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Research Article

Nano-Diamonds Containing Chitosan Gels as Inter-Canal Medication: From Design to Application *In vitro*

V. Tamara Perchyonok[1*], Nicolaas Basson[2], Rafael Felitti[3] , Shengmia Zhang[4], Desigar Moodley[2] , Sias R Grobler[2]

[1]*VTPCHEM PTY LTD, Glenhuntly, 3163, Victoria, Australia,*

[2]*Oral and Dental Research Institute, Faculty of Dentistry, University of the Western Cape, Private Bag X1, Tygerberg 7505, Cape Town, South Africa*

[3]*Faculty of Dentistry, Catholic University, Montevideo, Uruguay.*

[4]*School of Material Science and Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China*

**Corresponding author: Dr. Tamara Perchyonok, VTPCHEM PTY LTD, Glenhuntly, 3163, Australia, Tel: +61414596304; Email: tamaraperchyonok@gmail.com*

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Abstract

In this study, we demonstrated that the newly prepared chitosan nano-diamond hydrogels are suitable novel antimicrobial drug delivery systems against *Enterococcus faecalis*, as well as, bio-active materials capable of efficiently counteracting potential free radical damage generated during the potential endodontic treatment *in vitro*. The chitosan hydrogels showed a high adhesive force and were only slightly swelled in the aqueous medium. Tetracycline release suggested prolonged release of the antibiotic from the hydrogels. All the test samples gave an average inhibition zone against *Enterococcus faecalis* larger than the tetracycline control disc. The hydrogels also had significant free radical defense capability.

Keywords: Chitosan Nano-Niamond Hydrogels; Antimicrobial Drug Delivery Systems; *Enterococcus faecalis*; Cytotoxicity

Introduction

During a root canal procedure disinfection by means of an irrigation solution and intra-canal dressing plays an important role in the success of endodontic treatment. While the microbiota in the root canal lumen is effectively reduced by means of a chemico-mechanical preparation, disinfection should eliminate the bacteria that have survived the canal preparation. However, disinfection resistance can occur in areas not reached by instrumentation ie. in the isthmus, dentin tubules, lateral canals, apical ramifications and non-mechanically prepared walls. Therefore it is imperative to place a disinfection medicament between visits which has the function of eliminating bacteria that have survived canal preparation and to prevent reinfection of the root canal during the treatment period [1].

Enterococcus faecalis, a member of the intestinal microflora, is commonly present in infections such as urinary tract infections and infections of the endocardium. Although not regarded as an oral commensal, E. *faecalis* can reside in the oral cavity, albeit at very low numbers and often below the detection level of the standard culture methods. It is rarely found in the primary endodontic infections, but in cases of retreatment it represents between 38 to 70% of the isolates. The high frequency of isolation of E. *faecalis* from infected and filled root canals is an indication that this organism is an opportunistic pathogen despite its low incidence in the oral cavity [2,3].

A variety of intra-canal dressings, as well as, irrigation solutions are available for disinfection of the root canal. Unfortunately, these substances do not guarantee complete

eradication of microorganisms from the root canal [2]. Furthermore, most of them also have some cytotoxic and potential harmful effects and may act as irritants to host tissue [1]. One of the better medicaments for example, calcium hydroxide, contributes effectively in anti-exsudative action, the repair of periapical lesions and the induction of mineralization and has been used in dentistry since the beginning of the twentieth century [4]. However, E. *faecalis*, the most important opportunistic pathogen in cases of retreatment, is the most resistant bacterium against calcium hydroxide. And while chlorhexidine and combinations of disinfectants show some promise, eradication of *E. faecalis* from the root canal still remains a challenge, especially in re-treatment and systematic apical periodontitis [2,3]. Retreatment may necessitate the use of systemic antibiotics to prevent spreading of the infection [3]. The use of a locally applied antibiotic would be more advantages in this regard.

Reactive Oxygen Species (ROS) are oxygen-derived molecules that are highly reactive, and which play an important role in the host defense response. They can derive from both the host and from infectious agents. When they are produced in excess, or when there are defective oxidant scavenging, ROS can destroy host tissue and have been implicated inseveraloral diseases like periodontitis, orofacialpain,temporomandibular disorders and oral cancer[5,6].

