RESEARCH ARTICLE

Biocompatibility of Chitosan Nanoparticle in Root Canal Sealant with Vero Cell Line

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Abstract

Root canal sealants placed in primary tooth are unique in various characteristics. Chitosan nanoparticles have gained an important milestone in research area due to its biodegradable and its bioavailability nature. Chitosan nanoparticles synthesized by ionic gelation method and studied for its physical characteristics when added with root canal sealant provides a promising result. In this study, biocompatibility nature of Chitosan nanoparticles with Vero cell line was being investigated. Several solution parameters of Chitosan nanoparticles and root canal sealants were investigated to optimize diameter of nanoparticles after being characterized to XRD, SEM, and FTIR. The Chitosan nanoparticles were found to be biocompatible to fibroblasts and on a dose-dependent manner, these can be used in combination with the root canal sealant in primary teeth.

Keywords: Biocompatibility, Cell line study, Chitosan nanoparticles, Vero cell line.

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Introduction

Chitosan is a cationic polysaccharide (poly (B-(1-4)-2-amino-2-deoxy-D-glucose))obtained by partial deacetylation of chitin, which is a co polymer of glucosamine and an N acetyl glucosamine units, which forms the major component of crustacean shells.[1] Chitosan has become the focus of interest of many researchers for biomedical applications due to its distinct biological property including good biocompatibility, biodegradability and nontoxicity.[2,3] The antimicrobial and anti biofilm efficacy of these nanoparticles have been demonstrated in another study.[5] Recently it was suggested that these intracanal medicaments also are capable to have its detrimental effects on human stem cells of the apical papilla and human dental pulp cells.[6] Hence it would affect the clinical outcome of the material also. The vehicle and form of delivery of nanoparticles makes a great influence.[7] On the other hand, calcium hydroxide when applied in root canals varies in its physical and chemical properties as a compound and is also said to reduce root canal dentin microhardness as endodontic pastes and hence thereby it can affect its clinical applications also.[8] Since both the constituents of our study material are vulnerable to alterations, the main aim of this study was 1) to prepare chitosan nanoparticles from chitosan by ionic gelation method 2) Characterize the prepared chitosan nanoparticles to XRD and FTIR analysis, SEM 3) Characterized chitosan nanoparticles added to Root canal sealant and subjected to MTT Assay with Vero cell line to assess the biocompatibility of the nanoparticles in root canal dentistry.

This will assess the best possible combination of the study material to be biocompatible to the area of study.

Materials and Methods

Formation of Chitosan Nanoparticles

Analytical grade chitosan purchased was used for nanoparticles synthesis. Nanoparticles were synthesized via the Calvo's ionotropic gelation method with Sodium tri poly phosphate.[9] Chitosan was dissolved in acetic acid at various concentrations (1, 2, 3 mg/mL). The rule of thumb is that the concentration of acetic acid in aqueous solution has to be 1.5 times higher than that of chitosan solution. The TPP solution (1 mg/mL) was prepared by double-distilled water. Chitosan nanoparticles were spontaneously obtained on the dropwise addition of 5 mL of the chitosan solution to 2 mL of TPP solution under magnetic stirring (1000 rpm, 1 hour) at room temperature. Suspensions of various consistencies were obtained of which the opalescent suspension formed under the same abovementioned conditions were selected to be the ideal choice of sample material which pertains to the nanometric measurement. The nanoparticles were separated by centrifugation at 20,000 rpm and 14°C for 30 minutes, freeze-dried at 5 ± 3°C. The freeze-dried nanoparticles were confirmed under SEM to nanometric measurements.

FTIR analysis - The samples were examined by Fourier transform infrared (FTIR) analysis with a Nicolet 17DSX FT-IR spectrometer (Thermo Scientific, Waltham, MA). For IR analysis, one mg of the sample was mixed with 300 mg of KBr (infrared grade) and pelletized under vacuum. Then, pellets between 500 and 4000 cm^{-1} were analyzed with 120 scans averaging 4 cm^{-1} resolutions. The FTIR analysis was used to characterize the presence of specific chemical groups of chitosan.

