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Research paper

Collagen-Chitosan Hydrogel Formed in Situ Via Enzymatic Crosslinking

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Abstract

In this research, injectable collagen-chitosan conjugates (Col-Chit-Ph) hydrogel was formed by oxidative coupling of phenolic hydroxyl (Ph) groups in polymer chain via peroxidase-catalysed crosslinking reaction. The resulting Col-Chit-Ph solutions formed rapid hydrogel at physiological conditions by utilizing H2O2. The amount of chitosan in the compositions of Col-Chit-Ph hydrogels influenced the mechanical properties of the hydrogels. Here, conjugates having Col:Chit ratio of 1:1 showed greater resistance to compression compared to conjugates having Col:Chit ratio of 3:2. Meanwhile, the efficiency of cell attachment for conjugates with Col:Chit ratio of 4:1 is higher than conjugates with Col:Chit ratio of 1:1. Although the biological properties of hydrogel was good at Col:Chit ratio of 4:1, this hydrogel easily destroyed by external force. However, with higher amount of chitosan in the composite, hydrogels were more mechanically stable. This indicates the importance of the chitosan ratio to the novel Col-Chit-Ph injectable hydrogel composite.

Keywords: *In situ hydrogel, composite, gelation, collagen, chitosan*

1. Introduction

The significance of hydrogel in various biomedical fields continues to grow due to its excellent properties, such as high water content, good biocompatibility and permeability to nutrients, oxygen or other water- soluble metabolites (Jin et al., 2007). Hydrogel possess hydrophilic polymeric network that able to absorb and retain high amount of water, thus resemble the native tissue in body. Over the past year, a lot of studies have revolved around the application of hydrogel as carriers for drug delivery. To date, hydrogel also has been extensively used as scaffold for tissue engineering.

A variety of methods have been studied to synthesis hydrogels in the last decade. The introduction of enzymatic crosslinking reaction is considered as an important achievement of hydrogel-based technology. It has received increasing attention as an effective route for development of in situ forming hydrogel due to its high specificity and mild reaction conditions that are suitable for living cells (Le Thi et al., 2017). The in situ forming hydrogels also regarded as injectable hydrogels. Injectable hydrogels are highly desirable than the conventional preformed hydrogels due to in situ gelation of polymeric solution at the injection site without requiring any surgical implantation of preformed hydrogel. The injectable hydrogel system is easy to use especially for wound healing and drug delivery by simply injection of polymeric solution and bioactive agents using syringe. One such agent is horseradish peroxidase (HRP), an enzyme that mostly used for oxidative polymerization of phenol derivatives. Through HRP catalysed crosslinking reaction, polyphenols on polymer chain linked at the aromatic ring give rise to in situ hydrogel formation.

In this research, collagen (Col)-chitosan (Chit) composite hydrogel conjugated with phenolic hydroxyl (Ph) groups is used as biomaterial for HRP-catalysed crosslinking reaction. To date, there is no other research being reported about this composite conjugated with Ph groups. Type I collagen is chosen due to its properties of biodegradability and biocompatibility (Jiang et al., 2016). The fibrils of collagen provide excellent scaffold for cell activity. However, collagen has poor physical characteristic in nature. The combination of chitosan to collagen improves the physical properties of injectable hydrogel for particular biomedical uses.

2. Problem Statement

Collagen is a highly attractive biomaterial for preparation of in situ forming hydrogels, owing to its desirable biocompatibility. However, the low mechanical strength of collagen hydrogel limits the further use of this biomaterial. The combination of collagen and chitosan meet both physical and biological criteria for potential biomedical applications.

3. The Aim of Research

This study was conducted to develop a novel in situ forming Col-Chit-Ph hydrogel through HRP-catalysed crosslinking reaction.

4. Method of Research

Morpholinoethanesulfonic acids (MES), 3-(4-Hydroxyphenyl)-propionic acid (ρHP), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), horseradish peroxidase (HRP, 293 unit/mg), hydrogen peroxide (H2O2, 30% concentration) were from Merck. The other chemicals such as N-hydroxysulfosuccinimide (sulfo-NHS), F12: Dulbecco's Modified Eagles's Medium (DMEM) and Vybrant MTT cell proliferation assay kit were from Thermo Scientific, Gibco and Invitrogen re-

spectively. Collagen sponge from ovine tendon was a gift from Tissue Engineering Centre, UKM Medical Centre, while chitosan (degree of deacetylation of about 95%) was obtained from Primex. Collagen was dissolved in 50 mM MES aqueous solution and added with chitosan under gentle stirring in ice bath. Composites with Col:Chit ratio of 4:1, 3:2 and 1:1 were produced. The 100% chitosan solution (Chit) was prepared by dissolving in 50 mM MES solution at room temperature without adding collagen.

