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Photopolymerizable thiol-acrylate maleiated hyaluronic acid/thiol-terminated poly(ethylene glycol) hydrogels as potential *in-situ* formable scaffolds

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ABSTRACT

Despite their potential in various biomedical applications, photocrosslinkable hyaluronate hydrogels have often been limited by weak formation and unsatisfied mechanical strength which can be attributed to insufficient substitution of photoactive groups on the hyaluronate backbone and the oxygen inhibition effect. In this study, a new approach for the production of hyaluronic acid (MHA) with high acrylate group substitution (*i.e.* 2.27) is developed. It is based on the reaction of sodium hyaluronate and maleic anhydride in dimethyl sulfoxide, which has never been reported previously. Furthermore, the thiol-acrylate photopolymerization approach is employed to prepare maleiated hyaluronic acid/thiol-terminated poly(ethylene glycol) (MHA/TPEG) hydrogels which can overcome the oxygen inhibition effect. And the hydrogels possess porous structures, high swelling ratio, and tunable degradation rate. Specifically, the hydrogels could gel quickly within 15 s and demonstrate improved stiff ($G' = 4100$ Pa). The *in vitro* cytotoxic evaluation demonstrates that the hydrogels are cytocompatible to L929 cells. As a result, the *in-situ* formable hydrogel scaffolds exhibit great potential for medical applications.
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1. Introduction

Hydrogel scaffolds have been widely investigated for tissue regeneration, mainly due to their similar structures to extracellular matrix of native tissues [1] and effective provision of biochemical signals to support cell attachment, proliferation and differentiation [2]. In addition, hydrogel scaffolds gelled *in situ* can form in defect sites in minimally invasive manners, and conform and adhere to tissue [3], avoiding the invasive surgical implantation and the subsequently potential infection as well as the inferior mechanical strength caused by the unfit interfacial binding [4]. *In situ* formable hydrogels can be fabricated by various chemical and physical crosslinking approaches [5–7]. Among these methods, photopolymerization is one of the most promising techniques to achieve the hydrogel formation at a defect site *in vivo*, attributed to its rapid and highly tunable gelation kinetics under mild aqueous conditions at physiological temperatures with spatial-temporal control [8–10].

For proper biomaterials in photocrosslinkable hydrogels, a number of synthetic and naturally derived polymers, including acrylated poly(ethylene glycol) [11,12], alginate [13], chitosan [14], and hyaluronic acid [15], are currently being employed as tissue scaffolds. Among these polymers, hyaluronic acid, a main component of the extracellular matrix found in various tissues of the body, is an attractive candidate for hydrogel scaffolds because of its biocompatibility and non-immunogenic property [16]. In addition, hyaluronic acid can be enzymatically degraded by hyaluronidases *in vivo* with a tunable degradation rate [15] and thus endowing the relevant materials with desirable capacity to accommodate the regenerated tissue. Photocrosslinked hyaluronate hydrogels have been commonly prepared by employing acrylation or methacrylation of hyaluronate as a photocrosslinkable precursor [17,18]. However, as-obtained photocrosslinked hyaluronate hydrogels usually exhibit unsatisfied mechanical property, probably resulting from low and narrow degree of substitution (DS) of acrylate groups on hyaluronate backbone chains [19]. Recently, maleiated hyaluronic acid has been synthesized by esterification between hydroxyl groups of hyaluronic acid and maleic anhydride using formamide as a reaction solvent, but the DS was still related low (0.07–0.75) [19]. Therefore, it is desirable to develop a new approach for the production of hyaluronate with high DS.

Furthermore, the traditional production approach of photocrosslinked hyaluronate hydrogels suffers from the drawback, *i.e.* the acrylate/methacrylate systems are sensitive to oxygen inhibition [20] due to the inherent nature of radical chain-growth polymerization reactions, resulting in heterogeneous crosslinked networks [21] and slow formation processes.

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Such slow liquid-to-gel transformation processes can thus limit their practical applicability *in situ* because the hydrogels may not be formed, displaced, or diluted at the injection/deposition sites [22].

To address these issues, in this study, a photoactive hyaluronic acid (MHA) with high acrylate group substitution is synthesized by using dimethyl sulfoxide (DMSO) as a solvent under mild and heterogeneous reaction conditions firstly. DMSO was a good choice for the modification procedure, mainly due to its relatively low toxicity and significant provision of a higher conversion for the modification of carbohydrates [23,24]. Secondly, maleiated hyaluronic acid/thiol-terminated poly(ethylene glycol) hydrogels are prepared *via* thiol-acrylate photopolymerization process which demonstrates low or even no oxygen inhibition effect and can form networks with excellent physical and mechanical properties [25]. Thiol-terminated poly(ethylene glycol) (TPEG) is selected for its highly reactive thiol groups and widely proved biocompatibility, and applicability in the biomedical field [26]. A series of properties of photocrosslinkable MHA/TPEG hydrogels including rheological property, swelling kinetics, morphology, crystallinity and *in vitro* degradation are investigated. The *in vitro* cytotoxic evaluation of the hydrogels in combination with mouse fibroblasts is also studied to demonstrate their potential as tissue engineering scaffolds.

