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Study of the effect of mixing approach on cross-linking efficiency of hyaluronic acid-based hydrogel cross-linked with 1,4-butanediol diglycidyl ether

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ABSTRACT

Regardless of various strategies reported for cross-linking hyaluronic acid (HA) with 1,4-butanediol diglycidyl ether (BDDE), seeking new strategies that enhance cross-linking efficiency with a low level of cross-linker is essential. In this work, we studied the influence of mixing approach on two cross-linked BDDE-HA hydrogels prepared by two different mixing approaches; the large-batch mixing approach in which the hydrogel quantities were all mixed as a single lump in one container (hydrogel 1), and the small-batches mixing approach in which the hydrogel quantities were divided into smaller batches, mixed separately at various HA/BDDE ratios then combined in one reaction mixture (hydrogel 2). The result showed that the cross-linking reaction was mixing process-dependent. Degradation tests proved that, in relation to hydrogel 1, hydrogel 2 was more stable, and exhibited a higher resistance towards hyaluronidase activity. The swelling ratio of hydrogel 1 was significantly higher than that of hydrogel 2 in distilled water; however, in phosphate buffer saline, both hydrogels showed no significant difference. SEM images demonstrated that hydrogel 2 composite showed a denser network structure and smaller pore-size than hydrogel 1. In comparison to native HA, the occurrence of chemical modification in the cross-linked hydrogels was confirmed by FTIR and NMR distinctive peaks. These peaks also provided evidence that hydrogel 2 exhibited a higher degree of modification than hydrogel 1. In conclusion, the small-batches mixing approach proved to be more effective than large-batch mixing in promoting HA-HA entanglement and increasing the probability of BDDE molecules for binding with HA chains.

1. Introduction

Hyaluronic acid (HA) is a high-molecular weight, poly-anionic polymer with many applications in human medicine (Šimkovic et al., 2000). It is composed of repeating disaccharide units of glucuronic acid (known as uronic acid) and *N*-acetyl-D-glucosamine linked by alternating glycosidic bonds β - (1, 4) and β- (1, 3) (Schanté et al., 2011a, b).

Naturally, HA is found in human skin, extra-cellular matrix (ECM) and synovial fluid of vertebrates (Zawko et al., 2009). Due to the unique structure of HA and its biological properties which are very similar of that in human tissues, it has gained great attention and interest over the years. These properties have allowed HA to be used in different biomedical applications such as wound-healing, osteoarthritis (Liu et al., 2007) and tissue-augmentation (Kenne et al., 2013).

In the past, scientists were able to extract HA from bovine vitreous humor and rooster combs. Lately, efforts have been focused on producing high yields of HA from genetically modified bacteria (Streptococcus) with low-cost methods (Schanté et al., 2011a). However, native HA has very limited applications because it does not remain in the human body for prolonged periods due to its poor mechanical properties (Liu et al., 2007; Jeon et al., 2007) and *in vivo* rapid degradation (Pitarresi et al., 2007).

It has been reported that the half-life of HA after injection into the skin or joints is no longer than 1 day (Brown et al., 1991). Consequently, it is not a suitable material for therapeutic action particularly with dermatological applications such as injectable fillers addressed to replace tissue lost by the aging process. This fast turnover forms a major obstacle to the treatment by HA injection. (Schanté et al., 2012). HA-based hydrogels have therefore been developed to increase HA persistence in tissue and provide space-occupying supplement which results in the correction of skin contour deficiencies (Manna et al., 1999). These

Abbreviations: HA, hyaluronic acid; BDDE, 1,4-butanediol diglycidyl ether; BTH, bovine testicular hyaluronidase; PBS, phosphate buffer saline.

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hydrogels are supported with 3-diensional network structures which provide a chemical shield against decomposition acted by hyaluronidase or reactive oxygen, so they maintain the volume of HA for a much longer time in human skin than does the native HA. For instance, some of HA-based fillers with improved stability may enhance hydrogel's residence after implementation up to 12 months (Sadick et al., 2009). Moreover, hydrogels with extended HA degradation provide a long-term *in vivo* stability which is potential for various medical applications including cardiovascular medicine, orthopedics and even for bone or cartilage tissue engineering (Fakhari and Berkland, 2013). In fact, hydrogel's resistance against decomposition forms a crucial part to its utilization in the clinical application.

Most of HA-based products currently available in the market are modified with chemical cross-linkers to improve their mechanical properties and duration *in vivo* (Edsman et al., 2012). Examples of cross-linkers include methacrylamide (Segura et al., 2005), hydrazide (Prestwich et al., 1998) carbodiimide (Lai, 2012), divinyl sulfone (DVS), 1,4-butanediol diglycidyl ether (BDDE) and poly(ethylene glycol) diglycidyl ether (PEGDGE) (Gatta et al., 2013; Schanté et al., 2011a, b).

As reported, the percentage of cross-linker is positively correlated with the degree of cross-linking (Lai, 2014). With the increase of cross-linker concentration, the degree of cross-linking is increased (Caillard et al., 2008; Wong et al., 2015), thus enhancing hydrogel stability and resistance towards enzymatic degradation.

However, as a health concern, several studies showed that the excessive amounts of cross-linking agents are often toxic and can give unwanted reactions with the bioactive substances present in the hydrogel matrix (Hennink, and van Nostrum, 2012; Boogaard et al., 2000). They may affect the integrity of the substances to be entrapped such as drugs or proteins. Treating patients with excessive amounts of cross-linker is not a healthy practice and may be associated with undesirable effects, particularly if the dose contains residues of un-reacted cross-linker. However, when an amount of a cross-linking agent is reduced, it is difficult to obtain such high resistance against degradation. In fact, preparing a cross-linked HA hydrogel with both a low amount of a chemical cross-linker and efficient cross-linking is difficult. This field of study is currently receiving considerable attention, and has become one of the major challenging issues in hydrogel manufacturing.

