Preparation of Nanocellulose-Alginate Nanocomposites for Chlorhexidine Digluconate Drug Carrier

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Abstract. Root canal treatment failures generally caused by microorganisms that have ability to invade dentinal tubules. Chlorhexidine is widely known to effectively eradicate broad spectrum bacteria that infected dentinal tubules. The delivery of chlorhexidine to the infected site of root canal still becomes an issue. In order to increase the stability and to regulate the release of chlorhexidine, encapsulation of chlorhexidine digluconate was conducted by employing nanocellulose and alginate nanocomposites. Nanocellulose was prepared from palm kernel cake using acid hydrolysis method. Transmission electron microscopy images showed the formation of whisker and fiber nanocellulose with the average diameter of 20 nm. Resulting nanocellulose was combined with alginate in the presence of Ca2+ crosslinker. Scanning electron microscopy images displayed sphere and oval morphologies of microcapsules with diameter of 500 nm. The microcapsules were further loaded with chlorhexidine digluconate 2% (w/v). In vitro drug release properties were evaluated in PBS at pH 7.4 (normal tooth environment) and at pH 5.5 (infected tooth environment). The UV-VIS results showed that chlorhexidine digluconate has higher release rate at pH 5.5 than at pH 7.4, suggesting that the microcapsule is a good candidate for the delivery of chlorhexidine digluconate to the infected root canal tooth environment.

Keywords: Root canal treatment, nanocellulose, chlorhexidine digluconate, microcapsules, alginate, drug delivery.

1. Introduction

The main purpose of root canal treatment in endodontic field is to disinfect the entire root canal system based on eradication of broad-spectrum of microorganisms [1]. Eradication of microorganisms from infected root canals is a complex task that involves the employment of various instrumentation methods, irrigation procedures and intra-canal medicaments. Preservation of microorganisms in dentinal tubules after root canal chemo-mechanical treatment has been reported [2]. The complex anatomy of the root canal and dentinal buffering ability lead to a complicated and challenging delivery of antimicrobial agents to the
infected site. Delivery systems of antimicrobial active agents are needed to guarantee efficient bacteria eradication. Chlorhexidine (CHX) is a strong antiseptic biguanide that widely used in dentistry. CHX is a cationic biguanide comprising a positively charged hydrophobic and lipophilic molecule that show a broad-spectrum bactericidal and bacteriostatic properties [3]. The translation of controlled CHX drug delivery system in intracanal medicament is still limited. There is a need in lengthening the time release of CHX beyond the current delivery systems to improve bacterial reduction in infected root canal systems and to create a suitable environment for periapical healing. Encapsulation of CHX could offer a better medicament release control while further prolonging the delivery period of the bactericidal action in dentinal tissues. Nanocellulose is a highly crystalline cellulose nanoparticle that can be employed as drug carrier to perform control drugs release [4, 5]. Formation of nanocellulose nanocomposites with other polymers such as alginate is important to increase the drug loading capacity. Drug carriers derived from nanocellulose-alginate nanocomposites showed pH-dependent and sustained drug release properties [6]. Therefore, the aim of this work was to obtain CHX controlled drug delivery system by encapsulating CHX in nanocellulose-alginate nanocomposite microcapsules. The release behavior of CHX was further evaluated.

2. Materials and Methods

Materials. Palm kernel cake (PKC) was obtained from the local market. Alginic acid sodium salt from brown algae was obtained from Fluka. Sulfuric acid was purchased from Smart Lab. Nitric acid, sodium sulfate, sodium hypochlorite technical grade 12% and sodium nitrite were obtained from Brataco. Chlorhexidine digluconate was obtained from PPH Cerkamed. Phosphate buffer saline pH 7.4 was obtained from Biogear. All chemicals were used without further purification.

Extraction of cellulose from PKC. PKC (75 g) was added into solution containing HNO3 3.5% and NaNO2, heated at 90°C for 2h. Resulting suspension was filtered and washed several times with water. The solid residue was treated with 750 ml of solution containing NaOH 2% and Na2SO3 2% at 50°C for 1h, followed by filtration and washing with water until pH suspension reached 7. The solid residue was subsequently bleached with 250 ml of NaOCl 1.75% at 100°C for 30 min. Resulting cellulose was washed with water for several times.

Isolation of nanocellulose. Cellulose (135 g, wet residue) was suspended in 500 ml of H2SO4 45% at 45°C for 45 min. Suspension was cooled down to RT by adding 500 ml of water and was stored at RT overnight. Resulting colloidal solution was washed with demineralized water and sonicated at pH 7. Colloidal solution was centrifuged at 10000 rpm for min 10 min.

Preparation of nanocellulose-alginate nanocomposites microcapsules. Nanocellulose-alginate nanocomposites was prepared according to Ning et al. [6] and our previous work [7] with modified procedure. A hundred ml nanocellulose 2wt% was mixed with sodium alginate 1wt% and stirred for 15 min to result in homogeneous colloidal solution. Resulting nanocellulose-alginate solution was sprayed into 800 ml CaCl2 2wt%, followed by centrifugation at 10000 rpm. The residue was freeze-dried overnight.

