HiFi Ultra Reaction Buffer	10 µl	1X
Forward Primer (10µM)	2.0 µl	400 nM
Reverse Primer (10µM)	2.0 µl	400 nM
Template DNA	1.0 µl	387ng
RadiantTM HiFi Ultra Polymerase (2u/µl)	0.5 µl	
PCR-grade water	34.5 µl	

Table 3.2. PCR cycling conditions used for amplification of the *cafA* gene.

Cycles	Temperature & Time	Notes
1	95°C 1 minute	Initial Denaturation
30	95°C 15 seconds 68°C 15 seconds 72°C 30 seconds per Kb	Denaturation Annealing Extension 1min 30s

PCR products were then concentrated using DNA Clean & Concentrator™-5 (Zymo Research). The insert and vector DNA (pQE-60; Fig 10) were then digested using restriction endonucleases (*Nco*I and *Bgl*II) to create sticky ends for ligation. The digested insert and vector DNA were then ligated using T4 DNA Ligase (New England BioLabs Inc.).
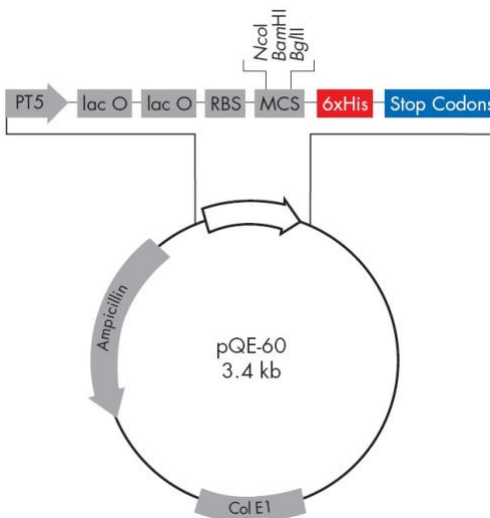
Fig 10. Vector map for pQE-60.

Vector containing the desired insert was then transformed into XL1 electrocompetent cells by electroporation. The cells were allowed to grow in SOC medium containing no antibiotics at 37˚C for 45 min. Following this the cells were streaked on a LB agar plate containing tetracycline and ampicillin (1µL/mL) and allowed to grow overnight at 37˚C (Fig 11). Individual colonies were selected and passaged in LB broth containing tetracycline and ampicillin (1µL/mL).
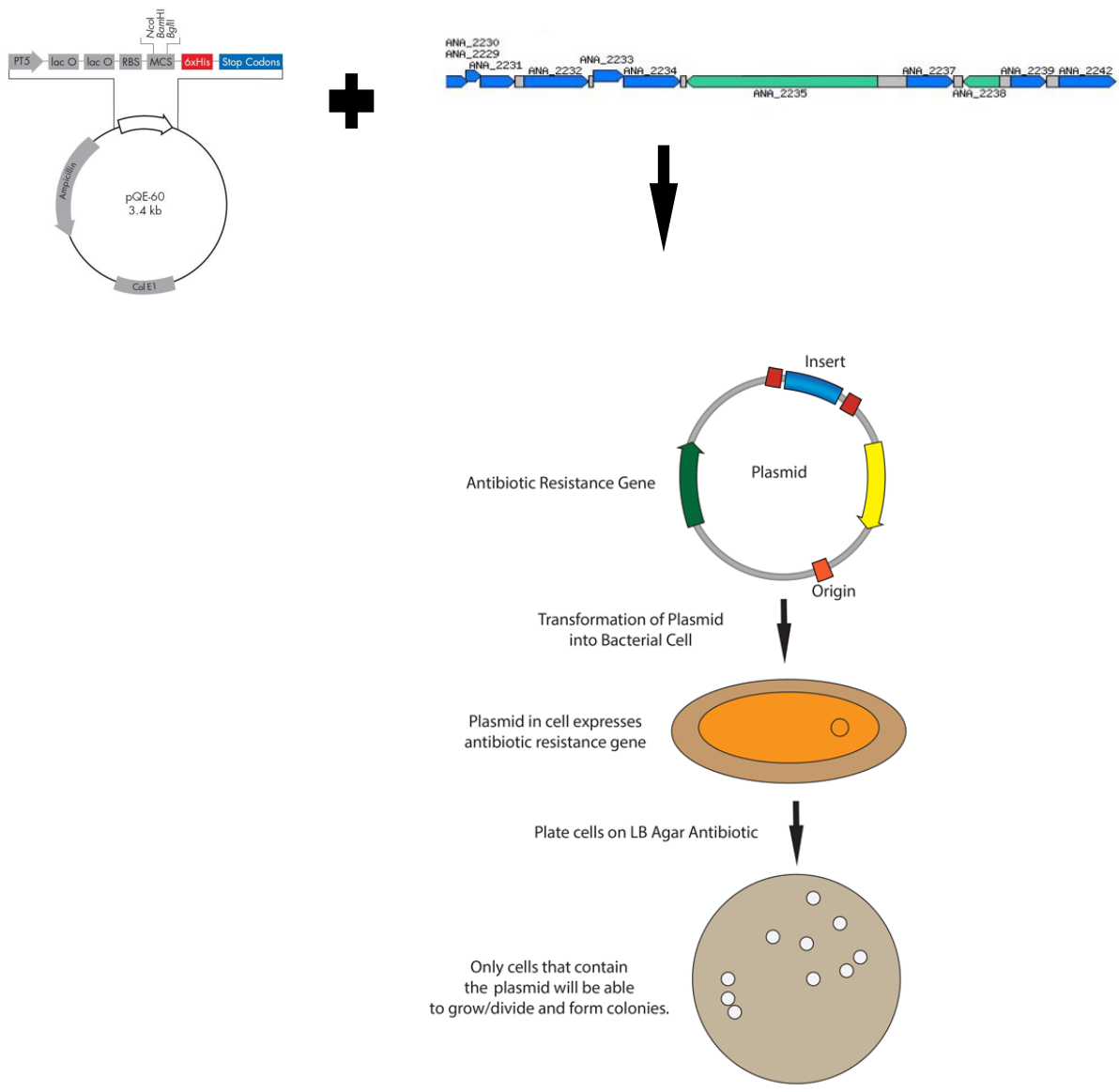
Fig 11. Schematic overview of bacterial transformation. (www.addgene.org)

