

Significance of Glucose Addition on Chitosan-Glycerophosphate Hydrogel Properties

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ABSTRACT

Chitosan-glycerophosphate hydrogel can be used as dental scaffold due to its thermosensitivity, gelation performance at body temperature, suitable acidity for body condition, biocompatibility, and ability to provide good environment for cell proliferation and differentiation. Previous study showed that glucose addition to the chitosan solution before steam sterilization improved its hydrogel mechanical strength. However, the effectiveness of glucose addition was still doubted because glucose might undergo Maillard reaction in that particular condition. The aims of this study are to confirm whether the glucose addition can increase the hydrogel mechanical strength and gelation rate effectively and also to compare their performance to be dental scaffold. This research was performed through several steps, namely preparation of chitosan-glycerophosphate solution, addition of glucose, gelation time test, gel mechanical strength measurement, functional group analysis, and physical properties measurements (pH, viscosity, and pore size). The result showed that glucose addition did not improve the hydrogel mechanical strength and gelation rate, neither when it was added before nor after steam sterilization. Glucose addition before steam sterilization seemed to trigger Maillard reaction or browning effect, while glucose addition after steam sterilization increased the amount of free water molecules in the hydrogel. Chitosan and glycerophosphate interact physically, but interaction between chitosan and glucose seems to occur chemically and followed by the formation of free water molecules. Glucose addition decreases the solution viscosity and hydrogel pore size so the hydrogel performance as dental scaffold is lowered.

Keywords: chitosan-glycerophosphate hydrogel; dental scaffold; glucose; thermogel property

ABSTRAK

Hidrogel kitosan-gliserofosfat dapat digunakan sebagai bahan perancah gigi karena bersifat termosensitif, mempunyai kemampuan gelasi pada suhu tubuh, mempunyai pH di sekitar pH fisiologis, bersifat biokompatibel, dan dapat menyediakan lingkungan untuk proliferasi dan diferensiasi sel. Penelitian sebelumnya menunjukkan bahwa penambahan glukosa pada larutan kitosan sebelum diautoklaf dapat meningkatkan kekuatan mekanik hidrogelnya. Namun, keefektifan tambahan glukosa ini masih diragukan karena glukosa dapat mengalami reaksi Maillard pada kondisi tersebut. Penelitian ini bertujuan mengonfirmasi apakah tambahan glukosa dapat meningkatkan kekuatan mekanik hidrogel dan laju gelasinya serta membandingkan potensinya sebagai bahan perancah gigi. Penelitian ini berlangsung dalam beberapa tahap, yaitu pembuatan larutan kitosan-gliserofosfat, penambahan glukosa, pengujian waktu gelasi, pengukuran kekuatan mekanik gel, analisis gugus fungsi, dan pengukuran sifat-sifat fisik (pH, viskositas, dan ukuran pori). Hasil eksperimen menunjukkan bahwa penambahan glukosa tidak meningkatkan kekuatan mekanik gel dan laju gelasinya. Penambahan glukosa sebelum sterilisasi uap menyebabkan terjadinya reaksi Maillard atau pencokelatan, sedangkan penambahan glukosa setelah sterilisasi uap ternyata meningkatkan jumlah molekul air bebas dalam hidrogel. Interaksi antara kitosan dan gliserofosfat terjadi secara fisik, sedangkan interaksi antara kitosan dan glukosa kemungkinan terjadi secara kimiawi. Walaupun tambahan glukosa menurunkan viskositas larutan, senyawa gula ini juga menurunkan ukuran pori hidrogel sehingga potensinya sebagai bahan perancah gigi berkurang.

Kata Kunci: bahan perancah gigi; glukosa; hidrogel kitosan-gliserofosfat; termogelasi

INTRODUCTION

Dental caries (cavities) may cause pain, infection, abscess, even tooth loss. Scaffold is one among the solutions to solve this problem. Scaffold which can be

used as a 3-dimensional framework for cell growth and migration provides an environment allowing cells proliferation and differentiation [1]. Scaffold can be used as a medium for dental pulp tissue growth in tooth cavities. So that, its function can be restored and able

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to repair itself after the injury or dental caries happened. The irregular shape of tooth cavities requires materials which are liquid at room temperature, but rapidly solidified into hydrogel when it is heated to body temperature. In spite of that, scaffold should meet several conditions, such as high pore size to facilitate the delivery and diffusion of nutrients through the whole cell structure ($> 20 \mu\text{m}$) [2]. They also should have a good biodegradability and biocompatibility [3].

