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Injectable collagen-chitosan hydrogel using ultrasonic pretreated ovine tendon collagen

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ABSTRACT

Normally discarded as waste, ovine tendon collagen has great potential for use as an injectable hydrogel. Typical collagen used for biomedical applications is derived from epidermal tissue. Compared to the epidermal collagen, tendon collagen consists of collagen fibrils with large diameters. This limits the conjugation of phenolic hydroxyl (Ph) groups to tendon collagen for the synthesis of an injectable hydrogel. In this study, pretreatment process by ultrasonication was used to prevent the aggregation of ovine tendon collagen fibril, thus reducing fibril diameters and increasing the surface area for effective conjugation of Ph groups to collagen-chitosan (Col-Chit) composite. In situ gelation of Col-Chit-Ph composite was achieved via peroxidase-catalyzed crosslinking reaction at physiological conditions. The collagen to chitosan (Col: Chit) ratio was found to significantly influence the physical, mechanical and biological properties of hydrogels. The higher composition of chitosan in the hydrogel with 1:1 Col: Chit ratio resulted in the shortest gelation time (< 1 minute) and higher mechanical strength (> 0.35 N) in all conditions. However, the poor cell growth rate of hydrogel at this Col: Chit ratio might limit its further use. The hydrogel with 3:2 Col: Chit ratio was mechanically stable and has the highest cell growth rate among others with difference of cell growth rate of about 94 % compared to the 1:1 ratio. Taking into account these biological features, hydrogel with 3:2 Col: Chit ratio is suggested for potential use in biomedical applications. This study shows the feasibility of using ultrasonic pretreatment method for collagen with large fibril diameter.

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1. Introduction

Injectable hydrogel, which is also known as *in situ* forming hydrogel, has the ability to undergo an *in situ* solution to gel transition when administrated into the body (Liu et al., 2016). When compared to preformed hydrogels transplantation, injectable systems allow accurate filling of irregular-shaped defects by simple injection of the hydrogel precursor solution to defect site. The challenges recently being addressed in the development of *in situ* forming hydrogels is the use of biomaterials with desired properties to meet intended biomedical uses. Collagen has been considered as the most popular biomaterials in biomedical fields due to its excellent biocompatibility as evidenced by extensive investigation on utilization of collagen as wound dressings, tissue engineered scaffolds or matrices for drug delivery (Ruszczak, 2003; Lee et al., 2001; Wallace and Rosenblatt, 2003).

One of the strategies to obtain injectable collagen hydrogel is by crosslinking of phenolic hydroxyl (Ph) groups conjugated to collagen. Here, the crosslinking reaction could be carried out using horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). Previous finding proposed an injectable collagen-phenolic hydroxyl (collagen-Ph) hydrogel derived from murine epidermal tissue (Kuo et al., 2015). However, in this study, the collagen was extracted from ovine tendon, which is typically discarded as waste. It is a cheap source of raw materials and large quantities can be isolated and purified for research purposes. Earlier studies reported that tendon collagen fibrils having large diameter than that of epidermal collagen fibrils (Gathercole et al., 1987).

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Hence, a pretreatment process was necessary for the tendon collagen to achieve effective conjugation of Ph groups for the synthesis of injectable hydrogels.

Since collagen is known for its weak mechanical strength (Yunoki and Matsuda, 2008), in the present work, collagen was combined with chitosan to improve its physical properties and degradation rate (Ma et al., 2003; Chen et al., 2005; Tangsadthakun et al., 2007). Other than being mechanically stronger than collagen, chitosan is non-toxic, contains antibacterial properties and has structural similarity to glucosaminoglycans (GAGs) of the extracellular matrix (Giri et al., 2012). The synthesis of injectable enzymatically crosslinked collagen-chitosan hydrogels was mentioned in our previous work (Lau et al., 2018). Since collagen-chitosan composite is formed by electrostatic interactions between carboxyl groups of collagen and amino groups of chitosan, the conjugation of Ph groups mostly occurred in the amino groups of collagen. In the presence of HRP and H_2O_2, composite hydrogel was formed by oxidative coupling of the Ph groups in polymer chains. Here, the influence of collagen: Chitosan (Col: Chit) ratio to the physical, mechanical and biological properties of enzymatically crosslinked hydrogels were reported.

2. Materials and methods

2.1. Ultrasonic pretreatment of collagen

Collagen was extracted and purified from ovine tendon according to the method described by Fauzi et al. (2016). The freeze-dried collagen sponge was cut into small pieces and added to 50 mM Morpholinoethanesulfonic acid (MES) solution to prepare a collagen solution with concentration of 0.5, 0.6 and 0.8 % (w/v). The mixture of collagen and MES solution was pretreated by ultrasonication at frequency 50/60 Hz and power 80 W for 10 minutes. The mixture was then transferred to ice bath and stirred at 500 rpm until homogenized.

