Photodynamically Activated Multifunctional Chitosan Nanoparticles to Disinfect and Improve Structural Stability of Dentin

by

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A thesis submitted in conformity with the partial fulfillment of the requirements for the degree of Doctor of Philosophy
Graduate Department of Dentistry
University of Toronto

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Doctor of Philosophy 2013

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Abstract

Bacteria have been confirmed as the main etiological factor for root canal infection as well as for root canal treatment failure. Thus the success of endodontic treatment depends on the complete elimination of bacteria and prevention of bacterial recolonization in the root canal system. The major challenge for conventional root canal disinfection strategies is the ability of bacteria to persist as biofilms within the anatomical complexities of the root canal system. In addition, the alterations in the ultrastructure of dentin tissue results in compromised structural integrity of root dentin leading to higher risk of fracture in root-filled teeth. The objectives of this study are twofold: 1) develop and test functionalized nanoparticles to eliminate biofilm bacteria and, 2) to stabilize and strengthen the dentin organic matrix by crosslinking collagen fibrils in the presence of biopolymeric nanoparticles. A bioactive polymeric nanoparticle functionalized with a photosensitizer may present as a single step treatment to achieve both the objectives. Chitosan a bioactive polymer was used owing to their inherent antibacterial and biocompatible characteristics. Chitosan micro-/nanoparticles were synthesized as well as functionalized with photosensitizer (rose bengal) for photodynamic activation. Bioactive chitosan nanoparticle functionalized with a rose bengal is expected to combine the properties of chitosan i.e., polycationic with higher affinity
to bacterial cell wall and alter membrane integrity; that of a photosensitizer i.e., to generate singlet oxygen when photoactivated; and the nano-form further potentiate these specific properties. These photodynamically activable chitosan nanoparticles showed the distinct characteristics of chitosan and rose bengal. The synergistic effect of the chitosan conjugated nanoparticles was able to eliminate monospecies and multi-species bacterial biofilms with complete disruption of the biofilm structure. The singlet oxygen generated during photoactivation produced photochemical crosslinking of dentin collagen and infiltration of chitosan nanoparticles. Following crosslinking the dentin collagen showed significantly improved mechanical properties (ultimate tensile strength and toughness) and improved resistance to degradation by bacterial collagenase. In conclusion, this study presents a potential photosensitizer functionalized chitosan nanoparticles based treatment strategy to improve the success of endodontic treatment to achieve complete disinfection of the root canal system and enhanced the mechanical/ structural integrity of the root-filled teeth.
Acknowledgements

With great pleasure in the completion of my project I would like to express my gratitude to all those who provided their kind support and motivation.

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The sincere help from all my group members, Dr. Suja and Arezou helped me a lot in working and learning more. My friend Gursonika whom I confided and shared good as well as bad times played an important role to help me unwind and relax. I am also thankful for the technical support provided by Dr. Milos Legner, Nancy Valiquette, Jian Wang, Henry Hong and Audrey Darabie.

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Annie Shrestha
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Preface

Dissertation format

This dissertation is a compilation of the research work that has been either been published or under review in peer reviewed indexed journals. Chapter 1 presents a general introduction to the subject, and a detailed literature review of the topics pertaining to the research problem. Chapters 2 to 7 are a compilation of the experimental data that have either been published or submitted for publication. They are presented in their published form, other than minor changes made to improve readability and reduce repetition. Chapter 8 serves a general discussion of all the experimental data obtained in the present study. Written permission for reproduction of all publications has been obtained, and is held on file.

Publications reproduced as dissertation chapters

Additional publications


Patent

Title: Multifunctional Advanced Nanoparticulate System for root canal treatment. Inventors: A Kishen, A. Shrestha. USPTO serial number of 61/614235.

Scholarships/ Awards

2011, 2012, 2013 American Association of Endodontics/DENTSPLY Best Paper Award
2012 3rd place poster award, Faculty of Dentistry Research Day
2012-2013 Ontario Graduate Scholarship
2011-2013 CIHR Strategic Training Program (“Cell Signals”) Fellowship (TGF-53877).

2011 1st place oral presentation, SRG Travel Awards, Faculty of Dentistry, University of Toronto
2011 Jean-Marie Laurichesse Research Award, International Federation of Endodontic Associations.

2009- 2013 Harron Scholarship Award, Faculty of Dentistry, University of Toronto
Abbreviations

Ca(OH)$_2$ Calcium hydroxide
CMCS Carboxymethyl chitosan
Cs-np Chitosan nanoparticles
CSRB Chitosan conjugated rose bengal
CSRB-np Chitosan conjugated rose bengal nanoparticles
DA Degree of acetylation
DD Degree of deacetylation
DNA Deoxy ribo-nucleic acid
DQ Degree of quaternization
EDTA Ethylenediamine tetra-acetic acid
EPS Extracellular polysaccharide
FITC Fluorescein isothiocyanate
GAGs Glycosaminoglycans
GD Glutaraldehyde
LD$_{50}$ Lethal dose
MMPs Matrix metalloproteinases
NaOCl Sodium hypochlorite
PDT Photodynamic therapy
PLGA Poly (lactic-co-glycolic) acid
PS Photosensitizer
RB Rose bengal
RNA Ribo-nucleic acid
ROS Reactive oxygen species
TBO Toluidine blue O
TiO$_2$ Titanium dioxide
TPP Sodium tripolyphosphate
VRF Vertical root fracture
ZnO Zinc oxide
“Research is formalized curiosity. It is poking and prying with a purpose.”

—Zora Neale Hurston
Chapter 1
Introduction
1.1 Background

The current success rate of root canal treatment is suggested to be 74%-95% (1-3). A conservative failure rate of approximately 13% in the average population has been estimated to result in 3.3 million failed root canal treatments in Australia and 54 million in the United States (4). Outcome studies confirmed that the prognosis of endodontic treatment depended on the stage of the disease process and whether the treatment is primary or retreatment (5-8). Follow up study after 4-6 years of initial endodontic treatment revealed that the presence of periapical infection at the time of treatment reduced the healing rate from 92% to 74% (5).

The principal objectives of root canal treatment are complete elimination of bacteria, prevention of reinfection of treated root canal, and preservation of structural integrity of dentin for a successful treatment outcome (9-11). However, complete elimination of bacteria from the root canals (9, 12, 13) and maintaining the structural integrity of dentin in endodontically treated teeth has been a daunting task in dentistry (9, 14). The limitation in the complete elimination of microbes has been attributed to the biofilm mode of root canal bacteria and complexities of the root canal anatomy (12, 13).

Mixed community of bacteria (facultative anaerobes, obligatory anaerobes, Gram-positive, and Gram-negative bacteria), existing as biofilms within the root canal system is the main cause of endodontic disease (13, 15-18). The root canal space physiologically is a sterile place, which does not contain a normal microbial flora. Therefore, any microbial species that are found in the infected root canals could be considered as an endodontic pathogen that plays a role in developing the ecology niche capable of causing endodontic disease. Endodontic infection of both primary and persistent nature demonstrated bacterial colonization as typical biofilms in the apical portions of root canal system. The morphology of these bacterial communities was consistent with the acceptable criteria so as to include endodontic disease in the category of biofilm-mediated diseases (15, 16). These infected root canals are conventionally managed using chemical antimicrobials in combination with mechanical instrumentation to achieve effective microbes free root canals prior to filling them with an inert filling material. The widespread recognition of biofilm as the main factor in dental infection has led researchers to work towards improved antimicrobial strategies (19-21) with little attention towards improving the structural integrity of the previously infected dentin. Disease-mediated degradation of the dental hard tissues caused by host/bacterial proteases
and treatment-mediated tissue damages have been reported in the past (23). The disease and treatment-mediated ultrastructural changes in the dentin resulted in a significant decrease in the mechanical integrity and chemical stability of dentin in root-filled teeth (14, 23-25).

Dentin forms the major bulk tooth structure. It is a biological composite consisting of inorganic phase (50 vol%), organic phase (30 vol%) and water (20 vol%) (26). The organic phase of dentin is chiefly type I collagen (90%) and rest are non-collagenous proteins. The collagen on the surface of root canal walls are exposed due to demineralization during disease process as well as due to the application of caustic chemicals during the root canal treatment (14, 27). The degradation of collagen by bacterial proteases and human matrix metalloproteinases have been suggested to result in large resorptive defects inside the root canals (22). Various degrees of collagen degradation were also reported from failed root canal treated cases (23). Surface modifications of the root dentin by crosslinking the collagen could strengthen and provide increased resistance to degradation (28). Crosslinking of dentin collagen using various chemicals have been reported to increase the mechanical properties and resistance to enzymatic degradation (29, 30). However, chemical crosslinking procedures require long periods of interaction (up to 8 hrs) to form stable covalent crosslinks in collagen (31-33) and this could be a major limiting factor in a clinical practice. Combination of a light-activable chemical (photosensitizer), irradiation using appropriate light and resultant singlet oxygen induced rapid and stable covalent crosslinking of collagen (34-36). Photodynamic crosslinking of proteins and collagen was achieved in the presence of appropriate photosensitizers such as riboflavin and rose bengal (RB) (34-37).

Currently, persistence of bacterial biofilm (9, 16) and fractures (38, 39) are two important reasons for extraction of root-filled teeth. This indicates that decontamination of bacterial biofilm and stabilization of previous infected hard tissue still remains to be the major challenges of root canal treatment. Despite many attempts to develop better root canal preparation and filling techniques, there has not been a scientific based approach that can resolve these two major issues of the root canal treatment. Therefore, it can be hypothesized that a treatment strategy that possess antibiofilm capability (to eliminate residual endodontic bacteria/biofilm), as well as stabilize the dentin matrix (to improve ultrastructural stability) will contribute significantly to overcome the shortcomings associated with the current endodontic treatment, and can be expected to improve the overall treatment outcome of root canal treatment.
Chitosan (poly (1, 4), β-d glucopyranosamine) a derivative of chitin, the second most abundant natural biopolymer has received significant interest in biomedicine (40-42). Chitosan and its derivatives show a broad range of antimicrobial activity, biocompatibility and biodegradability (43-46). Chitosan polymers have been considered structurally similar to extracellular matrix components and used to reinforce the collagen constructs (47). This hydrophilic polymer with large number of hydroxyl and free amino groups has been used for numerous chemical modifications and grafting (31, 33, 48). Chemical modifications of chitosan with photosensitizers and antibacterial peptides are also reported in the literature (49, 50). This biopolymer could also be synthesized into various nano-forms (41). Nanoparticles are microscopic particles with one or more dimensions in the range of 1-100 nm (51). Chitosan nanoparticles (Cs-np) by virtue of its charge and size are expected to possess enhanced antibacterial activity. In addition, Cs-np possess several characteristics such as non-toxic towards mammalian cells, color compatibility to tooth structure, cost effectiveness, availability and ease of modification. Cs-np can be delivered within the anatomical complexities and dentinal tubules of an infected root canal to enhance root canal disinfection (52). Incorporation of biopolymers such as chitosan into the dentin-collagen matrix could further reinforce and stabilize the collagen ultrastructure (53, 54). Chitosan possess free amine groups and this is known to amplify the number of crosslinking reaction sites forming ionic complexes with collagen (55, 56). Combining the chitosan-nanoparticles with photoactivable chemical would aid in developing a multifunctional particle that possess enhanced antibacterial efficacy as well as improve the structural integrity of dentin matrix in a root-filled teeth.

1.2 Hypothesis and Objective

It is hypothesized that photosensitizer conjugated chitosan-nanoparticles could markedly enhance the antibiofilm efficacy and crosslink dentin-collagen due to a synergistic mechanism contributed by high affinity of polycationic chitosan-nanoparticles to bacteria/biofilm/dentin and singlet-oxygen released by photodynamic effect (Fig. 1.1). Overall, if applied on the root canal dentin following routine cleaning and shaping and prior to filling, this treatment is expected to improve the antibiofilm efficacy and enhance the dentin structural integrity in a root-filled tooth.
1.2.1 Objectives

The objective of this study is to synthesize and characterize chitosan-nanoparticles and photosensitizer conjugated chitosan-nanoparticles and assess the ability of these cationic nanoparticles and singlet oxygen to (1) disinfect bacterial biofilms, and (2) enhance the resistance to enzymatic degradation and toughness of crosslinked dentin-collagen.

Specifically the proposed study aims to:

1) Synthesize and characterize polycationic chitosan-nanoparticles (Cs-np), rose bengal conjugated chitosan (CSRB) particles and CSRB nanoparticles (CSRB-np).

2) Evaluate and understand the antibacterial efficacy of Cs-np, CSRB and CSRB-np to disinfect *in vitro* monospecies (*Enterococcus faecalis*) and multispecies (*Streptococcus oralis, Prevotella intermedia and Actinomyces naeslundii*) biofilm bacteria.

3) Evaluate the mechanical properties (UTS and toughness) and resistance to enzymatic degradation dentin-collagen crosslinked and infiltrated with of Cs-np and CSRB-np.
1.3. Literature review

This section provides a detailed literature review for the background of this study. General introduction of the bacterial biofilms and details on its resistance mechanism as well as Endodontic biofilms are highlighted first. The dentin substrate changes and the risk factors are discussed thereafter. As the bacterial biofilms and the dentin substrate integrity are the two main issues, the treatment options that will be tested in this study are reviewed in detail. The two possible treatment strategies i.e., chitosan-nanoparticles and photodynamic therapy have been presented comprehensively.

1.3.1. Bacterial Biofilms

Bacteria have been associated with almost 80% human infectious diseases (21, 57). Bacteria persisting as biofilms have received a major attention due to the difficulty in the clinical management of biofilm mediated infections. Ranging from systemic disease conditions to localized oral infections antimicrobial resistance shown by the bacteria is an increasing area of concern (58). Biofilm is the preferred mode for bacterial growth and existence in the disease conditions of oral cavity like caries, periodontal disease and root canal infections (12).

As early as 1933, Arthur Henrici reported, “It is quite evident that for the most part the water bacteria are not free floating organisms, but grow on submerged surfaces; they are of the benthos rather than the plankton” (59). Nearly after 60 years, Costerton defined biofilms as “a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface” (60). Typically, bacteria occupy 10-20% of the total volume of the biofilm and the remainder is the extracellular matrix (61, 62). The extracellular matrix mainly consists of polyanionic exopolysaccharides secreted by bacteria itself. This hydrated envelope not only protects bacteria from noxious threats but also act as a scavenger to trap and concentrate nutrients for favorable growth (20). Dental plaque is one of the most common examples of bacterial biofilms.

Ultrastructure of biofilm consists of a heterogeneously arranged microbial cells in microcolonies that are adherent to a solid surface (Fig. 1.2) (12). These microcolonies are dense aggregates of bacteria arranged based on the physiological and metabolic states within the biofilms. As mentioned above, the polymeric matrix consists of polysaccharides, proteins, nucleic acids
(extracellular DNA) and salts making up to 95% of the biofilm volume (62). The advanced microscopy techniques have helped to determine the structure of the biofilms as “tower” or “mushroom” shaped structures adhered to a substrate. These hydrated structures are known to possess water channels that facilitate efficient interchange of nutrients and waste between bacterial cells and bulk fluid. The channels also act as primitive circulatory system and transfers information between bacterial microcolonies. Depending upon the environment and fluid shear forces, mature biofilms can detach microcolonies. Detachment could be either a continuous process such as erosion or rapid/massive loss of biofilm such as sloughing. This seeding dispersal can determine the morphology and structure of mature biofilms. The microcolonies that are shed from the biofilm possess traits of the parent biofilm and can lead to a distant infection (12, 63).

1.3.1.1 Development of Bacterial Biofilm

The development of bacterial biofilms is a time dependent process and has been well characterized (12). Prior to bacterial adherence to a surface, the substrate surface is coated with a conditioning layer that could be either organic or inorganic molecules. In case of dental plaque/biofilm, saliva forms a pellicle on the tooth surface. The bacteria adhere to this conditioned surface initiating the biofilm formation. Physicochemical properties of the surface; bacterial surface characteristics as well as environmental factors such as pH, temperature, fluid flow rate and nutrient availability all are known to play a part in the initial phase of bacteria-substrate interaction (64). This initial interaction is mainly physicochemical, that is followed by a molecular specific interactions. In this phase, the bacteria substrate interaction could be a combination of electrostatic attraction, covalent and hydrogen bonding, dipole interaction and hydrophobic interaction (64). These bonds gain strength and become irreversible with time. Finally specific microbial-substrate adhesion phase is produced via polysaccharide adhesin or ligand formation. The bacteria specific adhesin or ligand interacts with the receptors on the substrate and this adhesive potential is one of the determinants of biofilm development (65).

Once the bacteria are adhered firmly onto a substrate, the biofilm grow in size by colonization of secondary colonizers. The microcolonies formed by newly colonized bacteria could result in the lateral and vertical expansion of biofilm structures. The mature biofilm consists of microcolonies of bacteria that share specific roles in nutrition and support structures. At the cellular level there
Figure 1.2. Schematic diagram of stages of biofilm formation.
are two distinct types of bacterial interaction and are highly specific. Co-adhesion is the interaction of suspended or floating bacteria with the ones that are already adhered to a surface. Whereas, co-aggregation occurs between two genetically distinct bacterial cells in the suspension (65). One of the classic examples of co-aggregation is the association of long-filamentous bacteria *F. nucleatum* with spherical-shaped cocci resulting in a characteristic corncob structure in dental plaque (66).

1.3.1.2 Antimicrobial Resistance

Bacteria growing as a biofilm have 1000-1500 times greater resistance to antibiotics than planktonic bacteria (67). The biofilm resistance mechanism could be categorized as: (1) protection by the extracellular polysaccharide (EPS) matrix- diffusion barrier; (2) physiological state of bacteria- slower growth relative to planktonic cells; and (3) altered microenvironment and phenotype- differential expression of genes/proteins (12, 21, 58). The antimicrobial resistance in biofilms is a complex phenomenon, wherein one or more mechanisms go hand in hand (Fig. 1.3).

*Protection by EPS matrix:* EPS matrix is known to impart resistance to antimicrobials by acting as a physical barrier or neutralizing the chemicals applied. The diffusion of antimicrobials is limited to the surface of a biofilm structure enabling bacteria to survive deeper in the biofilms. The anionic EPS also act as a chemical barrier. The charge and interwoven dense structure reduces the penetration of antimicrobials ionic or electrostatic interactions (67, 68). Mostly the antimicrobials used are positively charged hydrophilic compounds that can bind with EPS. Antimicrobial agents such as iodine, chlorine and peroxygens have been shown to be neutralized by constituents of biofilm polymeric matrix (69).

*Physiological state of bacteria:* Persister cells are a small population of non-growing cells within a biofilm that contribute to antibiotic tolerance (70). These phenotypically different bacteria have very low metabolic activity, which allows them to enter a dormant state and survive with minimal nutrient requirement. As most antibiotics target actively growing cells, these persister cells are spared. However, once the antibiotic is stopped and given a favorable condition these persisters are known to repopulate the biofilm leading to treatment relapse (70).
Figure 1.3. Schematic showing mechanisms by which bacterial biofilms could resist antimicrobials. (1) Decreased penetration: presence of extracellular polysaccharide; (2) Decreased efficacy: altered environment (low O$_2$); (3) Trapped and destroyed by the enzymes; (4) Expression of genes: Efflux pumps; (5) Inactive: Persister cells.
**Altered microenvironment and phenotype:** The mature biofilm consists of multi-layered microcolonies of bacteria embedded in EPS matrix. This creates a gradient of nutrients and redox potential that offers surface bacteria advantage of higher growth rates (71). The deep-lying bacteria has limited access to nutrients and oxygen, the metabolic rates are reduced thus allowing bacteria to resist antimicrobials. The enhanced tolerance to antibiotics have been suggested as these chemicals target bacterial cellular processes such as DNA replication or translation as in actively growing cells. Thicker the biofilms with abundant EPS matrix, more tenacious they become to be removed by antimicrobials. In addition, exposure to stress or low levels of antimicrobials is known to result in the expression of certain stress genes, shock proteins and multi-drug efflux pumps in biofilm bacteria (72). The multi-drug efflux pumps are membrane bound active pumps found in both Gram-positive and Gram-negative bacteria and have been considered as one of the key factor in case of biofilm resistance (73, 74).

### 1.3.1.3 Endodontic biofilms

Endodontic infection or apical periodontitis has been established as a bacterial mediated infection as early as 1960s (75). Kakehashi confirmed that the presence or absence of a microbial flora is the major determinant in the healing of exposed rodent pulps (75). The causative bacteria exist within the apical region of root canals, release toxins and irritants and induce periapical inflammation in conjunction with the inflammatory and immune response (76, 77). However, there is no species specific correlation with apical periodontitis and bacterial communities are shown to follow particular patterns related to different clinical conditions (78). Despite the early recognition of bacteria as a culprit for apical periodontitis, it was not until 1987 that Nair showed bacteria existing as biofilms within the infected root canals using ultrastructural microscopic techniques (15). Current histopathological studies stresses that apical periodontitis is a biofilm-mediated disease (16, 79, 80).

The microbial flora of infected root canals has been determined based on various cultures, histological and molecular microbiology techniques. The earlier studies mainly used the first two methods with more and more research being carried out using PCR by newer studies. Infected root canals contain a restricted assortment of species (approximately 5-10) (81, 82) compared to more diverse and more than 500 species of the oral cavity. This has been mainly associated with the selective pressure exerted by the endodontic environment (83) (Fig. 1.4). Primary endodontic
Niches in the root canal and limiting factors

Coronal Third:
High O₂ tension
Nutrients available from the oral cavity

Main canal:
Low O₂ tension
Reduced nutrients from the oral cavity

Apical Segment:
Very low O₂ tension
Nutrients available from the periapical tissues
Bacterial by-products

Colonization will depend on:
• Redox potential
• Nutrients
• Symbiosis
• Antagonisms
• Host immunocompetence

Figure 1.4. The root canal space presents with selective pressures allowing specific bacteria to survive depending on their nutritional and environmental requirements. The schematic diagram is adapted and modified from Chavez (83).
infection generally showed presence of 5-7 species/canal (maximum 10) with bacterial numbers in the range of $10^2$-$10^8$ CFU/ root canal (84). The endodontic infection is more of polymicrobial in nature with Gram-positives (66%) dominant over Gram-negative species (34%) and higher percentage of strict anaerobes (84-86). Obligate anaerobes dominated in infected root canals up to 98% of the flora by day 1060 and facultative anaerobes decreased over time (87). In case of teeth with exposed pulp chambers due to caries, facultative anaerobic bacteria such as Streptococci may be present in significant numbers (86). Sundqvist et al. reported prevalence of these bacterial species: *Fusobacterium*, *Bacteroides*, *Eubacterium*, *Peptococcus*, *Peptostreptococcus* and *Campylobacter* in patients’ teeth with necrotic pulps when periapical destruction was present (81). Moreover in the apical region, fastidious, slow growing, obligate anaerobic bacterial cells clearly outnumbered the more rapidly growing, facultative anaerobic bacterial cells (82, 85). “Eight strain collection” of bacteria (*Streptococcus milleri*, *Streptococcus faecalis*, *Fusobacterium nucleatum*, *Fusobacterium necrophorum*, *Bacteroides oralis*, *Peptostreptococcus anaerobius*, *Propionibacterium acnes*, *Actinomyces bovis*) from auto-infected root canals were inoculated in sterile root canals and followed for microbiological assessment. The quantitative relative distribution of the bacterial strains was restored similar to the original proportion of the different bacteria belonging to the “eight-strain collection”. Bacteroides dominated and certain combinations of bacteria were more potent in inducing apical periodontitis than single strains (82). Prevalence of spirochetes in cases with larger apical lesions have been shown using electron microscopy studies (15). Other than these, studies have also reported more diverse and higher incidence of *Treponema* species in symptomatic root canals (88). The “community-as-pathogen” concept has been suggested to be more relevant with typical bacterial communities related to certain form of disease conditions than others (89). Role of microorganisms in the pathogenesis of endodontic disease has helped to develop a sound rationale for effective management of patients with endodontic infections. Consequently one of the main treatment goals of endodontic treatment is to eliminate microbes in an infected root canal.

The selection of particular combinations of bacteria could be attributed to the endodontic milieu that provides a selective habitat that supports the development of specific proportions of the anaerobic flora (81, 86). Oxygen deplete with time and zone of the root canal (apical portion), thus a proportionate decrease of facultative bacteria and concomitant increase of strict anaerobic bacteria with time and depth into the canals were observed. The strict nutrient supply favors
fastidious organisms those are able to utilize the tissue fluid and tissue disintegration products. Proteolytic bacteria also utilize amino acids and peptides as energy sources. As a biofilm community the bacteria inside the root canals show a metabolic synergism. In this case the metabolism of one species may supply essential nutrients for the growth of other members (86). Proteolytic enzymatic activity of Peptostreptococcus micros utilizes amino acids and peptides available from serum glycoproteins. Other bacteria without proteolytic activity could co-exist with P. micros, which also depend on the amino acids and peptides for metabolism. Another example is that of Campylobacter rectus that produces growth factors related to hemin and stimulates the growth of Porphyromonas species as they have specific nutritional requirements (vitamin K and Hemin).

Presence of microbes at the time of filling was one of the deciding factors for the successful outcome of endodontic treatment (17, 90). 79% of endodontically treated teeth with the presence of bacteria showed periapical lesions as compared to 28% in those without bacteria (17). Nair also showed that bacteria in anatomical complexities of root canals existed as biofilms and resisted the conventional chemo-mechanical preparation of root canals (13). Almost 88% of the cases showed persistent bacteria as determined using microscopic techniques. Recently Ricucci and Siqueira evaluated the prevalence of biofilms in primary and persistent infections using histopathological techniques (16). They reported intraradicular biofilms in almost 74% of the treated root canals with apical periodontitis at the apical segment. The biofilms from different individuals were different in terms of the relative proportions between bacterial cells and the EPS. The biofilms from the main canal were found to be invading into the dentinal tubules and apical ramifications. Though extra-radicular biofilms have been reported as a causative factor for persistent infections (9, 91), their prevalence has been reported to be on the lower side (6%). As bacteria persisting within the treated root canals have been considered as the major cause for treatment failure (13, 83, 92), research have focused on to characterize the specific bacteria associated with persistent infections and treatment modalities to improve their elimination.

The microbiota in cases of failed root canal treatment has been established to be markedly different to that of primary endodontic infection. As mentioned above, selective pressures exist in the untreated canals that favor the establishment of a very restricted group of the oral flora. For a microorganism to persist in the root filled canals more stringent conditions are applicable. It is highly plausible to conclude that those bacteria withstanding antibacterial measures during
cleaning and medication; and ability to adapt the nutrient starved environment inside the treated root canals could survive in the post-treatment cases (92). Facultative anaerobes (57.4%- 69%) & Gram positive organisms (83.3%) have been shown to predominate (93). Unlike the primary infection, the number of species detected inside the treated canals also is limited (1 to 3) (93). The facultative organisms such as Actinomyces, Enterococcus, Propionibacterium and Candida species were more prevalent. Sunde et al. evaluated the microbial population from 36 patients with refractory apical periodontitis (94). These cases were treated with conventional therapy along with calcium hydroxide medication. Almost 80% of the facultative anaerobic polymicrobial flora was composed of Gram-positive strains. Out of 36 lesions 27 revealed the presence of Staphylococcus, Enterococcus, Enterobacter, Bacillus, Pseudomonas, Stenotrophomonas, Sphingomonas, and Candida species. Although most of the cases were associated with polymicrobial flora, monospecies infection has also been reported consistently (9, 95). Among the monospecies bacterial infection, Enterococcus faecalis has often been found in the range of 12 to 77% of cases (93, 95, 96). E. faecalis is a Gram positive facultative anaerobe. It is not commonly found within untreated canals and make up a small proportion of the flora in primary infection (92). However, the frequent finding of these bacteria in persistent or refractory infections even as a monoinfection (87) had led researchers to explore the more specific characteristics of E. faecalis that enable them to survive in harsh environmental conditions.

One of the most common intra-canal medicaments, calcium hydroxide has been found to be ineffective in killing E. faecalis (97). E. faecalis had the ability to withstand high pH stress. Thus post calcium hydroxide treatment further provided the ecological selectivity for E. faecalis in reinfection (98). Other studies reported that E. faecalis formed biofilms of different nature under nutrient starvation and used dentin as a source of energy (99). These bacteria also invaded dentinal tubules up to 1500 μM (96, 99). In addition, specific stress response genes have been reported to provide E. faecalis the advantage of surviving inside a stringent environment (9, 100). The capability of enduring prolonged starvation by E. faecalis, as well as ability to foster once the nutritional requirements (serum) improve proved to be advantageous for their survival as a pathogen in persistent infections (101).
1.3.1.4 Treatment of Endodontic Biofilms

A thorough chemo-mechanical preparation of the root canal followed by a complete seal using an obturation material is desired for a successful root canal treatment. Various antimicrobial and chelating agents have been tried and tested to achieve this goal. Some requirements of the ideal root canal irrigants have been noted as: 1) broad antimicrobial activity to eliminate biofilms of anaerobic and facultative anaerobic bacteria and yeasts; 2) Dissolve necrotic pulp tissue; 3) Able to inactivate the endotoxins; 4) Able to remove smear layer following instrumentation and provide a clean root canal surface; and 5) Biocompatible to vital periapical tissues if extruded beyond the root canal space (102). Even the best of root canal irrigants cannot eliminate bacteria from areas where instruments are not able to reach. Till date, there is no antimicrobial irrigant that could perform all these functions single-handedly. Therefore, combinations of two or more irrigants are used to achieve a bacteria free and clean root canal surface prior to filling with gutta-percha (102).