Nowadays, there are many natural and synthetic hydrogel which can be used as a scaffold, include collagen/gelatin, chitosan, chondroitin sulfate, hyaluronic acid, agar/agarose, fibrin [4], alginate [5], and hydroxyapatite [6]. Among all of these materials, chitosan is preferable because of its abundance, biocompatibility, biodegradability, and its degradation products did not harmful to the body [7]. Chitosan-glycerophosphate (C-GP) hydrogel is a thermosensitive solution which will undergo gelation at body temperature (about 37°C) and has a pH around physiological condition. C-GP hydrogel is biocompatible and can also provide an environment for cells proliferation and differentiation [8-9].

Based on previous study, the higher concentration of GP used in C-GP scaffold the higher gelation rate of the solution. However, the high concentration of GP can reduce the hydrogel ability to support cell proliferation; it can even be cytotoxic. In contrast, low concentration of GP (5-10%) can support the proliferation of cells very well, but has a fairly long gelation time [10]. Gelation rate of C-GP hydrogel can be improved by adding polyols such as sorbitol, glucose, poly (ethylene glycol) (PEG), mannitol, triethylene glycol, and glycerol. Among those polyols, glucose is reported to give the best result in terms of gelation time and the pH [11].

Jarry et al. (2002) performed glucose addition to chitosan-glycerophosphate solution before sterilizing the chitosan. The result showed that the presence of glucose increased the solution viscosity and mechanical strength although it is still unclear how glucose can improve the gel mechanical strength [11]. Furthermore, the effectiveness of glucose addition was still doubted because glucose will normally undergo Maillard reaction in that condition. Maillard reaction happens when the nucleophilic amine group of the chitosan reacts with the reactive carbonyl group of the glucose as the reducing sugar and will rearrange to become new compounds. This reaction will change the structure, color, odor, and taste of the material [21]. This reaction can be inhibited by changing the glucose addition method.

This study aimed to confirm whether the glucose addition is able to increase the hydrogel mechanical strength and gelation rate effectively. In this experiment, the glucose was added in two methods, before and after the steam sterilization. The interaction between chitosan,

glycerophosphate, and glucose was observed. Other physical measurements were also performed, namely pH, viscosity, and pore size to determine their performance as dental scaffold. The hypothesis of this study was inability of glucose addition to improve the hydrogel mechanical property and gelation rate. It is also expected that glucose addition will increase its performance as dental scaffold because glucose will decrease the solution viscosity and increase the pore size without give any significant effect on the solution acidity. Hydrogel which has high gelation rate, high mechanical strength, good pore size and suitable to body's acidity and viscosity should be potential as dental scaffold material.

This paper reports the effect of glucose addition to the chitosan-glycerophosphate hydrogel properties, whether it will improve or reduce the hydrogel potential to be a dental scaffold based on some physical properties such as gelation time, pH, viscosity, and pore size. This paper also reports the interaction that happened between chitosan, glycerophosphate, and glucose in the hydrogel.

EXPERIMENTAL SECTION

Materials

The medical grade chitosan (molecular weight of $4.54 \times 10^5 \text{ g/mol}$ and deacetylation degree of 91.44%) were purchased from Biotech Surindo Inc. (Indonesia). The chitosan was stored in anhydrous conditions (humidity $< 10\%$) until used. β -GP disodium salt pentahydrate which has molecular weight of 306.0 and purity $> 98\%$ was purchased from Bioshop Canada Inc. and D-(+)-glucose powder was purchased from Sigma-Aldrich Pte Ltd (Singapore).

