Novel Endodontic Disinfection Approach Using Nanotechnology

Sarah Bukhari  
*University of Pennsylvania*, sara_m_bukhari@hotmail.com

Bekir Karabucak  
*University of Pennsylvania*

Hyun Koo  
*University of Pennsylvania*, koohy@upenn.edu

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Abstract
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Because iron oxides can be used as food additives, and iron oxide NP formulations are low-cost and FDA-approved for human use, it could be a safe and feasible approach to potentiate the effects of a commonly used antiseptic.

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Novel Endodontic Disinfection Approach Using Nanotechnology

Sarah Bukhari, BDS

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Thesis Supervisor

Hyun Koo, DDS, MS, PhD
Professor
Department of Orthodontics
Divisions of Pediatric Dentistry & Community Oral Health

Denis F. Kinane, BDS, PhD
Morton Amsterdam Dean
Professor
Departments of Pathology and Periodontics

Jonathan Korostoff, DMD, PhD
Professor
Department of Periodontics

Bekir Karabucak, DMD, MS
Interim Chair and Associate Professor
Department of Endodontics
Purpose

The aim of this *in vitro* investigation was to use a recently developed *Enterococcus faecalis* infection model using root canal for evaluating iron oxide (Fe$_3$O$_4$) nanoparticles (NP) with biomimetic (catalytic) properties as a new antimicrobial endodontic treatment. We compared iron oxide NP bioactivity with currently used chemical modalities using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) as analytical tools.

We hypothesized that iron oxide NP with enzyme-like (peroxidase) activity catalyzes H$_2$O$_2$ to promote bacterial killing within dentinal tubules (DT) via *in situ* production of free radicals. We further hypothesized that the NP is more effective than the conventional treatments (irrigants) used in the clinical endodontic practice.

Because iron oxides can be used as food additives, and iron oxide NP formulations are low-cost and FDA-approved for human use, it could be a safe and feasible approach to potentiate the effects of a commonly used antiseptic.
Introduction/ Literature review:

The fundamental role of microorganisms as the primary causative agents of endodontic infections and apical periodontitis have been well established (Kakehashi et al. 1965). Therefore, the effort has been directed towards eliminating those microorganisms for higher chances of successful root canal treatment (Bender et al. 1964; Bystrom et al. 1987; Sjogren et al. 1997). The root canal is a very complex system (Figure 1), with isthmuses, ramifications, deltas, and accessory canals and dentinal tubules (DT), all of which can harbor bacteria and biofilms (Trope and Bergenholtz 2002). Indeed, several studies have demonstrated the presence of biofilm inside the root canal (Ramachandran Nair 1987; Tronstad et al. 1990; Molven et al. 1991) with bacteria penetration of dentinal tubules (DT) at varying depth toward the cementum (Andreasen and Rud 1972; Ando and Hoshino 1990; Sen et al. 1995; Peters et al. 2001a). Among various microorganisms, Enterococcus faecalis has been frequently detected in persistent apical periodontitis infecting both the root canal and DT (Molander et al. 1998; Sundqvist et al. 1998; Gomes et al. 2008). Here, we present the available evidence in the literature of the many biological factors that make endodontic treatment and development of new therapies so challenging in the clinical setting.

Figure 1. Complexity of the root canal system: (A) A micro CT of an extracted tooth showing complex canal anatomy adapted from [http://rootcanalanatomy.blogspot.com](http://rootcanalanatomy.blogspot.com)
(B) Accessory canals: furcal canal indicated by red arrow and lateral canal indicated by blue arrow. (C) Isthmus, which is the channel connecting between two main canals (indicated by arrow). The image adapted from “Color Atlas of Microsurgery in Endodontology” (Kim et al 2001). (D) Ground histological section showing the DT following a S course shape (Chowdhary and Subba Reddy 2010).

Endodontic Infection and Apical Periodontitis as Biofilm-induced Conditions

Nair (1987) made the first observation of a biofilm structure attached to the root canal surface using light and electron microscope (Ramachandran Nair 1987). Several others who made the same observation followed this study (Tronstad et al. 1990; Molven et al. 1991), providing compelling evidence that endodontic infections and consequently apical periodontitis is a biofilm-induced disease (Figure 2. A and B). This might be one of the reasons that potentially explain the emergences of persistent apical periodontitis, which respond poorly to conventional root canal treatment. The mere existence of biofilm inside necrotic canal might not be the issue itself, rather the complexity of the root canal system (Figure 1), which make biofilm elimination challenging. Furthermore, the bacteria can also infect the DT (Figure 2. C), forming biofilms that are sheltered from the chemical action of irrigants and the mechanical removal by instruments. Because of the challenges associated with disinfection of DT, we have focused on this topic in our literature review.

![Figure 2. Microbiological challenges of root canal infections. (A) Histological](image-url)
section showing the main canal with biofilm attached to the dentinal wall (Siqueira et al. 2012). (B) Close-up view of the area indicated by arrow in (A), showing bacterial cluster typically found in biofilm structure attached to the canal wall. (C) Histological section showing DT infection with bacterial colonization deep at the DT (Ricucci et al. 2009)

**Dentinal tubules (DT) infection: general concepts.** The concept of dentin infection and bacterial invasion of DT has been controversial. It wasn’t until Chirnside work in 1958, that dentin infection concept was acknowledged. The author examined the radicular dentin of 50 extracted teeth with infected root canals after longitudinal sectioning and gram staining, and found that 62% of the teeth had bacterial penetration of what the author described as “frequently at considerable depth” (Chirnside 1958). Further clinical studies have clearly demonstrated that DT of infected root canals, with apical infection or heavily decayed crowns are loaded with microorganisms with varying depth of penetration (Andreasen and Rud 1972; Ando and Hoshino, 1990; Sen et al., 1995). Even though the clinical invasion of radicular dentin was disclosed, the clinical consequences of this bacterial penetration into DT remain unclear. However, if the root canal was inadequately filled, these microorganisms might creep back into the empty spaces, recolonize and become a focal source of persistent infection (Vieira et al. 2012)

**Characterization of DT infection.** Since the concept of dentin infection has been well-established in *in vivo* and *ex-vivo* settings, a great interest has been sparked to extensively study and characterize this phenomenon. While some have directed their effort towards identifying the nature of this infection in an *in vivo* setting, others have dedicated their work towards creating an *in vitro* model, to closely study the characteristics of dentin infection.

Several *in vitro* models to replicate dentin infection have been established. In attempt to shed the light on different aspects of the DT microbial invasion, several experiments were designed to assess: (i) the different pattern of invasion by different bacterial species (Akpata and Blechman 1982; Orstavik and Haapasalo 1990; Siqueira et al. 1996), (ii) the regional difference in tubular invasion (Zapata et al. 2008), (iii) the influence of the age of the biofilm and DT infection on their susceptibility to disinfecting
solutions (Wang et al. 2012), (iv) the effect of the age of the individual on the penetration capacity of bacteria (Nagayoshi et al. 2004), (v) the type of invading microorganisms (Ando and Hoshino. 1990), and (vi) susceptibility of bacteria within DT to intracanal antiseptics (Haapasalo and Orstavik 1987; Orstavik and Haapasalo 1990; Safavi et al. 1990).

In parallel, two methods for examining dentin infection and assessing the depth of penetration have been widely used in the literature: (1) histobacteriological examination by staining the specimens with Brown and Brenn or gram stain after sectioning them; (2) scanning electron microscopy (SEM) for visualization of the bacterial infection characteristics (Akpata and Blechman 1982; Haapasalo and Orstavik 1987; Orstavik and Haapasalo 1990; Safavi et al. 1990; Siqueira et al. 1996). However, these methods have limitations because of lack of information about the bacterial viability and quantitative analysis. To overcome these shortcomings, microbiological culturing methods were introduced.

