

Polylysine-functionalised thermoresponsive chitosan hydrogel for neural tissue engineering

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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

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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 (9L) 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 ¹³C 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 ddH₂O 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 K₂ source at a power of 180 W (12 kV \times 15 mA), a hemispherical analyser operating in the fixed analyser transmission mode and the standard aperture (1 \times 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.

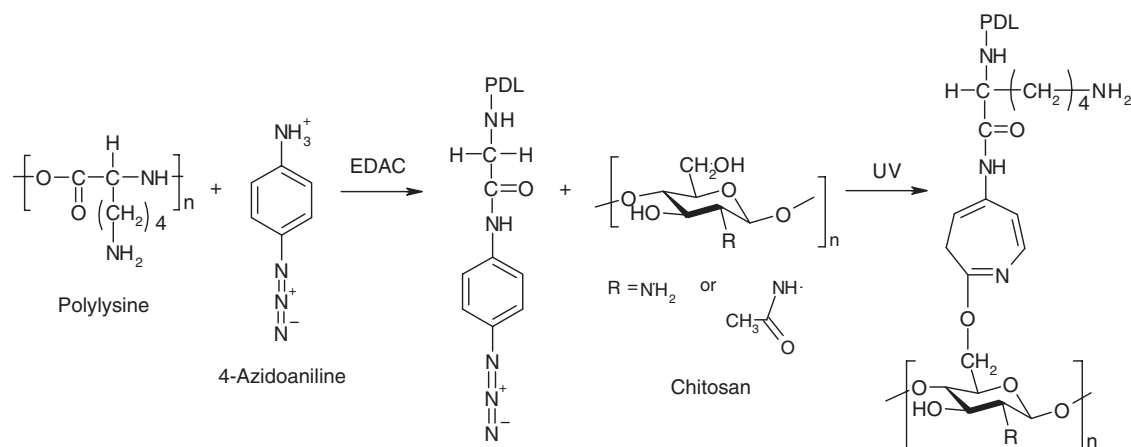


Fig. 1. Attachment of polylysine to photoreactive species, followed by attachment of both to chitosan.

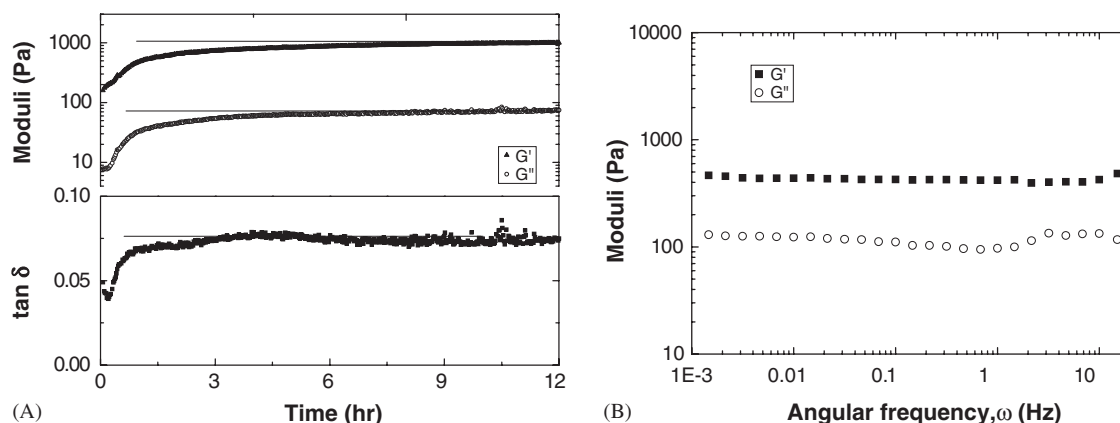


Fig. 2. An isothermal rheology scan (37 °C) of 1 w/v% chitosan/GP, showing gel evolution and stabilisation at 0.1 Hz (A) and final strength across a range of frequencies (B).

2.4. Sample preparation

Sample solutions were made by dissolving 0.8 or 0.5 w/v% chitosan in HCl to a molar ratio of 0.9:1 with chitosan amine group. While in an ice bath, 3.7 M GP was added drop wise to the solutions, to a molar ratio of 12:1 with chitosan and with isotonicity that was close to that of extracellular fluid. Solutions of different concentrations were made by mixing unreacted chitosan with the PDL-attached chitosan.

2.5. Rheology

Oscillatory rheology studies were performed on a Bohlin CS-50 rheometer in parallel plate configuration. 300 μ L chitosan/GP solution (see below for composition) was pipetted onto the plate and a single frequency of 0.1 Hz was used for the duration of a 12 h scan. After this period of time, the gel properties had stabilised and final measurements were taken from high (100 Hz) to low (0.001 Hz) frequency.

