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Antimicrobial Activity of Roselle-capped Silver Nanochip on *Aggregatibacter actinomycetemcomitans*

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Abstract

Objectives This article aimed to study the effects of the roselle-capped silver nanochip (SNP-Ro chip) against *Aggregatibacter actinomycetemcomitans*, and the toxicity of this film on fibroblast cells to develop this SNP-Ro chip into a local chemical for the treatment of periodontitis in the future.

Materials and Methods Using a microwave-assisted synthesis method, silver nanoparticles (SNPs) were prepared from a silver nitrate solution and roselle extract as a reducing and capping agent. Then, SNP-Ro chips were fabricated by mixing a solution of SNP-Ro with alginate gel. The antimicrobial effect of the synthesized SNP-Ro chips was performed by the disc diffusion technique and time kill assay. The cytotoxic effect was also determined by the MTS assay.

Statistical Analysis One-way analysis of variance (ANOVA) and Scheffe's method were used to analyze the data for this experiment.

Results All three ratios of the SNP-Ro chip produced inhibition zones ranging between 18.75 ± 2.08 and 19.03 ± 2.25 mm. In studying the killing time, the three groups of the SNP-Ro chips completely eradicated *A. actinomycetemcomitans* within 180 minutes. The percentage of the viable SNP-Ro chip-treated human gingival fibroblasts (HGFs) were significantly increased when compared with the alginate chip-treated cells ($p < 0.05$).

Conclusion This study developed a new method for the deposition of SNPs in alginate gel to make a thin small chip for the sustained release of the SNPs in a periodontal lesion. Therefore, the SNP-Ro chip has the potential to be developed as an adjunctive locally delivered antimicrobial agent in periodontal therapy.

Keywords
- *Aggregatibacter actinomycetemcomitans*
- microwave-assisted synthesis
- disc diffusion method
- time-kill assay
- MTS assay
- periodontal therapy
- antimicrobial

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Introduction

Periodontitis is an inflammatory disease initiated by specific bacterial species in dental plaque resulting in periodontal tissue destruction, tooth mobility, and finally, tooth loss.[1-4] Because of the fact that conventional treatment, such as scaling and root planing (SRP), does not completely eliminate periodontal pathogens, especially in deep periodontal pockets, antimicrobial agents can be used as an adjunctive therapy.[5,6] Local drug delivery is also highly attractive due to the ability to deliver the antimicrobial agent within the periodontal pockets, and the therapy is targeted on specific pathogens. However, the local application must reach the intended site of action, achieve therapeutic concentration, and last for a sufficient amount of time to achieve a positive effect. Currently, available local delivery drugs to satisfy the above criteria can be obtained in various forms such as, a chip, gel, and fiber.[7-9] Silver in the form of nanoparticles has been widely used in the medical and dental fields.[10,11] Previous studies indicated that SNPs have the ability to kill bacteria without causing bacterial resistance.[12] The synthesis of the SNPs can be done in three ways: physical, chemical, and biological. The method for the synthesis of SNPs is commonly used in chemical extraction but this method causes biological poisoning. Recently, the biosynthesis of SNPs with biomaterials, such as plant extracts, has been widely used. Roselle (*Hibiscus sabdariffa* L.) was found to have antioxidant and antibacterial activities.[13-15] Moreover, previous studies have reported that synthesized SNPs using roselle extract exhibited a 99.94% *Aggregatibacter actinomycetemcomitans* reduction.[16] Therefore, SNP-Ro was molded into thin film by using an alginate gel. The antibacterial activity against *A. actinomycetemcomitans* and cytotoxicity against the human gingival fibroblasts (HGFs) were evaluated.

Thus, this research aimed to develop an SNP-Ro chip to use as a local chemical for the treatment of periodontitis. The SNP-Ro chip was developed at different concentration levels. Alginate solution was used for the formation. Then, the film was tested for the activities against *A. actinomycetemcomitans* by a disc diffusion assay and time-kill assay. Toxicity against fibroblast cells was also tested by an MTS assay.