Studies have shown that despite thorough chemo-mechanical preparation of root canals, bacteria still exists in significantly high numbers within the uninstrumented areas of root canals such as isthmuses and lateral canals (13, 103-105). Peters et al. showed that 35% of the root canal surfaces remain untouched by the instruments invariable to the methods used (106). Histological sections of the apical portions of the chemo-mechanically prepared root canals revealed presence of bacteria in 86% of cases mainly as biofilms in the complex anatomical sites (13). In vitro studies have shown biofilm containing live bacteria within calcified structures in root canals which shows their ability to sustain in constrained environments (107). In terms of bacterial re-entry/recolonization studies comparing the efficacy of different obturation techniques and materials have shown that leakage occurs through the root filled canals (108). Therefore it may be concluded that even after application of various intra-canal medications, complete elimination of bacteria from the root canal system could not be achieved (90, 109).

1.3.1.5 Summary

Biofilm mode of bacterial growth within the root canals presents as one of the important limitations for effective endodontic disinfection. In addition, anatomical factors (root canal complexities, dentinal tubules and dentin composition), and chemical factors (concentration, penetration into dentin and buffering activity) are equally significant in determining effective biofilm elimination for treatment success (9, 14). Due to the shortcomings of the current anti-biofilm strategies during
root canal treatment, advanced disinfection strategies are being developed and tested in Endodontics. These newer disinfection strategies is aimed to eliminate biofilm bacteria not only from the main canals but also from the uninstrumented portions and anatomical complexities of the root canal system without inducing untoward effects on dentin substrate and periradicular tissue.

1.3.2 Dentin Tissue Changes

The root-filled teeth have been shown to be prone to fracture as compared to their vital counterparts. Gathering evidence from various clinical surveys, the prevalence of vertical root fracture (VRF) has not been well established till date. The prevalence of VRF in endodontically treated teeth was reported in wide range depending on the method of evaluation (39, 110-115). The studies that diagnosed VRF using radiographs (111, 114) reported the lower percentage of prevalence (2-5%) in contrast to the studies that used extracted root filled teeth to evaluate VRF (11-20%) (39, 115). VRF could be attributed to the compromised dentin structure that further could be caused by a multitude of factors. These factors can be broadly classified into iatrogenic and non-iatrogenic. Iatrogenic damage to the dentin occurs due to the strong and caustic root canal irrigants and medicaments used for cleaning of the root canals (24, 116). Dentin degradation/resorption during disease process (22, 117) and post-treatment degradation of dentin surface collagen by bacteria and MMPs (23, 118) are the non-iatrogenic damages.

Dentin is a composite material comprised of an inorganic component (50 vol%), organic component (30 vol%) and water (20 vol%) (26). Understanding the dentin microstructure in a hierarchical order is important to evaluate the structural and mechanical properties. The inorganic component is mainly carbonated nanocrystalline apatite minerals. These minerals are closely associated with the collagen scaffold either intrafibrillar or extrafibrillar (119). The apatite crystals (∼5 nm thick) are needle-like near the pulp and plate-like towards the dentino-enamel junction. Type I collagen is the major structural protein making up to 90% of the organic fraction (26). Type I collagen roughly 100 nm in diameter, exists as fibrils in a dentin which are stabilized by endogenous covalent intermolecular crosslinking (120). The collagen fibrils are randomly oriented in a plane perpendicular to the plane of dentin formation or dentinal tubules axis (121). In addition the three-dimensional fibrous polymer exists in an aqueous environment. The 10% of the organic phase consists of various phosphoproteins and other non-collagenous proteins. Proteoglycans and
glycosaminoglycans (GAGs) are known to be closely associated with collagen fibrils via hydrogen bonds. The proteoglycans and the hydrophilic anionic GAGs side-chains interact with one another forming interfibrillar bridges. These molecules absorb water and span the interfibrillar spaces thereby regulating mechanical properties of the dentin collagen matrix (122). The structural integrity of dentin provided by the inorganic and organic fraction is crucial to retain the function of a root-filled tooth (Fig. 1.5) (14). Other than the loss of tooth structure and altered proprioception, changes in the physical properties of dentin could contribute to the increased propensity of fractures in root-filled teeth (14, 123).

Dentin at a structural level exists as a fiber-reinforced composite. It is traversed by dentinal tubules that run continuously from the dentino-enamel junction towards the pulp canal in the root. The less mineralized intertubular dentin forms the matrix and the highly mineralized peritubular dentin forms the fiber reinforcements. The alignment of the dentinal tubules is also known to govern the mechanical properties depending upon the direction of force applied (121). The mechanical stability of biologic composites depends on the optimum balance between toughness and stiffness (124). The mechanical properties of dentin such as, Young’s modulus, tensile and compressive strength, and fracture toughness are the result of the complex interactions of its constituents as well as the microstructural arrangement. It is established that the collagen fraction of dentin contributes to its toughness and ultimate tensile strength, while the mineral fraction contributes to its elastic modulus and compressive strength (14, 125). The also act as a cushion in between the inorganic fractions of dentin (14, 126). Toughness is the total energy absorbed by a structure before it fractures (fracture resistance) whereas; stiffness is related to the elastic modulus of the material. The fracture mechanics approach has been suggested to be more appropriate to understand the failure of treated and untreated teeth as compared to the strength of this biological composite material (14, 121).

The water content of dentin exists as a free or “unbound” water and “bound” water. The unbound water is found in dentinal tubules and other porosities in the dentin (127). This water plays a role in keeping the dentin matrix washed in minerals such as calcium and phosphate. Free water is known to be lost by heating at 100 °C (128) and 85% of water is lost in the first 30 min of dehydration (129). As for the bound water, it is associated with the inorganic apatite crystals and organic phase (collagen and non-collagenous proteins). They are tightly bound in nature and are
Figure 1.5. The role of different constituents on the mechanical integrity of structural dentin (adapted and modified) (14).
reported to require much higher temperatures (600 °C) to be removed (128). The water forms a mono-layer of its molecules on the surface of hydroxyapatite via hydrogen bonds and then after van der Waals forces (130). Bound water also forms an integral part and stabilizes the triple helix of collagen molecule. Each tripeptide is known to consist of two water molecules (131). As the number of water molecule increase per collagen molecule, it swells laterally and water is known to act as a plasticizer. Dehydration of collagen leads to increased stiffness mainly due to formation of interpeptide hydrogen bonds that was previously inhibited by the hydrogen bonding with water. The overall diameter of the fibrils reduces as the interfibrillar spaces shrink and additional bonds are formed. Loss of water in dentin reduced the elastic modulus of 23.9 GPa to 20 GPa as determined by nanoindentation-based experiments (126). Other than increase in stiffness with dehydration of dentin at room temperature for 7 days, the toughness values also decreased significantly (127).

The iatrogenic factors could supplement the changes caused by the pathological process in dentin and further compromise the ultrastructural/mechanical integrity of teeth (14, 22, 39, 132). Clinical studies have reported internal resorption of root dentin in 75% of teeth with post-treatment disease (132). Bacterial collagenolytic enzymes and activation of host-derived MMPs has been suggested as the possible reason for such resorption/degradation (133). Commonly used root canal irrigants during treatment have been shown to remove the inorganic phase and/or the organic phase of the dentin (14, 24). Removal of the organic and inorganic fractions of dentin could compromise the mechanical integrity of teeth. Overzealous application of chemicals/medicaments has led to erosion and microcracking of dentin (14, 23, 134). Ethylenediamine tetra-acetic acid (EDTA) and sodium hypochlorite (NaOCl) are commonly used for root canal debridement. NaOCl is a deproteinating agent that results in the heterogeneous removal of organic substrate from dentin. Whereas EDTA is a chelating agent that demineralizes the dentin and exposes the surface collagen fibrils (26). The changes in the dentin substrate following application of common root canal irrigants resulted in almost 75% decrease in the mechanical strength (24). NaOCl resulted in the decrease of mechanical strength of root dentin up to 59% (135). Similarly, the common intracanal medicament Ca(OH)₂ also reduced the mechanical strength by 32% (135). The caustic and strong alkalinity of these chemicals may denature the carboxylate and phosphate groups, which leads to collapse of the dentin structure. The disruption could take place due to neutralization, dissolution or denaturing of the dentin organic proteins and proteoglycans, which serve as bonding agents
between the collagen network and the hydroxylapatite crystals in dentin (136, 137). The exposed and degraded dentin collagen would also serve as a good substrate for bacterial adherence and early biofilm formation (138).

In addition, routine instrumentation and filling procedures involving removal of bulk dentin may induce defects (micro-crack) in root dentin (139). Instrumentation of the root canal using rotary instruments is currently an important step in the shaping and disinfection of infected root canals. The wide range of rotary instrumentation systems available today has been tested in vitro to assess the damage to dentinal walls post-instrumentation. These in vitro experiments have revealed that canal preparations with rotary instruments resulted in defects on the root canal wall (139, 140). Additionally, these defects will serve as stress concentration zones that might further deteriorate during root filling procedures or function (chewing) (14, 141). Despite many attempts to improve the strength of root filled teeth with the help of a filling material (obturation), there is no such treatment available that restores the mechanical integrity of dentin.

Clinical studies have shown that dentin collagen from failed root canal treated cases presented with varying degrees of degradation depending upon the years of clinical function (23). Such dentin collagen revealed collagenolytic and gelatinolytic activities. These dentin surface changes could have further resulted in interfacial leakage that can aggravate the failure rates of root filled teeth (28). The enzymatic degradation of the exposed surface dentin collagen has been attributed to both extrinsic factors such as hydrolysis due to fluids leakage and intrinsic factors such as bacterial and human MMPs (23, 142-144). MMPs are zinc dependent endopeptidases that are capable of digesting extracellular matrix (ECM) in both physiological and pathological conditions (145). Studies have shown the presence and activity of MMPs-1, -2, -8 and -9 in human dentin and odontoblasts during various pathological processes (133, 146-148). These MMPs are thought to play a significant role in degradation of exposed collagen fibrils created within the hybrid layers during acid and ethylenediaminetetraacetic acid (EDTA) treatment (134, 149). Incompletely infiltrated collagen fibrils within the hybrid layers were degraded by the host-derived proteases in the absence of bacteria (23, 25). In addition proteolytic activation of latent pro-MMPs 2 and 9 by bacterial components has been associated with periapical bone resorption (143). Concerns of root dentin degradation following the use of adhesive restorative materials due to activation of MMPs have also been highlighted (23, 150). Commonly used acids such as EDTA and phosphoric acid have been shown to compromise the mechanical and biochemical properties of dentin due to host-
derived protease activity (151). Inhibition of MMPs activities has been studied extensively as an approach to control the pathological degradation of ECM. Synthetic peptides, chemically modified tetracycline, bisphosphonates, compound isolated from natural sources and chlorhexidine are being considered as potential MMPs inhibitors (151, 152). Although the iatrogenic and non-iatrogenic changes occur in the inner dentinal walls of root canals, they could act as vulnerable areas of stress concentration and fracture initiation eventually leading to vertical root fractures (14, 153).

Attempts to strengthen the surface dentin collagen have been investigated in dentistry (29, 125, 154). Crosslinking of collagen to produce additional crosslinks has been suggested as a potential method to increase the structural stability of dentin collagen (125). Crosslinking agents that are biocompatible with low cytotoxicity, forming stable crosslinked collagen in short reaction time is desired to crosslink root canal dentin during treatment. In vitro studies have shown increase in the mechanical strength of dentin collagen following glutaraldehyde crosslinking (155). On the similar lines, attempts to neutralize the dentin proteases as well as strengthen dentin collagen have been targeted in restorative and endodontic applications (30, 156, 157). Crosslinking of dentin collagen after chemo-mechanical preparation and prior to application of root filling material may aid in stabilizing the collagen matrix against enzymatic degradation as well as increase dentin mechanical properties of root treated tooth. However, limitations of crosslinking could not be overlooked. Dentin is a tough material owing to the organic, inorganic and water constituents. Formation of additional crosslinking could result in a brittle behavior that could predispose to higher fracture prevalence. Thus the degree of crosslinking is highly important. Incorporation of biocompatible polymers into the collagen matrix with ability to neutralize MMPs as well as enhance the mechanical property following crosslinking could be beneficial. One such bio-inert polymer, chitosan possesses the required characteristics. Collagen stability is a critical aspect in biological tissues including dentin and needs to be addressed in greater detail.

1.3.2.1 Summary

The compromised dentin structure in root-filled teeth reinforces the need for an alternative treatment strategy to enhance the ultrastructural stability of dentin matrix. One of the alternatives to increase the resistance of exposed collagen fibers is to crosslink the exposed dentin collagen. Depth and degree of crosslinking and time of interaction required to produce stable crosslinks are
few of the limiting factors. The more detailed description of collagen crosslinking will be provided in the photodynamic therapy: antibacterial and collagen crosslinking (1.3.4).

1.3.3 Chitosan: Nanoparticles and Functionalization

Chitosan (poly (1, 4), b-d glucopyranosamine) is obtained from deacetylation of the second most abundant polymer, chitin. Chitin was first isolated from fungi by Braconnot in 1811. It derives its name from its source i.e., “tunic or cover”. Odier extracted chitin from the elytrum of a beetle (158). The industrial extraction of chitin is generally obtained from the crustaceans such as crabs, lobsters and shrimps making up to $10^{13}$ Kg in the biosphere (159). The structure of chitin closely resembles that of cellulose and both act as a structural support and defense material in living organisms. The linear polysaccharide composed of $\beta$ (1 → 4) linked units of $N$-acetyl-2-amino-2-deoxy- D-glucose is insoluble in most of the common solvents (Fig. 1.6). The length of a chitin molecule could range from 5000 to 8000 glucosamine units in crabs and only 100 residues in case of yeasts (159). The rigid and highly ordered crystalline structures of chitin stabilized with hydrogen bonds are mainly between acetamido groups are responsible for the insolubility. The deacetylation of chitin resulted in chitosan with two $\beta$ (1 → 4) linked structural units viz. 2-amino-2-deoxy- D-glucose and $N$-acetyl-2-amino-2-deoxy- D-glucose. Chitin has two reactive groups: primary (C-6) and secondary (C-3) hydroxyl groups, allowing for various chemical modifications. Chitosan has additional amino (C-2) group on each deacetylated unit.

Chitosan is obtained following chitin deacetylation by the hydrolysis of acetamide groups to be replaced by amine groups at high temperature and strong alkaline medium. To obtain chitosan with higher degree of deacetylation a multistep process of deacetylation-washing-drying treatments has been suggested (160). The chitin is treated with 50% (w/v) NaOH, at temperatures ranging from 80-110°C under an argon atmosphere. This method of deacetylation presents disadvantage of long processing times and large of amount of alkali requirement. Despite attempts to increase the degree of deacetylation (DD) most commonly available commercial chitosan has 75-85% DD mainly due to the inaccessible amide groups in the chitin. The DD and molecular weight of chitosan are the two most important properties as physicochemical, functional and biological properties depend on them (161).

The degree of acetylation (DA) is the percentage of N-acetylated glycosidic units in chitin or chitosan. Degree of deacetylation is more commonly used and calculated as 100-DA percent.
Figure 1.6. Schematic of the chemical structure of (a) chitin; (b) cellulose; and (c) chitosan (completely deacetylated) (158).
Infrared spectroscopy is one of the simplest and reliable available techniques to determine DD. The higher the DD, more the number of primary amine groups that are free for chemical reactions, interaction with negatively charged particles and chelate metal ions. The charge density also depends on the available glucosamine units. Molecular weight could affect the properties of chitosan when designed for any specific applications. Mechanical properties of hydrogels, pore size of membranes and scaffolds, particles size of nanoparticles, permeability of epithelial cells, antimicrobial properties and drug releasing ability of micro capsules and nanoparticles all are known to depend on the molecular weight of the parent polymer (159, 162). Low molecular weight chitosan is soluble at neutral pH whereas, acidic pH is required for high molecular weight ones (>10^6). Chitosan is cationic at neutral or basic conditions with free amino groups (41). Low aqueous solubility of chitosan at physiological pH of 7.4 is a major limiting factor for its biomedical and pharmaceutical applications. Its pKa value is 6.3 and at acidic pH, it becomes soluble due to protonation.

\[
\begin{align*}
-NH_2 + H_2O & \rightleftharpoons -NH_3^+ + OH^- \\
\leftrightarrow
\end{align*}
\]

\[
\begin{align*}
\text{NH}_3^+ & \xrightleftharpoons[Ka]{\text{Ka}} \text{NH}_2 + H^+ \\
\text{Ka- dissociation constant}
\end{align*}
\]

The solubility of chitosan has been improved by modifying the reactive functional groups at C-2 (amino group); C-3 (primary hydroxyl) and C-6 (secondary hydroxyl) positions to obtain application specific chitosan derivatives (159, 163, 164) (Fig. 1.7). Several modified chitosans are being developed to suit a wide variety of applications. The most commonly used modifications are; carboxymethyl chitosan, highly cationic chitosan, polyurethane-type chitosan, hydrophobic chitosan and hydroxyalkyl chitosans. Carboxymethyl chitosan is obtained by using glyoxylic acid with end product of glucan carrying pendant glycine groups. Carboxylation of the hydroxyl or amine groups occurs during the process and could be controlled by reaction temperature and duration (163). This water-soluble form is commonly used as a valuable functional ingredient of cosmetic hydrating creams due to its moisturizing effect (159). The second modification, highly cationic chitosan could be obtained by quaternization of primary amine group of chitosan (164).
Chitosan is alkylated by reacting with excess of iodomethane using \( N \)-methyl-2-pyrrolidone as solvent in strong alkaline conditions. The degree of quaternization (DQ) could be controlled by varying reaction time and steps. DQ is an important characteristic as solubility, cytotoxicity, membrane permeability and antibacterial efficacy all depends on this. DQ of 40-50% has been suggested to be optimal to enhance the penetration of chitosan into the cells and tight junctions \((164, 165)\).

**Figure 1.7.** Schematic showing possible modifications on chitin and chitosan polymers where \( R_1, R_2 \) and \( R_3 \) are the reactive functional groups. These reactive functional groups could be replaced by \(-\text{COCH}, -\text{CH}, -\text{CHCOOH}, -\text{SO H}, -\text{PO (OH)}, \) etc.
Chitosan polymers are considered structurally similar to the extracellular matrix glycosaminoglycans (166). Extracellular matrix proteins such as proteoglycans and glycosaminoglycans are known to provide mechanical stability and compressive strength to the collagen by intertwining with the fibrous structure. These ECM proteins also influence cell behavior and growth. Chitosan composites with collagen could reinforce the collagen constructs as well as create a more suitable biomimetic environment for cells (47). Chitosan possesses low solubility at a physiological pH of 7.4 due to its rigid crystalline structure and primary amino group residues. However, conjugation of chitosan with rose bengal results in water-soluble particles even at higher pH (49). Of the various attractive biological properties, antimicrobial activity and accelerated wound healing are more researched upon. Chitosan is known to be a non-toxic, biologically compatible polymer allowing its widespread use in biomedical applications. Certain countries have approved its use in dietary supplements (167). FDA has also approved its use in wound dressings (159). Oral administration of chitosan has been suggested to chelate fat and reduce cholesterol, as it is not absorbed by the gastro-intestinal tract. The biodegradation in vivo takes place via breakdown of the polymer by lysozyme and bacterial enzymes in the colon. The lower molecular weight by products are then excreted via renal clearance (168). The intracellular distribution of chitosan is reported based on in vitro studies. The toxicity of chitosan has been compared that with sugar or table salt with similar lethal dose (LD50) (169). Mao et al showed that different molecular weight chitosans were relatively non-toxic at concentration up to 1mg/mL (170). The IC 50 values of 400 kDa and 150 kDa chitosans did not differ much in cytotoxicity tested in vitro on L929 fibroblasts suggesting that molecular weight played minimal role in cell viability. When used for gene delivery the chitosan-nanoparticles were uptaken by endocytic mechanism and majority was found in the cytosol that were further degraded by lysosomes (171).

As mentioned previously the rate and extent of chitosan degradation depends on DD and molecular weight of the polymer (168). Higher DD and higher molecular weight are known to slow the rate of degradation. However, introduction of additional crosslinks by using glutaraldehyde and anionic interaction reduced the rate of chitosan degradation by enzymes. This specific property of chitosan has been utilized for biomaterial and pharmaceutical applications (Table 1.1).

1.3.3.1 Antibacterial properties

Chitosan has been researched extensively owing to its excellent antimicrobial and antifungal
properties. It was found to be more effective against fungi and viruses by some studies (162). In case of bacteria, Gram-positive bacteria were more susceptible than Gram-negative ones. The minimal inhibitory concentrations ranged from 18-5000 ppm depending upon the organism, pH, DD, molecular weight, chemical modifications and presence of lipids and proteins (162, 172). The DD is known to influence the antibacterial activity. With higher DD, the number of amine group increases per glucosamine unit and thus chitosan showed higher antibacterial efficacy (163).

The exact mechanisms of antibacterial action of chitosan and its derivatives are still not vivid. However a more commonly proposed mechanism is contact mediated killing that involves the electrostatic attraction of positively charged chitosan with the negatively charged bacterial cell membranes (Fig. 1.8). This might lead to the altered cell wall permeability eventually resulting in rupture of cell and leakage of the proteinaceous and other intracellular components (162, 173). Rabea et al. also proposed that chitosan is able to chelate trace metals and essential nutrients; and inhibits enzyme activities essential for bacterial cell survival (162). Carboxymethylated chitosan showed a broad range of antimicrobial activity against both bacteria and fungi. Under transmission electron microscopy, the bacterial cells were seen to be completely enveloped in the chitosan forming an impermeable layer (44). This could have resulted in the prevention of transport of essential solutes leading to cell death. In case of fungi, chitosan was hypothesized to enter cell and reach nucleus, bind with DNA and inhibit RNA and protein synthesis.

Helander et al. showed that chitosan caused extensive cell surface alterations and covered the outer membrane of Gram-negative bacteria with vesicular structures resulting in the loss of the barrier function (174). This property makes chitosan a potentially useful indirect antimicrobial for food protection. When the mutants of Salmonella typhimurium with positive cell membrane were treated with chitosan, they were found to be more resistant than the parent strains. This further highlighted the role of electrostatic attraction in the antibacterial mechanism of chitosan. In addition, chitosan possesses several important properties for clinical application such as biocompatibility, acceptable color, cost effectiveness, availability and ease of chemical modification.
Table 1.1. Application of chitosan in various biomaterial and pharmaceutical fields based on their properties (158).

<table>
<thead>
<tr>
<th><strong>Applications</strong></th>
<th><strong>Rationale</strong></th>
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<tr>
<td>Biomaterial</td>
<td>Treatment of wounds and burns</td>
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<td></td>
<td>• Adhesive</td>
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<td>• Antibacterial</td>
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<td></td>
<td>• Permeability to $O_2$</td>
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<td>Tissue engineering</td>
<td>• 3-D support</td>
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<td></td>
<td>• Similar to GAGs</td>
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<td></td>
<td>• Encapsulation of cells</td>
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<tr>
<td>Pharmaceutical</td>
<td>1. Matrix in drug-release systems</td>
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<td></td>
<td>• Microspheres - protection against acidic pH</td>
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<td>• Nanoparticles - nasal delivery</td>
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<td>• Transdermal drug delivery - controlled release</td>
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<td>• Non-viral gene delivery</td>
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<td>2. Sustained release</td>
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Figure 1.8. A schematic diagram illustrating the antibacterial mechanism of nanoparticles with positive charge (A). Interfacial forces, especially electrostatic plays a role in the initial interaction between positively charged nanoparticles and negatively charged bacterial surface (B). Bacterial killing occurs upon contact mediated lipid peroxidation via production of reactive oxygen species (ROS) (C). The membrane damage induced by ROS eventually leads to ingress of nanoparticles into the cytoplasm (D). Extracellular polysaccharides secreted by bacteria in biofilm may prevent nanoparticles from interacting with bacteria and thus protect them from ROS (E). Adapted and modified from Neal (175).
1.3.3.2 Chitosan Nanoparticles

Nanotechnology, literally, is derived from the words ‘nano’ and ‘technology’ (176). Nano is defined as one billionth of a quantity, represented mathematically as $10^{-9}$. Chitosan is known as a versatile biopolymer that could be synthesized in various forms such as powder (micro- and nanoparticles), capsules, films, scaffolds, hydrogels, beads and bandages (41). Nanoparticles of chitosan have been mainly developed for drug and gene delivery applications. Nanocarriers made from chitosan demonstrated different physicochemical properties that could be controlled for various delivery capabilities (177). Nanoparticles of chitosan could be synthesized or assembled using many different methods depending on the end application or the features required in the nanoparticles (41).

Synthesis of nanoparticles by ionic gelation

There are different methods for synthesizing nanoparticles, which includes plasma-based/flame-hydrolysis method, chemical vapor deposition, electrodeposition, sol-gel processing, sonochemical processing routes, hydrodynamic cavitation processes and microemulsion techniques (41, 176). Nanoparticles of polymers and polysaccharides are most frequently prepared by four other methods: (1) dispersion of preformed polymers (“top down approach”), (2) polymerization of monomers (“bottom up approach”), (3) reverse micellar method and (4) ionic gelation or coacervation of hydrophilic polymers (41). In the current study, the method developed by Alonso and co-workers is utilized for preparing hydrophilic chitosan-nanoparticles (Cs-np) by ionic gelation with certain modifications (46).

The ionic gelation method involves a mixture of two aqueous solutions, one of which is chitosan polysaccharide and the other is a polyanion sodium tripolyphosphate (TPP). This synthesis process is extremely mild and involved mixing of two aqueous phases at room temperature. As the solutions are being mixed, the positively charged amino group of chitosan will interact with negatively charged tripolyphosphate to form coacervates in the nanometre range. Due to the ionic interaction conditions at room temperature, the material changes from the liquid state to the gel state (46). Alonso et al. reported that by altering the ratio of chitosan-TPP, a range of particle size (200-1000 nm) and zeta potential (between +20 and +60 mV) could be obtained. These Cs-np further showed protein loading capacity and continuous release of the entrapped protein for up to 1 week (46).
1.3.3.3 Functionalization

Following the Merriam-Webster dictionary meaning the word functionalize means to organize (as work or management) into units performing specialized tasks. Thus functionalization of chitosan could be combining or conjugating this versatile polymer with different reactive molecules depending on the application. These Cs-np have been modified for various protein, drug and gene delivery applications (Fig. 1.9) (177-180). The loading of Cs-np has been carried out by various methods such as embedding and encapsulation of drugs, proteins, dyes and genes.

Modifications of chitosan-nanocarriers with variety of ligands have been shown to increase the recognition and uptake of these nanocarriers into cells through receptor-mediated endocytosis. One of the common approaches to study the fate and interaction of nanoparticles with tissues and cells is by labeling the nanoparticles with fluorescein isothiocyanate (FITC) (181). FITC could be covalently bound to the amine groups in chitosan backbone using simple chemical reaction. These labeled Cs-np were predominantly internalized by endocytosis after a nonspecific interaction between the nanoparticles and cell membranes of A549 cells (human cell line derived from the respiratory epithelium). Another study also showed that labeling of Cs-np with Texas Red, complexes were found to enter the plasmid through endocytosis (182). In case of antibacterial applications, mostly Cs-np have been used as a vehicle for delivery of a range of antibiotics. The main advantage of using chitosan as a delivery agent is to circumvent the side effects of hepatic, nephrologic, hematologic or neurologic problems that are associated with systemic antibiotic administration (177, 183, 184). Functionalization of chitosan micro-/nanoparticles with various photosensitizers depending on the tissue specificity could provide targeted activities. Details on the conjugation of photosensitizer with chitosan could be found on the next section (1.3.4).

1.3.3.4 Summary

Chitosan presents as an attractive biopolymer with inherent antibacterial properties. The versatility to be modified into various sizes as well as chemical modifications could be advantageous for targeted antibacterial applications. Furthermore, the nanoparticles could be delivered into the anatomical complexities and dentinal tubules (52) were bacteria are known to escape the conventional disinfection strategies. Another salient property of chitosan is the biocompatibility or biodegradability that is of high priority to be used for any in vivo applications. Functionalization
Figure 1.9. Development of novel strategies for controlled release of drugs will provide nanoparticles with the capability to deliver two or more therapeutic agents. Multifunctional nanoparticles for drug delivery. Multifunctional nanocarriers can combine a specific targeting agent (usually an antibody or peptide) with nanoparticles for imaging (such as quantum dots or magnetic nanoparticles), a cell-penetrating agent (e.g. the polyArg peptide TAT), a stimulus-sensitive element for drug release, a stabilizing polymer to ensure biocompatibility (polyethylene glycol most frequently) and the therapeutic compound (adapted and modified) (185).
with photosensitizers could be used to specifically target bacteria over human cells. The release of singlet oxygen by the photosensitizers upon photoactivation could potentiate the antibacterial activity of the functionalized Cs-np. Such bioactive nanoparticles could perform multiple functions thereby help overcome the bacterial resistance to antimicrobials and disinfectants used currently for root canal disinfection.