2.2. Preparation of composite with phenol moieties

After collagen pretreatment, chitosan (Primex, degree of deacetylation≥ 95%) was added directly to the homogenous collagen solution under gentle stirring in ice bath. The composites with Ph groups at Col:Chit ratio 4:1, 3:2 and 1:1 were synthesized using method as decribed by Lau et al. (2018) and Sakai et al. (2009). Initially, 3-(4-Hydroxyphenyl)-propionic acid (ρHP) in 50 mM MES solution was added with N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS) before transferring to Col-Chit solution. After 3 hours stirring, the conjugate solutions were repeatedly suspended and precipitated using 90 % ethanol to remove the remaining ρHP, EDC and sulfo-NHS, followed by centrifuging at 10,000 rpm. The conjugate precipitates were air-dried and re-dissolved in 50 mM MES solution at 1 % (w/v). The conjugate solution was brought to pH 7.0 with 1 M NaOH for analysis.

2.3. Transmission electron microscope (TEM) analysis of collagen fibrils

The effect of ultrasonication on collagen fibrils was investigated using TEM (Zeiss Libra 120, Germany). A modified method has been adapted for sample preparation (Tsai et al., 2006). The collagen solution was prepared at concentration 0.4 % (w/v) and 0.15 % (w/v). A droplet of collagen solution was placed on 400 mesh size copper grid. Then, the fibrils were negatively stained by adding a droplet of 2 % phosphotungstic acid to the surface of copper grid. After TEM analysis, the percentage of reduction in fibril diameter of collagen was determined using ImageJ.

2.4. Gelation time of Col-Chit-Ph

The injectable properties of hydrogels were determined based on gelation time of conjugates using a test tube inverting method. The conjugate solutions were transferred to test tubes at 500 µl/ tube. To each tube, 50 µl HRP solution was added, followed by addition of 50 µl H_2O_2. The mixture was then mixed well. The effect of concentration of HRP on the gelation was studied by varying the concentration of HRP (1-5 unit/ml) at constant H_2O_2 (1 mM) concentration. On the other hand, the effect of H_2O_2 concentration were investigated by varying the H_2O_2 (1-25 mM) concentration at fixed concentration of HRP (3 unit/ml). The time when no fluidity was observed upon inverting the tube was regarded as gel formation.

2.5. Mechanical studies of hydrogels

The bloom strength measurement of hydrogels was performed with TA. XT-Plus texture analyzer (Stable Micro System, UK). Cylindrical gels were prepared in 12-well plate by addition of 3 ml conjugate solutions to each well. The concentration of HRP (1-5 unit/ml) added to samples were varied at fixed 1 mM H_2O_2. Hydrogels were left for 1 hour in incubator prior to compression by using 5 g cylindrical probe P/0.5 (0.5 mm diameter) at speed of 30 mm/min.

2.6. Studies on cell growth rate

Human dermal fibroblast (HDF) was seeded in each hydrogel samples in 96-well at 2,500 cells/well with 200 µl F12: DMEM culture medium. Hydrogels containing cells were incubated for 5 days at 37 ^oC in 5% CO_2. The cell growth rate was evaluated using MTT cell proliferation assay (Thermo Fisher Scientific, US) according to the manufacturer's instruction. The absorbance measurement was

performed using spectrophotometer (Bio-Tek Power Wave XS, US) at 570 nm. The cell growth rate was defined as follows:

$$\text{Growth rate } (h^{-1}) = \frac{In\ (Absorbance\ on\ 5th\ day / Absorbance\ 1st\ day)}{(Final\ culture\ time - Initial\ culture\ time)} \quad (1)$$

2.7. Porosity studies of hydrogels

The porosity of hydrogels was determined using solvent replacement method. After fabrication, the hydrogels were dried using freeze dryer. The dried hydrogels were weighted before being immersed in absolute ethanol for 24 hours. Excess ethanol on surface of hydrogel was blotted prior to weight measurement. The porosity was calculated as follows:

$$\text{Porosity} = \frac{M2 - M1}{\rho \times V} \times 100\% \quad (2)$$

where M_1 and M_2 were the mass of hydrogel before and after immersion in ethanol, respectively, ρ is the density of absolute ethanol, and V is the volume of the hydrogel.

