Published in final edited form as:

Biomaterials. 2008 November ; 29(31): 4149–4156. doi:10.1016/j.biomaterials.2008.07.026.

***In Vitro* and *In Vivo* Suppression of Cellular Activity by Guanidinoethyl Disulfide Released from Hydrogel Microspheres Composed of Partially Oxidized Hyaluronan and Gelatin**

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Abstract

This paper describes the preparation of oxidized hyaluronan crosslinked gelatin microspheres for drug delivery. Microspheres were prepared by a modified water-in-oil-emulsion crosslinking method, where 3-dimensional crosslinked hydrogel microspheres formed in the absence of any extraneous crosslinker. SEM analyses of the microspheres showed rough surfaces in their dried state with an average diameter of 90 μm . Lyophilization of fully-swollen microspheres revealed a highly porous structure. Guanidinoethyl disulfide (GED) was used as a model drug for incorporation into the microspheres; encapsulation of GED was confirmed by HPLC. There was an inverse correlation between the diameters of the microspheres with their GED loading. Macrophage was used as a model cell to evaluate the *in vitro* efficacy of GED release from the microspheres. The *in vivo* efficacy of the microspheres was further validated in a mouse full-thickness transcutaneous dermal wound model through suppression of cell infiltration.

Keywords

Hyaluronan; Gelatin; Microspheres; Guanidinoethyl disulfide; Cell infiltration

Introduction

Due to their potential in efficacy enhancement, biodegradable hydrogel microspheres formulated from either synthetic or natural polymers have attracted extensive interest as vehicles for delivery of both small and macromolecules [1–5]. Among natural polymers, hyaluronan (HA), a linear polysaccharide consisting of alternating β -1, 4-linked units of β -1, 3-linked glucuronic acid and *N*-acetyl-D-glucosamine, is a main constituent of extracellular matrix (ECM) which is known to play important roles in both cell differentiation/growth and wound repair [6,7]. Native HA is highly soluble and can be rapidly degraded *in vivo* [8]; crosslinking techniques have been developed to form HA based hydrogels, films and microspheres, where the physicochemical properties of HA have been enhanced without

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compromising its biocompatibility [9–13]. Partial oxidation introduces open ring structures into native HA and is a strategy to circumvent the chain rigidity of HA, which could enhance the elastic properties of the HA hydrogels formed [13]. In our previous work [14], a hydrogel amenable to cell migration and proliferation was prepared by combining oxidized HA (oHA) with gelatin. The system is self-crosslinkable and completely devoid of extraneous small molecular crosslinker, which are generally regarded as having cytotoxic potential. Importantly, the oHA/gelatin hydrogel was shown to be non-cytotoxic and biodegradable in both *in vitro* and *in vivo* models [14].

Macrophage plays significant roles in immunological reactions, allergy and inflammatory process against various implanted biomaterials; it also directs the progress of wound healing [15–17]. Activated macrophages not only inhibit the growth of a wide variety of microorganisms and malignant cells, but also involve in ECM production, cell proliferation and angiogenesis [18], thereby, contributing to the pathophysiologies of many inflammatory diseases [15]. Macrophages mediate inflammatory response and material degradation partly through nitric oxide (NO) [19–22]. NO produced by iNOS (inducible nitric oxide synthase) has multiple physiological and pathophysiological functions [24]. Recent evidence clearly implicates the pivotal role of NO in dermal wound healing [21,22,24,25]. It has been reported that application of a selective iNOS inhibitor can reduce inflammatory extravasations [26], which are closely associated with scar or polyposis formation in wound healing. Timely suppression of iNOS could hypothetically minimize scar formation. Guanidinoethyl disulfide (GED) is a highly potent inhibitor of iNOS [27] and it has been shown to significantly reduce experimental gingivitis linked to focal increase in iNOS [28].

To our knowledge, there was no report on coupling of GED to biomaterial-derived vehicles such as microspheres. This study aimed at developing an oHA/gelatin microsphere as a carrier for GED. The structural and physical properties of the microspheres were characterized by SEM, swelling and *in vitro* degradation, respectively. Parameters affecting the characteristics of microspheres and their *in vitro* drug release were investigated. The efficacy of the GED microspheres in suppressing macrophages infiltration was validated in mice transcutaneous full-thickness dermal wound models.

