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Oxidized dextran/amino gelatin/hyaluronic acid semi-interpenetrating network hydrogels for tissue engineering application

Xiaohua Geng[1,2,a], Liu Yuan[1,2,b] and Xiumei Mo[1,2,c*]

[1] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, College of Materials Science and Engineering, Donghua University, Shanghai 201620, China.

[2] Biomaterials and Tissue Engineering Lab, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China.

[a]xhgeng11@yahoo.com.cn, [b]lyuan6617@yahoo.com.cn

*Corresponding author: [c]xmm@dhu.edu.cn

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Abstract. In our previous study, oxidized dextran/amino gelatin (ODex/MGel) self-crosslinking hydrogels have been successfully prepared. Though their potential applications as in situ forming scaffolds for tissue engineering have been verified, the subcellular porosities of hydrogel networks which were induced by the intensity chemical crosslinking still pose a barrier for cells migration and proliferation within the hydrogels. The objective of this study was to develop an approach to accelerate cellular remodeling by preparing semi-interpenetrating networks (semi-IPNs) composed of ODex, MGel, and sodium hyaluronic (HA). Results showed that the addition of HA at the concentrations of 0.09% and 0.18% can greatly promote pre-osteoblast (MC3T3-E1) cells spreading throughout the ODex/MGel hydrogel networks. Therefore, these semi-IPNs hydrogels could be useful matrixes for cell transplantation in a variety of tissue engineering applications.

Introduction

Hydrogel networks have been widely investigated as three dimensional (3D) soft tissue engineering scaffolds to encapsulate cells due to their physical properties emulating the native extracellular matrix (ECM) [1, 2]. In the past few years, research interests shifted about the fabrication and application of injectable hydrogels [3]. Several advantages contribute for this phenomenon. First, as compared with conventional surgery methods, the injectable way only produces minimal invasiveness. Secondly, the flowing nature enable them fit the defect or cavity easily. Besides, various potential therapeutic agents, such as drugs [4], cells [5], and growth factors [6], could be incorporated by simple pre-mixing.

One challenge of injectable hydrogels being applied in tissue engineering and cells transplantation is that at early time points following encapsulation, the 15-35 Å mesh size of these highly crosslinking matrices has been shown to restrict cell spreading, resulting in a rounded morphology that can delay cell proliferation, migration, and matrix production [7]. Webb's group [8] engineered a semi-interpenetrating networks composed of hyaluronic acid and poly(ethylene glycol) diacrylate, even low levels of hyaluronic acid content could greatly facilitate 3D fibroblast spreading and proliferation through cell-mediated enzymatic degradation of hyaluronic acid. Therefore, through incorporate of fast degradation ECM components (collagen, hyaluronic acid, etc.) to form space and allowing cells to spread and proliferation could be a promising way to prepared hydrogels.

In our previous study [9], in situ forming hydrogels composed of ODex and MGel were successfully prepared. The objective of current studies was to prepared semi-IPNs of ODex/MGel with the incorporated of native, enzymatically degradable ECM component homogeneously dispersed in the ODex/MGel network.

Materials and Methods

Materials. HA (Mw 500,000) was purchased from Zhenjiang Dong Yuan Biotech Co., (Jiangsu, China). Dextran (Mw 100,000), sodium periodate, ethane diamine (ED), gelatin (type B, Mw 60,000) were purchased from Sigma-Aldrich. 1-ethyl-3-(3-dimethyl laminopropyl) carbodiimide hydrochloride (EDC) was purchased from GL Biochem (Shanghai, China). All other chemicals were of reagent grade and used as received.

Porcine iliac artery endothelial (PIEC) cell and mouse pre-osteoblast (MC3T3-E1) cells were obtained from the institute of Biochemistry and Cell biology (Chinese Academy of Sciences, China). Fetal bovine serum (FBS), methylthiazoleterazolium (MTT), and all other culture media and reagents were purchased from Gibco LifeTechnologies, CO.

Preparation of ODex. Oxidized dextran was prepared by according to the method we have introduced previously [9]. In brief, 6.34g of $NaIO_4$ (dissolved in 100 ml of water) was added dropwise to 100 ml of dextran solution (10 wt%), the solution was then stirred for 6 hours at room temperature and shielded from light. After that, 2 ml of ethylene glycol was added to terminate the reaction. Lastly, the ODex solution was dialyzed exhaustively (MWCO 3500) for 3 days against water, lyophilized to obtain the final product (yield: 70%). And the oxidized degree of dextran was determined to be approximately 42% by using the method report elsewhere [10].

