The Design and Physical Properties of An Optimized Chitosan Hydrogel for Potential Use in Endodontics

Hunter Housley
Marquette University

THE DESIGN AND PHYSICAL PROPERTIES OF AN OPTIMIZED CHITOSAN HYDROGEL FOR POTENTIAL USE IN ENDODONTICS

by

Hunter R. Housley, D.M.D.

A Thesis submitted to the Faculty of the Graduate School,
Marquette University,
in Partial Fulfillment of the Requirements for
the Degree of Master of Endodontics

Milwaukee, Wisconsin

May 2019

ABSTRACT
THE DESIGN AND PHYSICAL PROPERTIES OF AN OPTIMIZED CHITOSAN HYDROGEL FOR POTENTIAL USE IN ENDODONTICS

Hunter R. Housley, D.M.D.

Marquette University, 2019

Introduction: The aim of this study was to synthesize an optimized chitosan/carbon dot hydrogel and measure its effects on the proliferation of dental pulp stem cells.

Methods: 1.5% w/v chitosan hydrogel samples were fabricated with 300 mg/ml aqueous β-glycerol phosphate (β-GP) to create an optimized chitosan with a gelation onset temperature below 37°C. Spermidine-functionalized carbon dots (CDs) were added to test groups in concentrations of 25 µg/ml and 50 µg/ml. Complex shear viscosity of the prepared hydrogel samples was evaluated with a Kinexus pro+ shear rheometer (Malvern Panalytical). Samples were evaluated for cellular proliferation with dental pulp stem cells at days 1, 2, 5, and 14 using PrestoBlue and a Synergy HTX microplate reader (BioTek). Proliferation results were compared to a separate test groups with calcium hydroxide under the same test parameters.

Results: The optimized chitosan hydrogel samples had a gelation onset temperature of 34.6°C, which was not appreciably increased with the addition of CDs (34.7°C). All experimental chitosan hydrogels demonstrated superior cellular proliferation when compared to calcium hydroxide paste.

Conclusions: Chitosan/CD hydrogels may be a promising medicament for endodontic use and a viable alternative to either $Ca(OH)_2$ or TAP in regenerative endodontic cases. The CD structure contains surface characteristics that are open to customization for individual therapeutic needs. This hydrogel is thermally responsive and becomes a solid gel when administered at human body temperature. Further studies are warranted to determine its antibacterial properties, as well as other factors to understand how it may interact in the root canal space.

ACKNOWLEDGEMENTS

Hunter R. Housley, D.M.D.

I would like to thank the Endodontics faculty at the Marquette University School of Dentistry, specifically Dr. Lance Hashimoto, Dr. Sheila Stover, Dr. Joseph Gaffney, Dr. Mohamed Ibrahim, and Dr. Joseph DeGuzman in providing me a clinical foundation as I start my career in endodontics. By example, they have set the bar for excellence and helped me at every step.

I was fortunate to learn alongside with the following motivated, team-oriented co-residents: Alex Moore, Jon Irelan, Sukbum Yoo, Maksim Montatskiy, Abby Yavorek, Timothy Gainey, Michael Smoljan, and Igor Sulim. Thank you for making my residency enjoyable, collaborative, and engaging.

I also wish to thank Dr. Lobat Tayebi, Morteza Rasoulianboroujeni, Amir Yadegari, and Nikita Tongas for their assistance in study design and laboratory testing. Without their expertise, this study would not be possible.

Finally, I am most grateful for my family for their support and encouragement. My wife and children have stood by my side throughout my academic journey. In the moments I have wavered, they have helped me get back on my feet!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Among the medical and dental fields, a wide variety of biomaterials have been designed to improve treatment predictability and patient outcomes. When materials are engineered specifically for the body, one must consider all of the following as high priorities: safety, efficacy, and cost. It is often particularly challenging to meet each of these goals with the same biomaterial. A product may be safe and effective, but expensive; in contrast, a different product may be effective and low-cost, but unsafe. It is the pursuit of ideal materials that fuels innovation within healthcare.

Endodontics is the dental specialty devoted to maintaining health within the dental pulp, as well as treating diseases and/or injuries that occur within the tooth. Throughout the era of modern endodontics, materials used for the purpose of canal disinfection have largely remained the same. Canal disinfection with 5.25% sodium hypochlorite and interappointment calcium hydroxide, when indicated, have become the gold standard. These materials, however, are cytotoxic and necessitate careful use. Among the recently developed biomaterials, it is time to explore alternatives to common disinfectants in endodontics.

This study focuses specifically on the development of an optimized chitosan hydrogel, using spermidine-functionalized carbon dot (CD) nanoparticles as antibacterial additives. While currently in development, this material possesses promising attributes for potential use as an endodontic medicament. The following literature review and subsequent study will provide background knowledge of endodontic treatment, medicaments, and the potential role of this new material in clinical practice.

LITERATURE REVIEW

Goals of Modern Endodontics

As shown by the classic works of Kakehashi and Moller, bacteria within the dental pulp are the primary cause of apical periodontitis (1, 2). While not always painful, these diseases are commonly a result of complex biofilm invasion into the pulp tissue, which proliferates without proper dental intervention. As disease progression continues, biofilms become more established and increasingly resilient (3). Root canal therapy (RCT) is a commonly prescribed procedure with the fundamental aim to remove biofilm within the canal space and resolve dental infections (4). When performed to the appropriate standard of care, RCT has high success rates (5). This treatment is carried out by accessing the canal space, physically and chemically debriding the canal walls, and filling the space with material to provide a hermetic seal. While several factors contribute to the overall success of RCT, proper disinfection is paramount. It is commonly believed that residual biofilm is responsible for most endodontic failures (6, 7).

Regenerative Endodontics

Pulpal regeneration is a newer concept in endodontic therapy, based upon principles of tissue engineering. Just as in medicine, tissue engineering within the tooth requires an interplay of scaffolds, growth factors, and stem cells (8). Pulpal regeneration is not possible in all cases requiring RCT due to the lack of blood flow or stem cell availability. These techniques are reserved for necrotic teeth with apices larger than 1mm (9). When done correctly, the treatment can reestablish vascularized connective tissue within the tooth canal space and aid in continued root development. Since the newly

formed tissue lacks resemblance of healthy pulp, the term *regeneration* continues to be a topic of debate (10). This procedure is also referred to as revascularization, revitalization (11), or maturogenesis (12) among various experts.

There are often two or more steps involved in regenerative endodontics (13). The goal of the initial appointment is to access and adequately disinfect the canal space. Due to the high cytotoxicity of full-strength sodium hypochlorite, it is recommended to disinfect the canal with a 1.5% diluted concentration (14). At the end of the first appointment, either a calcium hydroxide ($Ca(OH)_2$) or triple antibiotic paste (TAP) medicament is placed. The goal of the second appointment is to remove the medicament, irrigate with ethylenediaminetetraacetic acid (EDTA) to release dentinal growth factors, and stimulate apical bleeding to create a scaffold. This process also facilitates stem cell migration (15), which is critical in the success of the procedure. A barrier is placed over the blood clot scaffold using mineral trioxide aggregate (MTA) or bioceramic putty and the tooth is permanently restored with resin or amalgam (16).

Both root canal therapy and pulpal regeneration require adequate disinfection for optimal results. In the case of regeneration, maintaining cell viability is also fundamental to success of the procedure. Using the currently available medicaments, disinfection is proportional to cytotoxicity—as the concentration of the disinfectant increases, cytotoxicity also increases. Thus, it desirable to understand the properties of currently available medicaments and test newer materials that carry an inherently lower cytotoxic effect.

Current Medicaments and their Use in Endodontics

According to the American Academy of Endodontists (AAE) Glossary of Endodontic Terms, medicaments are "interappointment agents used for palliative or antimicrobial purposes" (17). Though there continues to be a lack of consensus regarding single-visit versus multiple-visit therapy (18-21), medicaments still have their place in endodontics when additional measures for canal disinfection are indicated. The most commonly used medicaments are $Ca(OH)_2$ (with or without iodoform) and TAP. It is important to understand the characteristics of these materials in order to determine their best use in patient care.

First introduced by Hermann in the 1920's (22), $Ca(OH)_2$ is the most frequently utilized medicament in endodontics. It is strongly alkaline, with an approximate pH of 12.5 (23). When injected into the canal space, it separates into calcium and hydroxyl ions to increase the local pH (24). As long as the high pH is sustained, $Ca(OH)_2$ aids in bacterial destruction (23). Additionally, it has been shown to neutralize osteoclastic activity (25, 26)**,** dissolve necrotic tissue (27), and break down bacterial lipopolysaccharides (25). Paula-Silva also found that interappointment $Ca(OH)_2$ lowered bacterial prevalence, decreased matrixmetalloproteinase (MMP) expression, and reduced inflammatory cell infiltrate (21).

Despite the many purported benefits of $Ca(OH)_2$, its efficacy and biocompatibility are unclear. Many sources conclude that $Ca(OH)_2$ is ineffective as a medicament (28-30). In fact, some sources claim that it exhibits virtually no antimicrobial effect whatsoever (31-34). *Candida albicans* and *Enterococcus faecalis*, pathogens commonly associated with endodontic failures, are notably resistant to $Ca(OH)_2$ (35-38).

There is also some evidence that extrusion of $Ca(OH)_2$ may lead to delayed healing and apical tissue necrosis (39, 40). Due to its high pH, $Ca(OH)_2$ creates a zone of necrosis immediately adjacent to surrounding tissue and may disrupt cells that could differentiate into new pulp (41, 42). The work of Badr demonstrated that $Ca(OH)_2$ is highly toxic to stem cells (43), but other sources contradict this finding (44, 45). Based off animal studies, Andreasen concluded that prolonged use of $Ca(OH)_2$ increases the risk of root fracture (46). Thus, it is advisable to minimize the overall duration of the medicament use. Regardless of these risks, $Ca(OH)_2$ remains the current medicament of choice in endodontics.

TAP is an alternative endodontic medicament developed by Hoshino and colleagues (47). Traditionally consisting of metronidazole, ciprofloxacin, and minocycline, TAP uses a mixture of antibiotics to combat the broad spectrum of endodontic pathogens. The antibiotic paste has been shown to be more effective than $Ca(OH)_2$ against *E. faecalis* (48) and demonstrates promising outcomes when used in regenerative endodontics (49). TAP penetrates deep into the dentinal tubules and has a sustained antibacterial effect. This attribute leads to effective disinfection but presents a challenge in removal of the antibiotic (14). Berkhoff found that after various techniques of copious irrigation, 80% of the antibiotic remained tightly bound to dentin (50). In the same study, less than 15% of $Ca(OH)_2$ persisted along the canal wall (50). The inability to effectively remove TAP is thought to be an undesirable trait due to the medicament's cytotoxicity to stem cells (51, 52). TAP also has a short shelf life, tendency to stain dentin (53), and potential for allergic reactions, thus making it less appealing for use as a medicament.

It is clear that both $Ca(OH)_2$ and TAP have shortcomings when used as medicaments. For this purpose, disinfection by some other means continues to be investigated.

Polymers as an Alternative Medicament Vehicle

There are various alternatives to be explored as intracanal medicaments. Polymers are long molecules consisting of repeating sequences of covalently bonded chains. Among this family of materials, there are many choices with potential use in medicine and tissue engineering. Some polymers are naturally occurring, such as collagen, gelatin, chitin/chitosan, hyaluronic acid, and alginate. Natural polymers are inherently biocompatible and biodegradable (54, 55), which are ideal material characteristics for the endodontic uses previously described. Other polymers are made synthetically such as: polyethylene glycol (PEG), polyglycolic acid (PGA), and polycaprolactone (PCL). Though effective in various applications, these synthetic polymers may not be as ideal for endodontic use.

