ISSN 0973-2063 (online) 0973-8894 (print)

Bioinformation 22(8): 4737-4741 (2026)

BIOINFORMATION
Discovery at the interface of physical and biological sciences

BIOMEDICAL INFORMATICS

www.bioinformation.net

Volume 22(8)

Research Article

Received August 1, 2026; Revised August 31, 2026; Accepted August 31, 2026, Published August 31, 2026

DOI: 10.6026/973206300224737

SJIF 2026 (Scientific Journal Impact Factor for 2026) = 8.478
2022 Impact Factor (2023 Clarivate Inc. release) is 1.9

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Citation: Reddy *et al*. Bioinformation 22(8): 4737-4741 (2026)

Physicochemical evaluation of novel nano-calcium hydroxide-based intracanal medicaments: An *in vitro* study

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ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 22(8): 4737-4741 (2026)

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Abstract:
The optimal vehicle for enhancing the ion release and alkalinity of nano-calcium hydroxide remains unclear in endodontic therapy. Therefore, it is of interest to evaluate the effects of distilled water, propylene glycol, nano-chitosan and chlorhexidine as vehicles for nano calcium hydroxide in 60 extracted single-rooted teeth. Calcium ion release and pH were measured at 24 hours, 7 days and 15 days. Propylene glycol showed the highest and most sustained calcium ion release and alkalinity, while nano-chitosan exhibited a controlled release pattern. Thus, data shows the choice of vehicle significantly influences the physicochemical performance of nano calcium hydroxide and may affect its antimicrobial potential.

Keywords: Chitosan, calcium ion release, intracanal medicament, nano calcium hydroxide, pH and propylene glycol

Background:
A reduction in endodontic microbial load during root canal treatment is achieved through a combination of mechanical preparation and chemical disinfection procedures **[1].** Biomechanical preparation of the root canal reduces infection but due to the complex anatomy of the canal system, bacteria may persist **[2].** To enhance treatment outcomes, intracanal medicaments with antimicrobial properties are used to disinfect the canal further. Calcium hydroxide ($Ca(OH)_2$), first introduced by Hermann in 1920, is one of the most widely used intracanal medicaments due to its well-documented antibacterial properties and its ability to promote periapical healing. The therapeutic effect of calcium hydroxide is mainly due to its high alkaline pH (12.5–12.8) and its release of calcium and hydroxyl ions **[3]**. These hydroxyl ions act as highly reactive free radicals that damage bacterial cell membranes, denature proteins and disrupt DNA **[4].** Additionally, calcium hydroxide neutralizes endotoxins, contributes to the dissolution of necrotic tissue and stimulates hard tissue formation **[3]**. Despite its effectiveness, it has limitations against resistant bacteria such as *Enterococcus faecalis*, leading to the development of improved formulations **[4].** The vehicle used with calcium hydroxide plays a crucial role in controlling ion release and overall efficacy. Vehicles can be classified as oil-based (*e.g.,* eugenol, silicone oil, olive oil, metacresylacetate, camphor), viscous (*e.g.,* glycerin, propylene glycol), or water-soluble (*e.g.,* saline, distilled water). Oily vehicles exhibit the slowest ion release and longest retention **[5].** Aqueous vehicles allow rapid release of calcium and hydroxyl ions, resulting in a high initial pH but a short duration of action, necessitating frequent redressing **[6].** Viscous vehicles provide slower, sustained ion release due to their high molecular weight, prolonging medicament activity and reducing the number of clinical appointments **[7].** Additives such as chlorhexidine enhance antimicrobial activity and dentinal tubule penetration **[8]**. Recent advances include the use of chitosan nanoparticles and nano-calcium hydroxide to overcome limitations of conventional formulations **[9, 10].** Conventional calcium hydroxide particles have limited penetration into dentinal tubules due to their larger size, whereas nanoparticles (1–100 nm) possess greater surface area, improved penetration and enhanced antibacterial activity without compromising dentin properties **[11].** Therefore, it is of interest to describe and compare the physicochemical behavior of nano-calcium hydroxide formulations prepared with different vehicles through the assessment of calcium ion release and pH changes.