Preparation of MGel. MGel was prepared according to our previously described protocols [11]. Briefly, 5g of gelatin was dissolved in 100ml (0.1mol/l) sodium dihydrogen phosphate solutions (pH=5). Specific amount of ED and EDC were added, then the pH was adjusted back to 5 by hydrochloric acid (HCl). The molar ratio of carboxyl groups on gelatin chains, EDC, and ED was 1:2:40. The reaction was allowed to proceed at room temperature overnight. Hereafter, the result mixture was dialyzed against ultrapure water to remove the excess ED and EDC, the mixture was then freezed at -80℃, lyophilized and stored. The amino content in the MGel was determined by TNBS method according to our previously report [9].

Hydrogel preparation. 10 wt% of ODex solution was prepared by dissolve 1g ODex in 10 ml water at 80℃. 20 wt% of MGel solution was prepared by dissolve 1g MGel in 5 ml water at room temperature. 0.1g of HA was dissolved in 10 ml water to make a 1 wt% of HA solution. ODex solution and MGel solution were mixed at the volume ratio of 1:1, then HA solution of different volume was then added to research the final weight concentration of 0, 0.09%, and 0.18%, respectively. After homogenizing, the mixture were poured into molds and finally incubated at 37℃ to gelation. After 10 min of cure time, the hydrogels were removed from the mold.

Rheological measurements. Rheological measurements were performed on a rheometer (Thermo Haake Co., Germany). The aqueous polymer solution was mixed and pipetted directly onto the bottom plate, and the top plate was lowered to contact the gelling solution with a 1 mm gap size. For time sweeping tests, the storage moduli G' and loss moduli G" of hydrogels were monitored as a function of time at a frequency of 1 rad/s and a shear strain of 2% under a constant temperature of 37℃.

Equilibrium swelling ratio. For the swelling studies, samples were immersed in PBS and incubated at 37℃ for 24h to reach a swelling equilibrium. The samples were then removed from the PBS and the water on the surface was quickly wiped out, those hydrogels were weighted as Ws. After that, the samples were frozen at -80℃and dried by freeze drying to obtain the dry hydrogel weight (Wd). At least three specimens for each sample were measured. The swelling ratio (Qo) was calculated using the following equation:

$$Qo = Ws/Wd \quad (1)$$

Adhesion and spread of MC3T3-E1 cells on hydrogels. To investigate the effects of the hydrogel properties on cells adhesive and spreading, MC3T3-E1 cells were seeded on the surface of pure ODex/MGel and semi-IPNs hydrogels at a density of 10,000 cells $well^{-1}$. Cell viability was determined by using a Live/Dead Viability/Cytotoxiciy Assay Kit (Invitrogen, USA). After 24 h of culture, the medium were removed, and the hydrogels were washed twice with PBS. Then

specimens were incubated in “live/dead TM” dye solution (2 μm calcein-AM and 4 μm EthD-1) for 10 min for fluorescence microscope observation. Images of cells were acquired through fluorescent microscope (UL100HG Olympus Corporation, Japan).

MC3T3-E1 cells viability and proliferation in hydrogels. Viability and proliferation of MC3T3-E1 cells in D-G-4 hydrogels were assessed. Generally, MC3T3-E1 cells were trypsinized, counted, and quickly mixed with oxidized dextran/amino gelatin/hyaluronic (ODex/MGel/HA) polymer solutions at a density of 5×10^6 cell ml^{-1}. Then the mixtures were poured into 48-well cell culture plates for gel forming at 37℃ for 10 min. After that, complete medium was added, and the cell-laden hydrogels were incubated under standard culture conditions with medium exchanged every other day.

Live/Dead Assay Kit was used to verify the viability of MC3T3-E1 cells according to the method described above. Particularly, the incubated time was increased to 45 min to ensure the complete diffusion of staining solution. MTT assay (n=3) was used to determined cells proliferation within the hydrogels for 3, 7 and 14 days, respectively. In brief, cells were incubated with 0.5 mg/ml of MTT for 4 h at 37℃. The solution was then removed and DMSO was added, the hydrogels were left in the incubator for additional 10 hours to ensure the diffusion of purple formazan salts. Finally the absorbance of the resulting solution was measured at 492 nm using a multidetection microplate reader (MK3, Thermo, USA). Three parallels were averaged for each specimen.

