Application of Electrokinetic Principles for the Distribution and Delivery of Antibacterial Nanoparticles for Root Canal Disinfection

by

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A thesis submitted in conformity with the requirements for the degree of Master’s of Science

Faculty of Dentistry
University of Toronto

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Abstract

Endodontic disinfection is challenged by bacteria organized as biofilms in difficult to access areas including the extraradicular regions of teeth. The aim of this study was to propose and evaluate an electrokinetic delivery system capable of mobilizing charged antibacterial nanoparticles and inactivating biofilm located within the apical portions of the root canal and on the extraradicular root surface. A computational model was developed to predict electric field and current density distribution generated by the proposed intervention. Transport of CSnp was demonstrated using a polydimethylsiloxane (PDMS) microfluidic model. Antibacterial properties of electric current and CSnp were evaluated against Enterococcus faecalis in planktonic and biofilm forms. The proposed intervention demonstrated rapid CSnp transport with subsequent distribution and deposition in the periapical regions. The bacteriological study demonstrated enhanced antibacterial and antibiofilm effects. If translated clinically, this methodology may augment endodontic disinfection and offer the ability to treat extraradicular biofilms through a non-surgical approach.
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Andrei Ionescu, July 2015
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Chapter 1
Introduction
1.1 Current endodontic practice

1.1.1 Bacterial biofilm

The involvement of biofilms in endodontic infections has been well recognized and has gained much attention in recent years. Studies of endodontic microbiota in primary and persistent infections have demonstrated that bacterial colonization of the root canal system occurs mostly as polymicrobial aggregations, adherent to dentinal surfaces, and embedded in an extracellular polymeric substance (EPS), termed as biofilms (1–3). The EPS, with its highly charged and interwoven structure, deters penetration of antimicrobials by acting as a physical barrier and neutralizer for biochemical reactions (4). Such organization into communities of microorganisms has been shown to provide ecological advantages and offer protection from host responses as well as antimicrobials (5,6).

Multiple scanning and transmission electron microscopy studies (SEM, TEM) have shown that necrotic and infected canal systems may be coated with a bacterial plaque extending into canal intricacies (1,3) dentinal tubules (7) and on certain occasions extending externally onto the apical portion of the cementum (8). Bacterial colonies outside of the root canal system have been described as forming biofilm-like structures on the cementum adjacent to the apical foramen (2,8,9) or as forming cohesive colonies within the periapical lesion (10,11). A number of studies indicate that persistent/refractory apical periodontitis may be induced by bacterial biofilms on the external root surface that can effectively evade host defense mechanisms while remaining unreachable by endodontic irrigants (12–14). Moreover, recent studies utilizing advanced molecular techniques together with imaging by electron and light microscopy provide evidence that such extraradicular biofilms are not uncommon, especially in cases that did not respond to conventional endodontic treatment (15–18).

1.1.2 Goals of endodontics

Endodontic therapy aims to eliminate bacteria and prevent reinfection of a treated root canal while maintaining maximum structural integrity of the dentin. Root canal therapy may be performed on teeth with irreversibly inflamed dental pulps, in an attempt to prevent apical periodontitis, or on teeth with apical periodontitis aiming to treat infection. The condition in
which the root canal presents may therefore vary from that of an intact pulp–dentin complex, through a partially degraded pulp with areas of infection, to a system completely coated with mature bacterial biofilm (1). Complete elimination of bacteria from infected root canals while maintaining the structural integrity of dentin in endodontically treated teeth has proven to be a daunting task in dentistry (19,20). The limitation in the complete elimination of microbes has been attributed to the tenacity of bacteria in biofilm form, and localization of biofilm in root canal complexities or on extraradicular surfaces.

Current endodontic procedures utilize disinfectants which inevitably alter the dentinal structure through chemical or mechanical means (21). Any such treatment-induced changes that compromise the mechanical integrity of tooth structure may have a profound effect on the long-term survival of the tooth and should be minimized (22,22,23). As it stands, the major endodontic challenge involves removing bacteria that are responsible for apical periodontitis while minimizing any collateral damage to dentin.

1.1.3 Endodontic outcomes

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 (24–26). It is generally believed that the major cause of root canal failure is the persistence of microorganisms associated with root-filled teeth. However, as more and more evidence is uncovered that apical periodontitis is a biofilm-induced disease involving both the root canal system and the extraradicular surfaces (11,15–18), it can be postulated that clinical outcomes may be hindered by the limitations of current procedures in addressing biofilms present on the extraradicular surface. We suspect that the inherent plateau in endodontic outcomes may be in part due to our inability to address bacteria present on the external root surface during the initial endodontic therapy or retreatment.

1.1.4 Limitations of current techniques and advanced therapeutic options

Current non-surgical endodontic disinfection strategies are confined to the root canal system and focus predominantly on the chemical and mechanical debridement of internal contents. These strategies face several challenges posed by bacteria organized in biofilms, anatomical complexities of the root canal system, and factors associated with the toxicity of chemical
disinfectants. Furthermore, current non-surgical techniques do not attempt to eradicate bacteria located in the periapical region and on the external root surface. To overcome some of these limitations several advanced therapeutic options for endodontic biofilms have been proposed, all with the aim of disrupting the biofilm structure while simultaneously killing the resident bacteria even in locations untouched by root canal instrumentation procedures (4). A promising area of research looks at the potential use of antimicrobial nanoparticles for root canal disinfection and seeks to take advantage of their unique physical and chemical characteristics.

1.2 Nanoparticles for endodontic disinfection

Nanoparticulates exhibit high antibacterial activity as a result of their polycationic nature in combination with higher surface area and charge density. Their method of action relies on an entirely different mechanism of antibacterial activity than traditional antibiotics. Their nanometric size and increased charge density leads to a greater electrostatic force and intimate interaction with the negatively charged bacterial cell (27). The size of nanoparticulates plays an important role in their antibacterial activity, with smaller particles showing higher antibacterial activity than the microscaled ones (28–30). The accumulation of a large number of nanoparticles on the bacterial cell membrane has been shown to increase membrane permeability and a deterioration of membrane function (31,32). This phenomenon offers cationic nanoparticles broad spectrum antibacterial properties and may decrease the potential for bacterial resistance.

Previous studies carried out in our laboratory have characterized the effectiveness, stability and safety of chitosan nanoparticles (CSnp) (27,33–37). Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin, a major component of the shells of crustaceans such as crab, shrimp, and crawfish. CSnp have been shown to possess significant antibacterial (27) and antibiofilm (33) properties and were able to disrupt multilayered, 3-dimensional biofilm architecture in vitro (33). Their antibacterial activity 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 (38,39). Deposition on dentin surfaces has been shown to impede bacterial re-colonization (27). Despite numerous advantages, the effectiveness of CSnp remains limited by their ability to reach and interact with bacterial biofilms.
Effective inhibition of biofilm bacteria by CSnp requires a high concentration of nanoparticulates and a long duration of contact (33). If attempting to treat extraradicular biofilms in this manner, obtaining high concentrations of CSnp periapically is paramount. It is thought that in order for apical extrusion to occur, the apical fluid pressure must exceed the venous pressure of the facial veins (40). If the needle is intentionally made to engage dentin and the apical pressure is increased, the possibility exists for the CSnp suspension to be infused into the tissues causing uptake through portals of entrance into the facial venous vasculature. Thus, concentrating particles periapically may not be achieved with conventional positive pressure needle irrigation techniques. Furthermore, this increased apical pressure approach may also lead to tissue damage and post-operative pain.

1.3 Electricity in medicine and dentistry

In the medical sciences, electric protocols have received wide applications since the turn of the century (41). They have been used clinically for the treatment of bone non-union (42–44) to enhance drug transport across skin by iontophoresis (45) and for non invasive stimulation of central (46) or peripheral nerves (47). The mechanisms of action utilized in these protocols capitalize on neurostimulatory effects, electrophoretic and electro-osmotic driving forces, alteration to cell membrane permeability and modulation of metabolic and developmental processes of both prokaryotic and eukaryotic cells (48–50).