Instrumentation

The instruments used were Tomy High-Pressure Steam Sterilizer ES-315, TV-10 viscometer (Toki Songyo, Co. Ltd.), HM-205 pH-meter (DKK TOA Comp.), Christ Beta 1 freeze dryer, Shimadzu IRPrestige-21 FTIR spectrophotometer, M10 EVO SEM, and TA-XT2i texture analyzer.

Procedure

Preparation of chitosan/glycerophosphate solution

Chitosan solution 2.5% (w/v) was obtained by dissolving chitosan in acetic acid 0.14 M. The solution was stirred with a magnetic stirrer for 24 h. The chitosan solution was then steam sterilized in an autoclave at 121°C for 20 min. Cold glycerophosphate solution in concentration of 50% (w/v) which had been

filtered by Microfilter 0.22 μm was added dropwise to chitosan/glucose solution in an ice bath with a magnetic stirrer. The solution was stirred at 4 $^{\circ}\text{C}$ for 30 min [11-12]. The final control solution had chitosan concentration 2.0% (w/v) and glycerophosphate concentration 10% (w/v).

Addition of glucose

Glucose was added to chitosan solution in two different methods, before and after steam sterilization. For the first method, the glucose was added in powder form to the chitosan solution in various concentrations (from 0.5 up to 5.0% (w/v)). For the second method, the sterilized glucose solution in various concentrations (10, 30, and 50% w/v) were added to the sterilized chitosan and stirred in room condition for 30 min. Glucose addition was optimized by varying the volume ratios of chitosan 2.5% (w/v)-glycerophosphate 50% (w/v)-glucose 50% (w/v) to obtain the best gelation time. After the best volume ratio was identified, the glucose concentration was varied (0, 0.4, 1.2, and 2.0% (w/v)). The optimum glucose concentration was determined based on the best gelation time.

Time gelation test

Hydrogel gelation time was determined using an inverted tube test. One mL of each solution was poured into a vial. The vials were then kept at room temperature for 20-30 min to equilibrate. Gelation time was tested by putting the vial in a water bath at 37 $^{\circ}\text{C}$. Gelation process was observed by tilting the vial upside down slowly. The time required to transform a liquid solution to a solid hydrogel was recorded as the gelation time. The gelation was characterized by the flow rate of the hydrogel in the bottle when the tube is reversed and the color of the solution became opaque [10].

Gel mechanical strength measurement

Gel mechanical strength was measured using a texture analyzer TA-XT2i. The hydrogel sample was put in a tubular mold with a diameter and a height of 2 cm and then heated up to become a gel. The formed gel was placed in a sample compartment and analyzed for measuring the maximum force required to break the gel. The measurements were performed at least three times.

FTIR analysis. The interactions between chitosan, glycerophosphate, and glucose were observed on FTIR spectra. Some dry samples were ground with KBr powder and scanned at wave number range of 4000 to 400 cm^{-1} .

Viscosity and pH measurement. The viscosity of the solution was measured by using a digital viscometer while the pH was measured by using a pH-meter, both in room temperature. Each solution was measured at least three times.



Fig 1. Photos of chitosan-glycerophosphate solution before (left) and after gelation (right)

SEM analysis. Hydrogel samples were dried by freeze-drying process for 72 h. Dry hydrogel scaffold were cut and coated by Coater IB-2 Ion. Pore morphology of the hydrogel scaffold was observed in the SEM. Average pore diameter was measured based on the SEM images by image-G software.

RESULT AND DISCUSSION

Optimum Addition of Glucose

In this experiment, glucose was added in two different methods, before and after steam sterilization. Glucose addition before steam sterilization has been performed by Jarry et al. (2002), using concentration up to 5% (w/v). By using this method, our result showed that chitosan-glucose solution underwent Maillard reaction; the solution turned brownish and thick. This is observed even in the lowest glucose concentration (0.5% (w/v)). Based on this evidence, glucose addition before steam sterilization as performed by Jarry et al. (2002) could not be carried out for the next steps [11].

Glucose addition after sterilization was performed by adding a sterilized glucose solution to the sterilized chitosan solution. The Maillard reaction was successfully be prevented. Actually, increasing solvent acidity also could prevent the Maillard reaction, but it will disturb the hydrophobic interaction among chitosan chains and the gelation process was even longer. The glucose addition was optimized by varying the solution volume ratio and concentration. The best formula was chosen based on the highest gelation time.