Materials and Methods:
This *in vitro* experimental study was carried out at the Department of Conservative Dentistry and Endodontics, MNR Dental College and Hospital in association with the research lab of MNR Medical College and AMS Testing Laboratory, Hyderabad. Following the acquisition of patient consent, a total of 60 freshly extracted human mandibular single-rooted teeth, removed for periodontal or orthodontic reasons, were included in the study. Criteria for selection included teeth with single canals and fully formed apices. Teeth that showed signs of caries, cracks, fractures, calcifications, resorption, immature apices, or had previously undergone endodontic treatment were excluded from the study. An ultrasonic scaler was utilised to eliminate surface debris and soft tissue remnants following sample collection. Prior to use, the samples were maintained at room temperature in isotonic saline to prevent dehydration. Using G*Power software (version 3.1.9.6; Franz Faul, Mün Kiel, Germany), the sample size was determined with an effect size of 0.4067, a power of 80% and a significance level of 0.05, indicating a minimum requirement of 15 samples per group. To standardize the root length to 15 mm, the crowns were sectioned at the cemento-enamel junction with a diamond disc while being cooled with water. Canal patency was determined using size #10 K-file and the working length was determined by subtracting 1mm from the apical foramen. Protaper Gold rotary files up to size F3 were used for the biomechanical preparation. Irrigation was carried out using 2 mL of 2.5% sodium hypochlorite after each instrumentation, followed by 2 mL of 17% EDTA for 1 minute. Canals were dried using sterile paper points.

Grouping and medicament preparation:
The samples were randomly divided into four groups (n = 15 each):

[1] Group 1: Nano calcium hydroxide + distilled water
[2] Group 2: Nano calcium hydroxide + propylene glycol
[3] Group 3: Nano calcium hydroxide + 3% nano-chitosan
[4] Group 4: Nano calcium hydroxide + 2% chlorhexidine gluconate

For Groups 1, 2 and 4, 150 mg of nano calcium hydroxide powder was mixed with 0.15 mL of the respective vehicle. For Group 3, 150 mg powder was mixed with 0.20 mL of 3% nano-chitosan to obtain a homogeneous paste. Lentulo spirals were used to insert the prepared medicament into the canals and hand pluggers were used to compact it. Glass ionomer cement was used to seal the access cavity. Only the apical portion of each vertically mounted tooth was submerged in 6 millilitres of distilled water kept in glass vials. Ion diffusion through the apical area was made possible by this configuration, which replicated periapical circumstances. Throughout the investigation, samples were maintained at room temperature.

Measurement of calcium ion release:
One millilitre of the solution was taken out of each vial at 24 hours, 7 days and 15 days and it was replaced with fresh distilled water. An ultraviolet spectrophotometer operating at 220 nm was used to measure the concentration of calcium ion release based on a standard calibration curve.

pH measurement:
The pH of the solution was measured at the same time intervals using a pH meter. The solution was stirred for 5 seconds before measurement to ensure uniform ion distribution. The electrode was rinsed with distilled water and dried between readings.

Statistical analysis:
The data was tabulated and analyzed using version 23 of the Statistical Package for the Social Sciences (SPSS) software. Descriptive statistics like the mean and standard deviation were calculated for each variable at different time intervals. Calcium ion release and pH at 24 hours, 7 days and 15 days were compared intragroup using the Friedman two-way ANOVA by rank. Pairwise comparisons were conducted using post-hoc analysis where significant differences were discovered. To identify specific group differences, post-hoc testing was employed after intergroup comparisons using the Kruskal–Wallis test. A p-value of less than 0.05 was considered statistically significant.

Results:
Significant variations in calcium ion release were observed within all groups across the three time intervals ($p \leq 0.05$). Groups 1, 2 and 3 showed a progressive increase in calcium ion release from 24 hours to 15 days, whereas Group 4 showed an increase up to 7 days followed by a decline at 15 days. Intragroup pairwise comparisons revealed statistically significant differences for most time-point comparisons, particularly in Groups 2 and 3 **(Table 1).** Intergroup analysis showed significant differences in calcium ion release at all evaluated time points ($p < 0.05$). Group 2 consistently exhibited the highest calcium ion release, while Group 4 showed the lowest values. Pairwise comparisons indicated significant differences among most group combinations, except between Groups 1 and 3 at 7 and 15 days. Significant changes in pH were observed within all groups over time ($p < 0.05$). Groups 1, 2 and 3 showed a gradual increase in pH, whereas Group 4 exhibited a peak at 7 days followed by a decline at 15 days. Intragroup pairwise analysis showed significant differences in several time-point comparisons. Intergroup comparison of pH values revealed significant differences at all evaluated intervals ($p < 0.05$). Group 4 showed the highest pH at 7 days, whereas Group 2 exhibited the highest pH values at 24 hours and 15 days. Pairwise comparisons showed significant differences between multiple group combinations **(Table 2).**