2.6. Vapour pressure osmometry

In order to measure the osmolarity of the chitosan/GP solutions, a Vapro vapour pressure osmometer (Wescor 5520) was used. The sample chamber was cleaned with ddH₂O before dropping 10 μ L samples onto a paper filter at room temperature.

2.7. Preparation of hydrogels for cell culture

Solutions were sterilised by ultrafiltration, and either 15 μ L (for 2D culture on a film) or 150 μ L (for 3D culture in a gel) of each was laid into the bottom of a 48 well plate in triplicate. This was kept cool until cells were added.

Chitosan films were made by dissolving 0.8 w/v% chitosan in HCl as described above, coating the bottom of the culture well with this solution and then washing with 0.1 M NaOH to cause gelation of the film. This was then washed with sterile MilliQ water and then Kreb's solution (7.25 g/L NaCl, 0.4 g/L KCl, 0.14 g/L NaH₂PO₄ · H₂O, 2.6 g/L D-glucose, 0.01 g/L phenol red and 5.94 g/L Hepes in acid form).

2.8. 2-D cell culture on a film

Fourteen day pregnant, time mated C57 mice were anaesthetised (isoflurane, Isoflo, Abbott Laboratories) and then euthanized by cervical dislocation. Their embryos were removed, placed in Kreb's solution where the brain was dissected out and the olfactory bulbs and meninges removed. The cortex was then dissected from the fore brain and chopped into small pieces with a scalpel. A suspension of isolated FMCC was produced by trypsinising (125 μ g/mL Kreb's solution, Sigma) the pieces of cortex at 37 °C while shaking for 20 min, then mixing with a solution of soybean trypsin inhibitor (SBTI) and DNase (89 and 14 μ g/mL, respectively, both Sigma) to further separate tissue. The solution was

centrifuged for 1 min at 1,000 rpm to collect the tissue pieces, which were then resuspended in a more concentrated SBTI/DNase solution (2.75 and 0.42 mg/mL Krebs's solution, respectively) and triturated. This solution was allowed to stand for 10 min, and the isolated cells (which remain in suspension) were collected with the supernatant, which was centrifuged again (5 min, 2,000 rpm) to collect the cells. These cells were resuspended in 1 mL plating media (Dulbecco's Modified Eagle's Medium, Gibco, supplemented with 10% foetal calf serum) and the cells counted on a haemocytometer. From this, the final dilution of cells was calculated to get an end plating density of 150,000 cells/cm². A negative control substrate of tissue culture plastic (TCP) and a positive control substrate of TCP coated with an optimal concentration of 0.05 mg/mL PDL (40–70 kDa, Sigma) were used for comparison purposes. After 1 h incubation, the plating medium was replaced with culture medium (containing 1 μL/mL gentamycin, 0.5 mM glutamine and 20 μL/mL B27 supplement in neurobasal medium, all from Gibco). After 6 days incubation at 37 °C with 5% CO₂, the cells were fixed with 2.5% paraformaldehyde (Sigma) solution for 1 min and then rinsed in phosphate buffered saline (PBS). An Olympus IX71 microscope with attached digital camera (4 mega-pixel resolution, CAMELIA) was used to take three images (20 × lens) per sample, one in the centre of the well and two randomly between the centre and the well wall. The images were analysed through use of a template with five vertical lines, and cells or neurites in contact with a line were counted. A neurite was defined as a process that extended greater than one cell body distance from the soma and cell counts were analysed by a student *t*-test assuming equal variance.

2.9. 3-D cell culture in a gel

Cells were dissected as for 2D culture. A concentrated aliquot (~3 μL) of FMCC suspension was pipetted into each well and mixed thoroughly with the chitosan/GP solution so that 150,000 cells were distributed within the well while adding only minimum medium to give 10⁶ cells/mL. The culture plates were then incubated for 30 min to allow the solution to gel, before 0.3 mL plating medium was added. As for 2D culture, the serum-containing medium was removed after 1 h, to be replaced with a culture medium containing the same additives as for 2D culture. This method assumes diffusion of nutrients and waste products through the gel as the cells are not in direct contact with the medium. After 6 days culture, the medium was removed and each well was washed once with PBS. 250 μL of the assay solution, which contained 2 μM calcein acetoxyethyl ester (calcein AM, Molecular Probes) in PBS, was added to each well, and the culture plates incubated at 37 °C, 5% CO₂ for 30 min. The dye was then removed and the cells washed once with PBS. Five images (20 × lens) of each sample were taken at 100 μm from the well bottom. Cell counts were analysed by a student *t*-test assuming equal variance.

