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Impact of immobilizing of low molecular weight hyaluronic acid within gelatin-based hydrogel through enzymatic reaction on behavior of enclosed endothelial cells

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ABSTRACT

The hydrogels having the ability to promote migration and morphogenesis of endothelial cells (ECs) are useful for fabricating vascularized dense tissues *in vitro*. The present study explores the immobilization of low molecular weight hyaluronic acid (LMWHA) derivative within gelatin-based hydrogel to stimulate migration of ECs. The LMWHA derivative possessing phenolic hydroxyl moieties (LMWHA-Ph) was bound to gelatin-based derivative hydrogel through the horseradish peroxidase-catalyzed reaction. The motility of ECs was analyzed by scratch migration assay and microparticle-based cell migration assay. The incorporated LMWHA-Ph molecules within hydrogel was found to be preserved stably through covalent bonds during incubation. The free and immobilized LMWHA-Ph did not lose an inherent stimulatory effect on human umbilical vein endothelial cells (HUVECs). The immobilized LMWHA-Ph within gelatin-based hydrogel induced the high motility of HUVECs, accompanied by robust cytoskeleton extension, and cell subpopulation expressing CD44 cell receptor. In the presence of immobilized LMWHA-Ph, the migration distance and the number of existing HUVECs were demonstrated to be encouraged in dose-dependent and time-dependent manners. Based on the results obtained in this work, it was concluded that the enzymatic immobilization of LMWHA-Ph within gelatin-based hydrogel represents a promising approach to promote ECs' motility and further exploitation for vascular tissue engineering applications.

1. Introduction

The *in vitro* formation of vascular network-like structure tissue is initiated by migration of endothelial cells (ECs) as well as angiogenesis and microvascularization [1–3]. Migration of ECs is mainly mediated through cell interaction with cytokines and extracellular matrix (ECM) components [1–4]. Hydrogels synthesized from materials that are comprised of ECM substratum, such as hyaluronic acid (HA) and gelatin (hydrolyzed collagen), have emerged as an attractive building block to fabricate a vascular network-like structure in *vitro* through activating ECs from their normal quiescent state [1,2,4]. HA is a unique biopolymer composed of repeating disaccharide units of β-1, 4-linked glucuronic acid and β-1, 3-linked *N*-acetyl-D-glucosamine connected by glycosidic bonds which regulate ECs' behaviors [5,6]. This anionic biopolymer has a wide range of molecular weight with different properties during endogenous tissue remodeling [5–8]. While the high molecular weight HA is a natural barrier for migration and proliferation of ECs [5,6], the low molecular weight HA (LMWHA; $\leq 10^4$ Da) has been shown to up-regulate proliferation, migration, and sprout extension of ECs [5–7]. For instance, LMWHA enables ECs to migrate and initiate angiogenesis by primarily interacting with their receptors such as CD44, ICAM, and RHAMM [5,6].

Although LMWHA is a promising material for biological applications, due to its high solubility, it is impossible to obtain hydrogel from unmodified LMWHA [9] and even from modified LMWHA individually as a basal material of hydrogel construct [10]. The incorporation of modified LMWHA into gelatin as a robust base hydrogel construct can provide hydrogel matrix with cell interacting properties, which mimic ECM [11,12]. Gelatin is highly biocompatible and supports migration of ECs due to its cell adhesive functional groups [4,11,12]. The biopolymers having functional groups on their structures can be chemically modified and cross-linked to form hydrogel by different methods [4,13,14], including peroxidase-catalyzed enzyme reaction [14–18]. The peroxidase-

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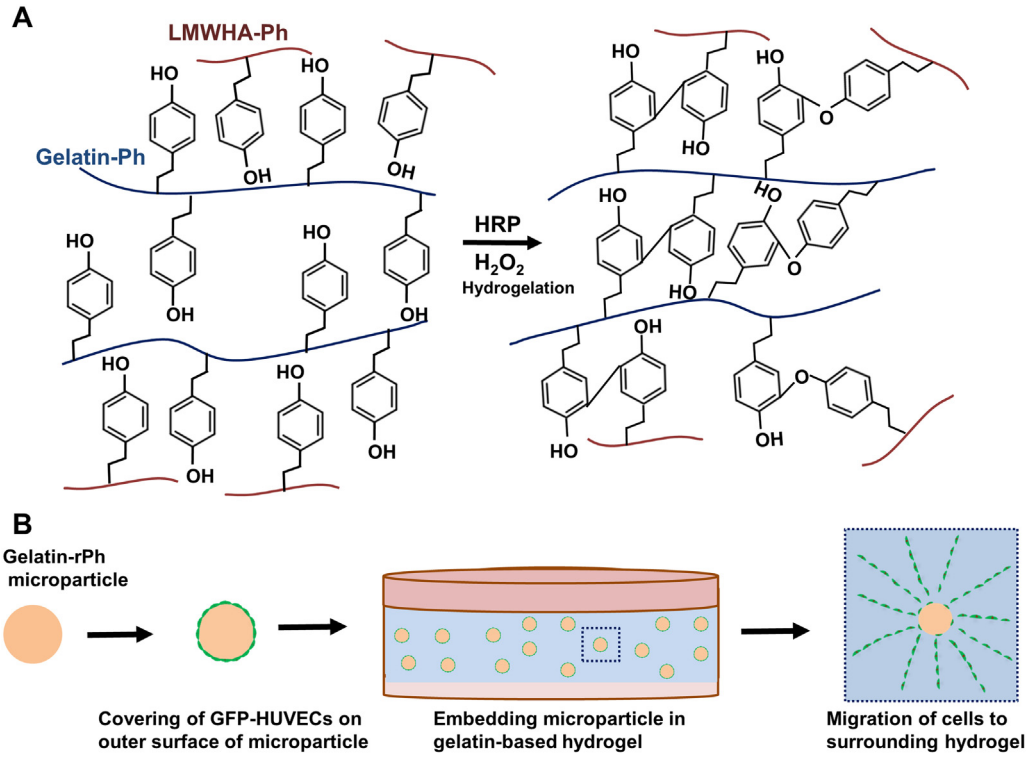

