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Soft hydrazone crosslinked hyaluronan- and alginate-based hydrogels as 3D supportive matrices for human pluripotent stem cell-derived neuronal cells

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ABSTRACT

Regenerative medicine, especially cell therapy combined with a supportive biomaterial scaffold, is considered to be a potential treatment for various deficits in humans. Here, we have produced and investigated the detailed properties of injectable hydrazone crosslinked hyaluronan-polyvinyl alcohol (HA-PVA) and alginate-polyvinyl alcohol (AL-PVA) hydrogels to be used as a supportive biomaterial for 3D neural cell cultures. To the best of our knowledge, this is the first time the polymerization and properties of hydrazone crosslinked AL-PVA hydrogel have been reported. The effect of the degree of substitution and molecular weight of the polymer components as well as the polymer concentration of the hydrogel on the swelling, degradation and mechanical properties of the hydrogels is reported. Furthermore, we studied the effect of the above parameters on the growth of human pluripotent stem cell-derived neuronal cells. The most neural cell supportive HA-PVA hydrogel was composed of high molecular weight HA component with brain-mimicking mechanical properties and decreased polymer concentration. AL-PVA hydrogel, with stiffness quite similar to brain tissue, was also shown to be similarly supportive. Neuronal spreading and 3D network formation was enhanced inside the softest hydrogels.

1. Introduction

Traumas and deficits in the human central nervous system (CNS) may have a permanent effect on the functionality of the patient and the prognosis in many cases is poor. Moreover, human CNS, as an organ, suffers from low inbuilt regenerative capacity, which makes healing with traditional medicine (drugs and surgical operations) insufficient. As a result, regenerative medicine is considered to be a potential treatment for CNS deficits. Regenerative medicine aims to restore normal functionality by enhancing the regeneration of tissue or by replacing the damaged parts with engineered biological transplants. One such strategy is cell therapy combined with a supportive biomaterial scaffold.

Biomaterial scaffold should fulfill specific criteria when they are used for neural tissue engineering with the aim of neuronal network regeneration. For example, the scaffolds should have similar mechanical properties to those of the brain or spinal cord, they should allow the infiltration of cells and axons, they should allow the transportation of nutrients and metabolites, they should integrate with the host tissue, they should exhibit a suitable degradation rate without any harmful degradation products, and they should not induce inflammatory and glial scar formation [1]. Based on these requirements, polymer-based hydrogels can be considered to be suitable biomaterial candidates for neural tissue engineering. A soft, hydrated form of three-dimensional crosslinked hydrogels resembles that of naturally occurring living tissue. The porous nature of hydrogels enables the transportation of waste, oxygen and nutrients. The mechanical and physical properties of hydrogels are tunable making it easier to mimic the living tissue [2]. Most hydrogels are also considered to be cyto- and biocompatible materials.

When designing hydrogels for this kind of application, a thorough knowledge of their various properties is important. Hydrogels have variable mechanical, physical and degradation properties that can be controlled, for example, by altering the molecular weight (M_w), the chemical structure and the number of available crosslinkable groups in the polymer, the ratio of gel components, the amount of water, and the crosslinking method. It is well known that, for example, crosslinking density can affect mechanical, swelling and degradation properties and the functionality of hydrogels. The difficulty lies in the altering of properties individually when needed without affecting the others [3]. Further modification of hydrogels can be carried out by incorporating extracellular matrix (ECM) molecules (collagen, laminin, *etc.*) or

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peptides to provide more anchoring sites for the cells [4,5].

