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Synergistic Effect of Linezolid Incorporated Chitosan Nanoparticles in Restorative Dental Cements – A Promising Dental Formulation.

Omar Farooq Burhanuddin Mohammed[1]*, Muthukrishnan Lakshmipathy[2],N. Somasundaram Mohan Kumar[3], and M.S. Nivedhitha[4]

[1] *Ph.D. Scholar, Saveetha Institute of Medical and Technical Science (SIMATS), Chennai*

[2] *Research Scientist, Dental Research Cell, Saveetha Dental College and Hospital (SIMATS), Chennai*

[3] *Professor, NMS Dental Clinic, #37, Modachur Road, Gobichettipalayam, Erode District, Tamil Nadu,*

[4] *Professor, Department of Conservative Dentistry and Endodontics, Saveetha Dental College and Hospital (SIMATS).*

Abstract: Chitosan is considered one of the best materials for use as a drug delivery vehicle. Chitosan is incorporated into dental cement to enhance its properties. Previous investigators have found its antibacterial activity near dental restorations, which was insufficient to produce a clinically significant effect. Hence, the incorporation of various antibacterial agents was proposed. Linezolid is one of the oldest drugs in the oxazolidinone antibiotics category, approved by the US FDA in 2000. It is used to treat various antibiotic-resistant bacterial infections. However, there needs to be more literature regarding using linezolid with chitosan. Current research aims to prepare and characterize chitosan nanoparticles with linezolid for use in dental cement. Chitosan nanoparticles were prepared using the ionotropic gelation technique. They were incorporated with Linezolid, and samples were analyzedby FTIR spectroscopy, Particle size analysis, Transmission Electron Microscopy, High-performance liquid chromatography analysis, antibacterial activity, and biocompatibility. Linezolid was successfully incorporated into chitosan. Chitosan's FTIR spectrum reflected the functional groups present in chitosan, and their peaks were superimposed in the presence of linezolid. Both chitosan and chitosan with linezolid had similar particlesizes (142nm). Linezolid was released from chitosan effectively on the 1st and 7th days (19.78 and 144.14µg/ml). The formulation showed antibacterial activity and was not cytotoxic to cells. Thus prepared linezolid incorporated chitosan is a promising material to be used as an antimicrobial agent in dental cement. Its bio-physico-chemical properties are promising for use in dental cement. In the future, these linezolid-loaded chitosan nanoparticles may be added to various restorative materials.

Keywords: Chitosan, Nanoparticles, linezolid, Antibacterial, Cements, Biocompatibility, Drug delivery.

***Corresponding Author**

Omar Farooq Burhanuddin Mohammed , Ph.D. Scholar, Saveetha Institute of Medical and Technical Science (SIMATS), Chennai

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1. INTRODUCTION

The Modern concept of pharmacotherapy involves the delivery of a drug to the location of its intended action. In this regard, nanoparticles(NP) have been applied with great success[1]. All conventional routes deliver these nanoparticles of drug delivery. Nanoparticle technology has also emerged as a viable drug delivery strategy, offering opportunities for controlled release, active component protection from enzymatic or environmental degradation, and localized retention. Nanoparticle fabrication methods are easily scalable and can be applied to various drugs[2].In this regard, the formulation of biocompatible and biodegradable materials has gained enormous attention. In addition, if the particle possesses tunable physical properties, it is seen as a greater advantage. In this regard, chitosan is considered one of the best materials for drug delivery[3]. Cancer, gastrointestinal diseases, pulmonary diseases, drug delivery to the brain, and ocular infections are some applications of chitosan-based nanoparticles. Recent research on chitosan-based NP for non-parenteral drug delivery is based on a growing understanding of chitosan properties. Chemical or physical modifications optimize the drug loading and release characteristics of nanoparticles[2].Concerning the origin and properties of chitosan, it is a biocompatible polymer derived from the chitin found in crustacean shells. It is widely used in various formulations and biomaterials[4]. It is a highly basic and cationic polymer approved by the United States Food and Drug Administration for tissue engineering and drug delivery. Chitosan is generated by N-deacetylating purified chitin. For example, controlling the degree of deacetylation with factors such as reaction conditions (concentration, chitin-to-alkali ratios, temperature), chitin source, and reaction extent can control the end product properties[5,6]. Therefore, Chitosan is incorporated into dental cements to enhance their properties[4]. Previous investigators have found its antibacterial activity near dental restorations better than conventional cements. However, it was insufficient to produce a clinically significant effect. Hence, the incorporation of various antibacterial agents was proposed, including linezolid. Linezolid is one of the oldest drugs in the oxazolidinone antibiotics category, approved by the US FDA in 2000. It is known to prevent bacterial protein synthesis via binding to rRNA on both the 30S and 50S ribosomal subunits. Therefore, it is used to treat various antibiotic-resistant bacterial infections[7]. The drug has been incorporated with chitosan for various purposes such as wound healing and wound dressings[8-10]. However, linezolid-incorporated chitosan nanoparticles need to be better studied in the literature. The system is intended to be used in restorative dentistry for caries prevention and control. Hypothetically, antibiotic linezolid, delivered through chitosan, can offer prolonged antibacterial activity in the clinical service of dental cements. This article describes the preparation, characterization, biological activity, and biocompatibility of linezolid-incorporated chitosan nanoparticles for potential dental cement application. Such material has yet to be prepared; this is a novel attempt to utilize linezolid in dental applications.