Due to its high water solubility and imbibition, PEG may not remain stable when left as a medicament in the canal space (56). PGA may also be an inappropriate material choice due to its acidic degradation and evidence of inducing inflammation *in vivo* (57). Though PCL is biocompatible, its use in endodontics has been a historic failure. Resilon, a product comprised of PCL, was widely touted as a replacement for gutta percha as an obturation material. After a few years of use, Resilon began to show a much higher failure rate. In a recent study, Barborka et al found that Resilon-treated teeth failed 5.7 times more frequently than those treated with gutta percha (58). Due to its history, the use of PCL as a carrier for intracanal medicament would be highly scrutinized.

Chitosan Hydrogels

Though several natural polymers have attributes that can be harnessed for endodontic use, in terms of disinfection and biocompatibility, none seem as inherently ideal as chitosan. Chitosan is produced through the deacetylation of chitin, a naturally available polymer found in the exoskeleton of crustaceans and in fungi cell walls. Naturally toxic to fungi, bacteria, and parasites, chitosan is biocompatible and minimally toxic to mammalian cells (59). When acting as an antimicrobial, chitosan binds to negatively charged cell surfaces to increase bacterial cell wall permeability and cause intracellular leakage (60). Chitosan is biodegradable and comes in several forms for use in regenerative medicine such as: hydrogels, fibers, sponges, and 3-dimensionally printed scaffolds (61-63).

For purposes of injectability, chitosan in hydrogel form is a logical choice for a potential medicament in endodontics. Hao et al developed a temperature-responsive hydrogel that showed promising healing properties within cartilaginous joint defects (64). Harnessing the properties of such a material for dental use would allow for the hydrogel to be injected into the endodontic canal space in a more aqueous sol phase. After reaching an optimized physiologic gelation temperature, the biomaterial could undergo a phase change into a solidified gel phase to enhance stability. When prepared at a low pH, chitosan hydrogels are also thermally reversible (65). This property would theoretically allow for easy removal within the root canal space by converting the hydrogel back to the aqueous sol phase.

Application of Nanomaterials

One major concern with antimicrobial drugs is the potential for developing drug resistance. Over the years of prescribing drugs to combat bacterial pathogens, stronger drug-resistant strains have shown an adaptive capacity to counteract antibiotic efficacy. These tenacious strains are prevalent and continue to present a medical challenge. As previously discussed, drug resistance has already been noted with the use of $Ca(OH)_2$ in endodontics (36). Nanomaterials appear to be a promising solution to enhance microbial effectiveness and decrease the potential for resistance. These microscopic particles carry many advantages because of their extremely large surface area. Due to their ability to disrupt multiple biologic pathways among microbes, experts suggest bacterial resistance to nanomaterials is unlikely (66).

Carbon dots (CDs) are a nanomaterial with fascinating potential in medical applications. CDs are generally defined as small carbon nanoparticles (smaller than 10nm) that contain various surface passivation schemes (67). Many techniques have been used to synthesize CDs, including chemical ablation (68-71), laser ablation (72-75), electrochemical carbonization (76-78), microwave irradiation (79-81), and hydrothermal/solvothermal treatment (82-86). Depending on the method of synthesis, particle size can either be highly specific or variable (87). CDs are relatively cost-effective and easy to synthesize, making them readily available (88).

A key characteristic to CDs effectiveness is the small particle size. Due to their microscopic nature, CDs drastically enhance the surface area to interact with the bacterial cell surface (59). When combined with other antibacterial reagents, CDs are shown to have a synergistic bactericidal effect (89, 90). The works of Omidi et al showed that a

carbon dot/chitosan nanocomposite was highly effective against *S. aureus* in a wound healing application (91).

It has been shown that CDs exhibit good water solubility, low toxicity, and high stability (92). CDs are inherently photoluminescent and such properties can be enhanced via chemical doping with elements such as nitrogen (93, 94), sulfur (95, 96), or phosphorus (97). As a fluorescent nanomaterial with excellent biocompatibility, CDs have shown great promise for fluorescent bioimaging (87). CDs have an abundance of oxygen-containing groups, providing a means for surface functionalization via covalent bonding (84, 98). This allows for vast customization of the CDs surface to alter and improve their properties for any given need.

Considering the body of literature surrounding these new materials and the challenges of root canal disinfection, this study was constructed to create an injectable CD/chitosan hydrogel that will encourage greater stem cell proliferation than $Ca(OH)_2$ paste. More specifically, the study was completed with the following aims:

1. To design a thermally responsive chitosan hydrogel with a gelation onset temperature below 37°C
2. To add CD nanoparticles to the hydrogel in order to enhance hydrogel qualities
3. To evaluate this novel hydrogel for its effect on dental pulp stem cell proliferation, in comparison with calcium hydroxide paste

EXPERIMENTAL DETAILS

Materials

The materials used throughout this study can be referenced in Table 1.

Table 1: Experimental materials

Material Description	Details	CAS #	Manufacturer
Chitosan	Medium molecular weight	9012-76-4	Sigma-Aldrich
Acetic acid (10%)	Glacial ACS grade	9012-76-4	VWR Amresco
β-Glycerol phosphate disodium salt	≥98.0% (NT)	13408-09-8	Sigma
Ammonium citrate	Tribasic	3468-72-8	Alfa Aesar
Spermidine	≥99% (GC)	124-20-9	Sigma-Aldrich
UltraCal XS	Calcium hydroxide paste	-	Ultradent
PrestoBlue™ Cell Viability Reagent	Resazurin-based reagent	A13262	Thermo Fischer Scientific
Dulbecco's Modified Eagle Media (DMEM)	High glucose	-	Corning
Phosphate-buffered saline (PBS)	Ultra Pure Grade	-	VWR
Corning™ 2.5% Trypsin	Without phenol red	25-054-CI	Mediatech, Inc.
Ethylenediaminetetraacetic acid (EDTA) 0.5M	Biotechnology Grade	20-158	EMD Millipore
Fetal Bovine Serum (FBS)	Corning cellgro®, Regular	-	Mediatech, Inc.
L-Glutamine	High Purity Grade	56-85-9	VWR
Penicillin, Streptomycin Mixture	10,000 µg/mL streptomycin, 10,000 µg/mL penicillin	-	QUALITY BIOLOGIC
Dialysis bags	MWCO ~1.0 Kda	-	-
Dental pulp stem cells	Harvested previously from extracted human third molars	-	-

Equipment

The equipment used throughout this study can be referenced in Table 2.

Table 2: Experimental equipment

Equipment Description	Model	Manufacturer
Rheometer	Kinexus Pro+	Malvern Panalytical
Centrifuge	Symphony 4417R	VWR
Vortex Mixer	Analog Vortex Mixer	VWR
Spectrometer	Nicolet iS 5N FT-NIR	Thermo Scientific
Transmitted Light Imaging System	EVOS FL Auto	Life Technologies
Microplate Reader	Synergy HTX	BioTek

Fabrication

A preliminary study was performed to determine the relative concentrations needed to create a chitosan hydrogel with a gelation onset temperature (T_{onset}) slightly lower than the average human body temperature, between 30°C and 37°C. A 3% weight/volume (w/v) chitosan solution was made by mixing 3 g of medium molecular weight chitosan in 100 ml deionized water, while adding 1.3 ml of 10% acetic acid dropwise (Figure 1). Various concentrations of aqueous β-glycerol phosphate (β-GP) were prepared and combined in a 1:1 ratio with the chitosan gel, using a vortex mixer and centrifugation (Figure 2). The samples tested in this initial study consisted of 1.5% w/v chitosan with β-GP concentrations of 175, 250, and 300 mg/ml.

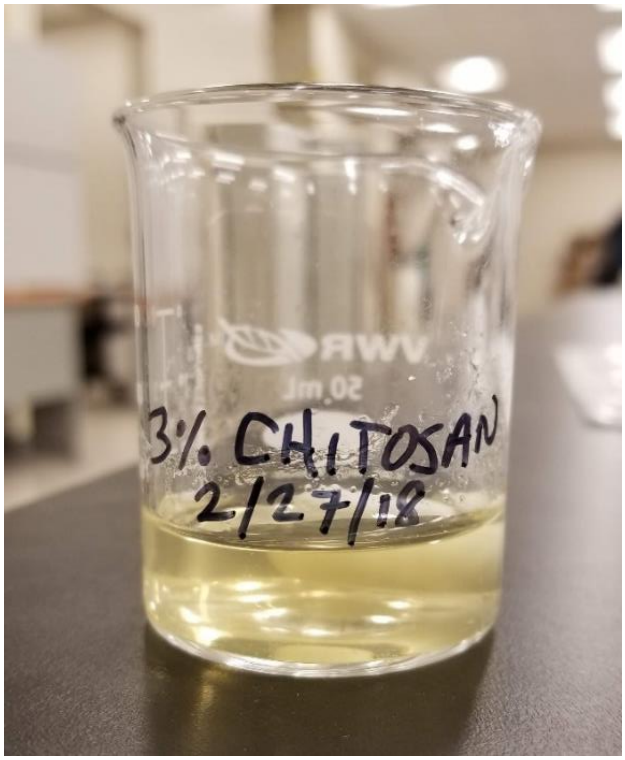

Figure 1: 3% w/v chitosan hydrogel prior to the addition of aqueous β-GP

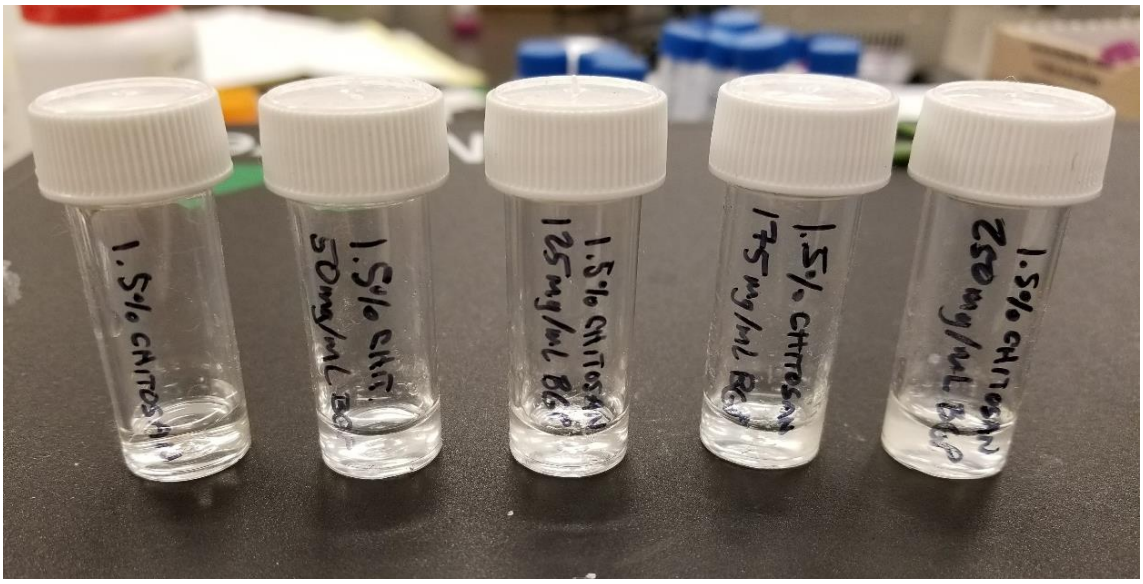

Figure 2: 1.5% w/v chitosan hydrogel samples fabricated for the preliminary study to determine the necessary concentrations of β-GP to reach an ideal T_{onset} (not pictured: 300 mg/ml β-GP).