1.4 Photodynamic Therapy: Antibacterial and Collagen Crosslinking

Photodynamic therapy (PDT) involves the use of nontoxic dye or photosensitizer (PS) in combination with visible light, which in the presence of molecular oxygen leads to the production of cytotoxic oxygen radicals such as singlet oxygen. This singlet oxygen is the responsible for the PDT cytotoxic action (186) and its production and activity depends on the PDT dose (187). PDT was discovered by chance during early 1900s, when a combination of nontoxic dyes and visible light resulted in killing of cells. Oscar Raab, used acridine dyes and showed that the combination of light and dyes was much more effective to kill a paramecium (188). Application of PDT as an alternative treatment for tumors has been explored and tested widely. For the last two decades, series of in vitro and in vivo studies have proven the efficacy of PDT in the management of various infectious and non-infectious diseases. The increase in the interest towards PDT can be seen by the exponential increase in the number of publications in the recent years (Fig. 1.10). Introduction of photosensitizers for in vivo applications and their approval in certain countries such as Canada, United States of America, European Union, Japan, Australia and New Zealand have led to a sudden surge in using PDT for various systemic and topical pathogenic conditions (189). In addition, PDT has also been applied to strengthen mechanical properties of collagenous tissues and artificial scaffolds for tissue engineering by photochemical crosslinking (32, 190, 191). The ongoing research is focused to bring about innovative improvements on PDT by increasing number of clinical trials and appropriate regulatory approvals for the usage of new photosensitizers. Cumulatively these efforts evince towards the increasing interest in the application of PDT for the coming years.

1.4.1 Antimicrobial PDT

Antimicrobial resistance is constantly on rise leading to a major hindrance in the treatment of many
infectious diseases (21, 192, 193). Emergence of resistant microbial strains, rise of transplants, medically compromised patients, advanced cancer patients and spread of infection due to increasing global travel between developed and developing nations are few of the major issues related to difficulties of managing infectious diseases (193, 194). The conservative management of such infections involving topical or systemic antibiotics has been shown to be ineffective owing to several factors (Table 1.2). Approximately 60% of the current human infections have been associated with the presence of bacterial biofilms, which includes both implant-related infections and chronic non-implant related infections (195). Additionally, widespread systemic use of antibiotics is a cause of multidrug resistance and superinfection due to untoward effects on normal ‘friendly’ flora (196).

The widespread recognition of biofilm as the chief contributory factor on human infection with the other mentioned criteria has led to dire situation in identifying a reliable and effective antimicrobial treatment strategy to combat infectious diseases which in the past had been trivial and easily treated (20, 21). PDT has been considered as one of the potential treatment modality for the treatment of localized infections irrespective of the causative microorganism, including those that are recalcitrant to conventional antimicrobial therapies (197-200). Furthermore development and testing of novel chemicals and peptides as well as complementary chemicals and physical methods such as sonication to enhance the existing antibiotic treatment has received significant focus (21).

**Mechanism of photodynamic inactivation of microbial cells**

PDT utilizing a suitable photosensitizer and irradiation conditions, to treat infections in cases where antibiotic-based therapeutic strategies have failed (198). Unlike in cancer therapy where PS is administered intravenously, for localized infections PS is delivered locally by various methods such as, topical application, instillation and interstitial injection or aerosol delivery. Selectivity of PS towards microorganisms over mammalian cells and effective removal of the causative microorganisms are the key points in achieving success of PDT to manage localized infections (197).
Figure 1.10. Number of publications (English language) on the PDT since 1980 till 2010 (Source: Pubmed).
Table 1.2. Limitations of current topical or systemic antimicrobial treatment strategies to manage infectious diseases.

| 1. Microbial factors | a. Antibiotic resistant mutant strains | MRSA (201)  
Vancomycin resistant enterococci (193) |
|----------------------|----------------------------------------|----------------------------------------|
|                      | b. Antibiotic resistance mechanisms     | Exchange of genetic materials  
Deficiency of specific porin channels  
Prevent influx of chemicals  
Promotion of active drug efflux  
Thickening of the peptidoglycan layer of the outer wall (202) |
|                      | c. Structure and organization of microbes | Variation in the outer wall in different classes of bacteria and fungi  
Formation of biofilms  
Protozoal existence as trophic feeding stage or resting cystic stage |
| 2. Host/human factors | Antibiotic misuse (197) | Excessive or inappropriate prescription  
Failure to complete treatment regimen  
Widespread use of antibiotics in livestock feedstuff |
Photosensitizers are chemicals capable of transferring the energy absorbed to other compounds that, in turn, generate metastable species that are very reactive. Mostly these PS are aromatic molecules that upon absorption of light undergoes an electronic transition to the singlet excited state (electron spins paired), which is highly reactive and unstable. The excited photosensitizer molecule can release its energy via different electronic or physical processes (intersystem crossing) and return to the stable ground state. It may undergo a transition to the triplet excited state (electron spins unpaired) and subsequently may gain the ground state via two specific mechanisms, Type I or Type II pathway (Fig. 1.11) (203). Type I pathway involves production of radical ions of oxygen due to electron-transfer from PS triplet excited state to the substrate. Radical ions such as superoxide, hydroxyl and lipid derived ions are the cytotoxic species responsible for Type I photoreaction (204). Type II pathway involves production of excited singlet oxygen due to energy transfer from PS triplet excited state to the ground state molecular oxygen, which is responsible for the oxidation of various cellular constituents (205).

In Type I pathway, oxygen present in a biological system is the electron acceptor, thus leads to the initial formation of the superoxide anion (O$_2^-$), which subsequently gets converted into the highly reactive hydroxyl ions (OH$^-$) specially in the presence of ferric ions (Fe$^{2+}$) (Fenton reaction) (203). In case of a bacterial cytoplasmic membrane, abstraction of allylic hydrogens from unsaturated molecules such as phospholipids takes place resulting in the formation of a radical species. The radical species further undergo reaction with the oxygen to yield lipid hydroperoxide. Membrane integrity is lost due to the lipid peroxidation, leading to loss of intracellular components and increased ion permeability. In addition, inactivation of membrane enzymes and peptides may also occur in Type I reaction (206).

In case of Type II pathway, singlet oxygen is produced utilizing the large concentration of oxygen present in many cell/tissue compartments. This is also facilitated by the low energy gap (22.5 kcal/mole) between the ground and first excited singlet state of oxygen. Type II reaction is considered as the predominant pathway to exert photodynamic inactivation of microbial cells. The short half-life and lack of diffusion of singlet oxygen, requires its generation in close proximity or even inside the cells to produce effective reaction with various cellular targets such as proteins, nucleic acids and lipids (Fig 1.11) (205-207). This may be a limiting factor for inactivation of Gram-negative bacteria, as the PS molecules need to interact or adsorb with the outer wall (198, 200, 208).
Figure 1.11. Schematic diagram of possible mechanism of photodynamic therapy where the excited photosensitizer (PS) after light irradiation could follow different pathways to return to its stable ground state. The PS upon photoactivation absorbs a photon and goes to the excited singlet state ($S_1$). The $S_1$ can come back to the ground state by releasing fluorescence or relax back to excited-triplet PS state ($T_1$). The long-lived $T_1$ state can phosphoresce and come back to $S_0$. Or else $T_1$ can interact with molecular oxygen following two specific pathways Type I and Type II mechanism, resulting in the generation of reactive oxygen species or singlet oxygen respectively. The singlet oxygen generated in Type II mechanism then interacts with biomolecules to produce the resultant PDT effect.
Photodynamic inactivation of bacterial cells can be described in a stepwise manner (Fig. 1.12). The differences in microbial cell wall characteristics need to be accounted for while determining the time of PS uptake before illumination (208, 209). The PS with slower uptake could result only in cell wall damage and with longer incubation times, other nuclear effects such as nucleic acid strand breakage might be apparent. The choice of PS is also critical in obtaining effective bacterial elimination. The commonly used phenothiazinium PS such as toluidine blue O (TBO) produced increased membrane permeability of *Escherichia coli*, whereas methylene blue produced DNA damage in the same organism (210, 211). Several *in vitro* and *in vivo* studies have shown the effectiveness of PDT in eliminating root canal biofilms (199, 212-216). Endodontic pathogen such as E. faecalis, P. intermedia, F. nucleatum, S. intermedius and A. actinomycetecomitans have been shown to be killed by using photosensitizers such as methylene blue, toluidine blue and rose bengal (217, 218). In the current study PDT in combination with chemomechanical preparation is expected to bring about the maximum reduction in root canal microbial loads. It is important to realize that PDT has been considered only as a possible supplement to the existing disinfection protocols to improve antibiofilm efficacy in root canal disinfection.

One of the significant advantages of PDT is the dual selectivity obtained firstly by targeting the PS, as it has high affinity for microbial cells, and secondly the light, as only the infected area is irradiated. As the PS typically shows a higher affinity towards microbial cells, the host cells could be affected less during PDT. The instant antimicrobial activity also offers added advantage as antibiotics take several days to produce comparable efficacy. The broad therapeutic window of PDT because of the high reactivity of ROS could effectively eliminate bacteria as well as the bacterial virulence factors such as endotoxins and proteolytic enzymes. Furthermore, due to the multiple targets of PDT on a bacterial cell, the probability of bacteria developing resistance to this treatment has been considered to be almost impossible (197, 198, 219).

The predominant type of photodynamic action is based on the class of compound (220). In addition, the site of action also governs the exact mode of action (208, 220). This, in turn, is a function of the physicochemical make-up of the photosensitizer as well as microbial conditions. These factors need to be considered, as PS based on the preliminary *in-vitro* testing of putative compounds with promising photochemical properties may not be effective against its microbial target due to metabolism, reduction and other factors (208, 221). This could be possible due to
Figure 1.12. Stepwise mechanism of photodynamic inactivation of microbial cells.
localization in a non-vital region of the target cell. For any new PS, physicochemical parameters such as lipophilicity ($\log P$), ionization ($pK_a$), light-absorption characteristics (the maximum wavelength of absorption, $\lambda_{\text{max}}$, and the intensity of the absorption, $\varepsilon_{\text{max}}$) and the yield of singlet oxygen ($\Phi_{\Delta}$) need special assessment prior to be used for antimicrobial PDT (222).

PS such as porphyrins, chlorins and phthalocyanines, for treatment of cancer or other diseases are chosen based upon their low dark toxicity to mammalian cells and ability to target tumor cells (198). The PS for antibacterial PDT are chosen based on their specificity to bacterial cells. The commonly used PS for antibacterial purpose are halogenated xanthenes such as rose bengal (223), phenothiazines such as methylene blue and toluidine blue (199, 224) and perylenequinones such as hypericin (225). These PS are delivered locally to manage localized infections rather than systemic injection as used for chemotherapy. The factors that determine the effective antibacterial PDT are: method/vehicle of topical application, effective time of interaction with the microbes at the site of infection, selectivity of PS to microbes, relative non-toxicity towards host tissues at the site of infection and ability to eliminate the microbes effectively to avoid regrowth of surviving pathogens following treatment (198).

Rose bengal is an anionic xanthene PS that have been used for various PDT-based applications. The water solubility (upto the concentration of $10^{-3}$M), absorption maxima in the visible region, high quantum yield of singlet oxygen ($\Phi_{\Delta}=0.76$) and relative inexpensiveness are some of the specific advantages of RB (226). The absorption maximum ($\lambda_{\text{max}}$) in water is at 550 nm wavelength. This peak is due to the monomeric forms of the dye, with dimer peaks appearing at 520 nm wavelength. With increase in the concentration of RB in water (>50 uM), the dye is known to aggregate by formation of dimers (35) and such phenomenon limits the use of RB due to impaired photochemical response (227). The effects of aggregation have been studies by using the ratio of peaks of monomer to dimer. Encapsulation into liposomes or binding with silica and chitosan has been tried to overcome the aggregation effects of RB (49, 227, 228). These modifications showed slight alterations in the absorption peaks of RB. However, the singlet oxygen yield was found to almost similar to the free RB in water (49). RB molecules also have fluorescence spectrum with excitation at 520 nm and emission at 583 nm wavelength.

RB has been used for antibacterial PDT against various gram positive and gram negative bacteria (208, 223, 228). There was a concentration and PDT dose dependence on the antibacterial efficacy
of RB (208, 229). However, this anionic PS has been shown to be less effective as compared to the cationic phenothiaziniums such as methylene blue (208, 230). The toxicity of RB towards cells has been debated by various studies as either non-toxic (36, 37) or toxic resulting in cell detachment (231, 232). Other than effects on bacterial and mammalian cells, the singlet oxygen produced has also been used to photocrosslink collagenous tissue and scaffolds (34, 36). More specific details on antibacterial and photocrosslinking properties will be provided in chapters 4-6.

Other than the limitations associated with interaction of photosensitizer with bacterial biofilms located in the root canals, tissue-specific challenging factors in the application of PDT for endodontic disinfection also needs consideration. The limited penetration of the activating light energy into the infected tissue, penetration of the optimum photosensitizer concentration into the infected tissue, low oxygen tension inside the root canals, and dentin discoloration by the photosensitizer are some of the issues that needs to be addressed before establishing PDT as a definitive treatment step in root canal disinfection (212, 233).

1.4.2 Photocrosslinking of Collagen

Photocrosslinking is a rapid process, which occurs primarily through photoactivation of sensitizers thereby inducing photooxidation of the amino acids, such as cysteine, histidine, tyrosine and tryptophan that led to the formation of covalent crosslinks in a light-independent manner (234). Covalent coupling between the free amino groups and photo-oxidized amino acids have been proven by the decrease in reactivity and available free amino groups following photo-crosslinking in the presence of a sensitizer (235). Photocrosslinking have been used in biological tissue engineering fields to stabilize biological tissues (34, 236). Proteins and collagen have been shown to be covalently crosslinked inter- and intramolecularly by illumination in the presence of appropriate photosensitizers such as rose Bengal (RB) (35, 37). Synthetic chemical agents such as glutaraldehyde (GD) are one of the commonest agents used to crosslink or fix tissues. However their use in vivo is limited due to the formation of highly cytotoxic reaction by-products (237) as well as longer duration of reaction time, which is a major limitation in clinical situations.

Photocrosslinking of collagen produces intermolecular and intramolecular crosslinks without incorporation of the photosensitizer used. This close crosslinking undoubtedly result in increased ultimate tensile strength and elastic modulus of collagen. However, the crosslinked collagen will present a stiff or brittle characteristic. Incorporation of polymers such as CMCS, will serve as
spacers in between the collagen fibrils preventing undesired zero-length crosslinking and subsequently improve fracture toughness (53). This would reinforce the collagen structure, ensuing in the increased post yield strain or toughness (55, 56, 238).

1.4.3 Role of Nanotechnology on PDT

Nanoparticles based treatment is gaining increasing attention in many areas of medical diagnostics and therapeutics. Likewise, nanoparticles based photosensitizers have been considered to potentiate the PDT of efficacy (239, 240). Functionalized nanoparticles containing various reactive molecules and decorated with peptides or other ligands have led to new possibilities of combating antimicrobial resistance (241, 242). Combination of photosensitizer and nanoparticles in cancer therapy has been covered in a vast literature with lesser attention to antimicrobial aspects of such combinations. Specific advantages have been quoted for using these modified nanoparticles based photosensitizers. They offer unique physicochemical properties, such as ultra-small sizes, large surface area/mass ratio and increased chemical reactivity. Further, by using a bioactive material for nanoparticles, these particles could display functions and properties similar to naturally occurring biomolecules present in cellular systems.

Some metallic nanomaterials, such as TiO$_2$, ZnO, and fullerenes as well as their derivatives, showed the ability to generate singlet oxygen and induced bacterial elimination (243). On the other hand different strategies to combine nanoparticles and PS have been suggested (19, 239). As mentioned in the review by Kishen, combination of nanoparticles with photosensitizers could be achieved by; (i) photosensitizers supplemented with nanoparticles; (ii) photosensitizers encapsulated within nanoparticles; (iii) photosensitizers bound or loaded to nanoparticles; and (iv) nanoparticles themselves serving as photosensitizers (19). These combinations of nanoparticles with photosensitizer have been proposed to enhance antimicrobial PDT (228, 239). Several factors could be attributed to this improved antimicrobial efficacy: 1) higher concentration of photosensitizer per mass with resultant production of ROS; 2) reduced efflux of photosensitizer from the target cell thereby decreasing the possibility of drug resistance; 3) possibility of targeting the bacteria due to greater interaction associated with the surface charge; 4) greater stability of photosensitizers after conjugation; 5) less physical quenching due to photosensitizer aggregation; and 6) controlled release of ROS following photoactivation (19, 228, 239).

The photosensitizer supplemented nanoparticles such as methylene blue-loaded poly(lactic-co-
glycolic) acid (PLGA) nanoparticles have been tested \textit{in vitro} on \textit{E. faecalis} biofilm and human dental plaque bacteria in combination with PDT (244). The cationic Methylene blue-loaded PLGA nanoparticles exhibited significantly higher bacterial phototoxicity in both planktonic and biofilm phases. These nanoparticles were seen enveloping the bacterial cells with higher concentration on the bacterial cell walls at all tested time points. Based on these observations the authors concluded that cationic methylene blue-loaded PLGA nanoparticles have the potential to be used carriers of photosensitizer PDT in within root canals. The photosensitizer bound polystyrene beads were used by Bezman \textit{et al.} wherein rose bengal was used as a photosensitizer (245). This modified photosensitizer was postulated to bind to the call membrane of bacteria with greater affinity as compared to the unmodified ones. The close association of photosensitizer following activation with light would favor improved bacterial elimination with generation of ROS. The binding of rose bengal with silica nanoparticles however resulted in slower yield of ROS as compared to the free rose bengal (228). This slower decay has been suggested (228) to result in longer period of antimicrobial efficacy and may be favorable for deeper penetration into the bacterial biofilms.

Although photosensitizers have been conjugated with different readily available synthetic polymers and liposomes, these constructs possess limited biocompatibility when applied \textit{in-vivo} (48, 49). The application of naturally occurring biopolymers such as chitosan may circumvent such issues of biocompatibility (49, 246). This hydrophilic biopolymer with a large number of free hydroxyl and amino groups has been used for numerous chemical modifications and grafting (31, 33, 48, 159). The biopolymer is wettable which favors intimate contact between photosensitizer and the aqueous suspension of microorganisms.

1.4.4 Summary

Research towards enhancing APDT for the effective biofilm elimination should be directed in future. The development of newer photosensitzers and nanoparticles formulations needs to be characterized with a well-defined protocol for endodontic applications for potent antibiofilm efficacy within root canals that too in the presence of tissue inhibitors such as serum.
1.5 References


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Chapter 2
Nanoparticulates for Antibiofilm Treatment and Effect of Ageing on its Antibacterial Activity

2.1 Abstract

The present endodontic disinfection strategies have been shown to be incapable of effective elimination of bacterial-biofilm due to antimicrobial-resistance. Nanoparticulates such as chitosan (Cs-np) and zinc-oxide (ZnO-np) are known to possess significant antibacterial-properties. This study aimed to test 1) the efficacy of Cs-np and ZnO-np in disinfecting and disrupting biofilm-bacteria and 2) the long term efficacy of these nanoparticulates following aging. Enterococcus faecalis (ATCC & OG1RF) in planktonic and biofilm forms were treated with different concentrations of Cs-np and ZnO-np. The treated bacteria were quantified using microbiological methods. The biofilm viability and structure following nanoparticulates treatment were assessed using confocal-laser-scanning-microscopy. The effect of ageing using sterile-saliva and phosphate-buffered-saline on the antibacterial-properties of the nanoparticulates was also determined. The rate of bacterial killing by the nanoparticulates depended on the concentration and time of interaction. Total elimination of planktonic bacteria was observed in contrast to the biofilm-bacteria which survived even after 72 hours. The confocal-microscopy images showed predominantly dead bacterial-cells and significant reduction in the thickness of biofilm (p<0.01) following nanoparticulates treatment in both the groups. Both the Cs-np and ZnO-np were found to retain their antibacterial-properties after ageing for 90 days. The present study highlighted the efficacy of the nanoparticulates, to reduce biofilm-bacteria, disrupt biofilm-structure and retain the antibacterial-property even following ageing. Cs-np and ZnO-np present a potential approach in biofilm disinfection.
2.2 Introduction

Endodontic infection is a biofilm-mediated infection and the success of an endodontic treatment will depend on the effective elimination of bacterial-biofilm from the root canal-system (1, 2). Resistance of bacterial-biofilm to present disinfectants such as sodium-hypochlorite and chlorhexidine is due to inherent microbiological factors and the complex anatomy of the root-canal-system (3, 4). It was reported that almost 35% of the root-canal surfaces remain untouched by the root-canal files regardless of the filing method (5). The bacteria present within the dentinal tubules are inaccessible to the currently used irrigants (eg. sodium-hypochlorite), medicaments (eg. calcium-hydroxide), and sealers (Grossman sealer) due to their limited penetrability into the dentinal tubules (5-7). The obturating materials are also reported to have a limited antibacterial-activity as most of the sealers lose their antibacterial-property by one week (8-10). All these factors contribute to the persistence of bacterial-biofilm within the root-canal-system (1, 4).

Biofilm mode of bacterial growth is one of the adaptive processes that allow bacteria to survive in nutrient depleted environment similar to the treated root-canals (11, 12). The apical portions of the root-canal treated mandibular molars revealed presence of bacteria in the form of biofilm mainly in the anatomical complexities (1). The biofilm-bacteria show higher antimicrobial resistance as compared to their free-floating “planktonic” counterparts (13, 14). The resistance of biofilm-bacteria has been attributed to the protective barrier provided by the extracellular polymeric matrix (EPM) (15). Therefore, agents which possess the ability to disrupt the EPM would allow better penetration of antibacterial-agents into the biofilm-structure and result in significant bacterial elimination. This approach would be advantageous in non-surgical root canal treatment (NSRCT). Various nanoparticles have gained popularity as antimicrobial-agents due to their broad-spectrum of activity and biocompatibility (16). Recent studies have focused on using nanoparticulate materials to disinfect root-canal (17, 18). Nanoparticulates exhibit higher antibacterial-activity due to their polycationic/polyanionic nature with higher surface-area and charge density, resulting in greater degree of interaction with the bacterial cell (17). It has been observed that the size of nanoparticulates play an important role in their antibacterial-activity with smaller particles showing higher antibacterial-activity than the macro-scaled ones (18-20).

Chitosan (poly (1, 4), b-d glucopyranosamine) is obtained from deacetylation of the second most abundant polymer, chitin. Chitosan is an excellent bioadhesive which possesses broad-spectrum
of antimicrobial properties. Nanoparticulates are those particles with diameter of 100 nm or less. Previous studies have reported significant antibacterial-efficacy of chitosan nanoparticulates (Cs-np) and zinc-oxide nanoparticulates (ZnO-np) against planktonic Enterococcus faecalis (17, 21). Antibacterial-activity of polycationic Cs-np and ZnO-np could be attributed to the electrostatic attraction with the negatively charged bacterial-cell, which might lead to the altered cell wall permeability, resulting in leakage of the proteinaceous and other intracellular components and death of the cell (22, 23). Certain metallic nanoparticulates (ZnO, MgO) are also known to cause membrane damage as a result of lipid peroxidation by the reactive oxygen species (ROS) such as superoxide (O$_2^-$) and hydroxyl radicals (OH$^-$) (24). Direct or close contact between the nanoparticulates and the bacterial membrane appeared to be essential for the ROS toxicity to be effective in peroxidation (16, 25). In addition, Cs-np and ZnO-np possess several important properties for clinical application such as biocompatibility, color (white), cost effectiveness (as compared to bioactive glass), availability and ease of modification.

E. faecalis is one of the primary organisms in patients with post treatment endodontic infection. It has been shown to exist as biofilm in the anatomical complexities and possess ability to penetrate dentinal tubules (26, 27). Even though nanoforms of bioactive glass have demonstrated significant antibacterial-properties, effects on biofilm-structure and effects of ageing have not been established (18, 28). The objective of this study is twofold: (a) to investigate the efficiency of Cs-np and ZnO-np to eliminate and disrupt the structure of a 7-days old E. faecalis biofilm in-vitro and (b) to evaluate the long-term antibacterial-activity of Cs-np and ZnO-np following ageing process.

2.3 Materials and Methods

All chemicals and bacteriologic media used in this study were purchased from Sigma-Aldrich Inc (St. Louis, MO), unless otherwise stated. Cs-np was synthesized according to the method reported in an earlier work (29). In brief, chitosan obtained from Sigma was dissolved in 1 v/v % acetic acid solution at a concentration of 0.1 w/v %, and the pH was raised to 5 with 1M NaOH. Cs-np was formed by adding 0.1% sodium tripolyphosphate in water to chitosan solution in a ratio of 3:1 under stirring at a speed of 1000 rpm. The Cs-np were separated by centrifugation at 15,000 rpm for 30 minutes. The supernatant was discarded and the Cs-np was extensively rinsed with
deionized-water to remove any residual NaOH and then freeze dried before further use. ZnO-np with a particle size of <100 nm was purchased and tested in this study.

2.3.1 Assessment of antibacterial-efficacy of nanoparticulates

Two strains of *E. faecalis* with biofilm forming capacity were tested in both planktonic and biofilm state. OG1RF strain is known to form aggressive biofilm compared to ATCC 29212 strain (30). *E. faecalis* was incubated overnight at 37°C under agitation in Brain-Heart Infusion (BHI) broth. The cultures were centrifuged (6000 rpm, 10 min, 4°C), supernatants discarded and the cells washed twice in sterile deionized-water. The cells were resuspended in deionized-water and adjusted to 10^7 cells/mL (optical density ≈ 0.1) at 600 nm (UV-VISIBLE Spectrophotometer, Shimadzu, Kyoto, Japan). 1 mL of the planktonic bacterial cells was inoculated into each well of the multiwell-plate. The bacterial cells were divided into two treatment groups (to test the effects of Cs-np and ZnO-np) and one control group. Three concentrations, 2, 5 and 10 mg/mL of both the Cs-np and ZnO-np were tested. At different time intervals, 100 µL of the bacterial inoculum was withdrawn and plated onto freshly poured BHI-agar plates following serial dilutions.

In order to test the antibacterial-efficacy of the nanoparticulates on bacterial-biofilm, 7-days old biofilm of *E. faecalis* (ATCC 29212 & OG1RF) were grown in multiwell-plates. Single colony from the agar-plate was inoculated into 50 mL of BHI-broth (Sigma, USA) and cultured overnight in an orbital incubator at 37°C at 100rpm. Fresh culture adjusted to an optical density of 1 at 600 nm, 1 mL of *E. faecalis* culture was added into each well of the multiwell-plates preconditioned overnight with sterile-saliva and incubated in an orbital incubator at 37°C, 100 rpm. Fresh media was replenished every 48 hours to provide a constant supply of nutrients and to remove dead bacterial cells. On the eighth day, the media was removed from the wells, and the biofilm was carefully washed twice with sterile deionized-water. The washed biofilm was maintained in 1 mL of sterile deionized-water. The bacterial cells were divided into two treatment groups (to test the effects of Cs-np and ZnO-np) and one control group. Three concentrations of 5, 10 and 20 mg/mL of both the nanoparticulates were tested. Nanoparticulates were added to the bacterial culture and incubated at 37°C, 100 rpm. The wells in the control group were maintained in sterile deionized-water. At different time intervals the nanoparticulates were removed from the wells and the biofilms washed gently using deionized-water. 1 mL of deionized-water was added, the biofilm was then disrupted and 100 µL of the biofilm-bacterial inoculum was plated onto freshly poured
BHI-agar plates following serial dilutions. Colonies were counted after 24 hours of incubation at 37°C and expressed as log colony forming units (CFU) per mL. The experiments were carried out in triplicates and the mean values were calculated.

2.3.2 Assessment of biofilm-structure following nanoparticulates treatment

The structure of the 7-days old biofilm following treatment with nanoparticulates was assessed using confocal-laser-scanning-microscopy (CLSM). *E. faecalis* (ATCC 29212) biofilm was grown on a glass coverslip that was fixed covering a circular hole (6 mm diameter) made in the base of a petridish (3 cm diameter). 1 mL of fresh culture of *E. faecalis* grown in BHI-broth was adjusted to optical density of 1 at 600 nm, and added into the petridish with the glass cover slip which was preconditioned overnight with 0.5 mL of sterile-saliva and incubated in an orbital incubator at 37°C at 100 rpm. Fresh media was replenished every 48 hours to provide a constant supply of nutrients and to remove non-adherent bacterial cells. Three petri dishes containing the biofilm were tested under each group. On the day of experiment, media was removed from the wells, biofilm washed gently twice with sterile deionized-water and was maintained in 1 mL of sterile deionized-water. Cs-np and ZnO-np (20 mg/mL) were added into the wells and incubated at 37°C at 100 rpm for 24 hours. After this period of nanoparticulates-biofilm interaction, the suspension was gently removed and the biofilms were washed with sterile deionized-water to remove the remaining nanoparticulates. The biofilms were then stained with 20 μL of Live/Dead *Baclight* stain (Molecular Probes, Eugene, OR) and incubated in the dark for 10 minutes. The biofilm-structures were then viewed under a confocal laser scanning microscope (CLSM) (Olympus, Japan). Kr/Ar laser was the source of illumination with 488 nm excitation and long-pass 500-523 nm and 622-722 nm emission filter settings for green and red signals respectively. Nine different areas were imaged from each sample using a 60x water objective. The optical sections of the biofilm-structure were recorded and analyzed using FluoView software (Olympus, Japan). Student *t*-test was used to compare the thickness of the biofilm before and after nanoparticulates treatment.