Nanodiamonds are carbon nanoparticles that in recent years have cemented its role into unique functional nanomaterials due to their superior physical properties such as small size, large surface area and high adsorption. A new area of research has been created due to numerous reports of applications of chitosan, as well as, nanodiamond-based films to build drug delivery systems in medicine [7-9]. Considering the importance of searching for new medicaments for endodontic therapy, the present study aims to design and evaluate the performance of newly prepared chitosan nano-diamond hydrogels as suitable novel antimicrobial drug delivery systems against *Enterococcus faecalis* and their ability to counteract potential free radical damage.

Materials and Methods

Preparation of various chitosan/nanodiamond hydrogels

Chitosan (Aldrich, Australia), glycerol (Sigma, USA) and glacial acetic acid (E. Merck, Germany) were used for this study. The degree of de-acetylation of typical commercial chitosan used in this study is 87%. Chitosan used in this study had a molecular weight 2.5 × 103 KD. The isoelectric point was 4.0 - 5.0. Nanodiamonds where purchased from Ebersoles25 carats, size 0 - 2 microns with a Grit of 14,000.

One milliliter of the therapeutic agent, tetracycline containing gel, was prepared by the dispersion of 200 mg of Tetracycline in glycerol (5% w/w) using a mortar and a pestle, as per the earlier reported generic protocol [7].Ten milliliters of glacial acetic acid (3% w/w) was then added with continuous mixing and finally nanodiamond:chitosan (10% nanodiamond: chitosan w/w) polymer was spread on the surface of the dispersion and mixed well to form the required gel. The strength of the prepared gel (10 gm) was 200 mg of tetracycline in each gram of the base. Asummary of the newly prepared materials is presented in Table 1.

Table 1. Gel formulations of chitosan:nanodiamond gels prepared for the study.

Gel Formulation		Nano diamond-Chitosan (10% ND:Ch) (w/w)	Tetracycline (w/w)	Hydroxyapaptite (w/w)	pH
NanoTetr	Gel-1	5	1	0	6.75
NanoChTetr	Gel-2	5	1	0	6.15
NanoHaTetr	Gel-3	5	1	1	6.20
NanoChHaTetr	Gel-4	5	1	1	6.00
NanoChHaTetrB12	Gel-5	5	1	1	6.21

Testing of the Nanodiamond Hydrogels

To test the properties of the newly designed hydrogels, an earlier reported general generic protocol was followed [7].

Bio-adhesive Test: Bioadhesion studies were done using a Chatillon apparatus for force measurement. [7] This method determines the maximum force and work needed to separate two surfaces in intimate contact. The hydrogels (0.1g) were homogeneously spread on a $1cm^2$ glass disc and then the discs were fixed to the support of the tensile strength tester using double sided adhesive. The gel was brought into contact with a slice of dentin in order to imitate adhesion of the gel to the tooth structure. After a preset contact time of 1 minute under contact strength of 0.5 N, the two surfaces were separated at a constant rate of displacement (1 mm/s). The strength was recorded as a function of the displacement, which allowed determination of the maximal detachment force, F_{max}, and the work of adhesion, W, which was calculated from the area under the strength-displacement curve [7].

Equilibrium swelling: The known weight-containing dry gels were immersed in pH 4.0 and pH 9.0 buffer solutions respectively and kept at 25°C for 48h until equilibrium of swelling had been reached. The swollen gels were taken out and immediately weighed with microbalance after the excess water lying on the surfaces was absorbed with a filter paper. The equilibrium swelling ratio (SR) was calculated using the

following equation:

$$SR = (Ws-Wd)/Wdx100\%$$

Where, Ws and Wd are the weights of the gels at the equilibrium swelling state and at the dry state, respectively. Experiments were repeated in triplicate for each gel specimen and the mean value was obtained [7].