These samples were tested with a Kinexus pro+ shear rheometer (Malvern Panalytical) (Figure 3). Gel samples were independently loaded onto the rheometer's thermoregulated platform and complex shear viscosity was measured as the temperature increased. Samples were evaluated using both single-frequency and multiple-frequency rheometry parameters. Since single-frequency settings yielded comparable results and allowed for more streamlined testing, this approach was used as the primary method for evaluating the T_{onset} throughout the later stages of this study.

The recorded data points were plotted to determine the T_{onset} of each sample. It was determined that 1.5% w/v chitosan with 300 mg/ml β-GP contained an ideal gelation temperature, which will be discussed later in further detail. This sample concentration was used throughout the remainder of the experiment and will hereafter be referred to as "optimized chitosan."

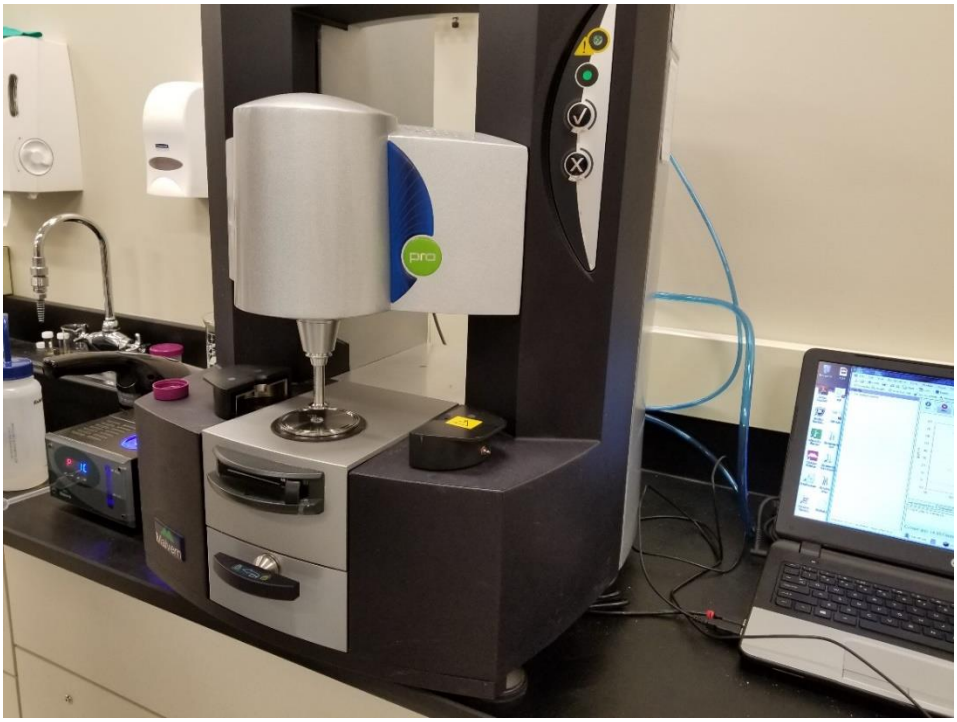

Figure 3: Loading hydrogel samples onto the rheometer

CDs were then synthesized with a simple heating method. This was done by placing 2 g of ammonium citrate powder in a beaker and heating the substance in an oven at 180°C for two hours, thus creating a dark brown residue. The resulting CDs were cooled to room temperature and ground into a fine powder, then stored for later use.

Functionalization

In order to functionalize the CDs with spermidine, 0.025 g of CDs powder was added to 1 ml of 0.1 M spermidine solution in a beaker. This mixture was heated to 260°C for two hours and subsequently cooled to room temperature (Figure 4). The remaining residue was dissolved in 5 ml of deionized water, then centrifuged for one hour at 6000 rpm to separate larger particles. Finer CDs were removed with the supernatant and poured into a dialysis bag (MWCO~1.0 Kda) for further filtration.

Dialysis was performed for five hours with water replacement every hour and the resulting carbon dot powder was stored at 4°C for later use.

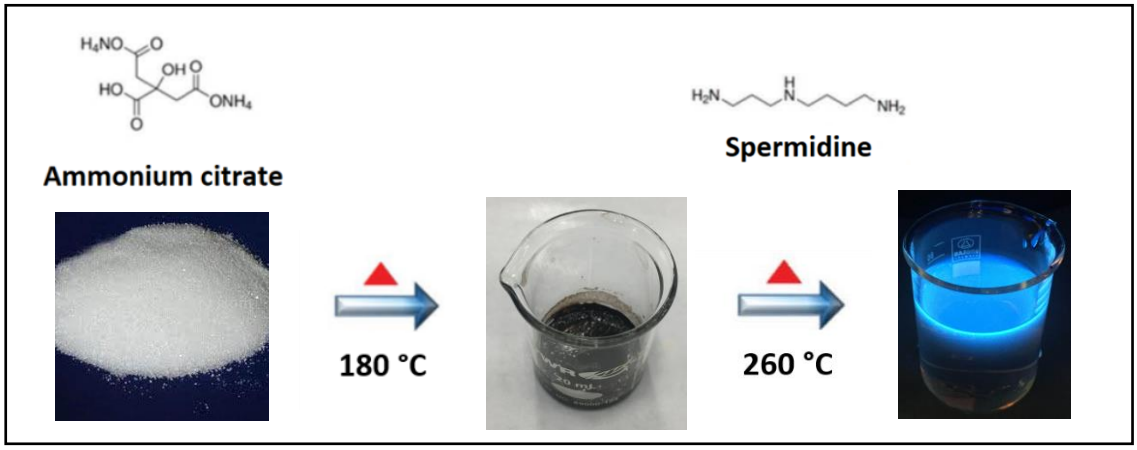

Figure 4: Synthesis process for spermidine-functionalized CDs via solid state pyrolysis

In order to form a homogenous mixture of CDs within the optimized chitosan, the hydrogel had to be refabricated. Slightly modifying the steps previously outlined in gel synthesis, spermidine-functionalized CDs were mixed into aqueous β-GP, then added to the chitosan (Figure 5). Samples were combined in a test tube and agitated by Vortex mixer, ultrasonic bath, and centrifugation. The final compositions of the experimental gels are as follows:

1) 1.5% w/v chitosan, 300mg/ml β-GP (optimized chitosan)
2) 1.5% w/v chitosan, 300mg/ml β-GP, 25 µg/ml CDs
3) 1.5% w/v chitosan, 300mg/ml β-GP, 50 µg/ml CDs

These resulting hydrogels were subjected to single-frequency rheometry, as previously described, to evaluate T_{onset}.

Figure 5: Addition of aqueous β-GP and CDs to chitosan gel. *Pictured from left to right: 600 mg/ml β-GP, 600 mg/ml β-GP with 50 µg/ml CDs, 600 mg/ml β-GP with 100 µg/ml, 1.5% w/v chitosan with 300 mg/ml β-GP and 50 µg/ml CDs.*

Cell Proliferation

De-identified dental pulp stem cells (DPSCs) were acquired from the research laboratory at the Marquette University School of Dentistry for testing cell proliferation of the experimental hydrogels. These cells were previously harvested from the pulp tissue of extracted human third molar teeth and were kept viable for research purposes. A growth media consisting of DMEM, 15% FBS, 1% L-glutamine, and 1% penicillin/streptomycin antibiotic was prepared. Cells were added to the media and incubated until the 3rd passage. They were visualized and counted using an EVOS FL Auto transmitted light imaging system (Life Technologies) (Figure 6). DPSCs were detached using a mixture of phosphate-buffered saline (PBS) with 0.25% trypsin/0.5mM EDTA and placed with fresh growth media into sterile well plates under a biosafety cabinet (Figure 7).

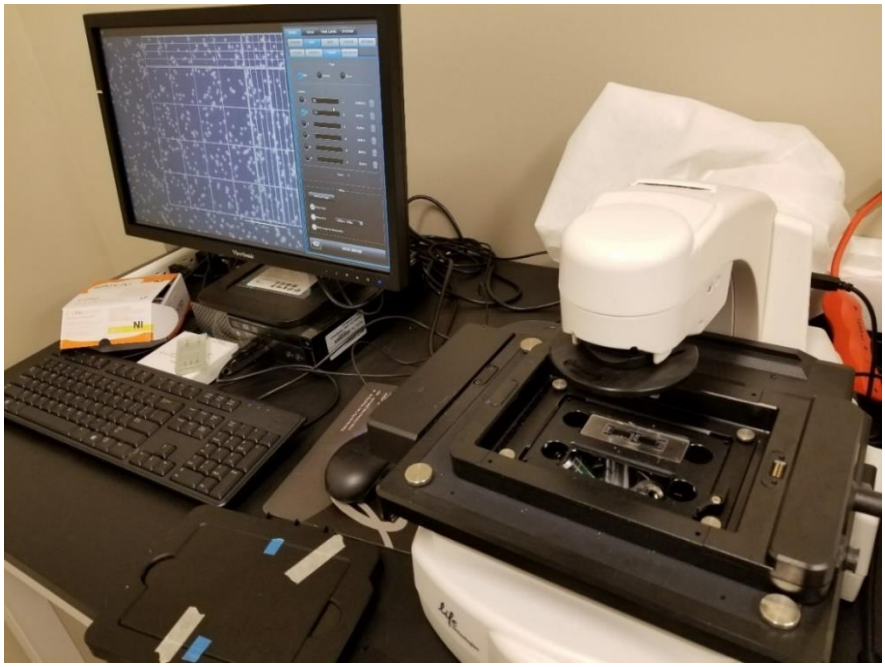

Figure 6: Cell counting to verify ideal concentration of DPSCs for cell proliferation testing

Figure 7: Detachment process of DPSCs using PBS with 0.25% trypsin/0.5mM EDTA

Experimental chitosan/CD hydrogels were exposed to UV radiation for 60 minutes for disinfection. 0.15 g of each sample were loaded, in triplicate, into porous inserts and submerged into the well plates containing DPSCs. The porous inserts provided a means for diffusion across the insert/media interface while minimizing material washout. A positive control was maintained to demonstrate cell proliferation solely within media, containing empty inserts with no gel. Sample well plates were incubated at 37°C and removed for testing at intervals of 1, 2, 5, and 14 days. At each of these intervals, the following steps took place:

1) Porous inserts and media were transferred to other wells (Figures 8, 9)
2) 100 µl of PrestoBlue and 900 µl of DMEM were added to the cellular wells to establish a fluorescent marker and maintain cell health, respectively
3) Cellular proliferation was evaluated using a Synergy HTX microplate reader (BioTek) to detect fluorescence, in triplicate of triplicated samples (Figure 10)
4) DPSCs were transferred from the microplate back to their original wells, along with the corresponding media and inserts

Every 3 days, half of the media was replaced with fresh media to maintain a healthy environment for cell growth while retaining the dissolved material that originated from the gel.