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Cell Proliferation and Assay

Cell proliferation and survival were determined in this study as proposed by Mosmann and later modified by Edmondson et al. using the MTT cell proliferation kit as per manufacturer's protocol. The National Center for Cell Science in Pune has launched VERO cell Lines (NCCS). The cells were kept at 37°C in a humid environment of 50 g/mL CO in a minimum primary medium associated with 10% FBS, penicillin (100 units/mL), and streptomycin (100 g/mL). Hi Media Laboratories provided MEM for purchase. Cistron laboratories provided the Fetal Bovine Serum (FBS). MTT (methylthiazolyldiphenyl tetrazolium bromide) and dimethyl sulfoxide (DMSO) were procured from Sigma-Aldrich (Cisco Research Laboratory Chemicals, Mumbai). Sigma Aldrich Mumbai provided all of the other chemicals and reagents.

In Vitro assay for Biocompatibility Activity (MTT Assay) (Mosmann, 1983)

Cells (well 1,105) were cultivated onto 24-well sections and refined for 30 minutes at 370°C with 5% CO. Tests were included various sums and brooded for 24 hours in the wake of arriving at cell intersection. Following brooding, the material is taken from the well and broke up in phosphate-cradled saline (pH 7.4) or MEM without serum for additional handling. It required 4 hours for the outcomes to show up and 0.5% 3-(4,5-dimethyl-2 thiazolyl)-2,5 tetrazolium bromide (MTT). Each well was then treated with 1 mL of DMSO after the incubation time. Absorption is measured as a control with DMSO using a UMS (universal measurement spectrophotometer) at 570 nm . The IC50 concentration was visually computed based on the measurements that were made. To figure out the viability percentage, the following formula was employed:

% cell viability = A570 of treated cells / A570 of control cells × 100

The percent of Cell Viability is displayed on the Y-axis, while the sample concentration is represented on the X-axis. To compare the whole cell viability evaluations, each experiment includes a cell control and a sample control.

Statistical Analysis

The data was gathered, tabulated, and the mean and SD for each group were determined. SPSS software used to perform statistical analysis (version 17; statistical package for the Social Sciences Corporation). The groups were compared using a one-way analysis of diversity, This is then followed by the Tukey's honest significant variance test at the 0.05 level of significance.

Results

MTT assay: The highest cell viability % significantly was different from that of the control and was recorded for 24, 48, and 72 hours. It decreases the cell viability % at maximum concentration of 30% and as dilution rate increases it, the viability increases. The cell viability was exactly 50% at 125 µg/mL (Figs 1 and 2).

Effect of time duration: Time was not a great factor in affecting cell viability %. Regardless of the particle size, mean values of cell viability % were decreased by time with 24, 48, and 72 hours with Chitosan nanoparticles, which were not that significant.

Effect of particle size: All the four different concentrations of nanoparticles recorded a statistically significantly higher mean values of cell viability % than the control group (of plain root canal sealant) at the three different time interval tested ($p < 0.05$). Hence we find that change in concentration of nanoparticles was not a factor affecting cell viability%.

