



## EVALUATION OF ANTIBIOFILM EFFICACY OF NANOPARTICLE AGENTS USING PHOTODYNAMIC THERAPY; AN IN VITRO STUDY

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### ABSTRACT

**Objective:** This study evaluated the antibiofilm efficacy of nanoparticle agents using photodynamic therapy. **Methods:** A total of Ninety dentin section blocks were used in this study. Dentin section blocks were prepared to be (4×4×1 mm) (Length× Width × thickness) respectively. Each dentin section was placed in 1.5 mL polypropylene tube filled with brain heart infusion (BHI) broth then underwent sterilization using autoclave at 121°C for 20 minutes and the sterilization was confirmed using bacterial count test. Cultivation of Standard strains of E. Faecalis ATCC 29212 and Staph. Epidermidis ATCC 12228 were prepared then inoculated on dentin section blocks and incubated for 3 weeks at 37°C in incubator (The medium was changed every 3 days). The specimens were grouped into 4 groups according to Final irrigant rinse were used: G1: final rinse using chitosan nanoparticles (CSnps)., G2: final rinse using Rose Bengal dye (RB), G3: final rinse using chitosan nanoparticles conjugated with Rose Bengal dye (CSRBnps) and G4: control group. Each group was activated using a diode laser 635 nm for 5 minutes at energy fluence 30 J/cm<sup>2</sup>. Then, the effect of tested final rinsing on multispecies biofilm was assessed with Confocal Laser Scanning Electron Microscope (CLSM) and data were statistically analyzed. **Results:** With regard to the antimicrobial effectiveness of tested irrigants, the highest mean percentage of dead bacteria was found in {CSRBnp (Group 3)} (88.38 ± 5.45) followed by {Csnp (Group 1)} (75.73 ± 5.15) followed by Rose Bengal dye (Group 2) (59.43 ± 8.14). The least mean percentage of dead bacteria was found in Control group (Group 4)} (7.25 ± 1.12). With a statistically significant difference between groups where (p<0.001). **Conclusion:** Functionalization of chitosan nanoparticles with Rose Bengal dye is a potent antibiofilm strategy for disinfection of the root canal system.

### INTRODUCTION

One of the most important goals of root canal treatment is to eliminate bacteria from the infected root canal systems through instrumentation and use of disinfecting agents<sup>(1,2)</sup>. Biofilm can be defined as a sessile multicellular microbial community characterized by cells that are firmly attached to a surface and enmeshed in a self-produced matrix of extracellular polymeric substance (EPS)<sup>(3,4)</sup>. Biofilm is considered one of the main etiological factors of endodontic infections. Its elimination from anatomical complexity of the root canal system is a challenging procedure. Thus; the use of a potent antibiofilm strategy has become mandatory to be able to resist endodontic infection<sup>(5,6)</sup>.

Photodynamic therapy (PDT) as an antibiofilm strategy is based on the use of a nontoxic dye (photosensitizer agent), that when activated by using a low energy light lead to production of free radicals such as singlet oxygen<sup>(7)</sup>. However; several studies have shown incomplete destruction of biofilms using PDT due to reduced penetration of the photosensitizer agent within the dentinal tubules<sup>(8)</sup>. Therefore, one of the promising method of enhancing antibiofilm action of PDT is its use in combination with nanoparticle agents as a drug delivery system.

Different nanoparticles have been introduced as an irrigant to control the bacterial biofilm in root canal system.

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The encapsulation of photosensitizer agent with nanoparticles may offer a novel design for enhanced drug delivery in the root canal system and photo destruction of root canal biofilms<sup>(9)</sup>.

So, the aim of this study was to evaluate the antibiofilm efficacy of nanoparticle agents either by themselves or in combination with photodynamic therapy using Confocal Laser Scanning Microscope (CLSM).