Figure 8: The transfer of inserts and media to new wells as part of the cell proliferation test protocol

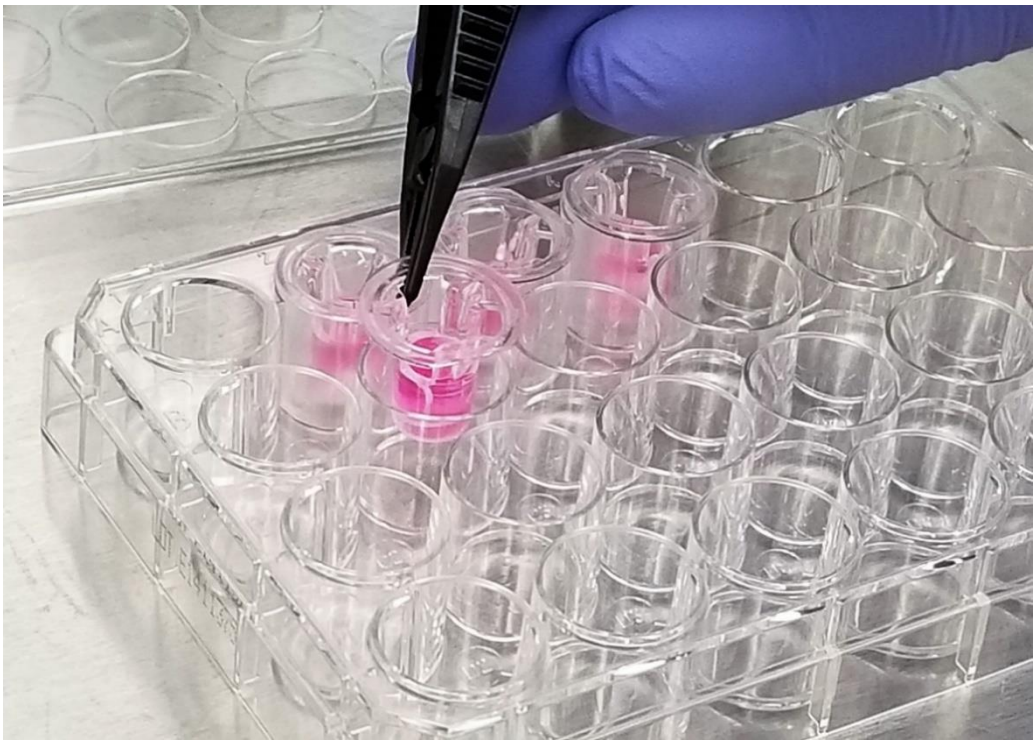

Figure 9: Porous inserts used in cell proliferation testing. *Samples were loaded in the inserts and submerged in cell growth media*

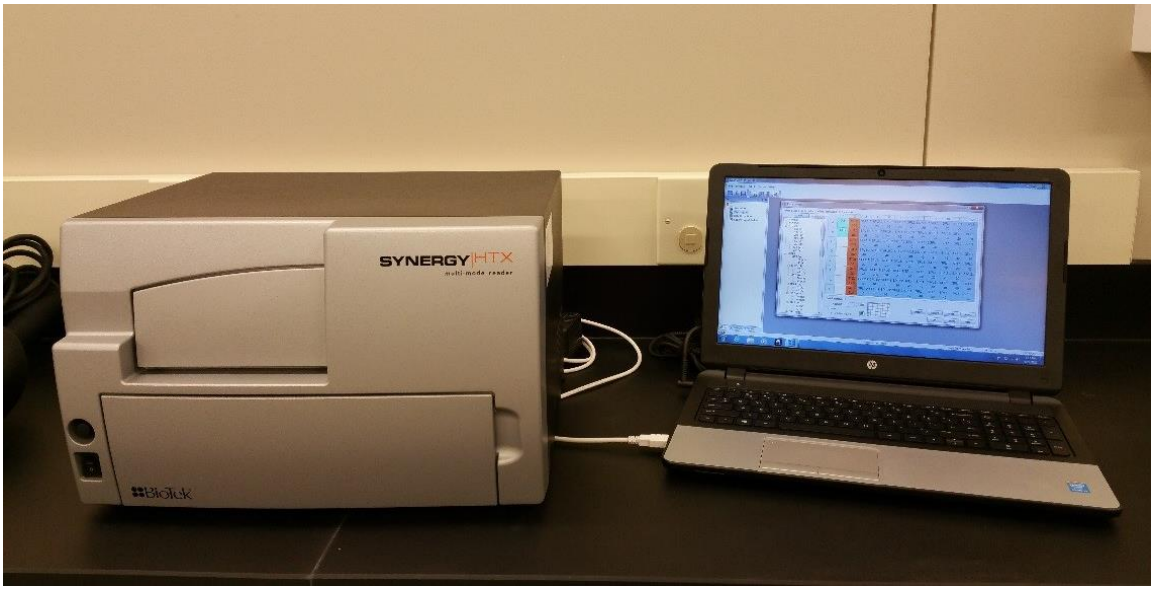

Figure 10: Synergy HTX microplate reader used to quantify cell proliferation

As a means for negative control comparisons, a supplemental experiment was performed using UltraCal XS $Ca(OH)_2$ paste (Ultradent). Test groups were prepared by measuring paste amount by weight, using groups with 0.15 g, 0.1 g, 0.05 g, and 0 g (control) of the $Ca(OH)_2$ paste. These samples were loaded, in triplicate, into the porous inserts using the cell proliferation protocol previously outlined (Figures 11, 12). Samples were tested at intervals of 1, 2, 5, and 14 days using a microplate reader and results were recorded.

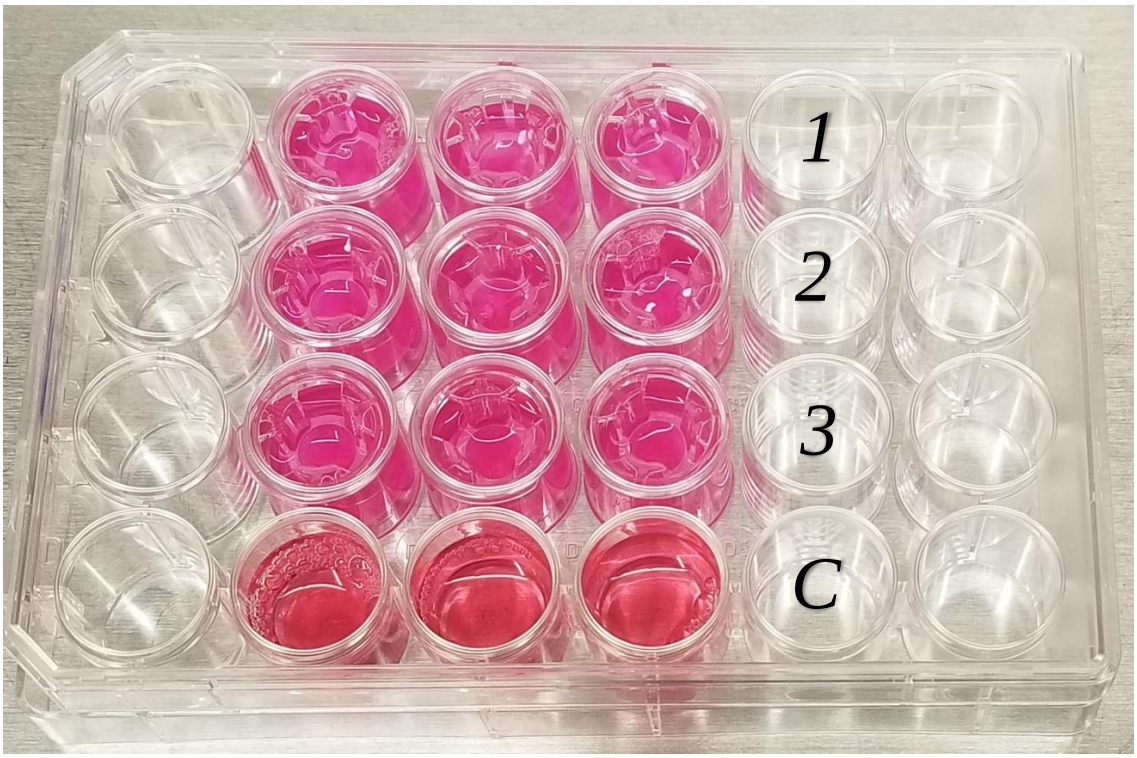

Figure 11: Porous inserts with media at 24-hour test period. *Rows are arranged in triplicate groups as follows: group 1 - 0.15 g $Ca(OH)_2$; group 2 - 0.10 g $Ca(OH)_2$; group 3 - 0.05 g $Ca(OH)_2$; group C - control without $Ca(OH)_2$. It was noted that the growth media in groups containing $Ca(OH)_2$ changed to a bright pink color.*

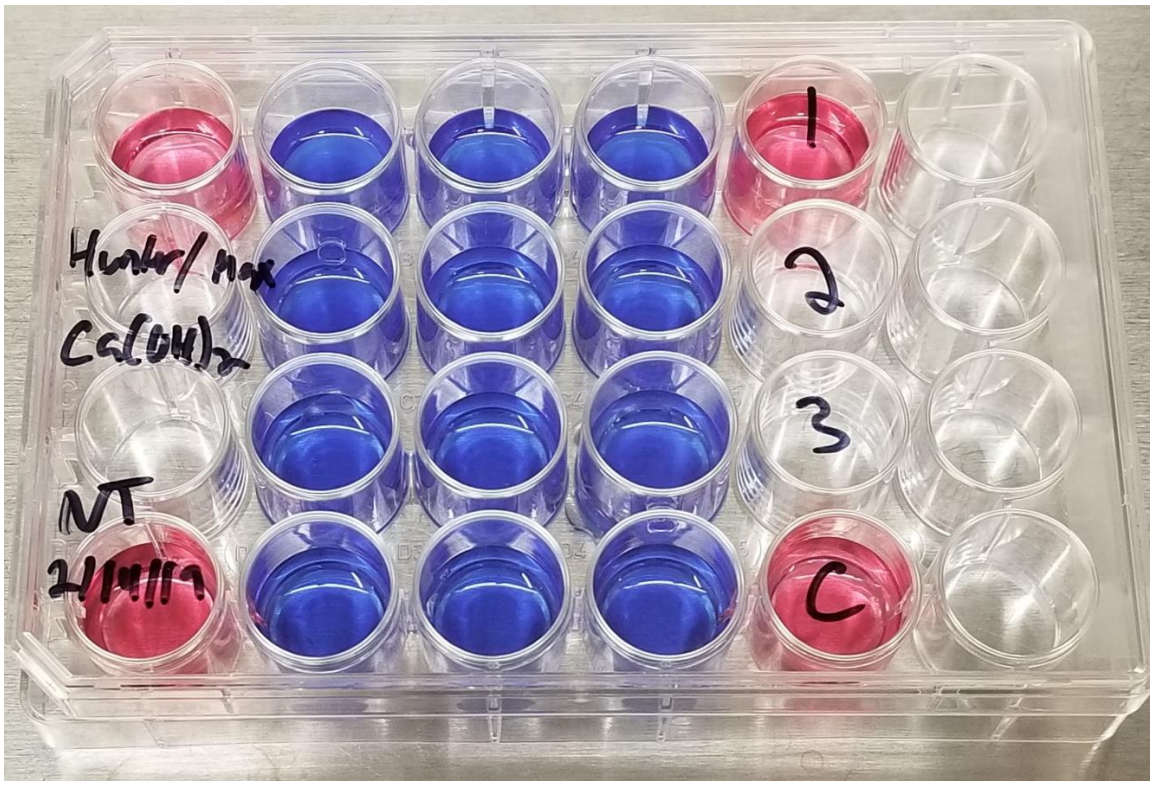

Figure 12: Wells containing DPSCs and PrestoBlue/DMEM at 24 hours. *After removal of porous inserts and corresponding media outlined in Figure 11, PrestoBlue/DMEM solution was added to samples containing DPSCs. Group 1 - cells from wells with 0.15 g $Ca(OH)_2$, group 2 – cells from wells with 0.10 g $Ca(OH)_2$, group 3 – cells from wells with 0.05 g $Ca(OH)_2$, group C – cells from control wells without $Ca(OH)_2$. Note: wells with fresh media were maintained around the perimeter to minimize evaporation. Each well with PrestoBlue was dispensed in triplicate into a microwell plate and placed in the Synergy HTX reader.*

RESULTS

Preliminary Study Data to Determine Gelation Temperature

As stated previously, initial rheometry tests were performed using both single-frequency and multiple-frequency settings. This provided a means for comparison between rheometry settings to determine an accurate approach for further tests. In addition, a broad range of β-GP concentrations were evaluated to determine the amount needed in order to reach an ideal T_{onset}.

