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Effect of hyaluronic acid initial concentration on cross-linking efficiency of hyaluronic acid – based hydrogels used in biomedical and cosmetic applications

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This work was aimed to explore the potential effect of hyaluronic acid (HA) initial concentration (7.0 – 14.0 % w/v) on cross-linking efficiency of HA hydrogels cross-linked with 1,4-butanediol diglycidyl ether (BDDE). The results revealed that the hydrogel prepared at 10.0 % HA concentration exhibited a slower degradation rate, a lower swelling ability and more regular porosity than those prepared at either lower or higher HA concentration. After four days incubating with hyaluronidase, the content of NAG (*N*-acetyl glucosamine) remaining in the 10.0 HA hydrogel was 25.1±1.9 % with respect to the total NAG content found in the original mass. In contrast, the hydrogels prepared at 7.0 % and 14.0 % HA concentration showed a less remaining content of NAG equaled to approximately 15.9±5.4 % and 19.5±2.6 % respectively. On the other hand, the swelling ability of tested hydrogels was steadily decreased with the increase of HA initial concentration until the 10.0 % HA hydrogel and then showed an opposite trend. Based on this finding, the 10.0 % HA hydrogel exhibited the lowest swelling ratio which was observed at 129±3.2 g/g in distilled water and at 116±2.4 g/g in phosphate buffer saline (PBS). The SEM images showed various morphologies within the entire range of tested hydrogels. However, the hydrogel prepared at 10.0 % HA concentration was more homogenous and appeared with narrower pore-size distribution ranged in diameter from less than 50 µm to approximately 300 µm. Finally, the effect of HA initial concentration was investigated by FTIR which confirmed that the 10.0 % HA hydrogel was subject to a greater loss of (- OH) at 3343 cm^{-1} than other hydrogels except the 11.0 % HA hydrogel. This phenomenon was probably attributed to the formation of pendants that allowed the 11.0 % HA hydrogel to appear with a lower peak intensity than the 10.0 % HA hydrogel in the FTIR spectra. In conclusion, the HA initial concentration plays a crucial role in determining the cross-linking efficiency of HA hydrogels cross-linked with BDDE.

1. Introduction

Hyaluronic acid (HA) is a high molecular weight and non-sulfated glycosaminoglycan composed of disaccharide repeating unit of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by alternating β-1,4 and β-1,3 glycosidic bonds (Schanté et al. 2011; Fan et al. 2006). HA is largely found in the extracellular matrix (ECM), vitreous humor of the eye and synovial fluid of vertebrates (Rhodes and Simons 2007; Zhu 2010; Zawko et al. 2009). The main function of HA is to create volume and provide lubricants to tissues, consequently preventing cell damages induced through various physical stresses (Romagnoli and Belmontesi 2008). HA is biocompatible, biodegradable and hydrophilic, so it allows the influx and retention of large amounts of water due to the abundant hydrophilic carboxyl groups (Allemann and Baumann 2008).

Due to its versatile features, HA has a wide-range of applications in the healthcare industry (Akdamar et al. 2009). HA plays important roles in cartilage matrix stabilization (Pitarresi et al. 2007). It is used in visco-supplement injections into the knee to increase the viscosity of the synovial fluid that helps cushion and lubricate the joint (Ruckmani et al. 2013). HA is also an important ophthalmic biomaterial (Lai 2014), it can be used in eye surgery such as corneal transplantation, cataract surgery and retinal detachment repairing (Ruckmani et al. 2013).

Moreover, products derived from HA are widely used in tissue engineering, because they provide three-dimensional networks that allow for nutrients and cellular waste to be diffused through them (Hoffman 2002). Recently, HA-based products have been investigated as polymeric drug carriers, the drugs can be easily loaded into the HA swollen matrices and released at a rate dependent on their physical properties and *in vivo* degradation rates. In cosmetics, it is known that older skin has less hyaluronic acid and therefore, less hydration and turgor due to the ageing process (Athre 2007), so the unique swelling of HA makes it an effective agent in dermal fillers to compensate loss of soft-tissues volume. Based on the aforementioned functions, HA has a large scope in biomedical and cosmetic applications. However, most of these applications are not addressed with native HA. The application of native HA is very limited due to its high water solubility and rapid degradation in the human body (Liu et al. 2007). The half-life of native HA is no longer than 24 h after injection into the skin (Brown et al. 1991).