In vitro **Tetracycline release**: The release study was carried out with USP dissolution apparatus type 1 (Copley U.K.) and slightly modified in order to overcome the small volume of the dissolution medium by using 100 ml beakers instead of the jars. The basket of the dissolution apparatus (2.5 cm in diameter) was filled with 1 gm of tetracycline gel on a filter paper. The basket was then immersed to about 1 cm of its surface in 50 ml of phosphate buffer pH 6.8, at 37˚C ± 0.5˚C and stirred at 100 rpm. 22 Samples (2 ml) were collected at 0.25, 1, 2, 3, 4, 5, 6, 7 and 8, 24, 48, 72, 96, 120, 144, 240 hours [23] and analyzed using a UV spectrophotometer (Cintra 5, GBC Scientific equipment, Australia). The UV-vis absorption spectrum of tetracycline hydrochloride in water is typicallyaround 361 nm. The eluted tetracycline hydrochloride was measured using a calibration curve for tetracycline hydrochloride in PBS at pH 6.8 and measured at an absorbance of $337cm^{-1}$.Three replicate measurements were performed for each designed formulation. Each sample was replaced by the same volume of phosphate buffer, pH 6.8, to maintain its constant volume and sinking condition [7].

Microbiological Investigations: The antibacterial activity of the hydrogels was tested against E. faecalis type strain (ATCC 29212) obtained from the American Type Culture Collection (ATCC, Manassas, USA). The tests were performed using the standard Kirby-Bauer agar disc diffusion method[10]. Muller-Hinton agar (Oxoid, Basingstoke, UK) plates were inoculated by streaking a standardized inoculum onto the plates with a throat cotton swab. The inoculum was prepared by suspending the bacterial cells from a single colony from an overnight culture into a sterile saline solution to match a 0.5 McFarland standard (containing about 10^7- 10^8 colony forming units/ml). From each test sample, 250µg of hydrogel was applied to a 6 mm diameter paper disc. A clean and sterile paper disc was used as a negative control. The paper discs were evenly spaced on the inoculated Muller-Hinton agar plates and incubated at 37^0C for 24 hours after which the diameter of the zones of growth inhibition was measured using a caliper. Each measurement was done in triplicate from three different angles and the testing of each sample was repeated 3 times. The antibacterial efficacy of the prepared gels was compared to antibiotic sensitivity discs (Mast Laboratories, Merseyside UL) containing 30 µg of tetracycline per disc [11].

Free radical scavenging ability: We adopted the method of Kyselova *et al*. (2003) to test for free-radical scavenging ability of the new hydrogels. This method records changes in water solubility of the model protein, bovine serum albumin (BSA) exposed to free radicals generated by the Fenton reaction system [12].

The incubation mixtures contained the following reagents: bovine serum albumin (0.8 mg/ml), phosphate buffer, pH 7.4 (10 mM), EDTA (4.8mM), $Fe(NH_4)_2(SO_4)_2$ (4 mM), ascorbate (4 mM) and H_2O_2 (0.2%) in water to reach a total volume of 2.5 ml. The hydrogels were added (0.2mg/ml) and the reaction mixture was incubated for 20 min at room temperature. After completion of the reaction, the mixture was centrifuged at 3500 rpm for 10 min. The supernatant was precipitated with an equal volume of trichloroacetic acid (10%) at 0^oC. The precipitate thus obtained was re-dissolved in 1 ml of Na_2CO_3 (10%) in NaOH (0.5 M) and the final volume made up to 2.5 ml by adding distilled water. An aliquot of the solution was used for protein determination using the method of Lowry et al. (1951) [13].

Statistics: The Student's T-test was used to analyze data were appropriate.

Cytotoxicity

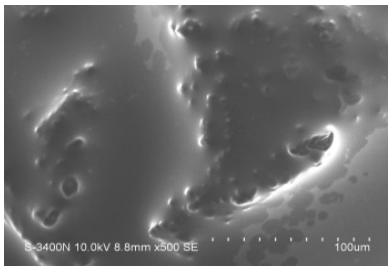
The cytotoxicity was done as previously reported [14]. Briefly, mouse fibroblast 3T3 cells were first grown to near confluency, diluted to a final cell suspension containing approximately 3 ×105 cells/ml and plated out in sets of 96 well plates. Chitosan or nanodiamands and the combination there of (chitosan/nanodiamond) were then added to the growth medium at a concentration of 1 mg/ml. Two hundred µl of each group was added to 20 wells in the 96 well plates. Medium without any gels was used as controls. After 24 hours the MTT colorimetric assay (15) was used to evaluate the cell survival rate. Absorbance was measured at a wavelength 540 nm on a spectrophotometer to determine the number of viable cells. Three replicates were done in each group.