1.3.1 Relevance of electrophoresis in medicine

The process of electrophoresis refers to the transport of charged colloidal particles under the influence of an electric field. When subjected to a non-uniform electrostatic field, particles in suspension will move at characteristic velocities determined by their charges, size and mobility (51,52). The subsequent ordered deposition of the particulate matter on the electrode towards which they are attracted is termed electrophoretic deposition (EPD). These phenomena are of relevance in many medical fields for manipulation of biological materials, e.g. proteins, enzymes, cells, as well as colloids, polymers and solid inorganic particles (53). Due to its simple and cost effective nature EPD has generated substantial attention for the fabrication of thin films and coatings of organic, inorganic and composite materials on substrates of complex shapes and surface areas (53). One common application of EPD is the generation of bioactive and biomimetic coatings on the metallic surfaces of implantable prosthetics (54,55). Electrophoretic
deposition of chitosan nanoparticles has been successfully achieved to form insoluble chitosan films of uniform thickness (56). Other recent research has been carried out to control the movement of biological entities such as enzymes, bacteria and cells (57,58).

1.4 Electrokinetic transport phenomena

The electrokinetic phenomena describe a family of effects that occur in heterogeneous fluids, or those containing particles, under the influence of an electric field. These effects are described in several textbooks and include electroosmosis, electrophoresis, streaming potential and sedimentation potential (59,60). These phenomena arise due to the interactions of electric charges and liquids, and are often characterized by the presence of an electrical double layer. Streaming and sedimentation potential are beyond the scope of this work and will not be discussed here.

1.4.1 The electrical double layer

Particles in suspension exhibit a zeta potential, or surface charge. This presence of a net charge affects the distribution of ions surrounding it, resulting in an increase in the concentration of counter-ions immediately adjacent to its surface. The region over which this influence extends is called the electrical double layer (EDL). First identified by Helmholtz circa 1879, this layer is thought to consist of two separate regions: an inner layer with strongly bound ions (stern layer) and an outer layer with loosely associated ions (diffuse layer). As the particle moves through solution, due to an applied voltage or gravity, the bound ions move along with it. At some distance from the particle there exists a “boundary” beyond which ions do not move along with the particle. This distance, known as the Debye length ($\lambda_D$), is located somewhere within the “diffuse layer” and demarcates the surface of hydrodynamic shear, or the slipping plane (60). A similar electrical double layer develops adjacent to any solid phase in contact with an aqueous medium (60). As such, an EDL with a characteristic Debye length is also expected to develop adjacent to the wall of a microfluidic system.
1.4.2 Electroosmosis

Electroosmosis refers to the movement of bulk liquid relative to a stationary charged surface due to an applied electric field (59). A tangentially applied electric potential gradient will cause the mobile portion of the EDL to migrate towards the cathode or anode depending on the polarity of the EDL. This electromigration of ions constituting the EDL causes viscous shearing of the adjacent bulk liquid molecules, ultimately resulting in bulk liquid motion. As the ions move, they draw water molecules along with them. The resultant fluid velocity may be described by the following formula,
\[ v_{eo} = \mu_{eo} E \]  

(1.1)

where \( E \) is the applied electric field and the \( \mu_{eo} \) describes the electroosmotic mobility, the magnitude of which can be a function of ion concentration, pH, and temperature. The electroosmotic flow velocity \( (v_{eo}) \) given in Eq. 1.1 may be estimated in terms of the zeta-potential using the Helmholtz-Smoluchowski equation given as

\[ v_{eo} = \mu_{eo} E = \frac{\varepsilon \varepsilon_0 \zeta_c}{\eta} E \]  

(1.2)

in an environment where \( \varepsilon \) is the dielectric constant of the solution, \( \varepsilon_0 \) is the permittivity of free space, where \( \zeta_c \) is the zeta potential of the micro channel and \( \eta \) represents viscosity of the solvent.

Figure 1.2. Schematic of electroosmotic flow in a microchannel. The Helmholtz-Smoluchowski relation (Eq. 1.2) predicts the magnitude of the plug-like electroosmotic fluid velocity. The inset diagram provides a schematic of the net charge distribution within the EDL located near the microchannel walls.

Adapted from Oddy 2005 (61)
1.4.3 Electrophoresis

Electrophoresis refers to the transport of charged particles relative to stationary bulk liquid due to an applied electric field (59). When an electric field is applied, the charged material will tend to migrate toward the anode or cathode depending on the polarity of the EDL. As the particle migrates through the liquid, counter-ions will move in and out of its charged ion cloud, or EDL. The electrophoretic velocity \( v_{ep} \) is proportional to the magnitude of the electric field \( E \) and the particle’s electrophoretic mobility \( \mu_{ep} \). This relationship can be explained utilizing Hückel’s approximation for thick double layers and small particles,

\[
v_{ep} = \mu_{ep} E = \frac{\varepsilon \varepsilon_0 \zeta_{np}}{1.5\eta} E \tag{1.3}
\]

where \( \zeta_{np} \) is the zeta potential of the particle. The resultant effects of electrophoresis and electroosmosis are responsible for particle transport and deposition. A particle’s total velocity \( v_{tot} \) as it moves through the microfluidic system is the sum of its electrophoretic velocity and the electroosmotic flow velocity.

\[
v_{tot} = v_{eo} + v_{ep} \tag{1.4}
\]
1.4.4 Electrophoretic deposition

The EPD process involves deposition of charged particles in a suspension under the influence of an applied electric field. The first model of EPD kinetics was proposed by Hamaker in 1940 (62) for electrophoretic cells with a planar geometry. It relates the deposited mass ($M$) with slurry properties, such as suspension concentration ($C_s$), and electrophoretic mobility ($\mu_{ep}$), with the physical conditions imposed on the system such as electric field ($E$), deposition surface area ($S$) and deposition time ($t$).

$$M = C_s\mu_{ep}SEt$$ \hspace{1cm} (1.5)
1.5 Factors influencing transport and electrophoretic deposition

Ideally in an EPD process, all of the applied electric field should be utilized to effect particle electrophoresis, the driving force for EPD. In reality an EPD suspension is far from ideal due to the presence of free ions in addition to the particles in suspension. A portion of the current is carried not only by the charged particles but also by the free ions co-existing in the suspension. The actual particle movement in a liquid media depends not only on the value of the current density passing through the suspension but also on the geometry of the electrophoresis cell and the physical and chemical parameters of the system. In their review, Besra and Liu (63) summarized various parameters influencing EPD in two groups; (1) suspension parameters, and (2) process parameters. These parameters are reviewed in detail by Dickerson and Boccaccini (64) and will be briefly summarized below with emphasis on those influencing the present application.

1.5.1 EPD suspension parameters

In general, the desired properties of the suspension vehicle are high dielectric constant, low conductivity and low viscosity.

**Dielectric constant of liquid:** The dielectric constant, or relative permittivity of a fluid is a quantity measuring the ability of a substance to store electrical energy in an electric field. The Helmholtz-Smoluchowski equation for electrophoretic mobility suggests that in general it is desirable to select a solvent with a high dielectric constant for maximum deposition mass (Eq. 1.3, 1.4, 1.5). However, Powers (65) studied the deposition of beta-alumina suspended in numerous organic media as a function of the dielectric constant and suggested that optimal deposition occurred when the dielectric constant was in the range of 12–25. It has been suggested that with too low a dielectric constant, deposition fails due to insufficient dissociative power; while with a high dielectric constant, the high ionic concentration in the liquid reduces the size of the double layer region and consequently the electrophoretic mobility (63).

**Conductivity of suspension:** The conductivity of the suspension is a key factor in EPD and needs to be taken into account. Ferrari and Moreno (66), demonstrated that if the suspension is too conductive, particle motion is very low, and if the suspension is too resistive, the particles
charge electronically and the stability is lost. They found the existence of an optimal band of conductivity range at various dispersant dosages and temperatures, in which a deposit is formed. However, increasing the current used during the EPD process can widen the useful conductivity range of the suspension (67). For practical purposes, the suspension vehicle may contain a deflocculant in order to decrease viscosity while increasing zeta potential of the particles. If so, a compromise must be made where the deflocculant concentration is kept as low as possible to minimize ionic charge, while still preventing sedimentation (68).

**Viscosity of suspension:** The Helmholtz-Smoluchowski equation suggests the electrophoretic mobility is inversely proportional to viscosity (Eq. 1.3,1.4,1.5). On this basis, it is wise to select a suspension vehicle with low viscosity to achieve maximum electrophoretic velocity and thus, deposition mass.