3. Results and discussion

3.1. Mechanical properties of chitosan/GP

Initial gelation of chitosan/GP takes around half an hour before it becomes solid-like (Fig. 2), but because gel

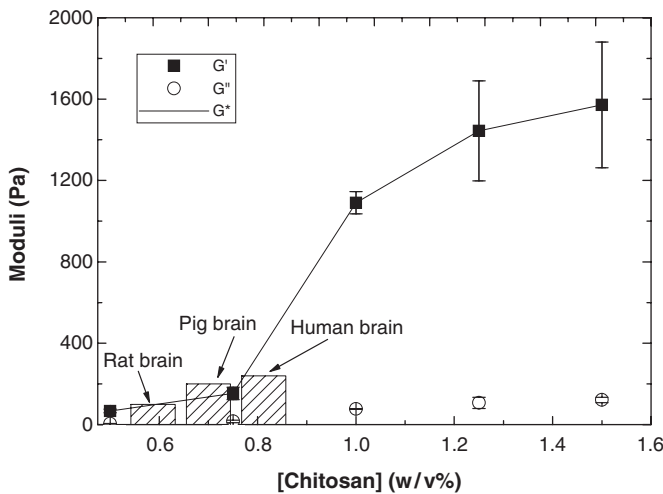


Fig. 3. Change in moduli with changing chitosan composition, compared to G* of rat [31], pig [32] and human brain [33].

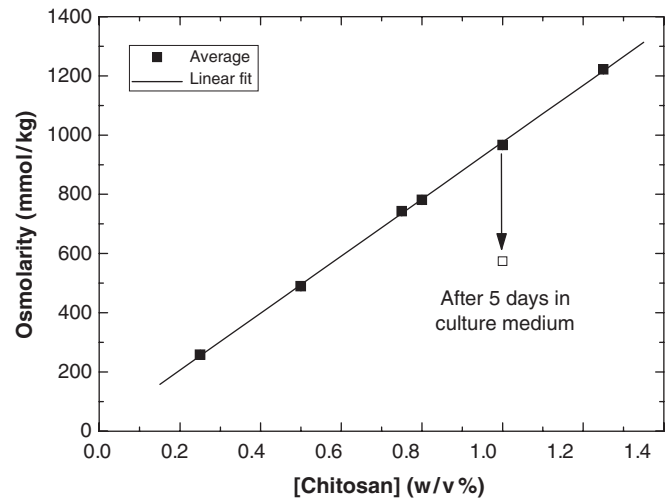


Fig. 5. Osmolarity of chitosan/GP of a range of concentrations.

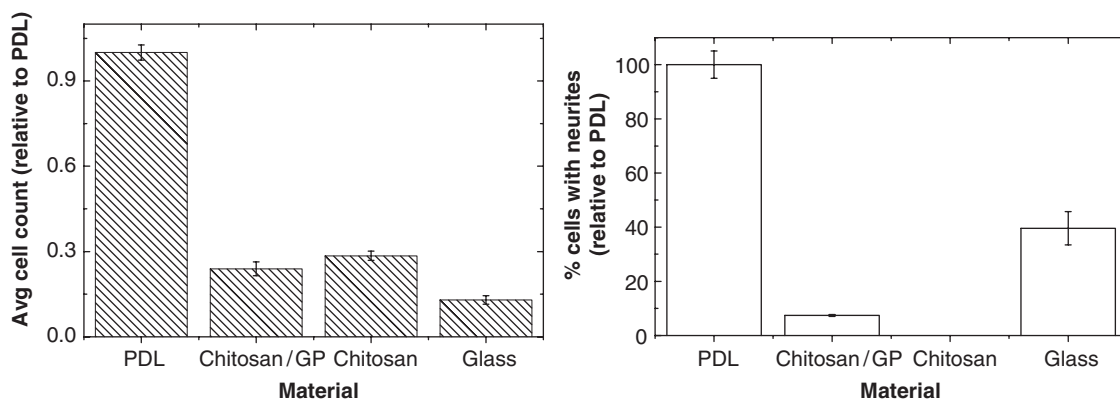


Fig. 4. Neuron affinity of 0.8 w/v% chitosan and chitosan/GP.

formation slows diffusion and mobility of the polymer chains, gel properties do not stabilise for another 9 h [30]. The strength of the gels varies non-linearly with chitosan concentration and ranges from 67 to 1572 Pa. Because the lower concentrations are of similar stiffness to brain tissue [31–33] they are suitable in vitro approximations of brain tissue when implanted into the brain as a scaffold and for this reason 0.8 w/v% chitosan/GP was chosen as the composition most suited for use with neural cells (Fig. 3).

3.2. Neuron affinity of chitosan/GP

Survival of FMCCs grown on 0.8 w/v% chitosan or chitosan/GP films was considerably less than on PDL, although significantly greater than on glass (Fig. 4). Cell survival was quantified by counting cell bodies in each field of view with an overlay template to simplify the task. The cells that did survive on 0.8 w/v% chitosan or chitosan/GP did not differentiate to send out neurites. Fig. 4 shows that neither of the samples support good attachment or growth of neurons, that is, they support markedly reduced neuronal numbers and the ratio of cells with neurites is reduced compared to polylysine.