Results and Discussion

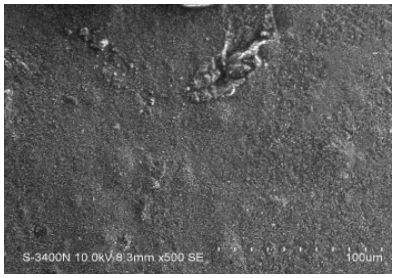
The characterization of nanodiamonds containing chitosan hydrogels: (Gel-1 to Gel-5)

SEM images were obtained to characterize the microstructure of the freeze-dried composite gels and are presented in Figure 1. It could be seen that the gels displayed a homogeneous pore structure. The micro-porous structure of the gels could lead to high internal surface areas with low diffusional resistance in the gels. The surfaces of the gels were also presented (Figure 1). The 'skin' of the gels can be seen, and the collapse of the surface pores may be due to the freeze-drying process.

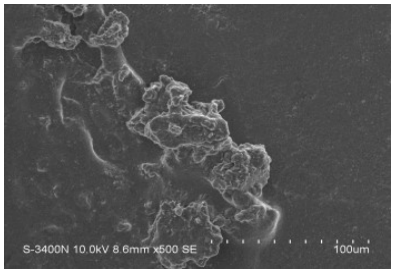
Figure 1. SEM photographs of interior morphology of the selected gels, (a) Gel-1, (b) Gel-2, (c) Gel-3, (d) Gel-4, (e) Gel-5 under investigation .



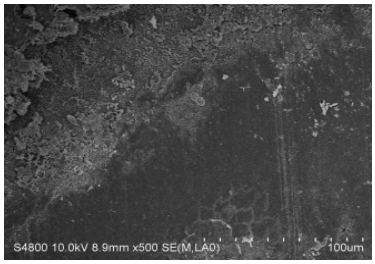
Gel 1



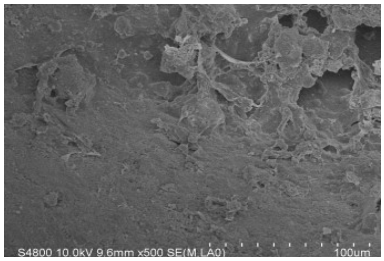
Gel 2



Gel 3



Gel 4



Gel 5

Bio-adhesion

High adhesiveness of the gels is desired to maintain an intimate contact with the tooth structure. The chitosan hydrogels showed a high adhesive force and work of adhesion. This can be expected due to the well-known intrinsic bio-adhesive properties of chitosan. The adequate water absorption capacity together with the cationic nature [9] that promotes binding to the negative surface of the dentin structure can also explain these results. The results are summarized in Table 2.

Table 2. Bioadhesion testing in vitro.

Hydrogel	Adhesive Force (**N**) ± SD (Dentin)	Work of Adhesion (**N cm**) ±SD (Dentin)
Gel-1	1.21±0.35	2.92±0.34
Gel-2	1.37±0.44	3.49±0.42
Gel-3	1.12±0.60	2.94±0.29
Gel-4	1.27±0.24	3.38±0.31

The presented values are an average (n=5)

According to Caffaggi, hydration of the polymer causes mobilization of the polymer chains and hence influences polymeric adhesion [16]. Appropriate swelling is important to guarantee adhesiveness; however, over hydration can form slippery non-adhesive hydrogels [17]. In addition, the molecular arrangement of the polymeric chains present in the new hydrogels, such as tetracycline, nanodiamond and hydroxyapatite can further enable interaction with the substrate. The correlation between the force and work of adhesion is noticeable in all the hydrogels.

Equilibrium swelling in the Nanodiamond-Chitosan Gels

These hydrogels are hydrophilic in nature due to the highly polar amide linkages, which can form efficient hydrogen bonding with the solvent, and can therefore swell in an aqueous medium. The average of swelling results of 3 comparable experiments for each sample of the hydrogels is summarized in Figure 2.

The hydrogels remained in the cylindrical form in which they were cast after swelling. All the hydrogels were only slightly swelled in the aqueous medium.