2. Experimental section

2.1 Materials

Sodium hyaluronan (M_w , 1.5×10^6) was provided by Engelhard, Inc. (now, BASF) (Stony Brook, NY, USA). Gelatin (Bloom 300, Type A, M_w , 100,000) and GED were purchased from Sigma-Aldrich (St Louis, MO, USA). Dialysis tube (MWCO 3,500) was obtained from Fisher (Hampton, NH, USA). oHA was prepared following the methodology we used previously [14]. The molecular weight oHA was determined (5 mg/mL in water) by HPLC (Waters 600e pump and controller, 715 Injector, 410 refractometer) coupled to Waters Ultrahydrogel 2000, 1000, and 500; 300 mm $\times$ 7.8 mm columns connected in series, using 0.1 M KNO₃ as a mobile phase at a flow rate of 0.8 mL/min at 50 °C. The oxidation degree of oHA was determined by ¹H NMR (Varian 500, Varian). All other chemicals were of reagent grade and deionized water was used.

2.2 oHA/gelatin microspheres preparation

oHA/gelatin microsphere was prepared using a modified emulsion-crosslinking method in the presence of a surfactant [4]. Briefly, 5 mL of a 2% (w/v) aqueous gelatin solution was mixed with 5 mL of a 2% (w/v) aqueous oHA solution at 37 °C. The mixture of gelatin and oHA was then added to 50 mL of soya oil with 1 mL of Span-80 pre-dissolved in it, maintained at 50 °C with a water bath, while under rapid agitation by a mechanical stirrer (LR400D lab stirrer,

Fisher Scientific, Pittsburgh, PA) at 900 rpm at 50 °C overnight. The microspheres were precipitated by first adding 60 mL of cold isopropanol to the mixture, and after an additional five minutes of stirring, the oil phase was removed by centrifugation (8,000 rpm). In order to extract the residual oil, the microspheres collected were washed three times with approximately 20 mL of an acetone/isopropanol (1:1 ratio) co-solvent mixture. The microspheres were recovered by drying overnight at room temperature.

2.3 Incorporation of GED into microsphere

Respectively, GED solutions (concentrations: 0.2%, 1% and 2%, w/v) were incorporated into the microsphere at the initial stages of the preparation. Briefly, 1 mL of a GED solution preheated to 37 °C was added to the gelatin and oHA mixture, and this reaction mixture was used to prepare microspheres following the procedure described above.

2.4. Microsphere morphological and size analysis

The morphology of both non-swollen and lyophilized swollen microspheres was evaluated by scanning electron microscopy (SEM) (SFEG Leo 1550, AMO GmbH, Aachen, Germany). Microsphere size distributions were determined under an optical microscope (Olympus, IX-71) via a QCapture 5 imaging software (Surrey, Canada).

2.5. Drug content of the microspheres

The amount of GED encapsulated in the microspheres was determined by the following method: 2 mg of microspheres were dispersed in 5 mL of 0.01 M HCl and agitated at 37 °C for 3 days. The final GED concentration in the PBS was determined by HPLC (column: Supelcosil LC-18DB, 250×4.6 mm; mobile phase: 3% acetonitrile in 20 mM KH₂PO₄, pH 3.0; flow rate: 0.8 mL/min; and detection: UV 245 nm).

2.6. Swelling analysis

The swelling studies of microspheres were performed in PBS (pH = 7.4) at 37 °C. Images were captured in succession under a microscope at the following time-points: 1, 3, 5, 10, 15, 30, 45 s; and 1, 2, 3, 5, 7, 10, 15, 20 and 30 min. The swelling kinetics was determined by monitoring the dynamics of the size of the microspheres. The swelling ratio (q) was calculated using eq. 1 assuming a spherical particle geometry:

$$q = V_{\text{wet}} / V_{\text{dry}} \quad (1)$$

where V_{wet} and V_{dry} are the volume of the swollen microspheres and the dried microspheres, respectively.