Microbial culturing of dentin samples has been used to examine the viability of microorganisms as well as to evaluate disinfection efficacy of different chemical agents. For example, treated or untreated dentin samples can be removed with sterile round burs sequentially increasing in size from the center closer to canal lumen to the periphery closer to the root cementum (Figure 3).

**Figure 3. Microbiological sampling.** Adapted from (Orstavik and Haapasalo 1990), the microbiological sampling technique using sterile round bur sequentially increasing in size from 023 to 031 and thereby increasing in the depth of sampling from the center closer to the canal lumen (labeled by green) to the periphery closer to the root cementum (labeled by red) as the size of the bur increases.
The dentin powder can then be incubated and assessed for viable bacterial population by determining the colony forming units (CFU) of cultivable bacteria and hence quantitative analysis can be performed (Orstavik and Haapasalo 1990; Siqueira et al. 1996). Alternatively, incubation of the whole specimen in a medium and analyzing the increased turbidity of the medium by spectrophotometry can be done to estimate bacterial viability (Heling and Chandler 1998) although limited in scope. However, these methods (albeit quantitative) provide little information about the spatial distribution of viable bacteria within DT. In an effort to overcome the limitations of aforementioned methodologies, a relatively recent article (Zapata et al. 2008) presented for the first time a method that allows for determination of bacterial viability within DT (live and dead as well as metabolically active) using Confocal Laser Scanning Microscopy (CLSM). This technique allows simultaneous analysis of the viability of the microorganisms and their spatial distribution within the infected dentin. Using these methods, different aspects of dentinal infection have been examined.

i) Depth of Bacterial Penetration: The depth of bacterial penetration might be clinically relevant, as the deeper the extension of the bacterial invasion, the more challenging their eradication would be. In this context, Peters et al (2001) examined 21 extracted teeth with apical periodontitis by grinding and culturing of root dentin after diving it into 3 layers: pulpal, middle and cemental to quantitatively evaluate the extent of infection (Figure 4).

Figure 4. Schematic diagram showing the layers from which dentin shavings were collected (Peters et al. 2001a). Layer A (indicated by red) is the closest to canal lumen and layer C (indicated by blue) is on the cementum side. Seventeen samples (81%) had bacteria invasion at layer A and 13 (62%) had bacteria at layer C.
In more than half of the infected roots, bacteria were present in the deep dentin close to the cementum (Peters et al. 2001a). This was in contrast to another study where they observed that bacterial penetration into DT was usually close to the main root canal. In few specimens some tubules were infected up to 300 μm deep (Siqueira et al. 2002). Interestingly, both bacteria and yeasts were found in the DT of extracted teeth with an average depth of penetration of 50 μm in the middle and apical thirds of the canal and sometimes as deep as 150 μm using SEM (Sen et al. 1995). The reasons for this variability in microbial penetration and composition remain unclear.

Furthermore, in vitro studies have shown that the depth of penetration is time dependent (Akpata and Blechman 1982). Deeper penetration was achieved in the specimens that were incubated for 3 weeks versus those that were incubated for 1 or 2 weeks. Notably, two obligate anaerobic bacteria used in this study Bacteriods melaninogenicus and Peptococcus asaccharolyticus failed to demonstrate any invasion of DT when compared to the facultative anaerobes Streptococcus faecalis (currently known as Enterococcus faecalis) and Streptococcus sanguis (now S. sanguinis). The authors concluded that the invasion was not only time dependent, but also appeared to be dependent on the growth rate of the bacteria, i.e. the faster the rate more effective invasion was observed. Hence, E. faecalis has been widely used as a test organism in dentin infection models. Another study re-tested the invasion of obligate anaerobes of DT using 5 different species and compared them with E. faecalis (Siqueira et al. 1996). Two species namely Propionibacterium acnes and Actinomyces israelii were able to demonstrate comparable heavy penetration to E. faecalis. Another two species (Porphyromonas endodontalis and Porphyromonas gingivalis) showed less dramatic penetration while one failed to invade the tubules (F. nucleatum). According to the study, their further cellular migration could have been prevented because of their spindle shaped cells clustered into tubules entrance forming bundles.

Penetration pattern and depth not only differs from one species to another, but also a regional variation in the root DT was found within the same strain (Love 1996). It was found that there is a significant difference between the bacterial invasions of the coronal 2/3, which reached a maximum of 200 μm and that of the apical third with a
maximum depth of penetration of 60 µm. This phenomenon appeared to be associated with several factors, including growth rate, size and the shape of bacteria, concentration gradient and tubule blockages such as reparative dentin and odontoblastic process.

**ii) Types of Bacteria Invading DT:** Only a few *in vivo* studies have investigated the types of bacteria infecting DT. Ando and Hoshino (1990) evaluated the depth and types of bacteria invading the root canal dentin by splitting 8 freshly extracted teeth with heavily decayed crowns. Bacteria were recovered from deep layers of infected root dentin, about 0.5-2.0 mm below the root canal-dentin boundary on split surfaces. Eighty percent of bacteria found were obligate anaerobes, suggesting an anaerobic environment within DT. Among the anaerobic isolates, strains belonging to *Lactobacillus* and *Streptococcus* were predominant, followed by *Propionibacterium* (Ando and Hoshino 1990). The morphologies of these detected bacteria were between cocci and bacillus similar to Chirnside findings (Chirnside 1958). It was observed in an *in vivo* study (Siqueira et al. 1996) that shape of bacteria could be conducive to their ability to penetrate DT. Spindle shape bacteria *F. nucleatum* failed to invade the DT and accumulated at the entrance of the DT openings. Furthermore, yeast cells were also found in DT (Sen et al. 1995).

In summary, the extensive characterization of DT infection revealed variable microbial composition and depth of penetration, while the bacterial penetration was found to be time, species and area dependent. The type of organisms invading DT was found to be mostly anaerobes. *E. faecalis* has been the microorganism of choice for dentin infection models as this bacterium is commonly detected in persistent endodontic canal infection and in DT. The ability of *E. faecalis* to invade and infect DT has been well-established (Haapasalo and Orstavik 1987) which might be attributed to their rounded shape and small size (Ma et al. 2011). Furthermore, this bacterium can adapt to harsh conditions (Portenier et al. 2001) due to its capacity to tolerate high pH levels (Chavez de Paz et al. 2007) and survive within DT in absence of nutrients (Haapasalo and Orstavik 1987) even after obturation (Shin et al. 2008). Moreover, it has been frequently isolated from persistent cases of apical periodontitis (Molander et al. 1998; Sundqvist et al. 1998; Gomes et al. 2008)
Current Dentin Disinfection Modalities

The presence of biofilms and microorganisms within DT of infected root canals has been clearly demonstrated clinically and \textit{in vitro} models. Since the primary goal of root canal therapy is achieved through eliminating as much microbes as possible from the root canal system, a combination of chemical agents with mechanical removal of infected dentin have been largely used in laboratory and clinical settings (Law and Messer 2004; Sathorn et al. 2007). Therefore, the disinfection efficacy of mechanical preparation and chemical irrigation has been tested to examine the viability of microbe-invading root canal.

