Acta Biomaterialia 27 (2015) 131–139

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Intrinsic and extrinsic determinants of central nervous system axon outgrowth into alginate-based anisotropic hydrogels

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ARTICLE INFO

Article history:
Received 19 March 2015
Received in revised form 12 July 2015
Accepted 21 August 2015
Available online 24 August 2015

Keywords:
Alginate hydrogel
Self-organization
Microchannels
Axon regeneration
Spinal cord slice culture
Entorhinal cortex slice culture
Astrocyte

ABSTRACT

Appropriate target reinnervation and functional recovery after spinal cord injury depend on longitudinally directed regrowth of injured axons. Anisotropic alginate-based capillary hydrogels (ACH) support *peripheral nervous system* derived axon growth, which is accompanied by glial supporting cell migration into the ACH. The aim of the present study was to analyze *central nervous system* (CNS) derived (entorhinal cortex, spinal cord slice cultures) axon regrowth into ACH containing linearly aligned capillaries of defined capillary sizes without and with gelatin constituent. Anisotropic ACH were prepared by ionotropic gel formation using Ba^{2+}, Cu^{2+}, Sr^{2+}, or Zn^{2+} ions resulting in gels with average capillary diameters of 11, 13, 29, and 89 μm, respectively. Postnatal rat entorhinal cortex or spinal cord slice cultures were placed on top of 500 μm thick ACH. Seven days later axon growth and astroglial migration into the ACH were determined. Axon density within capillaries correlated positively with increasing capillary diameters, whereas longitudinally oriented axon outgrowth diminished with increasing capillary diameter. Axons growing into the hydrogels were always accompanied by astrocytes strongly suggesting that respective cells are required to mediate CNS axon elongation into ACH. Overall, midsize capillary diameter ACH appeared to be the best compromise between axon density and orientation. Taken together, ACH promote CNS axon ingrowth, which is determined by the capillary diameter and migration of slice culture derived astroglia into the hydrogel.

Statement of Significance

Biomaterials are investigated as therapeutic tools to bridge irreversible lesions following traumatic spinal cord injury. The goal is to develop biomaterials, which promote longitudinally oriented regeneration of as many injured axons as possible as prerequisite for substantial functional recovery. Optimal parameters of the biomaterial have yet to be defined. In the present study we show that increasing capillary diameters within such hydrogels enhanced central nervous system axon regeneration at the expense of longitudinal orientation. Axon ingrowth into the hydrogels was only observed in the presence of glial supporting cells, namely astrocytes. This suggests that alginate-based hydrogels need to be colonized with respective cells in order to facilitate axon ingrowth.

1. Introduction

Biomaterial scaffolds represent a promising tool to replace lesion defects following spinal cord injury (SCI) and to provide a regeneration supporting matrix. The scaffolds aim to bridge the lesion defect within the spinal cord injury site, reduce the formation of scar tissue and guide outgrowing axons across the lesion, all of which are prerequisites for successful spinal cord repair [1–3].

Numerous natural and synthetic polymer-based biomaterials have been employed to promote regeneration after SCI [4,5]. Ideally, scaffolds should be immunologically inert, have a modifiable microstructure and should be biodegradable without generating toxic degradation products [6]. Furthermore, biomaterials should

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http://dx.doi.org/10.1016/j.actbio.2015.08.032
1742-7061/

match elastic properties of spinal cord tissue to prevent secondary neural damage. The integration and release of growth-promoting cells and factors substances is desirable [7]. Obviously, the integration of all these properties into one particular type of biomaterial is challenging, which makes the development of an effective biomaterial-based regeneration promoting therapy in the near future unlikely.

Hydrogels as specific type of biomaterial scaffolds, in which a hydrophilic natural or synthetic polymer network is swollen by an aqueous solution, are known to mimic the chemical, physical and mechanical properties of the natural extracellular matrix affecting cell behavior like adhesion, proliferation and differentiation [1,5]. Hydrogel scaffolds with an oriented microstructure are more advantageous than scaffolds with a random inner structure for a longitudinally directed axons regrowth across nerve gaps, which indicates that physical cues are essential for guiding axon regeneration [8,9]. Agarose-and gelatin-based scaffolds with oriented channels (200 and 20–60 μm in diameter, respectively) elicit directed axonal regeneration [10,11]. The natural polymer alginate can serve as the basis for an injectable scaffold [12] or as anisotropic capillary hydrogel (ACH) [13,14], which promote axonal regeneration following SCI. The characteristic longitudinally arranged capillary structure of ACH is facilitated by a self-assembling process during ion-driven gel formation. ACH elicit robust oriented axonal outgrowth both *in vitro* and after implantation in a rat SCI model [14]. In ACH as opposed to nanofiber-based scaffolds [15,16], injectable hydrogels [17–19], or extracellular matrix-based hydrogels [20–22], the geometry and elastic properties can be easily modified during the ACH self-assembly process. Especially the type of divalent cation used for ionotropic gel formation of alginate solutions strongly determines the size and the density of the capillary structure produced in ACH [23,24]. ACH with different capillary diameters (10, 18, and 55 μm) generated by introducing Cu^{2+}, Sr^{2+}, and Zn^{2+} cations during ionotropic gel formation were investigated using a postnatal dorsal root ganglion (DRG) axon outgrowth assay *in vitro*. DRG-derived axonal ingrowth into ACH was enhanced with increasing capillary diameter. Axonal outgrowth was always accompanied by Schwann cells [14].

