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Preparation and characterization of double macromolecular network (DMMN) hydrogels based on hyaluronan and high molecular weight poly(ethylene glycol)

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Contents

1 Experimental section

1.1 Cell culture

Porcine chondrocytes (PCCs) are isolated from porcine articular cartilage according to previously established protocol,[1] and cultured in chondrocyte culture medium (high glucose DMEM supplemented with 20% FBS ("Gold" Standard, PAA Laboratories), 0.4 mM proline (Sigma), 0.1 mM nonessential amino acids (Gibco), 50 mg/L ascorbic acid (Sigma), 10 mM HEPES (Gibco), and 1% penicillin-streptomycin (Invitrogen)) at 37 ^{o}C in 5% CO_2. PCCs at passage one are used herein. Mouse endothelial progenitor outgrowth cells (mEPOCs) are purchased from BioChain, and cultured in MCDB 131 medium (Sigma) containing 1.176 g/L sodium bicarbonate (Sigma), 1mL bovine brain extract (Hammond Cell Tech), 2% FBS ("Gold" Standard, PAA Laboratories), 1% penicillin-streptomycin (Invitrogen), 50 mg/L ascorbic acid (Sigma), 0.1 mg/mL heparin sodium salt (Sigma), and 10 ng/mL epidermal growth factor (EGF, Sigma) at 37 °C in 5% CO_2 atmosphere. mEPOCs at passage 14 are used in this study. Human mesenchymal stem cells (hMSCs) are purchased from Lonza and cultured in low-glucose Dulbecco's modified Eagle's medium (Hyclone) supplemented with 15% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (Invitrogen) at 37 °C and 5% CO_2. hMSCs at passage five are used in this study.

1.2 Cytocompatibility assay

An indirect contact method is employed to evaluate the cytotoxicity of DMMN gels.[2] PCCs and mEPOCs suspensions are seeded into a 24-well plate, respectively, at a density of 1.6×10^4 cells/well, and cultured in a 5% CO_2 atmosphere at 37 ^{o}C for 24 hours for cell adhesion (day one). Thereafter, 1.0 mL of fresh culture medium and freshly prepared DMMN-2-20 gel (diameter 6 mm, height 3.8 mm) is added into each well, at the same time, the culture medium is

used alone as the control. The culture medium is changed every other day. After one, three, and five days of culture, cell viability is measured with PrestoBlueTM cell viability reagent (Life Technologies). Briefly, the culture medium is replaced with 0.5 mL of DMEM (for PCCs) or MCDB 131 medium (for mEPOCs) containing 10% (v/v) PrestoBlue reagent and incubated at 37 ^{o}C for one hour in dark. The fluorescence intensity at 560 nm (excitation) and 590 nm (emission) is collected with the Fluorescence Microplate Reader (Bio-tek Synergy MX, USA). Cell viability is converted and expressed as the average fluorescence value with respect to that on day one.

1.3 Cell adhesion and proliferation

The RGD-modified HA-MA is synthesized by 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich)/N-hydroxysuccinimide (NHS, Sigma-Aldrich) coupling chemistry. Briefly, 0.2 g of HA-MA foam is dissolved in 20 mL of PBS, and 96 mg of EDC and 56 mg of NHS is added. The pH value of the mixture solution is adjusted to 5.5. After one hour of stirring, 40 mg of RGD-containing peptides (GGGGRGDY, GL Biochem, Shanghai, China) dissolved in 1 mL of DI water is added, followed by stirring for three days at ambient temperature. The reaction mixture is directly dialyzed in DI water for three days, and freeze-dried.

DMMN-2-20 gel sheet (thickness: 2.6 mm) synthesized from RGD-modified HA-MA is cut into round pieces with 8 mm of diameter. The gel pieces are placed in a 48-well plate, and hMSCs suspension is added into each well with cell density of 3×10^3 cells/cm^2. It is placed in a the incubator with 5% CO_2 at 37 °C. After two days of culture, the cytoskeletons of hMSCs are stained with Alexa Fluor® 546 phalloidin (Invitrogen) for labeling F-actin according to the manufacturer's protocol; cell nuclei are counterstained with DAPI (Sigma) solution (100 ng/mL). They are observed under a fluorescence microscope (OLYMPUS-LX71). The proliferation

activity of hMSC on gels is measured by above-mentioned PrestoBlue assay.

2 Results and discussion

Cytocompatibility of DN gels is a major concern in biomedical applications. The cytotoxicity of DMMN-2-20 gel is evaluated by an indirect contact method using PCCs and mEPOCs as model cells. As shown in Fig. S2a and b, Both PCCs and mEPOCs proliferate over culture time in control groups (culture medium) and experimental groups (culture medium containing DMMN-2-20 gel). No statistically significant difference in cell viability is observed between control groups and experimental groups throughout the culture period, except for the PCCs by day 3 of culture. The lower cell viability of PCCs in experimental groups on day 3 can be attributed to the presence of photoinitiator that is diffused out of DMMN-2-20 gel.

In order to meet the demand of cell adhesion as the regeneration substrate of some tissues, the DMMN-2-20-RGD gel is fabricated with 2% (g/mL) of RGD-modified HA-MA for the first network and 20% (g/mL) PEG20K-DA for the second network, which exhibits the comparable mechanical properties with DMMN-2-20 gel. As shown in Fig. S2c, the hMSCs adhere on DMMN-2-20-RGD and have quite good spreading after 24 hours of culture due to the recognition of RGD sequence by integrin on cell membrane; they exhibit normal polyhedral or spindle shapes with fine filopodia.[3] Moreover, the viability of hMSCs statistically increases with incubation time (Fig. S2d). These results indicate that, in addition to its extraordinary mechanical properties, the DMMN gels show excellent cytocompatibility, besides, when conjugated with cell signaling moieties, they can promote cell adhesion and proliferation.