CHAPTER 4

RESULTS

BAR requires prolonged exposure to inhibit established two species biofilm

Previous studies have shown that BAR requires prolonged exposure (>60 mins) to inhibit established three-species biofilm (29). To determine the time required by BAR to inhibit an established two-species biofilm, 3µM of free BAR was used to treat an established *P. gingivalils/ S. gordonii* biofilm for 1, 2 and 3 hours. *P. gingivalis* and *S. gordonii* microcolonies were then visualized by confocal scanning microscopy, and the number of microcolonies that formed per microscope field-of-view were recorded for each time point. As summarized in Table 4, treating the biofilm for 3 hours with BAR resulted in ~55% inhibition of the *P. gingivalis* microcolonies. In contrast, treatment for 1 and 2 hours showed no significant inhibition of *P. gingivalis* ($P > 0.05$). Figure 12 shows the ratio of *P. gingivalis/ S.gondonii* microcolonies were statistically significant between the control and treatment groups at 3 hour time point. This lower ratio means more inhibition of *P. gingivalis* colonies. This experiment provides preliminary data that supports the hypothesis that sustained release NPs can increase BAR effectiveness against established biofilms by reducing the amount of *P. gingivalis* bound to *S. gordonii.*

Time in hours	Ratio between *P. gingivalis* and *S. gordonii*	
	Control (Mean ± SD)	Treated (Mean ± SD)
1	0.23±0.009	0.21±0.019
2	0.33±0.013	0.31±0.013
3	0.33±0.006	0.15±0.011

Table 4. Effect of prolonged exposure of BAR on the ratio between *P. gingivalis* and *S. gordonii.*
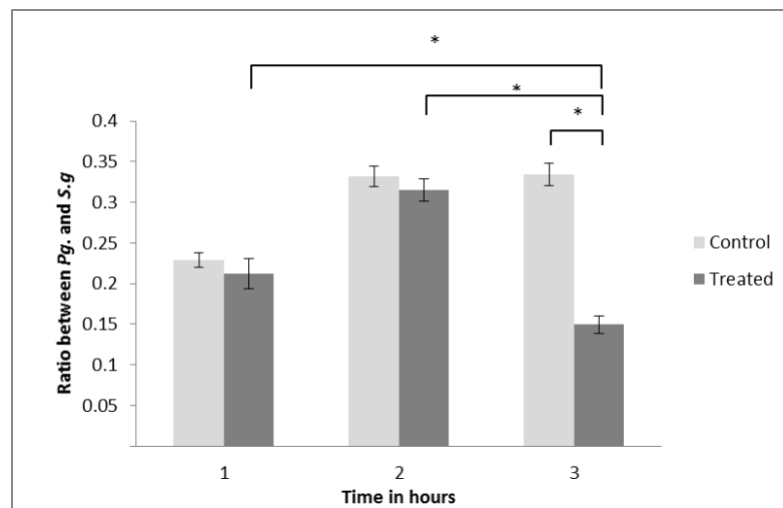

Fig. 12. Ratio of *P. gingivalis* and *S. gordonii* microcolonies treated with 3μM of BAR for 1, 2 and 3 hours. *, the ratio was statistically significant (P< 0.05)

Nanoparticle synthesis and characterization.

Analysis of SEM images (Fig. 13) revealed that the average size of NPs are 207±19nm and exhibit the expected spherical morphology. Zeta potential values for NPs were -15mV.
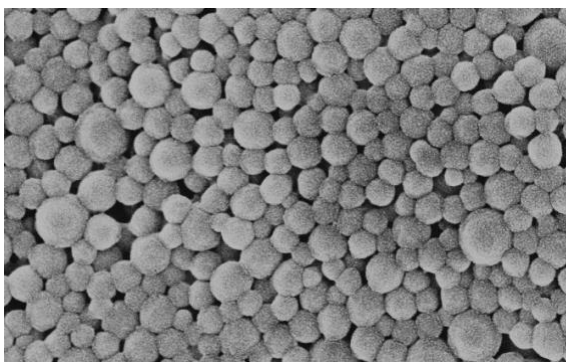
Fig 13. SEM image of BAR NPs

Loading experiments

In this experiment the amount of BAR encapsulated within the NPs was determined. Total fluorescence emitted by the NPs were converted to an amount of BAR by comparing the fluorescence to a known standard of F-BAR in TE. Loading experiments showed a total payload of 14.12±0.39 µg of BAR per mg of NP. This corresponded to an encapsulation efficiency of 33%.