To overcome these drawbacks, HA should be stabilized by chemical modification to ensure a longer residence time within the soft tissue after administration into the body. This process involves cross-linking of different HA chains by covalent bonds using chemical cross-linkers (Luo et al. 2000 ; Berkó et al. 2013; Masters et al. 2005; Kim et al. 2011; Yamanlar et al. 2011). The result of each cross-linking method is a cross-linked HA hydrogel with a three-dimensional network structure that retains water within its cross-linked network upon the hydration in an aqueous environment. The resulting hydrogel is more resistant than native HA towards enzymatic degradation due to the formation of bridges and intermolecular bonds between the HA chains and the cross-linker. A number of chemical cross-linkers have been addressed for HA cross-linking including 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. 2011). Crosslinking reaction can be performed on one of the HA functional sites; the carboxylic group (- COOH), hydroxyl group (- OH) or amino group (- $NHCOCH_3$). Modification targeting (- OH) group in the HA chains is widely employed, particularly for the development of HA dermal fillers, because it preserves the polyanionic nature of HA (Andre 2004; Chung et al. 2011; Hwang et al. 2012; Allemann and Baumann 2008).

Several studies showed that by increasing the chemical cross-linker content or concentration, the degree of cross-linking is increased (Caillard et al. 2008; Wong et al. 2015; Lai 2014), subsequently, slower degradation rate towards enzyme is exhibited. Hydrogels prepared with a higher cross-linking efficiency become stiffer and exhibit greater resistance against enzymatic degradation. A stronger HA-filler can also provide adequate force to lift the tissues and resist subsequent deformation. However, from a health perspective, incorporation of high contents of chemical cross-linkers in HA modification is not feasible. Separate studies have reported various complications and side-affect reactions correlated with the chemical cross-linkers used in HA modification, particularly for HA hydrogels treated with excessive amounts of the chemical cross-linkers (Alijotas-Reig and García-Giménez 2008; Micheels 2001; Loewe et al. 2001; John and Price 2009; Clark 2007; Hennink and Van Nostrum 2012; Boogaard et al. 2000; Ferretti et al. 2006; Tan et al 2009).

Some complications such as hypersensitivity reactions, nodules, induration, facial edema and localized cutaneous reaction have been widely reported in humans when treated with HA fillers (Alijotas-Reig and García-Giménez 2008; Micheels 2001; Loewe et al. 2001). Any allergic or side-affect reactions produced by HA-based fillers are thought to be caused by the chemical cross-linkers (Clark 2007). Furthermore, excessive amounts of cross-linkers are often toxic to the cells. They may affect the integrity of the substances to be entrapped such as drugs or proteins or produce unwanted reactions with the bioactive substances present in the hydrogel matrix (Hennink and van Nostrum, 2012; Boogaard et al., 2000 ; Sung et al. 1998; Ferretti et al. 2006; Tan et al 2009). In fact, the chemical cross-linkers are considered major obstacles in the use of injectable polymer scaffolds. The injectable fillers should be safe, non-allergenic, adverse-effect free and not associated with inflammatory response following their applications (Gatta et al. 2011; John and Price 2009).

Currently, there has been an increasing demand for HA-based hydrogels that offer advantages of lower chemical cross-linker. However, synthesis of hydrogels utilizing both low amounts of chemical cross-linker and high efficient cross-linking is complicated and challenging. Therefore, our study was aimed to explore the effect of HA initial concentration on cross-linking efficiency while maintaining a constant level of the chemical cross-linker. HA initial concentration is different from the total HA concentration expressed by the manufacturers. The total HA concentration usually includes the initial HA concentration bound with the cross-linker and the free HA solution which is added after chemical modification to facilitate the extrusion through fine-bore needles (Kablik et al. 2009).

In this work, the HA initial concentration represents the starting amount of HA powder involved in the gel formation process. We used 1,4-butanediol diglycidyl ether (BDDE) as the chemical cross-linker, because it has been widely employed in today HA-derived materials. The effect of HA initial concentration on cross-linking efficiency was evaluated through various analytical tests including *in vitro* degradation rate, swelling behavior, scanning electron microscope (SEM) and Fourier-transformed infrared (FT-IR).

2. Investigations and results

2.1. In vitro degradation rate

Table 1 displays the values of the *in vitro* degradation rates of formed hydrogels with 95% confidence interval for four days incubating with hyaluronidase, whereas Fig.1 shows a graph comparing their degradation profiles over the period of treatment.