Methods

Preparation of SNPs Capping with a Roselle Chip

A solution of silver nitrate ($AgNO_3$) was mixed with roselle extract to make final concentrations between $AgNO_3$ and roselle extract of 1:0.5, 1:1.5, and 1:2.5, respectively. All solutions were then heated in a microwave (800 W) for 2 minutes.[12] After 48 hours, the synthesized SNP-Ro was analyzed via ultraviolet–visible (UV-Vis) spectroscopy. The SNP-Ro chips were synthesized mixing each concentration of SNP-Ro and 10% of the alginate solution (w/v). The ingredients were added to the beaker and imported to the dryer at 60°C for 24 hours. The thin film was removed from the beaker then a punching machine was used to make a circular chip.

Analysis of the Antimicrobial Activities

Disc Diffusion Assay

The individual colony of *A. actinomycetemcomitans* was suspended in brain–heart infusion (BHI) broth and incubated for 24 hours. The density of the bacterial culture was adjusted to a 0.5 McFarland standard and diluted 1:100 times in nutrient broth. *A. actinomycetemcomitans* was swabbed uniformly on the BHI agar disc. Different concentrations of the SNP-Ro chip were then gently pressed in the designated position. Also, 0.2% of chlorhexidine gluconate (CHX) was used as the positive control and the alginate chip was used as the negative control. The culture plates were incubated at 37°C, 5% of CO_2 for 24 hours. After incubation, the diameters of the inhibition zones for each well were measured.

Time-Kill Assay

Different concentrations of the SNP-Ro chip were added to 1,000 µL BHI broth. Then, 10 µL of the prepared bacterial suspension was added to the nutrient broth containing each ratio of the SNP-Ro chip, as well as 0.2% of CHX and the alginate chip. Serial dilutions of the sample were performed from 1/10 to1/10,000, and 10 µL of the diluted sample was dropped over the nutrient agar dishes. The culture plates were incubated at 37°C for 48 hours. Colonies on individual plates were counted and expressed as CFU/mL. The experiment was repeated by changing the incubation time of the SNP-Ro chips and *A. actinomycetemcomitans* to 30, 60, 90, 120, 180, 240, 300, and 360 minutes, respectively.

Cytotoxicity to Human Gingival Fibroblasts

To detect the effect of the SNP-Ro chip on the HGFs, each chip was added to 1 mL of a serum-free medium for 30 minutes. The HGFs (~5 × 10^4 cells/well) were seeded into 24-well plates. After 24 hours of incubation, the cells were then treated for another 24 hours with a prepared solution of each concentration of the SNP-Ro chips and alginate chip. The cytotoxicity of the SNP-Ro chips was evaluated by the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (MTS assay; Promega, Wisconsin, United States) according to the manufacturer's protocol.

Results

Characterization of the SNP-Ro Chips

The synthesized SNP-Ro showed a specific pattern at 350 to 450 nm, which indicated the formation of the SNPs (►**Fig. 1A**). Plasmon resonance band spectra of all mixture ratios also displayed specific peaks at a similar wavelength. The absorbance spectra increased, which corresponded to the concentration of the extract in the mixtures.[17,18] When the SNP-Ro of the three proportions was fabricated with the alginic acid into the chips, it was found that the chips had a circular shape with a 3-mm radius and 0.01 ± 0.005 mm thickness. The colors of the chips were yellow to dark brown depending on the quantity of the roselle (►**Fig. 1B**). Using the scanning electron microscope (SEM) to examine the SNP-RO chips, the morphology of the synthesized SNP-Ro chips

had a flat surface with white circular particles diffused on the chips. In comparing the alginate chip without any SNPs, the surface was not flat and did not have any white circles appearing on it (►**Fig. 2**).

