Activity of Chlorhexidine Gluconate Loaded at Varying Polyelectrolyte Multilayers against Aggregatibacter Actinomycetemcomitans

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Abtract. Chlorhexidine gluconate (CHX) has a bactericidal effect to Aggregatibacter actinomycetemcomitans (Aa) which causes periodontal disease. Therefore the controlled loading and release of CHX from multilayer thin film is beneficial for the local treatment of periodontitis. This study used ten bilayers of PDADMAC/PSSMA and PDADMAC/AL and loaded CHX into each film. Both un-loaded and loaded thin films were analysed using UV-Vis spectroscopy and AFM. The loaded film was thicker and rougher than the un-loaded film for both PDADMAC/PSSMA and PDADMAC/AL film. The loaded films were released in H_2O_2 for 24 h, and UV-Vis spectroscopy was measured and compared with the standard calibration curve of CHX. Results indicated that PDADMAC/PSSMA released 0.00052 M and PDADMAC/AL released 0.00071 M respectively. Both the loaded films were tested for toxicity to Aa. Neither film killed all colonies of Aa, however they reduced colony growth by 99% compared to the blank condition.

1 Introduction

Periodontal disease can affect elderly people with the loss of teeth. Clinical findings show that a periodontal pocket depth of more than 3 mm results in horizontal or vertical bone loss, clinical attachment loss and gingival recession [1]-[3]. Following treatment by scaling and root planing of the deep pockets, the dentist usually irrigates the area with a local antibiotic such as chlorhexidine (CHX) solution and inserts a biodegradable periochip [4]. This local antibiotic has a bactericidal effect on bacteria remaining in the periodontal tissue and decreases inflammation and recurrence rate [4], [5]. CHX is an antibiotic agent in the bisbiguanide group. It is composed of two chains which are linked together with hexamethylene. The chemical name is 1,6-di [4-chlorophenyl diguanido] hexane [6] (Fig. 1).

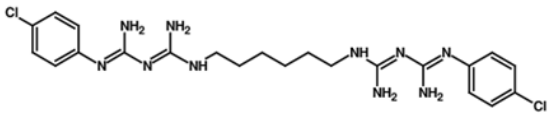

Figure 1. The chemical structure of chlorhexidine gluconate (CHX) [7].

CHX is effective against both gram-positive and negative bacteria, but shows little effect on fungi, viruses and tuberculosis (TB). It acts by destroying the cytoplasm of the bacteria which then leaks and the cell dies. Moreover high concentrations of CHX can bind with adenosine triphosphate and nucleic acid resulting in cell death after 8 –12 h. In the medical field, CHX is used as a mouth rinse for patients after surgery, rinsing during root canal treatment and a cleaning agent for medical equipment. An effective bactericidal concentration of CHX is between 0.12% and 0.20 % w/v. [8], [9].

In this study CHX was used as a model drug for loading and release into two types of polyelectrolyte multilayer (PEM) thin film. PEM thin films were developed by Decher and Hong in 1991 for modifying the surface of material [10]. PEMs were generated using the attractive force between positive and negative polyelectrolytes to form layer-by-layer thin film. The technique has been applied in many fields such as medical, engineering and industry as it is easy to use and inexpensive [11], [12]. Moreover the technique can be modified on a variety of materials including glass slides, silicon, gold, aluminium, wood and fibre [10], [13]. Positively and negatively charged polyelectrolytes are used to perform PEM. Examples of polyelectrolytes are polymers, proteins, nanoparticles and vitamins [14], [15].

This paper studied the ability of PDADMAC/PSSMA and PDADMAC/AL films to control the loading and release of CHX and their toxicity to anaerobic bacteria such as Aggregatibacter actinomycetemcomitans (Aa).