Gelation is the process of establishing a new network of many interacting molecules [14]. The process of gelation of chitosan-glycerophosphate solution characterized by turbidity and viscosity development of the solution along with the increase of temperature until the formation of a hydrogel (Fig. 1). The time required by the solution to turn into a perfect gel is referred as gelation time. The gelation process of

Table 1. Gelation time of chitosan/glucose-glycerophosphate hydrogel variation of volume ratio using chitosan 2.0% (w/v), glycerophosphate 10% (w/v), and glucose 2.0% (w/v)

Formula	Volume ratio of chitosan: glycerophosphate: glucose	Gelation time (min)
Control	4.00:1.00:0.00	9.0 ± 1.0
A	4.00:0.90:0.10	> 500
B	4.00:0.75:0.25	> 120
C	4.00:0.70:0.30	> 60
D	3.80:1.00:0.20	12.3 ± 2.5
E	3.60:1.00:0.40	49.5 ± 23.3
F	3.40:1.00:0.60	> 120
G	3.20:1.00:0.80	> 240

Table 2. Gelation time of chitosan/glucose-glycerophosphate hydrogel with variation of glucose concentration with volume ratio of 3.8:1.0:0.2

Formula	Chitosan (% w/v)	Glycerophosphate (% w/v)	Glucose (% w/v)	Gelation time (min)
D1	1.9	10	0	7.33 ± 0.58
D2	1.9	10	0.4	8.00 ± 1.41
D3	1.9	10	1.2	10.00 ± 1.00
D4	1.9	10	2.0	12.33 ± 2.52

C-GP solution is strongly affected by the pH and temperature of the environment [15]. The β -GP has three important roles in the formation of chitosan hydrogel, namely (1) raises the chitosan solution pH to near physiological pH (6.7-7.4); (2) prevents immediate gelation or precipitation; and (3) allows the formation of hydrogel that can be adjusted by increasing the solution temperature. The β -GP salt is a weak base that can neutralize the chitosan solution to achieve physiological pH and protect the chitosan chains with its glycerol groups to prevent the occurrence of instantaneous precipitation [8].

Glucose solution with a constant concentration (50% w/v) and various volume ratios were added to the chitosan solution (2.5% w/v) before the addition of glycerophosphate (50% w/v). The gelation time of the solution as seen in Table 1, shows that the addition of glucose decreases the rate of gelation process. It was antipodes with the report by Jarry et al. [11]. It happens because glucose has hydrophilic properties, which may disturb the hydrophobic interaction among chitosan chains so the gelation takes longer time. This finding proved that glucose is not able to improve the gelation time of C-GP hydrogel, neither added before nor after steam sterilization.

Among all of these formulations, the volume ratio of 3.80: 1.00: 0:20 showed the best result with gelation time about 12 min. It meets the dental scaffold requirement stated by Wang and Stegemann [16], that the C-GP hydrogel gelation time should be less than 30 min because the exposure of GP 2.5-15% for 30 min was not toxic to the cells. After 30 min, the excess GP

could be removed by rinsing after gel formation. It can reduce the cytotoxicity of the C-GP hydrogel. In this volume ratio, the final concentration of chitosan was 1.90% (w/v) and the GP was 10% (w/v).

Once the optimum volume ratio of chitosan, glucose, and GP obtained, the optimum glucose concentration was determined to evaluate the effect of glucose addition on the C-GP hydrogel gelation time. The result of solution gelation time is depicted in Table 2. The increasing glucose concentration increases the gelation time through a linear regression equation of $y = 0.1011x + 7.1412$ and the R^2 value of 0.9928. It shows that the glucose concentration addition slows down the gelation process. This phenomenon can be explained because the hydroxyl moiety in the glucose interacts with the hydroxyl moiety in the chitosan chain and prevent the chitosan interchain interactions.