Table 1: Average remaining NAG (%) of BDDE-HA hydrogels prepared at different HA initial concentration expressed with the 95% confidence interval for four consecutive days

Day	7.0 % HA	8.0 % HA	9.0 % HA	10.0 % HA
0	100	100	100	100
1	55.5± 5.7	58.9± 2.5	62.5± 3.4	68.4± 1.8
2	33.4± 1.7	35.9± 2.7	37.9± 2.0	42.9± 1.3
3	23.1± 2.8	26.9± 3.0	31.3± 4.6	34.2± 2.0
4	15.9± 5.4	18.6± 3.1	23.6± 3.8	25.1± 1.9
Day	**11.0 % HA**	**12.0 % HA**	**13.0 % HA**	**14.0 % HA**
0	100	100	100	100
1	66.6± 3.7	65.7± 6.3	64.7± 2.9	63.9± 2.0
2	41.4± 2.1	41.0± 5.5	38.9± 3.7	38.5± 3.5
3	32.5± 3.4	34.4± 4.0	30.5±4.2	31.3± 2.1
4	22.4± 2.2	21.4± 4.9	21.9± 2.9	19.5± 2.6

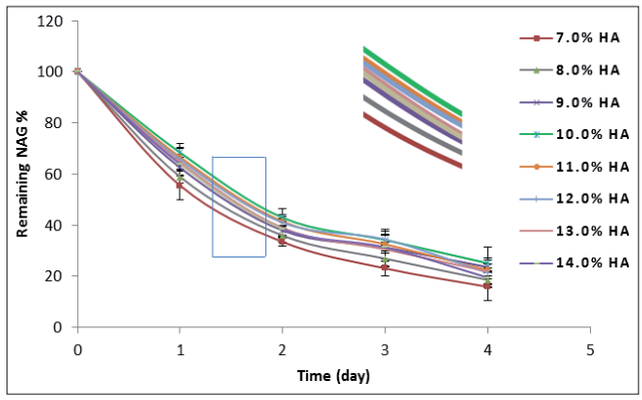

Fig.1: Degradation profiles of BDDE-HA hydrogels prepared at different HA concentrations, plotted as a function of time with the 95% confidence interval

The results verified that HA initial concentration had a substantial effect on hydrogel degradation during enzymatic incubation. A noticeable decline in the degradation rate was observed with the increasing of HA concentration from BDDE-HA hydrogel prepared at 7.0 % HA initial concentration to the hydrogel prepared at 10.0 % HA initial concentration. A slight increase in the degradation rate was then observed from the 10.0 % HA hydrogel to the hydrogel prepared at 14.0 % HA initial concentration. According to these data, the degradation rate was inversely proportional to the HA initial concentration to a certain level which was 10.0 % w/v in our experiments, and then relatively showed an opposite effect. This meant, that the 10.0 % HA exhibited an ideal level of HA initial concentration corresponding to 2.0 % (v/v) BDDE solution. Statistically, the degradation rates of hydrogels containing low HA concentration (7.0 % 8.0 %) and high HA concentration (13.0 % and 14.0 %) showed a significant difference ($p < 0.05$, n=3, ANOVA test) from the degradation rate of the 10.0 % HA hydrogel for four days incubated with hyaluronidase. However, the degradation rates of hydrogels prepared at moderate HA initial concentration (9.0 %, 11.0 % and 12.0 %) did not differ significantly from the degradation rate of 10.0 % HA hydrogel except on last day for the 11.0 % and 12.0 % HA hydrogels and on the first two days for the 9.0 % HA hydrogel.

As a comparison, the 10.0 % HA hydrogel exhibited much higher resistance against the action of enzyme than did hydrogels prepared at the lowest (7.0 %) or the highest (14.0 %) HA initial concentration, particularly on the first day of treatment. Approximately two-third of the 10.0 % HA hydrogel original NAG content (68.4±1.8 %) remained unreleased in the dry mass after one day compared to the 7.0 % or 14.0 % HA hydrogels which showed NAG remaining percentages of approximately (55.5±5.7 %) and (63.9±2.0 %)

Table 2: Average amounts of swelling ratios (g/g) of BDDE-HA hydrogels prepared at different initial HA concentrations expressed with the 95% confidence interval

Medium	7.0 % HA	8.0 % HA	9.0 % HA	10.0 % HA
Distilled	135 ± 6.5	134 ± 2.8	132 ± 5.1	129 ± 3.2
water PBS	124 ± 4.3	122 ± 2.4	120 ± 3.8	116 ± 2.4
Medium	**11.0 % HA**	**12.0 % HA**	**13.0 % HA**	**14.0 % HA**
Distilled	131 ± 6 5	132 ± 7.9	136 ± 6.2	137 ± 7.4
water PBS	117 ± 1.4	119 ± 3.S	121 ± 5.0	127 ± 6.2