Single-frequency rheometry demonstrated that 1.5% w/v chitosan with 300 mg/ml β-GP contained an ideal T_{onset} within the target range (Figure 13). In order to objectively

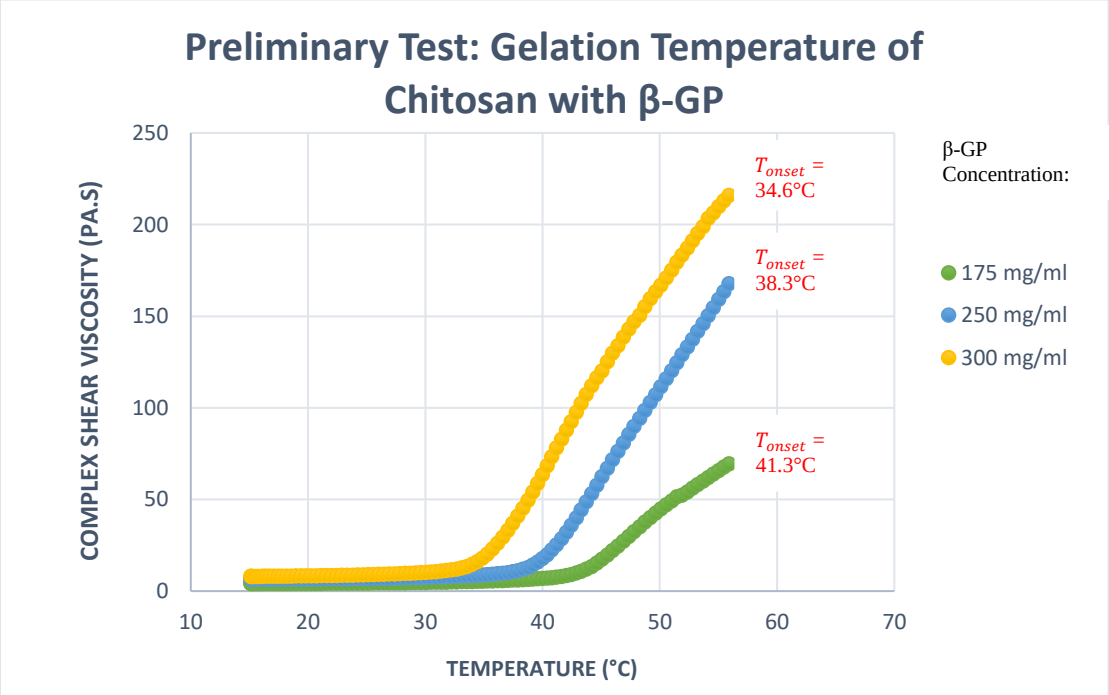

Figure 13: Single-frequency rheometric graph indicating the relative gelation temperatures of 1.5% w/v chitosan with various concentrations of β-GP. *Note: gelation onset temperature (T_{onset}) is indicated in red text. The only formulation with T_{onset} below 37°C contained 300 mg/ml β-GP.*

estimate T_{onset}, the steady slopes of complex shear viscosity in the sol phase were compared to those in the gel phase (Figure 14). The T_{onset} was determined by calculating the point of intersection with these two slopes.

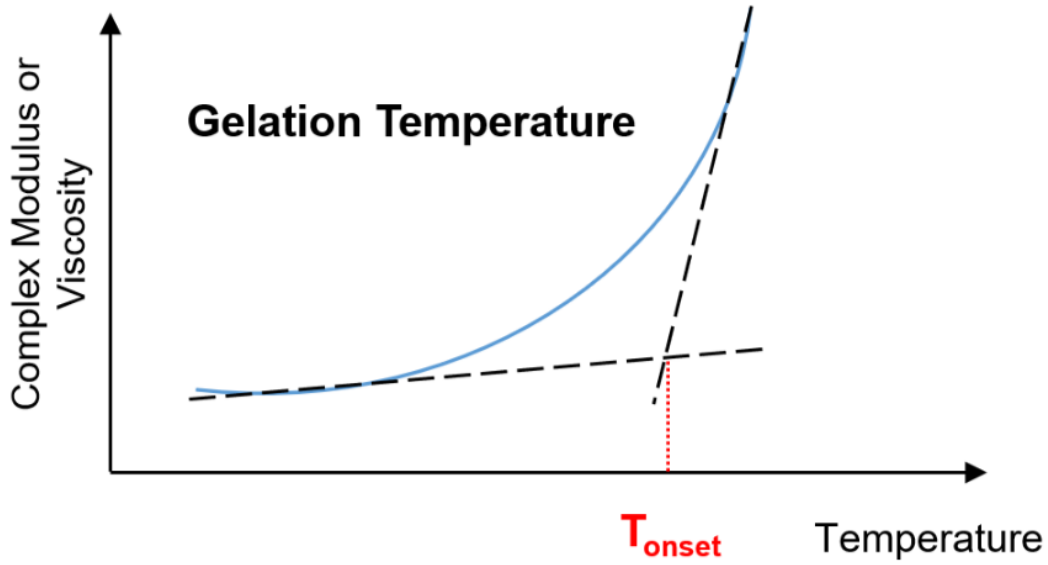

Figure 14: Diagram demonstrating the method for determining the T_{onset} for samples tested with single-frequency rheometry. *The T_{onset} is determined by calculating the point of intersection between sol phase and gel phase slopes.*

Considering the line equation $y = mx + b$, where m is the slope and b the y-intercept, the following mathematic reasoning was used to determine the gelation onset temperature:

$$T_{onset} = \left| \frac{b_{gel} - b_{sol}}{m_{gel} - m_{sol}} \right|$$

Using this formula, the T_{onset} for the optimized hydrogel was 34.6°C (Figure 15).

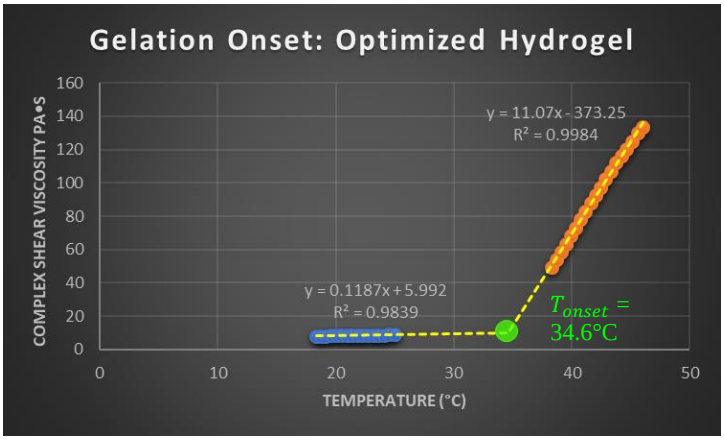

Figure 15: Gelation onset temperature for the optimized hydrogel using single-frequency rheometry*. Intersecting slopes (yellow lines) indicate that* T_{onset} *= 34.6°C, as shown by the green indicator.*

The results from evaluating the optimized chitosan at multiple frequencies showed that the T_{onset} was approximately 32°C-33°C. This was found by plotting the tan(δ) at corresponding temperatures between 30°C and 37°C among 11 frequencies ranging from 1.0 Hz to 10.0 Hz. The T_{onset} was analyzed visually along a graph by determining the range at which most of the points intersect (Figure 16).

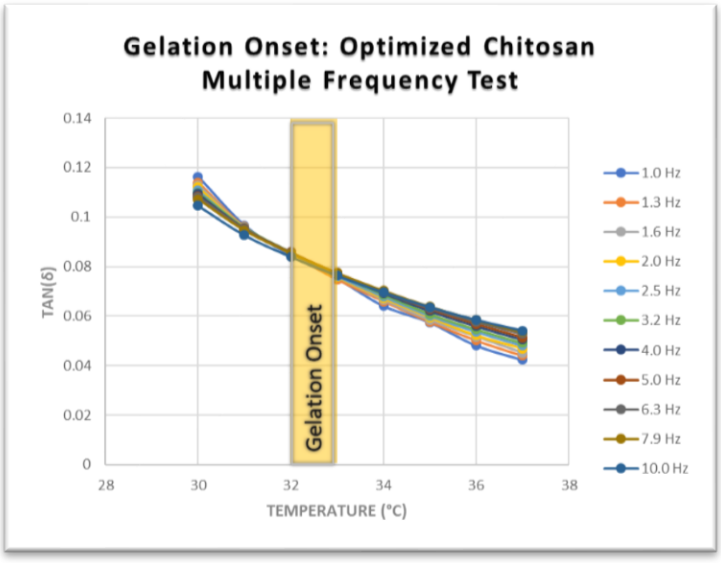

Figure 16: Multiple-frequency rheometric graph indicating gelation temperature of 1.5% w/v chitosan with 300 mg/ml β-GP.

In comparison of the two rheometer parameters (single vs multiple-frequency), both tests yielded comparable findings. Due to the simplicity of using single-frequency settings, it was determined to proceed with this method throughout the remainder of the study.

Gelation Properties of Experimental Hydrogels

Data was collected and plotted on each of the remaining samples. The addition of CDs had a negligible effect on the T_{onset} compared to that of the plain optimized chitosan. In concentrations of both 25 µg/ml CDs and 50 µg/ml CDs, the measured T_{onset} was 34.7°C (Figure 17).

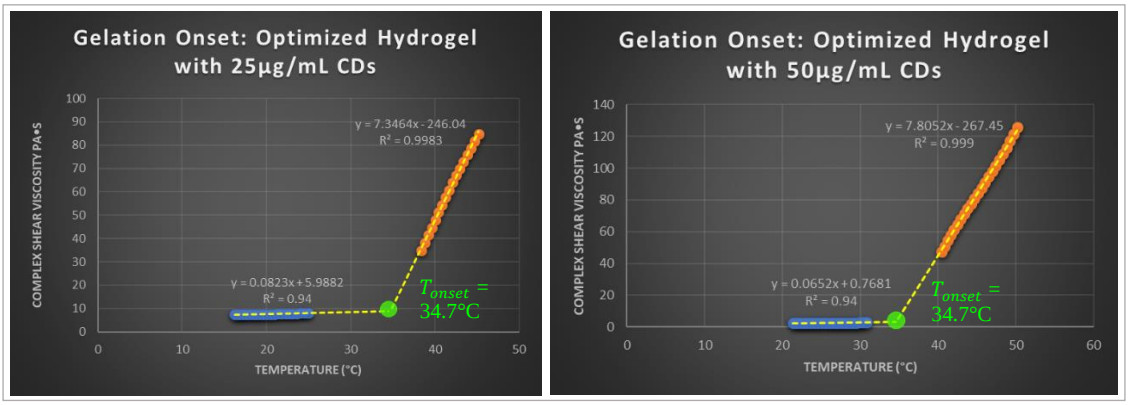

Figure 17: Gelation onset temperature for the optimized hydrogel with the addition of 25 µg/ml CDs and 50 µg/ml CDs. *Intersecting slopes (yellow lines) indicate that T_{onset} = 34.7°C for each sample, as shown by the green indicator.*

Cell Proliferation of Experimental Hydrogels and Calcium Hydroxide

Results from the cell proliferation tests were recorded and graphed for visual interpretation. Since the trials for experimental hydrogels and $Ca(OH)_2$ were performed separately, a method calculating percentage change was used to compare each sample to their control group (Table 3).