The equilibrium swelling ratio (SR) of newly developed biomaterials is important since it exerts an influence on their release rates. Environmental pH value also has a large effect on the swelling behavior of these gels. Such pH dependent properties

of the hydrogels come from the polyelectrolyte nature of chitosan segments in the hydrogel network.

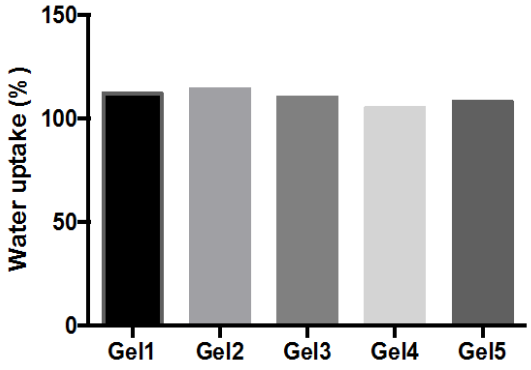


Figure 2. Water uptake by the newly prepared gels.

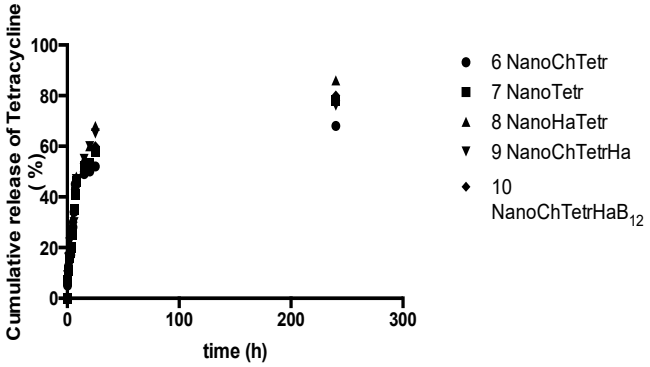


Figure 3. Tetracycline release from newly prepar

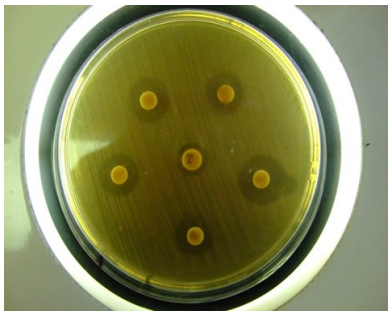


Figure 4. An example of inhibition zones produced by the different nano-diamond hydrogel mixtures.

In Vitro Release of Antibiotics from Chitosan Gels

The *in vitro* release of tetracycline hydrochloride quantified as the cumulative percentage of the therapeutic agent released as a function of time is presented in Figure 3.The tetracycline content of the hydrogels was detectable 240 hours. Significantly more tetracycline was detected between 96 hours and 240 hours than between 24 hours and 72 hours, suggesting the prolonged release of the antibiotic from the hydrogels.

The antimicrobial activity of the Hydrogels

All the test samples gave an average inhibition zone larger than the tetracycline control disc, thereby confirming the antibacterial activity of the different nano-diamond combinations against *E. feacalis* (Figure 4). Using the Student's T-test ($p<0.01$), no statistically significant difference was found between *NanoChTetr* and *NanoChTetrHaB*$_{12}$ and between *NanoTetr* and *NanoChTetrHa*. There was a significant difference between the rest of the samples when compared to each other and the positive control. The hydrogel with the highest antibacterial activity contained nano-diamonds and hydroxyapatite. By adding chitosan the activity was significantly reduced. By just adding nano-diamonds to the tetracycline the lowest antibacterial activity was obtained, however the antibacterial activity was still higher than the tetracycline control disc (Figure 5).

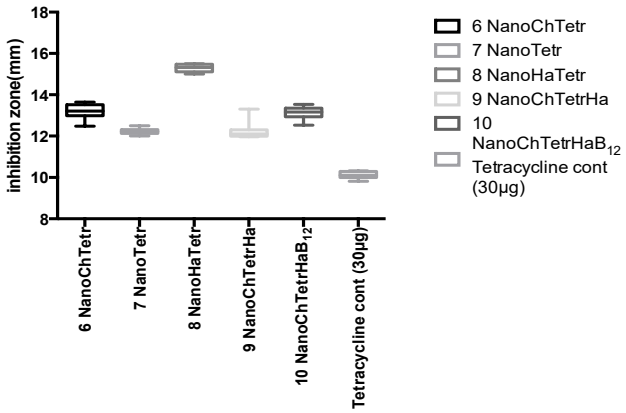


Figure 5. Antibacterial inhibition zones produced by the different nano-diamond combinations against *Enterococcus faecalis*.

