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Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering

K.E. Crompton[a,b], J.D. Goud[c], R.V. Bellamkonda[c], T.R. Gengenbach[d], D.I. Finkelstein[e], M.K. Horne[f], J.S. Forsythe[a,b,*]

[a]*School of Physics, Department of Materials Engineering, Monash University, Wellington Rd., Clayton, VIC 3800, Australia*
[b]*CRC for Polymers, 1st Flr., 8 Redwood Drive, Notting Hill, VIC 3168, Australia*
[c]*Neurological Biomaterials and Therapeutics, WHC Department of Biomedical Engineering, Georgia Institute of Technology, Emory University, Atlanta, GA 30332, USA*
[d]*CSIRO Molecular and Health Technologies, Bag 10, Clayton South, VIC 3169, Australia*
[e]*The Mental Health Research Institute, Locked Bag 11, Parkville, Victoria 3052, Australia*
[f]*Howard Florey Institute of Experimental Physiology and Medicine, The University of Melbourne, Parkville 3010, Australia*

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Abstract

Foetal mouse cortical cells were cultured on 2D films and within 3D thermally responsive chitosan/glycerophosphate salt (GP) hydrogels. The biocompatibility of chitosan/GP 2D films was assessed in terms of cell number and neurites per cell. Osmolarity of the hydrogel was a critical factor in promoting cell survival with isotonic GP concentrations providing optimal conditions. To improve cell adhesion and neurite outgrowth, poly-D-lysine (PDL) was immobilised onto chitosan via azidoaniline photocoupling. Increase in PDL concentrations did not alter cell survival in 2D cultures but neurite outgrowth was significantly inhibited. Neurons exhibited a star-like morphology typical of 2D culture systems.

The effects of PDL attachment on cell number, cell morphology and neurite outgrowth were more distinct in 3D culture conditions. Neurones exhibited larger cell bodies and sent out single neurites within the macroporous gel. Immobilised PDL improved cell survival up to an optimum concentration of 0.1%, however, further increases resulted in drops in cell number and neurite outgrowth. This was attributed to a higher cell interaction with PDL within a 3D hydrogel compared to the corresponding 2D surface. The results show that thermally responsive chitosan/GP hydrogels provide a suitable 3D scaffolding environment for neural tissue engineering.
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1. Introduction

Most neurons in the adult mammalian central nervous system (CNS) do not proliferate or renew themselves and consequently interest has focussed upon cell replacement therapies to repair damage in the CNS. Growth of implanted cells must be controlled in order to guide differentiation and neurite outgrowth, and hence study of suitable scaffolding materials to support cells on implantation is required. The materials must therefore provide appropriate chemical and spatial microenvironment for cell proliferation, differentiation and axon extension.

Hydrogels have many advantages as cellular scaffolds, because they have similar mechanical properties to soft tissue, have low interfacial tension which allow cells to move across the tissue-implant boundary [1] and use only non-toxic aqueous solvents that promote diffusion of oxygen, nutrients and waste throughout the scaffold. Both synthetic and naturally occurring injectable hydrogels have been deployed in the nervous system, and many provide support to cells cultured within the material. Dorsal root

*Corresponding author. Department of Materials Engineering, Monash University, Wellington Rd., Clayton, VIC 3800, Australia.
Tel.: +61 3 9905 9609; fax: +61 3 9905 4940.
E-mail address: john.forsythe@eng.monash.edu.au (J.S. Forsythe).

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ganglia (DRGs), PC12 cells or cortical cells grown within agarose will form neurites [2]. The ability for agarose to support neural cells was improved through optimisation of the gel in terms of porosity and gel stiffness [3], and was functionalised for in vitro culture of cortical or DRG cells [4,5]. Cortical cells that die on hyaluronic acid hydrogels survive and differentiate when poly-D-lysine (PDL) was covalently bound [6]. Differentiation and neurite outgrowth of forebrain neurons increases as poly(ethylene glycol) (PEG) based hydrogels degrades to create porosity [7].

Chitosan is a well-known biodegradable polysaccharide used in biomedical and cosmetic applications. It is derived from chitin found in crustacean shell which, together with chitosan, is the second most abundant polysaccharide in nature, after cellulose. Tissue engineering applications include dehydrated sponges that absorb fluid [8,9], material for encapsulating cells [10,11] or as a gel [12–14]. The latter relies on the pH sensitivity of chitosan solution: chitosan is soluble in dilute aqueous conditions but precipitates into a gel at neutral pH.

Chitosan supports attachment and growth of a range of cell lines [11,15–19] but does not offer the same support for neurons [17,20]. Gong et al. [17] cultured both gliosarcoma cells (9 L) and foetal mouse cortical cells (FMCC) on chitosan films to determine biocompatibility and nerve cell affinity, respectively. They found that both could be improved to be equivalent with the polylysine control by either coating or blending with polylysine. A follow-on study by the same group determined the optimum concentration of polylysine for cell attachment to be 3 v/v% with chitosan, both in serum and serum-free conditions. This was significantly better than the collagen control surface [21], and three times greater than for chitosan.