The osmolarity of chitosan/GP hydrogels was measured because the significant salt (GP) content may have produced ionic strengths unsuitable for cells. Ideally, the osmolarity of a hydrogel should be isotonic to the extracellular fluid and be around 300 mOsm. The osmolarity of 0.8 w/v% chitosan/GP was 781 mOsm (Fig. 5), more than twice that of physiological extracellular fluid. With time the osmolarity of a gel would be reduced due to flow of water into the gel and ions from the gel following the osmotic gradient, and after 5 days incubating 150 μ L of 1 w/v% gel with 0.7 mL medium at 37 °C, the osmolarity of the gel was reduced to 574 mOsm. Nevertheless, the hypertonic environment provided by 0.8 w/v% chitosan/GP may explain the low cell survival and neurite outgrowth. On the other hand, chitosan without GP has very low osmolarity.

This hypothesis was tested by culturing cells on 0.5 w/v% chitosan/GP. After time in culture media, this gel should be close to isotonic with extracellular fluid. Significantly more cells survived on 0.5 w/v% chitosan/GP than on either 0.8 w/v% chitosan/GP or the polylysine control ($p < 0.001$, Fig. 6). Neurite outgrowth was also superior to cells grown on 0.8 w/v% chitosan/GP, but not on polylysine, implying that while cell adhesion to chitosan/GP is improved, it does not promote extension of neurites from neurons to the same extent as does polylysine.

Osmolarity is a very important factor regulating biocompatibility of a material with cells either in vitro or in vivo. Thus it is appropriate to compose the hydrogel with 0.5 w/v% chitosan, even with the slightly less than ideal stiffness, in favour of the superior cell survival from optimal osmolarity. While the material promotes excellent cell survival, it could be improved by increasing neurite outgrowth of cells on the material. Functionalising biopolymers is an ideal way to modify the material's chemistry without altering other properties.

3.3. Analysis of functionalisation

After functionalising chitosan with PDL of average 2.5 kDa, XPS data clearly revealed the existence of PDL by the altered nitrogen: carbon ratio (XPS scans not shown). There are 5 ± 2 PDL molecules per 100 chitosan amines (see Table 1).

Table 1
Nitrogen to carbon ratio of various chitosans

Material	Chitosan	4-azidoaniline	PL
Theoretical N/C ratio	0.16 ^a	0.33	0.33
Experimental N/C ratio (attached to chitosan)	0.13		0.12
% chitosan mer with bound PDL	0		5 ± 2

^aThis was determined experimentally by ¹³C CP/MAS NMR spectroscopy.

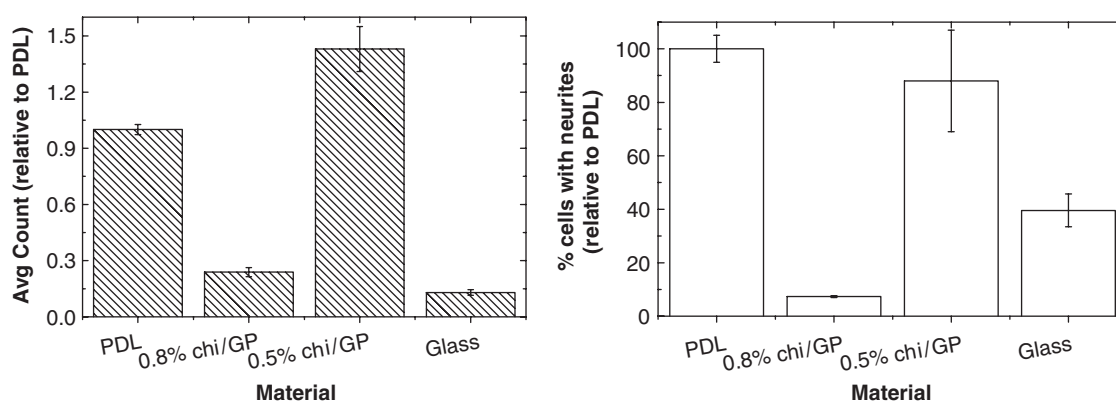


Fig. 6. Testing reduced chitosan/GP concentration (0.5 wt%) with FMCCs in culture on a film.