2 Experiment

2.1 Chemicals and methods

Poly(diallyldimethylammoniumchloride) (PDADMAC, medium molecular weight, 20% by wt in water, typical Mw=200,000 – 350,000), poly(sodium 4-

styrenesulphonic acid-co-maleic acid) (PSSMA, Typical Mw ~ 20,000), Alginic acid (Al, Typical Mw = 10,000 – 600,000) and poly(sodium 4-styrenesulphonate) (PSS, typical Mw = 70,000) were purchased from Sigma-Aldrich, (Thailand) Co., Ltd. Sodium chloride and sodium hydroxide were purchased from Labscan Asia Co., Ltd., Thailand. Chlorhexidine gluconate (CHX) was purchased from the Dental Hospital, Naresuan University. All chemicals and solvents were used as received without any further purification. Doubly distilled water was used in all experiments.

2.2 Multilayer thin films preparation

Ten millimolar PDADMAC and PSSMA were prepared at pH 9 and then added to 0.1M NaCl. A quartz slide was cleaned with piranha solution (H_2SO_4:H_2O_2 (70:30) and then coated with four layers of primer (PDADMAC/PSS). The multilayer thin film between PDADMAC and PSSMA was prepared by dipping the quartz slide in PDADMAC for 2 min, followed by three rinses in distilled water with pH adjusted at 9 by NaOH. The slide was then dipped in PSSMA for 2 min, followed by three rinses with distilled water. This step achieved the formation of one bilayer. Dipping was continued in PDADMAC and PSSMA until ten bilayers of PDADMAC/PSSMA had formed. The preparation of PDADMAC and Alginate thin film followed the same method [16].

2.3 Standard calibration curve of CHX

The concentration of CHX was varied at 0.00011 M 0.00056 M 0.00111 M 0.0017 M and 0.0022 M and measured by UV-Vis spectroscopy. The relation between concentration and absorbance value at 255 nm was plotted.

2.4 Loading CHX

To load of CHX in the PDADMAC/PSSMA and PDADMAC/AL films, the coated quartz slides were dipped into CHX solution (0.2% w/v, 50 ml). After dipping each film into CHX for 1, 2, 5, 10, 20, 30, 60 min and 24 h their wavelength was measured by UV-Vis spectroscopy. Finally, PDADMAC/PSSMA and PDADMAC/AL films both before and after loading were measured by atomic-force microscopy (AFM) to analyse their thickness and surface roughness.

2.5 Releasing CHX

Loaded PDADMAC/PSSMA and PDADMAC/AL films were dipped into H_2O_2 solution to release all CHX from the quartz slide. Then this solution was measured with UV-Vis spectroscopy to analyse the absorbance value at the maximum peak of CHX wavelength.

2.6 Antibacterial property against Aa

The antibacterial activity against Aa was tested using the standard method. The loaded films were exposed to 20 µl of Aa in brain heart infusion broth (2 ml). After 24 h incubation at 37 ◦C, 5% CO_2 condition, the bacteria/broth mixture was diluted five times. Then 50 µl of diluted bacteria was placed onto brain heart infusion agar using the spread plate method. After 24 h incubation the bacteria were counted. The result was corrected by the dilution factor to give the number of colony forming units (CFU) per millilitre. The percentage of bacterial reduction was then calculated and compared to the unloaded film.

3 Results and discussion

3.1 Multilayer thin film preparation

Twenty layers of PDADMAC/PSSMA and PDADMAC/AL were formed by the polyelectrolyte multilayer technique. The PEM films were then characterised via UV-Vis spectrophotometry and AFM. The wavelength of each PEM is shown in Fig. 2.

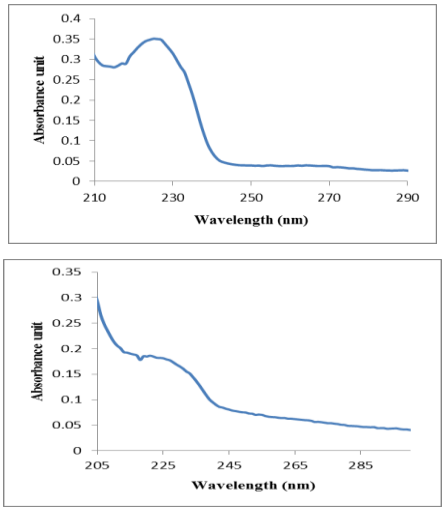

Figure 2. The UV–Vis absorbance spectra of PDADMAC /PSSMA film (upper) and PDADMAC/Alginate film (lower).