2.8. Swelling properties of conjugate hydrogels

Cylindrical gels were prepared in 24-well plate by addition of 1 ml of conjugate solutions to each well. Horseradish peroxidase (3 unit/ml) and H_2O_2 (1 mM) were added at 100 µl/well followed by mixing. The hydrogels were removed from well plate and water; the by-product of the gelation was absorbed using filter paper. The initial weight of hydrogels was recorded (W_1) before immersing in 5 ml simulated body fluid (SBF) at 37 °C. For every 10 minutes, the hydrogel samples were removed, then gently wiped with filter paper to expel the surface water and weighted (W_2). The percentage of swelling was being calculated as follows:

$$\text{Percentage of swelling} = \frac{W2 - W1}{W1} \times 100\% \quad (3)$$

where W_2 and W_1 are water-swollen and initial weight of hydrogel samples respectively.

2.9. Degradation studies of hydrogels

Cylindrical gels were prepared in 24-well plate by addition of 1 ml conjugate solutions to each well. Horseradish peroxidase (3 unit/ml) and 1 mM H_2O_2 were added to conjugate solutions at 100 µl/well and left to gel for 30 minutes before hydrogels were taken out from well plate. The initial weight of hydrogels was recorded. The hydrogels were transferred to containers having simulated body fluid and incubated at 37 °C. The hydrogels samples were taken out for weighing weekly until the hydrogel disintegrated. The degree of degradation was calculated as follows:

$$\text{Degree of degradation} = \frac{W1 - W2}{W1} \times 100\% \quad (4)$$

where W_1 and W_2 were the initial and final weight of hydrogel.

2.10. Statistical analysis

All data are expressed as mean values with standard deviation. The statistical significance between two groups of experimental data was assessed using Student's *t*-test.

3. Results and discussion

3.1. Ultrasonication effect on collagen fibrils

The TEM analysis showed that the diameter of collagen fibrils with ultrasonic treatment was significantly smaller as compared to collagen fibrils without ultrasonic treatment (Fig. 1).

The average reduction in fibril diameter of ultrasonic pre-treated collagen at 0.15 % (w/v) was 77.4 % whereas at 0.4 % (w/v) was 52.5 %. In addition, the ultrasonic treated collagen fibrils were homogeneously distributed and non-aggregated. The result is in concurrence with findings reported by Jiang et al. (2016) on collagen derived from grass carp skin. In that study, the collagen fibrils treated with ultrasonication possess smaller diameters and D-periodicity lengths compared to untreated fibrils. The ultrasonic energy transfers mechanical wave through a process called cavitation, where gas bubbles rapidly form and collapse in water. This cavitation could produce strong shock wave within the cavitation bubbles and surrounding area, thereby preventing the collagen fibrils from aggregating. Hence, this smaller diameter and uniformly distributed collagen fibrils effectively increase the surface area for attachment of Ph groups.

3.2. Gelation time of hydrogels

Previous work showed that Col-Chit-Ph was crosslinked to form hydrogels within 5 minutes via HRP-catalyzed crosslinking reaction (Lau et al., 2018). Here, the influence of Col: Chit ratio on gelation time of hydrogels was studied. The gelation time considerably decreased with increasing HRP concentration (Fig. 2), which was similar to those obtained by Jin et al. (2007) and Lee et al. (2013).

The gelation time increased linearly with the concentration of H_2O_2 (Fig. 3). The result was consistent with the findings of Kuo et al. (2015), which showed that increasing H_2O_2 concentrations resulted in a longer gelation time required for collagen Ph hydrogel formation. The longer gelation time was needed as H_2O_2 concentration increased due to inactivation of HRP by the excess amount of H_2O_2, thereby reducing the efficiency of crosslinking (Sakai and Kawakami, 2007; Jin et al., 2014). The heme group in HRP irreversibly destroyed when

H_2O_2 was in excess, causing HRP losing its catalytic activity (Lee et al., 2013).