Results and Discussion

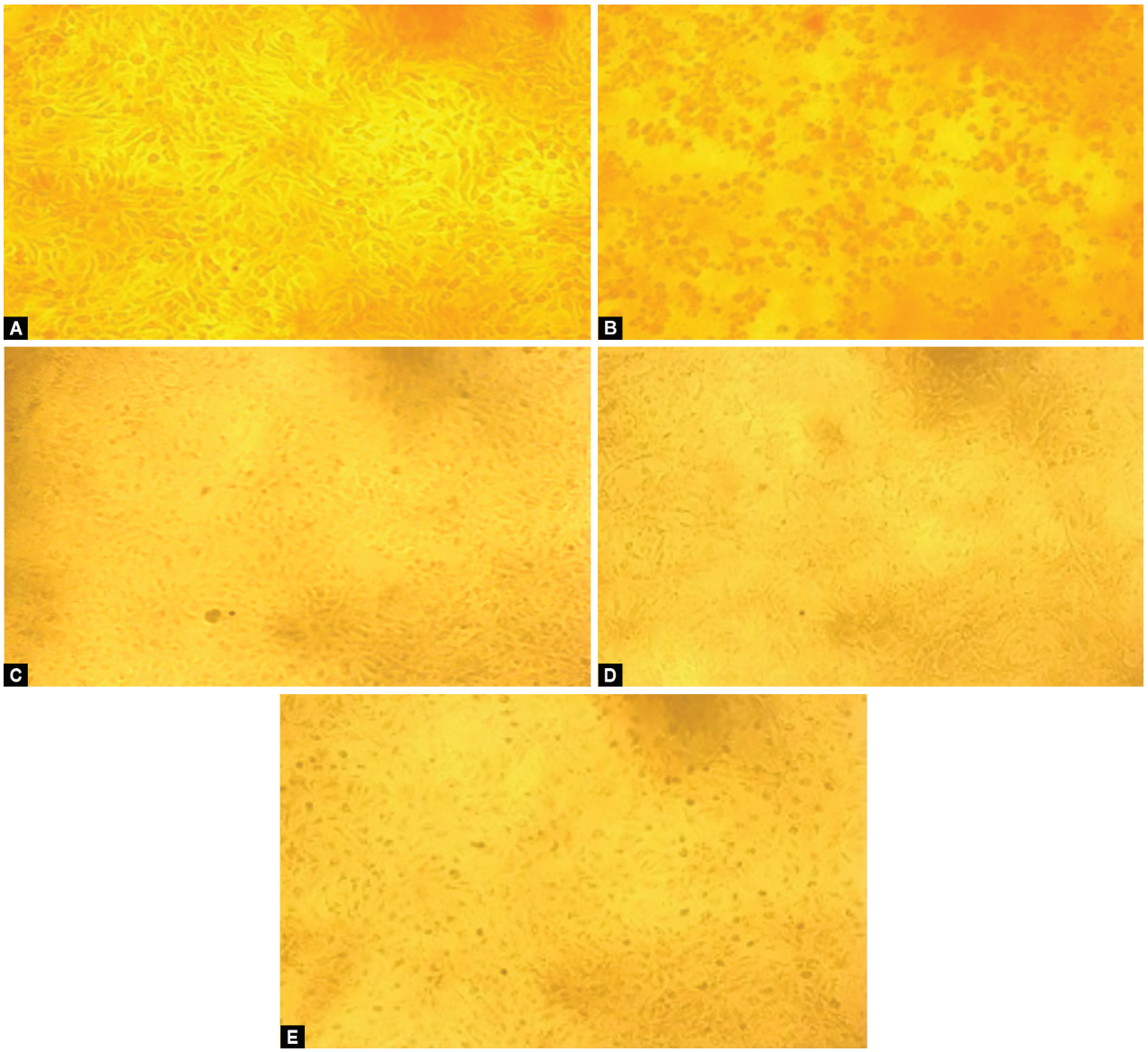
Between 1220 and 1020 cm^{-1} the dominant absorption band is assigned to the free amino group ($-NH_2$), was discovered in the generated chitosan by FTIR analysis (Figs 3 and 4). The sample also included absorption bands corresponding to the free amino group, which ranged in size from 1026 and 1259 cm^{-1}. The peak at 1374 cm^{-1} represents the -C-O stretching of primary alcoholic group (-CH2 - OH). The absorbance bands of 3644, 3464, 2963,2866,2284,1632, 1261 cm^{-1} indicated the N-H stretching, Symmetric CH_3 stretching and asymmetric CH_2 stretching, CH stretching C=O and C - N - stretching in secondary amide (amide II), respectively. The absorption peaks attached to N-H bound O-H of Chitosan in the spectra of nanoparticles move to a lower frequency, suggesting that the hydroxyl groups of Metapex (calcium hydroxide and Iodoform) have joined to the NH_2 groups of chitosan. Another sign of interaction evidence may be seen in the peak at 800 cm^{-1} which correlates with the normal methyl group absorption pattern (Fig. 5). Figure 6 shows the Chitosan XRD patterns. The XRD pattern of chitosan shows wide scattering peaks at $2\theta = 10^o$ and 21^o which are typical fingerprints of semi-crystalline chitosan (SEM shows particle size of 110° nm). The WAXD (Wide Angle X-ray Diffraction patterns of shrimp chitosan were discovered by Prashanth et al. to have two large distinctive peaks at $2\theta = 9.9\text{-}10.7^o$ and $19.8\text{-}20.7^o$ similar to our studies. Zeta potential determines the stability of a test sample. High zeta potential is favorable for controlling the rate of drug release. Particle surface charge is also important to denote the degradation of nanoparticles, due to interaction with lysozymes, which is crucial for drug delivery, and in turn dependent on the surface charge. The test material showed a zeta potential of -11.8 which shows its cationic nature and is of good quality as it does not aggregate due to weak repulsive forces and is found to be stable (Fig. 7)