2.7 In vitro GED release from oHA/gelatin microspheres

Release of GED from the oHA/gelatin microspheres was performed in PBS (pH = 7.4). oHA/gelatin microspheres with GED incorporated (0.2%, 1%, and 2%, $n = 3$) were dispersed in 1 mL of PBS, and the samples were incubated at 37 °C under constant agitation. Aliquots of 100 μ l were withdrawn at time points (0, 0.5, 1, 3, 5, 7, 10, 24, 72, 120, and 168 hr) after centrifugation, and replenished with equal volumes of fresh PBS. Samples of GED released from the oHA/gelatin microspheres were analyzed using HPLC (column: Supelcosil LC-18DB, 250×4.6 mm; mobile phase: 3% acetonitrile in 20 mM KH₂PO₄, pH 3.0; flow rate: 0.8 mL/min; and detection: UV 245 nm).

2.8 Cell culture

Macrophages (RAW264.7, ATCC, Manassas, VA, USA) was grown in high glucose DMEM (Gibco Grand Island, NY, USA) supplemented with 10% FBS (Hyclone, Logan, UT, USA)

and 1% Pen/Strep (Gibco Grand Island, NY, USA) at 37 °C in a humidified atmosphere of 5% CO₂. To test the efficacy of GED-loaded microspheres on macrophage inhibition, the microspheres were first dispersed and embedded in hydrogels also prepared by combining the oHA solution with the gelatin solution following our established methodology [14]. Briefly, 5 mg GED-loaded microspheres (1% theoretical GED content) were introduced into 1 mL mixed solutions of a 20% (w/v) oHA/gelatin (ratio 4:6) in borax. This mixture was incubated at 37 °C to form a hydrogel film with a thickness of 0.5 mm. 1×10^5 macrophages were seeded per well ($n = 3$) with either oHA/gelatin (coded as OG) hydrogel films or GED microsphere-loaded oHA/gelatin (coded as GOG) hydrogel films; macrophage cultured without hydrogels were used as negative controls. The media were changed daily and cell-laden hydrogels were retrieved on days 3 and 6 to observe the cell density, morphology and distribution with an Axiovert 200M microscope (Zeiss, Munich, Germany) via Axiovision 4 imaging software (Zeiss, Jena, Germany).

2.9 Inhibition of inflammatory cell infiltration in a mouse transcutaneous full-thickness dermal wound model

A mouse transcutaneous implant model was used to evaluate the efficacy of GED release from the oHA/gelatin microspheres *in vivo*. All animals received humane care in compliance with the protocols approved by SUNY-Stony Brook IACUC. Briefly, adult male mice (5 week old, Balb/cJ strain, Jackson Lab, Bar Harbor, ME) were anesthetized with isoflurane (5% for induction and 2.5% for maintenance). A round incisional wound was made in the dorsal midline skin on each mouse with a biopsy needle (diameter: 8 mm, Miltex, Japan). The implants ($n = 3$ per group), round disks of 0.5 mm in thickness and 8 mm in diameter, were prepared by embedding the 1% GED microspheres in the oHA/gelatin hydrogel films (see above) in a dose of 5 µg GED/piece; plain hydrogel films without GED microspheres were used as controls. All implants were first sterilized with 70% ethanol, followed by rehydration with sterile saline. One hydrogel disk was implanted into each fresh wound. The wound sites were plastered with a layer of Tegaderm™, and wrapped with a Band-Aid™. A dose of buprenorphine (0.05 to 0.1 mg/kg, sub-Q) was administered after the procedure.

Animals were euthanized at days 3 and 7 and the implanted hydrogel along with the tissues adjoining the wound sites were exercised, fixed in 10% neutral buffered formalin, processed and paraffin embedded; sections (10 µm thick) produced were stained with H&E.

2.10 Statistical analysis

Statistical differences between groups were discerned by Student's t-test. Differences were deemed significant at $p < 0.05$.

3. Results and Discussion

In this research, a microsphere system was developed using a facile water-in-oil emulsion process. HA was first reacted with sodium periodate to produce oHA with a 44.7% of oxidation at a molecular weight of 35.5 kDa. After emulsification, the oHA reacted with gelatin in the soya oil at 50 °C; the presence of multiple aldehyde groups and amino groups in the mixture enabled the production of hydrogel microspheres via Schiff base formation; this mechanism was revealed in our previous report [14]. GED loaded oHA/gelatin microspheres were prepared by incorporating GED solutions of (0.2%, 1%, and 2%), respectively. The encapsulation efficiencies of microspheres ranged from 37% to 76%, and apparently, and the amount of GED encapsulated could be adjusted; these results were summarized in Table I.