Antimicrobial Property of the SNP-Ro Chips

From the results of the disc diffusion screening, the SNP-Ro chips were shown to clearly possess antibacterial properties against *A. actinomycetemcomitans*. All three ratios of the SNP-Ro chip produced inhibition zones ranging between 18.75 ± 2.08 and 19.03 ± 2.25 mm with no statistically significant differences ($p > 0.05$) among the SNP-Ro chip groups. However, the inhibition zone diameters of each SNP-Ro chip showed a significant difference ($p < 0.05$) when compared with the alginate gel chip without SNPs[19-21] (►**Fig. 3**). In studying the killing time, the three groups of SNP-Ro chips completely eradicated *A. actinomycetemcomitans* within 180 minutes (►**Fig. 4**).

Cytotoxic Effect of the SNP-Ro Chips on Human Gingival Fibroblasts

The percentage of the viable SNP-Ro chip–treated HGFs was significantly increased when compared with the non-treated cells or alginate chip–treated cells ($p < 0.05$).[13] The comparison of the cytotoxic effect between each concentration of the SNP-Ro chip showed no significant difference ($p > 0.05$; ►**Fig. 5**).

Discussion

At present, SNPs have been developed for many medical uses and are mostly being applied as an antibiotic substance. However, the synthesis that uses chemicals to reduce the agents causes a biological toxin. As a consequence, the synthesis of SNPs with natural extracts has been found to reduce this problem. Roselle can be used as a reducing agent and glazing agent in replacement of chemical applications.[16] Additionally, apart from being a natural herb, Jung et al found that roselle has antimicrobial properties against *Bacillus subtilis* and *Staphylococcus aureus*.[22] Furthermore, the use of roselle as a glazing agent to prevent the precipitation of the SNPs corresponded to the study of Rodríguez-León et al that used ginseng extract to synthesize silver nanoparticles to counter precipitation.[23]

As for the absorption of the SNP-Ro using UV-Vis spectroscopy to measure the absorption of the solutions of the three proportions, the range was 350 to 450 nm which was the absorption of the SNPs. This conformed to the study of

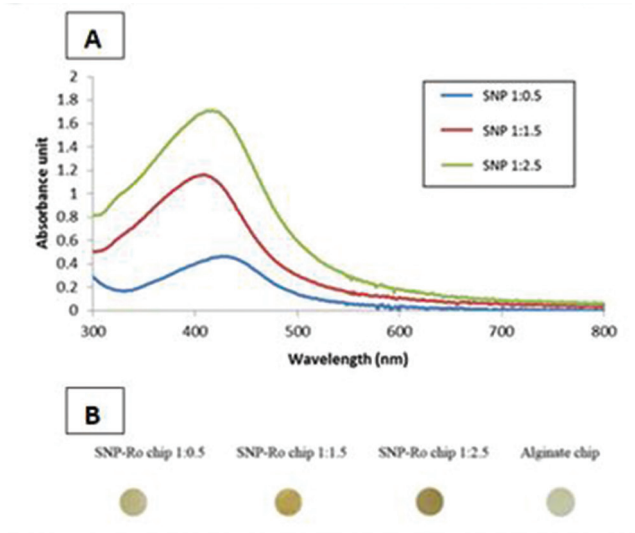

Fig. 1 (**A**) UV-visible absorbance peaks of the SNP synthesized using various concentration ratios of $AgNO_3$ and roselle extract as 1:0.5, 1:1.5, and 1:2.5. (**B**) SNP-Ro chips at different ratios and alginate chip. $AgNO_3$, silver nitrate; UV, ultraviolet.

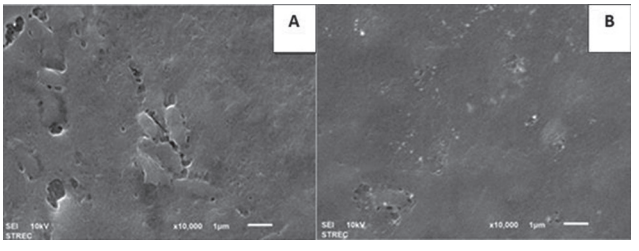

Fig. 2 Morphological characteristics of (**A**) alginate chip and (**B**) SNP-Ro chip (1:1.5).