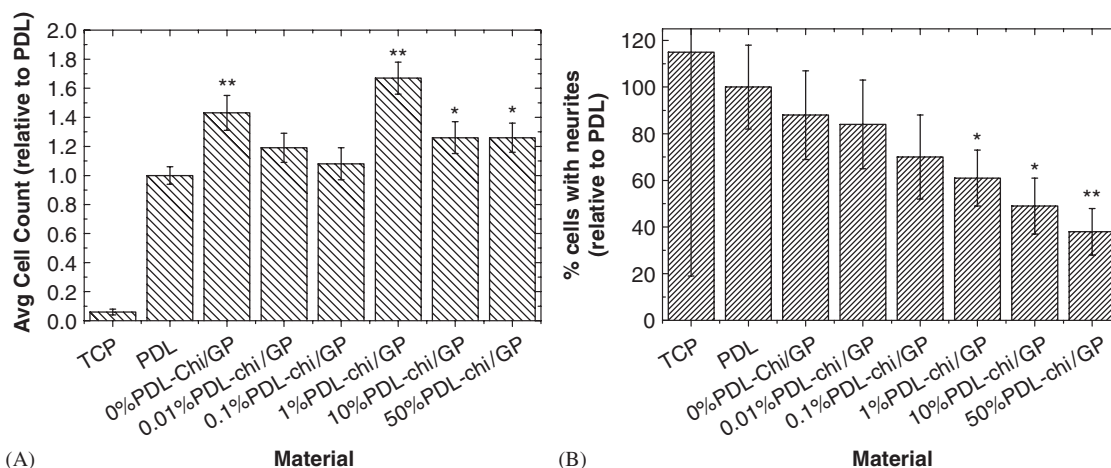


Fig. 7. Effect of PDL addition on 0.5 w/v% chitosan/GP biocompatibility with FMCC cells in 2D. (A) Average cell count normalised to poly-D-lysine; (B) average count of neurites per cell, normalised to poly-D-lysine. Tissue culture plastic (TCP) and poly-D-lysine (PDL) used as negative and positive controls, respectively, and the 0%PDL-chi/GP sample is plain chitosan/GP. The PDL concentrations given here are relative to undiluted PDL-chitosan/GP, but can be referred to Table 2 for absolute values. * indicates significant difference from PDL ($p < 0.05$), ** indicates ($p < 0.001$).

3.4. Culture on a functionalised film

Functionalising chitosan with polylysine did not alter cell survival, which was independent of polylysine concentration (Fig. 7A). However, with increasing PDL concentration, neurite outgrowth decreased (Fig. 7B). The toxicity of polylysine at high compositions [34] is attributed to cell membrane damage by the high positive charge [35]. However, this should affect cell survival more than neurite extension, suggesting that polylysine has another mechanism for inhibiting neurite outgrowth (and possibly other cellular functions). Biocompatibility (number of neurons supported and the ratio of the number of neurites per neuron) of chitosan was not improved by modifying it with any of the tested compositions of PDL (Table 2).

These results indicate that 0.5 w/v% chitosan/GP is highly biocompatible, to the extent that cell survival and neurite outgrowth cannot be improved by modifying the material with polylysine. There is not enough difference between the samples to determine the effect of the modifications. Moving to a 3D culture model, which distributes the same number of cells in a greater volume reduces cell density and therefore should reveal differences between PDL concentrations.

3.5. Culture in a 3D gel

About 0.5 w/v% chitosan/GP is biocompatible and neurite outgrowth cannot be improved by modifying the material with polylysine. However, 2D cultures do not represent the 3D environment of cells within scaffolds within the brain. In 3D culture, cells are completely surrounded in material rather than only having contact from below and the same number of cells as in 2D will have reduced “plating” density in 3D. However, since these

Table 2

Concentration of each of the PDL-modified hydrogels used in following studies

Material referred to as	100%	50%	10%	1%	0.1%	0.01%
Number of PDL molecules per 100 chitosan amines	5 ± 2	2.5	0.5	0.05	0.005	0.0005

effects are the same for each hydrogel tested they should not affect comparison of the materials. Nevertheless, there are marked differences between cells cultured in 2D and 3D [36]. We observed differences in the morphology of cells between these two culture systems. The cell bodies of cortical neurons cultured on a film are adherent and adopt the elongated spindle form typical of cells cultured on polylysine (Fig. 8A). Neurites are usually multiple, giving the cell a star-like shape. By comparison we (and others [37]) found cortical cells cultured in 3D within the chitosan/GP hydrogel had a round cell body and extended only a single neurite (Fig. 8B).

We found there were a significantly greater number of cells at a depth of only 200 μm from the bottom of the wells than higher in the hydrogels at 500 μm for all samples. Cells sink from their originally random positions within the chitosan/GP solution as it gels over half an hour. 3D culture was henceforth measured at a constant depth of 100 μm from the bottom of the well.

The cells survived well when cultured on the PDL-chitosan/GP films of all PDL compositions (Fig. 7A). In contrast, survival of cells cultured in 3D as revealed by calcein fluorescence was substantially greater when PDL concentration was at 0.01% and 0.1% and almost negligible at PDL concentrations higher than 50% (Fig. 9A).