The SEM images of the CS-np shows the particle size of the test sample which varies between 80 and 120 nm. Also, it shows the spherical nature of the particles and the particles are in the agglomerated state. Shows the homogeneity of the cells.Most CS nanoparticles synthesized for research purpose exhibited Agglomerative abilities. The porous nature harbors molecules effectively and can adsorb any drug added to it (Fig. 8).

The Biocompatibilty assay shows fibroblast like cells which appears thin and elongated (spindle shaped) which has a considerably smooth surface (Fig. 1A). Soon upon incubation and attributing to cell passages it becomes flattened and its contours are slightly defined (Fig. 1B). Soon they progress into flat islands of cells, which expands as time passes with granules which denotes the glycogen storage of the cells (Fig. 1C). With few more cell passages, these cells gets injured , and they start to shrink and they become round and lose their capacity to attach to the surface of the cultivation plate (Figs 1D and E).

Discussion

Chitosan nanoparticles were synthesized by ionic gelation method, which provides the more reactive amino groups.The main bioactivity of Chitosan is mainly due to the free electron pair of nitrogen in the amino groups, which is made available for interaction with other metals; electrostatic attraction of anions by means of deacetylation; which removes the acetyl group and provides us with functional amide groups. Chitosan is a biodegradable polymer that may be broken down by chemical or



Figs 1A to E: Biocompatibility effect of chitosan on VERO cell line, (A) Normal Vero cell line, (B) Biocompatibility effect of chitosan - 1000 µg/mL, (C) Biocompatibility effect of chitosan - 125 µg/mL, (D) Biocompatibility effect of chitosan- 62.5 µg/mL, (E) Biocompatibility effect of chitosan - 31.2 µg/mL

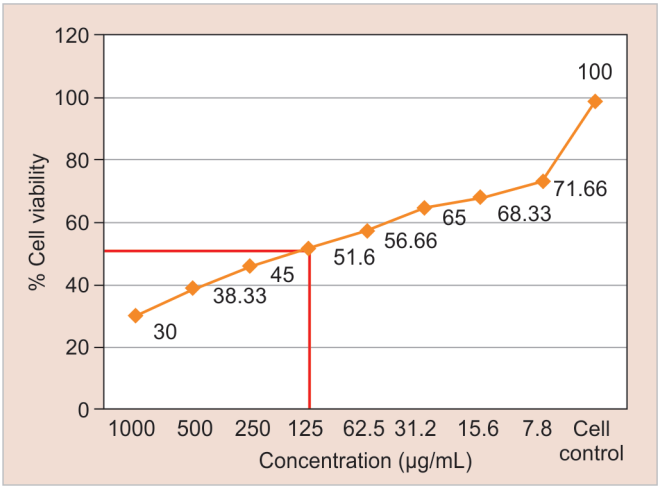


Fig. 2: MTT assay

enzyme catalysis, depending on the degree of deacetylation and the amino groups present. In neutral and alkaline pH , it has a poor solubility. Its adsorption and bioavailability into the human body vary according to the forms of chitosan nanoparticles employed, but so far its toxicity has not been recorded in any of the cell culture studies *in vitro* or *in vivo*. Chitosan has found to be an effective antibiofilm agent when used as root canal disinfectant and also has decreased the number of adhered bacteria in lateral canals without altering the flow of the root canal sealer in permanent teeth. Hence, in primary teeth, this material was chosen to prove its efficiency. There is a great interest these days for using dental material in its nano form in order to modify its physical properties. But still the cytotoxic adverse effects remain unclear.