2. Experimental

2.1. Materials

Sodium hyaluronate (HA, Mw = 1.5×10^6 Da) was purchased from Bloomage Freda Biopharm Co., Ltd., China. Thiol-terminated Poly(ethylene glycol) (TPEG, 20,000 ± 2000 Da) was obtained from Ponsure Biotechnology Co, Ltd., China. Maleic anhydride was supplied by Sinopharm Chemical Reagent Co, Ltd., China. Darocur 2959 (D-2959, 2-hydroxy-1-[4-(hydroxyethoxy) phenyl]-2-methyl-1-propanone) was donated from IGM Resins B.V. (Netherlands). Hyaluronidase (Type I-S) was purchased from Sigma-Aldrich. Mouse fibroblasts (L929 cells) were obtained from Procell Life Science Co., Ltd., China. Other reagents were all A.R. grade.

2.2. Synthesis water-soluble maleiated sodium hyaluronate (MHA)

For further photopolymerization, a modified sodium hyaluronate with photoactive groups was designed and synthesized. Briefly, 1.0 g sodium hyaluronate was suspended in 100 mL dry dimethyl sulfoxide (DMSO) in the flask. 3.5 g maleic anhydride was dissolved in 20 mL dry DMSO and then dropwise added into the flask within 20 min. The reaction was subsequently stirred for 24 h at 50 °C. After that, 1 mol/L $NaHCO_3$ solution was added to the reaction mixture to adjust the pH to 8–9 so as to convert the carboxylic acid to its sodium salt. The mixture solution was precipitated in acetone and then dialyzed (membrane molecular weight cut-off 12,000 $g \cdot mol^{-1}$) against water for 2 days. The dialysis solution was centrifuged to isolate the flocculent precipitate, and lyophilized to obtain pure MHA. FTIR and ^1H NMR spectra were recorded on a Bruker Tensor 27 instrument and Bruker AV 400 NMR instrument, respectively.

2.3. Preparation of photopolymerizable MHA/TPEG hydrogels

The blend solutions were achieved by mixing a MHA aqueous solution and a TPEG solution with a photoinitiator D-2959 at the concentration of 0.25% (w/v). The pre-solution composites are listed in Table 1. After stirring for 20 min, the blend solution was transferred into a disk-shaped mould consisting of two glass microslides separated by a spacer, then irradiated with an Omnicure Series 1000 UV light source (60 mW/cm^2, Exfo, Canada) for 15 min at ambient temperature to form hydrogels. The composite of hydrogels is listed in Table 1.

Table 1
Formulations and characteristics of MHA/TPEG hydrogels.

Sample no.	MHA conc. (w/v %)	Thiol:acrylate (mol ratio)	Sol content (g/g)
M3T-1	3	1.9:100	0.054 ± 0.021
M3T-2	3	0.9:100	0.061 ± 0.034
M3T-3	3	0.5:100	0.12 ± 0.03
M2T-1	2	1.9:100	0.17 ± 0.03
M2T-2	2	0.9:100	0.34 ± 0.04
M2T-3	2	0.5:100	0.37 ± 0.03

2.4. Rheological measurements

In situ dynamic photorheology is often used to measure the elastic and viscous moduli during photo-initiated gel formation processes [27]. A Haake Mars Rheometer (Thermo Fisher Scientific Inc.) equipped with a UV curing attachment and 20 mm parallel plate geometry was used to characterize the photocrosslinking kinetics in this study. The upper plate was made of optically transparent quartz acting as filter for UV light with a cut-off of 320–480 nm. The gap setting was fixed as 1.0 mm. UV light with the intensity of 60 mW/cm^2 was used for the crosslinking reactions of the MHA/TPEG precursors. Time-sweep oscillatory tests were performed at 25 °C at strain amplitude of 1.0% and a 6.28 rad/s, which was within the linear viscoelastic region [28]. The storage and loss modulus values were continuously recorded by Haake RheoWin measuring and evaluation software. Also, The MHA/TPEG solutions without the photoinitiator D-2959 were tested by the Rheometers with UV light off.

2.5. Swelling test

The dry photocrosslinked MHA/TPEG hydrogels (weighted as W_0) were soaked in phosphate buffered saline (PBS) at 37 °C. At regular intervals, the hydrogels were taken out of the PBS solution, and dried superficially with filter paper, weighted as W_S. The swelling ratio (Q) was determined as: $(W_S - W_0) / W_0$.

2.6. Sol determination

The dry photocrosslinked MHA/TPEG hydrogels (dry mass recorded as m_0) were swollen for three days in purified water at 37 °C, with the water replaced for 10 times every day. The swollen gels were lyophilized and the final mass recorded as m_1. The sol content was calculated as: $Sol = (m_0 - m_1) / m_0$.

2.7. Morphology of the hydrogels

To visually examine cross-section morphology of photocrosslinked MHA/TPEG hydrogels, a Jeol Model JSM-6510 scanning electron microscope (SEM) was used to analyze the pore structure. The freeze-dried samples were loaded on the surface of an aluminium SEM specimen holder and sputter coated with gold before observation. The accelerating voltage was 20 kV.

2.8. X-ray diffraction

X-ray diffraction (XRD) was used to investigate inter hydrogen bonding interaction between MHA and TPEG molecular chain. The XRD patterns of MHA, TPEG and MHA/TPEG hydrogels were performed *via* X-ray diffractometer (XRD2000, Shimadzu, Japan) with CuKa characteristic radiation (wavelength $\lambda = 0.154$ nm at 40 kV, 50 mA, and scan speed of 1°/min in the 2θ range of 5–60°).

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