Table 1: Intra-group pairwise comparison of Calcium ion release and pH change at different time intervals-post hoc analysis

Groups	Comparison between	P value (Calcium ion)	P value (pH)
Group-1	24hrs - 7days	0.006*	0.204
(Distilled water)	24hrs - 15days	<0.001*	<0.001*
	7days - 15days	0.134	0.010*
Group-2	24hrs - 7days	0.019*	0.362
(Propylene glycol)	24hrs - 15days	<0.001*	<0.001*
	7days - 15days	0.019*	0.024*
Group-3 (Nano-chitosan)	24hrs - 7days	0.019*	0.134
	24hrs - 15days	<0.001*	<0.001*
	7days - 15days	0.019*	0.006*
Group-4 (Chlorhexidine gluconate)	24hrs - 7days	<0.001*	0.003*
	24hrs - 15days	0.134	1
	7days - 15days	0.014*	0.003*

Table 2: Inter group pairwise comparison of mean Calcium ion release and pH change at three different time intervals-post hoc analysis

Comparison between		P value (Calcium ion)			P value (pH value)		
		24 hrs	7 days	15 days	24 hrs	7 days	15 days
Group 1	Group 2	<0.001*	0.002*	<0.001*	0.038*	1	1
	Group 3	0.013*	0.986	0.638	0.409	0.202	1
	Group 4	1	0.014*	0.036*	0.359	0.038*	0.133
Group 2	Group 3	0.484	0.171	0.051	<0.001*	0.033*	0.548

	Group 4	<0.001*	<0.001*	<0.001*	1	0.225	0.003*
Group 3	Group 4	0.002*	<0.001*	<0.001*	0.001*	<0.001*	0.403

Discussion:

Calcium hydroxide through the most widely used intracanal medicament, has limitations, including restricted penetration into dentinal tubules and potential weakening of dentin with prolonged use **[4, 12].** Therefore, nano-calcium hydroxide was tested in this study and the reduced particle size is expected to have enhanced penetration. At the same time, effectiveness of calcium hydroxide depends significantly on the vehicle used, which influences ion dissociation, diffusion and sustained release **[6, 13]**. This study evaluated the effect of different vehicles distilled water, propylene glycol, nano-chitosan and chlorhexidine gluconate on calcium ion release and pH when combined with nano calcium hydroxide. In the present study, the distilled water group showed an initial rapid release of calcium ions followed by a comparatively lower sustained release over time. This observation aligns with the findings of Esberard *et al*., who reported that aqueous vehicles promote rapid dissociation of calcium hydroxide, resulting in an immediate rise in pH **[14].** However, this effect tends to diminish over time, indicating that while aqueous vehicles are effective in achieving short-term alkalinization, they are less capable of maintaining prolonged therapeutic activity. The propylene glycol group exhibited the highest calcium ion release and a sustained increase in pH over the study period. These results are in agreement with previous studies by Simon *et al*., who showed that viscous vehicles enhance prolonged ion release due to their high molecular weight and reduced solubility **[7].** The hygroscopic nature of propylene glycol facilitates gradual water absorption, allowing controlled dissociation of calcium hydroxide. Estrela *et al.* also reported prolonged alkalinity with viscous vehicles, with sustained effects lasting several weeks **[15].** These findings suggest that viscous vehicles provide a more sustained therapeutic effect, which may be beneficial in managing persistent infections and large periapical lesions. Nano-chitosan showed a controlled and sustained release pattern of calcium ions along with a gradual increase in pH. These findings are consistent with the biphasic release behavior described by Ballal *et al*., where an initial burst release is followed by a prolonged diffusion phase. The mechanism is attributed to polymer swelling, gel formation and gradual matrix degradation, which regulate drug release **[16].** The combination of nano-calcium hydroxide with chlorhexidine showed comparatively lower calcium ion release and inconsistent pH changes. This finding is supported by studies suggesting that chemical interactions between chlorhexidine and calcium hydroxide may reduce ion dissociation and diffusion. The cationic nature of chlorhexidine can interfere with calcium hydroxide solubility, leading to reduced availability of ions over time **[8].** Freire *et al.* reported that chlorhexidine does not significantly enhance the pH of calcium hydroxide, while other studies have indicated that such combinations may alter physicochemical properties **[17].** However, despite reduced ion release, chlorhexidine contributes significant antimicrobial benefits due to its substantivity, as demonstrated by Delgado *et al*., who showed prolonged antibacterial activity against resistant organisms such as *Enterococcus faecalis* **[18].** In the present study, the pattern of pH changes differed slightly from the pattern of calcium ion release among the experimental groups. While Groups 1, 2 and 3 showed a gradual increase in pH from 24 hours to 28 days, Group 4 (nano calcium hydroxide mixed with chlorhexidine gluconate) showed a distinct pattern with a marked peak in pH at 7 days followed by a decline at 28 days. Similar variations in alkalinity patterns have been reported in studies by Kazemipoor *et al.* **[19]** and Shetty *et al.* **[20]**, evaluating different vehicles used with calcium hydroxide, where vehicle composition influenced the diffusion of hydroxyl ions and the resulting pH levels. The initial increase in pH observed in Group 4 may be attributed to the combined alkalinizing effect of calcium hydroxide and the chlorhexidine gel base. Chlorhexidine formulations often contain viscous gel vehicles such as hydroxyethyl cellulose or natrosol, which can retain hydroxyl ions in the surrounding medium and facilitate their diffusion into dentinal tubules. A study by Guerreiro-Tanomaru *et al.* reported that the vehicle used with calcium hydroxide can significantly influence hydroxyl ion diffusion and pH values in the surrounding environment. The temporary retention of hydroxyl ions by the gel base may therefore explain the sharp increase in alkalinity observed at the intermediate time interval of 7 days **[21].** However, the decline in pH observed at 28 days in Group 4 may be related to interactions between chlorhexidine (CHX) and calcium hydroxide (CH). Chlorhexidine is a cationic bis-biguanide compound and its association with calcium hydroxide may influence the physicochemical properties and ion diffusion capacity of the medicament. Guerreiro-Tanomaru *et al.* reported that the release and diffusion of hydroxyl ions from calcium hydroxide-based medicaments may be affected by association with other substances **[21].** Basrani *et al.* further showed that chlorhexidine alters certain physicochemical characteristics of calcium hydroxide formulations, including viscosity and contact angle, which may influence ion dissociation and diffusion over time **[22].** These interactions may contribute to reduced hydroxyl ion availability and decreased long-term alkalinity of the mixture. Another possible explanation is related to the substantivity of chlorhexidine, which is its ability to adsorb onto dental tissues and gradually release active molecules over time. According to Souza *et al*., chlorhexidine exhibits prolonged antimicrobial substantivity within the root canal system due to its adsorption to dentin and slow release properties. While this characteristic enhances antimicrobial activity, it may also influence the ionic equilibrium of the medicament system, thereby affecting the diffusion and availability of hydroxyl ions over extended periods. This phenomenon may explain why the pH values peaked at the intermediate time interval and subsequently declined in this group **[23].** In contrast, Groups 1, 2 and 3 showed a gradual and sustained increase in pH over time, which can be attributed to