Dose dependent loading of nanoparticles

To determine the dose dependent loading and encapsulation efficiency, NPs were synthesized using and initial concentration of 10µg, 21µg and 43µg per mg of PLGA. Fig 14 shows the total payload and the encapsulation efficiency for each batch of NPs. There is no significant difference in the percent encapsulation efficiency ($P>0.05$), but there is a dose-dependent increase in the total amount of BAR encapsulated. This suggests that NPs can be synthesized with varying concentrations of BAR to achieve optimal loading.
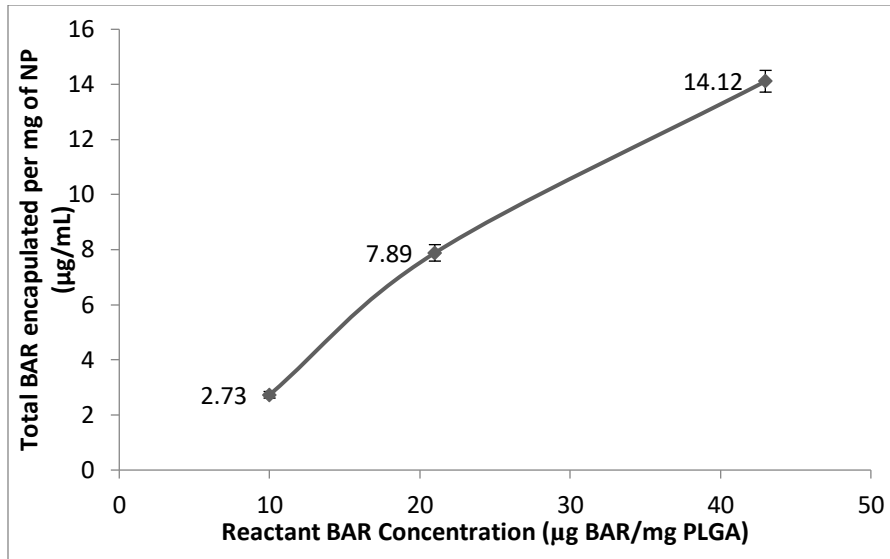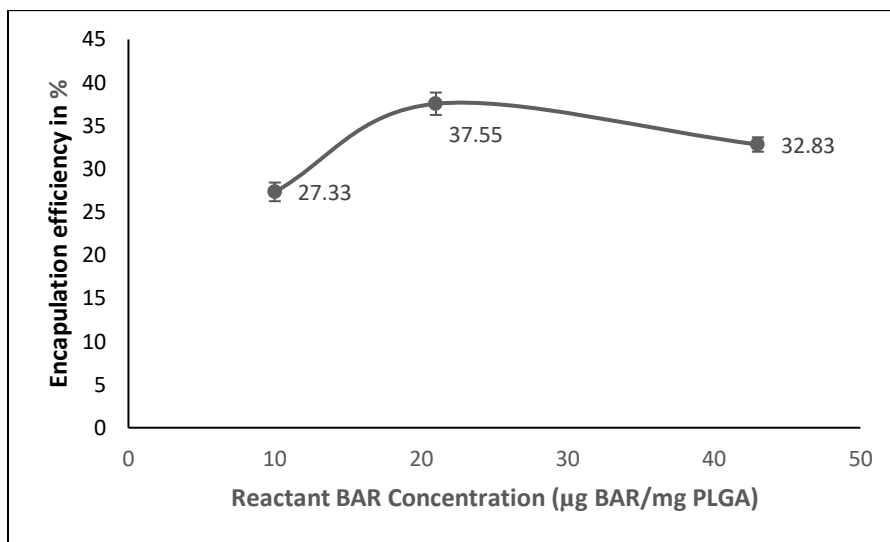
Fig 14.1. Dose dependent encapsulation of BAR.

Fig 14.2. Dose dependent encapsulation efficiency of BAR.
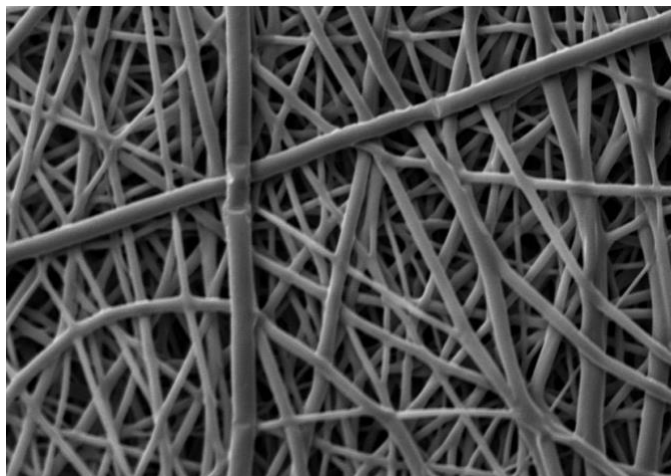
Nanofiber characterization

Fig 15. SEM image showing BAR NFs.

Similar to NPs, we validated the diameter and morphology of BAR NFs using SEM. Figure 15 depicts uniformly sized, well- delineated fibers with average size of 917±256 nm. To determine the total pay load aliquots of NFs were dissolved in DMSO and the amount of fluorescence emitted was converted to an amount of BAR by comparing to known standards of F-BAR in DMSO. Loading experiment to determine the total amount of BAR encapsulated in the fibers showed an encapsulation of 46.8± 0.63 μg of BAR per mg of NF, corresponding to an encapsulation efficiency of 92%.