Mechanical Preparation of Root Canal: The mechanical cleaning and shaping of the root canal is the first step of disinfection in root canal treatment. The aim of mechanical preparation is the removal of tissue remnants, bacterial biofilms and infected dentin as well as enlarging the canal space to allow for proper irrigation and close adaptation of the root filling to the canal walls (Metzger et al. 2013). However, this aim is not always attainable. Sen and coworkers (1995) stated that since the deepest extension of microorganisms is still considered in close proximity to the root canal wall, it should be easily removed considering that the difference between the diameters of two successive endodontic files is 50 μm (Sen et al. 1995). This assumption would have been true if the current file systems used could predictably and uniformly prepare the root canals. Unfortunately, that is not the case as observed clinically. In a series of studies using the micro-computed tomography (MCT) to analyze the effect of 6 Ni-Ti file systems on root canal geometry, it was found that 40% or more of the canal wall was left untouched when the canals were instrumented to an apical sizes ranging between #20 and #45 (Peters et al. 2001b; Hubscher et al. 2003; Peters et al. 2003; Paque et al. 2009). Gutierrez et al (1990) findings supported the aforementioned study by showing that bacteria might be left up to 250 μm inside the DT after instrumentation with reamers up to size Nr.80 (Gutierrez et al. 1990). Furthermore, it was shown that routine canal preparation of an apical size #30 failed to eliminate the bacteria from the tubules and the average penetration depth was 151 μm (Harrison et al. 2010). Thus, chemical agents (e.g. irrigants) need to be used in combination with mechanical preparation to improve the disinfection process (Dalton et al. 1998; Shuping et al. 2000).
**Chemical Irrigation:** There are many different chemical irrigants used in disinfection of the root canals. Sodium hypochlorite (NaOCl) with different concentrations (0.5% - 6%) is the single most commonly used agent. It is the only irrigant capable of dissolving organic tissues (such as pulpal tissue, necrotic tissues, biofilm and organic part of smear layer); (Haapasalo et al. 2010), while exhibiting potent and rapid antibacterial effect *in vitro* (Gomes et al. 2001). However, its potency is somewhat reduced when tested in a clinical setting. This might be due to the presence of organic matter such as tissue remnants, biofilm mass and inflammatory excaudate that react with NaOCl, thereby weakening its killing effect (Haapasalo et al. 2010). Furthermore, dentin appear to have an inhibitory effect on NaOCl (Haapasalo et al. 2000). However, these factors can be addressed to some extent by continuous replenishing of the irrigant.

Another commonly used chemical agent is chlorhexidine (CHX), a potent broad-spectrum antimicrobial. However, it lacks tissue dissolving properties of NaOCl, hence it is capable of killing bacteria, but unlike NaOCl it can't remove the biofilm (Chavez de Paz et al. 2010). CHX has gained popularity as an additional endodontic irrigant due to its substantivity as it binds to the dental hard tissues and continue to exert antimicrobial effect long after application (Mohammadi 2008). However, similar to NaOCl, its antimicrobial activity is negatively affected by the presence of organic matter (Russell and Day 1993). Other endodontic irrigants includes, hydrogen peroxide ($H_2O_2$) which has weak antimicrobial activity when used alone (Zehnder 2006), iodide based compounds, which imposes a risk of allergic reactions (Popescu et al. 1984) and other combination products such as MTAD (mixture of doxycycline, citric acid, and a detergent) and Qmix (mixture of bisbiguanide antimicrobial agent, a ployaminocarboxylic acid calcium-chelating agent and a surfactant). MTAD and Qmix have been promoted as the final irrigant and they were equally effective or less effective than conventional NaOCl and CHX (Kho and Baumgartner 2006; Liu et al. 2015). In summary, NaOCl remains the ‘gold standard’ in endodontic irrigation, while others have been used as an adjunct for enhanced disinfection.

Disinfection of DT depends not only on the antimicrobial activity but also the ability to penetrate the microscopic tubules to effectively kill the invading bacteria.
Several irrigants and intracanal medicaments have been evaluated for their antibacterial activity against DT invading bacteria. Camphorated paramonochlorophenol (CMCP) has shown to rapidly and completely disinfected DT in comparison to calcium hydroxide Ca(OH)$_2$, which failed to eradicate even superficially located *E. faecalis* within the tubules (Orstavik and Haapasalo 1990). Moreover, iodine potassium iodide (IKI) appeared to be more potent than 5.25% NaOCl and 2% CHX. However, CMCP is highly toxic and rarely used in the clinical setting; similarly, IKI has become less popular due to high risk of allergic reactions in patients (Popescu et al. 1984; Messer and Feigal 1985). Thus, most of the studies focused on optimizing the use of NaOCl and CHX.

Vahdaty and colleagues (1993) examined the antimicrobial effect of high (2%) and low (0.2%) concentrations of NaOCl and CHX on dentin blocks infected with *E. faecalis* by microbiological sampling using 3 round sterile burs sequentially (increasing in diameter to collect dentinal powder; see Figure 3) and then determining the number of viable cells. The authors found that both irrigants at both concentrations had a significant reduction in bacterial count at the superficial layers of dentin (100 μm). Nevertheless, both were less effective in disinfecting deeper layers (300-500 μm), while 50% of the samples remained infected (Vahdaty et al. 1993). Using a similar methodology, Heling and Chandler (1998) investigated the dentin disinfection potential of NaOCl (with/without EDTA), CHX and H$_2$O$_2$, either in sequence or using different combinations by incubating the dentinal powder sampled from different layers and measuring increase in turbidity. NaOCl and CHX were equally effective and significantly superior in killing *E. faecalis* at the layers closer to the canal lumen. NaOCl killed significantly more *E. faecalis* than H$_2$O$_2$ at superficial layers (equivalent to 100 μm depth). However, the differences in bacterial killing between the irrigants were not significant at the deeper layers. Notably, H$_2$O$_2$ in combination with CHX was more effective than CHX alone (Heling and Chandler 1998). Buck et al (2001) compared the efficacy of 0.5% NaOCl to 0.2% EDTA and 0.12% CHX by incremental sampling corresponding to three different dentinal depths and incubating the dentinal shavings for viable cell counting (Buck et al. 2001). In contrast to both previous studies (Vahdaty et al. 1993; Heling and Chandler 1998), NaOCl was found to be superior to CHX. Recently, it was demonstrated that a higher concentration of NaOCl (6%) and Qmix
(17% EDTA + 2% CHX) performed significantly better bacterial killing against *E. faecalis* within DT than lower concentration of NaOCl (2%) and 2% CHX, as determined by CLSM (Wang et al. 2012). However, the percentage of viable bacteria remained intact after treatment with 6% NaOCl and Qmix was between (46%-65%) and (60%-74%) respectively. Thus, new methodologies are needed to either enhance current chemical modalities or develop an alternative disinfection approach.

**New Technologies for Enhanced Disinfection Efficacy**

Recent advances in nanotechnology provide new and promising opportunities to kill bacteria, disrupt biofilms and control DT infection. A wide range of nanoparticles have been developed that display antimicrobial activity including- but not limited to- Silver Nanoparticles (AgNPs), Zinc Oxide-Based Nanoparticles (ZnONPs), Titanium Dioxide-Based Nanoparticles (TiO$_2$NPs) and Chitosan Nanoparticles. Due to their promising antibacterial potential, these nanoparticles have been incorporated in the oral health care as an innovative ingredient in mouthwash, toothpaste, composite fillings, implants and other dental materials, and even used as endodontic irrigants (Padovani et al. 2015). The bioactivity is governed by a variety of mechanisms. AgNPs bind to the cell membrane proteoglycans affecting bacterial viability (Chaloupka et al. 2010), while also hindering DNA replication process (Radzig et al. 2013). ZnONPs also exert antimicrobial effect through interaction with the bacterial membrane via lipid and protein binding leading to osmotic imbalance (Hajipour et al. 2012). TiO$_2$NPs, on the other hand, need to be photocatalyzed by UVA radiation, which enable them to produce reactive oxygen species that alter the bacterial membrane osmolality, causing bacterial death (Blecher et al. 2011). Chitosan nanoparticles act in similar fashion to silver and zinc oxide nanoparticles, by binding to the membrane of bacterial cells and increasing its permeability (Blecher et al. 2011).