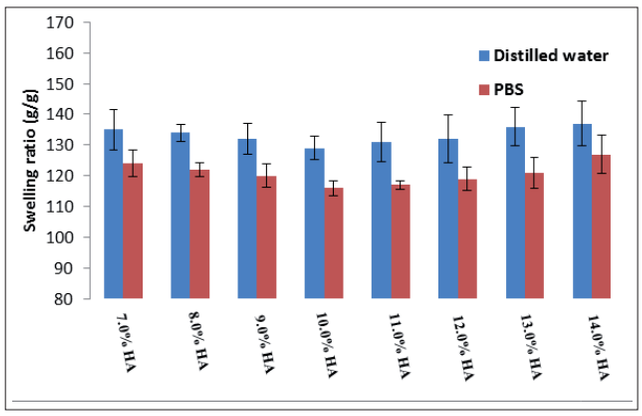

Fig.2: Swelling ratios (g/g) of all tested hydrogels in distilled water and PBS

respectively. Furthermore, the 7.0 % and 14.0 % HA hydrogels appeared to have lost more than 80 % of their NAG contents within the whole period of treatment in comparison to the 10.0 % HA hydrogel which lost approximately 75 % after similar period.

2.2. Swelling ratio

It is vitally important to measure the swelling capacity of formed hydrogels due to its major role in solute diffusion through the swollen network and in the release of entrapped molecules including drugs used for tissue engineering. Moreover, this property forms a fundamental feature in cosmetic applications including biomaterials used in soft tissue augmentation or in the industry of dermal fillers.
The results of welling ratios are shown in Table 2 with the 95 % confidence interval, a bar graph is displayed in Fig. 2 comparing the swelling ratios of formed hydrogels in distilled water and phosphate buffer saline (PBS). The results of the swelling ratios were in good agreement with the results of the *in vitro* degradation tests and the 10.0 % HA- based hydrogel proved the most stable hydrogel.
The results confirmed that the swelling ratios of tested hydrogels in distilled water and PBS were inversely correlated with the HA initial concentration from the 7.0 % HA hydrogel to the 10.0 % HA hydrogel. Pattern was then changed and the swelling ratio was steadily increased with the increase of HA concentration until the 14.0 % HA hydrogel. The hydrogels prepared at low (7.0 % and 8.0 %) and high (13.0 % and 14.0 %) HA initial concentration differed significantly from the 10.0 % HA hydrogel. The hydrogels with moderate HA: 9.0 %, 11.0 % and 12.0 % HA swelled to extents close to that reached by 10.0 % HA hydrogel in distilled water, however, in phosphate buffer saline (PBS), they showed different water-absorption ability. Generally, the data of swelling ratios ranged from maximum values at 137±7.4 g/g