BAR nanoparticles provide sustained release of BAR

For this experiment aliquots of BAR were incubated in PBS at 37 ºC. Supernatant from various time points were used to measure the fluorescence. Triplicate fluorescence readings at each time points were compared to a known standard of F-BAR in PBS. Fig 16.1 shows the total concentration of BAR released at each time point over a 24 hour

period. Fig 16.2 shows the percentage of encapsulated BAR released at each time point over a 24 hour period.
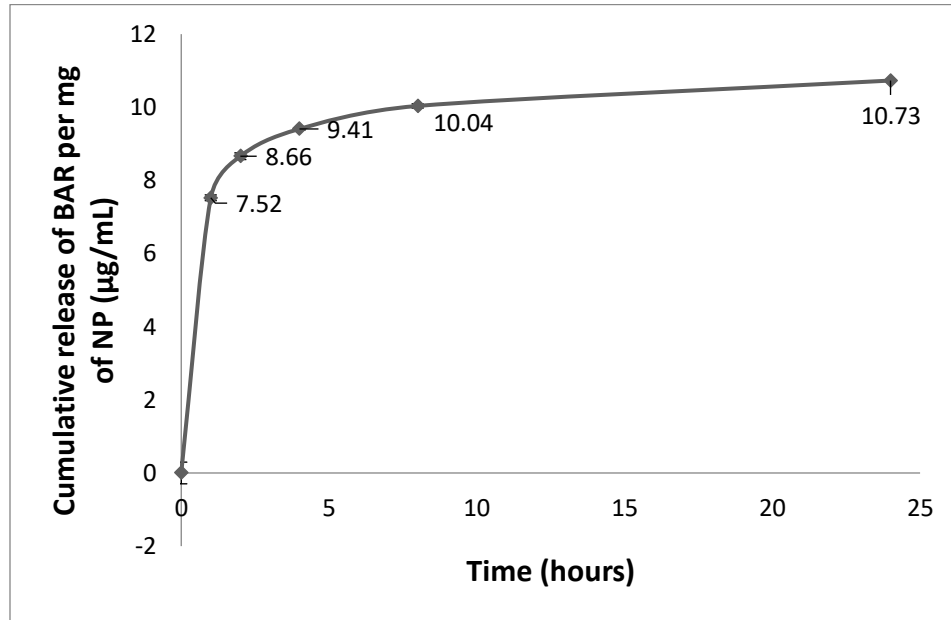

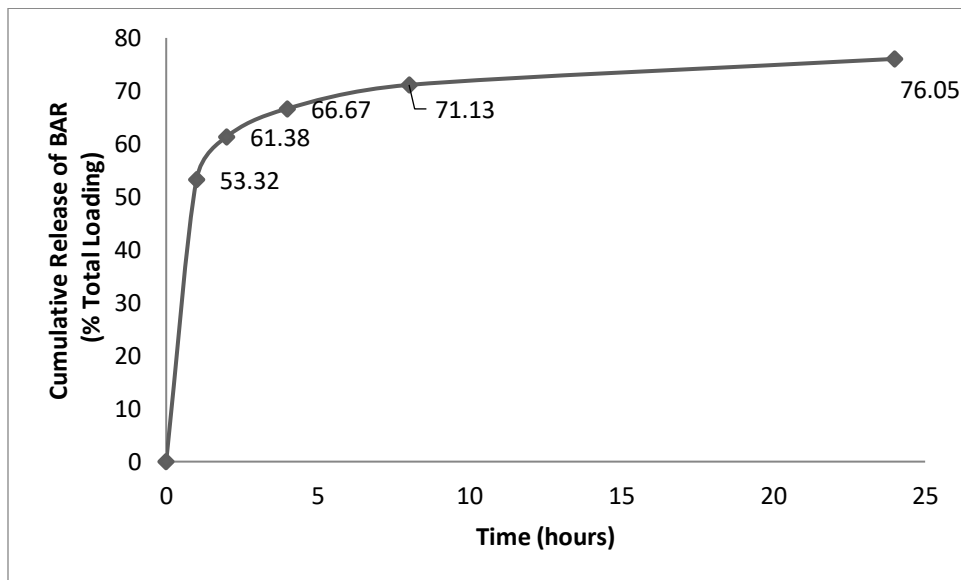**Fig 16.1.** Total amount of BAR released per mg NPs over a 24 hour period.

Fig 16.2. Percent of encapsulated BAR released per mg NPs over a 24 hour period.

Nanofibers provide sustained release of BAR in lower concentrations than NPs

To determine the sustained-release of BAR, NFs were incubated with in PBS at 37ºC. Similar to NP sustained-release experiments, the supernatant from each release time point was assessed to determine the amount of BAR released. The amount of F-BAR released at each time points was compared to a known standard of F-BAR in PBS. Fig. 17.1 and 17.2 show the total amount of BAR and the percent encapsulate BAR, released at each time point over a 24 hour period, respectively. Although NFs showed a higher encapsulation per mg of fiber (46.8± 0.63 µg/mg), relative to NP loading (14.12±0.39 µg/mg), less BAR was released over a 24 hour time period (1.23 µg/mg vs. 10.7 µg/mg)
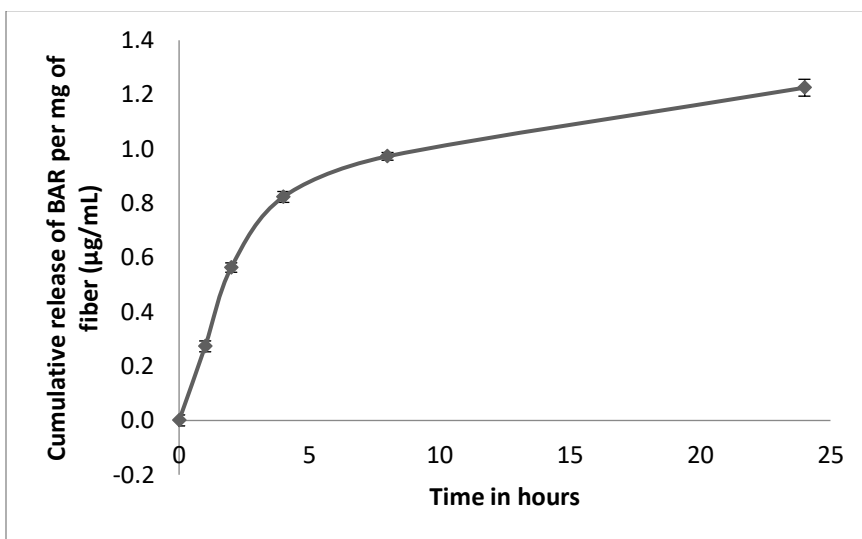