The formula D1 having glucose concentration of 0% showed very fast gelation time, even at room temperature and the gel formed was less homogenous. The formula D2 to D4 were stable in solution form at room temperature and solidify at 37 °C. They also gave good homogenous hydrogels. It suggests that D2, D3, and D4 formulas are more potential as a dental scaffold. Thus, the optimum glucose concentration is 0.4% (w/v) because it provided the most rapid gelation time, the uniform gel, and stability at room temperature.

Based on all the collected data, the optimum formulation for the glucose addition is chitosan:glycerophosphate:glucose 3.8:1.0:0.2 with the glucose concentration of 0.4% (w/v). This concentration was lower than the concentration of the glucose addition used by Jarry et al. (1-5% (w/v)) [11]. After all, it can be concluded that the optimum mass ratio for the glucose addition is chitosan: glycerophosphate: glucose of 4:25:1.

Mechanical Properties of Chitosan/Glucose-Glycerophosphate Hydrogel

Glucose is polymer additive which commonly used in chitosan to protect chitosan chain from head-induced degradation during heating or irradiating process in sterilization. Glucose will modify the structuring effect of water molecules around chitosan chains so it will become a more compact conformation and may be less prone to hydrolysis upon heating. It will improve the hydrogel mechanical strength [11,22]. Changing the glucose addition methods, from before steam sterilization to after steam sterilization, will dismiss the glucose role as the protect agent for chitosan during steam sterilization.

The mechanical property of chitosan-glycerophosphate and chitosan/glucose-glycero phosphate hydrogels can be seen in Table 3. The result

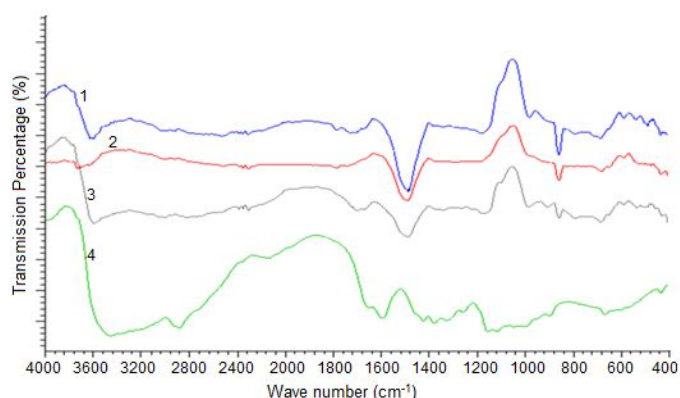


Fig 2. FTIR spectrum of chitosan-glycerophosphate hydrogel (1), chitosan-glycerophosphate with glucose 0.4% (w/v) hydrogel (2), chitosan-glycerophosphate with glucose 2.0% (w/v) hydrogel (3), and chitosan (4).

Table 3. Mechanical Property of Chitosan/Glucose-Glycerophosphate Hydrogel

Formula	Gel Mechanical Strength (gf)
Chitosan-glycerophosphate	29.40±16.54
Chitosan/glucose 0.4%-glycerophosphate	23.47±16.14*
Chitosan/glucose 2.0%-glycerophosphate	16.90±4.91*

* significantly different based on ANOVA test result with $\alpha = 0.05$

showed that glucose addition decreases the mechanical strength of the formed gel. It seems that glucose molecules cover the chitosan chains, and disrupts the balance of hydrophobic interactions and hydrogen bonds which were formed between the chitosan chains. This balance disruption might decrease the mechanical strength of the hydrogel, since the balance is a major factor in the chitosan-glycerophosphate hydrogel formation [20]. In spite of that, the presence of a number of free water molecules in the solution can also cause this reduction, the solution becomes less concentrated and the resulting gel is less strong.