2. MATERIALS AND METHODS

Chitosan and Sodium tripolyphosphate were purchased from Sisco Research Laboratories Pvt limited. India and used to prepare nanoparticles. Acetic Acid was purchased from Merck. These chemicals were used as received. All chemicals used were of analytical grade.

2.1. Synthesis of chitosan nanoparticles

Chitosan(CH) nanoparticles were prepared using procedures reported by Papadimitriou et al. (2008)[11]. The Ionotropic gelation technique was used with slight modifications. Briefly, Chitosan nanoparticles obtained by the addition of Sodium tripolyphosphate (TPP) (0.4 wt.%) to a chitosan aqueous solution (1 wt.% in 1% V/V acetic acid) in a weight ratio of CH: TPP, 2.5:1. Then, the mixture was kept under the vigorous stirring for 40 min. The formed nanoparticles were collected by centrifugation at 9000 rpm for 30 min, and the supernatant was discarded. After that, prepared nanomaterials were washed with double distilled water, re-suspended in distilled water, ultrasonicated for 30 s to get a homogenous suspension, and then freeze-dried and stored dry conditions at room temperature until further use.

2.2. Preparation of linezolid loaded Nanoparticles

Linezolid-loaded chitosan nanoparticles were prepared by adding linezolid into CH solution (30µg/ml) before adding TPP solution. Chitosan nanoparticles are hereafter referred to as C, and linezolid-incorporated chitosan is called CD. Samples were submitted for FTIR spectroscopy, Particle size analysis, and TEM.

2.3. Physicochemical Characterization of the Material

For FTIR Spectroscopy, Samples were mixed with FTIR Grade potassium bromide (Sigma Aldrich, Bangalore, India) (1:100 ratio) and made into pellets at 35MPa pressure using a hydraulic press. Thus, pellets were placed in the Bruker alpha 2 FTIR-ATR spectrometer, and the FTIR spectrum was recorded from 400 to 4000 cm^{-1} with a resolution of 4 cm^{-1}. The suspension of nano-chitosan (1 mg/ml) was prepared and sonicated for 5 mins and subjected to particle size analysis using Dynamic Light Scattering analysis with a Nanoplus analyzer (Micromeritics Gmbh). For TEM Analysis, Transmission electron microscopy (TEM) was used to analyze the size and morphology of prepared nanoparticles. TEM image of nanoparticle dispersion dried on copper grids was made using JEOL 120 CX microscope (Japan), operating at 120 kV. HPLC analysis was carried out for HPLC (High-Performance Liquid Chromatography) using the WATERS - 2695 HPLC system, consisting of an auto-sampler, quaternary pumps, column oven, and UV detector. All data were analyzed using Empower-3 software. Separated using Shim pack C18 column (250 mm X 4.6 mm, 5 µm) with a mobile phase of methanol: water (80:20, v/v) at a 1.0 ml/min flow rate. Detection was carried out at 256 nm. The column was equilibrated at 25 ^{0}C. The injection volume was 20 µl. The method was carried out for each sample at the 24th hour (1st day) and 7th day. The area under the curve was found, and the concentration of the drug was calculated using the standard formula.

2.4. Antibacterial activity studies

Antibacterial activity of chitosan nanoparticles against the strain *Streptococcus mutans* (ATCC 25175). *Lactobacillus acidophilus* (ATCC 314) and *Actinomyces viscosus* (ATCC 15987) were evaluated, *and for S. mutans* and *L. Acidophilus*, Mueller Hinton agar was used to determine the zone of

inhibition. Muller Hinton agar medium was prepared and sterilized for 15 minutes at 121° C for 15 lbs. Media was poured into the sterilized plates and allowed for solidification. The wells were cut using a sterile 9 mm polystyrene tip, and the test organisms were swabbed. The samples with different concentrations were loaded, and the plates were incubated for 24 hours at 37 °C. After the incubation time, zones of inhibition were measured. Brain heart infusion agar was used for *Actinomyces viscosus*, incubated in anaerobic condition at 37 °C for 24 h using a modified McIntosh and Fildes jar with Anerogaspak (HIMEdia, India) system. 3 groups were studied for C and CD (1mg/ml solution) in 50, 100, and 150 µl volumes.

2.5. *In vitro cytotoxicity assay*

The cytotoxicity was studied on Human Osteosarcoma cell lines (MG63) obtained from NCCS, Pune, India. The cells were grown in T25 culture flasks containing DMEM supplemented with 10% FBS and 1% antibiotics (100U mL−1 penicillin and 100µg mL−1 streptomycin). The cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Upon reaching confluence, the cells were detached using Trypsin–EDTA solution and were sub-cultured at a density of 5000 cells per well. The cells were treated with C and CD (25 µl from 1µg/ml) for 24 h at 37 °C in the CO_2 incubator. Later, cells were incubated with MTT (4 mg/ml) for 3 hours. The absorbance was measured at 540 nm with a standard microplate reader.