The Col-Chit composites and Chit having phenolic hydroxyl (Ph) groups were synthesized with modifications from Sakai et al. (2009). Initially, ρHP in 50 mM MES solution was added with EDC and sulfo-NHS before transferring to solution of Col-Chit and Chit. After 3 hours stirring, the conjugate solutions were repeatedly suspended and precipitated using 90% ethanol to remove the remaining ρHP, EDC and sulfo-NHS, then centrifuged at 10000 rpm. The conjugate precipitates were air- dried and re-dissolved in 50 mM MES solution at 1% (w/v).

The conjugate solutions were brought to pH 7 using 1 M NaOH and then injected into test tubes. Gelation of conjugates was based on the enzyme- catalysed crosslinking reactions using 3 units/ml of HRP and 1 mM of H2O2. The formation of conjugate hydrogels started when no fluidity was observed upon inclining the tube.

The bloom strength measurement of conjugate hydrogels was performed with TA. XT-Plus texture analyser (Stable Micro System). Cylindrical gels were prepared in 12-well plate by addition of 3 ml of conjugate solutions to each well. This was followed by the addition of 3 unit/ml HRP and 1 mM H2O2 and left for 1 hour in incubator prior to analysis. Hydrogels were analysed by compressing using 5 g cylindrical probe P/0.5 (0.5 mm diameter) at speed of 30 mm/min.

Human dermal fibroblast (HDF) was seeded in each 96-well of hydrogel samples at 2500 cells/ well with 200 µl F12: DMEM culture medium for 5 days at 37 oC in 5% CO2 incubator. The efficiency of cell attachments was evaluated using MTT cell proliferation assay according to the manufacturer's instruction. After incubation, the absorbance measurement was performed using spectrophotometer (Bio-Tek Power Wave XS) at 570 nm. The efficiency of cell attachments was determined using the following formula, based on the absorbance value of cell attached to conjugate hydrogels (Asample) relative to two dimensional (2D) polystyrene surfaces (A2D).

Cell attachment (%) = A_sample/A_2D x 100%

5. Analysis and Discussion

Initially, it was determined whether Col-Chit-Ph solutions able to form hydrogels at neutral pH via peroxidase-catalysed oxidative reaction.

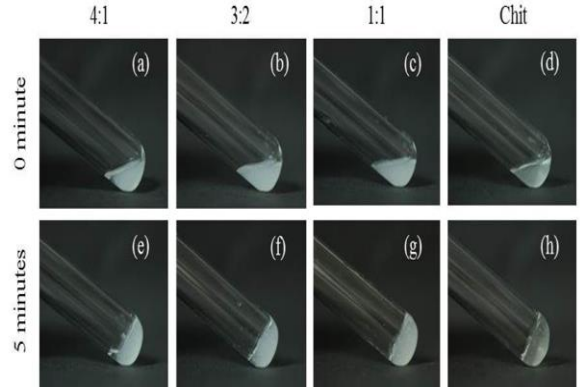

Figure 1. The images show the gelation of conjugates at 37 oC immediate after addition of HRP and H2O2 for samples with collagen to chitosan ratio of (a) 4:1, (b) 3:2, (c) 1:1 and (d) chitosan sample without collagen. Image of gelation at 5 minutes for the respective samples are shown in (e), (f), (g) and (h).

As shown in Figure 1, Col-Chit-Ph and Chit-Ph were successfully cross-linked via enzyme-mediated crosslinking reaction in the presence of HRP and H2O2. Stable hydrogel was formed within 5 minutes for all conjugates. The conjugates were crosslinked through oxidative coupling reactions of phenol groups, either carbon-carbon bond between ortho-carbons of the aromatic ring or carbon-oxygen bond between ortho-carbon and phenolic oxygen (Lee et al., 2008). The ability of Col-Chit-Ph to from rapid hydrogel is critical for intended biomedical applications.