Evidence of Interaction between Chitosan, Glycerophosphate, and Glucose

The interaction between chitosan, glycerophosphate and glucose can be observed on FTIR spectra (Fig. 2). The spectra were interpreted by comparing the absorption peaks with the correlation table compiled by Pavia et al. [17]. The absorption in 3400 cm^{-1} indicates the presence of primary amine moiety on chitosan chains [18]. The absorptions in $1500\text{--}1650\text{ cm}^{-1}$ and $1000\text{--}1200\text{ cm}^{-1}$ also indicate the existence of amine moiety on the chitosan chain. Those peaks become weaker, or even disappear after the solution transformed into hydrogel. It suggests that the primary amine moiety on chitosan chain is protonated and become positively charged ammonium ions, as

indicated by medium absorption in $2500\text{--}3300\text{ cm}^{-1}$ and a strong absorption around 1500 cm^{-1} . The absorptions in $1300\text{--}1240\text{ cm}^{-1}$ and $845\text{--}850\text{ cm}^{-1}$ indicate the presence of phosphate ester moiety on the chitosan-glycerophosphate hydrogel. This evidence showed that chitosan and glycerophosphate was interacted physically to become hydrogel. This result was suitable with the explanation of Chenite et al. (2001) who said that the gelation mechanism was depend on some physical interaction between chitosan, glycerophosphate, and water molecules [23].

Wide absorption in 3600 cm^{-1} indicates the presence of free hydroxyl group of water molecules which is released during the formation of glycoside bond between the hydroxyl group on chitosan chains and the hydroxyl group on glycerophosphate or glucose. Around 3600 cm^{-1} , band 2 had corrected peak area about 0002 and band 3 about 0085. It suggests that the increase of glucose addition would increase the amount of free hydroxyl group or the increase of the formed water. It is also supported by the moderate absorption in the region $1100\text{--}1300\text{ cm}^{-1}$, indicating the presence of glycoside bond. Band 2 had corrected peak area about $1100\text{--}1300\text{ cm}^{-1}$ of 2.147 and band 3 about 0.801. Absorption of band 3 and 4 in this area should be higher than band 2. This lower absorption peak can be caused by the heterogeneity of sample quantity which was analyzed, so that the quantitative analysis was difficult to do. It could be concluded that the addition of glucose increases the number of glycoside bonds formed, increases the amount of free water molecules, and decreases the solution viscosity. But the mechanism was still doubted because the formation of glycoside bonds usually need more heat treatment, while in this experiment the solution only heated up to $37\text{ }^{\circ}\text{C}$.

Significance of Glucose Addition to Chitosan-Glycerophosphate Hydrogel Properties as Dental Scaffold

Table 4 shows the measurement results of viscosity and pH of chitosan-glycerophosphate hydrogel and chitosan/glucose-glycerophosphate hydrogel. Glucose addition seems to reduce the viscosity of the formed solution significantly without giving any significant effect on its pH. The pH values meet the scaffold requirement, i.e. 6.7-7.3. Low viscosity can help the scaffold to interact easier with body fluids. The low viscosity can reduce the risk of cell death due to osmolality difference between the hydrogel solution and the body environment. The decrease of viscosity occurs due to many hydroxyl groups on glucose that can interact with the hydroxyl groups on the chitosan chain and form free water and

Table 4. Effects of glucose addition to hydrogel characteristics

Formula	Solution Viscosity (cP)	Solution pH
Chitosan-glycerophosphate	4.55±0.33	6.97±0.04
Chitosan/glucose 0.4%-glycerophosphate	2.66±0.34*	7.00±0.11
Chitosan/glucose 2.0%-glycerophosphate	2.04±0.37*	6.84±0.05