2.6. *Statistical Analysis*

Relevant data were entered in Microsoft Excel 2010, and statistical analysis was carried out. Mean and Standard deviation gave descriptive statistics.

3. RESULTS

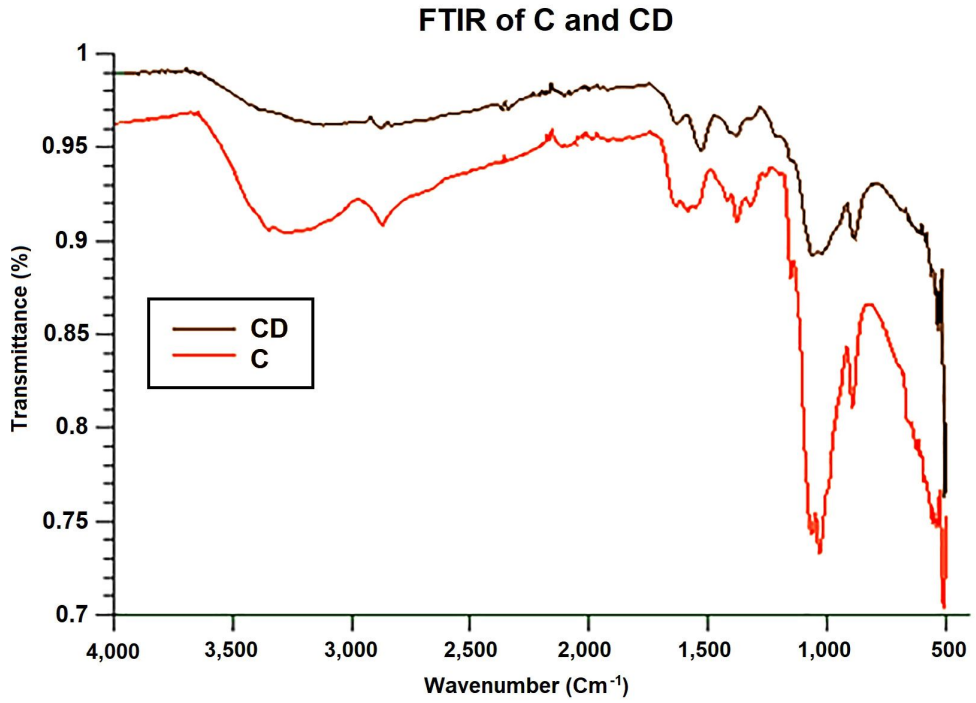
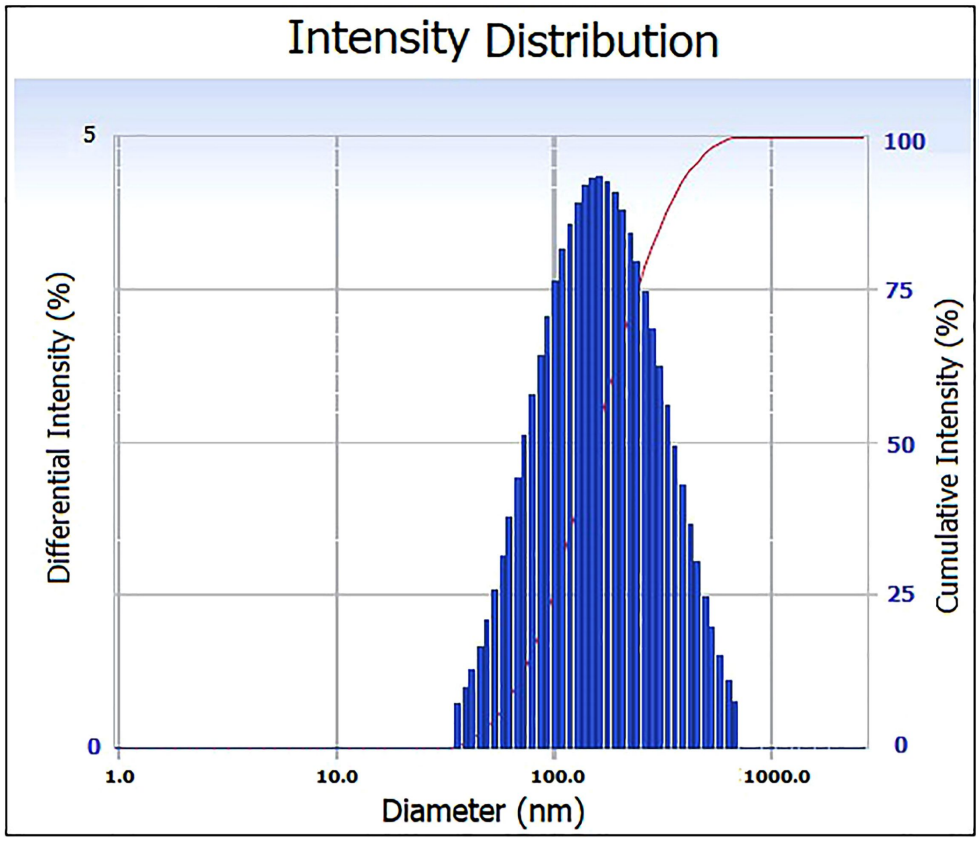
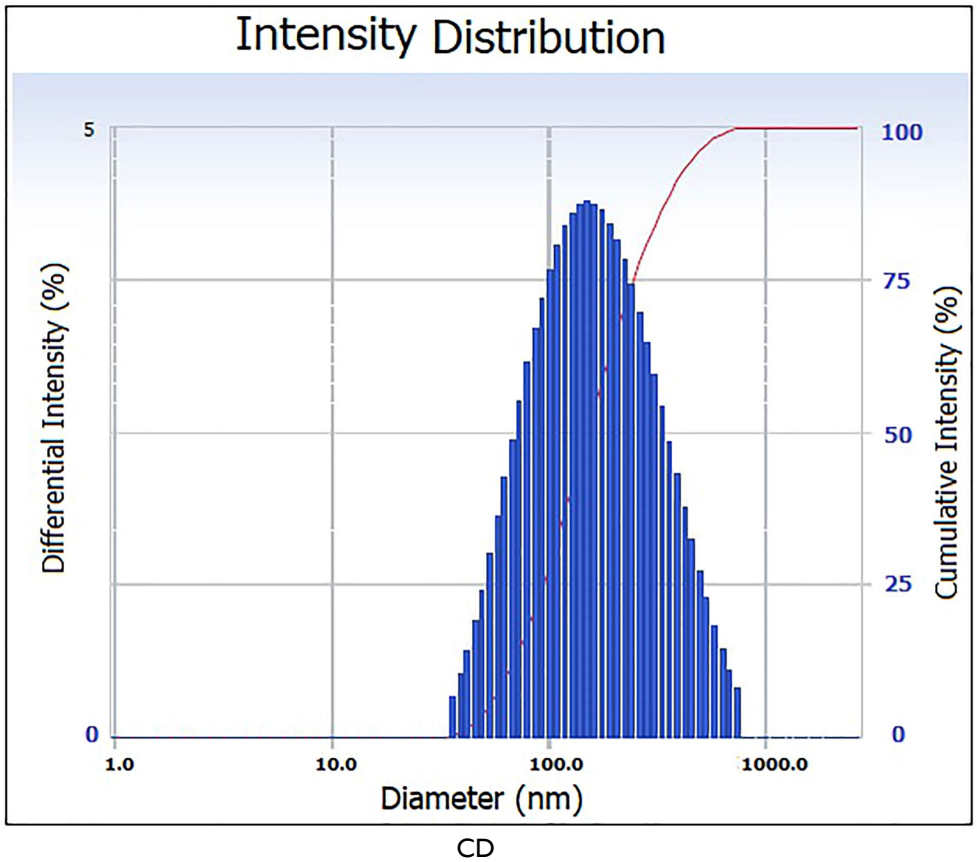