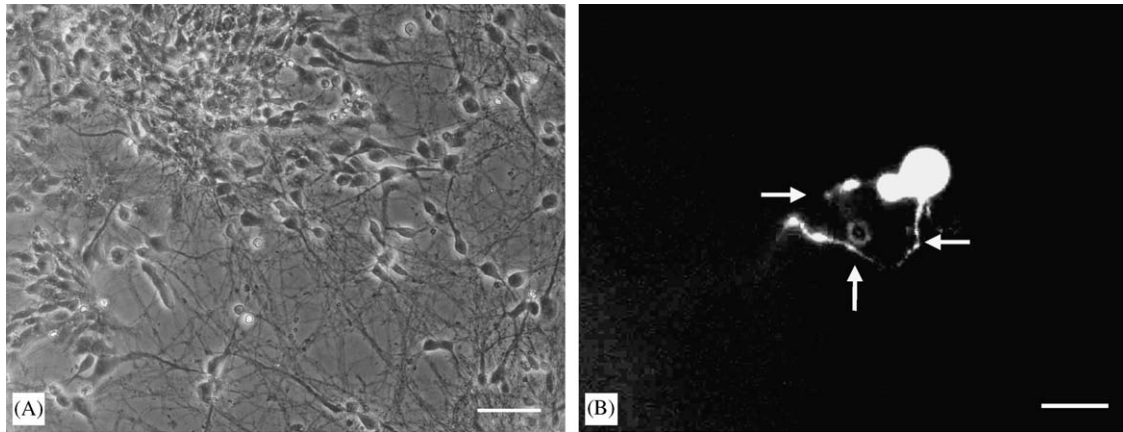


Fig. 8. FMCCs cultured 2D (A, phase contrast optical microscopy) and 3D (B, epifluorescence microscopy using the same lens) with 0.5 w/v% chitosan/GP. Arrows point to neurites extending in 3D, and scale bars represent 50 μm .

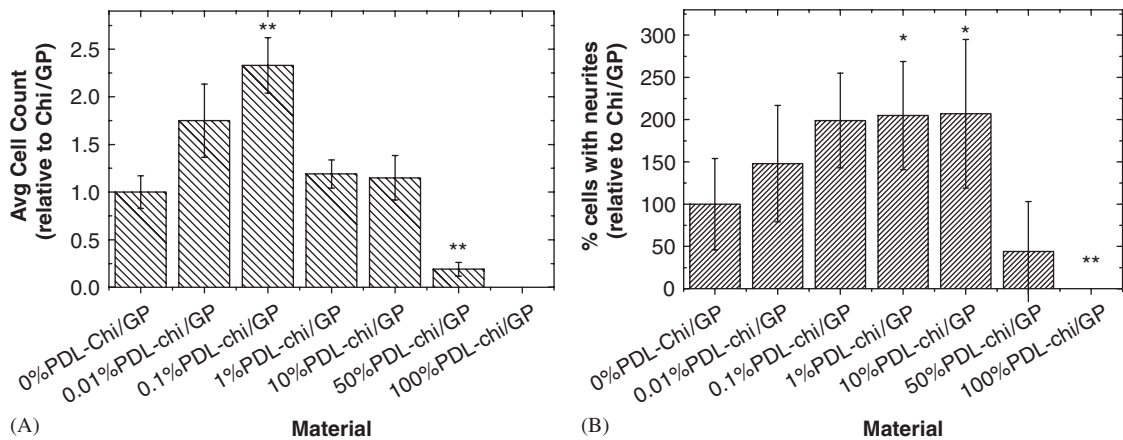


Fig. 9. Effect of PDL addition on 0.5 w/v% chitosan/GP biocompatibility in 3D. (A) Average cell count normalised to chitosan/GP; (B) average count of neurites per cell, normalised to chitosan/GP (0% PDL-chi/GP). The PDL concentrations given here are relative to undiluted PDL-chitosan/GP, but can be referred to Table 1 for absolute values. * indicates significant difference from Chi/GP ($p < 0.05$), ** indicates ($p < 0.001$).