* significantly different based on ANOVA test result with $\alpha = 0.05$

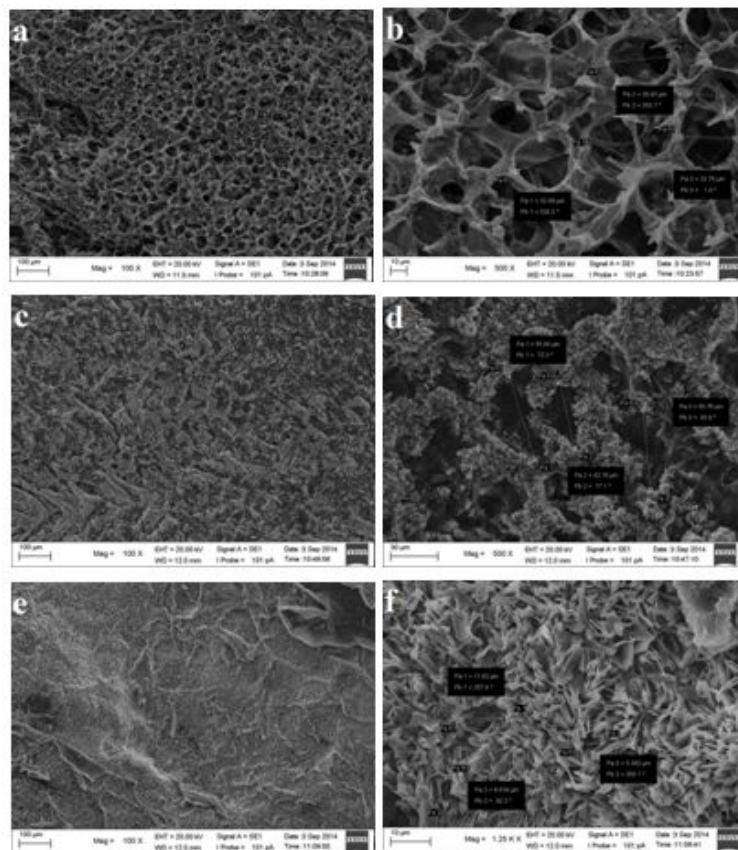


Fig 3. SEM images of chitosan-glycerophosphate hydrogel in magnificient of 100× (a) and 500× (b); chitosan/glucose 0.4%-glycerophosphate in magnificient of 100× (c) and 500× (d); and chitosan/glucose 2.0%-glycerophosphate hydrogel in magnificient of 100× (e) and 1250× (f)

also reduce the amount of hydrogen bonding and hydrophobic interactions which formed in chitosan interchain. It is almost similar with the role of glycerol on chitosan chain as reported by Chenite et al. [8].

The morphology of the chitosan-glycerophosphate hydrogel with and without glucose can be seen in Fig. 3. The hydrogel of chitosan-glycerophosphate had numerous spherical pores with size about 35.36 μm (Fig. 3a and 3b). The hydrogel of chitosan/glucose 0.4%-glycerophosphate exhibits larger pores, around 49.57 μm , but less in the number of pores (Fig. 3c and 3d). Glucose seems to make the hydrogel pore denser because glucose has the same hydroxyl groups such as that in the chitosan chains. These hydroxyl groups can interact each other to form a glycosidic bond between chitosan and glucose, resulted in water release and close gaps, so it looks denser. The effect of glucose

addition is more visible in the chitosan/glucose 2.0%-glycerophosphate hydrogel (Fig. 3e and 3f) where the pores are very dense, with the size about 7.8 μm in diameter. According to a study by Horst et al. [2], the diameter of human cells are about 10-30 μm , nerve fiber are about 0.2-20 μm , and most of the pulp arteries are smaller than 100 μm . Thus, chitosan/glucose 0.4%-glycerophosphate hydrogel is potential to support the regeneration of dental pulp tissue, as well as that of the chitosan-glycerophosphate hydrogel.

In addition to the pore size, another important morphology for cell growth is interconnectivity between the pores. This interconnectivity allows cells and nutrients for the growth and migration of cells [19]. From those hydrogels, the best interconnectivity is revealed by the chitosan-glycerophosphate hydrogel because in terms of the higher number of fine and

uniformly distributed pores than that of the chitosan/glucose-glycerophosphate hydrogel.

CONCLUSION

Glucose addition reduces the hydrogel mechanical strength and gelation rate, either when it was added before or after steam sterilization. Glucose addition before steam sterilization lead to Maillard reaction or browning effect, while glucose addition after steam sterilization increase the amount of free water molecules. The interaction between chitosan and glycerophosphate occurred physically, but there is a chemical interaction between chitosan and glucose and form free water molecules. Glucose addition decreases the solution viscosity and hydrogel pore size so it will decrease hydrogel performance as dental scaffold. Further study should be conducted to determine the interaction mechanism of chitosan and glucose.

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