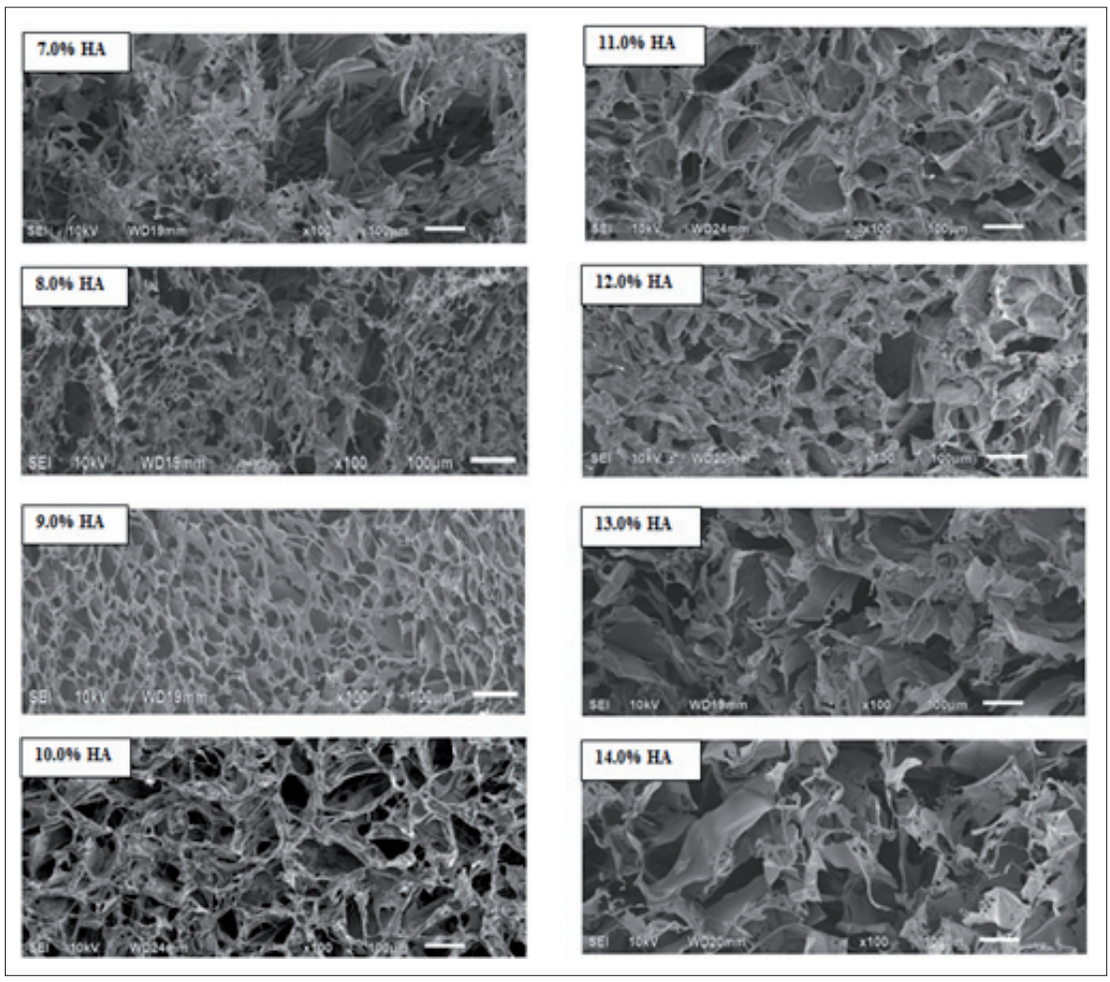

Fig. 3: Scanning electron micrographs of the surface of lyophilized BDDE-HA hydrogels prepared at different HA initial concentration

in distilled water and 127±6.2 g/g in PBS reached by the 14.0 % HA hydrogel to minimum values at 129±3.2 g/g in distilled water and 116±2.4 g/g in PBS reached by the 10.0% HA hydrogel.

2.3. *Scanning electron microscopy (SEM)*

The SEM images of the tested hydrogels showed different morphologies and pore-size distribution. Hydrogels with moderate HA initial concentration exhibited better network interconnection than hydrogels with low or high HA initial concentrations. The hydrogels with moderate HA initial concentration appeared with dense surface structures. In contrast, the low and high HA hydrogels showed less cross-linking density and appeared with some collapses occurred probably during the freeze-drying process.
The hydrogels with moderate HA had also narrower pore-size distribution. For instance, the 10.0 % HA hydrogel showed a pores-size distribution ranged in diameter from less than 50 µm to approximately 300 µm compared to the low or high HA hydrogels which showed much wider distribution ranges. Furthermore, the hydrogels with moderate HA were more homogenous and showed more regular porous networks. Figure 3 shows SEM surface images of lyophilized hydrogels captured at similar (x100) magnification parameter using the Secondary Electron Imaging (SEI).

2.4. *Fourier-transformed infrared (FT-IR)*

The results of FTIR (Fig. 4) show several characteristic peaks that could differentiate cross-linking efficiency occurred among tested hydrogels. However, the main characteristic peak was the one observed at 3343 cm^{-1}, this peak was assigned to the hydroxyl group. As reported, at high pH values or above the pKa value of the hydroxyl groups, the hydroxyl groups become almost deprotonated. The deprotonated hydroxyls groups are stronger nucleophiles than both the anionic carboxylic group and the amide.
Hence, the epoxide groups of BDDE react preferentially with the hydroxyl groups of HA to form stable ether bonds (Schanté et al. 2011). Therefore, under alkaline conditions, the BDDE targets one of the reactive hydroxyl groups in native HA. This means that the total (– OH) amount found in native HA decreases with the increase of cross-linking efficiency and subsequently appears with smaller peak in the FTIR spectra. Based on our results, hydrogels with moderate HA initial concentration showed larger loss of – OH (smaller peaks). This evidenced that hydrogels with moderate HA initial concentration had undergone a higher degree of modification with the BDDE molecules than those prepared at lower or higher HA initial concentration. In general, the largest downward peak was assigned for the hydrogel prepared at 7.0 % HA initial concentration, whereas the smallest peak was assigned for the hydrogel prepared at 11.0 % HA initial concentration, these data were relatively inconsistent with previous measurements where the 10.0 % HA hydrogel could not maintain its lead.