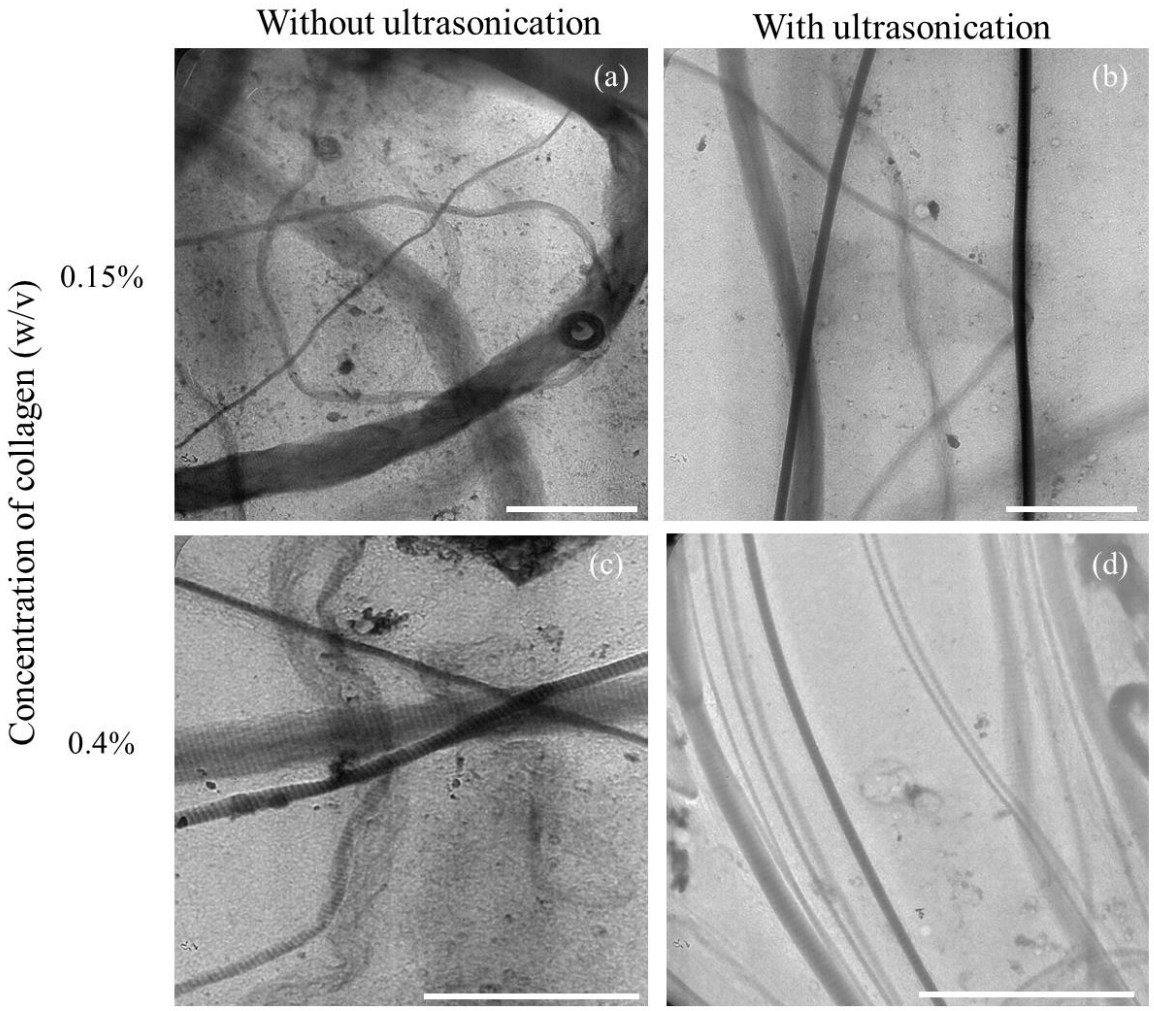

Fig. 1: The effect of ultrasonication on collagen fibrils at collagen concentrations (w/v) of 0.15% (a, b) and 0.4% (c, d). The collagen fibrils as shown in (a, c) were without ultrasonic treatment and (b, d) were treated with ultrasonic. The scale bar shows 2 µm

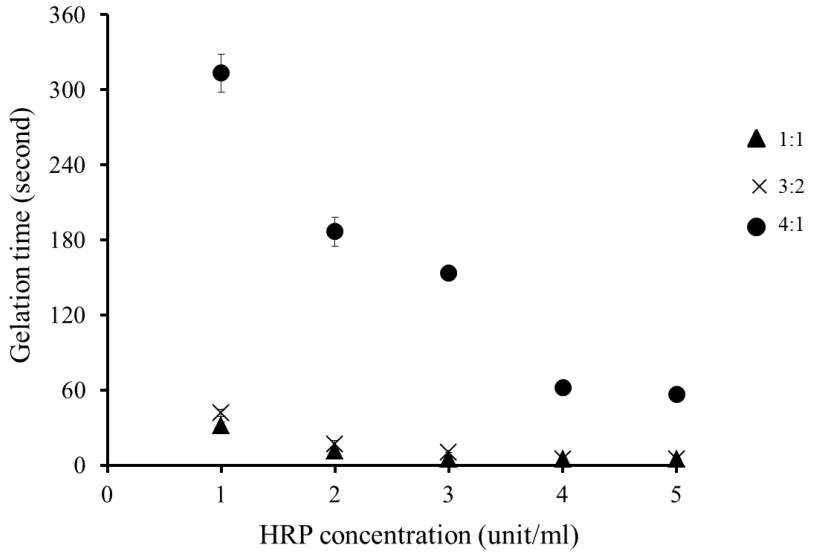

Fig. 2: The gelation time of conjugate hydrogels with 4:1, 3:2 and 1:1 Col: Chit ratios at different concentration of HRP