The action of tetracyclines is bacteriostatic. Bacteria actively pumps tetracycline into their cytoplasm, even against a concentration gradient. The tetracycline then binds to ribosomes and blocks the attachment of transfer RNA and thereby inhibits protein synthesis [18].

A number of mechanisms explaining the antimicrobial activity of chitosan have been postulated [16]. One of the proposed mechanisms (almost similar to tetracycline activity) is that the cross-linker moieties incorporated into hydrophilic chitosan increase their solubility and ease of penetration of the hydrogels into the cells of microorganisms. The chitosan then binds to microbial DNA, inhibits the transformation of mRNA and protein synthesis, and thereby inhibits metabolism [17].

Another suggested antibacterial mechanism of chitosan is the interaction between positively charged protonated NH_3^+ groups of the chitosan molecules and negatively charged

microbial cell surfaces. The electrostatic interaction results in changes in the properties of the cell wall permeability with leakage of the intracellular electrolytes causing internal osmotic imbalance that inhibit the growth of the microorganisms [18].

One of the advantages of nanodiamonds is that they are completely inert, optically transparent and seemingly bio-compatible. Although their in vivo toxicity depends on their particular surface characteristics, nanodiamond particles do not induce significant cytotoxicity in a variety of cell types [19, 20]. However, more recently antibacterial activity of partially oxidized diamond particles has been observed. Nanodiamonds can kill Gram positive as well as Gram negative bacteria, a property which is directly linked to their surface chemistry. The antibacterial properties can however be masked by proteins covering the surface groups responsible for their bactericidal activity [20].

Insight into free radicals involved during *in vitro* endodontic treatment

Without adequate endodontic treatment, infection of the root canal and peri-radicular tissue may result in persistent root canal infection. Reactive oxygen species (ROS) produced by leucocytes plays an important role in the host defense response against invading microorganisms, and is crucial in the pathogenesis of pulpal infections [21, 22]. However, excessive amounts of ROS may also destroy the adjacent host tissues and medications that generate excessive amounts of ROS such as the hydroxyl radical, the superoxideradical, and H_2O_2 may be cytotoxic [22]. In selecting a root canal medicament, it is necessary to weigh their therapeutic benefits against their potential harmful effects [11].

Chlorhexidinegluconate is an effective antimicrobial agent with potent antimicrobial and anti-inflammatory properties and has been widely used as an antiseptic agent for the irrigation of root canals as well as a medicament during root-canal treatment [1]. It has also been found that in combination with $Ca(OH)_2$ and H_2O_2, CHX is more effective against *Streptococcus* species and in eliminating *Enterococcus faecalis* from root canal infections [23, 24]. However, recent reports suggested CHX as a potential genotoxic agent toward leucocytes, oral mucosal cells and lymphocytes and the use of CHX on its own, or in combination with these products may be potential genotoxic and cause tissue damage [25].

The amount of uncontrolled ROS is the main cause of the inability of the healing process to continue and therefore it would be ideal to utilize the antioxidant capacity of the "designer hydrogels" to detect and fight the free radical excess.

It is well established that $HO^{\bullet}$ can be generated from a reaction known as the Fenton reaction in the presence of H_2O_2 [26, 27] and the generation of $HO^{\bullet}$ has been shown to be a critical factor in various ROS-induced oxidative stresses [28, 29].

Protein cross-linking can be used as a model for detection of free radical activity [8]. Water soluble Bovine Serum Albumin (BSA) can be polymerized by hydroxyl radicals generated by the Fenton reaction system of Fe^{2+}/EDTA/ H_2O_2/ascorbate [1]. As a result, the protein loses its water solubility and the polymerized product precipitates. The decrease in the concentration of the water-soluble protein can then be detected [12].

With this study we used the well calibrated model of Kyselova [8] to evaluate the chitosan: antibiotic: nanodiamond containing hydrogel for possible use in dental treatment where it can act as a defense material where interactions with CHX and $Ca(OH)_2$ may generate toxic ROS (Results presented in Figure 6).