In our study we had synthesized the nanoparticles by ionic gelation method and the accuracy of the particle size was examined by subjecting the material to SEM, XRD etc. FITR shows the different active methyl peaks removed by deacetylation and the presence of bio active amino groups in the nanoparticle

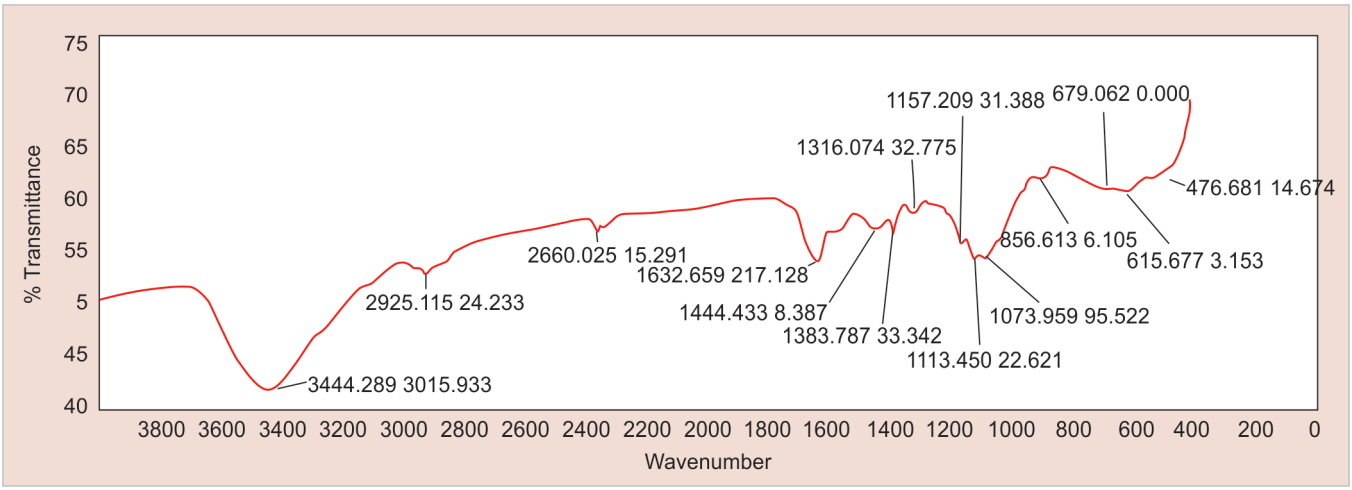


Fig. 3: FTIR absorption bands of chitosan

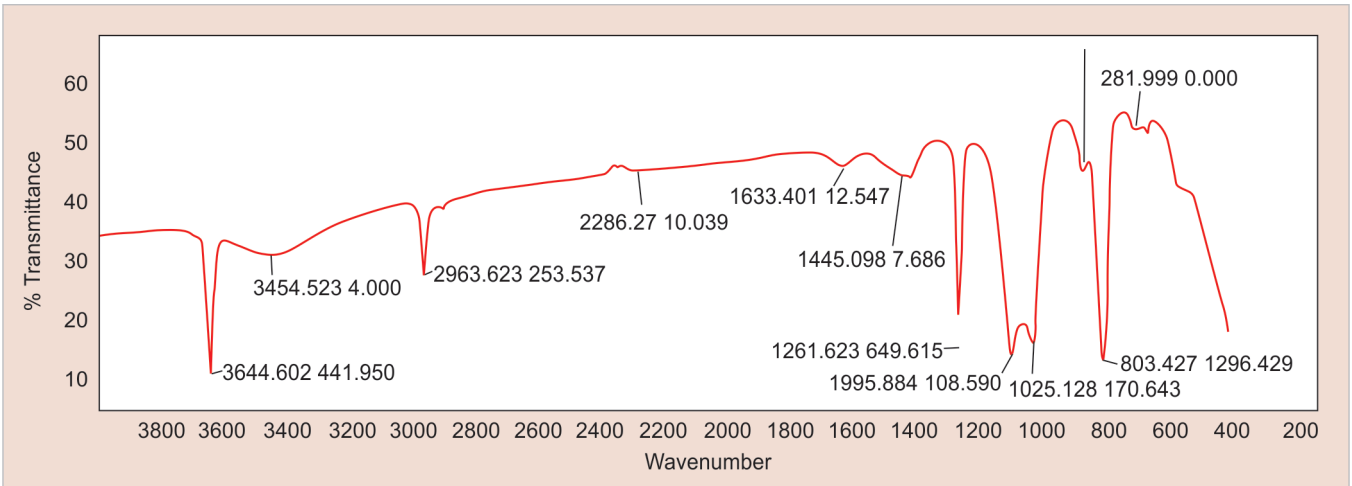


Fig. 4: FTIR absorption bands of chitosan-nanoparticles

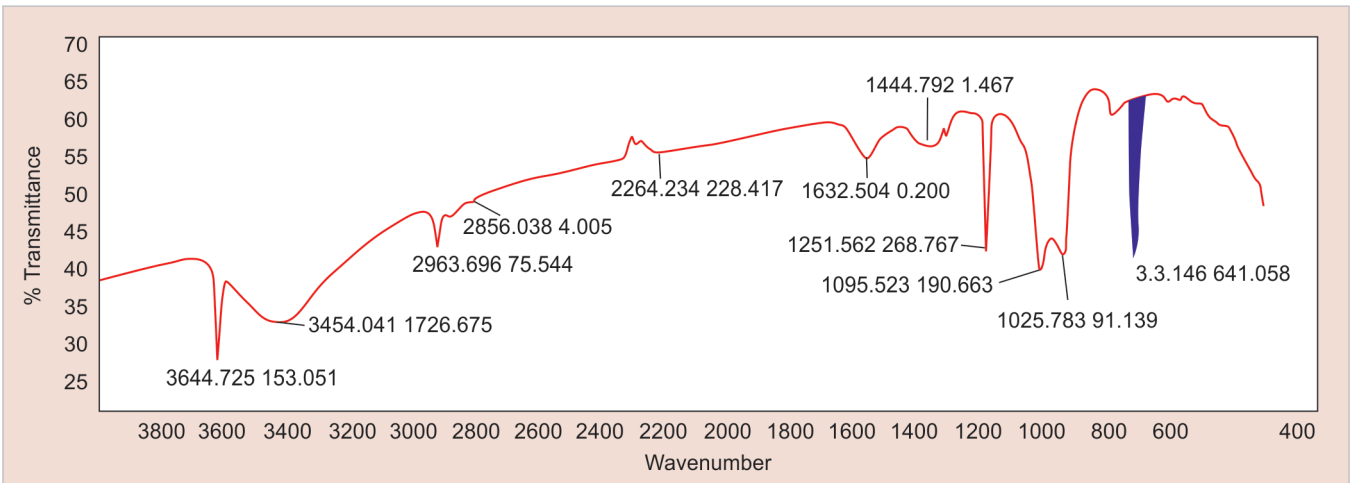


Fig. 5: FTIR absorption bands of chitosan-nanoparticles with Metapex

synthesized. Further when added with the root canal sealant we could see a chemical interaction in the OH group alone, which shows that both materials are not chemically reacting to produce a new material and chitosan acts as a carrier for the functionally active calcium and hydroxyl ions of root canal sealant Metapex. In our study we had followed the methodology developed by Mossman 1983. The basic mechanism in this technique is to convert water soluble methylthiazol tetrazolium to insoluble purple formazan; the concentration is determined spectrophotometrically, Apoptosis is the way by which the cell