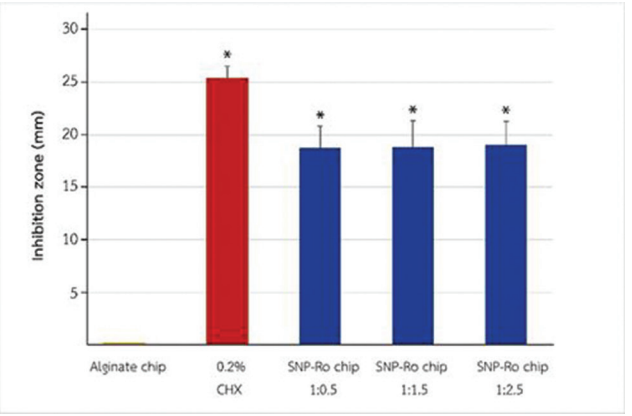

Fig. 3 Graph representing antibacterial activity of SNP-Ro chips by disc diffusion assay. CHX, chlorhexidine gluconate.

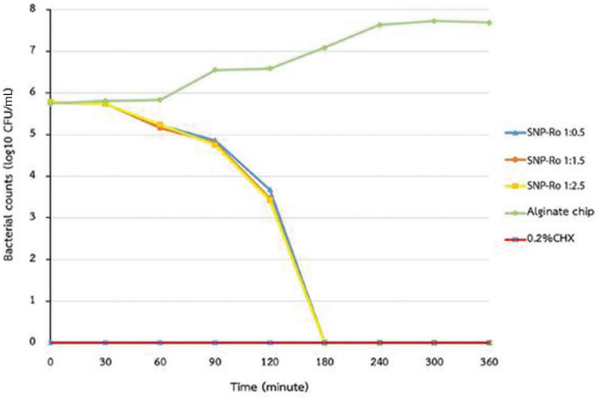

Fig. 4 Graph representing antibacterial activity of SNP-Ro chips by time-kill assay.

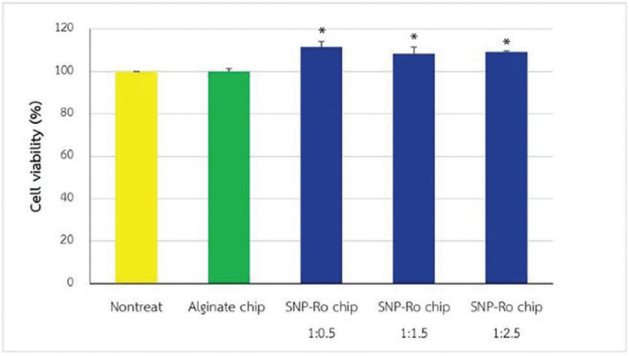

Fig. 5 Effects of SNP-Ro chips are shown with various concentration ratios on viability of HGFs. CHX, chlorhexidine gluconate; HGFs, human gingival fibroblasts.

Prakash et al on the synthesis of silver nanoparticles using the leaf extract of Spanish cherry as a reducing agent which showed a result of 434 nm.[24] Additionally, the absorption in this research might be slightly different when compared with other studies due to the different types of extracts; however, the range of absorption was similar.