3.1 Characterization of microspheres

Fig. 2 showed the SEM images of plain oHA/gelatin microspheres and microspheres loaded with 1% GED in the original dried state and swollen state, respectively. The elliptical appearance of the microspheres was likely due to the drying process. The surface of the microsphere in the dried state was moderately coarse (Fig. 2A and C) with small pores scattered across the area examined. Moreover, the diameter of the microspheres with 1% GED was considerably smaller than those without GED. However, the surface of the microspheres in their swollen state exhibited a very different morphology; a highly porous structure was observed.

Fig. 3 showed the size distributions of four oHA/gelatin microspheres formulations investigated. The spread in the microspheres' size distribution broadened considerably with a decrease in the GED contents. The mean sizes of the plain oHA/gelatin microspheres, and oHA/gelatin microspheres with 0.2%, 1% and 2% GED incorporated were, respectively, 91.6 ± 18.8 (Fig. 3A), 86.3 ± 19.7 (Fig. 3B), 56.4 ± 9.4 (Fig. 3C), and 31.4 ± 8.7 (Fig. 3D) μm , as summarized in Table I. The mean size of the GED-loaded microspheres was smaller when compared to its counterpart containing no GED; and it decreased from 91 μm to 31 μm , with the concentration of GED solution incorporated during the preparation increased from 0 to 2%. This could be attributed to the formation of smaller emulsion droplets caused by a decrease in solution viscosity with the increasing concentration of the small molecular GED during emulsification [3].

3.2 Swelling of the microspheres

Fig. 4 showed the image of fully swollen microspheres and the swelling kinetics in PBS at 37 °C. Fig. 4A depicted fully-swollen oHA/gelatin microspheres with 1% GED at its swelling equilibrium state; the appearances of blank microspheres were comparable but they are larger (not shown). Similar to the SEM results shown in Fig. 2, the microspheres were generally elliptical. All microspheres reached their swelling equilibrium state very rapidly (within 500 s). Compared to the plain oHA/gelatin microspheres, the GED loaded oHA/gelatin microspheres have higher equilibrium swelling ratios, and this ratio increased with an increase in their GED contents. The swelling ratio q increased from 6.02 to 19.82 when the concentration of the GED solution added during microsphere preparation increased from 0 to 2%. It could be postulated that the higher GED content reduced the interactions between the amino residues on the gelatin and the aldehyde residues on the oHA. Increasing the GED content during microsphere preparation resulted in decreasing of crosslinking density, thus, increasing in swelling.

3.3 In vitro release of GED

In vitro GED release was performed in 0.01 M PBS at 37 °C. Fig. 5A and B showed the typical HPLC eluent time profiles of a GED solution and a sample from the releasing study after 3 hr of incubation, respectively. GED has a retention time of approximately 13 min, while multiple peaks appeared on the eluent profile of the release sample injected into the HPLC column in the vicinity of 13 min. The direct implications were first, GED was successfully encapsulated in the microsphere and released, and second, the release of GED was accompanied by the release of matrix materials from the microspheres.

Fig. 6 showed the release kinetics of oHA/gelatin microspheres prepared by using 0.2%, 1% and 2% GED solutions in one week. The initial release kinetics (first 7 hr) depicted in the inset suggested that the release of GED was dependent on the microspheres' initial GED contents. Similar release kinetics had been observed in several comparable microspheres systems where physical entrapments were involved [29,30]. An initial burst release was observed in all cases, which could be attributed to the discharge of the free residual GED associated to the

microsphere surface that was not removed during the washing steps in the formulation process as well as release of the GED located near the microsphere surface [31,32]. The total amount of GED recovered in each formulation was proportional to the total amount of GED originally incorporated into the microspheres. For example, at 24 hr, the cumulative amount of GED released from microspheres prepared with 0.2%, 1% and 2% GED solutions were 4, 10 and 30 $\mu\text{g}/\text{mg}$ microspheres, respectively. Furthermore, swelling of the matrix is known to play a key role in drug release. As depicted in Fig. 4, the results of the swelling tests showed that the microspheres (i.e., 2% GED) had the highest rate of swelling among the three formulations. After the burst phase, GED continued to be released within one week, albeit at a modest pace, from the microspheres.