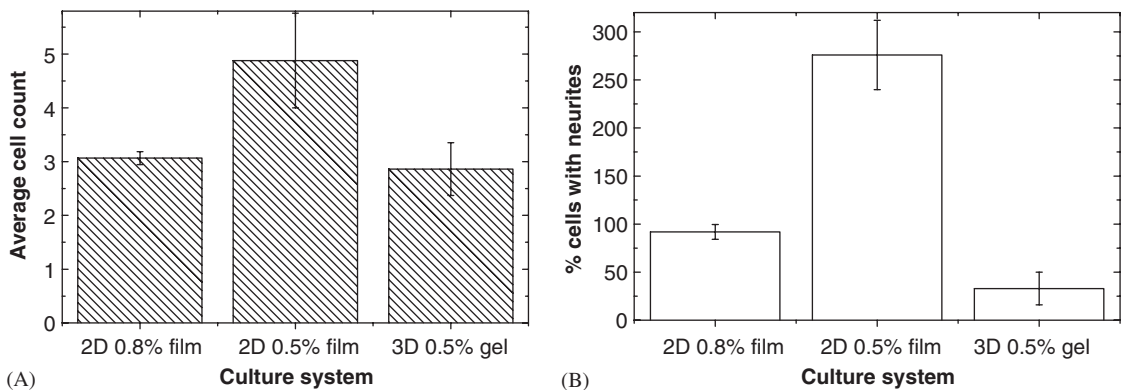


Fig. 10. Summary and comparison of the culture systems used to assess (A) FMCC survival and (B) neurite outgrowth chitosan/GP hydrogels.

The effect of PDL concentration on neurite outgrowth in 3D cultures was not clear although outgrowth was least at very high and very low concentration of attached PDL (Fig. 9B), and in contrast to the effects of PDL concentra-

tion on 2D cultures (Fig. 7B). This implies that the effect of PDL on cell survival is more acute when neurons are entirely surrounded by chitosan/GP hydrogel. When surrounded by hydrogel, cells are effectively exposed to

higher, and possibly toxic concentrations of PDL, reflected in increased cell death [34,38,39]. The effect on 2D cultures would be more subtle and the increase in PDL concentrations may only be sufficient to affect neurite outgrowth and not cause cell death.

In the 3D culture model, the survival of cells on chitosan/GP was less than the PDL-modified chitosan/GP and highlighted differences between samples. More cells survived when cultured 2D rather than 3D (an average count of 4.9 decreased to 2.9 in each field of view, Fig. 10 open bars) although this includes the effect of changed cell density, and neurites were inhibited in 3D (2.8 neurites/cell when cultured in 2D on 0.5 w/v% chitosan/GP, compared to 0.3 neurites/cell when cultured in 3D, Fig. 10 hashed bars).

The primary physical difference between chitosan and chitosan/GP is the presence of salt. However, at 0.5 w/v% the gel is isotonic to extracellular fluid and similar to chitosan immersed in physiological solutions. The GP itself is not likely to interact with cells and therefore interactions with chitosan are more likely to govern the cell response. GP will form secondary bonds with chitosan, altering the polarity and availability of the chitosan chain. Neurons are sensitive to charge [40] and hence it is possible that altering the chemistry of the substrate through addition of GP affects neuronal affinity.

4. Conclusion

Chitosan/GP has good cell adhesion properties and good neuron compatibility at low concentrations. It also supported neurons cultured three-dimensionally within the hydrogel, a better approximation to an ECM-like environment. Neuron survival was improved with the covalent attachment of polylysine to chitosan: cell survival doubled although neurite outgrowth did not change at an optimal concentration of 0.05% of chitosan mers with an attached polylysine molecule. Chitosan/GP functionalised with polylysine is an excellent *in vitro* substrate and scaffold for cortical cells, and may be useful for neural tissue engineering as an injectable scaffold.