Table 3: Proliferation results for experimental gels and $Ca(OH)_2$

Mean Fluorescence of Triplicated Samples and Percent Change							
Experimental Gels							
	Day 1	**Day 2**	**Day 5**	**Day 14**	**% Change Days 1 & 2**	**% Change Days 2 & 5**	**% Change Days 5 & 14**
Optimized Chitosan, 25µg/ml CDs	760.9 ± 39.6	886.3 ± 34.9	1802.4 ± 290.1	4666.8 ± 737.5	16%	103%	159%
Optimized Chitosan, 50µg/ml CDs	752.6 ± 33.2	950.1 ± 67.6	2018.2 ± 68.9	4747.1 ± 613.4	26%	112%	135%
Optimized Chitosan	818.6 ± 95.7	917.9 ± 26.2	2779.7 ± 216.7	5768.2 ± 1097.7	12%	203%	108%
Control (No Gel)	924.8 ± 62.1	843.3 ± 52.9	3936.1 ± 75.5	5411.2 ± 122.2	-9%	367%	37%
Calcium Hydroxide							
	Day 1	**Day 2**	**Day 5**	**Day 14**	**% Change Days 1 & 2**	**% Change Days 2 & 5**	**% Change Days 5 & 14**
UltraCal XS 0.15 g	527.7 ± 7.9	541.2 ± 7.0	517 ± 7.9	518 ± 4.8	3%	-4%	0%
UltraCal XS 0.10 g	528.6 ± 4.6	536.3 ± 3.4	514.7 ± 0.6	518.2 ± 5.8	1%	-4%	1%
UltraCal XS 0.05g	529.9 ± 2.4	538.6 ± 6.3	520.7 ± 0.6	550.3 ± 34.3	2%	-3%	6%
Control (No Paste)	783.3 ± 28.4	854.1 ± 31.0	1721.6 ± 136.5	1296 ± 64.0	9%	102%	-25%

The experimental hydrogels exhibited a more favorable environment for cell proliferation. In general, all samples remained relatively unchanged between days 1 and 2. As evidenced by Table 3, the material with the greatest overall proliferation was the plain optimized chitosan. Cells exposed to this gel proliferated early and rapidly. Between days 2 and 5, a 203% increase in fluorescence values was detected. These values increased an additional 108% between days 5 and 14. The gels containing CDs performed nearly as well, with steady percentage increases exceeding 100% at these same intervals.

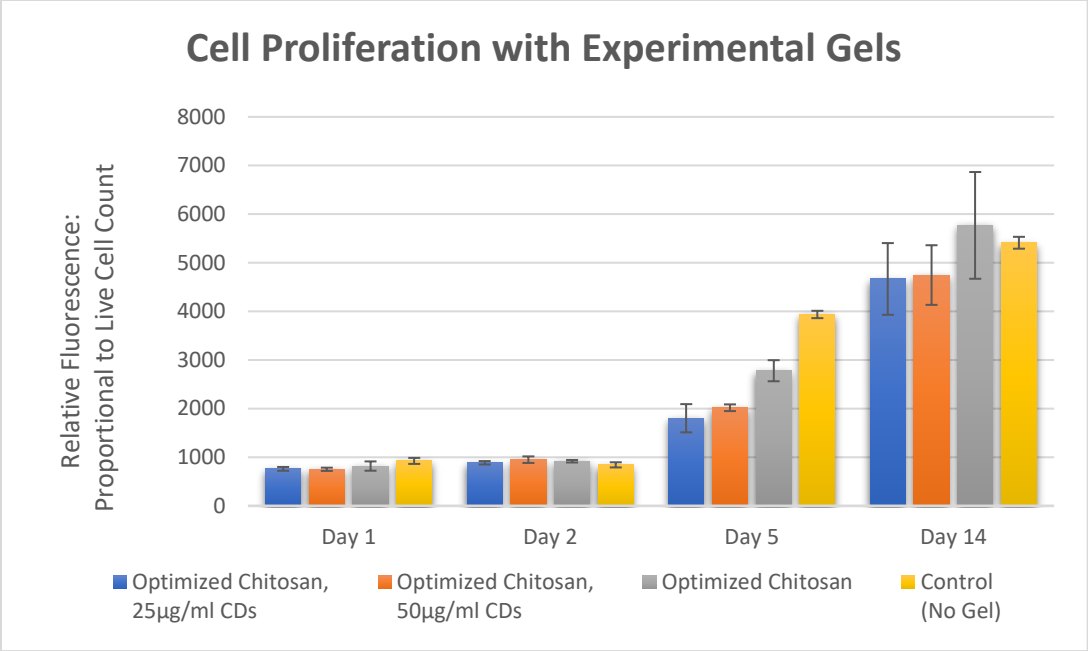

Figure 18: Cell proliferation results for the experimental gels. *Recorded data points are based off fluorescence readings of cellular interaction with PrestoBlue. Error bars are indicative of the standard deviation of the gel samples.*

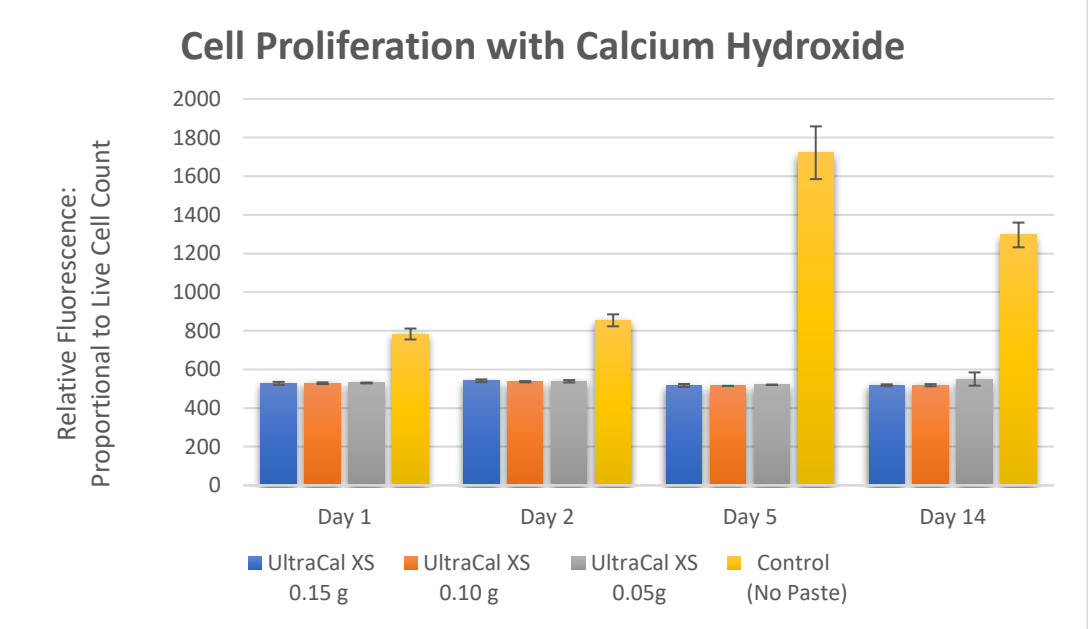

Figure 19: Cell proliferation results for UltraCal XS $Ca(OH)_2$ paste. *Recorded data points are based off fluorescence readings of cellular interaction with PrestoBlue. Error bars are indicative of the standard deviation of each group.*

In each $Ca(OH)_2$ sample, results showed little or no proliferation of DPSCs at each of the tested intervals (Figure 18). Between days 1 and 2, the percentage increase in proliferation was 1-3%. Each sample decreased in proliferation by 3-4% from days 2 to 5. At the final two time intervals (day 5 to 14), cell proliferation remained low at 0-6%.

DISCUSSION

$Ca(OH)_2$ and TAP have long been used as medicaments in endodontics. These materials, however, have some disadvantages that were previously discussed. In short, such drawbacks include pathogen resistance, tight adherence to dentin, and/or cytotoxicity concerns (35, 36, 39-42, 50). Though TAP is often regarded as cytotoxic to stem cells, there is no consensus on cytotoxicity with $Ca(OH)_2$ (43-45).

In all tested concentrations, $Ca(OH)_2$ drastically suppressed cell proliferation throughout the 14-day test period. This indicates that $Ca(OH)_2$ paste possesses a potent and long-lasting inhibitory effect on DPSCs. As it relates to regenerative endodontics (when proliferation of stem cells is paramount), these qualities are seemingly detrimental. Chitosan hydrogels could provide a vehicle for disinfection within the canal space that is "more kind" to stem cells, creating a potential for improved healing outcomes.

There is a reasonable explanation for the claims made by some researchers that $Ca(OH)_2$ is non-toxic, considering this study shows the contrary. To our knowledge, this study is the first to evaluate cell proliferation with $Ca(OH)_2$ using porous inserts. When testing proliferation, this method provides a more realistic representation of testing the impact of the paste on stem cell growth over time. Porous inserts facilitate indirect contact of $Ca(OH)_2$ with the cells via diffusion through media. At each tested interval, there is little risk of removing the original paste. This model simulates a single administration of $Ca(OH)_2$ paste, similar to the manner in which it is delivered in most clinical applications. Additionally, the inserts allow for consistent evaluation at each test interval without disrupting the cell culture. This method seems more accurate than the common approach of mixing $Ca(OH)_2$ powder into the growth media.

The positive control groups used in these proliferation tests demonstrated the growth pattern of DPSCs without the influence of a test gel or paste. It is noteworthy to address the phenomenon that cell growth was hindered between days 5 and 14 within each control group. This is likely due to cells reaching confluency within their respective well plates. Cells require room to proliferate and the population is negatively impacted without adequate space for expansion.

Within each control group, cell counts increased most rapidly between days 2 and 5. Throughout this same time period, the optimized chitosan group promoted exceptional proliferation. Based upon the findings presented in this study, this formulation seems to be the most biocompatible of all those tested. When CDs are added to the optimized injectable chitosan, cellular growth may be slightly lessened. This limitation seems minimal in comparison to the drastic inhibition presented by $Ca(OH)_2$.

In this study, CDs were functionalized with spermidine. The results presented indicate that this combination is relatively biocompatible when incorporated into a chitosan gel. Without further testing, it is difficult to quantify the proper therapeutic concentrations for endodontic use, as well as the factors that contribute to the slight decrease in cell proliferation. Given that CDs are inherently biocompatible (99, 100), it is logical that the level of cytotoxicity is more related to the functionalized surface than the CD nanoparticles themselves.

The use of CDs for dental purposes is exciting and warrants continued study. CDs are incredibly versatile nanoparticles, containing a peripheral surface that can be functionalized with countless surface schemes. Previous researchers have outlined the benefits of conjugating antibiotics with the CD surface, including prolonged drug release

and enhanced efficacy (90, 101). Future experimentation may explore a wider spectrum of functional surface groups, particularly antibiotics and antibacterial agents.

This study also demonstrates that chitosan hydrogels, with or without carbon dots, can be optimized to a specific T_{onset}. Creating a hydrogel with a T_{onset} between 30°C and 37°C has promising implications, particularly for biomedical use. In concept, this would allow for the hydrogel to be injected into the human body in an aqueous sol phase, then undergo a transition to a more rigid and stable gel at body temperature. As this applies to endodontics, it is thought that such a hydrogel could function as a means for medicament delivery that resists washout.