Fig 17.1. Total amount of BAR released per mg fiber over a 24 hour period.
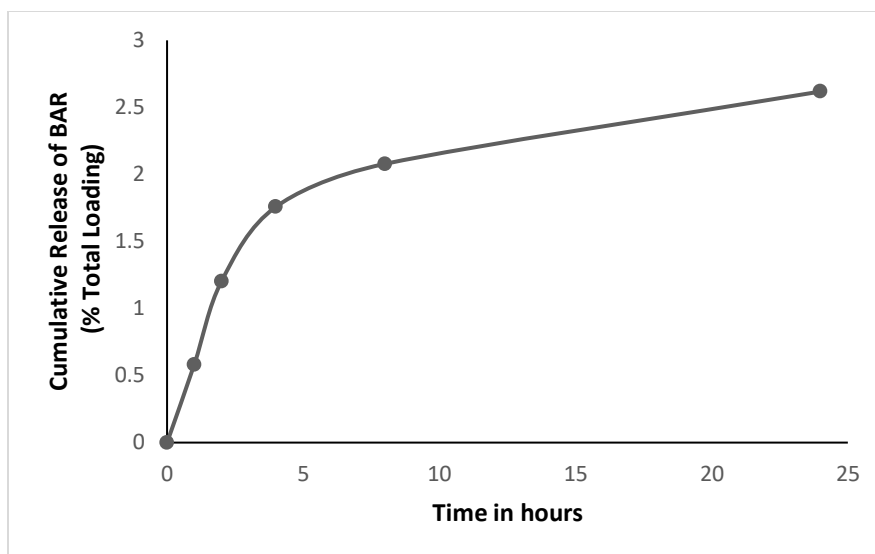
Fig 17.2. Percent of encapsulated BAR released per mg fiber over a 24 hour period.

Sustained release BAR-NPs inhibit established *P. gingivalis/ S. gordonii* biofilms.

To determine if BAR-encapsulated NPs inhibit *P. gingivalis* on an established *P. gingivalis/ S. gordonii* biofilm, *P. gingivalis* biofilms were formed on immobilized streptococci. Various molar concentrations of BAR-NPs in PBS were used to treat the established biofilm for 1 hour. Sufficient amounts of NPs to deliver BAR peptide concentrations ranging from 0.3 to 3 µM were tested and compared with 3 µM of free soluble BAR. Only one concentration of soluble BAR (3 µM) was used because the previous experiment showed 3µM of soluble BAR having no significant effect on established biofilms when treated for 1 hour. *P. gingivalis/ S. gordonii* microcolonies were visualized using confocal scanning microscopy and the ratio of green (*P. gingivalis*) and red (*S. gordonii*) fluorescence was quantified using Volocity image analysis software. For control reactions, the biofilms were treated with blank NPs for the same amount of time. The images of biofilms treated with BAR-NPs, soluble BAR or buffer alone are shown in Fig.18. As summarized in Table 5, BAR-NPs exhibited dose dependent inhibition of the

dual species biofilm with the ratio of green to red fluorescence being significantly reduced (P<0.05) at 1.7 µM and 3 µM. As expected 3 µM soluble BAR had no effect on the ratio of green and red fluorescence. The striking result was that while soluble BAR required a prolonged exposure of 3 hours, BAR-NPs showed a significant reduction in the ratio of green and red fluorescence in just 1 hour.

Control

Free BAR 3µM

BAR-NP 0.3 µM

BAR-NP 1.7 µM
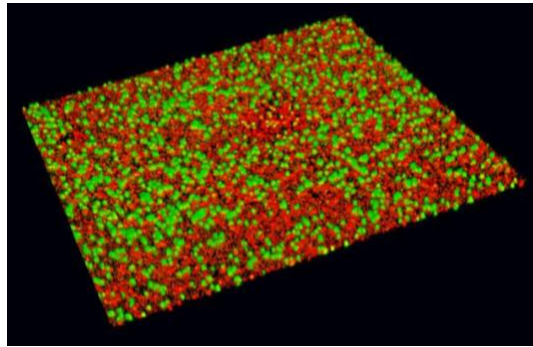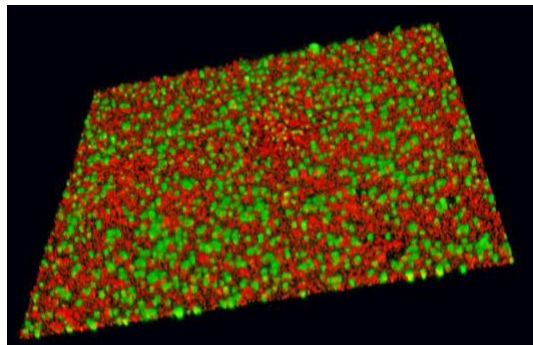
BAR-NP 3 µM

Fig. 18. Confocal microscopy images showing untreated, free BAR treated and BAR-encapsulated NP treated biofilms.

Table 5. Effect of free BAR, sustained release-NPs and BAR-modified NPs on the ratio between *P.gingivalis* and *S. gordonii.* Percentage inhibition in relation to the control is shown in ( ).