Results and discussion

Preparation of hydrogels. Fig. 1 shows the formation of semi-IPNs hydrogels. Dextran was oxidized with sodium periodate to acquire the aldehyde groups, while gelatin was reacted with ED in the appearance of EDC to replace the carboxyl groups on gelatin chains to amino groups. Finally, due to the happening of Schiff based reaction between aldehyde groups and amino groups, semi-IPNs hydrogels can be formed. Here, the HA act as the medium to connect the ODex/MGel groups together.

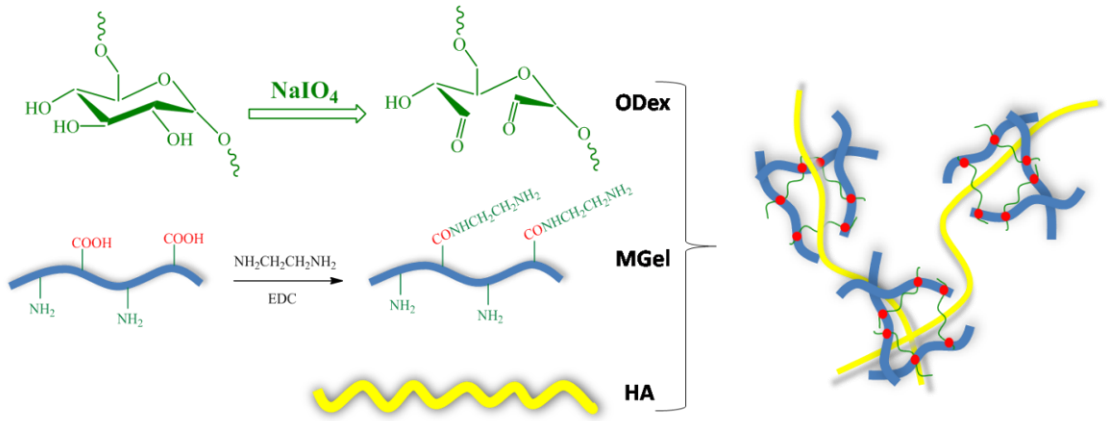

Fig.1 Scheme of the preparation of ODex and MGel and the quickly formation of ODex/MGel/HA semi-IPNs hydrogel via Schiff based reaction at 37℃.

Rheological analysis. The gelation process was monitored by rheological measurements. Fig. 2a shows variation of G’ of semi-IPNs during the time sweep measurement. From gross observation, there was not much difference between the three hydrogels. At the beginning, the G’ was remain in low level which can be ascribe to the fluid state of the initial mixture solution. Then, both of the G’ increased dramatically during the initial 500 seconds. After approximately of 1000 seconds, the G’ gradually levelling off. Eventually, well-developed 3D networks were formed.

As for G”, very different rheological behavior have shown among the three hydrogels. Fig. 2b shows that initially, the G” for all hydrogels was at the low level which means that viscosity of the hydrogels were belong to the low level. However, with the progress of crosslinking, the G” for

hydrogel without HA experience a little increase then gradually decreased to the originally level. Which means the ultimately formed ODex/MGel hydrogel was dominated by elastic properties and limited energy can be stored by viscous deformation. Conversely, for semi-IPNs hydrogels, the G" increased gradually with the increase of time. Semi-IPNs hydrogel with the HA content of 0.18% shows the highest G" value of approximately 27. This fact demonstrated that the ultimately formed semi-IPNs hydrogels shows both elastic properties and considerable viscous properties.

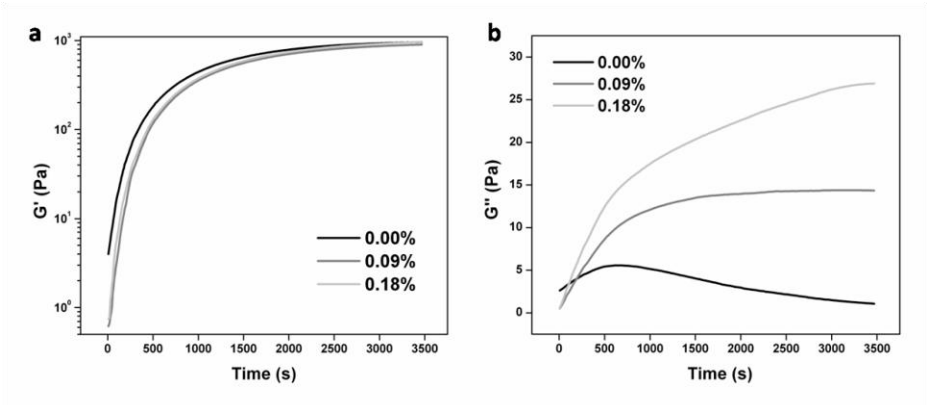

Fig.2 Time evolution of storage modulus (G') (a) and loss modulus (G") (b) of ODex/MGel hydrogels with different HA content (0.00 wt%, 0.09 wt%, and 0.18 wt%).