Although these nanoparticles are potentially effective and promising technology for endodontic disinfection, there are some limitations. The synthesis of these nanostructures can be complex and costly (Besinis et al. 2015). Furthermore, toxicology and environmental studies indicate the potential of AgNPs to cross the blood-brain barrier with subsequent accumulation in the brain (Yang et al. 2010), while
accumulating in the environment (Walser et al. 2012). ZnONPs have also shown significant cytotoxicity on human fibroblast and further chemical treatment is required to reduce its toxicity, which means extra steps on synthesis and added cost (Srakaew et al. 2012). Although chitosan nanoparticles are nontoxic (Aliasghari et al. 2016), their synthetic process is technique sensitive and careful purification steps are needed to avoid inclusion of toxins (Padovani et al. 2015). Recently, low-cost and biocompatible iron oxide nanoparticles have been developed that exhibits potent antibiofilm properties following topical oral applications (Gao et al., 2016).

Iron oxide nanoparticles have been widely used as contrast agents in MRI because of their high biocompatibility and ability to penetrate tumor and atherosclerotic plaque, resulting in many FDA-approved formulations (Corot et al. 2006). Fascinatingly, the iron oxide nanoparticles display an intriguing biomimetic activity by displaying enzyme-like (peroxidase) activity, and thereby have been termed nanocatalysts. In a seminal work, Gao et al. (2007) demonstrated that iron oxide nanoparticles (NP) possess an intrinsic peroxidase-like activity, which enable them to catalyze the breakdown of H$_2$O$_2$, and rapid production of bioactive free-radicals (Figure 5). Hydrogen peroxide (H$_2$O$_2$) is a commonly used low cost antiseptic for general disinfection purposes or as tooth-whitening agent (at concentrations as high as 10%) because it generates free radicals that exhibit antibacterial activity or stain removal (through degradation of polymeric substances). However, the process is slow and H$_2$O$_2$ by itself has modest anti-biofilm or endodontic disinfection effects when used alone. The catalytic nanoparticles could potentiate the efficacy of hydrogen peroxide by enhancing the production of free radicals locally for improved antibacterial effects. Recently, this concept was proven successful to kill bacterial embedded within biofilms using short-term topical applications with exceptional efficacy (5,000 times more effective than H$_2$O$_2$ alone) (Gao et al., 2016).
Figure 5. Catalytic Nanoparticle. (A) SEM image of spherical and homogenous iron oxide nanoparticles at nanoscale level. (B) The mechanism by which NP catalyzes free radical generation in the presence of $\text{H}_2\text{O}_2$. (C) Demonstrating the catalytic activity of NP via colorimetric reaction by using 3,3',5,5'-tetramethylbenzidine (TMB) as a substrate which produces a blue color after reacting with the free radicals catalyzed by nanoparticles in the presence of $\text{H}_2\text{O}_2$. (D) This catalytic activity is rapid and pH dependent activity (Gao et al. Nature Nanotechnol, 2007).
Given this exceptional antimicrobial activity, there is a potential application of NP-H$_2$O$_2$ system for therapeutic application against pathogenic endodontic biofilms. Considering the complex endodontic environment and difficulty of penetrating DT, there is an enormous potential to exploit these nanoparticles for in situ activation of H$_2$O$_2$ as an improved and feasible therapy against biofilms and invading bacteria in the root canal and DT. In this study, we propose a novel concept of using nanocatalysts to activate H$_2$O$_2$ generating free-radicals on site that can effectively kill bacteria within DT. Furthermore, iron oxide NP is biocompatible and biodegradable material, which can be scaled up at very low cost for affordable endodontic applications.

**Materials & Methods**

**Materials**

Dentin blocks prepared from extracted teeth, *E. faecalis* OG1RF, a gift from Dr. Brenda Gomes, Piracicaba School of Dentistry, State University of Campinas. 0.5 mg/ml iron oxide nanoparticles (NP), Sodium Hypochlorite 3% (NaOCl), Chlorhexidine 2% (CHX), Phosphate-Buffered Saline (PBS), Hydrogen peroxide 3% (H$_2$O$_2$), Brain Heart Infusion broth (BHI) (Dot Scientific, Inc. Burton, MI, USA) and LIVE/DEAD® BacLight™ Bacterial Viability kit (Molecular Probes, Eugene, OR, USA).

**Sample Preparation**

Single rooted intact extracted teeth were used in this study. The extracted teeth were kept in 0.01% NaOCl solution before use to prevent dehydration. A dentin block was prepared from the root of extracted teeth as described previously (Haapasalo and Orstavik 1987). Each tooth was horizontally sectioned at 1 mm level below the cemento-enamel junction (Figure 6. A) using a 0.6-mm-thick precision diamond saw (Isomet 5000; Buehler Ltd, Lake Bluff, IL) at 1000 rpm under water cooling to produce a standardized length of 4 mm dentin blocks (Figure 6. B). The root canals were then enlarged to a standardized size of Gates Glidden drill #6 (1.5 mm in diameter) (Tulsa Dentsply, Tulsa, OK) at 300 rpm under water-cooling (Figure 6. C). Each cylindrical dentin block was then carefully split by a sharp blade and a hammer into 2 semi-cylindrical halves (Figure 6. D). Furthermore, the outer surfaces of the semi-cylindrical
halves (the cemental side) were ground by a water-cooled low-speed hand-piece with a fine carbide bur (Tulsa Dentsply) at 300 rpm to achieve a standard thickness of 2 mm and to remove the root surface cement (Figure 6. E).

Figure 6. Schematic diagram of root canal sample preparation. (A) Single rooted teeth; the area from which the dentin block is prepared marked with red. (B) Standardized 4 mm root dentin block. (C) Standardized root canal diameter preparation with size 6 Gates Glidden (1.5 mm diameter). The vertical direction of block splitting into semi-cylindrical halves. (E) The outer surfaces of the semicylindrical halves (the cemental side) were ground to achieve a standard thickness of 2 mm and to remove the root surface cement.

Each specimen were then treated with 5.25% NaOCl followed by 17% EDTA for 4 minutes using an ultrasonic bath in order to remove the organic and inorganic parts of smear layer (Zapata et al. 2008; Ma et al. 2011). Two specimens were examined under SEM, at magnifications ranging between 500x to 5000x, to confirm a successful smear layer removal and achievement of widely open DT. Samples were rinsed in sterile water.
for 10 minutes after smear layer removal to eliminate any residual chemicals. Specimens were then sterilized by autoclave for 20 minutes at 121°C.

**Dentin Infection with *E. faecalis***

The strain *E. faecalis* OG1RF was used as a test organism for this study. The bacterium was grown on BHI agar plates aerobically at 37°C in an atmosphere of 5% CO₂. Isolated colonies (24 hours) of pure cultures of *E. faecalis* were suspended in 10 mL BHI and incubated overnight as described above. Five hundred μl of the bacterial suspension was re-suspended in BHI and standardized spectrophotometrically to 3X10⁶ CFU/mL matching an optical density of 0.5 absorbance at 600 nm (OD₆₀₀ = 0.5) (Zapata et al. 2008). Five hundred microliters of the adjusted *E. faecalis* suspension was then added to each well (Olympus 24 well plate, 3.5 ml, Genesee Scientific. San Diego, CA, USA) containing 2 ml of fresh BHI medium and the dentin specimen at the bottom of the well (Figure 7). All cell-well plates were then incubated at 37°C in BHI broth for 3 weeks, and the culture medium was changed every 48 h as described elsewhere (Ma et al. 2011) with some modifications. Four samples were used to verify the infection both on the canal surface and within DT using SEM, while two samples were used to verify nanoparticles binding to canal surface and its distribution using Environmental SEM (ESEM) and Energy Dispersive X-ray Spectroscopy (EDS).