3. Discussion

As mentioned earlier, HA cross-linking is crucial for providing a chemical shield against decomposition acted by hyaluronidase and producing HA-based hydrogels with longer residence. The faster release of NAG fragments (low cross-linking efficiency) observed with the hydrogels of low HA initial concentration was more likely attributed to the insufficient quantity of HA to form covalent bridges with BDDE molecules. In contrast, the slower release of NAG fragments (high cross-linking efficiency) with the moderate HA hydrogels was resulted from an adequate abundance of HA chains within their network matrices. It was evident, that incorporation of a larger HA content in the reaction medium resulted in a more exposure of HA chains to BDDE molecules, thus more covalently ether bonds were formed. The bovine testicular hyaluronidase failed then to cleave such linkages. By increasing HA concentration, the distance between HA chains become even shorter and the probability of BDDE molecules to bind with HA chains becomes larger.
This phenomenon could also be assigned to the physical cross-linking occurred between polymer chains as a resultant of increased HA contents. As stated, HA is a linear polymer, however, in solution it shows extensive intermolecular hydrogen bonding that induce distinctive secondary (helical) and tertiary (coiled coil) interactions (Prestwish et al. 1998; Scott et al. 1989). At high HA concentration, the chains show greater entanglements and self-association of HA random coils in solution which relatively work as an enzyme-attack inhibitor. The larger HA concentration, the

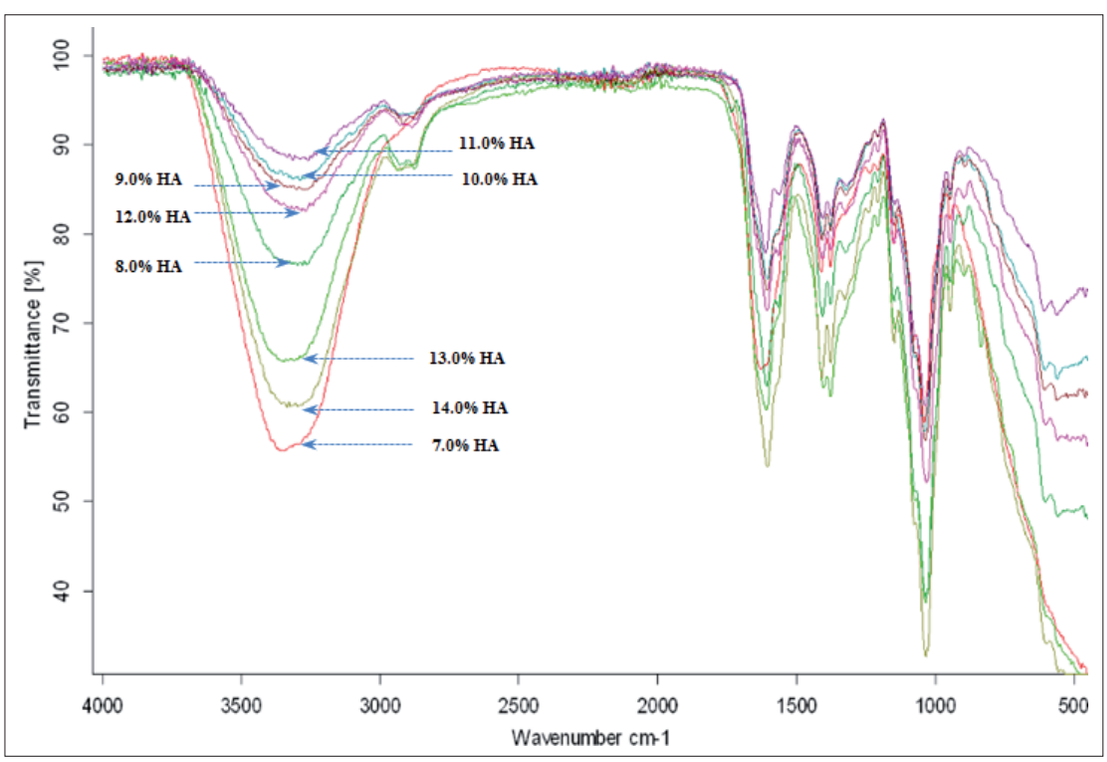

Fig. 4: FTIR spectra for tested hydrogels comparing the – OH peak for each hydrogel in response to HA initial concentration

larger quantity of HA "monomer unit" available in the reaction medium, subsequently higher tendency of HA chains to entangle. Furthermore, studies suggest that hydrogel stiffness may be affected by HA viscoelasticity; a rheological characteristic depending also on HA concentration and chains cross-linking (measurements were not carried out in this study). The hydrogen bonding between adjacent chains occur electrostatic repulsion between the carboxylic groups, subsequently the HA network is stiffened (Fakhari and Berkland 2013; Necas et al. 2008; Vejlens 1971). Although the occurrence of physical cross-linking as opposed to the chemical cross-linking is not entirely clear during the synthetic method, the HA concentration is postulated to play a significant role in the HA-HA polymer interconnection.