**Particle size:** Although no definitive limits exist in regard to particle size, it is important that the suspension is stable and the particles remain completely dispersed for homogeneous and smooth deposition. As particles increase in size they begin to sediment and electrophoretic mobility is hindered by gravity (63).

**Zeta potential:** The zeta potential of particles in suspension is a key factor in the electrophoretic deposition process. A large zeta potential plays a key role in: stabilization of the suspension by determining the intensity of repulsive interaction between particles, determining the direction and migration velocity of particle during EPD, and determining the density of the deposit. Particles with high surface charge during deposition will repulse each other, occupying positions which will lead to high particle packing density (69). The zeta potential can be controlled by addition of a variety of charging agents such as acids, bases and specifically adsorbed ions or polyelectrolytes, to the suspension (70). While the deposition rate is directly dependent on the zeta potential, the influence of the additives is exerted by its effect on the ionic conductivity of the suspension.

**Stability of suspension:** A key requirement in preparing suspensions for EPD is to achieve a well stabilized, unagglomerated, and homogeneous slurry. This colloidal stability is governed by the total interparticle potential energy. The dominating forces in most EPD system are: (i) the van der Waal attractive force, (ii) double layer (electro-static) repulsive force, and (iii) steric (polymeric) forces. In order to obtain a well-stabilized suspension, particles dispersed in the
suspending medium must exhibit sufficient repulsive forces to offset the van der Waals attraction (63). However, when the suspension is too stable, the repulsive forces between the particles will only be overcome by high electric field, and typical EPD condition deposition may not occur (71). Particle size also plays a role in this case as smaller colloidal particles tend to remain in suspension for longer periods of time due to Brownian motion, while larger particles require continuous agitation to remain in suspension.

1.5.2 EPD process parameters

Effect of applied electric fields: In general particles can be deposited faster by increasing the magnitude of the applied fields, however, the quality of the deposit can suffer. In 2001 Basu and colleagues (72) observed that less intense fields (25–100 V/cm) generated more uniform coatings while higher applied fields (> 100 V/cm) caused the film quality to deteriorate. It is postulated that the accumulation rate of the particle deposition influences their packing behavior. A higher applied field may cause turbulence in the suspension, disturbing the deposition process. In addition, particles may be moving so fast that they may not find enough time to settle in their optimal positions to form a close-packed structure (63).

Effect of deposition time: Researchers examining the role of time on EPD kinetics have observed a linear relationship between time and deposition mass during the initial period. This high deposition rate is subsequently decreased and reaches a plateau after very long deposition times (72–74). This observation can be explained due to the insulating properties of the deposit on the electrode surface causing the electric field influencing electrophoresis to decrease despite the voltage remaining constant (75).

Concentration of solids in suspension: Although the Hamaker equation (Eq 1.5) predicts a linear increase in deposit mass with increasing solute concentration, yield measurements for chitosan-based nanocomposites demonstrated a greater than expected increase in deposition yield with increasing particle concentration (56,76). It was suggested (77) that increasing particle concentration could result in enhanced depletion forces at the electrode surface, which promotes particle coagulation. Such interactions can result in attraction of similarly charged particles and explain the formation of thick films from the solutions of higher concentrations (78).
Conductivity of substrate: In general, the uniformity and conductivity of substrate electrode may also affect the quality of the deposited film. It has been suggested that low conductivity substrates lead to non-uniform green film and slow deposition (74,79).

The above literature provides evidence that careful control of multiple inter-related parameters is required to regulate the kinetics of electrophoretic deposition and the quality of deposit. In general, a well-dispersed stable suspension will provide a better deposition during EPD compared to an unstable or agglomerated powder suspension. The zeta potential is an important parameter that relates to suspension stability, particle mobility and provides information on the agglomeration of the particles in suspension. In general, as the absolute value of the measured zeta potential is increased, a better dispersion of the particles in the suspension is achieved. The electrical conductivity of the suspension also plays an important role in the process. Ions in the suspension carry most of the current when an electric field is generated during EPD. If the suspension is too conductive, particle motion becomes very slow, on the other hand if the suspension is too resistive, the particles charge electronically and the stability is lost.

1.6 Electrically-induced bactericidal effects

In recent years, a significant body of research has developed, examining electrical methods of controlling the growth of microorganisms. A comprehensive review by Freebarin and colleagues (80) describes many of the recent advances in this field. The following two sections will briefly describe the understanding of the effect of electric fields on bacteria, the “electricidal effect” and the electrical enhancement of biocide and antibiotic efficacy or the “bioelectric effect”.

1.6.1 Electricidal effect

The observation that electrical current can impart a bactericidal activity is a longstanding one. As early as 1919 it was reported that sterilization of milk could be achieved using low alternating current (AC), a process now known as “ohmic heating” (81). In 1965, Rosenberg et al. found that platinum electrodes immersed in a medium would inhibit the process of cell division in E. coli when a low-frequency AC was applied (82). Since this time various other studies have demonstrated that electric fields, both AC and DC, can disrupt bacterial integrity and possess quantifiable antibacterial properties (50,83). This direct killing of bacteria by electric current has been referred to as the “electricidal effect” and has been previously demonstrated against Escherichia coli in salt solutions (84) and water (85), Candida albicans in simulated urine (86),
Staphylococcus aureus in agar (87), Bacillus subtilis in water (88) and S. aureus and Pseudomonas aeruginosa in culture medium (50). In literature, electrically induced bactericidal effects refer predominantly to the control of planktonic bacteria or prevention of biofilm formation. Another area of research involves the reduction and eradication of the bacterial biofilm in situations where it has already been established, predominantly through electrically enhanced action of antimicrobials. This is referred to as the “bioelectric effect” (80,89).

1.6.2 Bioelectric effect

It is now well established that the concentration of antibiotics and biocides required to kill bacteria residing with a biofilm matrix can be 500–5000 times those needed to kill planktonic cells of the same species (90–92). Researchers have demonstrated that electric currents can augment the activity of some antimicrobial agents against certain bacteria in biofilms, a concept now known as “bioelectric effect” (93–95). It has been shown that electric current can reduce the very high concentrations of antimicrobials needed to kill biofilm bacteria to levels close to those needed to kill planktonic bacteria of the same species (93,96,97). In the last decade, several steps have been taken to advance this field of research toward the vision of a controlling bacterial adhesion and biofilm formation on implanted device surfaces and developing a variety of infection-resistant medical devices (80,83). Although few attempts have been made to reproduce the promising in vitro bacterial killing results on human patients, a recent animal model has suggested benefits in its application for the treatment of foreign body osteomyelitis (83).

1.6.3 Bacterial detachment

A series of recent experiments have demonstrated that electric fields can “detach” biofilms from various surfaces (98–100). Van der Borden and colleagues demonstrated that low electric currents ranging from 15 µA up to 125 µA (1.5–1.7 V) can successfully detach bacterial strains of S. epidermidis and S. aureus from surgical stainless steel anode surfaces within a parallel plate flow chamber over an extended period of time. Within their experimental parameters they observed a 7-fold increase in the initial bacterial detachment rate as the current was increased from 15 µA to 125 µA. Increasing the iconicity of the solution, however, led to a decrease in initial detachment rate. They recognized that the so called “detachment force” is proportional with the applied potential and needs to overcome attractive Lifshitz–Van der Waals and acid–base forces between the adhering bacteria and the surface. They postulated that higher ionic strengths yield a higher conductivity of the fluid and hence requiring a lower voltage to establish a certain electric current.
This lower voltage causes a decrease in the detachment-force and a lower detachment rate (99). Subsequent studies demonstrated added benefits to using block currents over DC including increased bacterial motion and decreased viability of remaining bacteria (98, 100).

1.7 Research problem

Current techniques for mechanical and chemical debridement of the root canal system are unable to reach and eradicate bacterial biofilm localized on the extraradicular cementum surface when utilizing a non-surgical treatment approach.

1.8 Specific aims

This study proposes a novel technique for non-surgical targeting of bacterial biofilms located within the root canal and on the external root surface. We propose to develop a protocol for the delivery of charged antimicrobial nanoparticles in suspension, through the portals of exit of a root canal system, and into the periapical tissues using custom electric fields.