![Figure 7. Diagram of dentin specimen within *E. faecalis* suspension.](image) The specimen was inoculated with an overnight suspension of *E. faecalis* in BHI (2 ml) adjusted spectrophotometrically to OD₆₀₀ of 0.5, which was grown for 3 weeks.
SEM and ESEM/EDS Analysis

Ten root dentin samples were prepared for SEM analysis. We selected two samples to examine whether smear layer was successfully removed, while four samples were used to verify both biofilm formation on the canal surface and bacterial infection of DT. Furthermore, additional 2 specimens were used to examine NP binding following topical treatment (see later) and compared to 2 untreated specimens. Samples for SEM were fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer at a physiological pH of 7.4, overnight at 4°C. The goal of buffering the fixative is to provide an isotonic solution and to protect the sample from becoming acidic, and thereby alter the apatite structure. After 3 series of phosphate buffer washes, the samples were post-fixed in 2.0% buffered osmium tetroxide for 1 hour on ice, washed again in the same buffer, and dehydrated in a graded ethanol series that begins at 30% and proceed at 20% steps up to 70% followed by 10% changes to 100% ethanol using anhydrous ethanol. The gradual ethanol-based dehydration served to chemically extract the water from the samples. Then, the samples were treated with two changes of hexamethyldisilazane (HMDS) (50% and 100%) and then allowed to air dry prior to mounting. Specimens were mounted on stubs using double-stick, electrically-conductive carbon tape. Before specimens were examined by SEM, they were coated with 60% gold and 40% palladium using a sputter coater. SEM images were acquired with an FEI Quanta FEG 250 scanning electron microscope at the Electron Microscopy Resource Lab (University of Pennsylvania).

To confirm NP binding to the canal surfaces, each of the root dentin samples was treated with 0.5 mg/ml NP for 5 minutes followed by washing for 1 minute with sterile water. Following treatment and washing samples were examined with ESEM/EDS. Every element has a unique atomic structure, which when excited by an electron beam, emits a specific set of characteristic peaks on its X-ray emission spectrum. These x-rays detected and analyzed by EDS can be used to identify, map out and quantify the element of interest (i.e., iron). Based on this principle, FEI Quanta 600 FEG ESEM coupled with EDS was used to visualize/identify iron oxide nanoparticles on treated samples at Singh Center of Nanotechnology, department of nanoscale characterization, University of Pennsylvania. The EDS elemental analysis post-NP treatment was
performed to visualize the spatial distribution of iron on the same image area taken by ESEM. The iron distribution was displayed as yellow color based on EDS identification of the characteristic x-ray emitted by the iron when subjected to electron beam from ESEM.

**Catalytic Activity of NP-Bound On Root Canal Surface**

The bioactivity of iron oxide nanoparticles relies on its inherent catalytic (peroxidase-like) activity. Therefore, it is important to examine whether the NP bound on the canal surface remains active. To test NP enzyme-like property, we used a well-established colorimetric method using 3,3,5,5-tetramethylbenzidine (TMB) (Gao et al. 2007). TMB serve as a substrate for reaction with reactive oxidative species generated by peroxidase-mediated catalysis of hydrogen peroxide (H$_2$O$_2$), producing a blue color that can be visualized and measured spectrophotometrically as detailed previously (Gao et al., 2007). We first tested the colorimetric reaction in solution phase. Briefly, twenty μl of 816 μM TMB was added to an eppendorf tube and adjusted to 1 ml with sodium acetate NaAc buffer (pH 4.5). The solution was thoroughly mixed, and 50 μl of 1% H$_2$O$_2$ was then added and mixed. The reaction mixture (1.7 ml) was then added to an eppendorf tube containing fifty μl of NP (1 mg/ml), and then mixed. The time kinetics of blue color development was measured with Beckman DU 800 spectrophotometer (Beckman Coulter, Inc. Brea, CA, USA).

For the NP-bound on the canal surface, the same colorimetric reaction was performed on site. The infected root canal surface was first treated with iron oxide NP for 5 minutes. Followed by the addition of 50 μl of the reaction mixture (20 μl of 816 μM TMB adjusted to 1 ml with NaAc buffer with the addition of 50 μl of 1% H$_2$O$_2$). After confirming binding of NP and their catalytic activity, NP-H$_2$O$_2$ dentin disinfection efficacy was tested and compared to other irrigants.

**Nanoparticle Treatment of Infected Dentinal Tubules (DT)**

The infected root dentin blocks were transferred to the bottom of cell culture wells (Olympus 24 well plate, 3.5 ml, Genesee Scientific. San Diego, CA, USA), followed by rinsing in sterile water for 1 minute. The outer surface (cemental side) of the specimens
was covered with nail varnish (Ma et al. 2011), and then randomly divided into 6 irrigant solution groups, as follows: 1) PBS (negative control), 2) 3% H₂O₂, test control), 3) Iron oxide nanoparticles (0.5 mg/ml) (test control), 4) Iron oxide nanoparticle (0.5 mg/ml) + 3%H₂O₂ (NP-H₂O₂; test ‘new irrigant’), 5) 3% NaOCl, positive control), and 6) 2%CHX, positive control.

To assess the efficacy of the treatment, we used a single topical application for 10 min (total exposure time). For the ‘new irrigant’ test solution, a droplet of 50 μl of NP was placed on the infected dentinal surface of the root canal wall for 5 minutes. Then, the excess NP solution was washed with sterile water and the same volume of 3% H₂O₂ was place for additional 5 minutes. For all others, each irrigation solution was placed as described above for 5 minutes, then treated with additional 5 minutes of sterile water to standardize the treatment time to a total of 10 minutes. After treatment, the specimens were vertically split through the root canal into 2 halves (as described previously), and longitudinally visible dentin canals were examined via confocal laser scanning microscopy (Figure 8. A and B).

**Figure 8. Sample treatment and CLSM imaging.** (A) Disinfecting the sample with different control and test irrigants. (B) Splitting the canal surface to stain freshly exposed DT with LIVE/DEAD baclight staining and examine them under CFLM.

**Analysis of DT Treatments using Confocal Laser Scanning Microscopy (CLSM)**

In this study, CLSM was used as an analytical tool to examine both bacterial
viability and their distribution across the DT length. This method can quantify the viability of bacteria before and after the use of different irrigants while providing information about the spatial distribution of infection (Nagayoshi et al. 2004; Zapata et al. 2008; Ma et al. 2011). Nagayoshi et al used LIVE/DEAD® BacLight™ staining and conventional wide field fluorescence microscopy to evaluate the effect of ozonated water on bacteria invading dentinal tubules (Nagayoshi et al. 2004). Because of limited visualization with conventional microscopy, samples required demineralization prior to analysis, which might have created artifacts. Thereby Zapata et al (2008), modified this method and introduced CLSM for examination of dentin infection. CLSM provides high resolution at a single cell level and eliminates the need of the demineralization process, while successfully showing the viability of bacteria invading DT (Zapata et al. 2008).

Following treatment with the 6 different irrigation solutions, the split dentin samples were stained with fluorescent LIVE/DEAD® BacLight™ Bacterial Viability kit (Molecular Probes, Eugene, OR) containing SYTO®9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide (PI), according to the manufacturer’s instructions. The SYTO 9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, PI penetrates only bacteria with damaged membranes, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. The excitation/emission maxima for these dyes are about 480/500 nm for SYTO 9 stain and 490/635 nm for PI. Prior to CLSM examination, each of the samples were rinsed with PBS for 1 minute and mounted on the microscope stage. Dentin surfaces were examined using multi-photon Leica SP5 microscope (Leica Microsystems GmbH, Mannheim, Germany) at the imaging core facility, School of Veterinary Medicine, University of Pennsylvania. The mounted specimens were viewed using with 20 LPlan (numerical aperture, 1.05) water immersion objective lens with no additional zoom. The border of the root canal and the dentin surface was first located within the field of view of the microscope. Then, the dentin surface was divided into 3 zones: inner (the closest to canal lumen), middle and outer. Each specimen was scanned at 6 positions randomly selected at the microscope stage and confocal image series were generated by optical sectioning at each of these positions. Forty-micrometer-deep scans (0.5-µm step size, 82 slices/scan) were
obtained at a resolution of 512×512 pixel.