2.3.3 Assessment of ageing effect on antibacterial-properties of nanoparticulates

Thin coatings of ZnO-np and Cs-np were prepared on the base of a 24 well-plate by dehydrating the suspension (10 mg per well) in a hot air oven (70°C) for 6 hours. The wells were divided into
three groups: group (1) nanoparticulates aged using sterile filtered saliva (1 mL/well); group (2) nanoparticulates aged using sterile phosphate buffered saline (PBS) (1X) (1 mL/well) and group (3) nanoparticulates without ageing- control group. All the groups were incubated in a CO$_2$ incubator at 37°C simulating the root-canal environment for different time intervals of 1, 7, 30 and 90 days. After different time intervals, *E. faecalis* (ATCC 29212) was incubated overnight at 37°C under agitation in the BHI-broth. The culture was centrifuged (6000 rpm, 10 min, 4°C), supernatants discarded and washed twice in sterile deionized-water. The cells were resuspended in deionized-water and adjusted to 10$^7$ cells/mL (optical density ≈ 0.1) at 600 nm. 1mL of the culture was inoculated into each well of the multi-well plate and incubated at 37°C, 100 rpm. The bacterial reduction was assessed after 24 hours of incubation by plating the cells onto freshly poured BHI-agar plates following serial dilutions. Colonies were counted after 24 hours of incubation at 37°C and cell survival fraction was expressed as the ratio of the CFU of bacteria treated with nanoparticulates to the CFU of bacteria that did not receive any treatment.

### 2.4 Results

**2.4.1 Assessment of antibacterial-efficacy of nanoparticulates**

Fig. 2.1 and 2.2 show that the rate of reduction of the bacterial CFU upon treatment with the nanoparticles depends on the state of the bacteria (planktonic or biofilm), duration and concentration of the nanoparticulates used. Cs-np showed total bacterial elimination within 8 to 12 hours of interaction with both *E. faecalis* strains. However, ZnO-np showed total elimination of only ATCC 29212 strain after 12 hours of interaction. The highest concentration (10 mg/mL) of ZnO-np showed maximum reduction up to 4 log of OG1RF strain (Fig. 2.1 C). The antibacterial-efficacy of both Cs-np and ZnO-np decreased in the case of biofilm-bacteria when compared to the planktonic bacteria (Fig. 2.2). At lower concentrations Cs-np and ZnO-np (5 and 10 mg/mL) showed a maximum reduction of 3-4 log after 72 hours. However, in the case of ATCC 29212 strain, after the initial decrease, there was a slight increase in bacterial numbers (Fig. 2.2 A & 2.2 B). Significant reductions of biofilm-bacteria of both strains were observed with the highest concentration used (20 mg/mL). In the case of the OG1RF strain, a maximum reduction of 3-4 log was observed (Fig. 2.2 C & 2.2 D). However, complete elimination of ATCC 29212 strain was observed only with ZnO-np after 72 hours of interaction (Fig. 2.2 A). The biofilm-cells...
Figure 2.1. Antibacterial-efficacy of Cs-np and ZnO-np using three different concentrations on planktonic *E. faecalis* strains ATCC 29212 (A & B) and OG1RF (C & D).
in the control group maintained in sterile DI water showed approximately 1 log reduction bacterial count after 72 hours (data not shown).

### 2.4.2 Assessment of biofilm-structure following nanoparticulates treatment

**Fig. 2.3** shows the CLSM images of the bacterial-biofilms before and after nanoparticulates treatment. In the untreated control, the biofilm-structure consisted of both live (green) and dead (red) bacterial-cells in a multilayered architecture. The number of live bacterial-cells was observed to be higher as compared to the dead cells. The thickness of biofilm-structure was found to be variable at different locations (30.4±10.8 μm). Following treatment with Cs-np and ZnO-np for 24 hours the biofilm-architecture was altered. Distribution of viable bacteria reduced significantly and the multilayered structure as observed in the control biofilm was disrupted. Bacterial-biofilms exposed to ZnO-np were completely disrupted with conspicuous loss of the intricate three-dimensional form. In case of Cs-np, the biofilm-bacteria were found to be aggregated in clusters of dead cells with few live bacterial-cells in the deeper layers of the biofilm. The thickness of the biofilm reduced significantly to 9.33±2.42 μm (p<0.001) and 16.88±11.7 μm (p<0.01) after 24 hours treatment with ZnO-np and Cs-np respectively.

### 2.4.3 Assessment of ageing effect on antibacterial-properties of nanoparticulates

The findings from this experiment showed that Cs-np and ZnO-np demonstrated antibacterial-activity even after ageing with PBS and saliva (**Fig. 2.4**). Total bacterial inhibition by both Cs-np and ZnO-np was observed till 30 days without ageing. Cs-np showed bacterial inhibition after 30 days of ageing in PBS. However, the antibacterial-effect of Cs-np aged in saliva showed pronounced reduction in antibacterial-efficacy after 90 days. The survival fraction of Cs-np aged in saliva was 0.6 as compared to less than 0.1 in the Cs-np aged in PBS and control groups. ZnO-np showed faster reduction in antibacterial-efficacy after ageing in PBS than in saliva. The survival fraction of bacteria was 0.4 in case of ZnO-np aged in PBS and less than 0.1 in case of ZnO-np aged in saliva on day 90. After 90 days, the nanoparticulates without ageing from the control group resulted in minimal increase in the survival fraction of less than 0.1.
Figure 2.2. Antibacterial efficacy of Cs-np and ZnO-np using three different concentrations on biofilm of *E. faecalis* strains ATCC 29212 (A & B) and OG1RF (C & D).
Figure 2.3. The three-dimensional confocal-laser-scanning-microscopy reconstruction of *E. faecalis* (ATCC 29212) biofilm (A & B) and following treatment with antibacterial ZnO-np (C & D) and Cs-np (E & F). The number of live bacterial-cells were reduced significantly and the three dimensional structure was also disrupted. (B, D and F show the sagittal sections of the biofilm-structure) (60x)
2.5 Discussion

This study demonstrated that the Cs-np and ZnO-np possessed significant antibiofilm properties and were able to disrupt the multilayered, three-dimensional biofilm-architecture. The antibacterial-property was retained even after ageing in saliva and PBS for 90 days. The planktonic bacterial-cells were eliminated more rapidly and at lower concentrations of Cs-np and ZnO-np when compared to the biofilm-bacteria. Direct contact-dependent inhibition of planktonic bacteria might be the main killing mechanism by these nanoparticulates. Whereas, resistance to penetration of the nanoparticulates due to negatively charged biofilm EPM could be the cause of, higher concentrations and a longer duration of contact required for elimination of biofilm-bacteria (15, 31). In addition, the EPM may also serve as a chemical barrier by adsorbing the harmful ROS from reaching the cell surface thereby decreasing the effect of ROS (32). The higher reduction of biofilm-bacteria by ZnO-np compared to Cs-np could be due to ROS production by ZnO-np, which was able to diffuse into the biofilm-structure. The presence of moist or aqueous environment of the biofilm might augment the production of ROS by ZnO-np (21).

The bacterial-biofilm structures demonstrated antimicrobial-resistance even with higher concentrations of antimicrobials (15). The disruption of biofilm-structure following treatment with the nanoparticulates was evident from confocal-microscopy. The confocal images based on the *Baclight* staining indicated predominantly dead cells with disruption of the biofilm-structures after 24 hours of treatment with both Cs-np and ZnO-np. However, the microbiological quantification showed 4-5 log of cells surviving after 24 hours. As discussed earlier, EPM acting as a physical barrier may result in a gradient of nanoparticulates affecting the biofilm-bacterial cells (15, 33). In addition, the green-fluorescence of the fewer number of surviving bacterial-cells within the biofilm-structure will be overwhelmed by the red-fluorescence of the larger number of dead cells. Even though the number of live bacterial-cells is reduced following treatment with the Cs-np and ZnO-np, they may possess the ability to grow and multiply once the exposure is eliminated and nutrients are provided. Therefore the findings from this study highlight the importance of comparing the CLSM information with direct cell viability assay for better picture of biofilm-disinfection.

The retention of the antibacterial-activity of Cs-np and ZnO-np for up to 90 days presents a
Figure 2.4. Effect of ageing using saliva and PBS in the antibacterial-efficacy of (A) Cs-np and (B) ZnO-np as a function of time. The graphs show the survival fraction of bacteria in contact with the nanoparticles at different time intervals following ageing.

Groups: ageing of Cs-np with saliva (CS-SA); with PBS (CS-PA) and ageing of ZnO-np with saliva (ZnO-SA); with PBS (ZnO-PA).
promising advantage for biofilm-elimination. In order to mimic interfacial fluid leakage in root-filled teeth we have used saliva in addition to the routinely used PBS to induce ageing of the Cs-np and ZnO-np. The Cs-np and ZnO-np showed good antibacterial-property till 30 days of ageing. Since the charge of nanoparticulates play an important role in bacterial elimination, ageing with saliva and PBS could have resulted in reduced efficacy due to interaction of ions present in these media with the nanoparticulates. However, Cs-np aged in PBS showed better antibacterial-efficacy than Cs-np aged in saliva. The use of saliva would help to evaluate the antibacterial-efficacy of these nanoparticulates even in worst case scenario of leakage in a root-filled tooth in-vivo. Saliva is known to consist of different types of ions (calcium, phosphates, potassium, etc.) and these may be responsible for the earlier loss of activity in the ageing group using saliva (34). In NSRCT, these nanoparticulates could be delivered into the anatomical complexities and dentinal tubules using high intensity focused ultrasound where conventional disinfectants are unable to reach (35). This could be highly beneficial since studies have shown that a significant portion of the root-canal surfaces remain untouched by instrumentation and bacteria survive in these favorable niches (1, 5). Use of liquids with higher diffusibility might further enhance diffusion of nanoparticulates into the biofilm structure and anatomical complexities within the root-canals.

In summary the present study highlighted the efficacy of the Cs-np and ZnO-np to reduce biofilm-bacteria and disrupt biofilm-structure. The antibacterial-property of these nanoparticles was retained even after ageing for 90 days. Therefore, Cs-np and ZnO-np possess a potential antibiofilm capability and further studies using ex-vivo or in-vivo models are required to validate its potential application in NSRCT.

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2.7 References


Chapter 3
Effect of tissue inhibitors on the antibacterial activity of chitosan nanoparticles and photodynamic therapy

3.1 Abstract

Newer antibacterial alternatives such as chitosan nanoparticles (Cs-np) and photodynamic therapy (PDT) have been investigated to achieve effective root canal disinfection. The current study aims to assess the effect of various tissue inhibitors such as dentin, dentin-matrix, pulp tissue, bacterial lipopolysaccharides (LPS), and bovine serum albumin (BSA) on the antibacterial activity of Cs-np and PDT. The antibacterial effect of Cs-np and PDT using photosensitizers, rose bengal (RB) and methylene blue (MB) were tested on planktonic Enterococcus faecalis ATCC 29212 with or without pre-treatment using different tissue inhibitors for an hour. Bacterial survival was assessed after 1, 8 and 24 h of incubation with Cs-np, and after PDT using RB and MB. Pulp and BSA inhibited the antibacterial effect of Cs-np significantly (p<0.05). The antibacterial effect of Cs-np was not affected by dentin, dentin-matrix and LPS. Antibacterial activity of PDT using MB and RB was inhibited in a decreasing order by dentin-matrix, BSA, pulp, dentin and LPS (p<0.05). The tissue inhibitors effect was higher in case of PDT with RB. Depending on the antibacterial mechanism of Cs-np and PDT, different inhibitory patterns were observed with different tissue inhibitors. The tissue inhibitors present within the root canal affected the antibacterial activity at varying degrees of Cs-np and PDT. Further research is required to enhance their antimicrobial efficacy in an endodontic environment.
3.2 Introduction

Efforts to achieve effective elimination of intracanal bacteria from the infected root canal system have received tremendous focus in Endodontics. Both primary and retreatment endodontic failures highlight the shortcomings of the current root canal disinfectants (1, 2). Other than the conventional antimicrobial agents such as sodium hypochlorite, chlorhexidine and calcium hydroxide; photodynamic treatment (PDT) and nanoparticles based disinfection strategies have been investigated recently (3-7). Despite the high antimicrobial efficacy of conventional disinfectants in vitro, clinical studies have demonstrated bacterial persistence within the root canal system after cleaning and shaping procedures (2, 8). In addition to the bacterial persistence, complex root canal anatomy, as well as the presence of various tissue inhibitors are known to impede the efficacy of conventional disinfection strategies (9-11).

The infected root canal system is known contain bacteria and its by-products, tissue fluids, dentin, dentin matrix and pulpal tissue remnants. These root canal constituents have been shown to reduce or completely inhibit the antibacterial activity of CHX, potassium iodide and calcium hydroxide (9, 12). Pulpal tissues are found in the apical ramifications and lateral canals even after cleaning and shaping procedures (13). The inorganic fraction of dentin is also known to play an important role as an inhibitor due to their buffering action (14). However, no specific mechanism has been suggested for the inhibitory effect of different organic components. On similar lines, it is important that all the newly developed disinfection strategies are tested in the presence of these tissue inhibitors to evaluate their intracanal antibacterial efficacy.

Nanoparticles based on metals or polymers are being assessed for augmenting the endodontic disinfection methods (3, 5). Biopolymeric nanoparticles such as chitosan (Cs-np) offers different advantages such as broad spectrum of antibacterial activity, biocompatibility and its ability to resist aging for longer periods when compared to conventional sealers (5, 15). The antibacterial effect of sodium hypochlorite irrigation has been reported to be limited to 130 microns into the dentinal tubules (16). These nanoparticles could be delivered deeper into the dentinal tubules using improved ultrasonic activation where bacteria are not effectively removed by routine cleaning and shaping procedures (17).

PDT is based on the use of a non-toxic photosensitizer, which when activated using a low energy
light results in the production of free radical species such as singlet oxygen (18). Singlet oxygen generated is highly reactive and is known to target various bacterial sites such as cell wall, nucleic acid as well as membrane proteins (19). This mechanism of antibacterial activity confers PDT with the advantages of broad spectrum antibacterial activity and lower risk of developing resistance. Phenothiazines and xanthenes are the two class of photosensitizers commonly tested for antibacterial efficacy (20). Methylene blue (MB) falls under the category of cationic phenothiazines and is effective against various endodontic bacteria (21, 22). MB possesses greater antibacterial effect due to its hydrophilicity, low molecular weight and cationicity (20). RB is an anionic photosensitizer with lesser antibacterial effect compared to MB (15). The antibacterial effect of PDT is compromised when photosensitizers are mixed in media containing serum products (22). Considering the influence of tissue inhibitors on the endodontic disinfectants this study aims to examine the effect of various tissue inhibitors on the antibacterial activity of Cs-np and PDT.

3.3 Materials and Methods

Chitosan nanoparticles (Cs-np) were synthesized following the previous literature (23). RB, MB and lipopolysaccharide (LPS) from *Escherichia coli* were purchased from Sigma Aldrich (St. Louis, MO, USA). Following inhibitors were tested against Cs-np and PDT using RB and MB; (a) 28 mg of dentin powder (9); (b) 10 mg of fresh bovine pulp, frozen and powdered (24); (c) 5 mg of dentin-matrix (9); and (d) 2% and 18% bovine serum albumin (BSA) (9) and LPS (1 μg/ml) (25).

Extracted human third molars and bovine teeth from the slaughterhouse were obtained following approval from the Research Ethics Office, University of Toronto. Dentin powder was prepared from third molars using a mechanical grinder. The dentin powder (28 mg) was treated with 17% EDTA (pH 7.0) for 5 days followed by washing and centrifugation 3 times (10,000 x g, 2 m) with deionized-water. The dentin-matrix thus obtained was lyophilized and used in the study as an inhibitor. Bovine teeth were decoronated; their pulps removed, frozen, and held at -80°C. The frozen pieces of pulp were chilled further in liquid nitrogen and broken into smaller pieces with a mortar and pestle. The chilled powdered pulp was placed in a glass vial, lyophilized, and the vial was tightly capped and stored at 5°C. *Enterococcus faecalis* was used as a test organism as it is found in high prevalence in the retreatment cases (26, 27). *E. faecalis* ATCC 29212 was grown
overnight on brain heart infusion (BHI) broth (Bacto, DIFCO Laboratories, NJ). The culture was centrifuged (4500 rpm, 10 m), washed twice in sterile deionized-water and adjusted spectrophotometrically to a cell density of approximately $10^8$ colony-forming units per mL (optical density= 0.7).

3.3.1 Effect of Tissue Inhibitors on the Antibacterial Properties of Cs-np

The experimental inhibitors; dentin, dentin-matrix, pulpal and bacterial remnants, and BSA were added into 500 μL of the 10 mg Cs-np and incubated in sealed test tubes at 37°C for 1 h. This concentration of Cs-np was chosen based on the previous study (5). The control group consisted of 500 μL of water, instead of an inhibitor. After 1 h of incubation, 500 μL of bacterial suspension was added and the incubation continued at 37°C. Fifty micro liter samples were taken immediately after adding the bacteria and after 1 h, 8 h and 24 h intervals. The samples were serially diluted, plated onto freshly poured BHI agar plates and incubated for 24 h at 37°C.

3.3.2 Effect of Tissue Inhibitors on the Antibacterial Properties of PDT

In case of PDT the inhibitors (dentin, dentin-matrix, pulpal and bacterial remnants, and BSA) were assessed on two photosensitizers (10 μM of RB and MB). The concentration of RB and MB was chosen based on the previous work (15). 1 mL of photosensitizers was incubated with tissue inhibitors for 1 h, 37°C. The photosensitizers and inhibitors mixture was then added into the cell pellet of *E. faecalis* and photosensitized for 15 min in dark. Following the photosensitization, bacterial cells were centrifuged to remove the unbound photosensitizers and subjected for PDT at 5 and 10 J/cm$^2$ energy dose. The samples were serially diluted, and various dilutions were plated onto freshly poured BHI agar plates for 24 h at 37°C.

3.3.3 Statistical Analysis

Statistical analysis of *E. faecalis* percentage survival rates under the different inhibitors treatment conditions was performed by using a one-way analysis of variance and Tukey test. P<0.05 was considered to indicate statistical significance.
Figure 3.1. Killing of *E. faecalis* ATCC 29212 by Cs-np, (A) in the presence of pulp, dentin, dentin matrix, and (B) LPS and BSA.
3.4 Results

3.4.1 Effect of Tissue Inhibitors on the Antibacterial Properties of Cs-np

Cs-np showed complete killing of *E. faecalis* without any inhibitors by 24 h (Fig. 3.1). Presence of dentin, dentin-matrix and LPS did not inhibit the antibacterial efficacy of Cs-np. The pulpal tissues showed highest inhibitory effect with only 27% bacterial reduction (Fig. 3.1 A) (p<0.05). BSA also produced strong inhibitory effect with only 40% bacterial reduction (p<0.05).

3.4.2 Effect of Tissue Inhibitors on the Antibacterial Properties of PDT

*E. faecalis* was completely eliminated with both photosensitizers, RB and MB following PDT in the absence of any inhibitors (Fig. 3.2). Dentin-matrix, BSA, pulp, dentin and LPS inhibited the PDT mediated antibacterial action of RB and MB in descending order. Photoactivation of MB produced significantly (p<0.05) better antibacterial activity than photoactivation of RB in the presence of dentin (58 vs. 88%), pulp (62 vs. 92%), BSA (71 vs. 98%) and LPS (50 vs. 61%). Presence of dentin-matrix significantly inhibited the antibacterial activity of photoactivated MB (98%) and RB (98%) (p<0.05).

3.5 Discussion

This study evaluated the influence of common tissue inhibitors on the antibacterial effect of Cs-np and PDT with different mechanisms of bacterial killing. Cs-np is known to possess inherent antibacterial property that is effective against *E. faecalis* (5, 23). The mechanism of action though not specific, has mainly been attributed to the electrostatic interaction with bacterial surface leading to increase in permeability and release of cell constituents (28). The inhibitory effect of pulp and BSA on Cs-np was significantly high. Periapical tissue exudates are rich in proteins and enter the apical aspect of the root canals through the apical foramen in purulent infections (29). Chitosan has been shown to bind with glycosaminoglycans and enhance the antimicrobial activity of other peptides (30). The cationic Cs-np is highly reactive and interacts physico-chemically with other charged particles. The components of pulp and BSA could also interact physico-chemically with Cs-np compromising its antibacterial efficacy. However, dentin and dentin-matrix showed lesser inhibitory effect. Previous study has shown that when dentin is treated with Cs-np with and without EDTA pretreatment, bacterial adherence was reduced significantly (23). The weaker
Figure 3.2. Killing of *E. faecalis* ATCC 29212 by PDT using (A) MB and (B) RB in the presence of pulp, dentin, dentin matrix, LPS and BSA.
chemical interaction of Cs-np with dentin matrix components could have resulted in the lack of inhibition of its antibacterial efficacy. CS is also known to form electrostatic complexes with LPS (25). The concentration of LPS might be a factor to consider for its inhibitory effect, as in this study the Cs-np used was effective enough to eliminate bacteria in the presence of LPS.

The antibacterial activity of both RB and MB were reduced at a varying degree in the presence of tissue inhibitors. Photosensitizers possess specific charges that could interact with the inhibitors used in the study. Presence of various ions also alters the photosensitizer uptake and antibacterial mechanism of PDT (20). Reduction in the uptake of photosensitizers into the bacterial cell could be one of the clinical factors in compromising the antibacterial effect of PDT (20, 31, 32). Bhatti et al. showed that the presence of serum reduced the uptake of toluidine blue in Porphyromonas gingivalis, by five times and inhibited the killing efficacy after PDT (31). In addition, quenching of singlet oxygen further undermines the antibacterial effect of PDT. The tissue inhibitors could also act as a potential substrate for highly reactive singlet oxygen (32). The singlet oxygen generated has been shown to crosslink the collagen from dentin as well as other proteins (33, 34).

The half-life of singlet oxygen has been shown to be compromised in the presence of serum proteins (31). The effect of pulp, BSA, dentin and LPS on MB was lesser when compared to RB. This could be due to the difference in the mechanism of photosensitizer uptake by the bacteria.

The uptake of RB into the bacterial cells are known to occur via protein transporter in contrast to electrostatic interactions and self-promoted uptake pathways in MB (20). Presence of tissue inhibitors in the vicinity of bacterial cells may have hindered the uptake of RB into the cell and reduced its efficacy. In case of MB, the cationicity and higher amount of reactive singlet oxygen could have resulted in moderate degree of bacterial killing even in presence of inhibitors.

In conclusion, dentin, dentin-matrix, pulp tissue, bacterial lipopolysaccharides, and bovine serum albumin (BSA) present within the root canal affected the activity of newer antibacterial approaches such as Cs-np and PDT. Further research of such treatment options within the root canals and improvements on their antibacterial efficacy is warranted before considering them for clinical application.

3.6 Acknowledgement

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3.7 References


Chapter 4
Photodynamically Crosslinked and Chitosan-incorporated Dentin Collagen

4.1 Abstract

A lingering concern with restored root-filled teeth is the loss of structural integrity of the dentin and dentin-sealer interface over time. We hypothesize that crosslinking of dentin collagen with simultaneous incorporation of a biopolymer into collagen matrix will improve its structural stability. This study aimed to investigate the effects of combining chemical/photodynamic crosslinking of dentin collagen with the incorporation of carboxymethyl-chitosan (CMCS) on the resistance to enzymatic degradation and mechanical properties of dentin collagen. Ninety-six demineralized dentin collagen specimens (human, n=72 and bovine, n=24) were prepared and crosslinked chemically/photodynamically, with/without CMCS. Glutaraldehyde and carbodiimides were used for chemical crosslinking, while rose bengal activated with a non-coherent light (540 nm) at 20 J/cm² was applied for photodynamic crosslinking. The crosslinked human dentin collagen was subjected to chemical characterization, 7-days enzymatic degradation and transmission electron microscopy (TEM), while the bovine dentin collagen was used for tensile-testing. Crosslinked collagen showed significantly higher resistance to enzymatic degradation (p<0.01), stable ultrastructure and increased UTS (p<0.05). Crosslinking CMCS with collagen matrix as observed in the TEM further improved the mechanical properties of dentin collagen (p<0.01). This study highlighted the possibility of improving the resistance and toughness of dentin collagen by chemically/photodynamically crosslinking collagen matrix with CMCS.
4.2 Introduction

Dentin is a composite material comprised of an inorganic component (50 vol%), organic component (30 vol%) and water (20 vol%) (1). The inorganic component contributes mainly to the stiffness of dentin (2). The main organic component of the dentin is type I collagen (90%) (1). These type I collagen fibrils are stabilized by endogenous covalent intra and inter-molecular crosslinks that contribute significantly to the mechanical properties of the dentin, such as fracture toughness, ultimate tensile strength (UTS) and viscoelasticity (2, 3). The structural integrity of hard tissues, such as dentin, depends on the optimum balance between toughness and stiffness (4).

When non-surgical root canal treatment (NSRCT) is performed, the canal space is disinfected by mechanical and chemical means, and subsequently filled with a core filler and sealer cement to prevent bacterial ingress for the long-term service of the tooth. The chemicals used during NSRCT may induce physical and chemical changes in the root dentin (2, 5, 6). Demineralization of dentin with exposed collagen fibrils has been reported after treatment with 17% EDTA (1-2 μm) or MTAD (5-6 μm), contributing to interfacial nanoleakage at the dentin-sealer interface (6). Further, 75% reduction in the mechanical strength of dentin was reported following exposure to NaOCl (7). Dentin collagen degradation due to bacterial enzymes and host-derived matrix metalloproteinases (MMPs) is another factor that can lead to the loss of structural integrity of root-filled teeth (2, 8).

In recent years there has been an increased focus on crosslinking of collagen and neutralization of MMPs to stabilize dentin collagen (9, 10). Increase in the number of inter- and intra-molecular bonds by crosslinking of collagen has been suggested to enhance the mechanical properties and to increase resistance of dentin collagen to enzymatic degradation (9, 11). Glutaraldehyde (GD) is commonly used fixative that induces crosslinking within collagen-fibrils (12, 13). However, the cytotoxicity of GD hampers its applicability in vivo (14). Water-soluble carbodiimides are biocompatible agents that induce the formation of amide-type crosslinks in collagen (12). Alternatively, photodynamic crosslinking has been reported to induce rapid and stable covalent crosslinking of collagen by exposing photosensitizers such as rose bengal (RB) to appropriate wavelength of light (540 nm) (15-17). Biocompatible methods and natural cross-linkers that can form stable crosslinked collagen in a short time may be beneficial for stabilizing the root dentin collagen after NSRCT.
In addition to crosslinking, reinforcement of the collagen matrix can be achieved by incorporating biopolymers that can be crosslinked with collagen fibrils (18, 19). Chitosan is a hydrophilic biopolymer with a large number of free hydroxyl and amino groups (20). Incorporation of chitosan has been suggested to improve the biological and mechanical properties of collagen constructs (19, 21). It is biocompatible and possesses a broad range of antimicrobial/antibiofilm activity (20, 22, 23). The purpose of this study was to investigate the effects of chemical and photodynamic crosslinking and simultaneous incorporation of water-soluble carboxymethyl-chitosan (CMCS) on the resistance to enzymatic degradation, ultrastructure and mechanical properties of dentin collagen. The null hypothesis was that collagen crosslinking in the presence of CMCS would have no effect on the resistance to enzymatic degradation and the mechanical properties of dentin collagen.

4.3 Materials and Methods

Rose Bengal (RB), 1-ethyl-3- (3-dimethyl aminopropyl)-carbodiimide (EDC) and N-hydroxysuccinimide (NHS) were obtained from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade (purity≥95%). CMCS was synthesized from chitosan powder as described by Laudenslager et al. (24).