Specifically the proposed study aims to:

1. Optimize parameters that would guide and deposit the nanoparticles in extraradicular areas.
2. Evaluate the safety and identify potential limitations of the proposed mechanism.
3. Experimentally visualize CSnp trajectories and deposition
4. Evaluate the antibacterial effect of the proposed approach on planktonic and biofilm bacterial models

1.9 Hypothesis

We hypothesize that the principles of electrophoresis can be used to deliver antibacterial bioactive polycationic nanoparticles through the root canal portals of exit to achieve deposition on the adjacent external root surface.

1.10 Chapter outline and research design

To overcome some of the limitations posed by current non-surgical endodontic strategies, this study proposes a novel technique for non-surgical targeting of bacterial biofilms. It will combine
the advantages of bioactive polycationic nanoparticles with the principles of electrokinetics to achieve predictable particle transport through the root canal system with subsequent deposition on dentinal surfaces and periapically. In addition, this treatment modality may benefit from the intrinsic antibacterial properties of electric currents (electricidal effect) and the ability of electric currents to enhance the activity of antimicrobial agents against biofilm (bioelectric effect). This protocol could lend itself to minimally invasive endodontic treatment requiring smaller canal preparations, a concept which is rapidly increasing in popularity. This proposed technique will aim to address intra- as well as extraradicular biofilm in a non-surgical fashion.

CSnp was used in this study because its intrinsic positive charge makes it attractive for electrophoretic transport. Furthermore, this natural biopolymer has been highly characterized and found to be antibacterial, biocompatible and exhibit excellent film-forming properties.

The next two chapters will present a series of studies designed to evaluate the feasibility, and antibacterial effects of such an approach. Chapter 2 describes a series of computational and microfluidic experiments aimed at optimizing the parameters for particle transport. The microfluidic studies provide qualitative assessment of particle transport and deposition. In this chapter, the proposed electrical parameters are compared to those utilized for other bioelectric applications. Chapter 3 describes a set of experiments that evaluate the effects of the proposed electrokinetic delivery system against *Enterococcus faecalis* when utilizing cationic CSnp.
Chapter 2
Electrokinetic Delivery System for the Distribution of Antibacterial Nanoparticles for Endodontic Disinfection: A Computational and Microfluidic Model
2.1 Abstract

**Introduction:** Current therapeutic strategies for endodontic disinfection are challenged by bacteria that can organize to form biofilms in difficult to access areas including the extraradicular regions of the teeth. Cationic chitosan nanoparticles (CSnp) have recently been proposed as an alternative to conventional disinfectants and have been shown to be biocompatible and to possess significant antibiofilm properties.

**Aim:** The aim of this study was to propose non-invasive intervention capable of mobilizing charged antibacterial nanoparticles and inactivate biofilm located within the apical portions of the root canal and on the extraradicular root surface. The proposed intervention aims to combine the principles of electrokinetics with the antibacterial properties of cationic nanoparticles.

**Methods:** A computer model (COMSOL multiphysics, Palo Alto, CA) was created to predict and visualize the electric field and the current density distribution generated by the proposed intervention. Transport of CSnp was demonstrated using a microfluidic model. Transparent devices simulating the root canal anatomy of a central incisor were created by polydimethylsiloxane (PDMS) micro-molding of a silicon master fabricated by conventional photolithographic techniques. Fluorescin isothiocyanate (FITC)-labelled CSnp were delivered and deposited into the simulated periapical regions using controlled electric fields. Experimental parameters were compared to other bioelectric applications.

**Results:** The results of the computer simulations predict an electric field of 5-50v/cm and maximum current density of 0.05-0.22 A/m². The observed particle trajectories closely followed the electric field lines depicted by the computer simulation. The microfluidic experiments demonstrated rapid and reproducible FITC-CSnp transport through the root canal anatomy with subsequent distribution and deposition in periapical regions.

**Conclusion:** The parameters required for achieving the suggested nanoparticle transport in a simulated model were comparable to those used for other bioelectric applications. A close correlation was noted between the electric field lines observed in the computer simulations and the nanoparticle transport visualized in the microfluidic models.
2.2 Introduction

As more evidence is uncovered that apical periodontitis is a biofilm-induced disease, which involves both the root canal system and the extraradicular surfaces (11,15–18), it can be postulated that the clinical success may be hindered by a clinician’s inability to address these areas with current disinfection protocols. Current non-surgical endodontic disinfection strategies are confined to the root canal system and focus predominantly on chemical and mechanical debridement of the internal contents. These strategies face several challenges posed by bacteria organized in biofilms within the anatomical complexities of the root canal system and factors associated with the toxicity of chemical disinfectants. Furthermore, the current non-surgical approach does not attempt to eradicate bacteria located in the periapical region and on the external root surface. To overcome some of these limitations several advanced therapeutic options have been proposed, all with the aim of disrupting the biofilm structure while simultaneously killing the resident bacteria in locations untouched by root canal instrumentation procedures (4). A promising area of research looks at the potential use of antimicrobial nanoparticles for enhanced canal disinfection and seeks to take advantage of their unique properties.

Previous studies carried out in our laboratory have characterized the effectiveness, stability and safety of chitosan nanoparticles (CSnp) (27,33–37). CSnp have been shown to possess significant concentration-dependent antibacterial (27) and antibiofilm (33) properties and were able to disrupt multilayered, 3-dimensional biofilm architecture in vitro (33). Their targeted antibacterial activity could be attributed to their electrostatic attraction to the negatively charged bacterial cell, which might lead to an altered cell wall permeability, resulting in leakage of the proteinaceous and other intracellular components causing death of the cell (38,39). Deposition on dentin surfaces has been shown to impede bacterial re-colonization (27). Despite numerous advantages, the effectiveness of CSnp remains limited by their ability to reach and interact with bacterial biofilms.

When subjected to an electrostatic field, particles in suspension will move at characteristic velocities determined by their charges, size and mobility (51,52). The process of electrophoresis refers to the transport of charged colloidal particles under the influence of an electric field (64).
Intimately related is the process of electroosmosis, the flow produced by an electric field acting on the net mobile charge in the fluid within the electric double layer surrounding the charged surface of the microchannel (101). The resultant motion generated by these forces is referred to as electrokinetic flow, while the subsequent deposition of the particulate matter is termed electrophoretic deposition (EPD). The deposition kinetics is dependent on time, applied voltage and particle concentration (63). Due to its simple and cost effective nature, EPD has generated substantial attention for the fabrication of thin films and coatings on substrates of complex shapes and surface areas (53).

Previous reports have shown chitosan to be an excellent film-forming polymer capable of generating uniform coatings isolated (102,103) or in conjunction with inorganic components (56,76,104,105). In this context, the principles of electrokinetics may be useful in achieving CSnp transport and deposition in a clinical setting aimed at delivering CSnp through the root canal system and subsequent deposition on the dentinal surfaces and at the periapex. This study is the first in a series suggesting a novel technique for non-surgical targeting of bacterial biofilms located on the external root surface. We propose to develop a protocol for the delivery of charged antimicrobial nanoparticles in suspension, through the portals of exit of a root canal system, and into the periapical tissues using electrokinetic principles.

2.3 Materials and Methods

A vector drawing designed to resemble a coronal cross-section of a maxillary central incisor and periodontal ligament were created in AutoCad (2013, Autodesk Inc., San Rafael, CA). The shape depicts a central incisor with a lateral canal in the apical 1/3 and an area of periapical bone loss. Anatomical dimensions were based on an extracted central incisor. The width of the periodontal ligament (PDL) space selected was 0.2mm (106), and the length of the canal was 13mm. The size of the root canal space was designed to accommodate a size 25, 0.06-tapered file. The size of the periapical lesion was selected arbitrarily, but designed with an epicenter encompassing the apical foramen and the lateral canal.

2.3.1 COMSOL simulation

A computer model was generated to illustrate the electric fields in our proposed experimental set-up. The 2D geometry was imported into COMSOL multiphysics software (Version 4.3, Palo
Alto, CA). Two electrodes were drawn as circles in the cervical portion of the PDL ligament space and a third in the coronal portion of the root canal. All boundaries were defined as insulators and the electrodes were defined as stainless steel (UNS S17600). The luminal contents were defined as water with a relative permittivity ($\varepsilon=80$), equal to that of water at 20°C (107). An extra fine mesh was used. The model assumed a closed microfluidic system and electric potential of 50V DC. The electrostatics (es) module was used visualize the electric potential generated, and predict the electric field distribution and the electric field lines. The second order current distribution module (siec) was used to simulate the current density distribution.