## MATERIALS AND METHODS

### 1. Selection of the teeth

A total of one hundred and twenty non-carious recently extracted human single rooted teeth with fully formed root apices were collected from patients with ages ranging between 20 and 45 years old. The selected teeth were cleaned of calculus and soft tissue remnants using a hand curette. A dental operating microscope DOM (8x) were used to examine the selected teeth. Also, to confirm the presence of type I root canal morphology in each tooth according to Vertucci's classification. The teeth that had caries, deep cracks, attritions, fractures or restorations would be excluded from the study.

### 2. Preparation of the samples

The dentin section blocks were prepared and standardized to be (4x4x1 mm) (Length x Width x thickness) according to Haapasalo & Orstavik D.<sup>(10)</sup> technique through the following steps: - Teeth were demarcation were done at CEJ and at the last 3mm of the roots. After that the crowns of the teeth were cut off at the level of C.E.J by using a diamond disc mounted in a straight hand piece under water coolant. Root canal patency for each sample was done using a size #10 and #15 K file. Root canals instrumentation were done using Revo-S rotary NiTi file system according to the manufacturer's instructions. Rotary files were mounted in a torque limited control motor at a torque 1.8 Ncm and speed 400 rpm as recommended by the manufacture starting from

SC1(25 taper 0.06) to AS40(40 taper 0.06). The apical 3mm of the roots were sectioned off and the roots were longitudinally sectioned in bucco-lingual direction along the midsagittal plane into two semi cylindrical halves and the cementum was removed from the root surface using Isomete saw at 1000 rpm under water cooling. Each samples of the roots were shaped and refined to be (4x4x1 mm) (Length x Width x thickness) respectively using Isomete saw at 1000 rpm under water cooling.

### 3. Sterilization of the samples

Each dentin section block was placed in 1.5 mL Eppendorf tube filled with brain heart infusion (BHI) broth and sealed within sterilization pouches then underwent sterilization using autoclave at 121°C for 20 minutes.

After complete the sterilization the Eppendorf tubes opened in a sterile air laminar flow cabinet and a sterile paper points were inserted into the Eppendorf tubes for 1 minute until it completely saturate and absorbed the broth media, then the paper point was transferred and spreader on brain heart infusion (BHI) agar plate. The plate was incubated for 24 hours at 37°C and 100% humidity for bacterial count. After 24 hours the plate was inspected and checked for no inhibition zones were founded around the incubated paper point indicating a sterility of the samples before inoculating the multispecies microorganisms.

### 4. Preparation of the microorganisms

Cultivation of Standard strains: *Enterococcus Faecalis* (E. Faecalis) American Type Culture Collection (ATCC 29212) and *Staphylococcus epidermidis* (Staph. epidermidis) American Type Culture Collection (ATCC 12228) were done and Each Eppendorf tube contain sterile dentin section block was opened in a sterile air laminar flow cabinet. Eppendorf tubes were filled with 0.5ml of E. faecalis suspension plus 0.5ml of *Staph epidermidis* suspension using a sterile micropipette tips for each

organism. Eppendorf tubes were closed tightly and shaken well then incubated for 21 days at 37°C in incubator. After incubation period, the samples were examined for confirmation of multispecies biofilm formation on dentin section blocks.

### 5. Confirmation of multispecies biofilm formation.

Three randomly samples were selected after finishing of sterilization and before bacterial inoculation. Also, others three randomly samples were selected after 21 days after incubation of dentin section blocks with multispecies microorganisms. These samples were inspected to confirm the presence/absence of multispecies bacterial biofilm formation using Scanning Electron Microscope (SEM).

### 6. Preparation of the irrigant solutions.

#### *I-Chitosan nanoparticles.*

Chitosan nanoparticles (CSnps) were prepared at 2 mg/ml according to Minimum inhibitory concentrations (MIC) for each microorganism (*E. faecalis* and *Staph. epidermidis*). Before using the CSnps, the antimicrobial activity was tested. Particles size analysis of the prepared CSnps were recorded within (50 nm) using Transmission electron microscope (TEM).