Fig. 1. FTIR Spectroscopy of C and CD

FTIR Spectra of C reflected that of chitosan, and CD showed superimposed peaks of chitosan and drug (Figure 1). The characteristic peaks of chitosan have bands corresponding to N-H and O-H stretching, intramolecular hydrogen bonds, C-H symmetric and asymmetric stretching, N-acetyl groups, and amide I, II, and III were visible in the spectra. Peaks of linezolid included N-H stretching, aromatic C-H stretching, aliphatic C-H stretching, C=O stretching, aromatic C=C stretching, C-N stretching, aliphatic C-H bending, C-F stretching, C-O stretching, C-N bending and aromatic C-H bending.



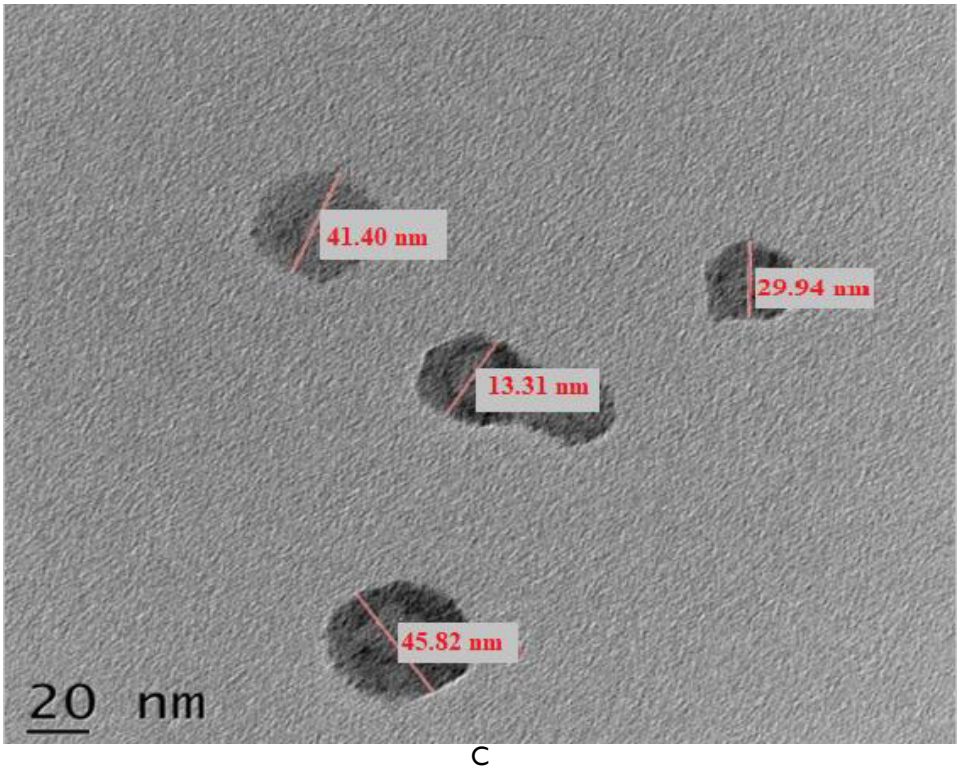
C



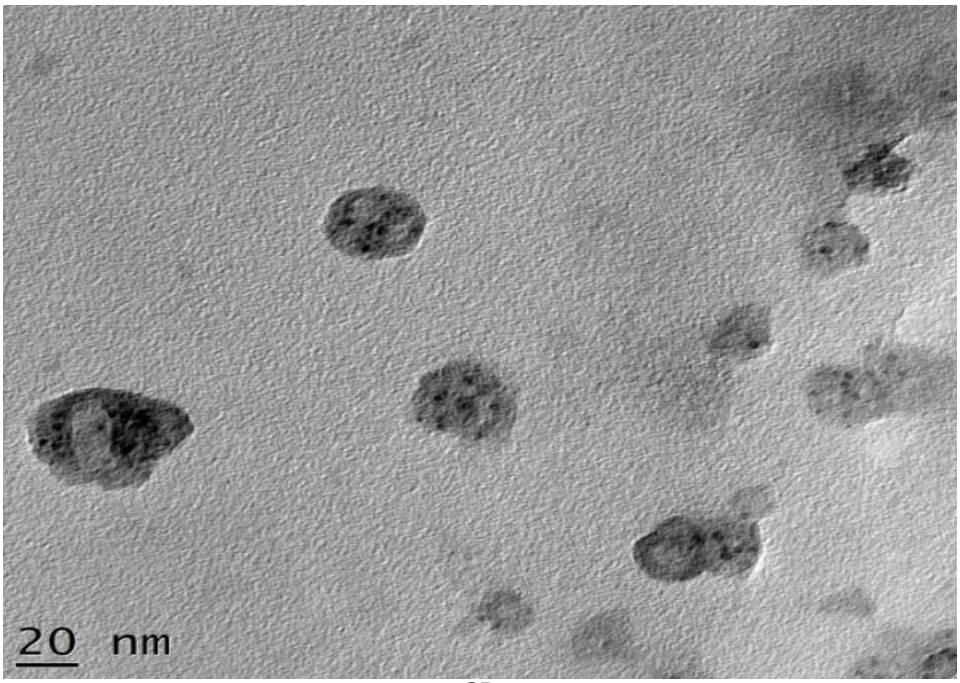
CD

Fig. 2. Dynamic Light Scattering of C and CD

Dynamic Light Scattering studies on the prepared nanoparticles(C) showed a particle size of 144nm. After the drug (CD) was incorporated, the size was 142 nm (Figure 2). The particle sizes were normally distributed around the mean value (Bell shaped curve). This mild difference is negligible, and hence they can be called as having the same particle size. However, it needs further evaluation in TEM.



C



CD

Fig. 3. Transmission Electron Microscopy

According to TEM, particle size is around 40 and 50nm (hydrodynamic diameter) for C and CD, respectively (Figure 3). Particles were spherical. The micrograph shows exact shape and size variation and a mild agglutination tendency. Therefore, sonication is a must for all physicochemical characterizations. The shape, though called spherical, has mild and negligible deviations in all particles.