2.3.2 PDMS Microfluidic device fabrication

Three-dimensional drawings were generated by extending the original vector design by 500µm in the Z-plane. Three cylindrical reservoirs were added in continuation with the root canal and PDL space to aid in solution delivery and electrode placement. Microfluidic devices were created using a conventional microfabrication technique based on a soft-lithography process (108,109). The 3D drawings were used to create a “mask” of the desired canal shapes. SU-8 Photoresist (MicroChem, Newton, MA) was spin-coated on the surface of the silicon substrate, covered by the “mask”, and the assembly was exposed to ultraviolet light at 300 nm for polymerization. The unexposed photoresist was removed by immersing in an organic solvent (1-methoxy-2-propyl acetate) for 30 minutes. The remaining polymerized material was dried at 90°C for one hour, and served as the masters for all further device fabrication. The polydimethylsiloxane (PDMS) insulating microstructures were manufactured by casting from the silicon masters and bonded to a glass slide using a plasma-bonding technique. Once the device fabrication was completed, 28-gauge side vented needles, serving as electrodes, were inserted into various locations of the canal system by puncturing through the PDMS material.

2.3.3 CSnp synthesis and FITC-labeling

FITC labeled CSnp were synthesized according to a previously reported protocol (110). In brief, chitosan (1 g) was dissolved in 100 mL of 0.1 M acetic acid. Dehydrated methanol (100 mL) was added and stirred. About 50 mL FITC (2.0 mg/mL) in methanol was slowly added to the chitosan solution. After 4h reaction in the dark at ambient temperature, the FITC-labeled chitosan was precipitated in 0.2 M NaOH raising the pH to 8-9. The precipitate was centrifuged (40,000g, 10 min) and washed by acetone:water (3:1 v/v) repeatedly until no fluorescence was detected in the
supernatant (Perkin Elmer Inc., luminescence spectrometer LS50B, kexc 485 nm, kemi 520 nm). The FITC-CS was then dissolved in 0.1 M acetic acid and dialyzed in water for 3 days in the dark and freeze dried (Vitris Co. Inc., Gardiner, New York). FITC-CS was converted to nanoparticulate form (FITC-CSnp) by ionic gelation with negatively charged tripolyphosphate (TPP) sodium ions (111). The synthesized FITC-CSnp were evaluated for their charge using a Zetasizer (Malvern Instruments Ltd, Malvern, UK).

2.3.4 Microfluidic experiments

Twenty-eight gauge side-vented needles, serving both as FITC-CSnp delivery portals and electrodes, were inserted into the cylindrical reservoirs by puncturing through the PDMS material. The microfluidic system was filled entirely with deionized (DI) water. FITC-CSnp were suspend in deionized water to a final concentration of 3mg/ml and sonicated in an ultrasonic bath for 5 minutes. Particles were injected into the device reservoirs and 50V DC was applied between sets of electrodes. Particle trajectories were visualized and recorded using a fluorescence microscope (Vert.A1, Carl Zeiss, Germany).

2.4 Results

2.4.1 COMSOL simulations

In the computer simulation, application of 50V DC generated a current of 1.7 mA, an electric field (E) between 5 to 50 V/cm and a current density (J) of 0.05-0.22 mA/cm² (Fig. 2.1 a, c) The electric field distribution suggested a 10-fold increase in E through the narrowest portions of the root canal system (minor apical foramen) and a drastic drop in E as the cross-sectional area increases in the region of the apical lesion (Fig. 2.1a).
Figure 2.1. Comsol simulation illustrating the effects of 50 V DC applied between 3 electrodes in a microfluidic system filled with deionized water. 

- a. the electric field distribution 
- b. electric field lines indicating possible particle trajectory 
- c. current density distribution. 
- d. photomicrograph of a microfluidic device.
2.4.2 Microfluidic studies

The application of 50V in the microfluidic model generated a current ranging between 0.5-1mA. Microfluidic experimentation revealed the particle trajectories closely followed the electric field lines depicted in the numerical model (Fig. 2.1b). In areas with “focused” electric fields, such as at the apical constriction, the lateral canal, and the periodontal ligament space, particles demonstrated rapid, streamlined flow (Fig. 1a). After 2-3 minutes, particles concentrated and trapped at the apical foramen causing chaotic flow characteristics and luminal obstruction (Fig. 2.2b and c).

Areas with diffuse electric fields, such as the coronal 1/3 of the root canal or the periapical lesion demonstrated slower but streamlined particle flow. As particles reached the anode, electrophoretic deposition was noted (Fig. 2.2d).
Figure 2.1. 10x mag. fluorescence microscopic stills showing several areas of the lumen of a microfluidic device containing FITC-CSnp suspended in deionized water while subjected to an electric field generated by a 50V potential difference. **a.** laminar flow in the apical 1/3 of the canal. **b.** particles becoming trapped in a narrow channel. **c.** particles trapped around the apical constriction. **d.** electrophoretic deposition on the cathode. Black arrows indicate the direction of CSnp flow.
2.5 Discussion:

The electrical properties of any material, including biological tissue, can be broadly separated into two categories: conductor and insulator. In a conductor, the electric charges move freely in response to the application of an electric field, whereas in an insulator (dielectric), the charges are fixed and not free to move. In reality, most materials, including biological tissue, actually display some characteristics of both insulators and conductors because they contain dipoles as well as charges that can move, but in a restricted manner. Significant variation exists for reported values of electrical properties of biologic tissues due to the inherent inhomogeneity and anisotropy of cellular structures and extracellular matrices (112). The dielectric properties of hard tissues are generally correlated with their density and mineral content (113). Generally, heavily mineralized tissues such as enamel, dentin and cortical bone can be classified as insulators (114,115) in comparison, cancellous bone, connective tissues, blood, muscle, and organ parenchyma are superior conductors (116).

The PDMS model fabricated in this study simulates the anatomical relationship of insulating and conductive structures, as they are situated in the periodontium. The model generates an array of insulating conduits that direct and focus the electric field. The surface charge and insulating properties of PDMS makes it suitable for electrokinetic experiments (117), while its optical transparency is ideal for direct visualization fluorescently-labeled particle trajectories and deposition kinetics (109).

The rationale for selecting CSnp for these experiments is that this natural bioactive biopolymer has been well characterized and exhibited excellent thin film-forming properties. Its intrinsic positive charge makes it attractive for electrophoretic transport. Previous studies have successfully demonstrated EPD of chitosan nanoparticles to form insoluble chitosan films of uniform thickness on substrates of complex shapes (56). CSnp are also easily modifiable to achieve desired electrochemical and biological properties.

The computational model illustrates that upon application of electric potential a non-uniform electric field distribution is generated. Simulated electric field lines pass through the apical constriction and the lateral canal running perpendicular to the simulated canal walls, and thus possibly perpendicular to most biofilm surfaces (Fig. 2.1b). Areas of higher magnitude or “focused” electric fields develop in regions with narrow cross-sections such as the apical
foramen, the lateral canal and the periodontal ligament space, while other “unfocused” areas
develop where the insulating surfaces are further apart such as the large area representing apical
bone loss (Fig. 2.1a). As described by the Hückel approximation, magnitude of the electric field
is proportional to the velocity of particle transport. The electrophoretic velocity \( v_{ep} \) is
proportional to the magnitude of the electric field \( E \) and the particle’s electrophoretic mobility
\( \mu_{ep} \). This relationship can be explained as

\[
v_{ep} = \mu_{ep}E = \frac{\varepsilon\varepsilon_0\zeta_{np}}{1.5\eta}E
\]  

(2.1)

in an environment where \( \varepsilon \) is the dielectric constant of the solution, \( \varepsilon_0 \) is the permittivity of free
space, \( \zeta_{np} \) is the zeta potential of the particle and \( \eta \) represents viscosity of the solvent (118). A
further effect of applying voltage potential across a conductive substrate causes positively
charged ions to move in the direction of the negative electrode. As the ions move they draw
water molecules along with them. This phenomenon is termed electroosmotic flow and is
particularly notable in tubes of narrow diameter. The velocity due to electroosmosis \( v_{eo} \) can be
explained by the Helmholtz-Smoluchowski equation as

\[
v_{eo} = \mu_{eo}E = \frac{\varepsilon\varepsilon_0\zeta_c}{\eta}E
\]  

(2.2)

where \( \mu_{eo} \) is the electroosmotic mobility and \( \zeta_c \) is the zeta potential of the microchannel.
Combined, the resultant effects of electrophoresis and electro-osmosis are responsible for
particle transport and deposition.