#### *II-Rose Rose Bengal dye (Photosensitizer agent).*

Stock solution of Rose Rose Bengal dye was prepared at concentration 10 µM/mL. The absorption spectra of diluted solution of the prepared RB dye was recorded within 550 to 750 nm using double beam UV-Visible Spectrophotometer and the maximum absorption peak was recorded at 650 nm.

#### *III-Chitosan nanoparticles-conjugated with Rose Rose Bengal dye (CSRBnps).*

The CSRBnps was prepared using a chemical crosslinking by a carbodiimide (N-ethyl-N-3-dimethyl aminopropyl carbodiimide) (EDC) at final concentration of CSRBnps was (0.3 mg/mL). The

absorption spectra of diluted solution of the prepared CSRBnps was recorded within 550 to 750 nm using double beam UV-visible Spectrophotometer and the maximum absorption peak was recorded at 665nm.

### 7. Grouping of the samples

All dentin section blocks were randomly classified according to the final rinse into 4 groups (n=20). **Group (1) (CSnps Group);** Final rinse using chitosan nanoparticles and activated with diode 635 nm for 5 minutes at energy fluency 30 J/cm<sup>2</sup>. **Group (2) (RB Group);** Final rinse using Rose Rose Bengal dye as a photosensitizer agent and activated with diode laser 635 for 5 minutes at energy fluency 30 J/cm<sup>2</sup>. (Photodynamic Therapy). **Group (3) (CSRBnps Group);** Final rinse using chitosan nanoparticles conjugated with Rose Rose Bengal dye (CSRBnps) and activated with diode laser 635 nm for 5 minutes at energy fluency 30 J/cm<sup>2</sup>. **Group (4) (control Group);** No final rinse was used for dentin section blocks contaminated with multispecies biofilm (Non-treated bacterial group) (Negative control group).

### 8. Application of the irrigant solutions and activation.

**Group (1):** Each sample was rinsed with 1 ml of chitosan nanoparticles solution (2mg/ml) concentration within the Eppendorf tube and left for 15 minutes (prior to irradiation) for allowing the irrigant to interact with biofilm. **Group (2):** Each sample was rinsed with 1 ml of Rose Rose Bengal dye solution (10 µM) concentration within the Eppendorf tube and protected from ambient light then left for 15 minutes (prior to irradiation) for allowing the dye to interact with biofilm. **Group (3):** Each sample was rinsed with 1 ml of CSRBnps (0.3 mg/mL) concentration within the Eppendorf tube and protected from ambient light then left for 15 minutes (prior to irradiation) for allowing the irrigant to interact with biofilm. **Group (4):** There is no final

rinse was used in this group. Each sample was remain within the Eppendorf tube without any treatment (Negative control group) until time of evaluation. Irradiation was performed in all groups except the control group using a diode laser 635 nm for 5 minutes at energy fluence 30 J/cm<sup>2</sup>.

### 9. Evaluation of the sample by Confocal Laser Scanning Microscope (CLSM).

After completion of the treatment protocol, the samples were prepared to evaluation by CLSM. The irrigant solution was aspirated from each Eppendorf tube with a sterile 3 ml plastic syringe. The dentin section blocks were washed gently with 100 µl phosphate buffered saline (PBS) solution to remove the excess of irrigant solutions. The samples were stained with Fluorescence-based live/dead stains to detect the viability of bacterial cells within multi-species biofilm. Fluorescein diacetate (FDA) and propidium iodide (PI) were used for staining the dentin section blocks to detect the live/dead bacteria respectively. After staining, CLSM analysis was done to discriminate between viable bacteria (green in color) and dead bacteria (red in color) within the biofilm and infected dentinal tubules.

After finishing the staining procedure, each dentin section block was placed on a sterile glass slide and covered with cover slide (0.13mm thickness). The cover slides were fixed on the glass slides at periphery with glue for allowing the stability of the samples during examination.

Slides were immediately mounted on a modified chamber device of CLSM and examined using Confocal Laser Scanning Microscopy (CLSM).