3.4 In vitro cell morphology and distribution

Macrophage is regarded as a very important cell type in mediating post-implant tissue/ biomaterial inflammatory and wound healing responses [33–35]. NO production by macrophages rises sharply in the presence of inflammatory stimuli [36,37]. Modulating NO secretion through suppression of iNOS activity could influence inflammatory responses. Therefore, macrophage was selected as a model cell type to validate the efficacy of the GED loaded microspheres in vitro.

Macrophages were cultured directly with the GOG hydrogel films. In parallel, the same amount of macrophages was seeded either in cell culture medium (i.e., background, control) or in the presence of OG hydrogel films (i.e., negative control, blank). Three and six days after cell seeding, the effects of GED-loaded microspheres on macrophages growth and their distribution patterns were visualized under a microscope; the results were shown in Fig. 7. Evidently, the presence of GED-loaded microspheres retarded the macrophage proliferation. In particular, on day 3, cells were found attached to the surface of the culture well (bottom) as well as the hydrogel films. Macrophages seeded on culture wells (Fig. 7A) and on the OG hydrogel (Fig. 7C) assumed a characteristic round pattern and a considerable number of them also spread out, indicative of activation (i.e., the inflammatory response) [34]. In contrast, virtually all the cells in the presence of the GOG hydrogel maintained their round shape (Fig. 7E), implicating a full suppression of activation. Furthermore, cells proliferated rapidly in the control group (Fig. 7B), whereas a more moderate cell growth was observed in the OG (Fig. 7D); proliferation of macrophages in the presence of the GOG hydrogel was suppressed (Fig. 7F). These results were summarized in Fig. 8. The cell numbers determined in the control group were significantly higher than those in either the group incubated with OG hydrogel or the group incubated with the GOG hydrogel, suggesting the release of GED leading to suppression of macrophage proliferation. By day 6, cells in the control group have reached full confluence growing over each other (Fig. 7B); the cells in the blank groups have reached 80% confluence (Fig. 7D). However, there was only a modest increase in cell number in the group of GOG hydrogel (Fig. 7F). Despite daily change of culture medium, continual suppression of cell proliferation was self-evident and this implied continual sustained release of GED from the microspheres embedded in the oHA/gelatin hydrogels. Lastly, the patterns manifested by Fig. 7E and 7F suggested apoptosis and this issue is currently being investigated independently.

3.5 Suppression of cell infiltration into hydrogel in mice full-thickness transcutaneous wound models

A full-thickness incisional wound was created on a mouse after hydrogel implantation and covered by the transparent Tegaderm™ dressing. Throughout the entire course of wound healing, the cell types interacting strongly with implants are predominantly macrophages and fibroblasts; the two cell types also interact and modulate the functions of each other [38,39]. In a typical wound healing response, the number of macrophages in the wound bed surges almost immediately, signifying the initial inflammatory reaction, peaks at days 3 to 4, and

begins to subside at days 6 to 7; conversely, the number of fibroblasts starts to surge at day 1, peaks at days 5 to 6, and begins to decline at days 7 to 8 [40,41]. Therefore, days 3 and 7 were chosen as the optimal time-points to observe the GED suppression of cell activities.

All animals tolerated the implant well without showing any apparent adverse reactions. After 3 days of implantation, gross anatomical observation of the wound bed showed tissue formation, manifested as a white ring surrounding the edge of the wound characteristically observed during the initial stage of healing, in both control and blank (results not shown). However, this phenomenon was not observed in the mice implanted with the GOG hydrogels, suggesting the suppression of wound healing. Fig. 9 depicted the typical cross-sections of the implanted hydrogels with their adjoined tissues, 3 days after implantation. We had previously demonstrated that the oHA/gelatin hydrogel was very amenable to cell infiltration and proliferation in subdermal implantation [14]. The pattern of cell infiltration depicted in Fig. 9A (OG hydrogel) was clearly consistent with the results of our previous studies [14]. However, the amount of cells infiltrated into the GOG hydrogel (Fig. 9B) were substantially lower than their counterparts depicted in Fig. 9A, with their maximum penetration generally reaching a depth of approximately half the thickness of the implanted hydrogel. Morphological observations of the 3-day specimen at high magnification showed that the great majority of the infiltrated cells were macrophages (marked with “♥”) and some neutrophils with occasional fibroblasts, moreover, fibroblasts were residing mostly along the adjoining fibrous tissues. It could thus be inferred that the presence of GED was capable of suppressing cell infiltration; ten random microscopic fields were selected on each implanted hydrogel and the numbers of cells penetrated were manually counted; the ratio of cells present in the GOG and OG hydrogels were 4.8:1. Moreover, there was also a clear disparity between the thicknesses and densities (as indicated by the intensity of staining) of the two hydrogel formulations after 3 days of implantation. Macrophages are known to produce a plethora of enzymes capable of digesting implanted materials [37], and both hyaluronan and gelatin are very susceptible to enzyme-mediated degradation. Therefore, a partial digestion of the implanted oHA/gelatin hydrogel with breakage of crosslinks, causing gradual loss of its structural integrity leading to its less resistant to water uptake and thus, swelling. This was likely the cause of the increased thickness of the OG hydrogel on the histology section. Conversely, the presence of GED suppressed the activities of the macrophages and led to a greater degree of preservation of the structural integrity of the implanted hydrogel. Seven days after implantation, the OG was almost completely infiltrated by cells with evidence of full-integration into the surrounding tissues being regenerated (not shown); in contrast, the GOG hydrogel film could still be clearly distinguished from tissue with moderate numbers of infiltrated cells. This suggested a continual suppression of cell activities.