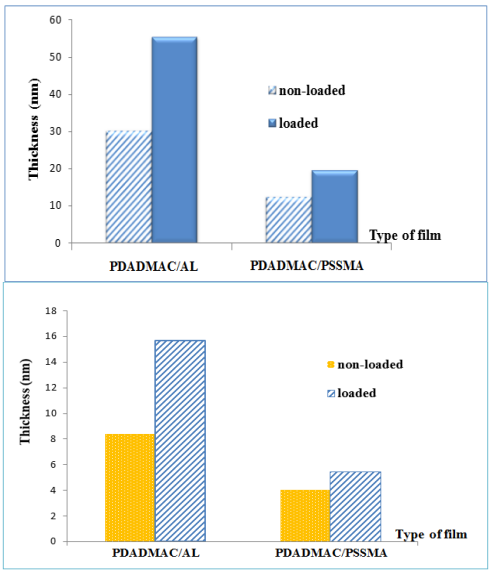

Figure 3. The thickness (upper) and surface roughness (lower) of loaded and non - loaded PDADMAC/PSSMA and PDADMAC/AL films.

The maximum absorbance value for 20 layers of PDADMAC/PSSMA and PDADMAC/AL was 226 and 210 nm as the wavelength of PSSMA and AL respectively. The PSSMA and AL were both present in the PEM thin film. [17], [18]. AFM was used to analyse the thickness and surface roughness of the multilayer thin film. Unloaded PDADMAC/AL film was thicker and rougher than PDADMAC/PSSMA film. The thickness of the PDADMAC/AL film was 30.39 ± 0.28 nm, whereas the thickness of the PDADMAC/PSSMA film was 12.54 ± 0.48 nm. The roughness values were similar to their thickness and the surface roughness of PDADMAC/AL was higher than PDADMAC/PSSMA (Fig. 3)

AFM indicated that the PDADMAC/AL thin film was thicker than the PDADMAC/PSSMA thin film as the charge density of Alginate was less than the PSSMA. Thus, the polyelectrolyte chain of Alginate had a lower negative charge than the chain of PSSMA. For this reason the chain of Alginate showed more elasticity and looping than the PSSMA chain. The PSSMA chain had a higher negative charge, so the repulsive force was higher, resulting in a straighter chain. The Alginate chain was adsorbed on the surface of the quartz slide more closely because of the lower repulsive force between the chains. Moreover, the looped Alginate chain made the multilayer thin film rougher and thicker. On the other hand, the PSSMA chain contained a higher negative charge which made this film smoother and thinner [19]. The characteristics of both chains and their adsorption on the surface are shown in Fig. 4 and the patterns of both multilayer thin films are presented as Fig. 5.

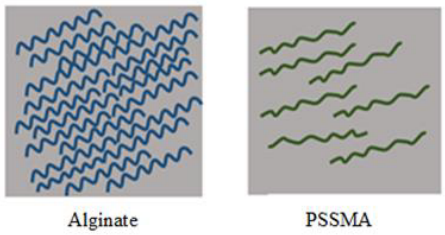

Figure 4. The characteristics of both alginate and PSSMA chains and their adsorption on the surface

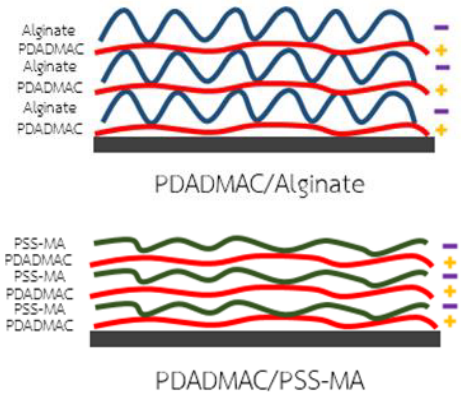

Figure 5. The patterns of multilayer adsorption of both PDADMAC/Alginate and PDADMAC/PSSMA.