	0.3 µM	1.7 µM	3 µM
Control			0.28±0.01
Free BAR peptide			0.26±0.02 (5%)
Sustained-release BAR-NPs	0.25±0.01 (6%)	0.22±0.01 (17%)	0.13±0.00 (51%)
BAR-modified NPs	0.27±0.01 (4%)	0.19±0.01 (27%)	0.11±0.01 (59%)

BAR-modified NPs inhibit established *P. gingivalis/ S. gordonii* biofilms.

Previously it was shown that BAR modified NPs competitively bind to *P. gingivalis* and prevent its initial colonization of the oral cavity (49). However in the oral cavity more complex and established biofilms are present. To determine if BAR modified NPs inhibit *P. gingivalis* on an established *P. gingivalis/ S. gordonii* biofilm, *P. gingivalis* biofilms were formed on immobilized streptococci. Various molar concentrations of BAR-modified NPs in PBS were used to treat the established biofilm for 1 hour. Sufficient amounts of NPs to deliver BAR peptide concentrations ranging from 0.3 to 3 μM were tested. *P. gingivalis/ S. gordonii* microcolonies were visualized using confocal scanning microscopy and the ratio of green (*P. gingivalis*) and red (*S. gordonii*) fluorescence was quantified using Volocity image analysis software. These results were then compared to control biofilms and biofilms treated with 3 μM of soluble BAR. The images of biofilms treated with BAR modified NPs are shown in Fig.19. and quantified in Table 5, BAR-modified NPs exhibited dose dependent inhibition of the dual species biofilm with the ratio of green to red fluorescence being significantly reduced ($P<0.05$) at 1.7 μM and 3 μM. Yet again the striking result was that while soluble BAR required a prolonged exposure of 3 hours, BAR-NPs showed a significant reduction in the ratio of green and red fluorescence in just 1 hour.
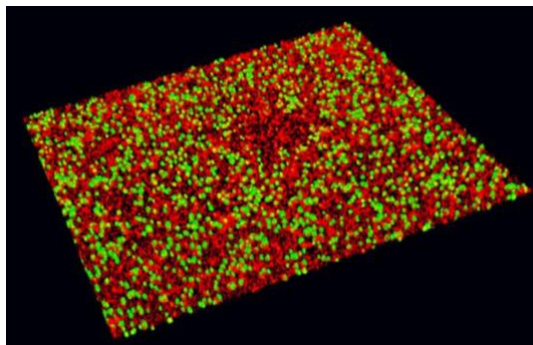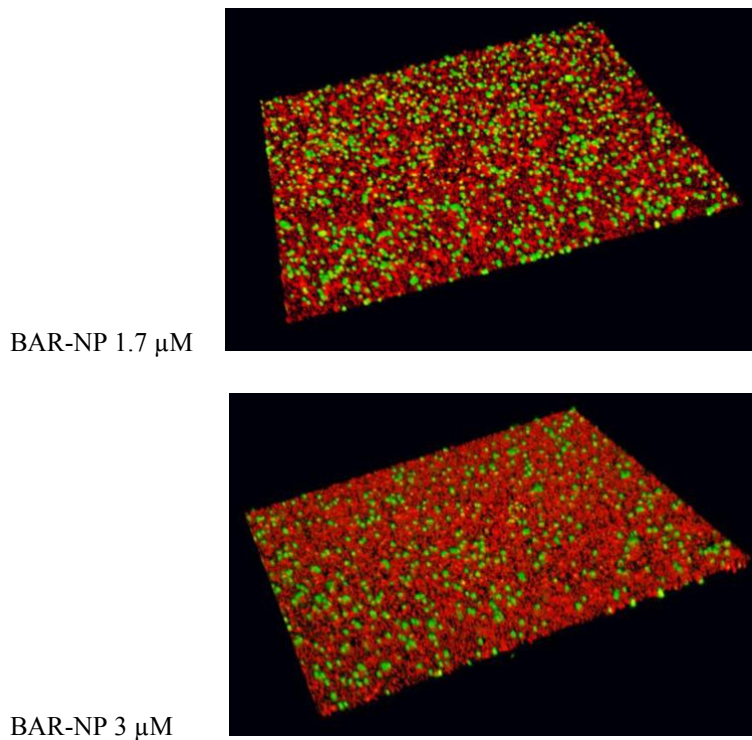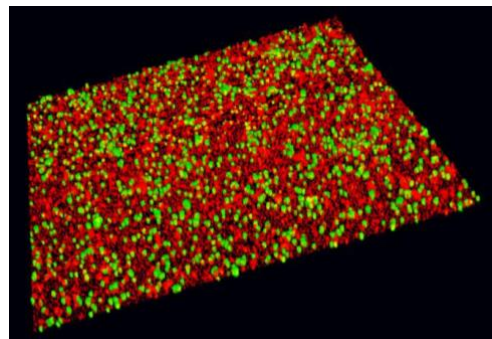

BAR modified NP 0.3 μM

BAR modified NP 1.7 μM
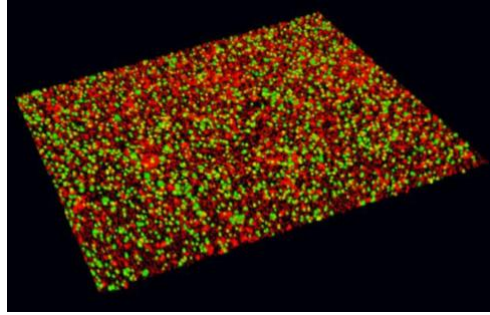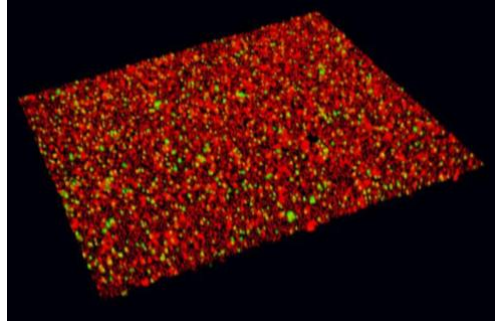
BAR modified NP 3 μM