To analyze the bacterial killing efficacy of the irrigants within the infected DT, all acquired images were processed by ImageJ software (https://imagej.nih.gov/ij/) and merged into a 2D stack at the maximum pixel intensity. ImageJ converts pixels from confocal image stacks into pixels with numerical values, allowing quantitative analysis of the images. The area of interest was set (750 µm²) and the threshold of the fluorescence intensity was adjusted manually by two independent examiners. The pixels corresponding to bacteria were then analyzed to numerical values by the two different examiners and the amount of total dead bacteria image (stained by PI) pixels was calculated. The average reading of the two examiners was then taken. All the data were normalized against PBS (negative control) and presented as fold-change (vs. PBS).

The data were statistically analyzed using GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Two tailed un paired t-test was used to compare two sets of data at a time with an end results of 15 sets of data compared/zone at a significance level of P < .05 with 95% CI.

Results

In this study, we first used a combination of environmental/scanning electron microscopy (E/SEM) and energy dispersive spectroscopy (EDS) to assess bacterial infection and nanoparticle binding in the root canal. Subsequently, we used confocal laser scanning fluorescence microscopy (CLSFM) to evaluate the antibacterial activity of NP-H₂O₂ and compare its efficacy to positive controls (CHX and NaOCl), test controls (H₂O₂ and NP each used separately) and to the negative control (PBS).

E/SEM-EDS Analysis of E. faecalis Infection and Iron Oxide NP Binding. Smear layer removal is an imperative step to provide the bacteria with a clear path for invasion of DT. The smear layer removal was verified via SEM examination after sample was immersed in 5.25% NaOCl followed by 17% EDTA each for 4 minutes in ultrasonic bath. A successful removal of smear layer was observed and the DT openings appear to be clear and unclogged (Figure 6). Then, the treated surfaces were used for E. faecalis.
biofilm formation using our modified in vitro model. Initially, SEM analysis of the infected surfaces was conducted to verify whether the model can recapitulate the biofilm formation on the canal surface as well as the DT infection.

The data reveal bacterial clusters (typically found in biofilms) bound to the dentinal wall of the canal surface after 3 weeks of infection with *E. faecalis* (Figure 7. A). In parallel, another sample, which has been infected using the same procedure, was carefully split to verify DT infection. SEM images clearly show DT heavily infected by *E. faecalis* (Figure 7. B). At a higher magnification (10,000 X), the morphology of the enterococci and their spatial distribution along the tubules the can be visualized in detail (Figure 7. B Circle). Consistent with previous studies, a heavy DT infection was observed at the inner zone closer to the canal lumen (Figure 7. C). The model successfully recapitulated both the biofilm formation on the canal surface and bacterial infection of DT.

**Figure 6: Verification of smear layer removal using SEM.** Five hundred times magnification. DT opening appears to be clear and unobscured.

**Figure 7. SEM examination of Biofilm formation and Dentine infection.** (A) Bacterial cluster typically found in biofilm structure attached to the dentinal wall of the canal surface at 5,000 X magnification (red circle). (B) SEM micrograph showing heavily infected DT at 5,000 X magnification. At a higher magnification 10,000 X (the area marked by circle) the morphology of enterococci can be visualized in details. (C) Heavy infection is noticed more at the inner zone of the root dentin at 2,000 X magnification.
Subsequently, we examined whether iron oxide nanoparticles can be retained following short-term, topical application (5 minutes) akin to clinical situation for endodontic treatment. A higher SEM magnification (20,000X) coupled with ESEM/EDS and elemental analysis technology was used to visualize and detect the NP bound to the canal surface. The root canal preparations were infected with *E. faecalis* as described above and then treated with nanoparticles. Then, the treated-samples were analyzed and compared to untreated samples. We observed small particles at nanometer scale (Figure 8. A at 20,000X) bound to the bacterial surface (highlighted in the figure). In contrast, these particles were absent on the bacterial surfaces of untreated canal (Figure 8. B). To further confirm whether these nano-size structures are in fact iron oxide nanoparticles, ESEM/EDS elemental analysis was carried out.

![Figure 8. SEM analysis of NP-treated and untreated surfaces. (A) An infected specimen treated with NP. Closer look at selected area, we observed small particles at nanometer scale (in blue) compared to the bacteria (in red) at microscale (B) NPs were absent on the bacterial surfaces of untreated canal.](image)

A randomly selected area of the root canal treated with nanoparticles was subjected to ESEM scanning followed by elemental analysis to verify the presence of iron, the main element of the nanoparticle used in this study (Figure 9. A). Because every element has a unique atomic structure, when excited by an electron beam released by ESEM, it emits a specific set of peaks on its X-ray emission spectrum, which is characteristic to that of the particular element, i.e. iron. In turn, the iron signature x-ray is detected by the EDS. The element then is identified and its
distribution can be mapped out and quantified. In this case, the yellow color scale represents the iron element concentration in the imaged area (Figure 9. B), which correlates well with the nanoparticle location observed in ESEM image (Figure 9. A). Figure 9 C and D show the quantitative value of iron in NP treated sample compared to that of untreated sample. The results obtained here further validate nanoparticle binding to the bacterial cells in the NP treated canal surfaces.

Figure 9. ESEM and elemental analysis by EDS of NP-treated surfaces. (A) A randomly selected section of the infected canal surface (3000 x) that was treated with
NP, closer look at the circular area (blue) we observed small particles at nanometer scale bound to the bacterial cluster. (B) elemental analysis, in which the yellow scale indicates the iron concentration, the brighter the pixel, the more concentrated the iron. the yellow color distribution correlates to the nanoparticles location in (A). (C) and (D) show the quantitative value of iron in NP treated sample (D) versus that of untreated sample (C). This confirms binding of nanoparticles to the bacterial clusters in the NP treated canal surfaces.

Altogether, we were able to develop a model for simultaneous analysis of biofilm formation and dentinal tubule infection using a well-establish pathogen (E. faecalis) and de facto root canal surface. Using a combination of ESEM/EDS with elemental analysis, we demonstrated that the nanoparticles retain within the canal, while binding to the bacterial surface following a single, short-term topical treatment demonstrating its feasibility mimicking clinical situation.

Is the Iron Oxide NP bound on the Canal Surfaces Catalytically Active? For iron oxide nanoparticles, it is important to maintain the peroxidase-like activity in order to provide a therapeutic effect. To determine whether the surface-bound nanoparticles are catalytically active, we conducted the TMB-based colorimetric reaction. If active, iron oxide nanoparticles can catalyze H$_2$O$_2$ breakdown producing free radicals that oxidize TMB (a peroxidase substrate) generating a blue color reaction whose intensity over time can be measured using a spectrophotometer. Figure 10.A shows a typical colorimetric reaction via nanocatalysis of H$_2$O$_2$ showing the blue color as a result of reaction of TMB with free radicals generated. The reaction occurs very rapidly reaching the peak within seconds as determined spectrophotometrically. The peak of activity was found to be around 500 seconds (~8 minutes). Excitingly, we observed that the infected canal surface treated with iron oxide nanoparticles also rapidly generated a blue color following exposure to H$_2$O$_2$, reflecting peroxidase-like activity over time (Figure 10. B). The data show that the surface-bound nanoparticles within the infected canal are catalytically active, which in turn can generate reactive molecules on-site and cause bacterial killing.
Antibacterial Activity of NP-H$_2$O$_2$. Since the catalytic activity of the nanoparticles bound to canal surface was confirmed, their antimicrobial efficacy was tested and quantified in comparison to negative control (PBS), test controls (H$_2$O$_2$ and NP) and positive controls (NaOCl and CHX). The tooth surface was divided into three dentinal tubule zones based on the distance from the root canal lumen (Figure 11), and each zone was analyzed separately. Representative CLSM images are presented in Figure 12, and the data from quantitative analysis are shown in Figure 13. Overall, NP-H$_2$O$_2$ was more
effective in killing *E. faecalis* in all 3 zones when compared to all other experimental groups, and the differences were statistically (P < 0.05-0.0005).