the continuous dissociation of calcium hydroxide particles and progressive diffusion of hydroxyl ions into the surrounding medium. Previous studies by Estrela *et al.*, Ballal *et al.* and Simon *et al.* have reported that the antimicrobial activity of calcium hydroxide is primarily related to the release of hydroxyl ions that create a highly alkaline environment capable of inactivating microbial enzymes and endotoxins **[7, 15 and 16].** Furthermore, viscous and polymer-based vehicles such as propylene glycol and chitosan have been shown to facilitate sustained ion diffusion, thereby maintaining prolonged alkalinity within the root canal system. Intergroup comparison revealed statistically significant differences ($P < 0.001$), with calcium ion release as follows: Propylene glycol > Nano-chitosan > Distilled water > Chlorhexidine. Clinically, propylene glycol appears to be the most effective vehicle for prolonged disinfection, particularly in cases of persistent infection, necrotic pulps and large periapical lesions. Nano-chitosan also shows promise as a controlled drug delivery system. Distilled water may be suitable for short-term applications, while chlorhexidine combinations may be beneficial for targeting resistant microorganisms despite reduced ion release. Although extensive research exists on conventional calcium hydroxide, studies on nano calcium hydroxide with different vehicles are limited. Due to its unique physicochemical properties, nano-calcium hydroxide may behave differently, warranting further *in vitro, in vivo* and clinical studies.

Conclusion:

Vehicle selection significantly influenced calcium ion release and alkalinity of nano-calcium hydroxide intracanal medicaments. Propylene glycol produced the highest and most sustained calcium ion release and alkalinity, while nano-chitosan demonstrated a controlled and prolonged release profile. Thus, viscous and polymer-based vehicles may improve the therapeutic potential of nano-calcium hydroxide as an intracanal medicament, warranting further clinical investigation.

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