Since H_2O_2 is an oxidant that could harm cells and surrounding tissues, a relatively faster gelation involving the minimum use of H_2O_2 is necessary for biomedical applications (Ogushi et al., 2007). For

instance, conjugate with Col: Chit ratio of 3:2 was able to achieve gelation within 10 seconds with the minimum use of H_2O_2 (1 mM) under fixed HRP concentration (3 unit/ml).

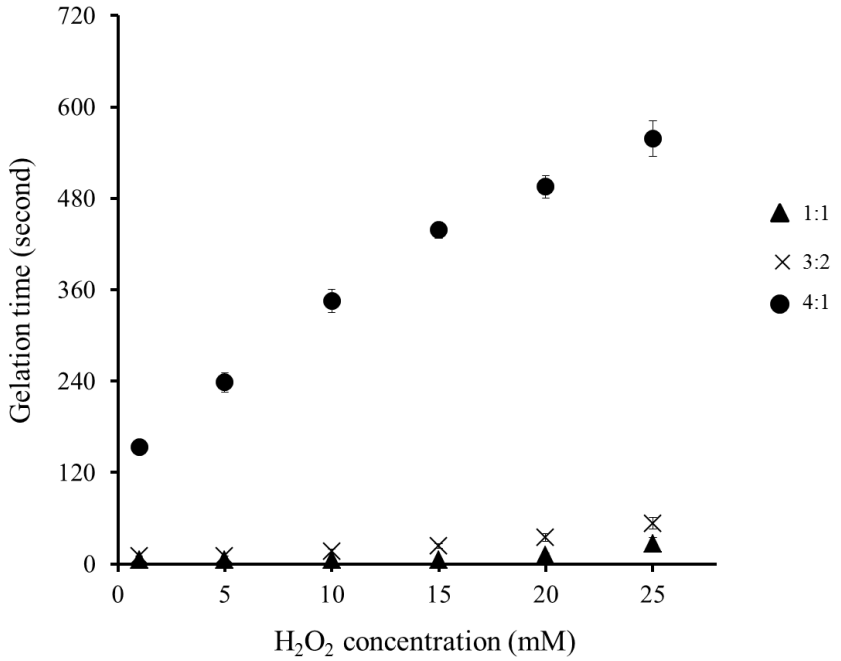

Fig. 3: The gelation time of conjugate hydrogels with 4:1, 3:2 and 1:1 Col: Chit ratios at different concentration of H_2O_2

Significant findings were obtained from the hydrogel with 1:1 Col: Chit ratio, which had the shortest gelation time (< 10 seconds) as compared to the other ratios in all parameter variations. This is because the higher composition of chitosan provides additional amino groups (Ma et al., 2003) to function as binding sites for phenolic hydroxyl groups, hence enhancing the hydrogel formation via HRP-mediated crosslinking reaction.

Hydrogel with 1:1 Col: Chit ratio could potentially be applied as haemostat as the gelation needed to be fast (< 5 seconds) in order to stop wound bleeding (Ghobril and Grinstaff, 2015). Meanwhile, the gelation time of 3:2 Col: Chit ratio (> 5 seconds) is attractive for application as instant wound dressing, in order to allow accurate filling of conjugate solutions to defect site before gelation and to achieve cohesion with surrounding tissue (Lee et al., 2008).

3.3. Mechanical properties of hydrogels

Mechanical properties are of great importance in maintaining structural stability of hydrogels for the biological functions of cells within the hydrogel matrix. The hydrogel with 4:1 Col: Chit ratio deformed and caused no resistance to trigger force. As shown in Fig. 4, the bloom strength of hydrogels with 1:1 Col: Chit ratio was significantly higher than 3:2 Col: Chit ratio. The greatest difference between these hydrogels was about 41 % at 3 units/ml of HRP.