### 10. Statistical analysis of data

The mean and standard deviation values were calculated for each group. Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests, data showed parametric (normal) distribution. Repeated measure ANOVA followed

by Tuckey post hoc test was used to compare between more than two groups in related samples.

## RESULTS

Data were explored for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and showed parametric (normal) distribution. The mean and standard deviation values were calculated for each group. One-way ANOVA followed by Tukey post hoc test was used to compare between groups in non-related samples. The significance level was set at  $P \leq 0.05$ . Statistical analysis was performed with IBM® SPSS® Statistics Version 20 for Windows.

Comparison between the percentage of dead bacteria in Control group and different experimental groups showed that the highest mean percentage of dead bacteria was found in Chitosan nanoparticles conjugation with Rose Bengal dye (Group 3) ( $88.38 \pm 5.45$ ) followed by Chitosan nanoparticles conjugation (Group 1) ( $75.73 \pm 5.15$ ), followed by Rose Bengal dye (Group 2) ( $59.43 \pm 8.14$ ). The least mean percentage of dead bacteria was found in Control group (Group 4) ( $7.25 \pm 1.12$ ). There was a statistically significant difference between all groups. Where ( $p < 0.001$ ).

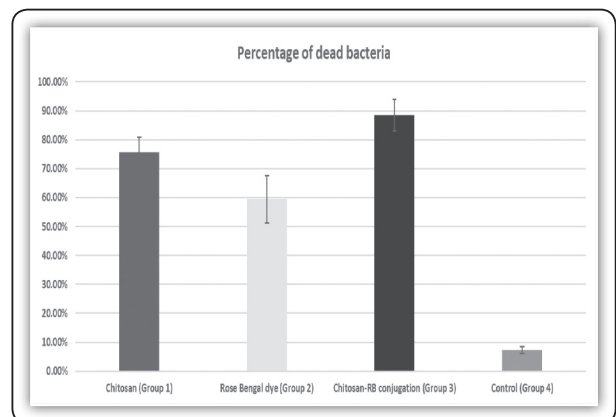


Fig. (1) A bar chart representing mean percentage of dead bacteria in control and all experimental groups.

## DISCUSSION

Single rooted teeth were selected in this study because of this tooth mainly have a single straight root canal in a bulky root that more suitable for dentin section blocks preparation with preset dimension. Others research done in this field also used the single rooted teeth as a biofilm model <sup>(11)</sup>. The teeth were collected from patients with ages ranging between 20 and 40 years to minimize variation in root dentin nature that may have effect on lumen size of the root canals <sup>(12)</sup>. The crowns of teeth were cut off to standardize a reference point and to facilitate the instrumentation of canals. Dentin section blocks were used in this study as a biofilms model to closely simulate as possible the in vivo root canal environment. The dentin section blocks were prepared with dimensions (4×4×1 mm) (Length × Width × thickness) respectively. These dimensions were standardized to be matched with the modified chamber room device of CLSM for ease of samples evaluation. Also, the laser source of CLSM cannot inspect the samples with dimensions more than 1 mm in thickness. Other researches were done in this field used these same parameters <sup>(13,14)</sup>. The *E. faecalis* bacteria was selected for this study because it is the most resistance bacteria associated with different forms of periradicular diseases including primary and persistent endodontic infections. Also, it has ability to attach itself to dentin wall and invade dentinal tubules forming a biofilm community able to resist irrigation solutions and intracanal medications <sup>(15)</sup>. *Staphylococcus epidermidis* was selected because it had been associated with endodontic infection, and for synergistic relationship with *E. faecalis* to form a multispecies biofilm <sup>(15)</sup>. Multispecies biofilm model was selected in this study to closely mimic in vivo biofilm where more than one type of bacteria was isolated from root canal infection. Using planktonic cell bacteria or monospecies biofilm doesn't actually reflect the endodontic infection conditions due to the microorganisms are