3.2 Loaded CHX into PEM

The relationship between the concentration of CHX and its absorbance value was measured by UV-Vis spectroscopy and plotted as a calibration curve (Fig. 6 upper). The absorbance value increased with increasing concentration of CHX. This linear relationship was demonstrated as Eq. (1).

$$y = 491.2x - 0.0327 \quad (1)$$

After loading CHX into both PEMs by dipping in 0.2% CHX solution for 24 h, the films were measured for absorbance as a function of time. Results showed that CHX adsorbed into both PEM thin films quickly within 20 min. The absorbance then increased up to 1 h and remained constant through 24 h. The kinetic of loading CHX into thin film as a function of time is shown in Fig. 6 (lower). The absorbance of the loaded PDADMAC/AL film after 24 h was higher than the loaded PDADMAC/PSSMA film.

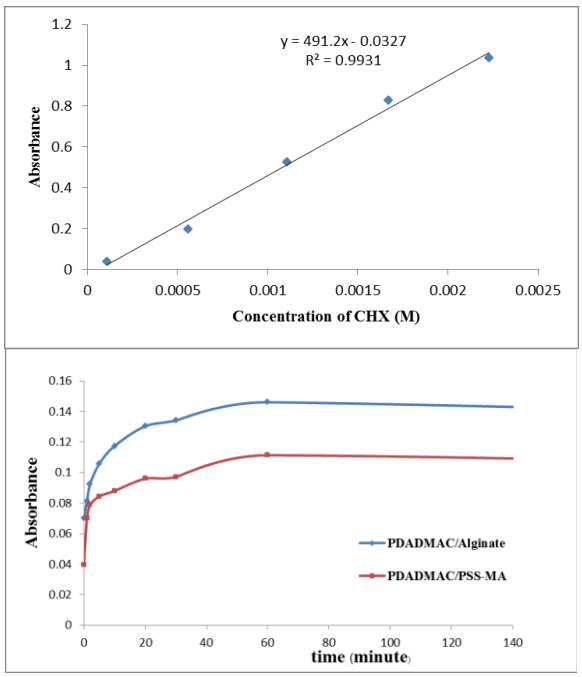

Figure 6. Standard calibration curve of CHX (upper) and kinetic curve showing the maximum absorbance of CHX when loaded into both thin films as a function of time (lower)

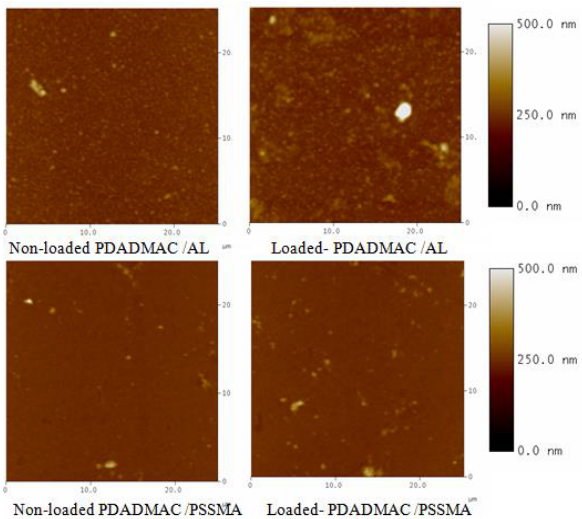

Figure 7. AFM picture showing the surface roughness of non-loaded and loaded PDADMAC/PSSMA and PDADMAC /AL films.

After loading CHX into the films, the thickness and surface roughness of both were characterized by AFM. The thickness of the loaded PDADMAC/AL film was 55.64 ± 1.25 nm, whereas the loaded PDADMAC /PSSMA was 19.73 ± 2.18 nm. The roughness of the loaded PDADMAC/AL was three fold higher than the PDADMAC/PSSMA film. The thickness and surface roughness between loaded and non-loaded PEM is shown in Fig. 3. The AFM picture comparing loaded and non-loaded film is shown in Fig. 7. After loading CHX into both films, their surface roughness increased.

