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The design and physical properties of an optimized chitosan hydrogel for potential use in endodontics

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Abstract
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.
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.

Keywords: chitosan, hydrogel, endodontics

Introduction

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 inter-appointment calcium hydroxide, when indicated, have become the gold standard. These materials, however, are cytotoxic and necessitate careful use.

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 [1]. When acting as an antimicrobial, chitosan binds to negatively charged cell surfaces to increase bacterial cell wall permeability and cause intracellular leakage [2]. Chitosan is biodegradable and comes in several forms for use in regenerative medicine such as: hydrogels, fibers, sponges, and 3-dimensionally printed scaffolds [1, 2, 3].

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 [4]. 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 [5]. This property would theoretically allow for easy removal within the root canal space by converting the hydrogel back to the aqueous sol phase.

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.

Methodology

A preliminary study was performed Department of Conservative Dentistry & Endodontics, Institute of Dental Sciences, Bareilly 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. 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.

These samples were tested with a Kinexus pro+ shear rheometer. 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."

Results

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 1). In order to objectively estimate T_{onset}, the steady slopes of complex shear viscosity in the sol phase were compared to those in the gel phase (Figure 2). The T_{onset} was determined by calculating the point of intersection with these two slopes.

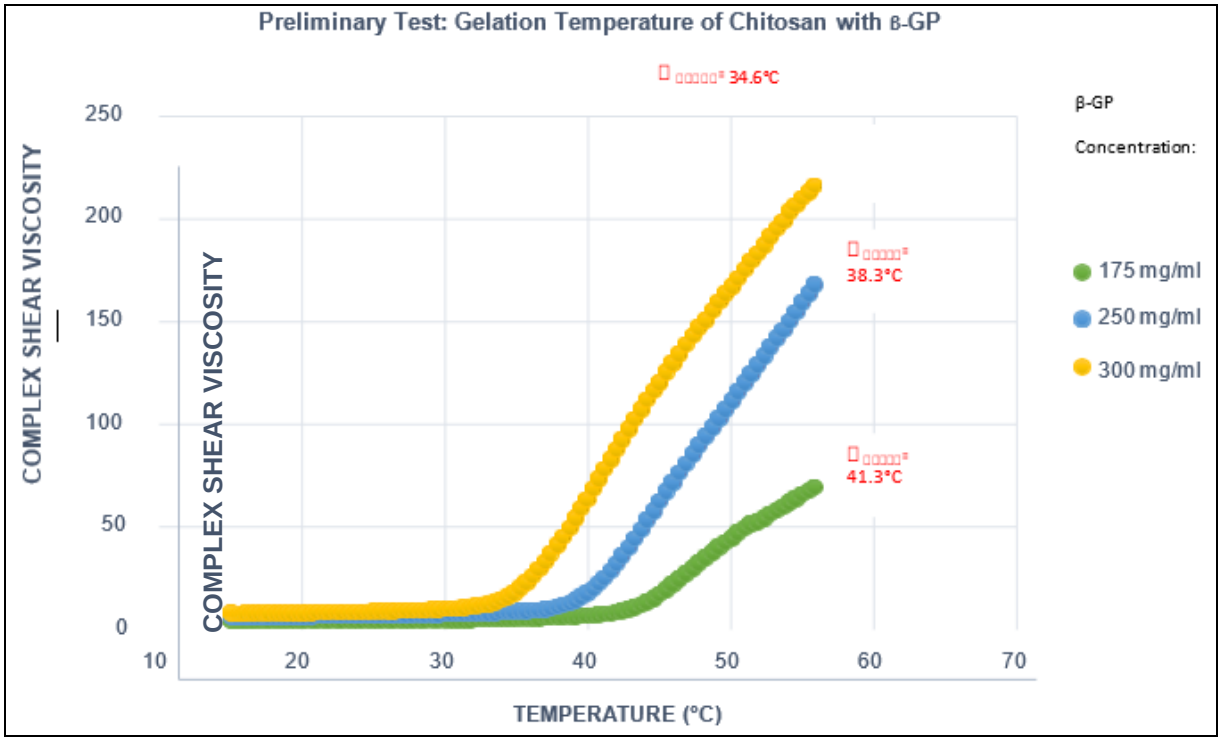

Fig 1: Single-frequency rheometric graph indicating the relative gelation temperatures of 1.5% w/v chitosan with various concentrations of β-GP. Note: gelation onset temperature (T_{onset}) is indicated in red text. The only formulation with T_{onset} below 37°C contained 300 mg/ml β-GP.