Numerous natural and synthetic polymer-based hydrogels have been used as scaffolds for the 3D culture of neural lineage cells as reviewed by, for example, Murphy et al. [2]. Two widely used polysaccharide polymers used in neural research are hyaluronan (HA) [6,7] and alginate [8,9]. HA is an anionic and hydrophilic polysaccharide composed of β-1, 4-D-glucuronic acid and β-1, 3-N-acetyl-D-glycosamine residues that is a major glycosaminoglycan component in the ECM of the brain. HA plays a vital role in the development of the CNS, and it is particularly abundant in the fetal brain and the surrounding immature neurons during differentiation in the spinal cord [10]. HA is produced by almost all the members of the animal kingdom as well as certain members of the streptococci species. It has a relatively simple repetitive chemical structure. The carboxyl and hydroxyl groups allow specific modification and the introduction of functional groups for crosslinking. Alginate (AL) is an anionic and hydrophilic polysaccharide composed of 1,4-linked β -D-mannuronic acid (M) and α -L-guluronic acid (G) residues that is obtained from brown algae. It is structurally similar to ECM. AL is inherently non-biodegradable, but it can be made degradable, for example, by replacing the divalent cations with monovalent cations or by oxidation [11]. The properties of natural polymer-based hydrogels can be improved and widened by combining them with a synthetic polymer such as polyvinyl alcohol (PVA). PVA is a hydrophilic polymer with good chemical, thermal and mechanical stability. Hydroxyl groups in the structure provide chemical versatility that enables further modification and functionalization [12]. Biocompatible PVA hydrogels have been used quite widely in several biomedical applications [13]. PVA has also been approved by the Food and Drug Administration (FDA) and Conformité Européenne (CE) for clinical use in humans [14].

HA-PVA and AL-PVA hydrogels can be fabricated by using hydrazone crosslinking, which is an aldehyde-hydrazide coupling reaction. Hydrazone crosslinking belongs to the group of pseudo click chemistry reactions together with the Michael addition reaction (pseudo is usually characterized by moderate orthogonality). The reactions are versatile, simple and reversible, and they have high reactivity and yield. Furthermore, no toxic reagents or side-products are produced [15]. This crosslinking method also enables the fabrication of injectable hydrogels. The injectability of the hydrogels is desirable for when the hydrogels are eventually transplanted into the injury site. To date, there have been several studies carried out on hydrazone crosslinked HA-PVA hydrogels [16,17]. The focus of these studies has, however, been on other areas of soft tissue engineering. To the best of our knowledge, this is the first time the polymerization and properties of the hydrazone crosslinked AL-PVA hydrogel have been reported.

In this study, we have produced injectable hydrazone crosslinked HA-PVA and AL-PVA hydrogels with variable properties. We studied the effect of the degree of substitution (DS%), the molecular weight of the polymer components and the polymer concentration of the hydrogel on swelling, degradation and the mechanical properties of the hydrogels. We also studied the effects of the above parameters on the growth of human pluripotent stem cell-derived neuronal cells.

2. Materials and methods

2.1. Materials and general methods

Hyaluronic acid sodium salt ($M_w = 1.5 \times 10^5$ g/mol) was purchased from Lifecore (Chaska, MN, USA). Hyaluronic acid sodium salt from streptococcus equi ($M_w = 1.5 - 1.8 \times 10^6$ g/mol), polyvinyl alcohol ($M_w = 27000$ g/mol, 98.0 –98.8 % hydrolyzed), t-butyl carbazate (TBC), 1,1 ′ - carbonyldiimidazole (CDI), glycine ethyl ester hydrochloride, hydrazine solution (35 wt % in H_2O), 1-hydroxybenzotriazole (HOBt), alginic acid sodium salt from brown algae (low viscosity), picrylsulfonic acid solution (5-% (w/v) in H_2O, TNBS), hyaluronidase from bovine testes (Type I-S, 400-1000 units/mg solid), ethylene glycol, dimethyl sulfoxide (DMSO), deuterium oxide (99.9 atom% D, contains 0.05 wt % 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt), sodium cyanoborohydride ($NaBH_3CN$), sucrose, sodium periodate, sodium acetate, acetic acid, sodium tetraborate decahydrate and boric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triethylamine was purchased from J.T. Baker (The Netherlands). Acetate buffer was prepared from sodium acetate and acetic acid, and borate buffer was prepared from sodium tetraborate decahydrate and boric acid. All solvents used were of analytical quality. Milli-Q water was used in synthesis and determinations. Dialysis membranes (Spectra/Por® cut-off 1000 and 25,000 g/mol) were purchased from Spectrum Laboratories, Inc. (Rancho Dominguez, CA, USA).