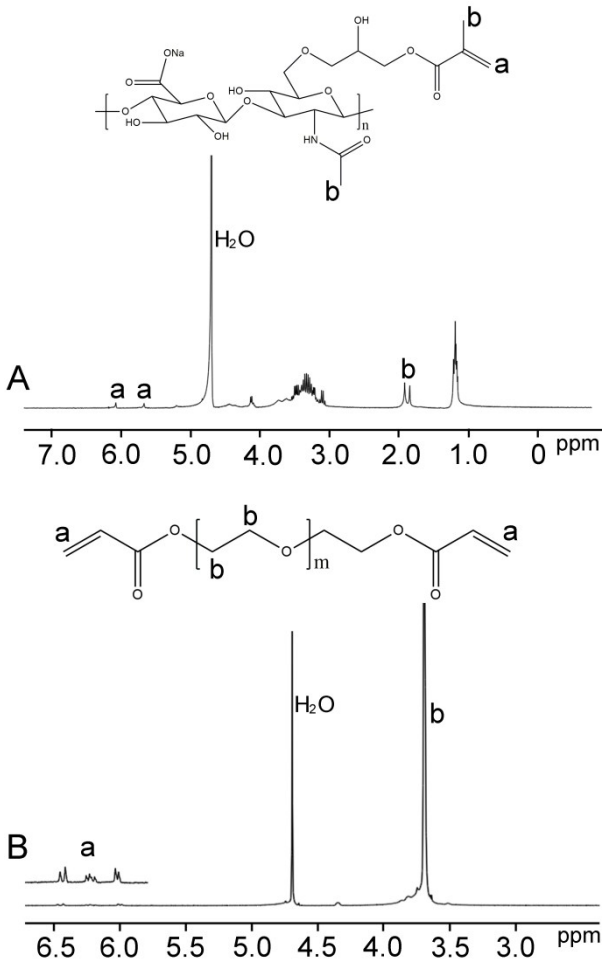

Fig. S1 (A) The ^{1}H-NMR spectrum of HA-MA. The methacrylation degree of HA-MA is generally defined as the amount of methacryloyl groups per disaccharide in HA-MA.[4] It is 17%, calculated based on the relative integral intensities of methyl protons (at δ 1.92 and δ 1.84 ppm) and acrylate protons (at δ 6.07 and δ 5.66 ppm).[4] (B) The typical ^{1}H-NMR spectrum of PEG20K-DA. The degree of acrylation is 78% and 95% for PEG20K-DA and PEG4K-DA, respectively, which is calculated from the relative integral intensities of methylene protons (δ 3.66 ppm) and acrylate protons (δ 5.86-6.44 ppm).[5]

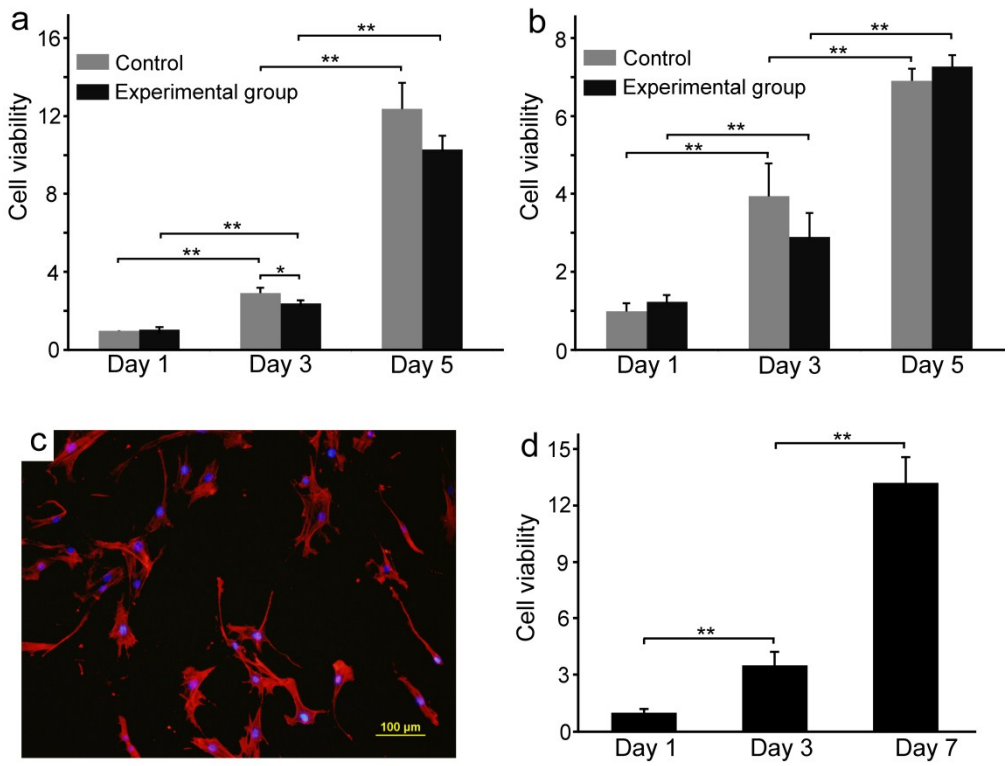

Fig. S2 Cell viability of PCCs (a) and mEPOCs (b) cultured without (Control) or with DMMN-2-20 gel (Experimental group) as a function of culture time; immunofluorescent staining for F-actin (c) and proliferation of hMSCs (d) on DMMN-2-20-RGD gel. The statistical significance is indicated with * ($p \leq 0.05$) and ** ($p \leq 0.01$). Scale bar is 100 μm.

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