In addition, the SNP-Ro chip was able to form a thin chip by using alginic acid which is a natural substance that causes no harm to humans, resulting in the capability of carrying the SNPs before being released on the specific sites for a particular treatment. After the analysis of the SNP-Ro chip using SEM, it was discovered that the surface was flat and there were white circular particles spread on the chip. This corresponded to the experiment of Lee et al that made a chip from poly(ether sulfone) with the characteristics of hybrid nanocomposite membranes by adding silver nanoparticles. Using the SEM, it was found that white circular particles of silver nanoparticles were spread all over the membrane.[25]

In the study of the antimicrobial effect of the SNP-Ro chips against *A. actinomycetemcomitans*, it was found that the SNP-Ro chips of the three proportions could release the SNPs which would destroy the bacterial cells. However, the antibacterial effect of the three chips displayed no statistical significance ($p \geq 0.05$). Alternatively, an alginate chip without SNPs was incapable of resisting *A. actinomycetemcomitans*. The antimicrobial results of the SNP-Ro chips from the experiment confirmed the study of Bindhu and Umadhevi who examined the antimicrobial effects of SNPs with the synthesis of plants. In that study, SNPs were developed using beetroot, and their antimicrobial activities against *Escherichia coli, Pseudomonas aeruginosa*, and *Streptococcus aureus* were indicated.[26] Two main mechanisms seemed to be responsible for the nanoparticles antimicrobial activities. Bindhu and Umadhevi presented that the silver particles released a positive charge to pair with the negative charge on the plasma membrane of the microorganism resulting in the plasma membrane's structural changes, building of small holes, and losing the ability to control the substance's output and input. This mechanism resulted in cell death.[26] Dakal et al demonstrated another mechanism in which the silver particles reacted with the chemical composition of the DNA resulting in the inability of the cell to process the cell's division and later dying.[27]

For the effect of the SNP-Ro chip on the HGFs, it was shown that all chips were not toxic to the HGFs. This corresponded to the experiment of Suwannakul et al who also found that synthesized SNPs, using *Glycyrrhiza glabra* root as a reducing agent to demonstrate an antimicrobial activity against *Streptococcus mutans*, were harmless to human gingival fibroblasts.[13]

A standard initial treatment for periodontitis is scaling and root planing (SRP). This method can efficiently remove the primary etiological factor, dental plaque and a local contributing factor, calculus[28] by using hand and ultrasonic scalers. Nevertheless, the hand instrumentation would smoothen the root better than an ultrasonic scaler. As a result, the hand instrumentation is a better method for reducing the adherence of subgingival plaque.[29] However, it was shown that pocket depths deeper than 6 mm were more difficult to scale. Some bacteria, such as *A. actinomycetemcomitans*, can invade the gingival tissue, which makes it impossible to eliminate completely from the pocket. Therefore, adjunctive treatment with antibiotics and antimicrobial agents may be required to overcome these bacteria. Thus, it has been found that probing depth and gain of clinical attachment level were improve significantly following a combination of SRP and locally delivered antimicrobials. A single episode of subgingival irrigation with tetracycline HCL was significantly altered the subgingival bacterial morphotypes towards one of periodontal health.[30] In this research, the silver nanochip could be another choice for local antimicrobial periodontal therapy because of its activities against *A. actinomycetemcomitans*, a key pathogen of periodontitis, within 180 minutes and was nontoxic to fibroblast cells. Furthermore, the manufacturing cost of silver nanochips was not high. However, experiments in animal models and clinical trials should be conducted before they are introduced to clinical practice for treatment of periodontitis.

Limitations

For the limitation in this research, the activities of the silver nanofilm were only tested against *A. actinomycetemcomitans*. Therefore, further studies should conduct testing on other types of bacteria, for example, *Porphyromonas gingivalis* and *Prevotella intermedia*.

Conclusion

This study developed a new method for the deposition of SNPs in alginate gel to make a thin, small chip for the sustained release of SNPs in periodontal lesions. All synthesized SNP-Ro chips containing different ratios of roselle extract demonstrated antimicrobial activity against *A. actinomycetemcomitans* without exhibiting cytotoxicity to HGFs. These findings suggested that the SNP-Ro chip has the potential to be developed as an adjunctive locally delivered antimicrobial agent for periodontal therapy.

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Conflict of Interest

None declared.

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