When applying a 50V potential difference across the microfluidic device, approximately 1-2 mA
of current was generated. In endodontics, electronic apex locators and electric pulp testers safely
utilize potential differences from 0-500V generating electrical currents from 0 to 3 mA (119–
121). The computational model in this experiment predicts an \( E \) of 5 to 50 V/cm suggesting a 10-
fold increase in \( E \) through the narrowest portions of the system. A drastic drop in \( E \) is expected
as the cross-sectional area increases in the region of the apical lesion (Fig. 2.1a).

In the literature, sensory perception thresholds are usually given in units of current, or charge
(122). In contrast, transdermal iontophoresis literature often refers to current density when
evaluating effects on drug permeation and tissue changes (123,124). To allow comparison to iontophoresis literature, in this study the current per unit area or current density (J) in Am$^2$ was modeled as

\[ J = \frac{E}{\rho} \]  \hspace{1cm} (2.3)

Assuming a homogenous resistivity ($\rho$) of $1.82 \times 10^5$ $\Omega \cdot m$ for water the electric field utilized in this computational analysis generates current densities between 0.05-0.22 mA/cm$^2$ (Fig. 2.1c). These values can be considered low when compared those established to be safe and clinically effective for transdermal iontophoresis of 0.1-1 mA/cm$^2$ (125).

Generally it is thought that the sensation caused by current application to the skin is caused by the direct electrical excitation of nerves (126). However the application of DC also causes net ion migration, with depletion or accumulation of ions near respective electrodes. This altered chemical composition will generate a sensation that will start under one of the electrodes (anode or cathode), where the chemical composition will slowly change according to polarity (126). Reported values for sensory threshold for DC applied on the hand was 5 mA and 45 $\mu$A if applied on the tongue (122). Pain thresholds are about 10-20 times higher (119).

A limitation of this computational model is that the inhomogeneity of dentin and bone cannot be taken into account when modeling electric fields and particle trajectories. As a non-conductive, porous, system filled with a conductive fluid, the dentinal tubule network also has the capacity to transport charges and sustain electrokinetic flow. In reality, a portion of the electric field can be expected to pass through the dentinal tubules, drawing particles laterally causing dentinal tubule penetration and occlusion, and similarly through bone. Charge flow through a porous substrate and electrophoretic infiltration of porous materials has been previously demonstrated in attempts to enhance material characteristics (127,128). Furthermore, this computational model does not take into account any potential influences of dentinal and pulpal tissue remnants and inflammatory exudate. These factors could potentially impact transport and electrophoretic deposition by increasing the conductivity and the viscosity of the suspension.
In brief, the proposed electrokinetic-based treatment modality provides the capability of directing antibacterial polycationic nanoparticles into periapical regions through any patent portals of exit targeting biofilms that are located extraradicularly and in other inaccessible areas. Subsequently, when encountering any resistance to flow, electrokinetically driven polycationic nanoparticles may infiltrate and coat these areas through electrophoretic deposition. Further investigations will be required to assess the effectiveness of the proposed therapy on bacterial biofilms pertinent to endodontics.

2.6 Conclusion

The series of experiments presented in this study demonstrated rapid and reproducible transport of CSnp through simulated root canal geometry with subsequent distribution and deposition in the periapical regions. The electrical parameters required for achieving the desired particle transport in the simulated model are comparable to those used for other bioelectric applications. If translated clinically, this treatment modality may provide the possibility to target intra- and extraradicular biofilms non-surgically.

2.7 Acknowledgments

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Chapter 3 Electrokinetic Delivery and Distribution of Antimicrobial Nanoparticles for Endodontic Disinfection: Antibacterial Effects
3.1 Abstract

**Introduction:** The concept of utilizing antibacterial nanoparticles to improve root canal disinfection has gained increasing attention. A recently described electrokinetic delivery method (Chapter 1) has the ability to target intracanal and extraradicular biofilms utilizing polycationic nanoparticulates. Previous *in vitro* experiments have shown that electric currents possess antibacterial properties (electricidal effect) and can enhance the activity of some antimicrobial agents against bacteria in biofilm (bioelectric effect).

**Aim:** The aim of this research is to evaluate the effects of the proposed electrokinetic delivery system against *Enterococcus faecalis* when utilizing cationic chitosan nanoparticles (CSnp).

**Methods:** The combined antibacterial properties of electric current and CSnp were evaluated on planktonic *E. faecalis* cells. Bacterial suspensions were treated with electric current, CSnp or a combination of the two modalities. At various time intervals, bacterial inoculum was withdrawn and plated onto BHI-agar plates after serial dilutions. Colonies were counted after 24 h of incubation at 37°C and expressed as log CFU per ml. To evaluate antibiofilm properties of the electric field, seven-week old *E. faecalis* biofilms grown on dentin slices were treated with electric current in a customized setup and compared to untreated controls. Samples were stained with a commercially available live/dead stain and examined under laser scanning confocal microscopy to assess the thickness of the biofilm structure.

**Results:** Electric current application for 10 minutes achieved approximately 1 log of bacterial reduction. The greatest reduction in bacteria was noted in the combined treatment group (50V+CSnp). Complete reduction of bacteria was observed after 24 hours in all treatment groups. Biofilms treated with 50V for 10 minutes demonstrated a statistically significant reduction (70%, P<0.001) in thickness when compared to untreated samples.

**Conclusion:** The current study highlighted that the proposed electrical parameters alone, exhibit significant antibacterial and antibiofilm effects. The application of electrical parameters in conjunction with CSnp, demonstrated a significant increase (p<0.01) in the antibacterial capacity of the polycationic antibacterial nanoparticles against planktonic *E. faecalis*. 
3.2 Introduction

Studies of microbiota in primary and persistent endodontic infections have demonstrated that bacterial colonization occurs mostly as polymicrobial aggregations, adherent to dentinal surfaces, and embedded in an extracellular polymeric substance (EPS), or biofilms (1–3). The EPS, with its highly charged and interwoven structure, deters penetration of antimicrobials by acting as a physical barrier and a chemical neutralizer (4). Such organization of microorganisms in disease environment provides biofilm bacteria ecological advantages and protection from host responses as well as antimicrobials (5,6).

The electrokinetic delivery system for nanoparticles described in the previous chapter, aims to overcome the challenges posed by bacteria in a biofilm. This treatment modality has the ability to transport and deposit charged antimicrobial nanoparticles into areas unreachable by conventional non-surgical disinfection methods. The protocol utilizes low intensity, direct current to drive and distribute nanoparticles through root canal complexities, targeting both intra- and extraradicular biofilm. An additional advantage of combining electrokinetics with antibacterial nanoparticles is the ability to disrupt biofilms through electrically induced bacterial detachment and killing, as well as through the electrical enhancement of antimicrobial efficacy of nanoparticulates.

Previous studies have demonstrated that electric fields can disrupt bacterial integrity and possess quantifiable antibacterial properties (50,83). The direct killing of bacteria by electric current has been referred to as the “electricidal effect”. Antibacterial activity of electric current has been previously demonstrated against *Escherichia coli* in salt solutions (84) and water (85), *Candida albicans* in simulated urine (86), *Staphylococcus aureus* in agar (87), *Bacillus subtilis* in water (88) and *S. aureus* and *Pseudomonas aeruginosa* in culture medium (50). Literature on this topic also extends the reduction and eradication of biofilm in situations where it has already been established. A series of experiments have demonstrated that electric fields can “detach” biofilms (98–100) and augment the activity of some antimicrobial agents against certain bacteria in biofilms, the “bioelectric effect” (93–95). It has been shown that electric current can reduce the very high concentrations of antimicrobials needed to kill biofilm bacteria to levels close to those needed to kill planktonic bacteria of the same species (93,96,97). These concepts have garnered interest for controlling bacterial adhesion and biofilm formation on implanted device surfaces (80,83).