Within the limitations of this study, many attributes of this novel material have not been investigated. The antibacterial properties of each prepared hydrogel are still unknown. Previous authors have found that chitosan, as well as spermidine-functionalized carbon dots, possess inherent antibacterial qualities (59, 60, 102). Though untested in this study, it is logical that the hydrogel formulations would perform favorably against bacterial and fungal pathogens. Antibacterial and antifungal tests could more definitively determine how these materials interact with such pathogens and indicate whether other chemical additives should be considered.

Further studies could also enhance what is known about the physical properties of the optimized chitosan hydrogel. Factors such as dimensional stability over time, thermoreversibility, and shelf life are still unknown. Despite these untested material attributes, the formulated chitosan hydrogels presented in this study possess qualities that are particularly exciting and should be studied in greater detail.

CONCLUSION

Chitosan/CD hydrogels may be a promising medicament for endodontic use and a viable alternative to either $Ca(OH)_2$ or TAP in regenerative endodontic cases. The CD structure contains surface characteristics that are open to customization for individual therapeutic needs. This hydrogel is thermally responsive and becomes a solid gel when administered at human body temperature. Further studies are warranted to determine its antibacterial properties, as well as other factors to understand how it may interact in the root canal space.

BIBLIOGRAPHY

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral surgery, oral medicine, and oral pathology 1965;20:340-349.

2. Moller AJ, Fabricius L, Dahlen G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. Scandinavian journal of dental research 1981;89(6):475-484.

3. Ricucci D, Siqueira JF, Jr. Biofilms and apical periodontitis: study of prevalence and association with clinical and histopathologic findings. J Endod 2010;36(8):1277-1288.

4. Sjogren U, Figdor D, Persson S, Sundqvist G. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997;30(5):297-306.

5. Sjogren U, Hagglund B, Sundqvist G, Wing K. Factors affecting the long-term results of endodontic treatment. J Endod 1990;16(10):498-504.

6. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 1998;85(1):86-93.

7. Nair PN, Sjogren U, Krey G, Kahnberg KE, Sundqvist G. Intraradicular bacteria and fungi in root-filled, asymptomatic human teeth with therapy-resistant periapical lesions: a long-term light and electron microscopic follow-up study. J Endod 1990;16(12):580-588.

8. Tollemar V, Collier ZJ, Mohammed MK, Lee MJ, Ameer GA, Reid RR. Stem cells, growth factors and scaffolds in craniofacial regenerative medicine. Genes & diseases 2016;3(1):56-71.

9. Kling M, Cvek M, Mejare I. Rate and predictability of pulp revascularization in therapeutically reimplanted permanent incisors. Endodontics & dental traumatology 1986;2(3):83-89.

10. Wigler R, Kaufman AY, Lin S, Steinbock N, Hazan-Molina H, Torneck CD. Revascularization: a treatment for permanent teeth with necrotic pulp and incomplete root development. J Endod 2013;39(3):319-326.

11. Lenzi R, Trope M. Revitalization procedures in two traumatized incisors with different biological outcomes. J Endod 2012;38(3):411-414.

12. Weisleder R, Benitez CR. Maturogenesis: is it a new concept? J Endod 2003;29(11):776-778.

13. Law AS. Considerations for regeneration procedures. J Endod 2013;39(3 Suppl):S44-56.

14. Diogenes AR, Ruparel NB, Teixeira FB, Hargreaves KM. Translational science in disinfection for regenerative endodontics. J Endod 2014;40(4 Suppl):S52-57.

15. Lovelace TW, Henry MA, Hargreaves KM, Diogenes A. Evaluation of the delivery of mesenchymal stem cells into the root canal space of necrotic immature teeth after clinical regenerative endodontic procedure. J Endod 2011;37(2):133-138.

16. Jung IY, Lee SJ, Hargreaves KM. Biologically based treatment of immature permanent teeth with pulpal necrosis: a case series. J Endod 2008;34(7):876-887.

17. AAE. Glossary of Endodontic Terms. In. 9th ed.; 2016.

18. Figini L, Lodi G, Gorni F, Gagliani M. Single versus multiple visits for endodontic treatment of permanent teeth: a Cochrane systematic review. J Endod 2008;34(9):1041-1047.

19. Nair PN, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after "one-visit" endodontic treatment. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 2005;99(2):231-252.

20. Paredes-Vieyra J, Enriquez FJ. Success rate of single- versus two-visit root canal treatment of teeth with apical periodontitis: a randomized controlled trial. J Endod 2012;38(9):1164-1169.

21. Paula-Silva FW, da Silva LA, Kapila YL. Matrix metalloproteinase expression in teeth with apical periodontitis is differentially modulated by the modality of root canal treatment. J Endod 2010;36(2):231-237.

22. Hermann BW. Calcium hydroxide as a mean for treatment and filling of the root canals. Würzburg University, Med Diss V 1920.

23. Siqueira JF, Jr., Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J 1999;32(5):361-369.

24. de Andrade Ferreira FB, Silva ESPA, do Vale MS, de Moraes IG, Granjeiro JM. Evaluation of pH levels and calcium ion release in various calcium hydroxide endodontic dressings. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 2004;97(3):388-392.

25. Safavi KE, Nichols FC. Alteration of biological properties of bacterial lipopolysaccharide by calcium hydroxide treatment. J Endod 1994;20(3):127-129.

26. Tronstad L, Andreasen JO, Hasselgren G, Kristerson L, Riis I. pH changes in dental tissues after root canal filling with calcium hydroxide. J Endod 1981;7(1):17-21.

27. Hasselgren G, Olsson B, Cvek M. Effects of calcium hydroxide and sodium hypochlorite on the dissolution of necrotic porcine muscle tissue. J Endod 1988;14(3):125-127.

28. Estrela C, Pimenta FC, Ito IY, Bammann LL. Antimicrobial evaluation of calcium hydroxide in infected dentinal tubules. J Endod 1999;25(6):416-418.

29. Valera MC, de Moraes Rego J, Jorge AO. Effect of sodium hypochlorite and five intracanal medications on Candida albicans in root canals. J Endod 2001;27(6):401-403.

30. Basson NJ, Tait CM. Effectiveness of three root canal medicaments to eliminate Actinomyces israelii from infected dentinal tubules in vitro. SADJ : journal of the South African Dental Association = tydskrif van die Suid-Afrikaanse Tandheelkundige Vereniging 2001;56(11):499-501.

31. Basrani B, Tjaderhane L, Santos JM, Pascon E, Grad H, Lawrence HP, et al. Efficacy of chlorhexidine- and calcium hydroxide-containing medicaments against Enterococcus faecalis in vitro. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 2003;96(5):618-624.

32. Gomes BP, Montagner F, Berber VB, Zaia AA, Ferraz CC, de Almeida JF, et al. Antimicrobial action of intracanal medicaments on the external root surface. Journal of dentistry 2009;37(1):76-81.

33. Ercan E, Dalli M, Dulgergil CT. In vitro assessment of the effectiveness of chlorhexidine gel and calcium hydroxide paste with chlorhexidine against Enterococcus faecalis and Candida albicans. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 2006;102(2):e27-31.

34. Lee JK, Park YJ, Kum KY, Han SH, Chang SW, Kaufman B, et al. Antimicrobial efficacy of a human beta-defensin-3 peptide using an Enterococcus faecalis dentine infection model. Int Endod J 2013;46(5):406-412.

35. Waltimo TM, Siren EK, Orstavik D, Haapasalo MP. Susceptibility of oral Candida species to calcium hydroxide in vitro. Int Endod J 1999;32(2):94-98.

36. Orstavik D, Haapasalo M. Disinfection by endodontic irrigants and dressings of experimentally infected dentinal tubules. Endodontics & dental traumatology 1990;6(4):142-149.

37. Evans M, Davies JK, Sundqvist G, Figdor D. Mechanisms involved in the resistance of Enterococcus faecalis to calcium hydroxide. Int Endod J 2002;35(3):221-228.

38. Kim D, Kim E. Antimicrobial effect of calcium hydroxide as an intracanal medicament in root canal treatment: a literature review - Part I. In vitro studies. Restorative dentistry & endodontics 2014;39(4):241-252.

39. Orucoglu H, Cobankara FK. Effect of Unintentionally Extruded Calcium Hydroxide Paste Including Barium Sulfate as a Radiopaquing Agent in Treatment of Teeth with Periapical Lesions: Report of a Case. Journal of Endodontics 2008;34(7):888-891.

40. Sharma S, Hackett R, Webb R, Macpherson D, Wilson A. Severe tissue necrosis following intra-arterial injection of endodontic calcium hydroxide: a case series. Oral surgery, oral medicine, oral pathology, oral radiology, and endodontics 2008;105(5):666-669.

41. Banchs F, Trope M. Revascularization of immature permanent teeth with apical periodontitis: new treatment protocol? J Endod 2004;30(4):196-200.

42. Hargreaves KM, Giesler T, Henry M, Wang Y. Regeneration potential of the young permanent tooth: what does the future hold? J Endod 2008;34(7 Suppl):S51-56.

43. Badr AE, Omar N, Badria FA. A laboratory evaluation of the antibacterial and cytotoxic effect of Liquorice when used as root canal medicament. International Endodontic Journal 2011;44(1):51-58.

44. Araújo LB, Cosme-Silva L, Fernandes AP, Oliveira TMd, Cavalcanti BdN, Gomes Filho JE, et al. Effects of mineral trioxide aggregate, BiodentineTM and calcium hydroxide on viability, proliferation, migration and differentiation of stem cells from human exfoliated deciduous teeth. Journal of applied oral science : revista FOB 2018;26:e20160629-e20160629.

45. Ji YM, Jeon SH, Park JY, Chung JH, Choung YH, Choung PH. Dental stem cell therapy with calcium hydroxide in dental pulp capping. Tissue engineering. Part A 2010;16(6):1823-1833.

46. Andreasen JO, Farik B, Munksgaard EC. Long-term calcium hydroxide as a root canal dressing may increase risk of root fracture. Dental traumatology : official publication of International Association for Dental Traumatology 2002;18(3):134-137.

47. Hoshino E, Kurihara-Ando N, Sato I, Uematsu H, Sato M, Kota K, et al. In-vitro antibacterial susceptibility of bacteria taken from infected root dentine to a mixture of ciprofloxacin, metronidazole and minocycline. Int Endod J 1996;29(2):125-130.

48. Adl A, Hamedi S, Sedigh Shams M, Motamedifar M, Sobhnamayan F. The ability of triple antibiotic paste and calcium hydroxide in disinfection of dentinal tubules. Iranian endodontic journal 2014;9(2):123-126.

49. Thibodeau B, Teixeira F, Yamauchi M, Caplan DJ, Trope M. Pulp revascularization of immature dog teeth with apical periodontitis. J Endod 2007;33(6):680-689.

50. Berkhoff JA, Chen PB, Teixeira FB, Diogenes A. Evaluation of triple antibiotic paste removal by different irrigation procedures. J Endod 2014;40(8):1172-1177.

51. Ruparel NB, Teixeira FB, Ferraz CC, Diogenes A. Direct effect of intracanal medicaments on survival of stem cells of the apical papilla. J Endod 2012;38(10):1372-1375.

52. Sabrah AH, Yassen GH, Liu WC, Goebel WS, Gregory RL, Platt JA. The effect of diluted triple and double antibiotic pastes on dental pulp stem cells and established Enterococcus faecalis biofilm. Clinical oral investigations 2015;19(8):2059-2066.