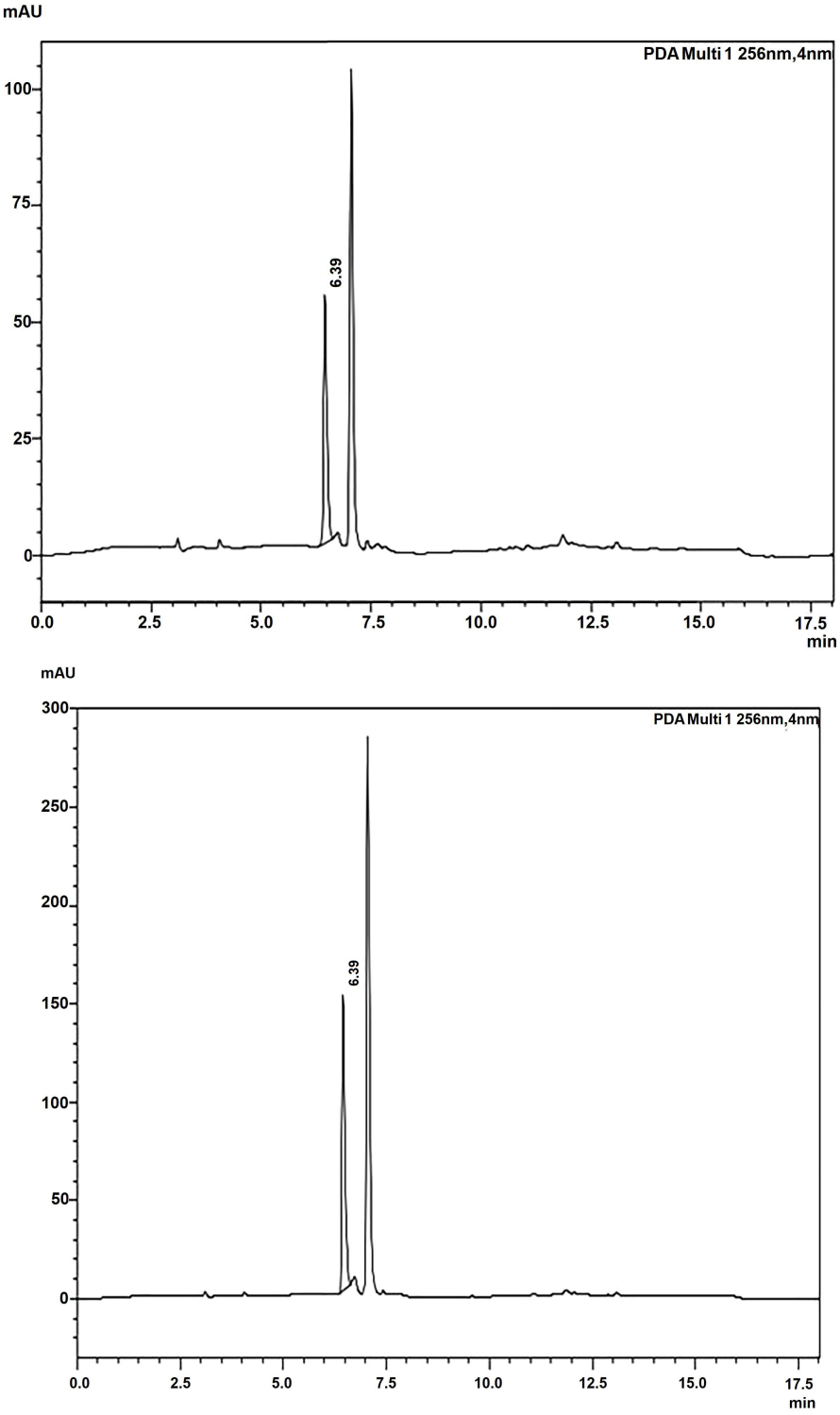


Fig. 4. HPLC of Drug delivered from chitosan nanoparticles at 1 and 7th days, whose concentrations were 19.78 µg/ml and 144.14 µg/ml, respectively. The HPLC showed an increase in the concentration of linezolid in the buffer testifying to the release of the drug from the loaded nanoparticles. This release is significant in terms of clinical use, and hence the linezolid release from chitosan nanoparticles is feasible for the current application.

Table 1: Antibacterial activity against dental pathogens by agar diffusion method

Microorganisms	The volume of the nanoparticles with the concentration of 1mg/ml						Control
	CD (Mean±sd) mm			C (Mean±sd) mm			Linezolid
	50µ l	100 µ l	150 µ l	50µ l	100 µ l	150 µ l	
S. mutans	30±2	34±1	35±2.5	8±0.5	10±0.5	13±0.5	11±0.5
L. acidophyllus	35±2.6	35±1.0	36±2.0	10±0.6	11±1.0	12±0.6	31±2.8
A.Viscosus	22±0.6	26±0.6	29±0.6	13±0.0	14±0.6	15±0.6	28±1.1

Antibacterial activity studies against three 3 dental pathogens using C and CD revealed that chitosan showed a small amount of antimicrobial activity, and drug addiction showed significant clearance zones (Table 1). This increase in antibacterial activity was approximately 2 to 3 times higher in CD than C for *S. mutans* and *L acidophilus* and double concerning *A, viscous.*