Cationic chitosan nanoparticles have been recently investigated as an alternative to conventional endodontic disinfectants and have been shown to be biocompatible and offer broad-spectrum
antibacterial properties (27,33). Their deposition on dentin surfaces has shown to impede bacterial re-colonization (27). CSnp are attractive candidates for electrophoretic transport and deposition due to their small size, positive charge, and excellent film forming properties. Previous studies have demonstrated successful EPD of chitosan nanoparticles to form insoluble chitosan films of uniform thickness on substrates of complex shapes (56). The aim of this investigation is to evaluate the effects of electrokinetically driven chitosan nanoparticles (CSnp) against *Enterococcus faecalis*.

### 3.3 Methods:

#### 3.3.1 CSnp synthesis

CSnp were synthesized in a similar fashion to previously published technique (35). In brief, chitosan commercially available chitosan (Sigma Aldrich, USA) was dissolved in an acetic acid aqueous solution (0.1%) at the concentration of 1.2 mg/mL and TPP (1%) solution was prepared in distilled water. Chitosan and TPP were mixed (3:1 v/v) under magnetic stirring at room temperature. The solutions were centrifuged (15,000 rpm/20 minutes/18 C) and washed twice using distilled water and freeze dried for 24 hours.

#### 3.3.2 Effects of electric current on planktonic *E. faecalis*

Overnight cultures of *Enterococcus faecalis* ATCC 29212 in brain heart infusion (BHI) broth were centrifuged (3,000 rpm, 10 min) and washed with deionized water, and the optical density was adjusted to 0.3 at 600 nm. To establish a suitable duration for the application of electric current, two 20 gauge needles serving as electrodes were fixed approximately 10 mm apart and submerged into 2 ml of bacterial suspension. After 2, 5 and 10 minute treatments with 50V (generating approximately 0.5 mA), 100 ml of the bacterial inoculum was withdrawn and plated onto freshly poured BHI-agar plates after serial dilutions. Colonies were counted after 24 h of incubation at 37°C and expressed as log CFU per ml. Experiments were repeated three times in triplicate.

#### 3.3.3 Effects electric current + CSnp on planktonic *E. faecalis*

To determine the influence of electric current on the antimicrobial activity of CSnp bacterial cells were divided into three treatment groups and a control group. Group-1 was treated with 50V (0.5 mA) for 10 minutes, group-2 combined 50V (0.5 mA) for 10 minutes with concurrent
application of CSnp to a final concentration of 3 mg/ml, and group-3 tested CSnp (3 mg/ml) alone. Group-4 served as the control and consisted of bacterial cells without any treatment. After 10 min, 4 h and 24 h, 100 ml of the bacterial inoculum was withdrawn and plated onto freshly poured BHI-agar plates after serial dilutions and cultured overnight. Colonies were counted after 24 h of incubation at 37°C and expressed as log CFU per ml. All experiments were carried out in triplicate and repeated three times. The results were subjected to one-way analysis of variance with a post hoc Tukey test. The difference between two groups was considered statistically significant if p<0.05.

3.3.4 Effects of electric current on E. faecalis biofilm structure

In order to evaluate the direct effect of current on bacterial biofilms, the proposed electrical parameters (50V, 10 minutes) were used to treat 7-week old E. faecalis biofilms grown on dentin slices. Serial sectioning through the cervical area of three human maxillary molars was carried out to generate 8 dentin/enamel slices of 0.5 mm thickness. One milliliter of overnight E. faecalis culture in BHI broth was added into each well and incubated at 37°C. Fresh medium was replenished every 48 h for 7 weeks to provide a constant supply of nutrients and to remove dead bacterial cells. Prior to any treatment, the medium was removed from the wells, and the biofilm was carefully washed to remove dead cells using sterile deionized water.

An apparatus designed to generate an electric field running parallel to the bacterial biofilm was fabricated by fixing two parallel needle electrodes approximately 10 mm apart on the bottom of a plastic dish. Dentin slices were laid horizontally with biofilm side up between the electrodes and submerged in 2 ml of sterile deionized water. Four samples were treated using 50V (generating approximately 2.0 mA) and 4 remained as untreated controls. Medium was removed and microbial biofilms were stained using 20µL of a LIVE/DEAD BacLight stain (Molecular Probes, Eugene OR) in the dark for 15 minutes. Dentin slices were inverted and placed on glass bottom petri dish for laser scanning confocal microscopy. Biofilm structures were imaged in four separate areas of each sample using a laser scanning confocal microscope (LSCM; Leica DMIRE2, Germany). Images were acquired using the 60x objective with oil as the immersion media. The student t-test was used to compare the thickness of the biofilm before and after treatment with electric current.
3.4 Results

3.4.1 Effects of electric current on planktonic *E. faecalis*

When planktonic *E. faecalis* were treated with 50V (0.5 mA) in the absence of CSnp, the number of viable bacteria decreased as the duration of current application was increased. A one-log reduction in viable planktonic bacteria was observed after 10-minute application (Fig. 3.1).

![Log CFU/ml vs Time](image)

**Figure 3.1.** Viable planktonic *E. faecalis* bacteria after application of 50V (0.5 mA) for various lengths of time.

3.4.2 Effects electric current + CSnp on planktonic *E. faecalis*

Figure 3.2 illustrates bacterial reduction achieved by the various treatment modalities. The 50V group demonstrated a significant immediate reduction of bacteria after the intervention (p<0.01), however the effect decreased with bacterial numbers reaching those of the control group at 24 hours. The CSnp group achieved approximately a 4-log reduction at 24 hours. The greatest reduction in bacteria was seen in the combined 50V+CSnp group with complete elimination of bacteria after 24 hours. The combined group demonstrated significantly more bacterial reduction than the CSnp, 50V or control groups at all time points (p<0.01).
3.4.3 Effects of electric current on *E. faecalis* biofilm structure

Figure 3.3, shows LSCM images of the bacterial biofilms before and after treatment with 50V (2.0 mA) for 10 minutes. Figure 3.3a, 3.3b show representative treated and untreated 3D biofilm structures while 3.3c shows all 32 imaged areas rotated to allow estimation of biofilm thickness (Z-axis). Both the treated and untreated samples exhibited biofilm structures consisting of both live (green) and dead (red) bacterial cells in a multilayered architecture. A 75% reduction in biofilm thickness was calculated after treatment with 50V (2.0 mA) for 10 minutes (P <0.001).
Figure 3.3. LSCM images of 8-week old *E. faecalis* biofilms containing both live (green) and dead (red) cells. Selected images show 3D biofilm structure of an untreated area (a) and one having received treatment with 50V (2.0 mA) for 10 minutes (b). (c) shows a streaking artefact caused by CSnp deposition on biofilm.
Table 3.1. Thickness of 8-week old *E. faecalis* biofilms grown on dentin slices after treatment with 50V (2.0 mA) for 10 minutes as measured by LSCM.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Area</th>
<th>Biofilm Thickness (µm)</th>
<th>Treatment Thickness (µm)</th>
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<tr>
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<td>120</td>
<td>30</td>
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<td>135</td>
<td>15</td>
</tr>
</tbody>
</table>

Average thickness 93.13 22.50

*a* untreated samples

*b* samples received 50V (2.0 mA) for 10 minutes

* treated samples demonstrated a statistically significant reduction (70%, P<0.001) in thickness when compared to untreated samples

3.5 Discussion

This study focused on the effects of the process and suspension parameters proposed in Chapter 2 against *E. faecalis* bacteria alone and in conjunction with bioactive polycationic CSnp. As apical periodontitis is a biofilm-mediated disease, it is important to not only evaluate the effects of the proposed treatment modality against planktonic bacteria but also bacteria adherent to dentin. Previous literature on this topic predominantly focuses on electrical control of bacteria in suspension or biofilm on implanted devices, indwelling catheters and other artificial surfaces such as surgical steel (80), as such it was important to customize an assay for this purpose. As a starting
point the effects of 50V, 50V+CSnp and CSnp were evaluated against planktonic *E. faecalis*. When *E. faecalis* in suspension were treated with 50V (0.5 mA) in the absence of CSnp, the number of viable bacteria decreased proportional with the application time. A one-log reduction in viable planktonic bacteria was observed after 10-minute application (Fig. 1). In subsequent experiments a 10-minute application of electric current was selected. The greatest reduction in planktonic bacteria was demonstrated in the combined (50V+CSnp) group at all time points, with complete elimination of bacteria after 24 hours. In this group 50V was applied for 10 minutes prior to application of CSnp. This synergistic result may be attributed to a combination of bulk removal of bacteria from solution within the initial 10-minute period followed by a subsequently increased efficacy of CSnp towards structurally or metabolically altered bacteria. In this study both electrodes were removed from the suspension following current application. Any viable bacteria electrophoretically attracted and bound to the electrodes would have been removed from the suspension. Poortinga et al (129) demonstrated this concept of attraction and electrophoretic deposition of planktonic bacteria onto electrodes forming a sessile film of microorganisms. The subsequent enhanced killing of bacteria by CSnp can be attributed to altered bacterial homeostasis. It has been shown that external electric fields can disrupt the delicate electrical equilibria of bacteria altering important membrane components (130) and influencing permeability (131), leading to leakage of cellular contents, causing morphological changes (132) and possibly increasing penetration of antibacterials (133).