The bioactivity of carbohydrate-based scaffolds such as alginate, chitosan, agarose, and dextran sulfate in respect to cell adhesion can be enhanced by adding gelatin. [25,26]. Hyaluronan and chitosan scaffolds containing gelatin constituent have been shown to induce superior integration of Schwann cells, neurons and olfactory ensheathing cells in comparison to respective scaffolds without gelatin [27,28]. Gelatin scaffolds supported the survival of stem cells after combined transplantation into spinal cord transections [29,30]. Gelatin constituent within ACH yielded superior dorsal root ganglion derived axon elongation *in vitro* without affecting axon density [14].

In the present study we aimed to investigate the capacity of ACH with variable capillary size and density to promote axon outgrowth of central nervous system (CNS)-derived axons – as opposed to previously investigated DRG-derived peripheral nervous system (PNS) axons – by employing postnatal day 4–6 spinal cord and postnatal day 2–6 entorhinal cortex slice cultures. ACH with variable capillary diameter were generated by introducing a variety of different cations. Furthermore, ACH were modified by introducing gelatin to further improve axon ingrowth.

2. Materials and methods

2.1. Preparation of alginate-based capillary hydrogels

Ultrapure sodium alginate with an average molecular weight between 150,000 and 200,000 g mol^{-1} and a guluronic acid content of 68% (PRONOVA UP LVG, Novamatrix FMC biopolymer, Norway) and type A porcine skin gelatin with a gel strength of about 300 g Bloom (Sigma Aldrich, Germany) were used for hydrogel synthesis. The protocol for preparation of anisotropic alginate-based hydrogels has been described previously [13,14]. Pure alginate hydrogels were prepared from aqueous solutions containing 20 g L^{-1} (2% w/w) sodium alginate and alginate/gelatin hybrid hydrogels from solutions containing 20 g L^{-1} sodium alginate and 2 g L^{-1} (0.2% w/w) gelatin. As gel-forming agents 1 mol L^{-1} solutions of $BaCl_2 \cdot 2\ H_2O$, $Cu(NO_3)_2 \cdot 3\ H_2O$, $Sr(NO_3)_2$, and $Zn(NO_3)_2 \cdot 6\ H_2O$ (all VWR International, Germany) were prepared. All solutions were prepared with purified water (Milli-Q®, Millipore, France) and filtered through a Nalgene® vacuum filtration system equipped with a polyethersulfone membrane with a pore size of 0.2 μm (VWR International). 65 g of alginate or alginate/gelatin solution were poured into anodized cylindrical aluminum moulds (5.5 cm in diameter and 4 cm in height, Schuett-Biotec, Germany) and carefully superimposed with 20 mL of electrolyte solution using pump spray bottles (VWR International). The moulds were covered by a lid and allowed to stand for at least 36 h until gel formation was finished. After removing from the moulds, the gels consisting of pure alginate were immersed in purified water and those consisting of alginate and gelatin in 2 g L^{-1} gelatin solution in order to remove excessive electrolyte; the gelatin solution was used to avoid leaching of the gelatin constituent during washing. The water or gelatin solution was changed at least 4 times after a minimum of 4 h. Gels were cut into 15 mm thick slices as previously described [13,14].

Gel slices were dehydrated by equilibrating in acetone/water mixtures of rising acetone content (25%, 50%, 75%) for a minimum of 4 h per step; then the gels were equilibrated in pure acetone and then in dry acetone (VWR International) twice for at least 4 h per step; solutions are slightly agitated by stirring. For crosslinking, dehydrated and acetone-soaked alginate gels were immersed in 0.1 mol L^{-1} solutions of hexamethylene diisocyanate (HDI; Sigma, Germany) in dry acetone for 4 h under slight stirring. Gels were removed from the HDI solution and immersed in dry acetone for 5 min to remove HDI from the capillary lumens. In order to remove acetone from the capillary lumens, the gel slices were put between two filter papers and dried in air for 10 min. The gels were immersed in water and heated to 70 °C for 2 h until carbon dioxide development stopped. Then, gels were immersed in 0.1 mol L^{-1} hydrochloric acid solution (VWR International) up to seven times for at least 2 h to remove the crosslinking divalent cations. After washing in sterile-filtered water until a neutral pH was reached, gels were finally sterilized and stored in 70% ethanol [14].

2.2. Ion exchange

To test the efficiency of the ion exchange process with HCl, gel specimens with a thickness of 2 mm and a diameter of 10 mm were prepared using a custom-made cutting machine and a hollow punch. The exchange was performed 0–7 times by immersing the small gel specimens in 0.1 mol L^{-1} HCl solution for a minimum of 2 h per step. At the end, all gel samples were washed with water until a neutral pH was reached, dried by lyophilisation (Edwards, UK) and weighted on a MT5 microbalance (Mettler-Toledo, Germany). Samples were decomposed by heating in 2 mL of ultra-pure, concentrated nitric acid (⩾69%, TraceSelect®, Fluka) at 120 °C in small porcelain cups (VWR International) until the liquid was completely vaporized. Then 2 mL of ultra-pure concentrated perchloric acid (⩾70%, TraceSelect®, Fluka) were added and the solution was heated again until the liquid was completely vaporized. The decomposition procedure using perchloric acid was repeated until a colorless remnant remained. This remnant was dissolved in 0.5 mol L^{-1} ultra-pure nitric acid solution and