The mechanism used for loading CHX into the multilayer thin film was electrostatic force. The top of the multilayer thin film was Alginate and PSSMA which had a negative charge. When loading CHX into these films, the attractive force between the negative and positive charge encouraged loading CHX into thin film multilayers. The adsorption rate of CHX was rapid during the initial 20 min, and then constant up to 24 h. This result agreed with Wei Yan et al. who studied the loading of drugs into nanoporous polymer thin films. They found that the initial absorbance value increased suddenly and then became constant [21]. Considering all the absorbance values, the thickness and roughness of both films were higher for PDADMAC/Alginate than for PDADMAC /PSSMA. The difference in the polyelectrolyte properties of the chain produced a PDADMAC/AL film with higher porosity and thickness and a greater surface area for loading CHX. PDADMAC/PSSMA had a straight chain and the multilayer thin film was smooth, thin and low in porosity, with less surface area for loading the drug.

3.3 Release of CHX in H_2O_2 after 24 h

CHX was released from both PEM thin films by immersion in H_2O_2. The released solution was measured by UV-Vis spectroscopy to calculate the remaining concentration of CHX in the PEM thin films. The absorbance of H_2O_2 after releasing CHX from PDADMAC/AL was 0.314 ± 0.013 and for PDADMAC/PSSMA was 0.225 ± 0.087. The absorbance results were calculated as concentrations using Equation 1, where y was the absorbance unit and x was the concentration of CHX. Finally the concentration of CHX loaded into PDADAMAC/AL was 0.00071 M (0.064%w/v) and in PDADMAC/PSSMA 0.00052 M (0.047% w/v). However, the effective concentration of CHX required to kill bacteria was 0.0034 M (0.12% w/v).

3.4 Antibacterial effect

After loading CHX into PDADMAC/PSSMA and PDADMAC/AL both multilayer thin films were tested for toxicity to Aa. CHX release from the multilayer thin films decreased the amount of Aa. In the blank condition, colonies of Aa increased to 7.7×10^9 CFU/ml. Loaded PDADAMAC/PSSMA decreased the growth of Aa to 1.64×10^6 CFU/ml with a percentage reduction of 99.98%. The loaded PDADMAC/AL reduced the growth of Aa to 1.02×10^7 CFU/ml, calculated at a percentage reduction of 99.87% (Fig. 8).

Figure 8. Colonies of Aa after exposure to both multilayer thin films after 24 h.

The result of the antibacterial testing determined that both loaded multilayer thin films decreased the growth of the Aa colony compared to the blank condition. CHX was released from both films to decrease the Aa colony. PSSMA is a polyelectrolytes composed of 50% of carboxylate groups(pK_a= 8.8) and sulfonic groups (pK_a nearly 1) [19]. Alginate is a polyelectrolytes contained carboxylic groups (pK_a= 3.2) [23], [24]. For both loaded multilayer thin film, when pH changed from 9 to 7.4 accelerate the rate of degradable of film due to the lower ionization of the carboxylate groups [19], [25], [26]. The attractive force between layer was lower resulting film was decompose and release CHX into broth. So when both loaded PEM thin films were at pH 7.4 in brain heart infusion broth, their charge and attractive force between the layers altered. This change made the film more susceptible to decomposition, and release of CHX.

However loaded film could not inhibit or bactericidal effect to Aa. They had just been decreased the growth of colony of Aa, due to their concentration of CHX which could loaded into both thin film was less than the effective concentration(0.12% w/v). Therefore they could not inhibit the Aa. So we can develop these multilayer thin films in their physical property such as the number of layer, increase dipping time or increase of concentration of CHX which used to load into film. This could be modified them more capacity to loaded concentration of CHX.

4 Conclusion

Smart PEM films can be manufactured to control loading and release of CHX. More CHX could be loaded into PDADMAC/AL than into PDADMAC/PSSMA thin films. Both loaded multilayer thin films decreased the growth of the Aa colony nearly 100 % compared to the blank condition. However, the concentration of CHX that could be loaded in both films was lower than 0.12% w/v. Thin films require further development to increase the release of drugs to inhibit bacteria. These smart thin films will be beneficial for the improved treatment of periodontal disease.

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