NP-H$_2$O$_2$ killed *E. faecalis* with a mean fold change of (24.76 ± 5.78 fold; P<0.0005), (42.78± 14.22 fold; P<0.005) and (8.6 ± 3.9 fold; P<0.05) compared with the negative control PBS in the inner, middle and outer zones respectively. NP-H$_2$O$_2$ was several fold more effective in killing *E. faecalis* at the inner zone (>15-fold; P<0.05-0.005), at the middle zone (~ 40-fold; P<0.05) and at the outer zone (~ 7-fold; P<0.05) when compared to test controls H$_2$O$_2$ or NP. This substantial enhancement of antibacterial activity of NP-H$_2$O$_2$ compared to H$_2$O$_2$ or NP when used alone, provides a strong evidence that the bioactivity is maximized when both are used together; furthermore, the robust antimicrobial efficacy cannot be attributed to either chemical (NP or H$_2$O$_2$) alone.

Altogether, our data suggest that the antimicrobial activity results from iron oxide nanoparticle activation of H$_2$O$_2$ through its intrinsic “peroxidase-like activity” catalyzing free radicals generation on site.
Figure 12: CLSM imaging of *E. faecalis* infected DT treated with different irrigants. Each of the treated samples was stained with LIVE/DEAD Baclight staining system. Each representative image panel are color-coded corresponding to the zones as shown in the Figure 11. Red, blue and yellow indicates the inner, middle and outer zones, respectively. (A1-A3) treated with PBS, (B1-B3) 3%H₂O₂, (C1-C3) NP, (D1-D3) NP- H₂O₂, (E1-E3) 2% CHX and (F1-F3) 3% NaOCl.
Figure 13: Quantitative analysis of CLSM imaging data. The amount of bacteria killed was measured by fold difference relative to PBS (negative control) at the inner zone (A), middle zone (B), and outer zone (C). NP-H$_2$O$_2$ significantly killed more bacteria than both the PBS base line and the other tested irrigants (CHX and NaOCl) in all zones (**$P < .005$, *$P < .05$ and **$P < .005$ and **$P < .05$).
Strikingly, the antibacterial activity of the novel NP-H$_2$O$_2$ approach was significantly better than the conventional irrigants throughout the DT length, particularly in the middle zone. NP-H$_2$O$_2$ was >9-fold more effective at the inner zone, while showing ~30-fold more *E. faecalis* killing at the middle zone when compared to positive controls CHX or NaOCl (P<0.05-0.005). Furthermore, NP-H$_2$O$_2$ also showed better antibacterial activity at the outer zone (~5-fold more killing vs. CHX or NaOCl; P<0.05) CHX or NaOCl displayed significant antibacterial activity compared to negative control (P<0.0005) and they were both significantly more effective than H$_2$O$_2$ alone in all zones(P<0.05-0.005). expect the outer for NaOCl. In contrast, there were no significant differences between CHX and NaOCl, which are consistent with the findings from previous published studies (Heling and Shapira 1978; Vahdaty et al. 1993).

In summary, NP-H$_2$O$_2$ has shown an exceptional antimicrobial activity against *E. faecalis* infection of DT, which was significantly superior to the currently used endodontic irrigants CHX and NaOCl. The outstanding disinfection capacity was achieved when NP was used in combination with H$_2$O$_2$ as each of these agents alone displayed minimal effects. The data indicates that the bioactivity is a result of H$_2$O$_2$ activation via iron oxide-mediated ‘nanocatalysis’, which produced free-radicals on-site that are capable of rapidly killing bacteria.
Discussion

Microorganism has been implicated as the primary causative agent for apical periodontitis (Kakehashi et al. 1965) by forming disease-causing biofilms (Ramachandran Nair 1987; Tronstad et al. 1990; Molven et al. 1991; Siqueira et al. 2002; Ricucci et al. 2009). It has been postulated that there exist a threshold for the bacterial infection load, which once exceeded, would tip the balance between the insults from bacteria and host defense, causing the onset of the disease (Siqueira and Rocos 2008). Furthermore, bacterial virulence also plays a key role in the pathogenesis (Smith 1937). Unfortunately, it has been difficult to determine such bacterial threshold for endodontic infection because of the variability of the composition of the microorganisms infecting the root canal (Siqueira and Rocos 2008). Since leaving viable bacteria inside the canal at the time of filling has shown to be detrimental to the outcome (Heling and Shapira 1978; Sjogren et al. 1997; Waltimo et al. 2005; Fabricius et al. 2006), the procedures for endodontic disinfection should ideally eliminate all intracanal microbes. However, complete disinfection is virtually impossible due to the physical limitation of the currently used mechanical techniques to reach all areas of the complex root canal system (accessory canals, isthmus) (Siqueira and Rocos 2008). Moreover, bacteria within biofilms tend to resist the antimicrobial action of irrigants and medicaments (Tronstad et al. 1990; Molven et al. 1991). Hence the contemporary disinfection techniques have fallen short from achieving that optimal goal of 'sterile root canal'. Thereby, realistically, the goal of endodontic treatment should focus on reducing the microorganisms as much as possible to prevent the onset or recurrence of the disease (apical periodontitis) (Siqueira and Rocos 2008).

Bacterial infection and biofilm formation on the root canal also facilitate microbial invasion of the dentinal tubules (DT). DT infection has been associated with endodontic treatment failure (Vieira et al. 2012), although its exact role remains unclear. Furthermore, DT infection has been reported to occur in 70% to 80% of the teeth with primary apical periodontitis (Peters et al. 2001a; Matsuo et al. 2003). Therefore, it is critical to achieve optimal dentin disinfection to maximize a successful outcome post-endodontic treatment. Unfortunately, the current mechanical and chemical disinfection
modalities have been sub-optimal in terms of eliminating viable bacteria from root canals and DT.

Mechanical instrumentation has shown limitations in preparing the entire root canal surface, leaving some areas untouched with tissue remnants that could potentially provide bacteria with nutrients source (Walton 1976; Zuolo et al. 1992; Siqueira et al. 1997; Peters et al. 2003; Paque et al. 2009). Current chemical irrigation solutions can reduce bacterial infection. Despite sodium hypochlorite being the ‘gold standard’ endodontic irrigant (Zehnder 2006; Haapasalo et al. 2010), it has been reported that 40%-60% of root canals still harbor viable bacteria after treatment with NaOCl (Bystrom and Sundqvist 1985; Siqueira et al. 2007a). Chlorhexidine, another clinically used irrigant, has been shown to have similar antimicrobial effect compared to NaOCl (Vianna et al. 2006; Siqueira et al. 2007b). Furthermore, both irrigants have demonstrated limited efficacy for DT disinfection, exhibiting antibacterial activity only at the superficial layers of the dentin (up to 100 μm in depth) (Vahdaty et al. 1993; Heling and Chandler 1998). Other chemicals, such as MTAD, Qmix and H2O2, have been used but with limited effect (Zehnder 2006; Haapasalo et al. 2010). Thus, there is a need for new approaches that enhance disinfection of root canal and DT. In an attempt to address these challenges, we proposed to use iron oxide nanoparticles (NP) in combination with H2O2 as a new chemical strategy to disinfect DT more effectively than current modalities.