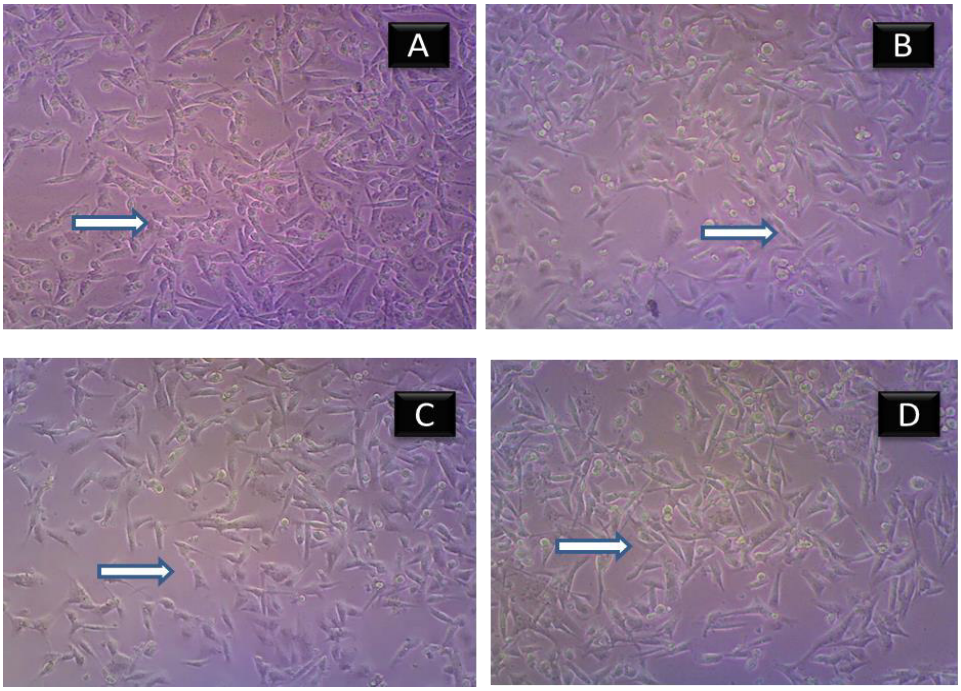


Fig.5. In vitro biocompatibility assay; A is Control (100%), B is chitosan (81% viability), C is Drug (75.4% viability), and D is chitosan Drug conjugate (77.4% viability). The arrow mark shows the cells with characteristic morphology.

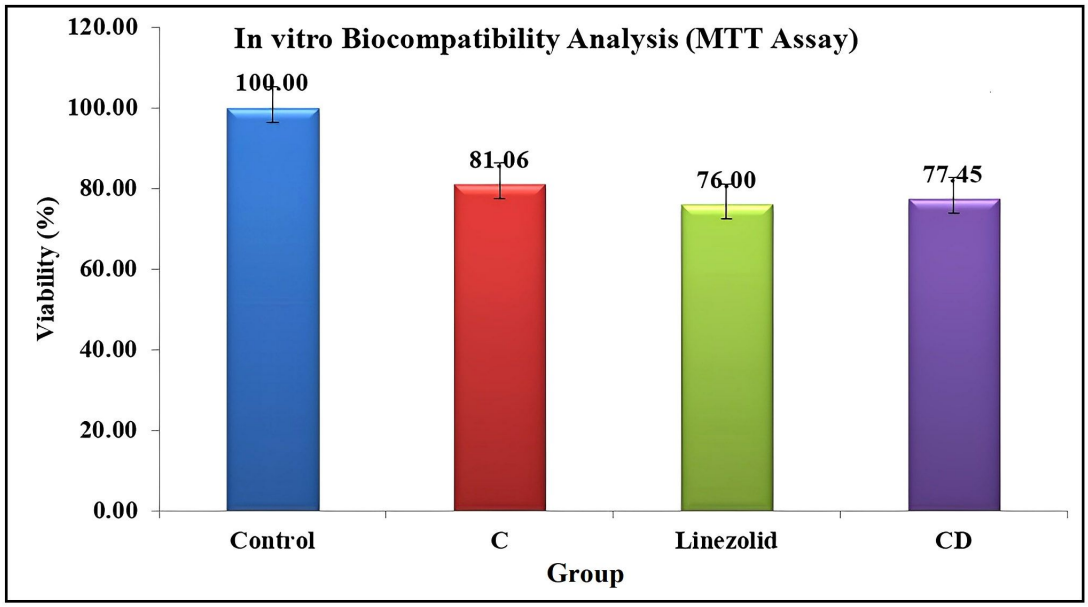


Fig. 6. Biocompatibility of the nanoparticles analyzed in the study. (% Viability)

The biocompatibility testing revealed that chitosan, linezolid and linezolid loaded chitosan has similar biocompatibility about 80%. This is a good percentage to accept the use of CD for linezolid release in said applications. The HPLC and antibacterial activity combined show the feasibility of using CD as a drug delivery system for dental cements. This combination will be added to dental cement in clinical applications to improve biocompatibility.

4. DISCUSSION

Linezolid-loaded chitosan nanoparticles have been fabricated successfully and studied for physio-biochemical properties. The ability of the system to deliver the linezolid through chitosan is studied using FTIR, HPLC, and TEM. Chitosan's FTIR spectrum is expected to reflect the functional groups in chitosan in a major way. A strong band in the region 3286–3349 cm^{-1} corresponds to N-H and O-H stretching and intramolecular hydrogen bonds. The absorption bands at around 2921 and 2869 cm^{-1} can be attributed to C-H symmetric and asymmetric stretching, respectively. These bands are typical of polysaccharides as they have an aliphatic chain. The presence of residual N-acetyl groups was confirmed by the bands at around 1632 cm^{-1} (C=O stretching of amide I) and 1321 cm^{-1} (C-N stretching of amide III), respectively. The band at 1550 cm^{-1} corresponds to the N-H bending of amide II. This is the third band characteristic of typical N-acetyl groups. A band at 1587 cm^{-1} corresponds to the N-H bending of the primary amine. The CH_2 bending and CH_3 symmetrical deformations were confirmed by the presence of bands at around 1416 and 1375 cm^{-1}, respectively. The absorption band at 1149 cm^{-1} can be