Recently, a pH-neutral chitosan solution was developed by Chenite et al. [22] by the addition of a polyol salt (glycerophosphate salt (GP) is most commonly used). The solution forms a macroporous gel scaffold when the temperature is raised to 37 °C [23]. This quality allows the material to be injected and to form a scaffold [24] in situ with minimal surgical destruction. While chitosan/GP has been used successfully in vitro [22,25], it has not been tested with nerve cells. However, since the physical properties of the fundamental material could be utilised in tissue engineering it would be of value to maximise the biocompatibility of chitosan/GP towards neurons. Currently, chitosan and chitosan/GP need improved neuronal compatibility if they are to be used with the nervous system. Rather than blending chitosan/GP with polylysine, leaving the polylysine free to diffuse away from the material, an examination of the effect of covalently binding polylysine to chitosan was undertaken with the intention of improving the biocompatibility and neuron affinity of the system. Polylysine was chosen for this purpose because its positive nature and high hydrophilicity is known to attract neurons and promote neurite outgrowth [26–29].

This work explores cell–hydrogel interactions in different in vitro environments, in order to improve neuron affinity of chitosan/GP. Specifically, we wish to work towards developing a biodegradable and injectable scaffold that can be used in conjunction with cell replacement therapies to help repair damaged neural pathways within the brain.

2. Experimental

2.1. Materials

The chitosan (Sigma) used was of degree of deacetylation (DD) 83% as determined by ^{13}C cross-polarisation magic angle spinning nuclear magnetic resonance spectroscopy (CP/MAS NMR, data not shown) and molecular weight 9.8×10^4 Da determined by gel permeation chromatography. It was purified by dissolving in 0.1 M HCl (BDH), filtering through grade 3 filter paper (Whatman), heating, and then when cooled, stirring with granulated carbon and refiltering. The chitosan was precipitated by adding 100 mL chitosan solution drop wise to 600 mL 0.1 M KOH (Aldrich). The precipitate was collected, rinsed twice with distilled deionised water, and freeze-dried for 48 h. (PDL MW 1–4 kDa) and β-glycerophosphate disodium salt (GP), *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) and 4-azidoaniline (all Sigma) were used as received.

2.2. Functionalising chitosan

PDL, EDAC and 4-azidoaniline were mixed in 20 mL ddH_2O in the molar ratios 40:1 EDAC:PDL and 4:1 4-azidoaniline:EDAC for 4 h at 4 °C in the dark (the first reaction in Fig. 1). The solution was collected and dialysed through a 500 Da MWCO cellulose ester membrane (Spectrum) to remove unreacted species. After dialysis, the solution was lyophilised for 48 h. The next stage of the reaction was attachment of the PDL-photoreactive species to chitosan (the second reaction in Fig. 1). This was accomplished by dissolving the reactive species in 6 mL PBS, which was then added to 0.12 g chitosan powder. The chitosan/PDL solution was placed in polystyrene petri dishes (Falcon) and irradiated about 5 cm from the UV light source (a Blak Ray lamp, model B100 AP, wavelength ~360 nm, 100 W) for 2 min. The chitosan-PDL was washed thoroughly five times to remove unbound reactive PDL, frozen and lyophilised. This product was used for the following characterisation and experiments.

2.3. Characterisation of peptide chemistry

Samples were prepared for X-ray photoelectron spectroscopy (XPS) by dissolving 1 w/v% of each chitosan in 0.05 M HCl, dropping the solution onto glass microscope slides and drying. XPS analysis was performed using an AXIS-HSi spectrometer (Kratos Analytical Inc.) with a monochromated Al K_α source at a power of 180 W (12 kV × 15 mA), a hemispherical analyser operating in the fixed analyser transmission mode and the standard aperture (1 × 0.5 mm). Each specimen was analysed at an emission angle of 0° as measured from the surface normal. A circular area with a diameter of approximately 1 mm was analysed on each sample, and two such spots analysed per sample other than for unmodified chitosan, which was used to determine uncertainty by measuring 10 spots.

All elements present were identified from survey spectra (acquired at a pass energy of 320 eV). High-resolution spectra were recorded from individual peaks at 40 eV pass energy (yielding a typical peak width for polymers of 1.0–1.1 eV). These data were quantified using a minimisation algorithm in order to calculate curve-fits and thus to determine the contributions from specific functional groups. The accuracy associated with quantitative XPS is ca. 10–15%. Precision (i.e. reproducibility) depends on the signal/noise ratio but is usually much better than 5%. The latter is relevant when comparing similar samples.

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