These results confirmed that a higher composition of chitosan in Col-Chit-Ph enhanced the mechanical properties of hydrogels. Besides, chitosan could act as GAG analog in intertwining with the fibrous collagen to attain mechanical stability (Tan et al., 2001). The mechanical strength of hydrogels correlates with its gelation time, whereby hydrogel with 1:1 Col: Chit ratio with the shortest gelation time (5 seconds) yielded the highest mechanical strength (0.49 N) as compared to the 3:2 Col: Chit ratio (0.28N).

3.4. Cell growth rate on hydrogels

The biological characterization of hydrogels showed that the cell growth rate of hydrogel with 3:2 Col: Chit ratio was 87 % higher than 4:1 Col: Chit ratio and 94 % higher than the 1:1 Col: Chit ratio (Fig. 5). The synergistic interaction between this hydrogel and secreted proteins from cells had created a favourable environment for cell growth (Tan et al., 2001).

Although previous work found that the hydrogel with 4:1 Col: Chit ratio presented the highest efficiency of cell attachment, the poor mechanical stability of this hydrogel limited the cell growth (Ma et al., 2003). The most significant difference was observed between hydrogel with 1:1 and 3:2 Col: Chit ratios due to the insufficient diffusion of nutrients and metabolites to cells within the high matrix density hydrogel with 1:1 ratio (El-Sherbiny and Yacoub, 2013). Hence, in term of biological features, hydrogel with 3:2 Col: Chit ratio is suggested for potential use in biomedical applications.

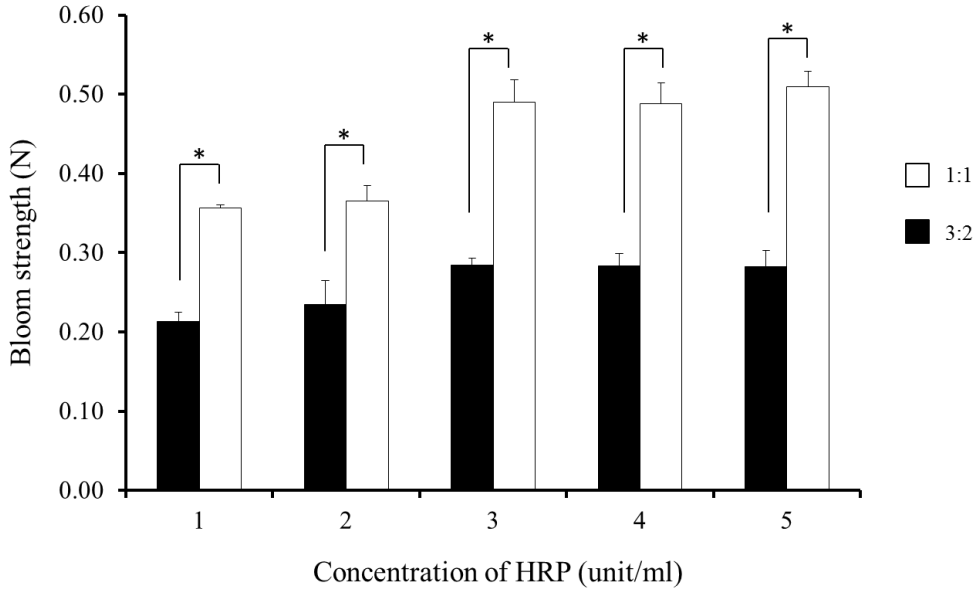

Fig. 4: The bloom strength of conjugate hydrogels with 3:2 and 1:1 Col:Chit ratios at different concentration of HRP. Student's t-test indicate a significant difference (*p < 0.01)