attributed to the asymmetric stretching of the C-O-C bridge. The bands at 1061 and 1028 cm^{-1} correspond to C-O stretching. All bands are found in the spectra of samples of chitosan reported by others[12, 13]. According to Reddy et al. (2013)' [14] IR peaks of linezolid include 3338 (N-H stretching), 3117, 3066 (aromatic C-H stretching), 2971, 2863, 2818 (aliphatic C-H stretching), 1738, 1662 (C=O stretching), 1545, 1516, 1453 (aromatic C=C stretching), 1425 (C-N stretching), 1381 (aliphatic C-H bending), 1334 (C-F stretching), 1274 (C-O stretching), 1198, 1177 (C-N bending), 1117, 1081 (aromatic C-H bending). Major peaks of linezolid include 1743, 1644, 1519, 1235, and 1117 cm^{-1} [15]. DLS studies on the prepared nanoparticles(C) showed a particle size of 144nm. After the drug (CD) was incorporated, the size was 142 nm. The change in size is not significantly different. Similar particle sizes (189nm) were reported by Khan et al. (2017)[16]and Kahdestani et al. (2021)[17]. However, Sreekumar et al. (2018)[18] have highlighted poor reproducibility in size due to many factors involving the formation of chitosan nanoparticles. However, the currently obtained particles are suitable for biomedical applications. TEM images were obtained to confirm the morphology of C and CD. In chitosan, Particle size is around 40 and 50nm for C and CD, respectively. Similar particle sizes were seen in TEM by various authors[19]. Nanoparticles tend to aggregate due to surface charge and other factors. In this study, only minimal attraction is seen. In this study, two different values are obtained for particle size. DLS showed a much higher value than TEM. DLS is an intensity-based technique, so larger particle sizes are prioritized. Since TEM is a numerical technique, it will emphasize the smallest components of the size distribution. Further, in the DLS technique, the diameter of the hydration sphere is measured. Greater charge density leads to greater deviation. But in TEM, physically observable particles are measured[20]. Therefore, one can deduce that the particle size is about 50nm, with a hydration sphere of about 144nm diameter. HPLC technique has been extensively used to study the delivery of drugs from their vehicles. This study showed a significantly increased release over 1 week in HPLC, indicating sustainable drug release. The chitosan nanoparticle alone served as a control, and the drug-loaded nanoparticle showed the release of linezolid from the nanoparticle (Figure 4). This shows that linezolid bound into chitosan was released into the system, indicating the suitability of the prepared nanoparticle to deliver linezolid into the surroundings. There was increased release over time. Tedizolid[21], Clarithromycin[22], Lemon grass oil[23], and Chitosan nanoparticles have been loaded for various applications, including ocular delivery. This study is novel in designing chitosan nanoparticles for delivery in dental cement. In the future, this formulation will be optimized and tested in vivo.

Antibacterial activity studies against three dental pathogens using C and CD revealed that chitosan showed a small amount of antimicrobial activity, and the addition of the drug showed significant clearance zones (Table 1). The linezolid control disk showed similar antimicrobial activity to C towards *S. mutans*. However, it was similar to CD towards *L. acidophilus* and *A. Vicous*. However, increased antibacterial activity towards *S. mutans* when released from CD implies its synergistic effect. Therefore, loading the drug can benefit cements, providing increased antimicrobial capability. The antimicrobial activity analysis shows that linezolid did not lose the structure-function relationship and is in the potent form inside the nanoparticle[21]. Mikusova et al. (2021)[24] have described the biocompatibility of chitosan nanoparticles used for drug delivery. From the biocompatibility results of this study, it has been deduced that both C and CD had good biocompatibility and less toxicity than expected. Previous authors have shown good biocompatibility of chitosan. In addition, given the anatomy of restored teeth, where cements contact only acellular hard tissue, the compatibility shown here is adequate. However, restorations that reach the gingival vicinity need extra care. The rationale for the biocompatibility study is to find the feasibility of using this material near gingival margins. From the results, it can be inferred that the drug-loaded nanoparticle can also be used in the restorative materials near the gingiva.

5. CONCLUSION

In this study, chitosan nanoparticles are prepared and loaded with linezolid for dental cements. Its bio-physico-chemical properties are promising for use in dental cements. Furthermore, these linezolid-loaded chitosan nanoparticles can be incorporated into various restorative materials to prevent secondary caries. However, further studies are needed to arrive at proper concentration and formulation.

6. AUTHORS CONTRIBUTION STATEMENT

Dr. M.S. Nivedhithaand Dr. N.Somasundaram Mohan Kumarconceptualized and designed the study, Dr. Omar Farooq Burhanuddin Mohammed discussed the methodology, curated the data, and prepared the original draft, Dr. Muthukrishnan Lakshmipathy analyzed the data and provided valuable inputs towards designing of the manuscript. All authors read and approved the final version of the manuscript.

7. CONFLICT OF INTEREST

Conflict of interest declared none.

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