Therefore, the objective of our study was to evaluate the influence of mixing procedure on HA cross-linking efficiency while maintaining a constant level of cross-linker. We modified a reported method described by (Malson and Lindqvist, 1986) and later improved by (Piron and Tholin, 2002) to synthesize two HA hydrogels cross-linked with 1, 4-butanedioldiglycidyl ether 1, 4-butanediol diglycidyl ether BDDE using two different mixing approaches. We selected BDDE because it is currently used in the majority of market-leading HA hydrogels. The reaction between HA and BDDE was performed in strong alkaline conditions to form a stable covalent ether bond (De Boulle et al., 2013). At a very high pH range, the epoxide groups of BDDE preferentially react with the hydroxyl groups of HA, because the deprotonated hydroxyls are much stronger nucleophiles than both the anionic carboxylic group and the amide (Kenne et al., 2013). The impact of the mixing approach on cross-linked BDDE-HA hydrogels was mainly evaluated *via in vitro* degradation rate and swelling measurements. The hydrogels' surfaces topography and their microstructures were characterized using scanning electron microscopy (SEM). Fourier transform infrared spectroscopy (FTIR) and nuclear magnetic resonance spectroscopy (NMR) were employed to confirm the occurrence of cross-linking reaction.

2. Materials and method

2.1. Materials

Sodium salt of hyaluronic acid (average molecular weight = 2,000,000 Da) was donated by Vivatis Pharma (Hamburg, Germany). The cross-linker 1,4-butanediol diglycidyl ether (BDDE), the enzyme hyaluronidase (3000 U/mg) and dialysis tube were purchased from Sigma-Aldrich Co (St. Louis, Missouri, USA). All other chemicals were of analytical grade and used as received without any purification.

2.2. Preparation of cross-linked hydrogels

Two cross-linking reagent solutions were prepared by adding 200 μl of 1,4-butanediol diglycidyl ether (BDDE) into 9.8 ml of 0.25 M NaOH, (pH 13). 1.0 g of HA powder was added to one reagent solution and mixed thoroughly at 40 °C for 2 h and then neutralized with 0.1 M HCl to a pH of approximately 7.0. The resulting hydrogel was labeled "hydrogel 1" and the mixing approach was referred to as "the large-batch mixing approach". Similar HA quantity was divided into smaller batches at different weights; 300 mg, 250 mg, 200 mg, 150 mg and 100 mg. Each batch was mixed with 2.0 ml of the second reagent solution in separate containers for 30 min; all batches were then combined in one reaction container and left under continuous mixing for 90 min at 40 °C. The combined mixture was then neutralized with 0.1 M HCl to a pH of approximately 7.0. The resulting hydrogel was labeled "hydrogel 2" and the mixing approach was referred to as "the small-batches mixing approach". Both hydrogels were then dialyzed for two days against distilled water to remove BDDE residue and non-reacted HA fragments. The cross-linked hydrogels were then lyophilized using (Labtech freeze-dryer LFD 5518 model, Daihan Labtech Co.); the samples were first frozen at −80 °C in an ultra-low temperature freezer (MDF-U3386S, SANYO Electric Co., Japan) for 4 h to ensure complete freezing and then sublimed at −50 °C for 24 h under a vacuum of 5 mTorr. When all free ice was removed, the temperature was increased to 25 °C and the samples were left for 2 h to remove trace water molecules bound to them. Finally, the products were stored at 8 °C until the characterization studies were carried out. For a comparison purpose, the cross-linked hydrogels were compared with native HA polymer.

3. Measurements

3.1. In vitro degradation rate

The *in vitro* degradation rates of cross-linked hydrogels were carried out according a colorimetric method described by (Reissig et al., 1995). The method was based on quantification of the colored *N*-acetyl glucosamine (NAG) resulting from the enzymatic digestion after a particular interval. The extracts were analyzed by a single-beam UV–Visible spectrophotometer (Spekol 1500, Analytik, Jena, Germany) and the absorbance was recorded at 585 nm. In brief, equivalent samples A, B, C, D and E were taken from the lyophilized native HA and cross-linked BDDE-HA hydrogels (the total was 15 samples) and placed in separate test tubes. Each sample was mixed with 500 μl of bovine testicular hyaluronidase (BTH) with an activity of 300 units/ml in 10 ml phosphate buffer saline (PBS) solution. Samples "A" were kept for one day of treatment whereas samples "B, C and D" were treated for 2, 3 and 4 days respectively. Samples "E" were left with hyaluronidase until complete digestion. The enzymatic reaction was stopped by heating each test tube in boiling water for 5 min. The resulting extracts were centrifuged and the supernatants were transferred into 10 ml volumetric flasks and diluted up to the mark. Approximately 1.0 ml from each filtrate was mixed with 0.1 ml of 0.25 M sodium carbonate and then boiled for 1 min in a water bath. Amounts 6:1 of glacial acetic acid and Ehrlich's reagent were added to the filtrates and left until a violet color was produced.

After confirming previously the time requiring for the color to reach its maximum intensity in native HA, the color for each sample was allowed to reach its maximum intensity and then all samples were prepared for the colorimetric assay. Measurements were carried out in triplicate and the results were presented as mean with 95% confidence interval.