CHX drug loading. Nanocellulose-alginate nanocomposite microcapsules (0.75 g) were immersed in 200 ml of CHX 2wt% and stirred for 48h. Microcapsules loaded with CHX were separated by filtration.

In vitro CHX release test. A twenty five mg of nanocellulose-alginate nanocomposite microcapsules was stored in cellulose dialysis membrane tubing (Sigma-Aldrich, MWCO 14000) and incubated in 50 mL PBS pH 7.4 at 37°C. At a predetermined time interval, 5 mL of incubated solution was taken out to determine the amount of CHX, at the same time 5 mL of fresh PBS solution was added into the solution containing loaded drug to keep the total
volume of solution constant. The amount of rutin released during incubation with specified time was measured using UV VIS spectrophotometer. In vitro release test was also conducted in HCl solution at pH 5.5 using the same procedure.

**Characterizations.** FTIR measurements were recorded with KBr pellets on Prestige 21 Shimadzu at wavelength 4500 to 400 cm\(^{-1}\). Spectra were measured at a resolution of 4 cm\(^{-1}\) with number of scan 40. Transmissions Electron Microscopy (TEM) images of nanocellulose were taken using HITACHI HT7700 with acceleration voltage of 80-100 kV. Sample was prepared by deposition of a small droplet of colloidal solution of nanocellulose in isopropyl alcohol on carbon coated copper micro grid (EM-japan) and allowed to air dry. Scanning Electron Microscopy (SEM) images were taken on Hitachi SU3500. The samples were dispersed in isopropyl ethanol, sonicated for 10 min, deposited on cover glass and dried in an oven. Afterwards the samples were gold-coated in a sputter coater MC-1000, Hitachi. X-Ray Diffraction (XRD) analysis, measured on Rigaku using Cu anode with wavelength of 1.5406 Å. The data were collected over the 20 range of 10°-60°. UV-VIS measurement was performed on Shimadzu UVmini-1240 at wavelength of 255.5 nm.

**Statistical Analysis.** Comparison between *in vitro* CHX drug release at pH 7.4 and 5.5 was evaluated using Independent T-test SPSS program. The differences were considered as statistically significant at p<0.05.

3. Results and Discussion

3.1 Preparation of nanocellulose

Nanocellulose was prepared from PKC using acid hydrolysis method. Pretreatment of PKC was first conducted to open the bulk structure of PKC using nitric acid, resulting in a brown solid product. The hemicellulose content was further removed using sodium hydroxide solution, followed by dissolution of lignin substance in NaOCl solution. Resulting cellulose was indicated by the formation of white suspension that purified cellulose suspension. A high concentration of sulfuric acid (45 wt%) was used to hydrolyze resulting cellulose, leading to the cleavage of cellulose microfibrils. The acid esterifies some of surface hydroxyl groups of cellulose to produce nanocellulose comprising negatively charged sulfate groups that help to form a stable dispersed colloidal solution [8] (Figure 1a). XRD diffractogram of nanocellulose reveals the crystalline nanocellulose with characteristic peaks of native cellulose as shown by diffractions at 15°, 16.5°, 20.5°, 22.5°, 34°, and 35° (JCPDS#030289). The value of crystallinity index of nanocellulose accounted for 60%. It was calculated from the ratio of the height of the peak at 22.5° and the height of the minimum (Intensity of amorphous area, which is at about 18°) [9, 10].

TEM images of nanocellulose in Figure 1c and 1d display various structure of nanocellulose, prepared using the same acid hydrolysis procedure but different purification processes were applied. Nanocellulose in Figure 1c was obtained after immediate washing of nanocellulose to reach neutral pH of filtrate. This treatment resulted in whiskers nanocellulose with diameter around 20 nm and length of 200-300 nm (ratio diameter to length= 1:10 ~ 1:15). Additionally, nanofibers structure was also observed with diameter of 20-50 nm. Nanocellulose in Figure 1d was obtained after washing nanocellulose that was stored at pH 2-3 at 4°C around two weeks. Afterwards, nanocellulose was washed to reach pH 7. During storage, the hydrolysis continued to occur, causing further cleavage of glycosidic linkage [11] and finally resulted in spherical nanocellulose with diameter around 20 nm. Beside the sphere structure, agglomerated nanofibers were also found. It is suggested that various morphologies of nanocellulose was affected by different particle size of PKC starting material.
3.2 Preparation of alginate-nanocellulose nanocomposite microcapsules