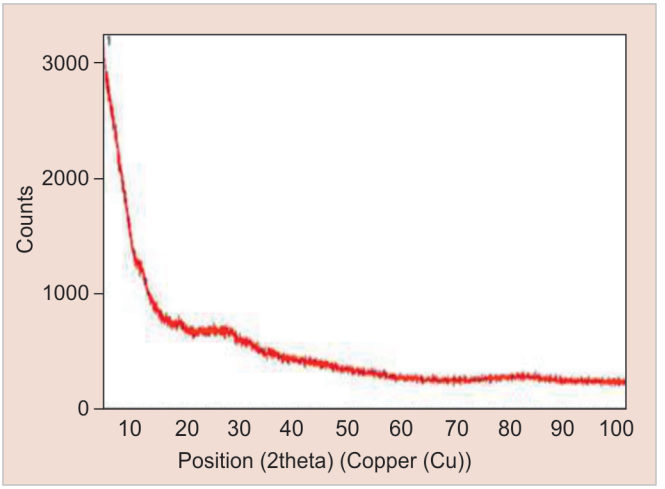


Fig. 6: XRD diffraction peaks of chitosan np

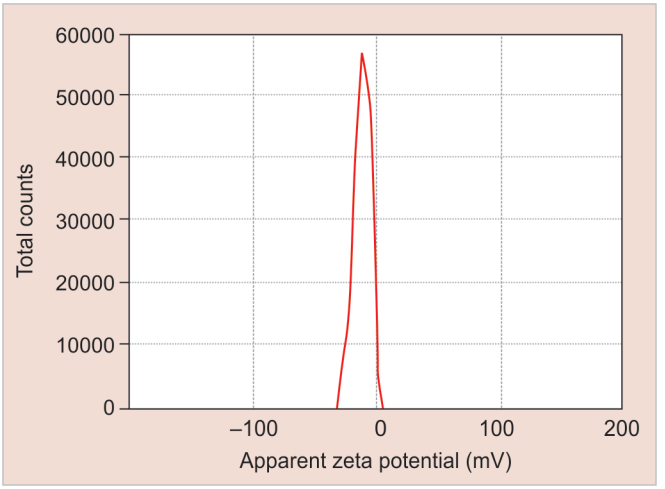


Fig. 7: Zeta potential of chitosan np

engulfs and causes changes in DNA and thereby leads to cell death. This is mainly characterized as membrane blebbing, chromatin condensation, and nuclear fragmentation. Since in different time durations half the cells was viable at around 125µg/ml, and there was not much a change during different time durations- it was observed that the chitosan nanoparticles are biocompatible to the fibroblast cells and in a safe concentration of 125µg/ml (Table 1) . Also there were no significant changes with variation in concentrations of the test material. A similar observation was by Omar Zaki et al. - who concluded that medium and large Chitosan nanoparticles are relatively non toxic when treated with mouse bone marrow derived hematopoietic stem and progenitor cells.[12] Hu B et al. in 2012 has observed that chitosan nanoparticles with particle size of 150 µg/mL they could enter the intestines.[13] Zhang et al. reported oleyl-chitosan nanoparticles exhibited no cytotoxicity on A549 cells.[14,15] Chitosan nanoparticles are highly biocompatible as seen in our study but the range varies according to the target area of delivery and also the type of material to which its conjugated.

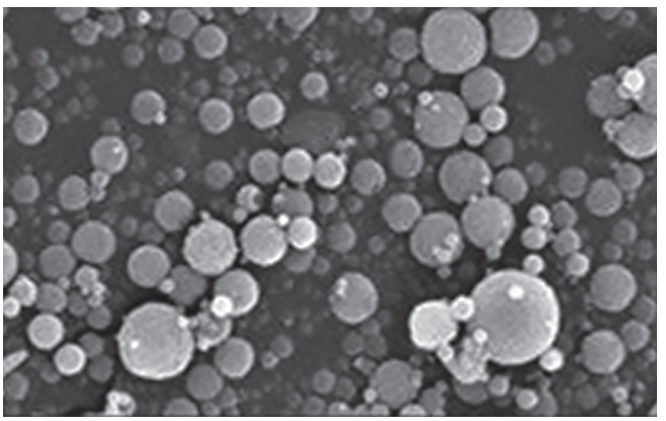


Fig. 8: SEM picture of the chitosan 2wt/vol% in 2x and 4x magnification

Table 1: Biocompatibility effect of chitosan on vero cell line

S. No.	*Concentration (µg/mL)*	*Dilutions*	*Absorbance (OD)*	*Cell viability (%) 12 hours*	*Cell viability (%) 24 hours*	*Cell viability (%) 48 hours*
1	10,000	Neat	0.18	30	35	38
2	500	1:1	0.23	38.33	38.33	38
3	250	1:2	0.27	45	48	49
4	125	1:4	0.31	51.66	53	56
5	62.5	1:8	0.34	56.66	56.66	58
6	31.2	1:16	0.39	65	65	66
7	15.6	1:32	0.41	68.33	68.33	68.33
8	7.8	1:64	0.43	71.66	71.66	73
9	Cell control	–	0.60	100	95	90

Conclusion

In this study, chitosan nanoparticles were prepared from shrimp shells chitin of approximate size 110 nm. These nanoparticles prepared from shrimp waste can be used as an intracanal medicament in dentistry, as its properties are highly complementary to the dental material and biocompatible to the tissues. Research is growing rapidly in the field of dentistry utilizing nanomaterials, still *in vivo* applications of these materials have to be proved successful.

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