However, if HA initial concentration has exceeded moderate level, the cross-linking efficiency weakens and the hydrogels start to degrade faster. Possible reason for this; the excess amount of HA in reaction medium will lack the BDDE molecules and form viscous aggregates. High HA concentration may restrict reaction movement or prevent HA powder to disperse thoroughly within the reagent solution. In addition, it may lead to inhomogeneity or irregular distribution of cross-linking density throughout hydrogel matrix, subsequently, different densely regions are generated. In our experiments, the solutions exposed to high HA content (13.0 % and 14.0 % HA) were more viscous and had slower gelation reaction than those mixed with moderate HA content.

The results of swelling ratios supported the results of *in vitro* degradation rates. All tested hydrogels showed remarkable affinities towards water and swelled to various extents depending on their cross-linking efficiencies. Due to the effect of ionic strength, the hydrogels in PBS expanded to lower extents than their expansions in distilled water. Generally, it has been described that an increased cross-linking results in decrease water content (Cardoso et al. 2014). The cross-linking efficiency usually determines the overall swelling of cross-linked hydrogel and penetration depth of water into hydrogel matrix, so it has a significant influence on the equilibrium state between extension and retraction forces.

Comparing to the swelling ratios of low and high HA hydrogels, the hydrogels prepared at moderate HA initial concentration (9.0 % - 12.0 %) showed a limitation on the extent to which they could swell due to the large formation of chemical cross-linkages between HA chains and BDDE molecules, subsequently they could not preserve a good water up-take ability. Moreover, the larger HA-HA entanglements occurred in the moderate HA hydrogels were proposed to contribute on the elastic network retraction force that oppose additional swelling and thus show less swelling capacity.

As shown in the SEM images, the moderate HA hydrogels appeared more rigid and cohesive throughout their surface sections. They showed continuous network structures with pores being very small and more regularly distributed, these small pores enabled the polymer matrix to swell to lower ranges. The hydrogels with low or high HA initial concentration did not show good morphology and appeared with softer surface microstructure, particularly the low HA hydrogels. They had weak scaffolds and large pores allowing more water to be absorbed into the hydrogels matrices, subsequently higher swelling ratios were obtained. Furthermore, the low and high HA hydrogels were less homogenous and their cross-linking densities were irregularity distributed, such inhomogeneity weakens the network structure of cross-linked hydrogel and contribute in the rise of swelling capacity. The high uncertainty level or wider 95% confidence intervals observed around the average swelling ratios of the 7.0 % , 13.0 % and 14.0 % HA hydrogels verified this conclusion. In fact, hydrogels with moderate initial HA concentration were more homogenous and more thoroughly mixed during gelation process.

The analysis of FTIR revealed that the loss of –OH was largely influenced by HA initial concentration. When two (– OH) groups in two adjacent HA chains are covalently blocked with BDDE epoxides, the total – OH will decrease. The 11.0% HA hydrogel showed lower peak intensity than 10.0% HA hydrogel indicating that the 11.0% HA hydrogel was subject to a greater modification. The reason beyond this could possibly be attributed to the reactive groups involved in the chemical reaction. During the cross-linking reaction, the BDDE reacts with HA chains at both ends forming the cross-linkages found in the hydrogel network. However, some portion of BDDE reacts with HA at one end leaving the other end pendent or reacted with water/hydroxide (Kenne et al. 2013) . The mechanism of this interaction is mainly dependent on reaction conditions or relative tendency of epoxides binding with the hydroxyl groups of HA.

The pendent or mono-linked chains, however, do not contribute in cross-linking efficiency or the total outcome of hydrogel properties including degradation and swelling ability. Data of FTIR describe the total change occurred in HA after chemical modification which includes both mono-linked BDDE molecules and those involved in the actual cross-linkages. The greater chemical modification occurred with the 11.0 % HA hydrogel was probably due to the large formation of mono-linked chains.

4. Experimental

4.1. Materials

Hyaluronic acid was donated by Vivatis Pharma (Hamburg, Germany) with an average molecular weight 1,000,000 Da. Hyaluronidase (3000 U/mg) and BDDE were purchased from Sigma-Aldrich Co (St. Louis, Missouri, USA). Ehrlich's reagent, phosphate buffer saline (PBS) and alkaline solutions were in-house prepared.