Microcapsules were prepared by combining nanocellulose and alginate. Nanocellulose was employed in order to stabilize the structure of alginate and to control the release properties of the microcapsule. Nanocellulose and alginate colloidal solution was sprayed into CaCl$_2$ solution to form cross-linked structure of alginate via ionic interaction between carboxylate group (COO$^-$) and Ca$^{2+}$ ions, while nanocellulose was entrapped between alginate networks [7]. SEM images of nanocellulose-alginate nanocomposites depicted in Figure 2 exhibit sphere and oval shape with an average diameter of 500 nm and a length of 1.2 μm. Some inhomogeneous, irregular and agglomerated particles are still observed. This might be caused by combined spraying technique (rate of spraying, interval time etc.) and the concentration of sodium alginate which was probably too high, making alginate polymer chains easily to interact and to cross-link each other. These parameters (spraying rate, time interval and alginate concentration) during crosslink process need to be investigated further.
Nanocellulose-alginate nanocomposite microcapsule was used to entrap CHX antibacterial agent for root canal medicament application. CHX has been proved highly effective as an intracanal treatment by inhibiting the growth of *Enterococcus faecalis* [12, 13]. To enhance bacterial elimination from infected root canal systems and to make an ideal environment, it is highly desirable to prolong the release of CHX *in situ*. The root canal drugs should be able to penetrate the dentine tubules canal that has diameter varies between 1 and 4 micrometers [14, 15]. It was hypothesized that drug carrier prepared with a suitable size and a controlled release behavior could deliver the medicament deep into dentin tubules, allowing for sustained inhibition of bacteria in the root canal system. Notably, SEM results show that the size of nanocellulose-alginate microcapsules (500 nm-1.2 μm) are considered small enough for penetration inside the dentin tubules.

### 3.3 In vitro drug release properties

Entrapment of CHX was conducted by immersing nanocellulose-alginate microcapsules in CHX 2%. FTIR spectrum of nanocellulose-alginate nanocomposite loaded with CHX in Figure 3a indicates characteristic absorptions of these three compounds. The presence of CHX is shown by absorption at 1529 cm\(^{-1}\) and 1411 cm\(^{-1}\) (C=C aromatic functional groups), 3213 cm\(^{-1}\) (N-H group) and 2931 cm\(^{-1}\) (C-H stretching vibration). Characteristic absorptions of nanocellulose are shown by peaks at 1000-1249 cm\(^{-1}\), attributed to C-O stretching vibration (overlapped with alginate functional groups), peak at 3213-3334 cm\(^{-1}\) from O-H hydrogen bonded stretching vibration (overlapped with peak of CHX and alginate). Alginate can be identified from the presence of absorptions at 1633 cm\(^{-1}\) and 1411 cm\(^{-1}\), originating from asymmetric and symmetric stretching vibrations of carboxylate salt ions. The N-H functional groups from alginate appear as overlapped peak at 3213 cm\(^{-1}\).
In vitro drug release was evaluated at two conditions, at pH 7.4 in PBS solution to represent normal tooth environment and at pH 5.5 in HCl solution to mimic the infected tooth condition (Figure 3c). Saphiro Wilk homogeneity test and normality test results showed that p value (sig.) in all sample groups is more than 0.05. It can be concluded that the data is homogeneous and normal. Statistical analysis using Independent T-test showed that all sample groups had p value less than 0.05. Statistically H0 is rejected, confirming a significant difference between the release rate of CHX in normal condition (pH 7) and infected condition (pH 5.5). The release rate of CHX is much higher in an infected condition than a normal condition.

Lin et al. [6] reported that nanocellulose incorporated with alginate polysaccharides nanocrystals had maintained the integrity the nanocomposites, attributed to the presence of rigid nanocrystals that improved the stability and prolong the degradation. They showed that the microcapsules performed better stability at pH 1 (HCl solution) than at pH 6.8 and 7.4. In our case, the release profile was higher at pH 5.5 than 7.4, which is contrary to the results of Lin et al. This can be explained by the properties of CHX loaded into nanocomposites. It is envisaged that CHX comprising amine groups and hydroxyl groups are able to form stronger hydrogen bonding with alginate and nanocellulose compared with theophylline drug model.
employed by Lin et al. [6] This hydrogen bonding will be easily disrupted in the presence of acid (at low pH), resulting in higher release CHX than at pH normal 7.4. This results show that nanocellulose-alginate nanocomposites is a promising candidate for delivering CHX for root canal medicament.

4. Conclusions
In this work, nanocellulose-alginate nanocomposites were prepared as drug carrier for CHX entrapment. It is shown that size of resulting nanocomposites is suitable for tubuli dentin penetration, although some irregular and agglomerated particles were still observed. In vitro drug release evaluation of nanocellulose-alginate loaded with CHX demonstrated controlled release properties, showing higher release properties of CHX at pH 5.5 (infected condition) than pH 7.4 (normal condition). This result suggests that nanocellulose-alginate nanocomposites drug carrier is a potential candidate for delivery CHX to tubule dentin. In vitro antibacterial properties of the nanocellulose-alginate loaded with CHX will be evaluated in the future work.

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6. References
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