4.3.1 Demineralized Dentin collagen Specimens

Thirty-six non-carious human incisors (freshly extracted for periodontal reasons) without any patient identifiers and 12 bovine incisors (from a slaughterhouse) were collected following the University of Toronto ethical guidelines and stored in 0.9% saline until use. The human teeth were used for chemical and enzymatic degradation analysis while the bovine teeth were used for mechanical testing. Bovine dentin collagen was used for the mechanical test, as it is easier to obtain freshly extracted uniformly sized incisors and standardize the specimens for tensile testing (25). Dentin sections were obtained as shown in Fig. 4.1 and demineralized in 1M EDTA (pH = 7.4) at 37°C for 7 days. The resulting dentin collagen specimens were rinsed for 10 minutes in deionized-water to remove residual EDTA, stored in sterile deionized-water at 4°C and used within one week of preparation.
Figure 4.1. From each tooth two dentin sections of 0.5 mm (labio-lingual) thickness were prepared from either side of the root canal lumen using a slow-speed diamond wafer blade (Buehler, Dusseldorf, Germany) under continuous water irrigation. Crown and the apical portion of the sections were removed. Final dentin sections of 12x2x0.5 mm (human) and 16x2x0.2 mm (bovine) were obtained by grinding in wet emery paper of grit sizes 400, 800 and 1000 under continuous water irrigation and demineralized in 1M EDTA (pH = 7.4) for 7 days. The dentinal tubules were aligned perpendicular to the specimen surface being treated.
4.3.2 Crosslinking of Collagen

Demineralized dentin collagen specimens, human (N=72) and bovine (N=24) were divided into six treatment groups with human (n=12) and bovine (n=4) giving a total of 16 samples per group. Three of the treatment groups were subjected to chemical crosslinking by treating the dentin collagen specimens for 6 hours with one of the following: 2.5% glutaraldehyde (GD group); EDC/NHS (2:1; EDC/NHS group); and CMCS 1% + EDC/NHS (CMCS-EDC/NHS group). The photodynamic crosslinking used RB 10μM (RB group) and CMCS 1% + RB 10μM (CMCS-RB group) where collagen specimens were placed in a 24 well plate. The specimens in the RB group were immersed in 1 mL of RB solution for 15 min; excess RB was removed and the photosensitized collagen specimen was activated with a non-coherent light for 6.5 min (540 nm, 20 J/cm²) (LumaCare Inc., NewPort Beach, CA, USA). In the CMCS-RB group, specimens were initially treated with 1 mL CMCS for 15 min, excess CMCS was removed and photodynamic crosslinking conducted similar to the RB group. After crosslinking the specimens were thoroughly washed in deionized-water three times, stored in a vacuum dessicator overnight and then tested for chemical analysis. For the enzymatic degradation analysis, the specimens were lyophilized for 24 hours. The bovine dentin collagen specimens were maintained in deionized-water for mechanical testing and used within a week. The control group in mechanical testing included bovine dentin collagen specimens without any treatment.

4.3.3 Chemical Characterization

The vacuum desiccated collagen specimens were treated with liquid nitrogen, ground and mixed with potassium bromide (1:100 w/w) for the fourier transform infrared (FTIR) spectroscopy (16 cm⁻¹ resolution, 100 scans per sample) (Shimadzu, Kyoto, Japan).

4.3.4 Enzymatic Degradation Analysis

Ninhydrin assay was used to quantify the amino acid release following enzymatic degradation of collagen as described by Mandl et al. (26). In brief, desiccated collagen specimens (5 mg) were subjected to enzymatic degradation using collagenase from *Clostridium histolyticum* with an activity of 125 CDU/mg solid (P/N C-0130; Sigma) in 50 mM HEPES buffer at 37°C. After 1, 2, 3 and 7 days of degradation, 200 μL of the solution was treated with ninhydrin reagent. The amount
of free amino acids released following degradation of collagen specimens after heating with ninhydrin, was proportional to the optical absorbance (560 nm) of the solution \( 13 \). The amount of amino acids released from the crosslinked and non-crosslinked dentin collagen specimens were quantified using the standard curve of L-Leucine.

### 4.3.5 TEM Evaluation

Four specimens from each group were processed for TEM evaluation after crosslinking and 5 days of enzymatic degradation. The collagen specimens were fixed in 2.5% glutaraldehyde (0.1M phosphate buffer) (overnight). All specimens for the TEM were prepared following previous protocol \( 8 \). The 90 nm thick sections were prepared along the cross-section of the specimens and examined under TEM (Hitachi H-7000, Tokyo) at 80 kV.

### 4.3.6 Mechanical Testing

The bovine dentin collagen specimens from all six groups were used for UTS testing (Instron 5544, Instron Corporation, Canton, MA, USA). The specimens were positioned in the loading jig by carefully gripping the two ends (4 mm), and were subjected to a tensile load at a crosshead speed of 1 mm/min until failure occurred. A 100 N load cell with sensitivity of ± 0.5% was used in this experiment. Care was taken to keep the specimens well hydrated at all times during the test. To avoid the tear of the samples at the jaw, the ends of the samples were covered using aluminum foil \( 27 \). The stress-strain curves of all specimens were plotted for all the groups. The UTS and toughness (MPa), represented by the area under the stress-strain curves, were calculated using OriginPro 8.1 software (OriginLab Corporation, MA, USA).

### 4.3.7 Statistical Analysis

Average and standard deviations were collected for each group and analyzed using one-way ANOVA and post hoc Tukey test to compare between groups at 95% confidence interval.

### 4.4 Results

#### 4.4.1 Chemical Characterization

The FTIR spectra obtained from the dentin collagen specimens are shown in Fig. 4.2 A & 4.2 B.
The amide I (1635 cm\(^{-1}\)), amide II (1542 cm\(^{-1}\)) and C-N (1458 cm\(^{-1}\)) bands were analyzed to assess the presence of crosslinking (21,28). Both the crosslinking methods resulted in a reduced amide I peak relative to the amide II peak in all the crosslinked collagen specimens. However, chemical and photodynamic crosslinking in the presence of CMCS showed broad amide peaks as well as shift to lower wavenumber (1512 cm\(^{-1}\)), which was due to the incorporation of CMCS into the collagen network.

4.4.2 Enzymatic Degradation Analysis

The amount of amino acids released following enzymatic degradation of the crosslinked and non-crosslinked dentin collagen was significantly different as a function of time (\(p<0.05\)) (Fig. 4.2 C). After 7 days the control group specimens disintegrated completely and released the highest amount of amino acid (5 \(\mu\)mol/mL). The amount of amino acid (\(\mu\)mol/mL) release on the 7\(^{th}\) day was in the ascending order of- GD group (0.096); EDC/NHS (0.25); RB group (0.25); CMCS-RB (0.91) and CMCS-EDC/NHS (0.98).

4.4.3 TEM Evaluation

TEM micrographs from the control group revealed a collagen matrix that contained intact, banded collagen fibrils (Fig. 4.3 A). The arrangements of the collagen fibrils in the GD and CMCS-RB groups were denser with smooth edges (Fig. 4.3 C & 4.3 E). Enzymatic degradation for 5 days resulted in irregular loosely arranged fibrils, loss of cross-banding and disorganized mesh of microfibrillar strands in the control group (Fig. 4.3 B). However, the treatment groups revealed stable collagen ultrastructure without any signs of degradation of collagen fibrils. A coating of CMCS on the surface and the incorporation of CMCS within the collagen matrix were evident (Fig. 4.3 E & 4.3 F).

4.4.4 Mechanical Testing

The stress-strain curves demonstrated increased UTS values and toughness in all the crosslinked dentin collagen groups except GD, compared with the control group (Fig. 4.2 D) (Table 4.1). Even though the GD group showed a greater increase in UTS, the percentage elongation decreased drastically indicating brittle behavior. The average initial toughness of collagen following demineralization was 17 MPa. All other treatment groups, EDC/NHS, CMCS-EDC/NHS, RB and
Figure 4.2. FTIR spectra of dentin collagen after chemical crosslinking (A), and after photodynamic crosslinking (B). In the crosslinked collagen the amide bands, especially amide I (1666 cm\(^{-1}\)), decreased relative to the amide II (1558 cm\(^{-1}\)) bands in the crosslinked groups. Spectral designations: (a) Control; (b) 2.5% glutaraldehyde (GD group); (c) EDC/NHS (2:1; EDC/NHS group); (d) CMCS 1% + EDC/NHS (CMCS-EDC/NHS group); (e) RB 10\(\mu\)M (RB group) and (f) CMCS 1% + RB (CMCS-RB group). (C) Graph showing release of amino acid from dentin collagen after chemical crosslinking and photodynamic crosslinking. Control group specimens showed the highest release of amino acids by day 7. All the crosslinked specimens showed significantly less degradation (p<0.01). (D) Stress-strain curve of dentin collagen specimens after chemical crosslinking and photodynamic crosslinking. Toughness values (area under the curve) were significantly higher (p<0.01) in all the treatment groups except GD when compared to the control group.
Figure 4.3. TEM micrographs of collagen fibrils before and after enzymatic degradation for 5 days. All the specimens were viewed along the cross-section. (A) Demineralized dentin matrix without any treatment showed the presence of well-arranged collagen fibrils. The edges of the specimens showed frayed open collagen fibrils due to the sample preparation (Open arrow). (B) After 5 days of degradation the collagen fibrils were denatured, the fibrillar arrangement was disrupted; the density of the fibrils had reduced with open spaces resembling a moth eaten appearance (Star). The frayed edges of the specimens had smoothened out. (C & D) The GD crosslinked specimens showed dense fibrillar arrangement and normal cross-banding of the fibrils. There was no change in the appearance and density of the collagen fibrils after degradation. (E) Crosslinking in the presence of CMCS resulted in the incorporation of CMCS into the collagen matrix. The fibrillar arrangement and density were similar to that of GD group. The CMCS formed a layer of polymeric film (Black arrow) on the collagen surface. (F) Following degradation the fibrillar arrangement and collagen architecture well maintained. Areas of CMCS polymer incorporated into the collagen fibrils were evident (White arrows).

Table 4.1. Changes in the ultimate tensile strength (UTS) and toughness of dentin collagen following different crosslinking procedures.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GD</th>
<th>EDC/NHS</th>
<th>CMCS-EDC/NHS</th>
<th>RB</th>
<th>CMCS-RB</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTS (MPa)</td>
<td>4.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>(MPa/m&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>(0.78)</td>
<td>(1.66)</td>
<td>(2.61)</td>
<td>(0.59)</td>
<td>(0.70)</td>
<td>(1.52)</td>
</tr>
<tr>
<td>Toughness</td>
<td>17.49&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.74&lt;sup&gt;d&lt;/sup&gt;</td>
<td>107.05&lt;sup&gt;d&lt;/sup&gt;</td>
<td>152.15&lt;sup&gt;d&lt;/sup&gt;</td>
<td>51.95&lt;sup&gt;d&lt;/sup&gt;</td>
<td>104.31&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>(MPa/m&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>(0.64)</td>
<td>(2.66)</td>
<td>(18.69)</td>
<td>(4.45)</td>
<td>(1.52)</td>
<td>(24.58)</td>
</tr>
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</table>

For each row, groups identified by different superscript letters are significantly different (p<0.05). Results are means (± 1 SD).
CMCS-RB, showed significant increases in toughness compared to the controls (p<0.05). The CMCS incorporation during collagen crosslinking resulted in higher toughness when compared to mere crosslinking of collagen (p<0.05).

4.5 Discussion

Chemical or photodynamic crosslinking methods can stabilize biological tissues by inducing various intra- and intermolecular crosslinks in collagen (12, 17, 21). Potentially, demineralized dentin can be stabilized using crosslinking strategies (9, 11). To evaluate the overall effects of crosslinking procedures on dentin collagen, chemical and mechanical properties need to be investigated. The findings from this study demonstrated that chemical and photodynamic crosslinking delayed the enzymatic degradation of dentin collagen, and at the same time increased the overall UTS and toughness. Thus the null hypothesis, that collagen crosslinking in the presence of CMCS would not affect the enzymatic degradation and mechanical properties of dentin collagen, was rejected. Even though chemically crosslinked collagen showed physical and mechanical effects similar to those of photodynamically crosslinked collagen, the treatment time required to establish stable crosslinks is much longer with chemical methods (9, 18, 28). This could be a major limitation clinically, where shorter treatment time is desirable. Photodynamic crosslinking is a rapid process that occurs via the production of singlet oxygen or radicals by the light activated photosensitizers. The singlet oxygen interacts with photooxidizable amino acid residues, such as Cys, His, Trp or Tyr in a protein molecule. The photooxidized products in turn, react with normal or photoaltered residues in another protein molecule resulting in a crosslink (29). Photoactivation could excite the highly reactive free amino groups in the CMCS and crosslink it with the adjacent collagen molecules. These CMCS incorporated collagen matrix was confirmed using the TEM analysis.

The FTIR spectroscopy confirmed the formation of additional collagen crosslinking with reduced amide I peaks relative to the amide II peaks due to conversion of free -NH$_2$ groups to –NH groups (21, 28). The increase in CN bands relative to amide I bands has been suggested to be due to the crosslinking between COOH and NH$_2$ groups (21). The incorporation of CMCS into collagen network was confirmed by the decreased peak heights as well as broad amide bands (19). The overlap of the amide bands of collagen and CMCS (1589 cm$^{-1}$) could have resulted in the broad amide peaks and shift in amide II following crosslinking of CMCS with dentin collagen (19). The
physical stability of crosslinked dentin collagen was evaluated by monitoring the release of amino acids following bacterial enzymatic degradation (12, 26). Bacterial collagenase degraded collagen by hydrolyzing the peptide bond on the amino-terminal side of Glycine (−X-Gly-Pro) (30). Following crosslinking of collagen, the sites of collagenase attack may be hidden or modified, and this contributes to the significant difference in the release of amino acid residues following enzymatic degradation as observed in this study (31). The enzymatic degradation for 5 days resulted in significant loss of fibrillar arrangement and density as evident from TEM images.

In this study bovine dentin collagen was used for mechanical testing. The study required dentin collagen specimens of prescribed dimension within specific time after extraction to carry out the mechanical testing. Previous studies have highlighted that bovine teeth are morphologically and histochemically similar to human teeth and have been considered as a suitable substitute for human teeth (25, 32). These studies have highlighted that the basic organic constituent in bovine and human dentin are similar, crosslinking and its effect could be correlated qualitatively even though the quantitative values of mechanical properties are different (32). It was observed that GD treatment increased UTS, but decreased toughness of the dentin collagen resulting sharp drop in strain beyond the elastic limit, characteristic of brittle behavior. This may be attributed to the random crosslinking between reactive groups (21). EDC can induce crosslinking by the formation of stable amide-bonds between carboxyl groups and primary amines (33). However, functional groups located on the adjacent collagen microfibrils are sometimes too far apart to be bridged by carbodiimides alone. The incorporation of CMCS in both EDC/NHS and RB crosslinked collagen specimens improved the mechanical properties and showed the highest values of toughness when compared to the merely crosslinked collagen specimens. The incorporation of CMCS may have reinforced the collagen structure by amplifying the number of amine reaction sites resulting in the formation of ionic complexes between CMCS and collagen during crosslinking (34). CMCS incorporation may also prevent undesired zero-length crosslinking and subsequently improve the fracture toughness (18). Chitosan polymers have been considered structurally similar to extracellular matrix materials and reinforced the collagen constructs (35).

This study highlighted the possibility of improving the chemical stability and toughness of dentin collagen by chemically/photodynamically crosslinking collagen matrix with CMCS. This approach could have potential application in improving the ultrastructural stability of root dentin following NSRCT.
4.6 Acknowledgment

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4.7 References

Chapter 5
Characterization of a Conjugate between Rose Bengal and Chitosan for Targeted Antibiofilm and Tissue Stabilization Effects as a Potential Treatment of Infected Dentin

5.1 Abstract

Bacterial biofilms and dentin structural changes are some of the major challenges in the management of infected dentin tissue. This study characterized a photosensitizer-conjugated chitosan with enhanced photodynamic efficacy against dental biofilms, as well as ability to reinforce the post-infected dentin matrix in order to improve its mechanical and chemical stability. Rose bengal conjugated chitosan (CSRB) was synthesized using a chemical crosslinking method, characterized for photophysical, photobiological and cytotoxicity properties. Its potential as an antibacterial and matrix-reinforcing agent on dentin-collagen were also evaluated. *Enterococcus faecalis* as planktonic and in vitro biofilms were treated with CSRB and photodynamically activated with 5-60 J/cm² green light. Dentin-collagen was used for the CSRB crosslinking experiments and evaluated for chemical changes, resistance to enzymatic degradation and mechanical properties. CSRB was a photosensitizer with efficient singlet oxygen yield. In vitro photoactivation gave higher fibroblast cell survival compared to RB alone. CSRB showed significant antibiofilm photoinactivation (p<0.01). The CSRB crosslinked dentin-collagen showed higher resistance to collagenase degradation and superior mechanical properties (p<0.05). In summary the photoactivated CSRB particles synthesized in this study may be a synergistic multifunctional treatment approach with lower cytotoxicity, effective antibiofilm activity as well as ability to reinforce the dentin-collagen to enhance resistance to degradation and improve mechanical properties. This may be a targeted treatment strategy to deal with infected dentin hard tissues in a clinical scenario, wherein both disinfection and structural integrity need to be addressed concomitantly.
5.2 Introduction

Bacterial biofilms and dentin structural changes are major challenges in the management of infected dentin tissue. Antimicrobials are traditionally used to for non-invasive management of infected hard tissue. Although chemical based disinfectants are important to reduce microbial loads and remove infected smear layer from root dentin, they have only a limited ability to eliminate biofilm bacteria especially from root dentinal complexities (8, 28). The chemical treatment of root dentin is known to produce irreversible hard tissue alterations such as demineralization or surface degradation (29). The combination of ultrasonic agitation with chemical irrigants further increased the degree of surface degradation on root dentin (9). In addition, degradation of the dentin matrix is also caused by host and bacterial proteases (14). Therefore pathologically and iatrogenically modified dentin may compromise the mechanical integrity of bulk dentin (22). The search for alternative antimicrobial approaches able to achieve effective biofilm elimination from root dentin has received considerable interest in recent times.

Photodynamic therapy (PDT) is under investigation for various purposes such as antimicrobial disinfection (15, 18), anticancer therapy (19), tissue welding and tissue engineering approaches (6). The combination of an effective photosensitizer, the appropriate wavelength of light, and ambient oxygen are the key factors in PDT (18). The singlet oxygen generated during the interaction of these three factors has been reported to have a broad range of antibacterial activity. The chances of developing microbial resistance to PDT are low as the oxygen-based free radicals act on multiple targets within the bacterial cell (15, 18, 36). Photodynamic inactivation of a wide variety of both Gram-positive and Gram-negative species in both planktonic and biofilm forms has been reported in the literature (12, 15, 16, 36). We had previously shown that modifying a photosensitizer with a cationic polymer resulted in much better photodynamic killing of bacterial biofilms than either the xanthene dye rose bengal or the phenothiazinium dye methylene blue (36). Tissue-specific optimization of PDT using modified methylene blue delivery medium improved elimination of bacterial biofilms from root canals (16).

A single step treatment to achieve effective biofilm elimination as well as to stabilize dentin tissue would provide an excellent strategy in the management of infected root dentin. Modifying photosensitzers with bioactive polymers presents as an attractive option to achieve this objective. Immobilization of photosensitzers on polymeric supports avoids the problem of removing
residual photosensitizers and provides advantages of reduced toxicity and improved stability in physiologic environments \((4, 26, 41)\). Although photosensitizers have been conjugated with various readily available synthetic polymers, biocompatibility becomes a significant limiting factor when applied \(in-vivo\). Use of a naturally occurring biopolymer such as chitosan has been proposed to counteract the biocompatibility issues of synthetic polymers \((26, 30)\).

Chitosan is a derivative of chitin, the second most abundant natural biopolymer, and has received significant interest in the biomedical literature \((10, 47)\). It shows a broad range of antimicrobial activity, and has biocompatible and biodegradable properties \((24, 37)\). Chitosan has been subjected to numerous chemical modifications and grafting procedures due to its large number of free hydroxyl and amino groups \((4, 24)\). The polymer is hydrophilic (wettable) which favors intimate contact between the photosensitizer functionalized surface and the aqueous environment of microorganisms. We formed a hypothesis that Rose Bengal conjugated chitosan (CSRB) could perform the dual function of enhanced antibiofilm photoinactivation efficacy and improved structural stability of dentin-collagen due to the synergistic effect of CSRB and singlet oxygen generated after photoactivation. In the present study we carried out characterization of the CSRB and evaluated its potential application as a dual-function antibacterial and crosslinking agent on dentin-collagen. We hypothesized that CSRB could be a light-activated dual-action synergistic photosensitizer delivery vehicle that enables enhanced antibiofilm efficacy as well as the ability to induce crosslinking of collagen matrices by photoactivation.

5.3 Materials and Methods

All the chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise.

5.3.1 Synthesis and Characterization of CSRB

The CSRB was synthesized using chemical crosslinking by a carbodiimide \((N\text{-ethyl}-N'-(3\text{-dimethyl aminopropyl})\ carbodiimide, EDC) based on a modified published protocol \((26, 36)\). In brief, 1% solution of chitosan was prepared in HCl (0.1 M) and stirred at room temperature in a shaker for 4 hours to obtain a clear solution. After the chitosan dissolved completely, pH was increased to 5 by adding 1 M NaOH and stirred at room temperature using a magnetic stirrer for 12 hrs. A solution of RB (0.05 g) was prepared in 10 mL of aqueous EDC (50 mM) and added
drop-wise to the chitosan solution over 30 min. The mixture was stirred for 12 hrs under dark conditions. The conjugated product was then dialyzed against deionized-water using a dialysis membrane (Sigma, cellulose tubing, cut off 12000-14000 g/mol). The water was replaced daily and dialysis was carried out for a period of 1 week. UV-absorption spectrum (550 nm) of the deionized-water used in dialysis was monitored for the presence of RB (UV-Visible spectrophotometer, Shimadzu 1100, Japan). The dialysis was stopped when no RB absorption was detected in the dialysate. The polymer solution was then freeze dried at -80°C. The CSRB powder obtained was stored in a cool and dark place until further use. The chemical structures of the reagents and product are shown in Fig. 5.1.

Figure 5.1. Schematic of the chemical reaction during conjugation of chitosan with RB in the presence of N-ethyl-N’-(3-dimethyl aminopropyl) carbodiimide (EDC). The formation of chemical bonds between the NH group of chitosan and photosensitizers are highlighted with dotted circle.
Photophysical characterization of conjugated (CSRB) and unconjugated photosensitizer (RB) solutions were conducted by using UV-Visible absorption spectroscopy. The ratio of monomer absorbance at 560 nm to dimer absorbance at 528 nm for different concentrations was calculated to assess aggregation in water. The effective concentration of CSRB was determined based on the highest monomer:dimer ratio. Chemical characterization of the conjugated CSRB was done using fourier transform infrared (FTIR) spectrophotometer (Shimadzu, Kyoto, Japan). The prepared CSRB was mixed with potassium bromide (1:100 w/w) to prepare pellets for the FTIR spectroscopy. The experiments were conducted in transmission mode (16 cm\(^{-1}\) resolution, 32 scans per sample).

Photo-oxidative characterization was conducted to assess the ability of CSRB to generate singlet oxygen. These experiments were conducted in 24 well plates according to a procedure described previously (17). The generation of singlet oxygen from photoactivated RB and CSRB was assessed spectrophotometrically using 1,3-diphenylisobenzofuran (DPBF) that reacts with singlet oxygen. 2 mL of DPBF (200 uM in ethanol) was added (corresponding to absorbance of 2 at 410 nm) to 100 uL of different photosensitizer solutions. A Lumacare lamp (LumaCare Inc., NewPort Beach, CA, USA) with 540+/-15 nm band-pass fiber optic probe was used as a light source (power= 50 mW/cm\(^2\)). In this experiment the rate of singlet oxygen production was related to the rate of decrease of DPBF absorbance at 410 nm (slope of linear trend line, \(k\) value) as a function of irradiation time. The decrease in absorbance was monitored as a function of time using a UV-visible microplate reader (Epoch, Biotek, USA).

### 5.3.2 Evaluation of cytotoxicity and phototoxicity of CSRB

Cytotoxicity of CSRB was assessed quantitatively using mitochondrial activity assay (spectroscopic) and qualitatively using trypan blue exclusion assay (light microscopic). Approximately 1x10\(^5\) NIH 3T3 mouse fibroblast cells (American Type Culture Collection CCL 1, Rockville, MD) were seeded into 24 well plates in Dulbecco’s Modified Eagle medium supplemented with 10% fetal bovine serum and antibiotics and incubated for 48 hrs in 5% CO\(_2\) incubator (Thermo Scientific, Waltham, MA). After incubation, the cells were treated either with CSRB or RB in DMEM at 37°C for15 min in dark. The cells were irradiated with 540 nm light with a total fluence of 20 J/cm\(^2\). RB and CSRB were also tested without light irradiation (dark toxicity). The cells were incubated in the media for 24 hours before evaluating for cytotoxicity.
The supernatant media was removed without disturbing the cells and washed with 1 mL of PBS. Cell survival was determined by the standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay that determines the mitochondrial activity (27). MTT was added at a concentration of 0.5 mg/mL in medium and incubated for 4 hours. After the incubation period, MTT medium was removed, and 1 mL dimethyl sulfoxide was added to dissolve the insoluble formazan crystals. The absorbance at 540 nm was measured photometrically by using a UV-visible microplate reader. Percentage survival of cells was calculated based on the control samples without any treatment as 100%. All analyses were repeated three times in triplicates (total of 9 observations), and the statistical significance was analyzed by one-way analysis of variance. The fibroblasts were also subjected to trypan blue exclusion assay to assess their morphology following treatment. Trypan blue, a vital dye, is excluded from the living cells but stains the dead cells (48). In this assay, the treated cells were washed with PBS and stained with 1 mL of 0.4% (w/v) trypan blue. After 5 minutes of incubation at room temperature, the excess dye was washed with PBS and examined under bright field microscopy (Leica DM IRB, Wetzlar, Germany).

5.3.3 Evaluation of antibacterial efficacy of CSRB

*Enterococcus faecalis* (ATCC 29212) was used to test the antibacterial efficacy of RB and CSRB in both planktonic and biofilm forms. *E. faecalis* is a Gram-positive, facultative aerobic bacteria found in high prevalence in persistent infections following root canal treatment (31). Overnight cultures of *E. faecalis* in BHI broth were centrifuged (3000 rpm, 10 min), washed with deionized-water and optical density adjusted to 0.7 at 600 nm (approx 10^9 CFU/mL). Cell pellets from 1 mL of the above suspension were collected by centrifugation and treated either with 1 mL of RB (10 μM) or CSRB (0.3 mg/mL) at 37°C for 15 min, and protected from ambient light. Dark toxicity was evaluated after 15 min of sensitization with the two treatment solutions. Control group consisted of bacterial cells without any photosensitizer or light treatment. In case of PDT, bacterial cells were centrifuged and excess photosensitizers removed. The sensitized planktonic-bacteria were irradiated using 540 nm fiber with doses of 5 and 10 J/cm^2. After treatment, cell pellets were resuspended in sterile deionized-water (1 mL) and 100 μL of the suspension were plated on freshly poured BHI agar after serial dilution. Colonies were counted after 24 hours of incubation at 37°C and expressed as log colony forming units (CFU) per mL.
In order to test the antibacterial efficacy of photosensitizers on bacterial biofilms, 7-days old biofilms of *E. faecalis* were grown in 24-well-plates. 1 mL of overnight culture in brain-heart infusion broth (BHI) was added into each well and incubated at 37°C, 100 rpm. Fresh media was replenished every 48 hrs to provide a constant supply of nutrients and to remove dead bacterial cells. On the eighth day, the media was removed from the wells, and the biofilm was carefully washed once with sterile deionized-water to remove the dead cells. The biofilm was sensitized with 1 mL of RB (10 μM) or CSRB (0.3 mg/mL) at 37°C for 15 min and irradiated at different doses (20, 40 and 60 J/cm²). Dark toxicity was evaluated after the sensitization period with the two photosensitizers. After PDT treatment, the biofilms were washed gently and 1 mL of sterile PBS was added, the biofilms disrupted mechanically, and plated on freshly poured BHI agar following serial dilutions. Control wells were maintained in sterile PBS. Colonies were counted after 24 hours of incubation at 37°C and expressed as log colony forming units (CFU) per mL. The experiments were carried out twice in triplicate and the mean values were calculated.

5.3.4 Evaluation of CSRB to induce photodynamic crosslinking of dentin-collagen

Sixteen non-caries human incisors and eight bovine incisors were collected according to an approved protocol from University of Toronto ethical guidelines committee (#26363) and stored in 0.9% saline, 4°C until use. The bovine teeth were used for mechanical testing while the human teeth were used to assess chemical composition and enzymatic degradation. Bovine teeth were used for the mechanical test, to avoid the difficulty of obtaining freshly extracted uniformly sized human incisors and to standardize the specimens for tensile testing (46). During experiments, dentin sections of 0.5 mm thickness were prepared from either side of the root canal using a slow speed diamond-wafering blade (Buehler, Coventry, UK) under continuous water irrigation (5). The sections were further ground into dimensions of 12x2x0.5 mm (human) and 16x2x0.2 mm (bovine) using wet emery paper of grit sizes 400, 800 and 1000 under continuous water irrigation. The dentin sections were demineralized in 1M EDTA (pH = 7.4) for 7 days. The resulting dentin-collagen specimens were rinsed for 10 minutes in deionized-water to remove residual EDTA and subsequently stored in sterile deionized-water at 4°C.

Demineralized dentin-collagen specimens, human (N=32) and bovine (N=16) were divided into four treatment groups with human (n=8) and bovine (n=4) giving a total of 12 samples per group:
1) Control group (No-treatment); 2) glutaraldehyde group (2.5%); 3) RB group (10μM); and 4) CSRB group (0.3mg/mL). The dentin-collagen samples were crosslinked with glutaraldehyde for a period of 6 hrs. In photodynamic crosslinking, collagen-samples were placed in a 24 well-plate (area of 2 cm²/well) and immersed in 1 mL of RB or CSRB solution for 15 min. After the sensitization period, excess RB and CSRB were removed, and the photosensitized collagen was activated with 540 nm light for 6.5 min (20 J/cm²). Crosslinked specimens were thoroughly washed in deionized-water three times, stored in a vacuum desiccator overnight and then tested for chemical analysis. For the enzymatic degradation analysis, the specimens were lyophilized for 24 hours. The bovine dentin-collagen specimens were maintained in deionized-water for mechanical testing and used within a week. The control group in mechanical testing included bovine dentin-collagen specimens without any treatment.