Cell culture reagents DMEM/F12, Neurobasal, GlutaMax, B27, N2 and penicillin/streptomycin were purchased from Thermo Fisher Scientific (Waltham, MA, USA), and basic fibroblast growth factor (FGF2) from Bio-Techne (Minneapolis, MN, USA). Rabbit anti-microtubule associated protein 2 (MAP-2, AB5622) was purchased from Merck Millipore (Darmstadt, Germany), monoclonal mouse anti-B-tubulin III (T8660) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and Alexa Fluor 488 or 568 conjugated secondary antibodies (1:400) from Thermo Fisher Scientific. VECTASHIELD mounting media containing 4 ′,6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, United Kingdom).

Nuclear Magnetic Resonance (NMR) spectroscopy experiments were measured with a Varian Mercury 300 MHz NMR Spectrometer (Palo Alto, USA). Polymer samples (5 mg) were dissolved deuterium oxide (600 μL) containing internal standard (0.05 wt % 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid, sodium salt). Fourier Transform Infrared (FTIR) spectroscopy experiments were collected on a Perkin Elmer Spectrum One FT-IR Spectrometer (Waltham, MA, USA) in the spectral range of 400 to 4000 cm^{-1}. Freeze-dried polymer and hydrogel samples (1–2 mg) were pressed into KBr (200 mg) tablets.

2.2. Synthesis of aldehyde-modified hyaluronan and alginate

Aldehyde groups were generated from the vicinal diol groups of HA and AL by using periodate oxidation (Fig. 1 (a), (b)) according to the previously reported method [18] that was slightly modified. Briefly, sodium hyaluronate or alginate (0.500 g) was dissolved in deionized water (100 mL). Sodium periodate (Table S1.) was dissolved in deionized water (2.7 mL), added dropwise and stirred for 2 h to 4 h (Table S1.) in the dark at room temperature under nitrogen. Ethylene glycol (4 equivalents) was added to inactivate any unreacted periodate and the solution was then stirred for 1 h. Derivatized polymers were dialyzed with MW cutoff 25,000 membrane against deionized water for three days. Purified polymers were lyophilized to obtain white cotton-like products (HAALD and ALALD). ^{1}H NMR (Fig. 1, D_2O, 300 MHz): HAALD: δ 4.47 (m, 1H, H1 of glucose unit), 3.82 –3.35(m, 5H, H2–5 of glucose unit), 2.03 (s, 3H, -NHC(O)CH_3). ALALD: 5.72 (s, 1H, H4-G), 5.26 and 5.18 (s, 1H, hemiacetal protons), 5.00 (s, 1H, H1-G), 4.59 (s, 1H, H1-M and H5-GM), 4.39 (s, 1H, H5-GG). FTIR (Fig. 2, KBr pellet): HAALD: 1720 (ν(C = O) of –C(O)H), 1618 (ν(C = O) of –NHC(O) – and –C(O)OH). ALALD: 1732 (ν(C = O) of –C(O)H)), 1634 (ν(C = O) of –C(O) OH).

The DS% of HAALD was determined using a TNBS method [16,19]. Briefly, HAALD (20 mg) was dissolved in acetate buffer (2 mL, 0.1 M, pH 5.2) and added to TBC solution in acetate buffer (1 mL, 0.0348 g, 10-fold excess per molar amount of sodium periodate used). The mixture was allowed to react for 1 h at room temperature. $NaBH_3CN$ (1 mL, 0.0166 g, equimolar amount to TBC) in acetate buffer was added and allowed to react for 24 h at room temperature under nitrogen. The polymer was dialyzed with MW cutoff 25,000 membrane against 0.1 M NaCl for 24 h and for a further 24 h in deionized water. Purified polymer was lyophilized to obtain a white cotton-like product. The ^{1}H-NMR spectrum was measured and the DS% was determined from the integration of the ^{1}H-NMR peaks. ^{1}H NMR (D_2O, 300 MHz): δ 1.9 (3H,