53. Nagata JY, Gomes BP, Rocha Lima TF, Murakami LS, de Faria DE, Campos GR, et al. Traumatized immature teeth treated with 2 protocols of pulp revascularization. J Endod 2014;40(5):606-612.

54. Taylor PM. Biological matrices and bionanotechnology. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 2007;362(1484):1313-1320.

55. Silva SS, Mano JF, Reis RL. Potential applications of natural origin polymer-based systems in soft tissue regeneration. Critical reviews in biotechnology 2010;30(3):200-221.

56. Liu X, Holzwarth JM, Ma PX. Functionalized synthetic biodegradable polymer scaffolds for tissue engineering. Macromolecular bioscience 2012;12(7):911-919.

57. Taylor MS, Daniels AU, Andriano KP, Heller J. Six bioabsorbable polymers: in vitro acute toxicity of accumulated degradation products. Journal of applied biomaterials : an official journal of the Society for Biomaterials 1994;5(2):151-157.

58. Barborka BJ, Woodmansey KF, Glickman GN, Schneiderman E, He J. Long-term Clinical Outcome of Teeth Obturated with Resilon. J Endod 2017;43(4):556-560.

59. Das B, Patra S. Chapter 1 - Antimicrobials: Meeting the Challenges of Antibiotic Resistance Through Nanotechnology. In: Ficai A, Grumezescu AM, editors. Nanostructures for Antimicrobial Therapy. Elsevier; 2017. p. 1-22.

60. Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydrate research 2004;339(16):2693-2700.

61. Miguel SP, Ribeiro MP, Brancal H, Coutinho P, Correia IJ. Thermoresponsive chitosan-agarose hydrogel for skin regeneration. Carbohydrate polymers 2014;111:366-373.

62. Qasim SB, Zafar MS, Najeeb S, Khurshid Z, Shah AH, Husain S, et al. Electrospinning of Chitosan-Based Solutions for Tissue Engineering and Regenerative Medicine. International journal of molecular sciences 2018;19(2).

63. Ye K, Felimban R, Traianedes K, Moulton SE, Wallace GG, Chung J, et al. Chondrogenesis of infrapatellar fat pad derived adipose stem cells in 3D printed chitosan scaffold. PLoS One 2014;9(6):e99410.

64. Hao T, Wen N, Cao JK, Wang HB, Lu SH, Liu T, et al. The support of matrix accumulation and the promotion of sheep articular cartilage defects repair in vivo by chitosan hydrogels. Osteoarthritis and cartilage 2010;18(2):257-265.

65. Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al. Novel injectable neutral solutions of chitosan form biodegradable gels in situ. Biomaterials 2000;21(21):2155-2161.

66. Huh AJ, Kwon YJ. "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era. Journal of controlled release : official journal of the Controlled Release Society 2011;156(2):128-145.

67. LeCroy GE, Yang S-T, Yang F, Liu Y, Fernando KAS, Bunker CE, et al. Functionalized carbon nanoparticles: Syntheses and applications in optical bioimaging and energy conversion. Coordination Chemistry Reviews 2016;320-321:66-81.

68. Tian L, Ghosh D, Chen W, Pradhan S, Chang X, Chen S. Nanosized Carbon Particles From Natural Gas Soot. Chemistry of Materials 2009;21(13):2803-2809.

69. Ray SC, Saha A, Jana NR, Sarkar R. Fluorescent Carbon Nanoparticles: Synthesis, Characterization, and Bioimaging Application. The Journal of Physical Chemistry C 2009;113(43):18546-18551.

70. Qiao ZA, Wang Y, Gao Y, Li H, Dai T, Liu Y, et al. Commercially activated carbon as the source for producing multicolor photoluminescent carbon dots by chemical oxidation. Chemical communications (Cambridge, England) 2010;46(46):8812-8814.

71. Dong Y, Zhou N, Lin X, Lin J, Chi Y, Chen G. Extraction of Electrochemiluminescent Oxidized Carbon Quantum Dots from Activated Carbon. Chemistry of Materials 2010;22(21):5895-5899.

72. Sun YP, Zhou B, Lin Y, Wang W, Fernando KA, Pathak P, et al. Quantum-sized carbon dots for bright and colorful photoluminescence. Journal of the American Chemical Society 2006;128(24):7756-7757.

73. Hu S-L, Niu K-Y, Sun J, Yang J, Zhao N-Q, Du X-W. One-step synthesis of fluorescent carbon nanoparticles by laser irradiation. Journal of Materials Chemistry 2009;19(4):484-488.

74. Li X, Wang H, Shimizu Y, Pyatenko A, Kawaguchi K, Koshizaki N. Preparation of carbon quantum dots with tunable photoluminescence by rapid laser passivation in ordinary organic solvents. Chemical communications (Cambridge, England) 2011;47(3):932-934.

75. Cao L, Wang X, Meziani MJ, Lu F, Wang H, Luo PG, et al. Carbon dots for multiphoton bioimaging. Journal of the American Chemical Society 2007;129(37):11318-11319.

76. Zhou J, Booker C, Li R, Zhou X, Sham TK, Sun X, et al. An electrochemical avenue to blue luminescent nanocrystals from multiwalled carbon nanotubes (MWCNTs). Journal of the American Chemical Society 2007;129(4):744-745.

77. Shinde DB, Pillai VK. Electrochemical preparation of luminescent graphene quantum dots from multiwalled carbon nanotubes. Chemistry (Weinheim an der Bergstrasse, Germany) 2012;18(39):12522-12528.

78. Bao L, Zhang ZL, Tian ZQ, Zhang L, Liu C, Lin Y, et al. Electrochemical tuning of luminescent carbon nanodots: from preparation to luminescence mechanism. Advanced materials (Deerfield Beach, Fla.) 2011;23(48):5801-5806.

79. Zhu H, Wang X, Li Y, Wang Z, Yang F, Yang X. Microwave synthesis of fluorescent carbon nanoparticles with electrochemiluminescence properties. Chemical Communications 2009(34):5118-5120.

80. Zhai X, Zhang P, Liu C, Bai T, Li W, Dai L, et al. Highly luminescent carbon nanodots by microwave-assisted pyrolysis. Chemical communications (Cambridge, England) 2012;48(64):7955-7957.

81. Gong N, Wang H, Li S, Deng Y, Chen X, Ye L, et al. Microwave-assisted polyol synthesis of gadolinium-doped green luminescent carbon dots as a bimodal nanoprobe. Langmuir : the ACS journal of surfaces and colloids 2014;30(36):10933-10939.

82. Yang ZC, Wang M, Yong AM, Wong SY, Zhang XH, Tan H, et al. Intrinsically fluorescent carbon dots with tunable emission derived from hydrothermal treatment of glucose in the presence of monopotassium phosphate. Chemical communications (Cambridge, England) 2011;47(42):11615-11617.

83. Zhu S, Meng Q, Wang L, Zhang J, Song Y, Jin H, et al. Highly photoluminescent carbon dots for multicolor patterning, sensors, and bioimaging. Angewandte Chemie (International ed. in English) 2013;52(14):3953-3957.

84. Yang Y, Cui J, Zheng M, Hu C, Tan S, Xiao Y, et al. One-step synthesis of amino-functionalized fluorescent carbon nanoparticles by hydrothermal carbonization of chitosan. Chemical communications (Cambridge, England) 2012;48(3):380-382.

85. De B, Karak N. A green and facile approach for the synthesis of water soluble fluorescent carbon dots from banana juice. RSC Advances 2013;3(22):8286-8290.

86. Zhang Z, Hao J, Zhang J, Zhang B, Tang J. Protein as the source for synthesizing fluorescent carbon dots by a one-pot hydrothermal route. RSC Advances 2012;2(23):8599-8601.

87. Wang Y, Hu A. Carbon quantum dots: synthesis, properties and applications. Journal of Materials Chemistry C 2014;2(34):6921-6939.

88. Baker SN, Baker GA. Luminescent Carbon Nanodots: Emergent Nanolights. Angewandte Chemie International Edition 2010;49(38):6726-6744.

89. Dong X, Awak MA, Tomlinson N, Tang Y, Sun YP, Yang L. Antibacterial effects of carbon dots in combination with other antimicrobial reagents. PLoS One 2017;12(9):e0185324.

90. Jijie R, Barras A, Bouckaert J, Dumitrascu N, Szunerits S, Boukherroub R. Enhanced antibacterial activity of carbon dots functionalized with ampicillin combined with visible light triggered photodynamic effects. Colloids and surfaces. B, Biointerfaces 2018;170:347-354.

91. Omidi M, Yadegari A, Tayebi L. Wound dressing application of pH-sensitive carbon dots/chitosan hydrogel. RSC Advances 2017;7(18):10638-10649.

92. Karfa P, Roy E, Patra S, Kumar S, Tarafdar A, Madhuri R, et al. Amino acid derived highly luminescent, heteroatom-doped carbon dots for label-free detection of Cd2+/Fe3+, cell imaging and enhanced antibacterial activity. RSC Advances 2015;5(72):58141-58153.

93. Zhou J, Yang Y, Zhang C-y. A low-temperature solid-phase method to synthesize highly fluorescent carbon nitride dots with tunable emission. Chemical Communications 2013;49(77):8605-8607.

94. Krysmann MJ, Kelarakis A, Dallas P, Giannelis EP. Formation mechanism of carbogenic nanoparticles with dual photoluminescence emission. Journal of the American Chemical Society 2012;134(2):747-750.

95. Sun C, Zhang Y, Wang P, Yang Y, Wang Y, Xu J, et al. Synthesis of Nitrogen and Sulfur Co-doped Carbon Dots from Garlic for Selective Detection of Fe(3.). Nanoscale research letters 2016;11(1):110-110.

96. Chandra S, Patra P, Pathan SH, Roy S, Mitra S, Layek A, et al. Luminescent S-doped carbon dots: an emergent architecture for multimodal applications. Journal of Materials Chemistry B 2013;1(18):2375-2382.

97. Prasad KS, Pallela R, Kim D-M, Shim Y-B. Microwave-Assisted One-Pot Synthesis of Metal-Free Nitrogen and Phosphorus Dual-Doped Nanocarbon for Electrocatalysis and Cell Imaging. Particle & Particle Systems Characterization 2013;30(6):557-564.

98. Yin J-Y, Liu H-J, Jiang S, Chen Y, Yao Y. Hyperbranched Polymer Functionalized Carbon Dots with Multistimuli-Responsive Property. ACS Macro Letters 2013;2(11):1033-1037.

99. Sahu S, Behera B, Maiti TK, Mohapatra S. Simple one-step synthesis of highly luminescent carbon dots from orange juice: application as excellent bio-imaging agents. Chemical Communications 2012;48(70):8835-8837.

100. Sk MP, Jaiswal A, Paul A, Ghosh SS, Chattopadhyay A. Presence of Amorphous Carbon Nanoparticles in Food Caramels. Scientific Reports 2012;2:383.

101. Thakur M, Pandey S, Mewada A, Patil V, Khade M, Goshi E, et al. Antibiotic Conjugated Fluorescent Carbon Dots as a Theranostic Agent for Controlled Drug Release, Bioimaging, and Enhanced Antimicrobial Activity. Journal of Drug Delivery 2014;2014:9.

102. Li Y-J, Harroun SG, Su Y-C, Huang C-F, Unnikrishnan B, Lin H-J, et al. Synthesis of Self-Assembled Spermidine-Carbon Quantum Dots Effective against Multidrug-Resistant Bacteria. Advanced Healthcare Materials 2016;5(19):2545-2554.