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Injectable hyaluronan-methylcellulose composite hydrogel crosslinked by polyethylene glycol for central nervous system tissue engineering

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ABSTRACT

Spontaneous recovery ability of central nerves has inspired researchers to focus on tissue engineering techniques, especially scaffolds. To obtain a material with an appropriate degrading rate, an injectable composite hydrogel HAMC consisting of hyaluronic acid and methylcellulose was prepared using polyethylene glycol as a cross linker in this study. HAMC combined the advantages of two components to be fast-gelling, injectable, degradable, biocompatible, and it was able to meet some special shape requirement for injured tissue by *in-situ* forming. Moreover, due to the crosslinking effects polyethylene glycol brought to methylcellulose, the rheological properties and stability of HAMC were greatly improved, which could prolong the residence time of this hydrogel effectively. Cell viability results showed HAMC was cytocompatible for further applications *in vivo*, and would be a promising choice for neural tissue engineering in the future.

1. Introduction

Since it was reported that central nerves showed potential spontaneous recovery ability under certain conditions, researchers have been trying to realize neuroregeneration using implanted neural stem cells through tissue engineering techniques [1,2]. As a carrier for cells and growth factors, a scaffold plays the role of artificial extra cellular matrix (ECM) that creates a highly biomimetic niche [3–5]. Therefore, natural polymers are always widely applied as tissue engineering scaffolds, such as collagen, chitosan, alginate and hyaluronic acid [5,6]. Due to the great challenges brought by the particularity of central nervous system (CNS), injectable hydrogel has become a more and more popular material in the field of CNS tissue engineering [7]. Besides the mechanical properties similar to soft tissues, it also meets the shape requirement well by *in-situ* forming and matching the injured part after injected, which conforms to a trend toward minimal invasion in clinical medicine.

Hyaluronic acid (HA), one of the typical hydrogel materials, is a kind of glycosaminoglycan abundantly distributed in extra cellular matrix. Its native molecular weight (MW) ranges from 10^3 to 10^4 kDa [8], and properly processed molecules can induce angiogenesis and cell proliferation [8,9]. Although HA hydrogel can be viscous after swelling, it degrades naturally and rapidly under the reactions with radicals, matrix metalloproteinases or hyaluronidases *in vivo*, perhaps destroying scaffold structure before the complete recovery of tissues. It is necessary to modify HA by chemical or physical crosslinking, or mixing with other polymers [10–12]. Methylcellulose (MC) is a thermosensitive hydrophilic compound derived from cellulose which exists in the cell wall of plants. The thermogelation phenomenon of MC depends on solution concentration, molecular weight, degree of substitution, additives, *etc.* [13–16]. It has been proven that MC could promote neural regeneration by connecting proteins and helping axon grow [17].

As a simple approach for injectable hydrogels, various thermosensitive materials were developed besides MC, such as poly(*N*-isopropyl acrylamide) (PNIPAAm)-based copolymers [18,19], poly[2-(dimethylamino)ethyl methacrylate] (PDMAEMA)-based copolymers [20], polyethylene glycol (PEG)/polypropylene glycol (PPG)-based systems especially PEG/PPG-based polyurethane [18,21–28] and so on, used for adipose regeneration, bone regeneration, skin wounds healing or neural repair [29–33]. However, in terms of neural repair, a contradiction between injectability and degradability have not been solved ideally yet. Hydrogel blend HAMC combines the advantages of HA and MC together to be fast-gelling, injectable, degradable and biocompatible. Gupta and Ballios reported successively that HAMC did not have an adverse effect on the spinal cord and improved cell survival of retinal stem cell-derived rods and neural stem cells [29,30]. The urgent

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problem at present is that unmodified HAMC degrades too fast and thus probably fails in repairing the injured tissue during its lifetime.

Crosslinking is a practical way to stabilize the structure of hydrogels at the molecular level, so that the scaffold will be able to serve for a long enough time till the completion of tissue regeneration. For instance, thiol, dihydrazide, carbodiimide and divinyl sulfone (DVS) are all frequently utilized for HA modification [34–37]. As for cellulose derivatives like MC, DVS and epichlorohydrin (ECH) were used [38–40], whereas considering the cytotoxicity, they are substituted by carbodiimide, citric acid or PEG now [15,41–43]. Studies showed that PEG not only accelerated the crosslinking of MC molecules, but also maintained the rheological properties of either MC or HA [15,43,44]. PEG as a synthetic polymer is bio-inert alone and non-immunogenic, but can provide defined ECM analogs with incorporated signals like growth factors [45,46]. So it was employed here to crosslink MC, and we also used dihydrazide/carbodiimide as a contrast, investigating their effects on materials from different aspects.

Objectives of this study were to prolong the residence time of HAMC in physiological environment, seek a properties-balance and obtain a hydrogel satisfying the needs of lifetime for tissue regeneration and potential for *in vivo* applications. Herein, HAMC hydrogel was prepared by blending HA (1% *w/v*) and MC (7% *w/v*), and cross linked with PEG. Control groups were set by (1) choosing a different cross linker (adipic dihydrazide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (ADH/EDC) crosslinking HA instead of MC) [47–49] or none; (2) changing MC concentration (MC%) to 5%, 9% and 11%. Gelation experiments, rheological tests, degradation measurement, scanning electron microscope observation and *in vitro* cell culture were conducted to characterize our materials. These results were expected to be references for the design of scaffold materials for CNS tissue engineering in the future.