Fig. 1. Schematic illustrations of (A) conjugated hydrogel formation through HRP-catalyzed cross-links between Ph moieties in gelatin-Ph and LMWHA-Ph. (B) Gelatin-rPh microparticle covered with layer of GFP-HUVECs, and embedding in gelatin-based hydrogel for microparticle-based cell migration assay.

catalyzed hydrogelation has attracted attention as a novel route for the fabrication of hydrogels because of its mild reaction conditions for mammalian cells [12,14–17]. In particular, horseradish peroxidase (HRP)-mediated hydrogelation systems have been extensively studied after the work by Kurisawa et al. [15] reporting the hydrogelation of an aqueous solution of hyaluronic acid derivative possessing phenolic hydroxyl (Ph) moieties. Based on that reaction, HRP catalyses the polymerization of Ph moieties on polymer chains in aqueous solution to form hydrogel [12,14–16]. In this study, the derivatives of gelatin and LMWHA both possessing Ph moieties (gelatin-Ph and LMWHA-Ph, respectively) were cross-linked through a coupling reaction of Ph moieties, which is catalyzed by HRP with H_2O_2 as an oxidant substrate (Fig. 1A) [15,17–19]. The aim of this study is to investigate the effectiveness of enzymatic immobilization of LMWHA-Ph by comparing bare LMWHA, as well as free LMWHA-Ph dissolved in culture media, with immobilized LMWHA-Ph on the motility of ECs. Moreover, the stability of covalent cross-linked LMWHA-Ph with gelatin-Ph hydrogel was examined to confirm the feasibility of LMWHA-Ph immobilization during the culture period. In addition, the efficient concentration of immobilized LMWHA-Ph within hydrogel was evaluated to stimulate ECs. The effectiveness of LMWHA-Ph on motility of ECs that regulate ECs' behavior were investigated, based on microparticle-based cell migration assay (Fig. 1B) and determination of CD44 cell receptor expression.

2. Materials and methods

2.1. Materials

Sodium HA (molecular weight: 1.79×10^6 Da) was obtained from the JNC Corp. (Tokyo, Japan). *N*-Hydroxysuccinimide (NHS), HRP (160 units/mg), sodium periodate, collagenase (170 units/mg) and H_2O_2 aqueous solution (30% (w/w)) were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Tyramine hydrochloride, 3-(4-hydroxyphenyl) propionic acid, sodium cyanoborohydride and ethyl carbazate were products of Kanto Chemical (Tokyo, Japan). Water-soluble carbodiimide hydrochloride (WSCD) and 2-morpholinoethanesulfonic acid (MES) were obtained from Peptide Institute (Osaka, Japan) and Dojindo Molecular Technologies (Kumamoto, Japan), respectively. Gelatin (Type B from porcine, 300 Bloom) was purchased from Sigma (St. Luis. MO, USA).

2.2. Cell culture

Green fluorescent protein (GFP)-expressing human umbilical vein endothelial cells (GFP-HUVECs; Angio-Proteomie, MA, USA) were cultured in MCDB 107 medium (Cell Science & Technology Institute, Miyagi, Japan) containing 10% (v/v) fetal bovine serum (FBS), 75 μg/mL penicillin, 50 μg/mL streptomycin, 10 ng/mL human epithelial growth factor (Sigma-Aldrich) and 10 ng/mL human recombinant fibroblast growth factor-2 (ReproCell, Kanagawa, Japan) under a humidified atmosphere of 5% CO_2/95% air at 37 °C.

2.3. Preparation of LMWHA and LMWHA-Ph

LMWHA was prepared using the periodate oxidation method [13,20]. Briefly, sodium HA was dissolved in distilled water at 10 g/L. Sodium periodate (21.3 g/L) was dropwisely added to the HA solution, followed by stirring for 12 h in the dark. Ethylene glycol (10 g/L) was added to stop the reaction and after dialyzing against a large amount of distilled water (molecular weight cut-off (MWCO): 3000 Da), the LMWHA product was obtained by freeze-drying. LMWHA was dissolved in 60 mM sodium acetate buffer (pH 5.5) at 1.0% (w/v). Sodium cyanoborohydride and tyramine hydrochloride were sequentially added to the solution at 1.49 and 0.63% (w/v), respectively. The molecular weight of prepared LMWHA was determined to be 6400 Da by gel permeation chro-