more likely to be organized in structures attached to each other and/or the root canal walls in form of a multispecies biofilms that make the bacteria 1000-1500 times greater resistant to phagocytosis, antibodies and antimicrobial agents than planktonic state bacteria <sup>(16,17)</sup>. Chitosan nanoparticles is a biocompatible and biodegradable polymer that exhibit significant antibacterial activity against Gram-positive bacteria, Gram-negative bacteria and multispecies biofilm during disinfection of root canals <sup>(18)</sup>. The concentration of chitosan nanoparticles in this study was 2 mg/ml which was chosen according to determine the minimal inhibitory concentrations (MIC) of Csnp to each microorganism (*E. faecalis* and *Staph. epidermidis*) separately, then the highest value of MIC was selected to use on multispecies biofilm <sup>(19)</sup>. The Rose Bengal dye (RB) was selected as a photosensitizer agent for Photodynamic therapy (PDT) due to its antibacterial activity against various gram positive and gram-negative bacteria. Also, RB dye is an anionic PS that has been shown excellent binding with cationic polymers such as chitosan nanoparticles. The concentration of anionic RB dye that used in the study was 10 µM. At this concentration, the singlet oxygen that produced was sufficient to induce significant biological activity against bacterial biofilm and this concentration also was founded to be below the cytotoxic limit <sup>(20,21,22)</sup>. Confocal Laser Scanning Microscope (CLSM) was used in this study because of it determine the microbial biofilms at different levels of the infected root canal. Also, it provides detailed information about the presence, distribution, viability status, and depth of penetration of biofilm inside dentinal tubules in accurate 3D reconstructions images. In the present study, the control group (group 4) showed the least mean percentage of dead bacteria when compared to other groups. The percentage of dead bacteria was found to be 7.25 % which is the normal for any untreated bacterial population. This result is in agreements with other researches done in this



filed<sup>(23&24)</sup>. This can be explained by the fact of the absence of disinfecting agent in the control group that has lethal effect on the multispecies bacterial biofilm. With regard to RB dye group (group 2), it showed the least mean percentage of dead bacteria when compared to other experimental groups. This result could be attributed to the fact of RB dye is an anionic photosensitizer (negatively charged photosensitizer) that does not interact electrostatically with the negatively charged bacterial cell membranes, resulting in the membrane barriers of the bacterial cells limit the simple diffusion of RB dye into the bacteria cells. This led to reduced penetration of the Rose Bengal dye into bacterial biofilm within dentinal tubules.<sup>(25)</sup> also, this result is in agreement with other researches done in this field<sup>(26)</sup>. With regard to CSRBnps group (group 3), it showed the highest mean percentage of dead bacteria when compared to other experimental groups. The exact mechanisms of antibacterial action of CSRBnps still are not perspicuous. However; it could be attributed to many reasons. Firstly, conjugation of the photosensitizer with a chitosan nanoparticle combining the properties of both antibacterial agents (synergism). This allowed photosensitizer molecules to enter the bacterial cells and increased penetration of the photosensitizer molecule into the biofilm structure. Secondly, the antibacterial properties of CSRBnps which lead to increased membrane permeability causing intracellular leakage, damage to the bacterial membrane and disruption of the biofilm structure<sup>(27)</sup>. With regard to CSnps group (group 1), it showed higher mean percentage of dead bacteria when compared to RB dye group (group 2). This can be attributed to the nanosized form of chitosan which allowed for more surface interaction and penetration within the biofilm structure. Besides, the electrostatic attraction of positively charged chitosan with the negatively charged bacterial cell membranes, which might lead to the altered cell wall permeability eventually resulting

in rupture of cell and leakage of the intracellular components leading to disrupt of biofilm surface<sup>(28)</sup>. In addition to, the antimicrobial activity of CSnps by itself which features them on RB dye group. By the matter of fact, CSRBnps group (group 3) were higher than CSnps group (group 1) due to its functionalization with photosensitizer agent.

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