Interestingly, when the sequence of treatment was reversed, i.e. CSnp were applied to the solution prior to the application of electric potential, no synergistic effect was observed (results not shown). This caused an instant unfavourable increase in conductivity of the suspension followed by electrolytic bubble formation and electrode fouling.

Based on our previous computational analysis described in Chapter 2 we would expect electric field lines running parallel to the plane of the dentinal biofilm. In order to achieve this, a customized apparatus was created by fixing two parallel needle electrodes approximately 10 mm apart on the bottom of a plastic dish. Dentin slices were laid horizontally with biofilm side up between the electrodes and submerged in 2 ml of sterile deionized water. LSCM analysis was utilized to evaluate changes in biofilm structure as this procedure can provide qualitative and quantitative information about biofilm when subjected to a specific treatment. It was the initial intent of this study to utilize LSCM to evaluate the effects of 50V and CSnp individually as well
as in combination against *E. faecalis* biofilm. This goal, however, could not be achieved using this methodology as the deposition of CSnp on top of biofilm samples created a disruptive streaking artefact preventing quantification of biofilm thickness (Fig. 3c). The effects of 50V alone, nevertheless, could be evaluated. Untreated dentin samples harbouring 8-week *E. faecalis* biofilm sowed 93 ± 27.50 µm thick biofilm with few dead (red) cells scattered in between the live (green) cells (Fig. 3a). Treatment with electric current resulted in a significant decrease in film thickness to 22 ± 6.58 µm (75%, P < 0.001). However no obvious difference in the live:dead ratio was observed. These trends were consistent with the findings of Zhang et al., who recently demonstrated that a repeated application of DC decreased the number of viable bacterial cells without significantly increasing the proportion of dead or membrane-compromised cells, thus indicating a possible anti-adhesive rather than bactericidal effect (94). The reduction in biofilm thickness observed can be attributed to electric current induced bacterial detachment. This phenomenon was previously demonstrated by van der Broden et al (100) who observed electric-current-induced detachment of *S. epidermidis* from surgical stainless steel and decreased viability of the remaining bacteria. Therefore it is speculated that the results in the current study may in part be attributed to the detachment of bacteria from biofilm and removal of planktonic bacteria from solution by rendering cells immobilized on the intracanal electrode surface through electrophoretic deposition.

Biofilm development begins with the physicochemical interaction between microorganisms and the substrate surface. This initial interaction lies in complex balance between attractive and repulsive forces including Van der Waals forces, acid-base interactions, and electrostatic forces (134), while the integrity of a more mature biofilm is maintained by the EPS charges. It has been proposed that repulsive forces between bacteria and substrate can be enhanced by modification of the substrate surface charge thus provocing surface detachment of bacterial biofilms (135). To date, the precise mechanism behind the effect of electric current on bacteria and the electrical enhancement of antibacterial agents has not been fully established. The suggested theories include, disruption of charged bacterial membranes, stimulating autolysis, electrolytic generation of oxygen, electrochemical generation of potential oxidants and the electrophoretic transport of antibacterial particles into and disruption of the charged EPS matrix by electric current (80,89,94).

The proposed therapeutic intervention in this study utilizes the non-uniform electric fields to generate electrokinetic transport of polycationic antibacterial nanoparticles for the purpose of
endodontic disinfection. The suggested electrical parameters have been shown to be capable of achieving predictable particle transport in a simulated root canal system with subsequent deposition at the periapex (Chapter 1). If translated into a clinical procedure this intervention will bring antibacterial particles into close proximity to inaccessible endodontic biofilms, including those located in dentinal tubules, apical ramifications, and on extraradicular surfaces. Subsequent deposition of antibacterial particles in these areas may disrupt existing biofilms, impede bacterial re-colonization or influence healing of periapical tissues.

In brief, this study demonstrates the ability of this intervention to capitalize on the antibacterial effects of electric currents including the electric-current-induced bacterial detachment and removal. One limitation of the current approach is the inability to evaluate the combined effects of 50V+CSnp on biofilm bacteria using. Further research is required to study the deposition kinetics and antibiofilm effects of the proposed modality in suitable endodontic models.

3.6 Conclusion
The proposed therapeutic intervention for endodontic disinfection utilizes electrokinetic principles to drive and deposit bioactive polycationic nanoparticles onto surfaces colonized by bacterial biofilms. This study demonstrates, that when the suggested electrical parameters were utilized in conjunction with CSnp, the antibacterial capacity of the nanoparticles was dramatically increased. Furthermore, this study elucidates that the proposed electrical parameters alone, exhibit both antibacterial and antibiofilm effects.

3.7 Acknowledgments
This study was supported in part by grants from the American Association of Endodontists Foundation, the Canadian Academy of Endodontics Endowment Fund, and the Alpha Omega Foundation of Canada.
Chapter 4
Conclusion
The proposed therapeutic intervention for endodontic disinfection described in this work utilizes electrokinetic principles to drive and deposit bioactive polycationic nanoparticles onto surfaces colonized by bacterial biofilms. The series of *in vitro* experiments presented here demonstrate that this methodology can successfully influence particle trajectories and deposition in a simulated root canal anatomy while simultaneously reducing planktonic bacteria and disrupting biofilm.

The experiments presented in Chapter 2 demonstrated rapid and reproducible transport of CSnp through a simulated root canal anatomy with subsequent distribution and deposition in the extraradicular regions. The computational analysis suggests that the electrical parameters required to achieve the desired particle transport in this simulated model are comparable to those used for other bioelectric applications.

The bactericidal effects of the proposed method for endodontic disinfection were further evaluated in Chapter 3. The parameters required for electrophoretic transport demonstrated an electricidal effect against planktonic *E. faecalis* in an *in vitro* model. When the electric fields were applied in conjunction with CSnp, a synergistic antibacterial effect was demonstrated. The proposed electrical parameters also exhibited antibiofilm effects causing a reduction in biofilm thickness, indicating a possible bacterial detachment effect.

If translated clinically, this treatment modality may provide the capability for enhanced endodontic disinfection. This methodology may also offer the ability to treat intra- and extraradicular biofilms through a non-surgical approach.
References


Appendix I: Research Ethics Board Approval
PROTOCOL REFERENCE # 29738

January 16, 2014

Dr. Anil Kishen
FACULTY OF DENTISTRY

Dr. Andrei Ionescu
FACULTY OF DENTISTRY

Dear Dr. Kishen, Dr. Andrei Ionescu,

Re: Your research protocol entitled, "Targeting extraradicular biofilms by delivering antibacterial nanoparticles through root canal portals of exit using customized electric field"

ETHICS APPROVAL

Original Approval Date: January 16, 2014
Expiry Date: January 15, 2015
Continuing Review Level: 1

We are writing to advise you that the Health Sciences Research Ethics Board (REB) has granted approval to the above-named research protocol under the REB's delegated review process. Your protocol has been approved for a period of one year and ongoing research under this protocol must be renewed prior to the expiry date.

Any changes to the approved protocol or consent materials must be reviewed and approved through the amendment process prior to its implementation. Any adverse or unanticipated events in the research should be reported to the Office of Research Ethics as soon as possible.

Please ensure that you submit an Annual Renewal Form or a Study Completion Report 15 to 30 days prior to the expiry date of your current ethics approval. Note that annual renewals for studies cannot be accepted more than 30 days prior to the date of expiry.

If your research is funded by a third party, please contact the assigned Research Funding Officer in Research Services to ensure that your funds are released.

Best wishes for the successful completion of your research.

Yours sincerely,

Elizabeth Peter, Ph.D.
REB Chair

Daniel Gyewu
REB Manager