Swelling analyses. The swelling characteristics of hydrogel networks are important in various applications as it affect solute diffusion, surface properties, and mechanical properties. The equilibrium swelling ratio was generally influenced by the crosslinking density, gel composition, etc.. As shown in Fig 3, hydrogels without HA shows the highest swelling ratio of about 32 which corresponding well with our previously report. For semi-IPNs hydrogels, the swelling ratio was apparently lower than that of hydrogels without HA. The swelling ratio for hydrogels with the HA content of 0.09% and 0.18% were 27 and 25, respectively.

It is assumed that the swelling ratio of semi-IPNs hydrogels were strongly affect by the introduction of HA macromolecule. The existed of HA chains interpenetrated within the ODex/MGel crosslinking networks can form a semi-interpenetrated networks. As a result, the interlock between macromolecule chains became stronger and more resistant for water came in. Therefore, swelling ratio for semi-IPNs hydrogels was lower than pure ODex/MGel hydrogels.

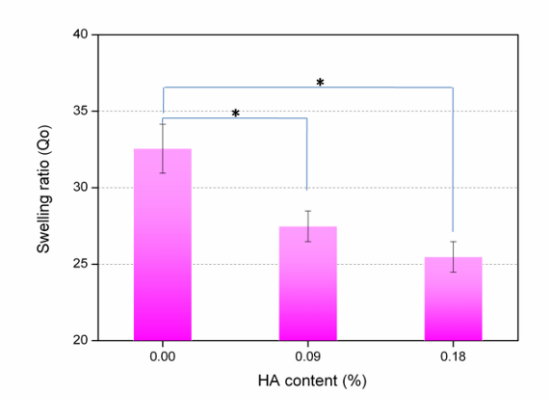

Fig.3 Swelling properties of ODex/MGel hydrogels and semi-IPNs hydrogels

Adhesive and spread of MC3T3-E1 cells on hydrogels. In order to testify the influence of the incorporation of HA on the biocompatible of ODex/MGel hydrogels, MC3T3-E1 cells were cultured on the surface of the hydrogels with different content of HA. As shown in Fig. 4, all of the cells have spread out on the surface of hydrogels after cultured for 24 h, which indicated that our ODex/MGel hydrogels possess good biocompatibility, and can support cells to adhesive and spreading. It is worth noting that in Fig.4a, the dead cells appeared (red cells) which demonstrated

that the pure ODex/MGel hydrogels may have some cell-cytotoxic for cells. Nonetheless, cells on semi-IPNs hydrogels were almost alive. One possible explanation is that the HA is one of the basic component of ECM; therefore, it can promote positive affect for the cells attaching on it.

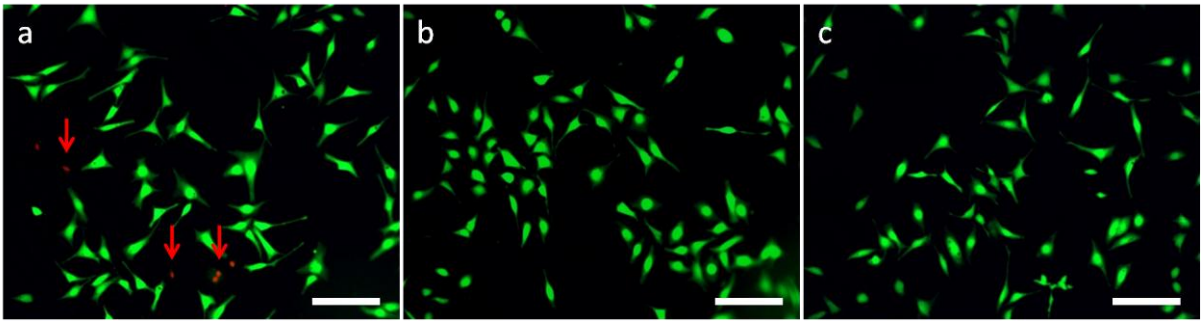

Fig.4 Fluorescence micrographs of MC3T3-E1 cells grown on the surface of ODex/MGel (a), semi-IPNs hydrogels with HA content of 0.09% (b) and 0.18% (c) after cultured for 24 h. Alive cells were stained to green, while dead cells were stained to red. Scale bar represents 50 μm. Cell seeding density is 10,000 cells ml^{-1}.