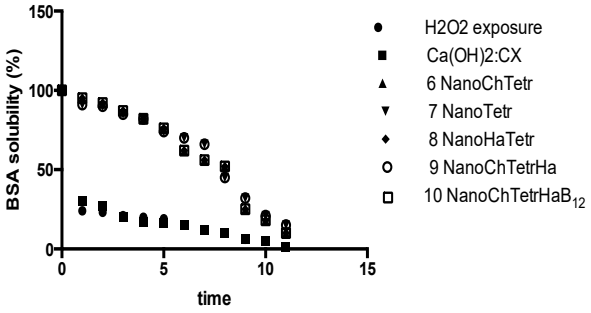


Figure 6. Free Radical Defense Capability of the Prepared Nanodiamond:Chitosan.

As clearly demonstrated by Figure 5, upon exposure to standard H_2O_2 in the form of a Fe^{2+}/EDTA/H_2O_2/ ascorbate solution as a baseline determinate for free radical generation under "prototypical in vitro free radical damage", or a mixture of the $Ca(OH)_2$:Chlohexadine paste as an alternative method of generating ROS under in-vitro conditions similar to the "endodontic treatment" in vivo, upon incorporation of the chitosan substituted hydrogels the built-in antioxidant capacity of the functionalized biomaterial activates free radical defense of the in vitro model system. Further in vivo investigations are currently on the way in our laboratories to highlight the importance of this observation. This model represents the practical approach of in situ monitoring and testing of the amount of the free radical production and synergistic antioxidant defense of the system. Further investigations with fine-tuning of the system are currently under way in our laboratory.

Cytotoxicity

The Friedman ANOVA test by ranks showed significant differences ($p<5\%$) between the controls and their 3 different additives gels.(Figure 7) The median cell survival rates were found to be: chitosan (113%), nanodiamond (92%), chitosan/

nanodiamond (93%). The ANOVA test showed significant differences (<5%) amongst the 3 different gels. Chitosan alone, using The Friedman ANOVA test by ranks, showed significant differences (p<5%) between the controls and their 3 different gels. The median cell survival rates were found to be: chitosan (113%), nanodiamond (92%), chitosan/nanodiamond (93%). The ANOVA test showed significant differences (<5%) amongst the 3 different gels. Chitosan alone was found to have a significantly (Bonferroni test) higher (p<0.05) cell survival rate than nanodiamonds or the chitosan+nanodiamond combination, but no significant difference (p>5%) was found between nanodiamonds and the chitosan+nanodiamond combination, although the chitosan+nanodiamond combination was slightly higher, which demonstrates the positive effect of chitosan. The maximum and minimum values were given. The intermediary box represents the position of 50% of the values and the line within the box shows the median values.

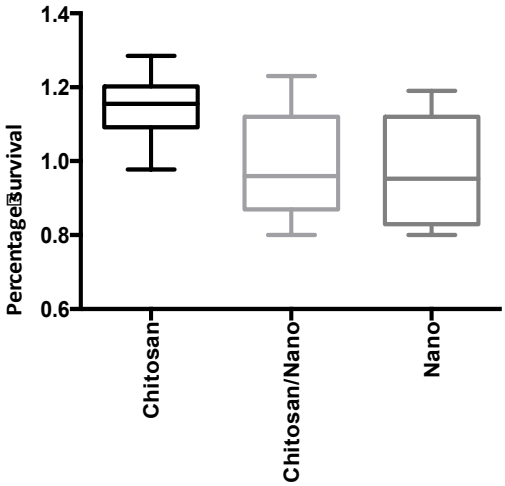


Figure 7. The box and whisker plots of the mouse fibroblast 3T3 cell survival rates for chitosan, nanodiamondsand chitosan/nanodiamonds, where 1.0 is equivalent to 100.0%.

Conclusion

In this study we demonstrated that the newly prepared chitosan nano-diamond hydrogels are suitable novel antimicrobial drug delivery systems against *Enterococcus faecalis*, as well as, bio-active materials capable of efficiently counteracting potential free radical damage generated during the potential endodontic treatment *in vitro.*

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