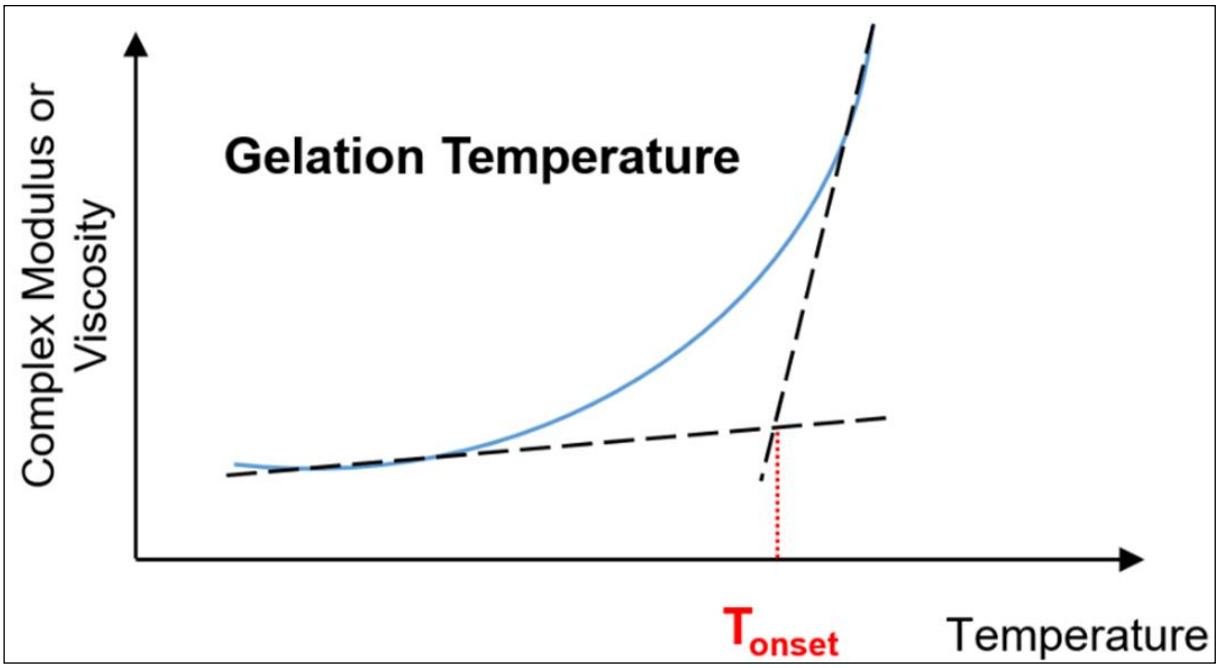

Fig 2: Diagram demonstrating the method for determining the T_{onset} for samples tested with single-frequency rheometry. The T_{onset} is determined by calculating the point of intersection between sol phase and gel phase slopes

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 3).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 3).