In this study, bloom strength is regarded as the maximum force reading obtained when a trigger force penetrates into the gel to compress at a depth of 4 mm. The bloom strength of Col-Chit-Ph hydrogels was measured after achieving gel stability. The hydrogel with Col:Chit ratio of 4:1 deformed and caused no resistance to trigger force.

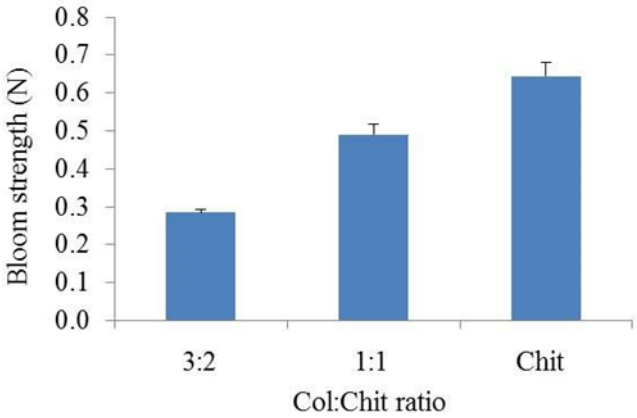

Figure 2. The bloom strength of conjugate hydrogels. The data represent the mean values with standard deviation from triplicate experiments. Conjugates with Col:Chit ratio of 4:1 could not withstand the trigger force and deformed, giving no value.

The data shown in Figure 2 suggested that higher composition of chitosan in Col-Chit-Ph enhanced the mechanical properties of hydrogels. This is likely due to the chitosan that winds around collagen triplex helix gives rise to increased NH2 groups available for oxidative coupling reaction of phenols (Sionkowska et al., 2004). As a result, the greater extent of crosslinks has effectively enhanced the mechanical stability of hydrogel during compression (Sakai et al., 2009). However, Chit-Ph has the highest reading probably due to Chit-Ph that contains only chitosan give rise to greater intermolecular interactions among chitosan chains and thus produce strongest hydrogel (Tahrir et al., 2015).

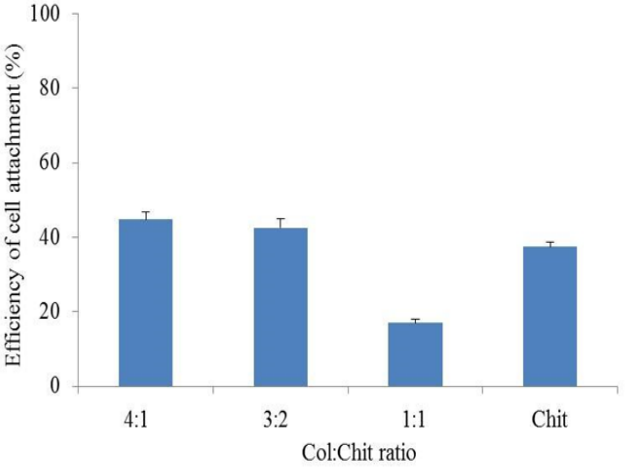

Figure 3. The cell attachment on conjugate hydrogels relative to 2D cell attachment

Figure 3 shows the HDF cell attachment on conjugate hydrogels relative to 2D surface after 5 days. The result implied that the efficiency of cells attached to conjugate hydrogels could be influ-

enced by the amount of collagen in conjugates. This result agreed with previous studies that collagen fibrils provide the major scaffold for cell attachment (Yunoki and Matsuda, 2008). The Chit hydrogels have higher efficiency of cell attachment than hydrogel with Col:Chit ratio of 1:1 due to the possibility of the large amount of chitosan has greatly disrupted the collagen fibrils (Wang et al., 2011). The result also indicated that higher composition of chitosan in Col-Chit-Ph hydrogel caused insufficient diffusion of nutrients and metabolites for cells within the hydrogel network. (El-Sherbiny and Yacoub, 2013).

6. Conclusion

In conclusion, we have developed fast in situ forming Col-Chit-Ph hydrogels via HRP-catalysed crosslinking reaction at neutral conditions. On basis of these results, Col-Chit-Ph hydrogels with greater composition of chitosan give higher mechanical strength but lower efficiency of cell attachment. This study provides directions on developing the novel Col-Chit-Ph injectable hydrogels, where the composition of Col-Chit-Ph could be tailored according to its applications.

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