Fig. 19. Confocal microscopy images showing untreated, free BAR treated and BAR-modified NP treated

biofilms.
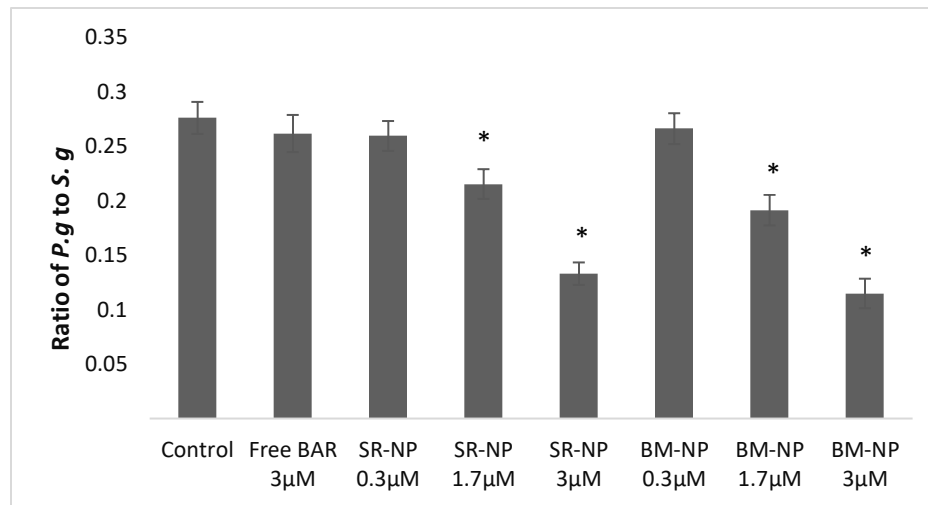
Fig. 20. Ratio of *P. gingivalis* and *S. gordonii* microcolonies treated with free BAR, Sustained released (SR) NPs, BAR modified (BM) NPs. *, the ratio was statistically significant relative to control and free BAR treated biofilms. (P< 0.05)

Expression of CafA adhesion for targeting nanoparticles

Insert gene was amplified using PCR. Gel electrophoresis of the PCR products showed an acceptable size of approximately 2500 base pairs (Fig 21)
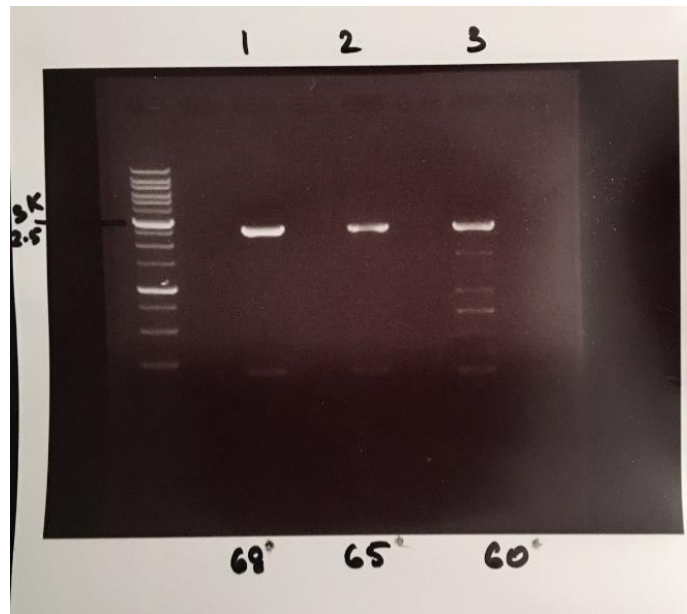
Fig 21. Gel electrophoresis showing PCR products amplifying *cafA* gene.

This insert DNA was the digested and ligated with PQE60 vector and successfully electroporated into XL1 competent cells. Plasmid from recombinant *E. coli* was sent to sequencing. A BLAST® search of the sequencing results showed that the recombinant *E. coli* was coding for collagen binding protein and not CafA.

CHAPTER 5

DISCUSSION

One of the most common infectious diseases of the oral cavity, periodontitis, results in tooth loss and significantly affects patient quality of life. Periodontitis is considered to be a public health problem and a significant economic investment is made on treatment and prevention. The multifactorial disease etiology and, treatments based on limiting the disease progression combined with the decreased bioavailability of antibiotics in the diseased site have all resulted in difficulty managing periodontal diseases effectively.

Periodontitis is an inflammatory disease most often associated with the overgrowth of *P. gingivalis*. The primary niche for *P. gingivalis* is in the anaerobic sub gingival pocket of the oral cavity; however the initial colonization occurs when *P. gingivalis* interacts with oral commensals. One such interaction important for *P. gingivalis* colonization is the species specific interaction with *S. gordonii*. Previous studies have identified that this interaction is mediated by Mfa1 of *P. gingivalis* and SspB polypeptide of *S. gordonii*. This initial interspecies interaction represents a potential target for limiting *P. gingivalis* colonization and prevent periodontal diseases. A synthetic analog of the SspB polypeptide designated BAR was identified. BAR was successful in limiting *P. gingivalis* colonization both *in vitro* and *in vivo*. While BAR is effective in limiting the initial colonization of *P. gingivalis*, it was shown to be less effective against well-established and complex biofilms. Our preliminary studies revealed that a prolonged exposure of BAR can significantly inhibit *P. gingivalis* in an established two species *P. gingivalis/ S. gordonii* biofilm. In this

study, we hypothesized that targeted PLGA nanoparticles encapsulating BAR peptide may increase the potency of BAR by providing a higher localized concentration of BAR for a prolonged duration of time.