4.2. Synthesis

A total of eight cross-linked BDDE-HA hydrogels were prepared by mixing 0.7, 0.8, 0.9, 1.0, 1.1, 1.2, 1.3, and 1.4 g of HA powder with 10 ml basic solution (0.25M NaOH) containing 200 µl (2.0 % v/v) of BDDE. So, the added quantities of HA formed various initial concentrations equaled to 7.0 %, 8.0 %, 9.0 %, 10.0 %, 11.0 %, 12.0 %, 13.0 %, and 14.0 % (w/v) respectively. Each mixture was allowed to mix thoroughly for 2h at 40 °C and then neutralized by adding equivalent amounts of 0.1M HCl to a pH of approximately 7.0. The resulting hydrogels were then dialyzed against distilled water for 3 days to remove un-modified HA and BDDE residues. Finally all hydrogels were lyophilized using LFD 5518, Labtech freeze-dryer (Daihan Labtech Co) and then all products were stored at 8 °C until the characterization studies were carried out.

4.3. Measurements

4.3.1. In vitro degradation rate

Five equivalent samples (A, B, C, D and E) were taken from each lyophilized hydrogel and placed in separate test tubes. An amount of 10 ml phosphate buffer saline (PBS) solution containing 500 µl of bovine testicular hyaluronidase (BTH) enzyme with an activity of 300 units /ml was added to each sample. Samples "A" were digested for one day whereas samples "B, C and D" were kept with enzyme solution for 2, 3 and 4 days respectively. Samples "E" were left with hyaluronidase until complete digestion. The *in vitro* degradation measurements were then performed in accordance with the colorimetric method reported by Reissig et al. (1995), with little modification. In brief, each sample was centrifuged at 2000 rpm for 1 min, the resulting supernatant was transferred into a 10 ml volumetric flask and then diluted with distilled water up to the mark. About 1.0 ml from each extract was boiled with 0.1 ml sodium carbonate solution for 1 min in a water bath. All samples were then allowed to develop a violet color by adding approximately 6.0 ml of glacial acetic acid and 1.0 ml of Ehrlich's reagent, the intensity of color depended on the amount of *N*-acetyl glucosamine (NAG) released in each extract. Under similar treatment, a series of standard solutions ranged between 20 µg /ml to 400 µg/ml were prepared.

All samples were then analyzed by a single-beam UV-Visible spectrophotometer (Spekol 1500, Analytik, Jena, Germany) and the absorbance was recorded at 585 nm. The *in vitro* degradation rate for each sample was expressed as the % NAG remaining in the non-digested fraction according to Eq. (1):

Remaining NAG = $(C_{NAG} - P_{NAG}) / C_{NAG} \times 100\%$ (1)

where C_{NAG} represents the NAG content after complete degradation (complete release) while P_{NAG} represents the NAG content after the first, second, third and fourth day of treatment.

4.3.2. Swelling ratio

Swelling measurements were carried out through a gravimetric method using a 4-decimal point analytical balance from Adam Equipment Inc. (USA). Equivalent portions from all lyophilized hydrogels were obtained and immersed in distilled water and phosphate buffer saline (PBS, pH = 7.4). The samples were left to reach their equilibrium states and then removed, excess water was gently dried by absorption paper. The swelling ratio was calculated by obtaining the final weight of each swollen sample in comparison with the starting weight according to Eq. (2):

Swelling ratio (g/g) = W_s / W_d (2)

where W_s is the weight of sample at equilibrium state and W_d is the weight of lyophilized sample.

4.3.3. Scanning electron microscopy (SEM)

The morphologies of cross-linked hydrogels were investigated by a scanning electron microscope (SEM) from JEOL (Tokyo, Japan). Initially, the lyophilized samples were coated with platinum using an ion sputter prior to visualization process. The SEM was adjusted to obtain images in the Secondary Electron Imaging (SEI) mode with a voltage of 10 kV. All images were captured at similar conditions and magnification.

4.3.4. Fourier-transformed infrared (FT-IR) analysis

Fourier transformed infrared (FTIR) spectra of hydrogels were measured using Bruker Tensor 37 Fourier Transform Infrared Spectrometer (Ettlingen, Germany). Spectra represent an average of 32 scans and they were recorded between 4000 and 400 cm^{-1} with a resolution of 4 cm^{-1}. Data were manipulated using OPUS software.

4.4. Statistics

Measurements were carried out in triplicate and the results were presented as mean with 95% confidence interval. Statistical analysis for significance between the cross-linked hydrogels was performed by means of Student's t-test, and analysis of variance ANOVA. Value of $P < 0.05$ was statistically considered significant.

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Conflicts of interest: None declared.

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