Conclusions

Crosslinked microspheres of oHA/gelatin with and without GED are prepared by a modified in-emulsion crosslinking method. Microspheres formed are nearly spherical with a rough surface, and the plain oHA/gelatin microspheres have a mean size of around 90 μm . The sizes of microspheres are strongly correlated with their GED incorporation and they reach their fully swollen state within half an hour. The duration of *in vitro* GED release is approximately 1 week. *In vitro* culture of GED microspheres with macrophages results in an inhibition of both their activity and proliferation. Implantation of GED microspheres embedded hydrogels in mice full-thickness transcutaneous models reveals the suppression of cell infiltration into hydrogels that are normally very amenable to cell infiltration and proliferation.

Acknowledgements

Funding of this study by the National Institutes of Health (DK068401, WC) is gratefully acknowledged. We would also like to thank Jim Quinn in the Department of Material Science and Engineering (SUNY-SB) for the SEM analysis.

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Fig. 1.
The structure of GED.

Fig. 2. Scanning electron micrographs of plain (A, C) and 1% GED loaded (B, D) oHA/gelatin microspheres. (A) and (B): original dry state.(C) and (D): lyophilized after fully swollen.

Fig. 3. Frequency and size distribution plots of oHA/gelatin microspheres.

Fig. 4.
The effect of GED content on the swelling dynamics of the oHA/gelatin microspheres.

Fig. 5. Typical HPLC elution curve of: (A) a standard GED solution, and (B) a sample from the release test of 1% GED loaded oHA/gelatin microspheres.

Fig. 6. GED release profiles of oHA/gelatin microspheres formulated with 0.2%, 1% and 2% GED solutions.

Fig. 7. Morphology of macrophages in the presence hydrogel. Control: (A) 3 days, (B) 6 days; OG hydrogel: (C) 3 days, (D) 6 days; and GOG hydrogel: (E) 3 days, (F) 6 days. The GED-loaded microspheres in the GOG hydrogel was formulated with a 1% GED solution.

Fig. 8.

The densities of macrophages after cultured for 3 days and 6 days, respectively. The GED-loaded microspheres in the GOG hydrogel was formulated with a 1% GED solution.

Fig. 9. H&E staining of typical wound after 3 days. The wound beds were: (A) implanted with OG hydrogel, and (B) implanted with GOG hydrogel containing GED-loaded microspheres formulated from 1% GED solution C: fibrous tissue formed; I: implant; L: Loose connective tissue; ▼:Macrophage; ↑:Fibroblast. The images were captured under 20×magnification.

Table I

Particle size distribution of plain and GED incorporated oHA/gelatin microspheres. GED encapsulation efficiency was also presented.

Microsphere composition	Mean diameter (μm) $\pm$ SD	GED encapsulation yield (%)
oHA/gelatin	91.56 $\pm$ 18.76	--
oHA/gelatin/0.2% GED	86.28 $\pm$ 19.69	76.64
oHA/gelatin/1% GED	56.39 $\pm$ 9.43	70.98
oHA/gelatin/2% GED	31.39 $\pm$ 8.67	37.4