5.3.5 Chemical Characterization

The vacuum desiccated collagen specimens were treated with liquid nitrogen, ground and mixed with potassium bromide (1:100 w/w) for the FTIR spectroscopy (16 cm⁻¹ resolution, 100 scans per sample).

5.3.6 Enzymatic Degradation

Enzymatic degradation analysis was conducted to quantify the amino acid release using ninhydrin assay as described by Mandl et al. (25). In brief, the dentin-collagen specimens were subjected to enzymatic degradation using collagenase from Clostridium histolyticum with an activity of 125 CDU/mg solid (P/N C-0130; Sigma). Desiccated collagen specimens (5 mg) were added into 5 mL of buffer solution (50 mM HEPES containing 0.36 mM CaCl₂) and incubated at 37°C for 30 min. 0.1 mL collagenase enzyme (0.1 mg/mL in HEPES buffer) was added into the collagen containing buffer solution and incubated at 37°C in an orbital incubator (100 rpm). After 1, 2, 3, 7 and 14 days of degradation, 200 μL of the solution was added into ninhydrin reagent (2 mL), mixed well and kept in boiling water for 30 min. The containers were allowed to cool to room temperature and 10 mL of 50% isopropanol was added. The amount of free amino acids released following degradation of collagen specimens after heating with ninhydrin was proportional to the optical absorbance (560 nm) of the solution (39). The total amount of amino acids released from
the crosslinked and non-crosslinked dentin-collagen specimens were quantified using a standard curve with L-leucine.

5.3.7 Determination of Mechanical Properties

The fully-hydrated bovine dentin-collagen specimens from all four test groups were used for tensile testing (Instron 5544, Instron Corp, Canton, MA) with a 100 N load cell. The specimens were positioned in the loading jig by gripping the two ends (4 mm) and subjected to tensile load at a crosshead speed of 1 mm/min until failure occurred. Care was taken to keep the samples hydrated at all times during the test. The stress-strain curve per sample was plotted for all the four groups. The ultimate tensile strength and toughness were calculated using OriginPro 8.1 software (OriginLab Corporation, MA). Toughness (MPa) was represented by the area under the stress-strain curves of each collagen sample and percentage change was used to compare before and after crosslinking of collagen.

5.3.8 Statistical Analysis

Average and standard deviations were collected for each group and analyzed using one-way ANOVA and post hoc Tukey test to compare between groups at 95% confidence interval.

5.4 Results

5.4.1 Characterization of CSRB

The absorption spectra obtained for CSRB displayed peaks characteristic of RB (Fig. 5.2 A). Approximately 10 nmol of RB molecules were attached to 1 mg of chitosan. Thus the concentration of RB in 0.3mg/mL of CSRB was calculated to be 3 μM. The absorption bands for CSRB were broader and red-shifted when compared to that of RB alone. These spectral changes confirmed that RB was attached to the polymer chain of chitosan (26). The monomer to dimer ratio showed a concentration dependent response (Fig. 5.2 B). With increase in the concentration of CSRB, the monomer to dimer ratio decreased linearly. Concentration of CSRB above 0.5 mg/mL showed dimer peak almost equal to that of monomer with ratio near to one indicating dimerization or aggregation. The FTIR spectra of conjugated CSRB showed bands, which could be assigned to the amide bonds between chitosan and RB (Fig. 5.2 C). Two characteristic peaks
Figure 5.2. Typical graph showing absorption spectrum of (A) RB and CSRB. The absorption peak 549 nm of RB shifted to 560 nm and became broader following conjugation with chitosan. (B) Ratio of monomer (560 nm) to dimer (528 nm) peak decreased linearly with increase in the concentration of CSRB ($R^2=0.95$) in the solution suggesting aggregation/dimerization. (C) FTIR spectra of chitosan and CSRB (400-4000 cm$^{-1}$ wavenumber).

at 1651 (amide I, carbonyl stretching vibration) and 1558 cm$^{-1}$ (NH$_2$ bending) were prominent in chitosan and CSRB spectra (26). However, the ratio of intensities at 1558 and 1652 cm$^{-1}$ was higher in CSRB when compared to chitosan, suggesting the reduction of amide I bonds due to utilization of the free amine groups of chitosan to form bonds with carboxyl group of RB. The peak (900-1100 cm$^{-1}$) corresponding to the saccharide group of chitosan was also prominent in the CSRB. The 1388 cm$^{-1}$ peak (-CH$_2$ bending) characteristic to the glucosamine units of chitosan reduced in CSRB.

Fig. 5.3 shows decrease in the DPBF concentration with oxidation, which indicated the rate of singlet oxygen production upon photoactivation of CSRB and RB. It was observed that the singlet oxygen release was high enough to convert all the available DPBF for both the photosensitizer by 5 min. However, singlet oxygen release in CSRB was faster in the first 1 min followed by a slower rate. The rate of singlet oxygen generation increased with increase in the concentration of both the photosensitizer used. CSRB 0.3 mg/ml ($k=1.342$) showed twice the production rates of singlet oxygen compared to RB at 10 uM ($k=0.695$). Based on the monomer to dimer ratio and singlet oxygen yield, CSRB at the concentration of 0.3 mg/mL was used in all the subsequent experiments.

5.4.2 Evaluation of cytotoxicity and phototoxicity of CSRB

Fig. 5.4 shows the percentage cell survival after different photosensitizer treatments both in dark and light conditions. CSRB at lower concentration (0.3 mg/mL) showed cell survival of 75% in dark and 48% in light. At higher concentration of CSRB (0.5 mg/mL), the dark toxicity was higher (60% survival) but phototoxicity was unchanged. RB showed higher dark toxicity but similar
**Figure 5.3.** The oxidation of 1,3-diphenylisobenzofuran (DPBF) due to singlet oxygen generation following photoactivation of RB and CSRB measured as the reduction of DPBF absorbance.

**Figure 5.4.** Graph showing cell survival following treatment with RB and CSRB with and without photodynamic treatment (PDT). PDT resulted in significantly increased cytotoxicity as compared to CSRB treatment without PDT (p< 0.05).
levels of phototoxicity. Under microscopic examination, cells were observed to uptake CSRB into the cytoplasm (Fig. 5.5 A), whereas RB showed aggregation at the cell membrane (Fig. 5.5 C). Following irradiation, almost all the cells showed uptake of trypan blue. However, RB+PDT treated cells showed altered cell morphology and dark blue nucleus (Fig. 5.5 D) as compared to the CSRB+PDT. CSRB+PDT cells showed pinkish hue in the cytoplasm, and the morphology was maintained in most of the cells (Fig. 5.5 B).

5.4.3 Evaluation of antibacterial efficacy of CSRB

Fig. 5.6 shows the antibacterial efficacy of RB and CSRB on planktonic and biofilm-bacteria in both dark and light conditions. CSRB showed a much higher level of dark I killing of planktonic bacteria than RB (Fig. 5.6 A) as expected from the antibacterial properties of chitosan. There were more than 7 logs of killing at 0.1 mg/mL CSRB and complete eradication at 0.3 mg/mL. By contrast RB showed dark toxicity of only 0.5 CFU log reduction. After illumination complete eradication was obtained with both CSRB concentrations and both fluences, while surviving bacteria were seen with RB and 5 J/cm².

In case of biofilm bacteria, both CSRB and RB showed at least 3 logs of PDT-mediated bacterial killing but in the case of CSRB the killing was fluence dependent and was significantly higher than RB at 40 and 60 J/cm² (Fig. 5.6 B). The biofilms with only light irradiation did not show any bacterial killing.

5.4.4 Evaluation of CSRB to induce photodynamic crosslinking of dentin-collagen

Fig. 5.7 A shows the FTIR spectra obtained from dentin-collagen. The amide I bands (1666 cm⁻¹), amide II bands (1558 cm⁻¹) and CN (1458 cm⁻¹) bands were analyzed to assess the presence of crosslinking (33). Amide I bands (1666 cm⁻¹) was attributed to C=O stretching vibrations coupled to N-H bending vibration. The amide II bands (1566 cm⁻¹) were due to the N-H bending vibrations coupled to C-N stretching vibrations (43). Following crosslinking of collagen, the amide I bands decreased compared to the amide II bands in all the crosslinked samples. The conversion of the free -NH₂ groups in collagen to N-H groups would result in reduced amide I peak relative to the
Figure 5.5. The trypan blue staining pattern of cell line subjected to (A-B) CSRB and (C-D) RB treatment with and without photodynamic treatment (PDT). The cells subjected to CSRB showed presence of photosensitizer (pink) within the cytoplasm (arrow) of the viable cells with clear round nuclei (A). RB did not show any uptake into the cells and were found outside as dark pink aggregates (arrow) (C). Following PDT cells from both the groups showed increased uptake of trypan blue uptake (nuclei of cells are stained dark bluish-purple). However, RB+PDT (D) resulted in disruption of cell morphology and irregular cell membrane, which was less in cells subjected to CSRB+PDT (fibroblast cells with extensions) (B).
Figure 5.6. Log number of *E. faecalis* in planktonic (A) and biofilm (B) forms surviving the PDT conducted in a multiwell plate. There was a significant difference in the killing of biofilms by CSRB compared to RB (*p*<0.01). Error bars show the standard deviation from average value.
Figure 5.7. FTIR spectra of dentin-collagen (A); amino acid released following enzymatic degradation of dentin-collagen (B); and stress-strain curve after mechanical testing of dentin-collagen following crosslinking (C). GD- glutaraldehyde.

Amide II peak. Crosslinking between COOH and NH$_2$ groups would also result in increase in C-N bands relative to amide I bands (43).

The amount of amino acids released following enzymatic degradation of the crosslinked and non-crosslinked dentin-collagen was significantly different as a function of time ($p<0.05$) (Fig. 5.7 B). After 7 days, the control group specimens disintegrated nearly completely and released 9.8 µmol/mL of amino acid. Further degradation till day 14 did not show any significant increase in the amino acid release. The glutaraldehyde group showed the highest resistance to collagenase degradation even on the 14th day (0.41 µmol/mL). In case of photodynamically crosslinked dentin-collagen samples using RB, resistance to degradation was comparable to the glutaraldehyde group till day 7, and showed a slight increase on day 14 (1.58 µmol/mL). CSRB crosslinked dentin-collagen showed a gradual increase in the release of amino acid as compared to crosslinking with RB.

The stress-strain curves demonstrated increased ultimate tensile strength of all the crosslinked dentin-collagen samples compared with the non-crosslinked control samples (Fig. 5.7 C). The toughness was calculated for each sample as described in the methods section. The glutaraldehyde crosslinked dentin-collagen samples showed a higher increase in ultimate tensile strength, however the percentage elongation of the collagen-samples decreased drastically indicating a brittle behavior. The average initial toughness of demineralized dentin-collagen was 17 MPa. The glutaraldehyde crosslinked reduced toughness by almost 28% (10.74±2.6 MPa). CSRB crosslinking reinforced the dentin-collagen as compared to the RB photocrosslinking. The toughness of CSRB (66.67±13.41 MPa) (281%) crosslinked samples was significantly higher as compared to the RB (51.95±1.52 MPa) (196%) ($p<0.05$).
5.5 Discussion

Conjugation of chitosan with RB showed properties of both a bioactive polymer and a photosensitizer as determined by the FTIR and UV-visible absorption spectra of CSRB. Due to the presence of chitosan, the CSRB conjugates would be cationic in nature (abundant free amine groups). The net charge of CSRB was positive (+15 mV) but was lower as compared to the unmodified chitosan particles (+20 mV) as determined previously in our lab (36). Since higher concentration (>0.5 mg/mL) of CSRB showed aggregation as judged by the low monomer to dimer ratio, a concentration of 0.3 mg/mL was used in the present study. The presence of a saccharide peak in FTIR and the decreased amide I peak indicated chemical conjugation of chitosan with RB (26). At neutral or basic pH conditions, chitosan with free amino groups was insoluble in water (1) but at low pH conditions, the amino groups were protonated thus making chitosan water-soluble. Therefore, the solubility of chitosan depends on the balance between free amino and N-acetyl groups (1, 23). Conjugation of reactive amino groups with RB resulted in CSRB conjugate that is soluble in water (pH 7.2) (24). The ability of CSRB to produce singlet oxygen was confirmed by the indirect method of DPBF consumption assay. The rate of singlet production by CSRB was higher and biphasic as compared to RB.

Increases in the uptake of photosensitizers by colorectal cancer cells following conjugation with cationic molecules have been reported in the past (11). Similarly, CSRB particles synthesized were cationic and were therefore uptaken into the cytoplasm of the fibroblasts in contrast to RB that is anionic. The biocompatibility of RB is somewhat unclear, as the dye has been described by certain group as cytotoxic (20, 38), and by other groups as non-cytotoxic (6, 7). Unmodified chitosan has been reported to favor cell growth (10, 43), and preliminary tests conducted in our lab also showed 100% fibroblast cell survival in the presence of chitosan (data not shown). In the present study, RB showed higher dark toxicity that did not increase significantly following PDT. However, the conjugation of RB with chitosan decreased the dark cytotoxicity of RB, and even after PDT there was 50% cell survival. In another study, photosensitizer-doped conjugated polymers have been shown to show lower cytotoxicity even though they entered the cells via endocytosis (34). The polymer conjugate reduced the aggregation at the cell surface and reduced dark cytotoxicity. If CSRB would be applied to decontaminate the root-canal dentin, even if CSRB extruded into the apical tissues, because the fiber optic delivery system would be confined within the root canal the amount of light energy penetrating beyond the apical foramen would be insufficient to release a
harmful amount singlet oxygen to damage apical cells. Furthermore, presence of proteins in tissue fluids at the apical region may also lead to lessening of PDT mediated host damage (3).

Chitosan is highly reactive towards anionic particles/surfaces such as bacteria and biofilms (32). The bacterial membrane is negatively charged owing to the lipopolysaccharide (gram negative) and lipotechoic acid (gram positive) present on the surface. In the case of bacterial biofilms, the extracellular polysaccharides of the biofilm matrix are negatively charged favoring cationic molecules to have higher binding and uptake. The hydrophilic nature of chitosan further favors intimate binding of photosensitizer to bacterial cells. Although both chitosan and its modifications have been shown to have antibiofilm properties they work somewhat slowly; the time taken for effective elimination was a minimum of 48 hours (37). Conjugation of a photosensitizer with a positively charged molecule would allow photosensitizer molecules to enter the bacterial cells and resulted in a rapid and increased killing efficiency at a lower concentration than the neutral and anionic photosensitizer molecules (12, 36). CSRB combined with PDT completely eliminated the biofilm bacteria, which could be due to the better uptake of photosensitizer into the bacterial cells. Subsequent photoactivation of the will result in the production of singlet oxygen. The slower singlet oxygen release by CSRB as seen in the biphasic pattern could provide an advantage giving deeper penetration of singlet oxygen by not exhausting all the available ambient oxygen. The high antibacterial effect could be due to the synergistic activity of chitosan and PDT. Even though complete elimination of planktonic bacteria was observed with CSRB treatment alone, biofilm showed a much higher degree of resistance. A previous study has shown that RB could not completely eliminate biofilm bacteria (36).

The findings from this study demonstrated that, crosslinking delayed the enzymatic degradation of dentin-collagen, and at the same time increased the overall ultimate tensile strength and fracture toughness. The chemical composition and presence of collagen crosslinking were confirmed using FTIR spectroscopy (33, 43). During PDT, the singlet oxygen produced by the activated photosensitizers is known to facilitate formation of inter- and intra-molecular covalent crosslinks in collagen molecules and other available reactive sites (6, 35, 38). Coupling between the free amino groups and photo-oxidized amino acids has been proven by the decrease in both reactivity and available free amino groups following photodynamic crosslinking (42). The tensile testing provided information on the mechanical properties after chemical/photodynamic crosslinking of dentin-collagen (2, 41). Chitosan from the CSRB could form additional bonds with the collagen
and act as spacers to prevent undesired zero-length crosslinking, subsequently reinforcing the mechanical properties (13). Bacterial collagenase enzyme degraded collagen by hydrolyzing the peptide bond on the amino-terminal side of Glycine (–X-Gly-Pro) (44). Use of a commercially available purified bacterial collagenase has been used previously to degrade collagenous tissues (25, 40). Following crosslinking of collagen, the sites of collagenase attack may be hidden or modified, and this contributes to the significant difference in the release of amino acid residues following enzymatic degradation (21). Degradation of dentin collagen is a time dependent slow process as long as there is a fluid tight seal. The crosslinking of dentin collagen is expected to further delay any type of degradation in case of interfacial leakages. In this study, untreated control specimens showed the highest overall release of amino acid at all-time points in the degradation analysis.

The CSRB particles synthesized and characterized in this study may present a synergistic multifunctional treatment approach with lower cytotoxicity to effectively eliminate bacterial activity as well as the ability to crosslink and reinforce the dentin-collagen matrix to enhance resistance to degradation and improve its mechanical properties. CSRB may provide a potential single step treatment strategy to deal with infected hard tissues in a clinical scenario wherein both disinfection and structural integrity needs to be addressed concomitantly.

5.6 Acknowledgement

Funding from University of Toronto, Canadian foundation of innovation and CIHR Training Fellowship, TGF-53877 is gratefully acknowledged. M.R. Hamblin was supported by US NIH (grant R01AI050875).

5.7 References


Chapter 6
Functionalized photoactivated nanoparticles that target bacterial-biofilms and tissue ultrastructure
6.1 Abstract

Treatment of infected teeth presents two major challenges: (1) persistence of the bacterial biofilm after treatment and (2) compromised structural integrity of the dentin hard tissue. In this study, bioactive polymeric chitosan nanoparticles functionalized with rose bengal, CSRB-np was developed to produce antibiofilm effects as well as stabilize structural integrity by crosslinking dentin-collagen. CSRB-np was non-toxic to fibroblasts and had significant antibacterial activity even in the presence of serum albumin. The CSRB-np showed targeted antibacterial efficacy by adhering to the bacterial cell surface, permeabilizing and lysing the cells after photodynamic treatment (PDT). CSRB-np significantly eliminated *Enterococcus faecalis* biofilms and disrupted their structure as well. Incorporation of CSRB-np and crosslinking resulted in significantly improved resistance to degradation and mechanical strength of dentin-collagen. This functionalized bioactive nanoparticle provided a single step treatment of infected teeth with combined properties of chitosan and that of a photosensitizer to eliminate bacterial biofilms and stabilize dentin-collagen.
6.2 Introduction

Approximately 60% of human infections are associated with bacterial-biofilms, which include both implant-related infections and chronic non-implant related infections (1). Likewise the rate of root canal treatment failure of infected teeth has not decreased below 18-26% even with advanced therapeutic options to improve the treatment outcome (2, 3). The widespread recognition of biofilm as the main factor in dental infection (4) has led research towards improved antimicrobial treatment strategies. Little attention has been paid towards improving the chemical stability and mechanical properties of these previously infected hard tissues. Compromised mechanical integrity and chemical stability due to disease-mediated degradation of the dental hard tissues such as dentin caused by host/bacterial proteases (5), and treatment-mediated tissue damages have been reported in the past (6). The two major challenges in the management of infected dental hard tissue are (1) decontamination of bacterial-biofilm from the root canals and (2) repair of disease-mediated hard tissue changes. Although approaches that counter these challenges should lead to improved treatment outcomes, currently there is no treatment in dentistry that would produce significant antibiofilm efficacy and at the same time enhance the ultrastructural integrity of dentin tissue in infected teeth.

PDT has been applied in biomedicine owing to its broad-spectrum antimicrobial activity (7) and ability to produce crosslinking of proteins and collagen (8, 9). The singlet oxygen produced facilitates the formation of inter- and intra-molecular covalent cross-links in collagen molecules and other available proteinaceous active sites in the presence of appropriate photosensitizers such as rose bengal (RB) (9). Incorporation and crosslinking of biopolymers such as elastin and chitosan with collagen has been reported to reinforce the collagen scaffolds (10-12). Another significant issue in antimicrobial treatments is that the agents should be selective in eliminating bacteria while sparing the adjacent mammalian cells (targeted antibacterial activity). Immobilization of photosensitizers on polymeric supports avoided the toxic side effects of residual photosensitizers and provided an added advantage of stability in the physiologic environment (13, 14).

Functionalized nanoparticles containing various reactive molecules and decorated with peptides or other ligands have led to new possibilities of combating antimicrobial resistance (15, 16). These modified nanomaterials offer unique physicochemical properties, such as ultra-small sizes, large surface area/mass ratio and increased chemical reactivity. Further, by using a bioactive material
for nanoparticles, these particles could display functions and properties that are similar to naturally occurring biomolecules present in cellular systems. Nanoparticles either encapsulating or surface modified with photosensitizer have been proposed to enhance antimicrobial photodynamic therapy (PDT) (17, 18). However, the compromised efficacy of PDT in the presence of protein rich media or serum (19, 20) needs to be addressed prior to in vivo application.

Although photosensitizers have been conjugated with different readily available synthetic polymers and liposomes, naturally occurring biopolymers such as chitosan provide clear advantage of biocompatibility when applied in vivo (13, 14). Chitosan (CS) is a derivative of chitin, the second most abundant natural biopolymer, and has received significant interest in the fields of biomedicine, food industries, agriculture and environmental science (21). It shows a broad range of antimicrobial activity, and has biocompatible and biodegradable properties (22-24). This hydrophilic biopolymer with a large number of free hydroxyl and amino groups has been used for numerous chemical modifications (12, 14). CS polymers have also been considered structurally similar to extracellular matrix components (25). CS nanoparticles showed reduction of bacterial-biofilms but were not able to penetrate into the biofilm structure resulting in prolonged interaction time to produce antibiofilm activity (23).

The aim of the current work was to develop CS nanoparticles functionalized with photosensitizer (RB), which could exhibit the dual function of eliminating biofilms as well as stabilizing the dentin-collagen matrix. Nanoparticles of CS chemically conjugated with RB (CSRB-np) are expected to possess a broad-spectrum photoantimicrobial effect. Further the nanoparticles with higher reactive surface area would have increased interaction with bacteria in the root canal and dentin-collagen. We formed the hypothesis that CSRB-np when photoactivated could perform the dual function of targeted elimination of bacterial-biofilms and improved structural stability of dentin-collagen matrix. Towards this goal we synthesized and characterized CSRB-np, and evaluated its potential application for antibacterial/antibiofilm efficacy and collagen matrix stabilization for management of infected dental hard tissue. Ultrastructural evaluations were carried out to understand the interaction of these nanoparticles with bacterial cells as well as collagen fibrils, and antibacterial activity was also assessed in the presence of tissue inhibitors such as bovine serum albumin.
6.3 Methods

All the chemicals used in this study were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, USA) unless noted otherwise.

6.3.1 Synthesis of CSRB-np

CSRB-np was synthesized by conjugating Cs-np with RB (Figure 6.1 A). Cs-np was synthesized according to the method reported in an earlier work (45) and chemically crosslinked to RB using \(N\)-ethyl-\(N'\)-(3-dimethyl aminopropyl) carbodiimide (EDC 5 mM) and \(N\)- Hydroxysuccinimide (NHS 5 mM). The CSRB-np formed was dialyzed (Sigma, cellulose tubing, cut off 12000-14000 g/mol) for 1 week, the filtrate was then freeze-dried starting at -80 °C.

6.3.2 Characterization of CSRB-np

Size of the synthesized CSRB-np was determined using transmission electron microscopy (TEM). Absorption spectra for conjugated (CSRB-np) and unconjugated (RB) photosensitizer solutions were recorded using UV-Visible spectroscopy (Epoch, Biotek, USA). Photophysical characterization of CSRB-np to determine the ratio of monomer to dimer (absorbance at 550 nm to 528 nm) at different concentrations was also carried out. The effective concentration of CSRB-np was determined based on the highest monomer:dimer ratio (least aggregation). The conjugated CSRB-np was analyzed for their chemical composition using Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu, Kyoto, Japan) (16 cm\(^{-1}\) resolutions, 32 scans/sample). Photo-oxidative characterization was conducted to assess the ability to generate singlet oxygen by CSRB-np as described previously (26). A broad-spectrum Lumacare (LumaCare Inc., Newport Beach, CA, USA) lamp fitted with a 540+/−15 nm filtered fiber (output power= 50 mW) was used as a light source.

6.3.3 Evaluation of cytotoxicity of CSRB-np

Approximately \(10^5\) NIH 3T3 mouse fibroblast cells (American Type Culture Collection CCL 1, Rockville, MD) were seeded into 24 well plates in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% bovine serum and antibiotics and incubated for 48 h in 5% CO\(_2\). After incubation, CSRB-np and RB dissolved in DMEM were added to the cells and incubated for 15
minutes in dark. The cells were irradiated for a total dose of 20 J/cm$^2$. RB and CSRB-np were also tested without irradiation. Cell survival was determined by the standard 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (0.5 mg% MTT) assay that determines the mitochondrial activity (27). Percentage survival was calculated based on control sample without any treatment as 100%. All analyses were repeated three times in triplicate, and the statistical significance was analyzed by one-way analysis of variance.

6.3.4 Mechanism of antibacterial effect of CSRB-np

Bacterial membrane permeability and release of cytoplasmic contents were assessed using absorbance at 260 nm and transmission electron microscopy (TEM). Overnight cultures of *E. faecalis* (ATCC 29212) was washed twice in sterile deionized-water (4000 rpm, 10 minutes, 4 °C) and adjusted to $10^8$ CFU/mL (optical density ≈ 0.7) at 600 nm. Aliquots of cell suspension (1 mL) were then centrifuged and the cell pellets were treated with different photosensitizer solutions (37 °C for 15 minutes), protected from ambient light. The release kinetics of intracellular contents was measured using the absorbance of the bacterial cell filtrate at 260 nm (OD$_{260}$). For PDT, the photosensitized cells were centrifuged and cell pellets irradiated (5 J/cm$^2$, 540 nm). The % change in OD$_{260}$ at 15 minutes post sensitization and after PDT was calculated with respect to the OD$_{260}$ of the sample measured at 0 minute. The time dependent effect of CSRB-np without light activation was also monitored at different time intervals. All specimens for the TEM were prepared following previous protocol (6). The bacterial cells were pelleted and fixed in 2.5% glutaraldehyde (0.1 M phosphate buffer) (overnight). The 90 nm thick sections were prepared and examined under TEM (Hitachi H-7000, Tokyo) at 80 kV.

6.3.5 Uptake of CSRB-np by bacterial-biofilm

Uptake of RB and CSRB-np by 7 day old biofilms of *E. faecalis* was evaluated. 1 mL of overnight culture was added into each well of the multiwell-plates and incubated at 37 °C, 100 rpm (media was replenished every 48 h). Different concentrations of CSRB-np (0.3, 0.5 & 1 mg/mL) and RB (10, 25, 50 & 100 μM) were added to the biofilm and incubated at 37 °C for 15 minutes, protected from ambient light. Three samples were used for each concentration. Excess photosensitizer solutions were removed leaving behind the bound RB and CSRB-np in biofilm, washed once and the cell-bound photosensitizers extracted using 2% sodium dodecyl sulfate. Quantification of
photosensitizer was done spectrophotometrically at the absorption maxima of the RB (550 nm). Uptake values were expressed as the total RB concentration (µM) extracted from biofilm bacteria.

6.3.6 Effect of BSA on the antibacterial efficacy of CSRB-np

RB and CSRB-np were evaluated for the antibacterial efficacy in the presence and absence of 2% bovine serum albumin (BSA) (28). Two concentration of CSRB-np (0.1 and 0.3 mg/mL) was tested with PDT dosage of 2 and 5 J/cm². The BSA effect was tested by adding BSA into 1 mL of RB and CSRB-np and incubated at 37°C for 1 h. The photosensitizers with and without BSA were added to the cell pellets of *E. faecalis* (10⁸ CFU/ml) and photosensitized for 15 minutes in dark. Following the photosensitization, bacterial cells were centrifuged to remove the unbound photosensitizers and subjected for PDT (5 and 10 J/cm²). The samples were quantified immediately after PDT and continued incubation for 24 h. Bacterial survival was quantified by plating 50 µL of samples onto freshly poured BHI agar plates.

6.3.7 Assessment of antibacterial efficacy of CSRB-np

Monospecies biofilms of *E. faecalis* were grown for 21 days as mentioned above. The biofilm-bacteria was sensitized either with CSRB-np (0.1 & 0.3 mg/mL) or RB (10 µM) for 15 minutes and exposed to PDT with different doses. In case of PDT, the sensitized biofilm bacteria were irradiated using 540 nm fiber, with dosage of 20, 40 and 60 J/cm²; and fractionated dosage of 10 and 20 J/cm² twice. After treatment, biofilm bacteria were disrupted mechanically and quantified using culture method. Colonies were counted after 24 h of incubation at 37 °C and expressed as log CFU/mL. The experiments were carried out in triplicates and the mean values were calculated.