Iron oxide NP display peroxidase-like property, which enable them to catalyze the free radical generation in the presence of H2O2. The exact mode of catalytic action is currently unknown although it involves H2O2 binding into the iron oxide nanostructure with subsequent activation of H2O2 by ferric and ferrous ions in situ, generating free radicals ·OH (hydroxyl radical) and O2− ·/HO’2 (superoxide anion radical/perhydroxyl radical) (Wang et al. 2012). This mechanism is pH dependent, so that the catalysis is activated at acidic pH while greatly diminished at pH 6.5 and completely abolished above 7.0 (Gao et al., 2007; Gao et al., 2016). Importantly, iron oxide NP substantially enhanced bacterial killing and oxidative cleavage of biofilm components in the presence of H2O2, potentiating its anti-biofilm efficacy in vitro and in vivo (Gao et al. 2007; Gao et al. 2014; Gao et al. 2016).
In the present study, both biofilm formation and DT infection were successfully recapitulated using in vitro models. Moreover, iron-oxide NP was capable of binding to the infected canal surfaces despite a single, short-term (5 min) treatment when analyzed by SEM and ESEM/EDS. Importantly, the surface-bound NP was catalytically active as demonstrated via the colorimetric reaction using TMB (a substrate for determination of peroxidase activity). We then tested whether iron oxide NP activation of H$_2$O$_2$ could effectively kill *E. faecalis* present at different depth of DT (inner, middle and outer). Excitingly, NP-H$_2$O$_2$ displayed potent antimicrobial effects across the entire tubule length, and it was more effective in killing *E. faecalis* (at each of the DT 3 zones) when compared to currently used irrigants NaOCl and CHX (positive controls). NP and H$_2$O$_2$ were also tested to examine the antimicrobial effect of either of them when used alone. As expected, NP or H$_2$O$_2$ by itself showed minimal effects, reinforcing the concept of NP-H$_2$O$_2$ combination for optimal *E. faecalis* killing though nanocatalysis. Interestingly, both CHX and NaOCl performed poorly against *E. faecalis* at the middle and outer zones, especially when compared to NP-H$_2$O$_2$. Although Ma and co-workers have demonstrated that 6% NaOCl performed significantly better in killing *E. faecalis* within DT than 2% NaOCl and 2% CHX (Ma et al. 2011), this study show a highly effective and superior DT disinfection approach to current chemicals.

It is important to note that other nanoparticle-based technologies have been tested for antimicrobial purposes in treating root canal infection. Nanoparticles could be of a great value due to their nanoscale size that theoretically enables them to penetrate DT which have a diameter ranging between 2.4 to 2.9 μm (Lopes et al. 2009). Among them, silver nanoparticles have been the most widely investigated agent. The antibacterial activity of a silver nanoparticle solution was evaluated and compared to 5.25% NaOCl against *E. faecalis* and *Staphylococcus aureus*. The nano silver-based irrigant was as effective as NaOCl in preventing the bacterial growth of the root canal bacteria (Moghadas 2012). However, Wu et al. (2014) found that silver nanoparticles solution is a weak irrigant when tested in vitro against *E. faecalis* biofilms on root canals and compared to 2% NaOCl (Wu et al., 2014); the reasons for this discrepancy remain unclear, but likely due to different formulations and biofilm models. In contrast to these previous findings, iron oxide NP (single treatment) was substantially more effective than
5.25% NaOCl. Thus, NP-H₂O₂ could be potentially superior to both silver nanoparticles and current chemical irrigants, although a direct comparison needs to be conducted both in vitro and in vivo.

At the same time, we also recognize the limitations of the data derived from our in vitro studies. We used a single species infection model using E. faecalis as the test organism. Although endodontic infection is polymicrobial, E. faecalis strain has been selected because of their frequent isolation from persistent case of apical periodontitis (Molander et al. 1998; Gomes et al. 2008). Moreover, E. faecalis ability to invade DT has been well established in in vitro infection models (Haapasalo and Orstavik 1987). Furthermore, E. faecalis has been shown to survive harsh conditions (Portenier et al. 2001), high pH levels (Chavez de Paz et al. 2007), starvation (Haapasalo and Orstavik 1987) and entombment (Shin et al. 2008). However, future studies should be directed toward building multispecies model that better recapitulate the complex nature of endodontic infections as well as testing NP-H₂O₂ antimicrobial efficacy in these models.

Another important limitation is the autofluorescence properties of the dentin, because of the presence of organic matter with endogenous fluorophores in the dentinal structure (Konig et al. 1998). The autofluorescence of dentin depends on age and degree of mineralization (Lutskaya 2012), with the highest intensity measured in poorly mineralized dentin (young patients) between 460–500 nm. This range is very close to that of SYTO9, which has an excitation/emission maxima of about 480/500 nm, but far from that of the PI (490/635 nm) (Figure 14). Interestingly, Ma et al (2011) indicated that this autofluorescence was minimal and caused no interference with their CLSM-based bacterial viability quantification. In this study however, the background autofluorescence—interfered with the fluorescent signal generated by the bacteria stained with STYO 9 that accounts for live bacteria, and thereby it could provide erroneous quantification of total viable cells. However, the dead cells (labeled with PI) were precisely visualized and quantified. Thus, we focused on measuring total dead bacteria within a standardized area of the each of the treated dentin relative to PBS (negative control) treatment (data as fold-difference vs PBS control). The limitation of quantifying only the signal produced by the bacteria stained by PI (dead) is that the determination of the live/dead bacteria ratio is not possible. Although this method of
quantification can measure and compare killing efficacy of the test irrigants, it is unable to measure how much viable bacteria remained in the DT. In the future studies, the CLSM method should be improved to allow determination of the ratio live/dead cells. This can be achieved by using different combinations of fluorescent probes that has excitation/emission spectra away from that of the tooth autofluorescence wavelength (Figure 14). For example, Syto40/PI or Syto82/7-aminoactinomycin D can be used to determine live/dead cells (figure 14).

Altogether, our study provides tenable evidence of a potent therapeutic approach for endodontic disinfection using nanotechnology. Furthermore, we uniquely analyzed the antimicrobial efficacy of different irrigants by determining bacterial killing spatially at different DT depth using CLSM. To our knowledge, this is the first study exploring the nanocatalysis concept for enhanced bacterial killing across the dentinal tubule. Iron oxide NP may also have additional advantages compared to silver and other nanoparticles as endodontic irrigants. Iron oxide is highly biocompatible, and a recent in vivo study (using the same NP) demonstrated no toxic effects soft oral tissues following topical NP-H$_2$O$_2$ treatments (twice-daily) for up to 21 days in a rodent caries model (Gao et al., 2016). Iron oxides are commonly used as food additives and many iron oxide NP formulations are FDA-approved for chronic treatment. Furthermore, it is a sustainable material that can be synthesized on large scale using simple chemical synthesis methods at very low cost (couple cents per mg) (Wei and Wang, 2013). The flexibility of iron oxide chemistry allows the production of new nanoparticle shapes and sized that may further improve DT penetration. Furthermore, the concentration of H$_2$O$_2$ can be adjusted. Thus, future studies should be directed towards further optimization of the NP-H$_2$O$_2$ system as well as incorporation in other endodontic materials such as intracanal medicaments and sealer. Moreover, the efficacy of NP-H$_2$O$_2$ system need to validated using appropriate rodent models and further evaluated in clinical studies.
Figure 14. Tooth enamel and dentin autofluorescence. (A) Adapted from (Matosevic et al. 2010) showing laser induced fluorescence of different part of extracted carious tooth illuminated by the 405 nm laser, notice the dentin fluorescence with a maximum emission at 500nm which overlaps that of SYTO9 shown in (B). (B) Range of emission wavelengths of different fluoroprobes, its noteworthy that proposed combination SYTO40/PI or Syto82/aminoactinomycin D are all away from the emission maximum of the SYTO9 and consequently the dentin.
References