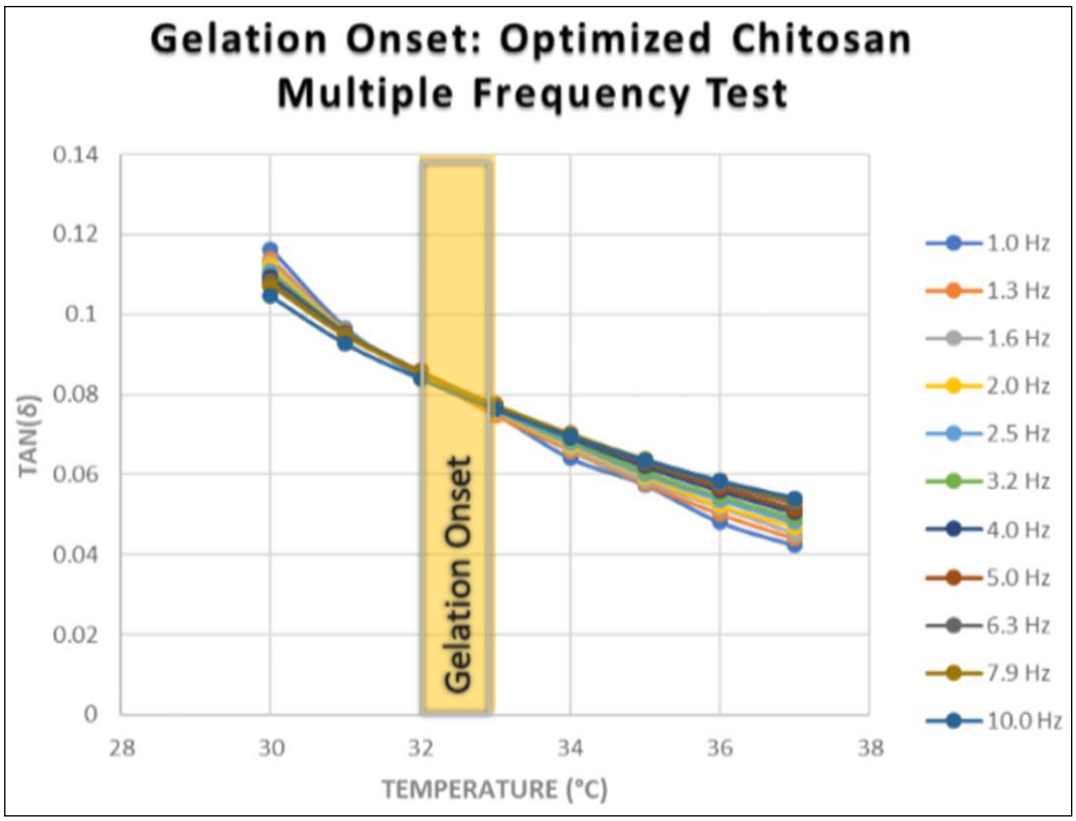

Fig 3: Gelation onset temperature for the optimized hydrogel with the addition of 25 µg/ml CDs and 50 µg/ml CDs. Intersecting slopes (yellow lines) indicate that $T_{onset=}$ 34.7°C for each sample, as shown by the green indicator.

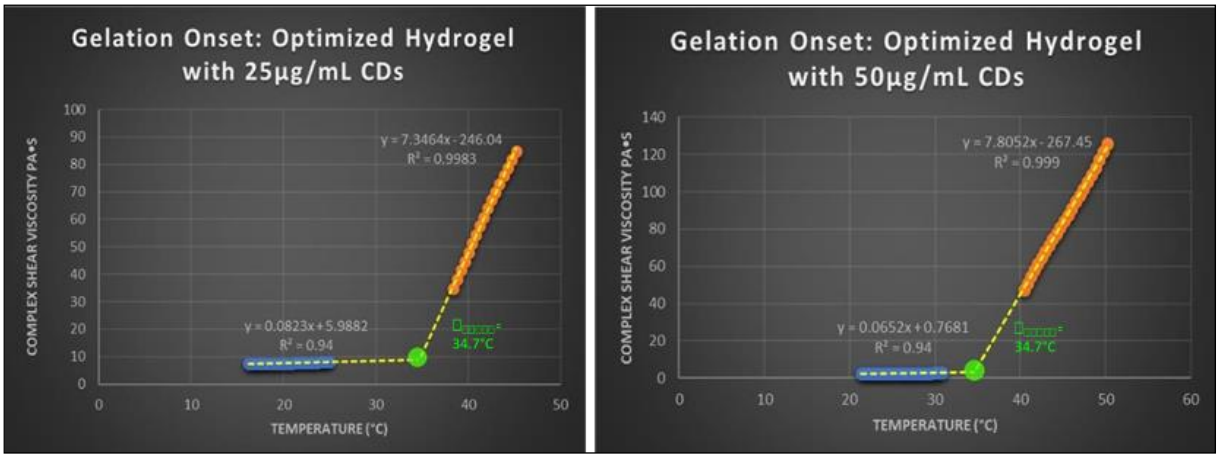

Fig 4

Table 1: Proliferation results for experimental gels and Ca (OH)2

Mean Fluorescence of Triplicated Samples and Percent Change							
Experimental Gels							
					% Change	**% Change**	**% Change**
	Day 1	**Day 2**	**Day 5**	**Day 14**	**Days 1 & 2**	**Days 2 & 5**	**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							
					% Change	% Change	% Change
	Day 1	Day 2	Day 5	Day 14	Days 1 & 2	Days 2 & 5	Days 5 & 14
UltraCal XS	527.7 ± 7.9	541.2 ± 7.0	517 ± 7.9	518 ± 4.8	3%	-4%	0%
0.15 g							
UltraCal XS	528.6 ± 4.6	536.3 ± 3.4	514.7 ± 0.6	518.2 ± 5.8	1%	-4%	1%
0.10 g							
UltraCal XS	529.9 ± 2.4	538.6 ± 6.3	520.7 ± 0.6	550.3 ± 34.3	2%	-3%	6%
0.05g							
Control	783.3 ± 28.4	854.1 ± 31.0	1721.6 ± 136.5	1296 ± 64.0	9%	102%	-25%
(No Paste)							

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 [6, 7, 8, 9-12]. Though TAP is often regarded as cytotoxic to stem cells, there is no consensus on cytotoxicity with Ca (OH)2 [13, 14, 15].

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 pop Within each control group, cell counts increased most rapidly between days 2 and

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 [16, 17], 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 (1, 2). 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.

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