The structure of the biofilms following CSRB-np treatment was assessed using confocal laser scanning microscopy (CLSM) (Olympus IX81 inverted fluorescence microscope SU X1 with spinning disk confocal scan head). The biofilm-structures were assessed after staining with Live/Dead *Baclight* stain (Molecular Probes, Eugene, OR) in the dark for 10 minutes. Diode-pumped solid state laser lines (Spectral Applied Research,) was the source of illumination with 491 nm excitation and long-pass 525 nm +/-50, and 700 nm +/-75 emission filter settings for green and red signals respectively. Nine different areas were imaged from each sample using a 60x oil immersion objective. The optical sections of the biofilm-structure were recorded and analyzed using velocity software. Student *t*-test was used to compare the thickness of the biofilm before and
after nanoparticles treatment.

6.3.8 Effect of CSRB-np on photodynamic crosslinking of dentin-collagen

Sixteen freshly extracted human incisors and eight bovine incisors were stored in 0.9% saline until use. The bovine teeth were used for mechanical testing while the human teeth were used for chemical and enzymatic-degradation analysis. Dentin sections of $12 \times 2 \times 0.5 \text{ mm}^3$ (human) and $16 \times 2 \times 0.2 \text{ mm}^3$ (bovine) were prepared from either side of the root canal lumen (29). The dentin sections were demineralized in 1M ethylenediaminetetraacetic acid (EDTA, pH = 7.4) for 7 days. The demineralized dentin-collagen specimens (total - 48) were randomly divided into four treatment groups ($n = 12$): 1) No-treatment - (Control); 2) 2.5% glutaraldehyde (GD); 3) RB 10 $\mu$M (RB); and 4) CSRB-np 0.3 mg/mL (CSRB-np). The dentin-collagen samples were crosslinked with GD for a period of 6 h. In photodynamic crosslinking, collagen-samples were immersed in 1 mL of RB or CSRB-np solution for 15 minutes and the photosensitized collagen was photoactivated (20 J/cm$^2$). Crosslinked specimens were thoroughly washed in deionized-water three times and lyophilized for 24 h, for the enzymatic degradation analysis. The bovine dentin-collagen specimens were maintained in deionized-water to be used for mechanical testing.

Enzymatic degradation of the dentin-collagen specimens using collagenase from Clostridium histolyticum with an activity of 125 CDU mg$^{-1}$ solid (P/N C-0130; Sigma) was conducted to quantify the amino acid release using a ninhydrin assay as described by Mandl et al. (30) at 1, 7 and 14 days. The fully hydrated bovine dentin-collagen specimens from all four test groups were used for tensile testing (Instron 5544, Instron Corporation, Canton, MA) with a 100 N load cell, at a crosshead speed of 1 mm/minute until failure occurred. Care was taken to keep the samples hydrated at all times during the test. The ultimate tensile strength and toughness (MPa), were calculated using OriginPro 8.1 software (OriginLab Corporation, MA). Four specimens from each group were fixed in 2.5% glutaraldehyde and processed for TEM evaluation (6).

6.4 Results

6.4.1 Synthesis and characterization of CSRB-np

Fig 6.1 B showed the aggregates of spherical CSRB-np under TEM. The absorption spectra obtained for CSRB-np displayed bands characteristic of RB (Fig. 6.1 C). The amount of RB bound
in the conjugated CSRB-np was calculated to be 14 μM per 0.1 mg/mL. Based on the absorption spectra the monomer to dimer ratio was calculated for various concentration of CSRB-np. Since higher concentration (>0.5 mg/mL) of CSRB-np showed aggregation with a low monomer to dimer ratio, CSRB-np at a concentration of 0.3 mg/mL was used for further experiments in the present study. FTIR spectra of conjugated CSRB-np showed bands, which could be assigned to the amide bonds between CS and RB (Fig. 6.1 D). Two characteristic peaks at 1651 (amide I, carbonyl stretching vibration) and 1558 cm\(^{-1}\) corresponding to (NH\(_2\) bending) were prominent in the CS spectra. (14) The ratio of intensities at 1558 and 1652 cm\(^{-1}\) was lower in CS as compared to the ratio at 1446 and 1582 cm\(^{-1}\) in CSRB-np, which is suggestive of the utilization of free amine groups of Cs-np to form bonds with the CO- group of RB. The peak (900-1100 cm\(^{-1}\)) corresponding to the saccharide group of CS was also prominent in the CSRB-np. The presence of saccharide peak and decreased amide I peak indicated chemical conjugation of RB with Cs-np. A shift from 3348 to 3459 cm\(^{-1}\), and a sharper peak in the CSRB-np indicated that the hydrogen bonding was enhanced. The CSRB-np possessed a zeta potential of +30±0.8 mV as a result of the free amine groups from the Cs-np. CSRB-np produced singlet oxygen upon photoactivation similar to RB, as observed by the decrease in the DPBF concentration (Fig. 6.1 E).

6.4.2 Cytotoxicity assay using RB and CSRB-np

CSRB-np did not exhibit dark toxicity after 15 min exposure with 95.5±12% cell survival. However, following irradiation cell survival reduced to 72.86±9%. As reported previously, the microparticles of CSRB showed higher toxicity (<50%) after PDT (31). RB showed significantly higher dark toxicity (55.8±2% cell survival) and further reduction of cell survival (51.23±3%) after PDT.

6.4.3 Mechanism of antibacterial effect of CSRB-np

Following PDT with both RB and CSRB-np bacterial suspensions showed increased absorbance at 260 nm compared to the dark value (Fig. 6.2 A). Interaction of CSRB-np and bacteria in the dark resulted in bacterial membrane damage and subsequent leakage of cellular constituents (Fig. 6.2 B). With increase in interaction time (2 and 8 h), CSRB-np 0.3 mg/ml showed a higher ability to induce bacterial membrane damage as compared to RB. TEM images further provided information on the bacterial morphology at the ultrastructural level wherein details of membrane
A) Chitosan

[Chemical structure of sodium tripolyphosphate (TPP)]

Sodium tripolyphosphate (TPP) → Ionic gelation → Chitosan nanoparticles

[Chemical structure of Rose Bengal (RB)]

EDC/NHS

[Diagram of CSRBnp]

B) TEM image of CSRBnp with a scale bar of 100 nm.

C) Absorbance spectra of RB (10uM) and CSRBnp (0.1mg/mL) against wavelength (nm).

Absorbance (au)

Wavelength (nm)

- RB 10uM
- CSRBnp 0.1mg/mL
Figure 6.1. Chemical reaction during conjugation of CS nanoparticles with rose bengal (RB) in the presence of EDC (N-ethyl-N’-(3-dimethyl aminopropyl) carbodiimide) and NHS (N-Hydroxysuccinimide) (A). Transmission electron microscopy (TEM) image of CSRB-np (scale bar= 100nm). The CSRB-np were of 60±20 nm in size (B). Absorption spectra of RB and CSRB-np with peak maxima at 550 nm (C). FTIR spectra of chitosan and CSRB-np (400 to 4000 cm\(^{-1}\) wave number) (D). The singlet oxygen yield monitored photometrically using oxidation of 1,3-diphenyisobenzofuran (DPBF). The rate of singlet oxygen yield in case of CSRB-np was slower than that of RB (E).
integrity and its disruption could be evaluated. Following CSRB-np treatment in the dark for 15 min, the nanoparticles were found to adhere to the bacterial cell walls (Fig. 6.3 A & B). The well-defined cell borders and dark cytoplasm were observed. Subsequent exposure to PDT resulted in mostly dead bacterial cells. The cell surface was irregular and various stages of cell membrane damage and leakage of cell constituents were evident (Fig. 6.3 C & D). In case of RB as the photosensitizer, following PDT both dead and viable cells could be found in approximately equal numbers (Fig. 6.3 E).

6.4.4 Uptake of CSRB-np by bacterial-biofilm

Bacterial-biofilms showed significantly high uptake of CSRB-np as compared to RB (Table 6.1).

6.4.5 Effect of BSA on the antibacterial efficacy of CSRB-np

CSRB-np showed excellent antibacterial efficacy with complete bacterial elimination at 2 J/cm² (Fig. 6.4, A). The dark toxicity of CSRB-np also was significantly higher as compared to RB and increased with increase in the CSRB-np concentration. RB required higher PDT dosage as compared to CSRB-np for complete bacterial elimination. BSA inhibited antibacterial effect of both RB and CSRB-np even after PDT with 10 J/cm² (Fig. 6.4, B). When the irradiated bacterial cells were incubated for 24 h, CSRB-np produced significant bacterial reduction which was not seen with RB.

6.4.6 Assessment of antibacterial efficacy of CSRB-np

CSRB-np demonstrated photodynamic antibacterial efficacy against planktonic (data not shown) and biofilms of E. faecalis (Fig. 6.4, C and D). Both CSRB-np and RB did not show complete killing even after 60 J/cm² (Fig. 6.4, C). Fractionation of PDT dosage was found to be more effective with complete elimination of biofilm bacteria with CSRB-np (0.3 mg/mL) and in case of RB (Fig. 6.4, D). Fig. 6.5 showed the CLSM images of the bacterial-biofilms structure before and after PDT treatment using RB and CSRB-np. The initial thickness of biofilm-structure was found to be 39.2±7.3 μm. The distribution of viable bacteria was reduced significantly and the multilayered biofilm-architecture was completely disrupted following CSRB-np PDT. The thickness of the biofilms reduced significantly to 23.1±5.57 μm (p<0.05) and 13.1± 4.3 μm (p<0.05) for RB and CSRB-np respectively. In case of RB the biofilm architecture was not
Figure 6.2. Graph showing release of cell constituents (absorbance at 260 nm) following treatment with RB and CSRB-np with and without PDT (A). Time dependent release of cell constituents following treatment with RB and CSRB-np (B). With increase in interaction time, CSRB-np showed highest cell membrane damage.
Figure 6.3. Transmission electron microscopy images for planktonic *E. faecalis* after treatment with CSRB-np for 15 min (A and B). Aggregates of CSRB-np could be seen surrounding the bacterial cell. Nanoparticles were found attached to the bacterial cell surface and forming an envelope (⏎) (B). The cells did not show any disruption of morphology. Following PDT of the sensitized bacteria, various stages of membrane damage as well as release of cell constituents were evident (C and D). Most of the bacteria showed some kind of cell membrane disruption (★), and release of cell constituents (✪) at higher magnification (D). However, the bacterial cells after PDT with RB as the photosensitizer showed both live (¤) and dead (⌘) cells (E).

Table 6.1. Uptake from bacterial cells obtained after incubation with different photosensitizers.

<table>
<thead>
<tr>
<th>Photosensitizer</th>
<th>Photosensitizer (μM) uptake mL⁻¹ of <em>E. faecalis</em> cells</th>
</tr>
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<tbody>
<tr>
<td>RB 10 μM</td>
<td>2.72 ± 0.15</td>
</tr>
<tr>
<td>RB 25 μM</td>
<td>2.80 ± 0.09</td>
</tr>
<tr>
<td>RB 50 μM</td>
<td>3.01 ± 0.11</td>
</tr>
<tr>
<td>RB 100 μM</td>
<td>3.68 ± 0.17</td>
</tr>
<tr>
<td>CSRB-np 0.3 mg mL⁻¹</td>
<td>16.15 ± 5.82</td>
</tr>
<tr>
<td>CSRB-np 0.5 mg mL⁻¹</td>
<td>24.06 ± 9.77</td>
</tr>
<tr>
<td>CSRB-np 1.0 mg mL⁻¹</td>
<td>40.68 ± 4.32</td>
</tr>
</tbody>
</table>

Values represent the uptake in μM mL⁻¹ of cells obtained after incubation of biofilm bacteria with RB and CSRB-np. Values are the means of three readings ± standard deviations. There was a significant increase in uptake of photosensitizer by bacterial cells when conjugated with CS (p<0.05).
**Figure 6.4.** Bacterial survival of planktonic *E. faecalis* after PDT using RB and CSRB-np (A). Bacterial survival of planktonic *E. faecalis* in the presence of bovine serum albumin (BSA) after treatment with different nanoparticles with and without PDT (B). CSRB-np after PDT and incubation for 24 hrs showed the best results as compared to CSRB-np and RB with/without PDT. Antibiofilm effect of CSRB-np and RB on *E. faecalis* biofilm (C and D). Complete elimination was obtained only after fractionation of PDT dose in case of CSRB-np at the higher concentration (D) in contrast to both the photosensitizers even after PDT dosage of 60 J/cm² (C). Error bars show the standard deviation from average value.
disrupted and pockets of live cells were still present within the dead cell aggregates.

### 6.4.7 Effect of CSRB-np on photodynamic crosslinking of dentin-collagen

The quantity of amino acids released following enzymatic degradation of the crosslinked and non-crosslinked dentin-collagen was significantly different as a function of time (p<0.05) (Table 6.2).

After 14 days the control group specimens disintegrated completely and released the highest amount of amino acid (11.74 µmol/ml). The GD group showed the highest resistance to collagenase degradation even on the 14th day (0.41 µmol/ml). CSRB-np crosslinked dentin-collagen showed slightly faster degradation as compared to RB but was not statistically significant on day 14.

The tensile testing used provided information on the mechanical properties such as UTS and toughness after chemical/photodynamic crosslinking of dentin-collagen specimens (32, 33) (Table 6.3). GD crosslinked dentin-collagen samples showed brittle behavior due to increased UTS and decreased percentage elongation. The samples crosslinked using GD showed reduction in toughness whereas RB and CSRB-np showed significant increase in toughness compared to the control group samples (p<0.05). TEM micrographs from the control group revealed a collagen matrix that contained intact, banded collagen fibrils (Fig. 6.6, A). Following crosslinking using CSRB-np, the arrangements of the collagen fibrils were denser with smooth edges (Fig. 6.6, C). The surface of the crosslinked collagen showed a layer of collagen fibrils and nanoparticles incorporated within the collagen mesh (Fig. 6.6, D).

### 6.5 Discussion

The primary focus of this study was to further improve the antimicrobial photodynamic treatment of biofilms as well as stabilize dentin-collagen by synthesizing a CS nanoparticles functionalized with photosensitizer (CSRB-np). CSRB-np showed characteristics of both polymer and photosensitizer as determined by the absorption and FTIR spectra and released singlet oxygen upon photoactivation. The binding of RB to the polymeric Cs-np as well as oxygen scavenging ability (34) of CS could have contributed to the reduced rate of singlet oxygen yield. This slower release of singlet oxygen as observed with CSRB-np could provide sufficient time for molecular
Figure 6.5. The three-dimensional confocal laser scanning microscopy reconstruction of the biofilms subjected to PDT using RB and CSRB-np. (A) The biofilm receiving no treatment showed a multilayered three dimensional structure with both live (green) and dead (red) cells. (B) The biofilms subjected to sensitization with RB and PDT (40J/cm\(^2\)) showed significantly higher number of dead cells. The mat like biofilm structure was not disturbed. (C) In case of CSRB-np the biofilm structure was completely disrupted with only few live and dead cells remaining on the substrate.

Table 6.2. The enzymatic degradation of dentin-collagen with time following different crosslinking procedures. The values represent the amount of amino acid released (µmol ml\(^{-1}\)) following degradation of dentin-collagen determined using Ninhydrin assay.

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.660</td>
<td>9.81</td>
<td>11.74</td>
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<tr>
<td></td>
<td>(0.71)</td>
<td>(2.17)</td>
<td>(1.34)</td>
</tr>
<tr>
<td>GD</td>
<td>0.034 [b]</td>
<td>0.32 [d]</td>
<td>0.41 [f]</td>
</tr>
<tr>
<td></td>
<td>(0.012)</td>
<td>(0.003)</td>
<td>(0.003)</td>
</tr>
<tr>
<td>RB</td>
<td>0.06 [b]</td>
<td>0.48 [d]</td>
<td>1.58 [f]</td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>(0.31)</td>
<td>(0.71)</td>
</tr>
<tr>
<td>CSRB-np</td>
<td>0.027 [b]</td>
<td>1.59 [d]</td>
<td>2.51 [f]</td>
</tr>
<tr>
<td></td>
<td>(0.003)</td>
<td>(0.32)</td>
<td>(0.47)</td>
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</tbody>
</table>

The results are averaged values obtained with standard deviations in the parentheses. For each column, groups identified by different Roman letters are significantly different (p<0.05).
Figure 6.6. Transmission electron micrographs of dentin-collagen without any treatment (A and B) and following photocrosslinking treatment with CSRB-np (C-F). CSRB-np were incorporated on the collagen and could be seen as aggregates on the surface (White arrows). Following treatment with collagenase, the control samples degraded and lost the normal fibrillar pattern (B). The CSRB-np crosslinked dentin-collagen showed resistance to enzymatic degradation (E and F). The scale bars in A-D represent 100 nm and in E-F are of 500 nm.

Table 6.3. The ultimate tensile strength (UTS) and toughness of dentin collagen following different crosslinking procedures.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GD</th>
<th>RB</th>
<th>CSRB-np</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(0.78)</td>
<td>(1.66)</td>
<td>(0.70)</td>
<td>(1.76)</td>
</tr>
<tr>
<td>Toughness [MPa]</td>
<td>17.49 [c]</td>
<td>10.74 [d]</td>
<td>51.95 [d]</td>
<td>74.31[d]</td>
</tr>
<tr>
<td></td>
<td>(0.64)</td>
<td>(2.66)</td>
<td>(1.52)</td>
<td>(14.58)</td>
</tr>
</tbody>
</table>

The results are averaged values obtained with standard deviations in the parentheses. For each row, groups identified by different Roman letters are significantly different (p<0.05).
oxygen to be replenished in the site of PDT and prolong the antibacterial effect (17). Replenishment of molecular oxygen during this lag phase as shown during fractionation of light dosage (35) could provide possible explanation to the enhanced antibacterial PDT effect. The findings from the current experiments, confirmed that conjugating RB with CS nanoparticles abrogated the toxicity towards fibroblasts, as CS has long been known to be highly biocompatible (36).

Damage to bacterial membrane, increased membrane permeability and subsequent intracellular leakage are the suggested antibacterial effects of CS (24). The amount of cytoplasmic content released subsequent to CSRB-np at higher concentration and treatment for prolonged time was comparable to that of PDT. Cationic micelles when interacting with bacteria have shown similar membrane lysis and release of cell constituents that has been mainly attributed to the electrostatic interaction and steric hindrance imposed by the micelles surrounding the cells (37). The increased uptake of CSRB-np into bacterial cells and biofilm structure is mainly attributed to their positive charge and nano-size, which led to not only greater interaction between bacterial cell and CSRB-np but also deeper penetration into the biofilm structure.

The cationic CS nanoparticles and CSRB-np are highly reactive and interacts physico-chemically with other charged particles in the solution. The tissues and particles present in the vicinity could also act as a potential substrate for highly reactive singlet oxygen (38). Other than direct inhibition of these nanoparticles, presence of tissue fluid or BSA is known to reduce the efficacy of antibacterial PDT due to crosslinking action or the compromised half-life of singlet oxygen (20). The CSRB-np and PDT showed the best results to eliminate bacteria even in the presence of BSA.

Conjugation of photosensitizer with a cationic molecule allowed photosensitizer molecules to enter the bacterial cells and resulted in increased killing efficiency at lower concentration than neutral and anionic photosensitizer molecules (39, 40). Similarly, in this study cationic CSRB-np bound to the negatively charged bacterial surface and diffused through the extracellular polymeric substance, thereby facilitating the penetration of the photosensitizer molecule through bacterial membrane and biofilm structure (41). CSRB-np combined with PDT showed complete elimination of the biofilm structure. Singlet oxygen is known to diffuse approximately 50 nm (42), and the closer proximity of the photosensitizer molecule to the cell surface might have allowed the diffusion of singlet oxygen into the resident bacterial cells. Irradiation for prolonged time periods
would deplete the molecular oxygen available in the immediate surroundings of the cells (35). The slower release of singlet oxygen by CSRB-np as reported in this study and oxygenation of biofilm during fractionation resulted in the complete elimination of biofilm.

Chemical crosslinking requires longer treatment time to establish stable collagen cross-links as compared to the photodynamic process (10, 43). This is a major limitation especially for clinical applications, where shorter treatment time is highly desirable. Photodynamic crosslinking is a rapid process that occurs via the production of singlet oxygen or radicals by the light excited photosensitizers. The photo-oxidized amino acids react with normal or photo-altered residues in another protein molecule resulting in a crosslink (44). Addition of biopolymers such as CS nanoparticles during collagen crosslinking would further enhance the biological and physical properties of collagen matrix.

Bacterial collagenase degrades collagen by hydrolyzing the peptide bond at the amino-terminal of glycine (–X-Gly-Pro) (45). Following crosslinking of collagen, the sites of collagenase attack may be protected or modified, and this may contribute to the significant difference in the release of amino acid residues following enzymatic degradation (46). In this study, crosslinked collagen showed significantly higher resistance to degradation. Crosslinked collagen-specimens demonstrated improved mechanical properties and CSRB-np group resulted in incorporation of CS nanoparticles into the collagen architecture. The CS nanoparticles in the collagen matrix may have reinforced the collagen structure by increasing the number of amine reaction sites resulting in the formation of ionic complexes between CS and collagen during crosslinking (47).

CSRB-np synthesized and characterized in this study possessed the following beneficial characteristics: (1) CS as an effective broad spectrum antibacterial with biocompatibility; (2) RB as a photosensitizer with photodynamic antibacterial and crosslinking ability; (3) the nano-size further enhanced the photoactive and antimicrobial properties due to high surface area to mass ratio resulting in increased interaction with the substrate. Nanoparticles in general possess stability limitations that restrict application in vivo (48). Due to their strong self-reactivity they form aggregates or flocculates thus compromising the available reactive surface area. Furthermore, the root canal system presents a complex anatomical challenge for effective delivery of antimicrobial agents. Efforts to reach these deep niches in the root dentin that are not accessible to mechanical instruments or liquid chemicals are continuously being investigated. The stability and delivery of
these antibacterial nanoparticles into infected dentin tissue needs to be addressed and this is a separate avenue for biophysical research.

As hypothesized, the CSRB-np displayed properties of both CS and RB in a photoactivated nano-
structure that performed the dual function of targeted elimination of bacterial-biofilms and improved mechanical and chemical stability of dentin organic matrix. The present study provides a novel nanoparticle based approach to enhance biofilm elimination and simultaneously restore the ultrastructural integrity of infected dental hard tissue, both of which hold equally pivotal status for the long-term treatment success of infected teeth.

6.6 Acknowledgement

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6.7 References


Chapter 7

Antibacterial activity of photosensitizer functionalized chitosan nanoparticles: Effect of tissue inhibitors
7.1 Abstract

Application of antibacterial nanoparticles to improve root canal disinfection has received interest recently. The current study aims to assess the antibacterial effect of a novel photosensitizer (rose bengal) functionalized chitosan nanoparticles (CSRB-np) to eliminate bacteria in the presence of various root canal constituents that are known to inhibit the antibacterial efficacy of root canal disinfectants. The synthesized CSRB-np was evaluated for size, charge and singlet oxygen release. The antibacterial effect of CSRB-np was tested on planktonic Enterococcus faecalis with or without pre-treatment using different inhibiting agents such as dentin, dentin-matrix, pulp tissue, bacterial lipopolysaccharides, and bovine serum albumin (BSA). Bacterial survival was assessed in a time dependent manner. The antibacterial effects following photodynamic activation on CSRB-np, a cationic photosensitizer (methylene blue-MB) and an anionic photosensitizer (rose bengal-RB) in the presence of inhibitors were also evaluated. CSRB-np was 60±20 nm in size and showed reduced rate of singlet oxygen release as compared to MB and RB. Pulp and BSA inhibited the antibacterial effect of CSRB-np (without photoactivation) significantly ($p<0.05$) even after 24 h of interaction. In case of PDT, the pulp and BSA significantly inhibited the antibacterial activity of all three photosensitizers. However, CSRB-np showed residual effect and completely eliminated the bacteria following 24 h of interaction after PDT. The inherent antibacterial activity of polycationic chitosan nanoparticles and the singlet oxygen released following photoactivation of RB synergistically provided CSRB-np the potential to achieve significant antibacterial efficacy even in presence of tissue remnant within the root canals.
7.2 Introduction

Despite the high antimicrobial efficacy of conventional disinfectants in vitro, clinical studies have demonstrated bacterial persistence within the root canal system even after cleaning and shaping procedures (1, 2). The effectiveness of antimicrobial irrigants are known to be compromised under in vivo conditions, due to the complex root canal anatomy that permits localization of bacteria in the inaccessible areas. In addition the effectiveness of antibacterial chemicals depends on the concentration, time and volume of irrigants inside the root canals. It is challenging task to ensure optimum concentration/volume of irrigants for sufficient time in all location of root canal system. The recent advances towards achieving predictable endodontic disinfection have focused on newer alternatives such as photodynamic therapy (PDT). Polycationic conjugates (3, 4) and nanoparticles (5, 6) have potentiated the antimicrobial effect of PDT. In this situation, it is highly pertinent to realize the importance of dentin constituents, tissue remnants and serum products present within the root canals and their ability to neutralize the commonly used antibacterial disinfectants (7-9). Similar reduction in the antibacterial activities of newer disinfectants (chitosan nanoparticles and PDT) were also reported (10). Considering the negative effects of tissue inhibitors on the currently available root canal disinfectants, it is important to develop antimicrobials that are effective even in their presence.

Nanoparticles are insoluble particles that are no greater than 100 nm in size. They exhibit novel and significantly improved physical, chemical and biological properties due to their nanoscale (11). They could be used to deliver various antibacterial agents including photosensitizers. Nanoparticles based on metals or polymers are also being assessed for augmenting the current endodontic disinfection methods (5, 12). Coating or surface attachment of photosensitizers (example: rose bengal or toluidine blue) to nanosized glass beads or gold resulted in significantly improved antibacterial properties (13, 14). Chitosan, a bioactive polymer offers an attractive material for conjugation with other reactive molecules due to the free amine and hydroxyl groups. This versatile polymer can be synthesized into nano-forms for various biomedical and pharmaceutical applications (15). Broad spectrum antibacterial activity, biocompatibility and ability to resist aging for longer periods provide antibacterial nanoparticles significant advantages in root canal disinfection (12, 16). Nevertheless, their antibacterial activity was found to be seriously compromised in the presence of various root canal constituents, mainly pulpal tissues and bovine serum albumin (BSA) (10).
PDT is based on the use of a non-toxic photosensitizer, which when activated using a low energy light results in the production of free radicals such as singlet oxygen (17). Singlet oxygen generated is highly reactive and is known to target various bacterial sites such as cell wall, nucleic acid as well as membrane proteins (17, 18). The antibacterial activity of PDT was shown to be compromised in the presence of root canal constituents such as pulpal tissues, serum, dentin matrix and bacterial remnants (lipopolysaccharides) (10, 19). The degree of inhibition also depended on the class of photosensitizer used, either phenothiazines with positive charge (methylene blue-MB) or xanthenes with negative charge (rose bengal-RB). The reduced efficacy of antibacterial PDT was mainly attributed to the interaction of photosensitizers with the tissue inhibitors, leading to reduced binding to the bacterial cell, reduced uptake into bacterial cells, and decreased half-life of the singlet oxygen produced upon photoactivation (20, 21). The antibacterial effect of PDT was found to be significantly improved when chitosan was used along with PDT (22). This was mainly attributed to the membrane destabilizing/permeabilizing effect of chitosan that could subsequently enhance the effect of singlet oxygen on bacterial cells. Conjugating the commonly used photosensitizer with chitosan and further making them into nanosize offers an attractive single-step option for disinfection. The photosensitizer rose bengal with a free carboxyl groups forms chemical crosslink with the amine groups of chitosan nanoparticles. The rose bengal functionalized chitosan nanoparticles is hypothesized to offer the following advantages: 1) affinity to bacterial cells, interaction resulting in bacterial elimination by cationic chitosan; 2) further increased interaction and uptake due to the nano-sized particles; and 3) singlet oxygen released following photoactivation of rose bengal. Based on the above hypothesis rose bengal functionalized chitosan nanoparticles (CSRB-np) were synthesized, characterized for size, charge and ability to yield singlet oxygen and their antibacterial efficacy was assessed in the presence of various tissue inhibitors with and without photoactivation.

7.3 Materials and Methods

Chitosan, RB, MB, lipopolysaccharide (LPS) from *Escherichia coli* and 1,3-diphenylisobenzofuran (DPBF) were purchased from Sigma Aldrich (St. Louis, MO, USA). Following agents were tested for the inhibitory effects; (a) 28 mg of dentin powder; (b) 10 mg of fresh bovine pulp, frozen and powdered; (c) 5 mg of dentin-matrix; and (d) 2% and 18% bovine serum albumin (BSA) and LPS (1 μg/ml) (7, 10). Extracted human third molars and bovine teeth from the slaughterhouse were obtained following approval from the Research Ethics Office,
University of Toronto. Dentin powder, dentin-matrix, pulpal tissues were obtained as mentioned in previous study (10). *Enterococcus faecalis* was used as a test organism as it was found in high prevalence in the persistent and retreatment cases (23). *E. faecalis* ATCC 29212 was grown overnight on brain heart infusion (BHI) broth (Bacto, DIFCO Laboratories, NJ). The culture was centrifuged (4500 rpm, 10 m), washed twice in sterile deionized-water and adjusted spectrophotometrically to a cell density of approximately $10^8$ colony-forming units per mL (optical density= 0.7).