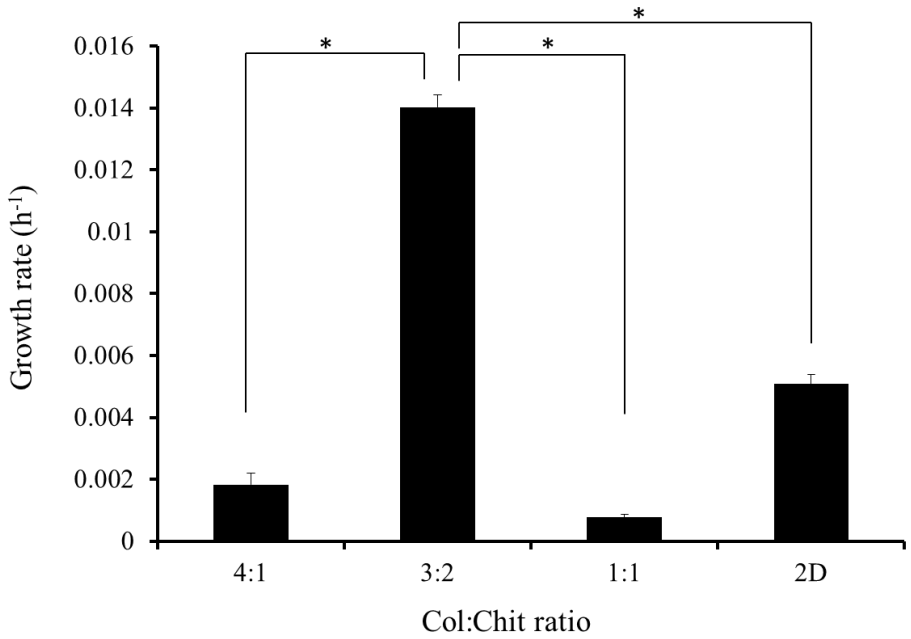

Fig. 5: The cell growth rate on conjugate hydrogels after 5 days incubation. Student's t-test indicate a significant difference (*p < 0.01)

3.5. Porosity of hydrogels

The porosity of hydrogels has a prominent effect on cell functions, such as cell attachment, migration and proliferation. The hydrogel with 3:2 Col: Chit ratio had porosity 42 % higher than 1:1 ratio (Fig. 6).

The porous hydrogel enabled more effective diffusion of nutrients and metabolites to cells. The porosity of hydrogel with 1:1 Col: Chit ratio was found to be 28 % lower than 4:1 Col: Chit ratio. This could be due to the higher composition of chitosan in hydrogel contributed to higher degree of crosslinking, hence resulted in a lower porosity of the hydrogel (Tan et al., 2001). The lower porosity in hydrogel matrix with 1:1 Col: Chit ratio had minimized its cell proliferative capacity (Heydarkhan-Hagvall et al., 2008).

3.6. Swelling properties of injectable hydrogels

The swelling behaviour of injectable hydrogels was measured every 10 minutes (Fig. 7) to evaluate its potential to build up pressure if injected in the wound area.

In general, all conjugate hydrogels showed a low percentage of swelling, which ranged from 2 % to 6 %, with conjugate hydrogel with Col: Chit ratio of 3:2 showed the highest percentage of swelling (5.3 %) in initial 10 minutes. This result revealed that conjugate hydrogels did not swell markedly after soaking in SBF. The low percentage of swelling

indicated that the injectable hydrogels would not cause the loss of a large amount of body fluid at the desired site, hence preventing the build-up of pressure around the wound that might cause pain to the patient (Muktar et al., 2018). In addition, the hydrogels could maintain almost similar size and shape without generating shear stress to cells around wound as cells are easily damaged by forces caused by swelling of hydrogels (Sakai and Kawakami, 2007).

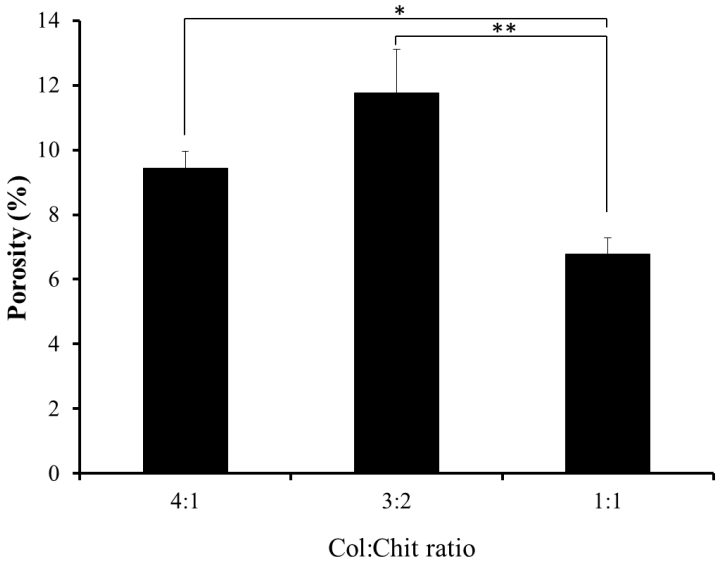

Fig. 6: The porosity of conjugate hydrogels, where hydrogel was formed using 3 unit/ml HRP and 1 mM H_2O_2. Student's t-test indicate a significant difference (*p < 0.01, **p < 0.05)