MC3T3-E1 cells encapsulation within hydrogels. Fig.5 shows that the fluorescence micrographs of cell-laden hydrogels. After 4 days of culture in vitro, most of cells inside ODex/MGel hydrogels were round (Fig. 5a), while within ODex/MGel/HA hydrogels (Fig.5 b, c), some of cells had started to form extensions, indicating that the cells were starting to spread. By 8 days of culture, more cells in ODex/MGel/HA hydrogels with HA content of 0.09% (Fig. 5e) and 0.18% (Fig. 5f) were in spindle shape, while in ODex/MGel hydrogels (Fig. 5d), almost all of the cells were still round and no cells have developed out extensions.

Cell spreading is highly important for obtaining cell-cell contacts, and hence the outcome of tissue [12]. In 3D hydrogel system, cell spreading may be hampered by the physical obstruction of hydrogel networks. It was supposed that inclusion of fast degradation component will permit formation of 3D cell extensions, since degradation will generate space for cells to make their way through the matrix, spreading, migrating and establishing cell-cell contacts. In this work, the HA acted as the fast degradable component, by degraded to form spaces for cells spreading. With the content of HA increased from 0 to 0.18%, more cells can spread out within the hydrogels.

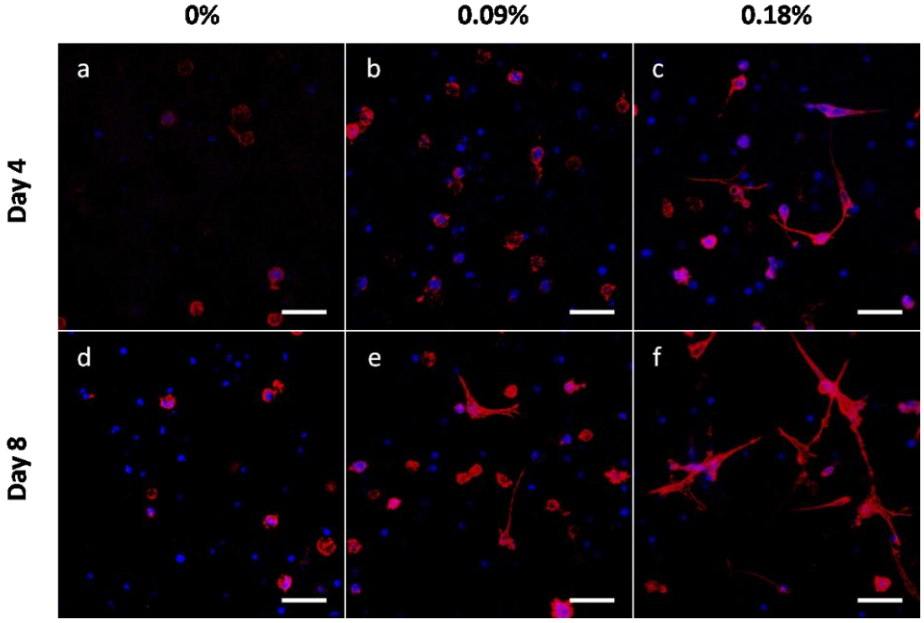

Fig.5 Fluorescence micrographs of MC3T3-E1 cells encapsulated within ODex/MGel (a, d) and semi-IPNs hydrogels with HA content of 0.09% (b, e) and 0.18% (c, f) after cultured for 4 days and 8 days. The cytoplasm was stained by phalloidin-Alexa568, and the nucleus was stained by DAPI. Scale bar represents 100 μm. Cell seeding density is 5×10^6 cells ml^{-1}.

Conclusion

Biodegradable hydrogels were successfully prepared by using oxidized dextran, amino gelatin, and hyaluronic. With the increasing of the content of HA, the prepared hydrogels showed more elastic characterize, and lower ability to absorb water. Cell adhesive and spreading experiments demonstrated that the addition of HA can improved the livability of MC3T3-E1 cells on the hydrogels surface. More importantly, the cell-encapsulate experiments show that the ODex/MGel/HA hydrogels can support MC3T3-E1 cells to survive and spreading within them. Those characterizes indicate that our ODex/MGel/HA semi-interpenetrating network hydrogels could have a great potential to be used as tissue engineering scaffolds in the further.

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