### 7.3.1 Synthesis and Characterization of CSRB-np

Rose bengal functionalized chitosan nanoparticles (CSRB-np) were synthesized following the previous literature (4, 12). The synthesized CSRB-np were evaluated for their size using transmission electron microscopy (TEM) and charge using zetasizer. Photo-oxidative characterization was conducted to assess the singlet oxygen yield upon photoactivation. Measurements were carried out in a 24 well plate according to a procedure described previously (4). Generation of singlet oxygen by photoactivation of CSRB-np 0.3mg/mL, RB10 μM and MB 10 μM was studied photometrically using DPBF, a singlet oxygen scavenger. 2 mL DPBF (200 μM) was added to 100 μL of different PS solutions. A broad-spectrum Lumacare (LumaCare Inc., NewPort Beach, CA, USA) lamp fitted with a 540 or 660 nm filtered fiber was used as a light source. The decrease in absorbance intensity at 410 nm was monitored as a function of time using a UV-Visible microplate reader (Epoch, Biotek, USA) and was proportional to the rate of singlet oxygen production.

### 7.3.2 Time Dependent Evaluation of Antibacterial Activity in the Presence of Inhibitors

The experimental inhibitors were added into 1mL of the 0.3 mg CSRB-np and incubated in sealed test tubes at 37°C for 1 h (7, 10). This concentration of CSRB-np was chosen based on the characterization experiments conducted in our laboratory. The control group included CSRB-np without any inhibitors. After 1 h of incubation, the CSRB-np and inhibitor solution was added to the bacterial cell pellets and vortexed to ensure uniform interaction. The antibacterial activity was monitored at different time intervals by taking 50 μL samples of bacterial suspension that were serially diluted and plated onto freshly poured BHI agar plates and incubated for 24 h at 37°C to
determine the colony forming units (CFU) of viable bacteria.

### 7.3.3 Effect on Antibacterial Photodynamic Activity in the Presence of Inhibitors

In case of PDT the two experimental inhibitors (pulpal remnants and BSA) that showed highest inhibitory effect were taken and pre-incubated with photosensitizers (CSRB-np, RB and MB) as previous (4, 24). The photosensitizers and inhibitors mixture was then added into the cell pellet of *E. faecalis* and photosensitized for 15 min in dark. Following this the bacterial cells were centrifuged to remove the unbound photosensitizers and subjected for PDT at 5 and 10 J/cm² energy dose for 1.66 and 3.33 min respectively. The samples were evaluated immediately after PDT as well as after further interaction for 24 h. The samples were serially diluted, and various dilutions were plated onto freshly poured BHI agar plates for 24 h at 37°C.

### 7.3.4 Statistical Analysis

The experiments were carried out in triplicates (CFU counts) with three samples per group each time, a total of nine observations per treatment. Data values were transformed into percentage survival to reduce variance heterogeneity. Statistical analysis of *E. faecalis* percentage survival rates under the different inhibitors treatment conditions was performed by using a one-way analysis of variance and Tukey multiple comparison test. P value <0.05 was considered to indicate statistical significance.

### 7.4 Results

#### 7.4.1 Synthesis and Characterization of CSRB-np

**Fig. 7.1A** shows the spherical aggregates of CSRB-np under the TEM with smooth surface and in the 60±20 nm size range. The charge of CSRB-np was positive 30±.06 mV. These average values were obtained from six independent measurements of nanoparticles. CSRB-np produced singlet oxygen upon photoactivation similar to RB, as observed by the decrease in the DPBF concentration (**Fig. 7.1B**). RB and MB showed rapid release of singlet oxygen.
Figure 7.1. (A) Transmission electron microscopy image of rose bengal functionalized chitosan nanoparticles (CSRB-np). (B) The graph shows the decrease in the absorbance of 1,3-diphenylisobenzofuran due to the singlet oxygen release following photoactivation of CSRB-np, RB and MB.
7.4.2 Time Dependent Evaluation of Antibacterial Activity in the Presence of Inhibitors

CSRB-np showed complete killing of *E. faecalis* in the absence of inhibitors after 24 h interaction (Fig. 7.2). Presence of dentin, and LPS reduced the rate of antibacterial efficacy in the initial hours of interaction (1 and 8 h) and complete elimination was obtained after 24 h. The pulpal tissues showed highest inhibitory effect followed by BSA with only 65% bacterial reduction even after 24 h (p<0.05).

7.4.3 Photodynamic Effect on Antibacterial Activity in the Presence of Inhibitors

*E. faecalis* was completely eliminated with all the photosensitizers, CSRB-np, RB and MB following PDT in the absence of any inhibitors (Fig. 7.3). BSA and pulp inhibited the PDT mediated antibacterial activity of all three photosensitizers. The 24 h interaction of CSRB-np after PDT resulted in complete elimination of bacteria even in the presence of inhibitors (Fig. 7.3A). The CSRB-np interaction without PDT also showed 50-65% bacterial reduction. This post PDT complete reduction of bacterial viability was not seen in cases of RB and MB. MB in the presence of BSA and RB in the presence of pulp showed increased killing following PDT and 24 h interaction.

7.5 Discussion

The Cs-np are highly reactive cationic nano-scaled particles that interact physico-chemically with other charged particles as well as bacteria. The cationicity and the quantum size effect of nanoparticles could be further supplemented with singlet oxygen release in the presence of a photosensitizer. Conjugation of chitosan nanoparticles with rose bengal could provide the combined activity of Cs-np (bioactivity, quantum size effect, high affinity to bacterial cells and biocompatibility and photosensitizer (singlet oxygen release) thereby potentiating the antibacterial efficacy. CSRB-np possessed strong antibacterial properties against the *E. faecalis* in a time dependent manner, which is further, enhanced upon photoactivation. This could be mainly contributed to the affinity of Cs-np towards bacterial cell surface and its ability to permeabilize cell walls combined with the singlet oxygen produced by PDT (12).
Figure 7.2. Killing of *E. faecalis* ATCC 29212 by CSRB-np in a time dependent manner (A) in the presence of pulp, dentin, dentin matrix, and (B) LPS and BSA.
**A**

Percentage survival

- CS2R Bnp
- CS2R Bnp + BSA
- CS2R Bnp + BSA + 24h
- CS2R Bnp + pulp
- CS2R Bnp + Pulp + 24h

Legend: 0, 5 J, 10 J

**B**

Percentage survival

- MB
- MB + BSA
- MB + BSA + 24h
- MB + Pulp
- MB + Pulp + 24h

Legend: 0, 5 J, 10 J
Figure 7.3. Killing of *E. faecalis* ATCC 29212 by CSRB-np and PDT using (A) CSRB-np, (B) MB and (C) RB in the presence of pulp and BSA. The bacterial survival was assessed immediately after PDT and after 24 hours of post-PDT treatment to evaluate residual activity.
Presence of charged particles in a solution could negatively impact the interaction and uptake of nanoparticles or photosensitizers into the bacterial cell, which in turn compromised the antibacterial efficacy. An infected root canal system will invariably contain necrotic pulpal tissue, bacterial by-products and dentin debris after instrumentation. BSA has been used as a substitute for albumin that is the main protein in human serum and inflammatory exudates (25). Purulent infected root canals may present with periapical tissue exudates in the apical portions of the root canal (26). Pulp tissues consist mainly of organic material such as cells and extracellular matrix proteins. These organic materials and dentin could act as a buffer against most commonly used root canal irrigants and medicaments (7, 27). Other than the direct electrostatic interaction of the charged nanoparticles with these inhibitors, the singlet oxygen produced after photoactivation could also react these components (20). The singlet oxygen is the main cytotoxic agent in PDT, which induces oxidation of the biological substrates. The singlet oxygen interacts on a non-specific manner with the cells and its half-life is known to be diminished in the presence of serum (21).

The CSRB-np showed the best ability to eliminate bacteria even in the presence of BSA and pulp, which further highlighted the advantage of combining the activity of PDT with bioactive photosensitizer functionalized nanoparticles. The antibacterial treatment that can retain its efficacy in the presence of such inhibitors would provide an attractive alternative in endodontic disinfection.

Pulp and BSA strongly inhibited the antibacterial activity in both time dependent and PDT manner. The immediate antibacterial activity of CSRB-np after PDT in the presence of BSA and pulp was not significant and was similar to the conventional PDT with MB and RB. Upon prolonged interaction, CSRB-np was able to reduce the bacteria up to 65% even without photoactivation and completely eliminated (100%) bacteria after photoactivation. The bacteria with minor insult following PDT may recover if environment is conducive for growth. Further reduction of bacterial numbers as seen with MB and RB in the presence of BSA and pulp respectively, could be due to the inability of bacteria to grow that had undergone some damage due to the singlet oxygen. The presence of Cs-np in the suspension of bacteria following PDT enhanced antibacterial activity of CSRB-np as seen in the present study. Dentin, dentin-matrix and LPS did not affect antibacterial efficacy of CSRB-np. This could be mainly due to the weaker interaction of these inhibitors with the CSRB-np as shown previously in case of Cs-np (10).

The rate of singlet oxygen production by CSRB-np was lower when compared to the parent
photosensitizer RB. This difference in singlet oxygen production is mainly due to the binding of RB to the polymeric Cs-np. CS is known to scavenge oxygen resulting in reduced singlet oxygen efficacy (28). As the half-life of singlet oxygen is compromised in the presence of tissue fluids, prolonging the yield of singlet oxygen could be considered advantageous. In addition, the slow release of singlet oxygen is helpful in hypoxic root canal environment, since this will allow the molecular oxygen replenishment during fractionation of PDT dosage (29). Thus, the enhanced antibacterial effect of CSRB-np was a combined effect of Cs-np and singlet oxygen yield. Previously it has been shown that chitosan infiltrated and coated dentin collagen surface following photodynamic crosslinking process (30). The crosslinked and chitosan infiltrated dentin collagen demonstrated improved mechanical properties and stability against enzymatic degradation. The dentin surface treated with Cs-np after different root canal irrigants resulted in higher reduction of bacterial adherence (31). Similarly, CSRB-np in the root canal treatment could be applied to treat dentin surface that could prevent bacterial adhesion thereby preventing bacterial recolonization and biofilm formation. Other than this, leaving the Cs-np on the dentin surface could be beneficial for cell proliferation especially in regenerative endodontics.

The higher affinity of cationic Cs-np to bacterial cells with resultant antibacterial activity and singlet oxygen release following photoactivation of RB provided a synergistic mechanism for CSRB-np to exert its antibacterial efficacy even in the presence of tissue inhibitors. In conclusion, CSRB-np presented a novel antibacterial treatment for achieving significant bacterial reduction within the infected root canals.

7.6 Acknowledgement

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7.7 References


Chapter 8
Discussion and Conclusion
8.1 General Discussion

The two major issues in the treatment of infected root canals, bacterial biofilms and stabilization of the infected and treated dentin were addressed in the present study. The study involved synthesis and characterization of various chitosan micro-/nanoparticles for antibiofilm and tissue stabilization effects. The chemical modifications and nanoparticles of chitosan were developed as the project evolved towards providing a targeted approach to eliminate bacteria, preserve mammalian cells and strengthen the dentin hard tissues.

Chitosan possess inherent antibacterial properties (1). The nanoparticles of chitosan are known to exhibit higher antibacterial efficacy owing to its quantum size effect. The main mechanism of killing by these Cs-np is suggested to be direct contact-dependent inhibition of bacteria (2). The cationic Cs-np is highly reactive towards anionic particles/surfaces such as bacteria and biofilms (3). The bacterial membrane is negatively charged owing to the lipopolysaccharide (gram negative) and lipotechoic acid (gram positive) present on the surface. In the case of bacterial biofilms, the negatively charged polymeric matrix favor cationic molecules to have higher binding and uptake (4). However, the charge and thickness of the EPM could limit the penetration of the antimicrobials requiring higher concentrations of antimicrobials and longer duration of contact for elimination of biofilm bacteria (4-6). The EPM acting as a physical barrier might also result in a concentration gradient of nanoparticulates within the biofilm (5, 6). Other than these general issues of antimicrobial resistance, root canal disinfection presents with additional limiting effect due to the root canal constituents. The antimicrobial efficacy of root canal disinfectants was compromised in vivo as compared to in vitro tests (7, 8). The root canal constituents such as bacteria and its by-products, tissue fluids, dentin, dentin matrix and pulpal tissue remnants all have been shown to compromise the antibacterial activity of commonly used antibacterial disinfectants to varying degree (8, 9).

Combination of different antimicrobials and delivery methods has been investigated to eliminate biofilm bacteria effectively (10-12). In addition to the reduction of viable bacteria within the biofilm, disruption of the biofilm structure consisting mainly of EPM should also be focused. Dunne et al. have shown that biofilm matrix could not be disrupted even after increased concentration and time of antibiotics treatment (5). The bacteria surviving within this biofilm matrix could re-grow in no time when conducive environment is provided. PDT due to its non-
specific antibacterial activity has been used for treatment of localized infection (13, 14) as well as to improve collagen stability (15, 16). The main toxic agent for the PDT action is singlet oxygen. Singlet oxygen is known to diffuse approximately 50 nm (17), and half-life is reported to vary based on the solvent used (18). In the physiological environment, the half-life of singlet oxygen is expected to be less than 1 μs (19). Therefore, the higher the uptake of photosensitizer into bacteria/biofilm, better would be the antibacterial effect following irradiation (18). The closer proximity of the photosensitizer molecule to the cell surface might allow the diffusion of singlet oxygen into the bacterial cells within the biofilm. Availability of molecular oxygen has also been shown to govern the singlet oxygen yield. Studies have reported that fractionation of PDT dosage would further potentiate the PDT effect by replenishing the molecular oxygen (20). The slower release of singlet oxygen could also be advantageous by similar mechanism of fractionation. Furthermore, slow singlet oxygen rate could lead to step-by-step destruction of biofilm structure due to deeper penetration of these reactive molecules.

The Cs-np synthesized in the initial phase of the study possessed significant antibiofilm properties (21). The antibacterial property of Cs-np was retained even after ageing in saliva and PBS for 90 days. Although the confocal microscopy of biofilms treated with Cs-np showed increase in the dead cells as compared to live cells, the multilayered, three-dimensional biofilm-architecture were not disrupted completely (21). Pockets of live cells were found within the aggregates of dead cells. Both chitosan and its modifications have shown antibiofilm properties that are time dependent and the time taken for effective elimination was a minimum of 48 hours (21). The few number of live bacterial-cells within the biofilm structure could grow and multiply once the exposure is eliminated and nutrients are available. Other than this, the antibacterial efficacy of Cs-np was found to be significantly compromised in the presence of tissue inhibitors (22). The inhibitory effect of pulp and BSA on Cs-np was significantly higher as compared to other inhibitors tested. This inhibition of antibacterial efficacy has been attributed to physico-chemical interaction of cationic Cs-np with charged particles of pulp and BSA. Based on these initial findings, the Cs-np was proved to be effective in bacterial elimination and selective killing of biofilm bacteria. However, the importance of complete disruption of biofilm structure could not be down played. The Cs-np presented with specific shortcomings such as: time taken for biofilm reduction was found to be significantly long; incomplete disruption of biofilm structure and inhibition of its efficacy in the presence of tissue inhibitors.
In order to overcome the shortcomings of the Cs-np and provide an effective single step treatment to achieve effective biofilm elimination as well as to stabilize dentin tissue modified chitosan micro-/nanoparticles were developed following repeated experiments to achieve the desired properties of CSRB and CSRB-np. CSRB-np combined with PDT and dose fractionation showed complete elimination of the biofilm structure. CSRB and CSRB-np both showed properties of bioactive polymer chitosan and rose bengal photosensitizer as determined by the chemical and photophysical characteristics. Even though the ability of CSRB to produce singlet oxygen was higher, CSRB-np were preferred over the microparticles. This was based on two main reasons: 1) higher cytotoxicity towards NIH3T3 fibroblast cell lines, and 2) slower $\text{^{1}O_2}$ yield could be desirable for antibiofilm efficacy where longer and deeper penetration of $\text{^{1}O_2}$ is desired. CSRB-np showed higher uptake into the bacterial biofilm and increased bacterial membrane damage as evident from various microscopic techniques at the ultrastructural level. Although Cs-np and CSRB reduced bacterial biofilms to significantly higher number, CSRB-np was able to completely eliminate and disrupt both monospecies and multispecies biofilms (Appendix: Figure 3). To further highlight the advantage of CSRB-np, these nanoparticles retained their antibacterial efficacy even in the presence of tissue inhibitors.

The dentin collagen cross-linking was achieved by using chemical as well as photodynamic crosslinking. The chitosan was found to form chemical bonds with collagen upon chemical and photodynamic crosslinking. Furthermore the toughness properties of the chitosan incorporated dentin collagen were significantly higher as compared to those with mere crosslinking. To further improve this crosslinking and chitosan incorporation into the crosslinked dentin collagen a single step photodynamic crosslinking using CSRB-np was investigated. The mechanical properties such as tensile strength and Young’s modulus of dentin depend on the orientation of sample along the load due to its structural anisotropy (not same in all directions) (23, 24). As compared to the bone, the magnitude of anisotropy (~10 %) is lesser. The perpendicular arrangement of mineralized collagen fibrils to the direction of dentinal tubules in root dentin emphasizes that care should be taken to obtain samples along the direction parallel to the collagen fibrils in case of tensile tests. The Young’s modulus and tensile strength were significantly lower in the direction of the tubules as compared to when force was perpendicular to the tubules (24, 25).

Chemical crosslinking required longer treatment time to establish stable collagen cross-links as compared to the photodynamic process (26, 27). This is a major limitation especially for clinical
applications, where shorter and simpler treatment steps are highly desirable. CSRB and CSRB-np upon photoactivation were able to produce additional collagen crosslinks due to the production of singlet oxygen or radicals. In addition, the chitosan that was conjugated with the photosensitizer further enhanced the resistance to degradation and physical properties of collagen matrix following crosslinking. In case of CSRB-np crosslinked collagen, there was incorporation of Cs-np into the collagen architecture as evident from TEM images and FTIR analysis. The Cs-np in the collagen matrix reinforced the collagen structure by increasing the number of amine reaction sites resulting in the formation of ionic complexes between CS and collagen during crosslinking. The findings from this study demonstrated that, crosslinking with CSRB-np also delayed the enzymatic degradation of dentin-collagen, and at the same time increased the overall ultimate tensile strength and toughness. The characteristic stress-strain curve of a hard tissue collagen such as bone showed three distinct regions when tensile stresses are applied (28). Similar observations were found for the dentin-collagen without any crosslinking and in photodynamically crosslinked samples with and without CS incorporation. The initial toe region is non-linear and occurs due to unwinding or unraveling of the hydrogen bonds stabilizing the collagen triple helices as well as microfibrils. The linear region represents the stretching of the collagen fibrils along the direction of force applied. The failure region gives the break point of the collagen fibrils. The dentin-collagen in all the chemically crosslinked groups showed change in the toe-region, suggesting of significantly increased crosslinking as compared to the photodynamic crosslinking groups. However, the toughness values obtained were higher in CS incorporated groups of both chemical and photodynamic crosslinking highlighting the role of CS and crosslinking in improving the structural stability of dentin. The protection from enzymatic degradation is highly desirable not only in root-filled teeth but also in cases of restored of carious lesions. The increased resistance to degradation of crosslinked dentin surface collagen would provide protection against interfacial leakage. The Cs-np that was incorporated on the dentin collagen could also act as an antibacterial coating thus preventing ingress of bacteria along the interface. Similar concepts of coating surfaces with chitosan have been tested to reduce bacterial adherence and biofilm formation (29).

CSRB-np synthesized and characterized in this study possessed the following characteristics: (1) CS with higher affinity to bacterial cell wall as well as biocompatibility; (2) RB as a photosensitizer with photodynamic antibacterial and crosslinking ability; (3) the nano-size further enhanced the photoactive and antimicrobial properties due to high surface area to mass ratio
resulting in increased interaction with the substrate; and (4) photochemical crosslinking of dentincollagen resulted in increased resistance to degradation and improved UTS and toughness properties (Fig. 8.1).

8.2 Future Studies

1. In non–surgical root canal treatment, these nanoparticulates could be delivered into the anatomical complexities and dentinal tubules using high intensity focused ultrasound where conventional disinfectants are unable to reach (30). This could be highly beneficial since studies have shown that a significant portion of the root canal surfaces remain untouched by instrumentation and bacteria survive in these favorable niches (1, 5). The stability and delivery of these antibacterial nanoparticles into infected dentin tissue needs to be addressed and this is a separate avenue for biophysical research.

2. The research towards delivery of these nanoparticles in sufficient concentration to exert effective antibacterial and collagen crosslinking activities is totally a different area. The dynamics of fluids flow inside the root canals and interaction with the root canal walls need to be studied in detail.

3. PDT to be effective inside the root canals, tissue specific such as delivery of optimum dose of light and time of photosensitization could be focused in future research. Use of liquids with higher diffusibility could enhance diffusion of nanoparticulates into the biofilm structure and anatomical complexities within the root canals. However, issues of restricting these treatment solutions within the root canal confines to avoid undue periapical immune response needs equal consideration.

4. Detailed study of CSRB-np interaction with eukaryotic cell lines would shed more light on the different mechanism of action on bacteria versus mammalian cells.

5. In vivo experiments using animal models to study the immune response following treatment of infected dentin tissue with these nanoparticles would help to elucidate information on the healing response. This could also provide evidence on the toxicity of these bioactive nanoparticles upon direct exposure to living cells.
Figure 8.1. Proposed mechanism for the synthesized chitosan conjugated rose bengal nanoparticles (CSRB-np) highlighting the specific advantages of each component and their role as a targeted antibacterial agent as well as interaction with collagen.
6. Clinical samples from failed root treated teeth could be obtained. The detailed analysis of the root canal surface of such failed cases should be done to evaluate the dentin substrate changes such as resorption pits and collagen degradation. Such evidence from clinical samples would highlight the importance of strengthening the dentin structure in addition to cleaning and shaping of root canals during endodontic treatment.

7. Newer high-throughput methodologies to characterize the microbiota associated normal and diseased oral sites such as next-generation sequencing of 16S rRNA amplicons and pyrosequencing has reported a vast increase of microbial taxa including root canals (31-33). These have significantly contributed to refining and augmenting the knowledge of the community membership and structure in and on the human body in healthy and diseased conditions. As most oral infectious diseases are non-species specific and rather biofilm-related polymicrobial infection, specific patterns related to health or disease could be detected by the high-throughput sequencing technologies. Despite many technological advances, systematic reviews have found that the reported success rates for both primary and secondary endodontic treatments have not improved over the last four or five decades (34-37). Further advances in technology may hold the perspective to have important implications in terms of accurate association or causal relationship of specific microbes to disease process as well as therapeutic measures during root canal treatment.

8.3 Challenges and Limitations

1. Synthesis of CSRB-np
   The synthesis of CSRB-np required modification as compared to the chemical conjugation of CSRB microparticles. Initially the CSRB-np synthesis was attempted based on the interaction of CSRB microparticles with sodium tri-polyphosphate (ionic gelation method). However, the nanoparticles obtained were non-uniform in size with large polymeric aggregates. Therefore, the synthesis was modified by chemically crosslinking the Cs-np that was freshly prepared as mentioned in Chapter 8.

2. Standardization of biofilm models
   The importance of obtaining relevant in vitro biofilm models to test antimicrobial could not be emphasized more. The review by Kishen and Haapasalo (2012) explained the requirements of biofilm models. In this study, most of the initial experiments were conducted on monospecies bacterial biofilm in 24-well plates before proceeding to the
multispecies biofilm model grown on dentin substrate. The multispecies biofilm model took a number of trial and error to obtain uniform biofilms each time. As well as, care was taken to transfer (in reduced transport fluid) these anaerobic biofilms for confocal microscopy that was located in another imaging facility.

3. Dentin-collagen samples and mechanical tests
The mechanical tensile test required uniform sample size of 16 mm length to be gripped by the loading jigs. The samples also needed to be prepared in the direction parallel to the direction of collagen fibrils i.e., along the long axis of the tooth. We performed initial tensile tests on dentin-collagen obtained from extracted human canines. Due to scarcity of healthy single-rooted human teeth with adequate length and large sample size, we used bovine dentin-collagen in rest of the experiments (Chapters: 4-6). The width of the collagen specimens is crucial to avoid any jaw break. Thus, we used 200 microns thick collagen samples; well hydrated with drops of deionized water and covered the ends with aluminum foil support. In case of jaw breaks during tensile tests, the samples were discarded and another fresh sample was tested to match the sample size.

4. Ultrastructural Microscopy
The transmission electron microscopy has been used to evaluate dentin-collagen ultrastructure by previous studies. TEM provided high-resolution images for both bacteria as well as dentin-collagen. However, the training time, tedious sample processing, sectioning and imaging costs involved in TEM need to be considered. Use of microscopic techniques such as two-photon microscopy and polarization microscopy could help obtain high-resolution images of collagen fibrils and assess degradation in a faster and less expensive manner.

5. Amino-acid analysis and HPLC
We tried to understand the mechanism of crosslinking and specific interaction of collagen with chitosan by using molecular techniques such as amino-acid analysis, high-performance liquid chromatography (HPLC) and SDS-PAGE analysis. Most of the previous studies used collagen gels or soluble collagen to understand the changes and interactions. In our case as the crosslinking might have occurred only on the surface of dentin-collagen, the changes detected in amino-acid analysis and SDS-PAGE was not significant other than glutaraldehyde crosslinked samples. Furthermore, the quantification of chitosan incorporated into collagen would be interesting. HPLC could provide valuable
information in this aspect. However, the quantity of chitosan incorporated on the dentin surface might be less to be detected by HPLC or amino-acid analysis.

8.4 Conclusion

As hypothesized, the CSRB-np displayed properties of both CS and RB in a nano-form that performed the dual function of eliminating bacterial-biofilms and improved mechanical properties and biochemical stability of dentin organic matrix. The current study provides a novel nanoparticle based approach to enhance biofilm elimination and simultaneously improve the structural integrity of dentin. This photosensitizer functionalized chitosan-nanoparticles will have potential application in the management of infected teeth.

8.5 References

8. Portenier I, Haapasalo H, Orstavik D, Yamauchi M, Haapasalo M. Inactivation of the antibacterial activity of iodine potassium iodide and chlorhexidine digluconate against


Appendix: Supplementary Data
Figure 1. 7-day old biofilms of *P. aeruginosa* (P.a. 14) was grown in multiwell-plates to simulate an in vitro biofilm situation. Bacterial survival expressed as log CFU/mL of *P. aeruginosa* in biofilm forms surviving the PDT conducted in a multiwell plate. The biofilms were sensitized with 10 μM of RB and MB; and 0.3 mg/mL of CSRB for 15 min. a) There was a significant difference in the killing by CSRB compared to RB and MB. CSRB showed complete killing at 40 J/cm². b) The antibacterial PDT effect of MB and RB did not show any improvement with increasing photosensitization time. Standard deviations are within the parentheses corresponding to the color of the data points.
Figure 2. The three-dimensional laser scanning confocal microscopy reconstruction of the *P. aeruginosa* biofilm subjected to PDT using CSRB, RB and MB. (Inlet shows the sagittal section) (60X). a) The biofilm receiving no treatment; b) the biofilm subjected to CSRB and irradiation (40J/cm²); c) the biofilm subjected to RB and irradiation (40J/cm²); and d) the biofilm subjected to MB and irradiation (40J/cm²).
Figure 3. For multispecies biofilm, *Prevotella intermedia* ATCC 25611, *Actinomyces naeslundii* ATCC 12104, *Streptococcus oralis* ATCC 35037 were grown on 0.5 mm thick dentin sections prepared from the coronal part of extracted molar teeth. All strains were grown and maintained on trypticase soy agar (TSA: BD, Franklin Lakes, NJ) supplemented with 5% sheep blood, hemin (5 μg mL⁻¹), vitamin K1 (0.5 μg mL⁻¹), and yeast extract (10 mg mL⁻¹) under an anaerobic atmosphere (10% CO₂, 5% H₂, and 85% N₂) in a COY Model 2000 Forced Air Incubator (Coy Laboratory Products, Grass Lake, MI). Bacteria were incubated at 37 °C in trypticase soy broth (TSB, BD) supplemented with hemin (5 μg mL⁻¹), vitamin K1 (5 μg mL⁻¹), and yeast extract (1 mg mL⁻¹) overnight to prepare inocula. Bacterial cultures were adjusted to 0.3 optical density and 0.5 mL of each of the bacteria were added in 24-well plates with dentin sections and incubated anaerobically. The media was changed every 4 days.

The SEM images showed a robust biofilm structure with three specific bacterial strains (based on the morphology) and abundant EPM (A-C). The biofilms subjected to sensitization with CSRB-np and PDT (40J/cm²) were completely disrupted and dentin surface with open tubules could be appreciated (D). The three-dimensional confocal laser scanning microscopy reconstruction of the biofilm with both live (green) and dead (red) bacterial-cells in a multilayered architecture (E). The biofilms subjected to sensitization with CSRB-np and PDT (40J/cm²) were completely disrupted with only dead cells (F).

The initial thickness of biofilm-structure was found to be 60.5±9.3 μm at 3 weeks. The distribution of viable bacteria was reduced significantly, the biofilm thickness was reduced and the multilayered biofilm-architecture was disrupted following CSRB-np PDT. The thickness the biofilms reduced significantly to 11.5± 5.57 μm (p< 0.05).