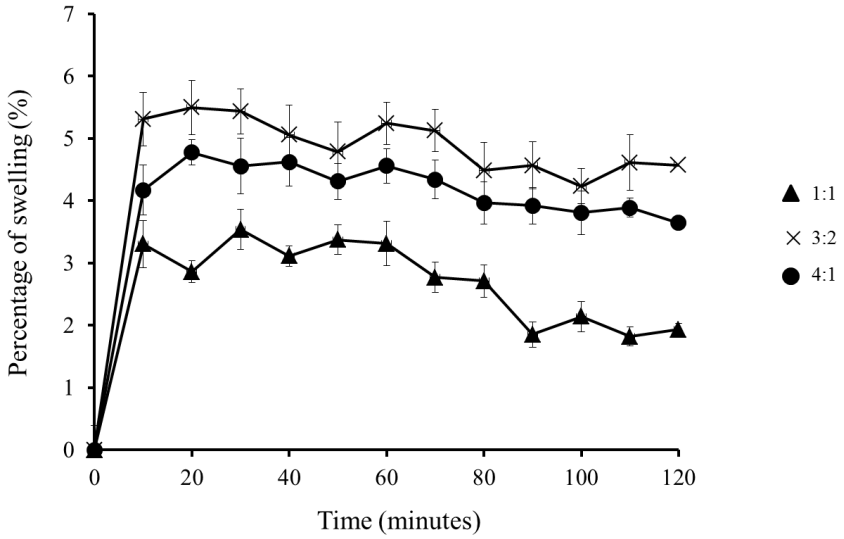

Fig. 7: The percentage of swelling of conjugate hydrogels with Col:Chit ratio of 4:1, 3:2 and 1:1 for period of 120 minutes. The data represent the mean values with standard deviation from triplicate experiments

3.7. Degradation of hydrogels

The injectable hydrogels should be designed to degrade within a period of time. The degradation measurement of hydrogel was performed per week as hydrogel showed insignificant degradation when measured per day. Since the hydrogel with 4:1 Col: Chit ratio was easily ruptured by trigger force, degradation study was only conducted for hydrogels with 3:2 and 1:1 Col: Chit ratios. The hydrogel with 3:2 Col: Chit ratio degraded significantly faster than that with 1:1 ratio for the 1st and 2nd weeks (Fig. 8), indicating that highly crosslinked hydrogel matrix could result in slower degradation rate because of limited accessibility to the cleavage sites of this hydrogels (Lee et al., 2013). Besides, chitosan degraded slower than that of collagen, thus the incorporation of chitosan not only improved the mechanical properties but also reduced the degradability of hydrogels (Ding et al., 2008). All hydrogels were ruptured in the 3rd week, where bulk degradation had occurred in hydrogels leading to the breakdown of the interior network structure of hydrogels.

4. Conclusion

Pretreatment by ultrasonication has successfully prevented aggregation of collagen fibrils, thereby resulting in smaller fibril diameter and increasing

the surface area for attachment of Ph groups. Furthermore, injectable enzymatically synthesized collagen-chitosan hydrogel with 3:2 Col: Chit ratio has potential to be used as biomaterial such as wound dressing due to its ability to form rapid gelation, biodegradability and mechanically stable for cell growth on hydrogel matrix.

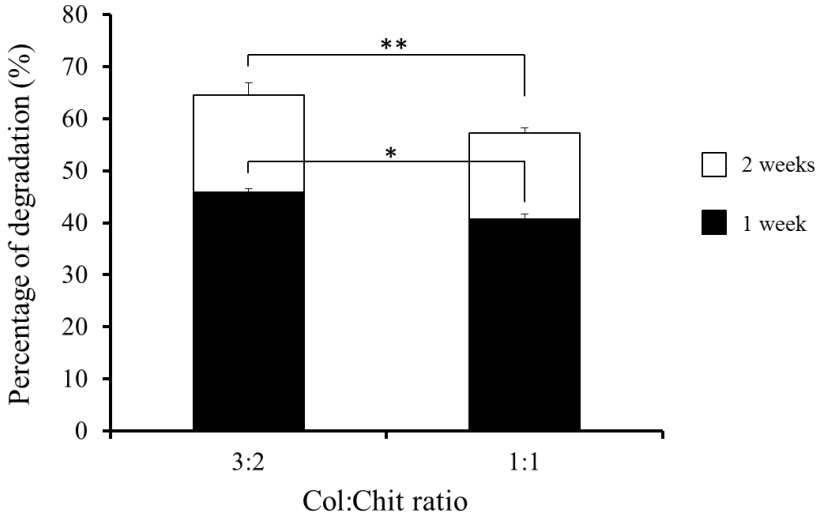

Fig. 8: The percentage of degradation of conjugate hydrogels with 3:2 and 1:1 Col:Chit ratios at 1st and 2nd week. Student's t-test indicate a significant difference (*p < 0.01, **p < 0.05)

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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