2. Materials and methods

2.1. Preparation of HAMC hydrogels

MC powder M20 (Sinopharm Chemical Reagent Co., Ltd., China) was used to prepare solutions in artificial cerebrospinal fluid (aCSF) in accordance with a reported method [17], containing 4% *w/v* NaCl as an electrolyte additive to lower the gelation temperature. The components of aCSF could be seen in details from published papers [29] and all water used in this study had been deionized. MC solutions were refrigerated at 4 °C overnight.

To prepare HAMC blends, 1% *w/v* HA (MW: 2.6–2.7 kDa, Freda, Shandong, China) powder was added into MC solutions, stirred till the powder was fully dispersed and dissolved. For blank control groups (hereafter as Blank), the sample was ready to be refrigerated again. For PEG-crosslinked groups (PEG), we dropped 2.5% *v*/v PEG solutions (0.4 mg/mL, PEG-600, Sinopharm Chemical Reagent Co., Ltd., China) into the stirring system and adjusted pH value to 7.4 with 1 M NaOH. For ADH/EDC-crosslinked ones (AE), the pH of HAMC was first adjusted to 4.5 with 1 M HCl, after which, 0.375% *w/v* ADH and 0.375% *w/v* EDC were added. After continuous stirring for 20 min, pH was adjusted back to 7.4 by addition of NaOH. All groups of HAMC were stored at 4 °C overnight prior to following experiments [29,44,48,50].

2.2. Gelation process

Gelation was assessed using the inverted tube test conducted at 37 °C to confirm if HAMC samples gelled at human body temperature and to measure the gelation time. 1 mL HAMC was injected into the bottom of an empty test tube in water bath at 37 °C by a micropipettor. The tube was inclined at 2, 3, 5, 10, 15 and 20 min intervals to observe if the gel flowed (observing time could be longer if necessary).

Table 1
Gelation time of HAMC hydrogels.

MC concentration (%)	Observation time (minutes)					
	2	3	5	10	15	20
5	○	○	●	●	●	●
7	○	●	●	●	●	●
9	●	●	●	●	●	●
11	●	●	●	●	●	●

○: unset sol, ●: solidified gel.

2.3. Rheology

Generally, hydrogels are viscoelastic materials, which exhibit mechanical relaxation responding to the time length or frequency (ω) of an applied field. Complex modulus G^* and complex viscosity η^* are usually used to describe their rheological properties, where $G^* = G' + iG''$ (G' for storage or elastic modulus and G'' for loss or viscous modulus).

In the fluid state, a hydrogel had a smaller G' than G''. As the temperature increased, G' would finally be greater than G'', indicating that materials had become solid-like. When $G' = G''$, that critical point corresponded to the gelation temperature (T_{gel}). The HAMC hydrogel was dropped on the surface of sample stage, then G' and G'' values were recorded from room temperature to 40 °C at a speed of 1 °C/min. Frequency = 1 Hz, and 72 data points were collected.

G-ω and η-ω relationships vary from material to material. So after the heating, samples were cooled down to 37 °C and equilibrated for 3 min. Shear frequency changed from 1 to 100 rad/s, dynamic deformation controlled under 1%, 30 data points were collected for $G'(\omega)$, $G''(\omega)$ and $\eta^*(\omega)$.

The fluidity of pseudoplastic fluid like HA will be enhanced under shear stress, which is called thixotropy. But the viscosity curve $\eta(\tau)$ during the decrease of shear stress τ didn't overlap that in the increasing process of τ, enclosing a thixotropic loop. The loop area was a measure of thixotropy. Shear stress on the sample was increased from 10 Pa to 300 Pa, and then dropped back to 10 Pa. 60 data of $\eta(\tau)$ were collected and marked in the same coordinate system.

The rheometer (Physica MCR300, Anton Paar, Austria) used here was connected to a dedicated computer with the software US200. After the hydrogel sample was in place, test head went down to 1 mm high and heat preservation cover was closed. All measurement was auto-conducted by computer once the test mode and parameters had been set.

2.4. Degradation in vitro

Hydrogel samples for degradation experiments were soaked in aCSF at 37 °C which imitates the physiological environment of central neural system for 3 weeks. Specific steps were as below: (1) a 2 mL microcentrifuge tube was weighed before (w_1) and after (w_2) injected with 400 μL HAMC (15 samples for each group, and w_2–w_1 was denoted as wet weight w_w); (2) 600 μL aCSF was added to the tube containing HAMC. (3) all samples were marked and placed on a tube rack in order, then put into a 37 °C incubator; (4) the aCSF in microcentrifuge tubes was changed daily; (5) at 1, 4, 7, 14, and 21 day (t), 3 samples of each group were taken out to get rid of the culture solution, and after a three-hour freeze at −26 °C they were lyophilized for 24 h and weighed again (dry weight $w_d(t)$).

According to the weight on the first day, dry to wet weight ratio of HAMC was $w_d(1)/w_w(1)$. So the theoretical initial dry weight of the sample at t day would be $w_d(t)^* = w_w(t) \times w_d(1)/w_w(1)$, assuming that HAMC hydrogel was water homogeneous. Degradation at t day was calculated as