We successfully and reproducibly synthesized NPs that encapsulate BAR. BAR encapsulated NPs resulted in a gradual release of BAR. This suggest a therapeutic approach to release BAR to inhibit *P. gingivalis*. NPs continued to release BAR up to 24 hours but the amounts were significantly low. We envision that surface modification of these NPs will help not just to target them but also prolong the release for up to 24 hours. Various polymer blends like PLA, PLCL or different lactide to glycolide ratio of PLGA can be used for synthesis of NPs to prolong the release beyond 24 hours. Our goal is to achieve an optimum concentration of BAR for 24 hours since our ultimate goal is to use these NPs therapeutically.

In addition to prolonging BAR delivery, another approach is to localize NPs in the oral cavity for longer duration. To achieve this and maximize NP efficacy, it is important that the NPs are present in areas of the oral cavity where *P. gingivalis* is present. To target NPs to these niches, a surface adhesin CafA found on *A. oris* can be used. CafA is a surface protein found on *A. oris* which helps it to coaggregate with streptococci. CafA reacts with a surface carbohydrate that is expressed by many of the *streptococcus* species that *P. gingivalis* interacts with.

One strategy is to surface modify NPs with CafA to target them to region where oral streptococci and *P. gingivalis* are present in the oral cavity. Since CafA is not commercially available, the gene that encodes for CafA was amplified and expressed in *E. coli*. The resulting recombinant *E. coli* was unsuccessful in expressing CafA. The potential

reason for the failure in exression of CafA was a mis-anotation of *cafA* gene in oralgen. Further experiments are underway to design new primers to try create a new recombinant *E. coli* to express CafA. If there is difficulty in expressing CafA other proteins like Fab fragments from existing anti-SspB antibodies can be used to surface modifiy NPs.

Nanofibers are polymeric fibers synthesized by electrospinning of the polymer. Electrospinning is rather inexpensive and user friendly process used to fabricate nanofibers. Fibers are traditionally used in gingival sulcus to provide a better localized concentration of drugs. Different fibers used in local periodontal therapy are tetracycline fiber, doxycycline polymer, chlorhexidine chip, etc. The ability to use BAR fibers within the sulcus which is the primary niche of *P. gingivalis* will help in improved local concentration of BAR and better inhibition of *P. gingivalis*.

Electrospun nanofibers encapsulating BAR had a higher payload but the release kinetics showed poor release in the first 24 hours. While this suggests that NFs are capable of releasing BAR over a period of weeks or months, they were unable to deliver at least the IC_{50} concentration of BAR. The reason why NFs were unable to release higher concentration of BAR is not known at this time. Various polymers can be tried to optimize the release kinetics of the NFs. The ease of synthesis and the ability to use in the subgingival pocket makes NFs a very good candidate for delivery of BAR.

Our results show that a 1 hour treatment using a molar equivalent of BAR-NP resulted in significantly higher inhibition of *P. gingivalis* in an established biofilm than a 3 hour treatment using free BAR. We believe that the greater efficacy of BAR-NPs will be helpful in developing NPs in therapeutic formulations such as a toothpaste, mouth rinse or

chewing gum. Surface modification to bind to other oral bacteria might further help in longer bioavailability and higher local concentration of BAR.

BAR-modified NPs have also shown to inhibit *P. gingivalis* in an established biofilm. Together these experiments suggest that NPs can deliver higher localized concentration of BAR sufficient to inhibit *P. gingivalis* in an established two species biofilm. Our results suggest that use of nanotechnology is a novel approach which can successfully be used to prevent oral diseases by limiting pathogen colonization of the oral cavity. We also understand that periodontal diseases have a multispecies etiology. The versatility of nanotechnology to use multiple proteins in the same nanoparticle formulation will allow to target other species of bacteria to limit periodontitis.

Future studies will focus on expression of CafA adhesin which will then be biotinylated and conjugated to avidin-NPs as done in BAR modification of NPs. If necessary, the level of surface associated CafA will be adjusted to increase or decrease the retention on streptococci. Once appropriate retention is achieved, the controlled release profile will be assessed to determine if surface modification has affected the release kinetics. Additional studies will focus on optimizing the total payload by various ratios of polymer formulations used for nanoparticle synthesis. Further experiments will test the ability of targeted sustained release BAR-NPs to reduce *P. gingivalis* colonization both *in vitro* and *in vivo*. Additionally, BAR-NPs can be tested on more complex three species biofilms. Following this, experiments will be done to examine the toxicity of targeted sustained release NPs against human gingival cells and methods to formulate NP preparations will be studied. We are also working on more economical alternatives like peptidomimetics of BAR. If successful in identifying a peptidomimetic for BAR the same

nanotechnology can be used as a delivery vehicle for these peptidomimetics to inhibit *P. gingivalis*. Our long term goal is to develop these NPs to be tested for efficacy in humans in a clinical trial. Studies will be focused to test the toxicity of NPs against human oral gingival and innate immune cells. Once NPs are shown to be non-toxic they will be tested for their efficacy to prevent *P. gingivalis* colonization and re-colonization after treatment.

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