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References

- [1] Ratner BD, Hoffman AS. In: Andrade JD, editor. *Hydrogels for medical and related applications*. Washington, DC: ACS; 1976. p. 1–36.
- [2] Bellamkonda R, Ranieri JP, Bouche N, Aebischer P. Hydrogel-based three-dimensional matrix for neural cells. *J Biomed Mater Res* 1995;29:663–71.
- [3] Balgude AP, Yu X, Szymanski A, Bellamkonda R. Agarose gel stiffness determines rate of DRG neurite extension in 3D cultures. *Biomaterials* 2001;22:1077–84.
- [4] Bellamkonda R, Ranieri JP, Aebischer P. Laminin oligopeptide derivatized agarose gels allow three-dimensional neurite extension *in vitro*. *J Neurosci Res* 1995;41:501–9.
- [5] Luo Y, Shoichet MS. A photolabile hydrogel for guided three-dimensional cell growth and migration. *Nat Mater* 2004;3:249–53.
- [6] Tian WM, Hou SP, Ma J, Zhang CL, Xu QY, Lee IS, et al. Hyaluronic acid-poly-D-lysine-based three-dimensional hydrogel for traumatic brain injury. *Tissue Eng* 2005;11:513–25.
- [7] Mahoney MJ, Anseth KS. Three-dimensional growth and function of neural tissue in degradable polyethylene glycol hydrogels. *Biomaterials* 2006;27:2265–74.
- [8] Lai HL, Abu-Khalil A, Craig DQM. The preparation and characterisation of drug-loaded alginate and chitosan sponges. *Int J Pharm* 2003;251:175–81.
- [9] Gingras M, Paradis I, Berthod F. Nerve regeneration in a collagen-chitosan tissue-engineered skin transplanted on nude mice. *Biomaterials* 2003;24:1653–61.
- [10] Li RH, White M, Williams S, Hazlett T. In: Shoichet MS, Hubbell JA, editors. *Polymers for tissue engineering*. Lincoln, RI, USA: VSP; 1998. p. 235–54.
- [11] Zielinski BA, Aebischer P. Chitosan as a matrix for mammalian cell encapsulation. *Biomaterials* 1994;15:1049–56.
- [12] Gutowska A, Jeong B, Jasionowski M. Injectable gels for tissue engineering. *Anat Rec* 2001;263:342–9.
- [13] Drury JL, Mooney DL. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* 2003;24:4337–51.
- [14] Khor E, Lim LY. Implantable applications for chitin and chitosan. *Biomaterials* 2003;24:2339–49.
- [15] Bumgardner JD, Wiser R, Gerard PD, Bergin P, Chestnutt B, Marini M, et al. Chitosan: potential use as a bioactive coating for orthopaedic and craniofacial/dental implants. *J Biomater Sci Polym Ed* 2003;14:423–38.
- [16] Zhu Y, Gao C, He T, Liu X, Shen J. Layer-by-layer assembly to modify poly(L-lactic acid) surface toward improving its cytocompatibility to human endothelial cells. *Biomacromolecules* 2003;4:446–52.
- [17] Gong HP, Zhong YH, Li JC, Gong YD, Zhao NM, Zhang XF. Studies on nerve cell affinity of chitosan-derived materials. *J Biomed Mater Res* 2000;52:285.
- [18] Dhiman HK, Ray AR, Panda AK. Characterization and evaluation of chitosan matrix for *in vitro* growth of MCF-7 breast cancer cell lines. *Biomaterials* 2004;25:5147–54.
- [19] Elçin AE, Elçin YM, Pappas GD. Neural tissue engineering: adrenal chromaffin cell attachment and viability on chitosan scaffolds. *Neurol Res* 1998;20:648–54.
- [20] Mingyu C, Kai G, Jiamou L, Yandao G, Nanming Z, Xiufang Z. Surface modification and characterization of chitosan film blended with poly-L-lysine. *J Biomater Appl* 2004;19:59–75.
- [21] Cheng MY, Gong K, Li J, Gong YD, Zhao NM, Zhang XF. Surface modification and characterization of chitosan film blended with poly-L-lysine. *J Biomater Appl* 2004;19:59–75.
- [22] Chenite A, Chaput C, Wang D, Combes C, Buschmann MD, Hoemann CD, et al. Novel injectable neutral solutions of chitosan form biodegradable gels *in situ*. *Biomaterials* 2000;21:2155–61.
- [23] Chenite A, Buschmann M, Wang D, Chaput C, Kandani N. Rheological characterisation of thermogelling chitosan/glycerol-phosphate solutions. *Carbohydr Polym* 2001;46:39–46.
- [24] Crompton KE, Prankerd RJ, Paganin DM, Scott TF, Horne MH, Finkelstein DI, et al. Morphology and gelation of thermosensitive chitosan hydrogels. *Biophys Chem* 2005;117:47–53.
- [25] Hoemann CD, Sun J, Legare A, McKee MD, Buschmann MD. Tissue engineering of cartilage using an injectable and adhesive chitosan-based cell-delivery vehicle. *Osteoarthritis Cartilage* 2005;13:318–29.
- [26] Grinnel F. Cellular adhesiveness and extracellular substrata. *Int Rev Cytol* 1978;53:65–144.

- [27] Blau A, Weini C, Mack J, Kienle S, Jung G, Ziegler C. Promotion of neural cell adhesion by electrochemically generated and functionalized polymer films. *J Neurosci Methods* 2001;112:65–73.
- [28] Jacobson BS, Branton D. Plasma membrane: rapid isolation and exposure of the cytoplasmic surface by use of positively charged beads. *Science* 1977;195:302–4.
- [29] Vogt AK, Lauer L, Knoll W, Offenhäusser A. Micropatterned substrates for the growth of functional neuronal networks of defined geometry. *Biotechnol Prog* 2003;19:1562–8.
- [30] Cho J, Heuzey M-C, Begin A, Carreau PJ. Physical gelation of chitosan in the presence of b-glycerophosphate: the effect of temperature. *Biomacromolecules* 2005;6:3267–75.
- [31] Woerly S, Pinet E, de Robertis L, Bousmina M, Laroche G, Roitback T, et al. Heterogeneous PHPMA hydrogels for tissue repair and axonal regeneration in the injured spinal cord. *J Biomater Sci Polym Ed* 1998;9:681–711.
- [32] Brands DWA, Bovendeerd PHM, Peters GWM, Wismans JSHM. The large shear strain dynamic behaviour of in vitro porcine brain tissue and a silicone gel model material. *Stapp Car Crash J* 2000;44:249–60.
- [33] Hirakawa K, Hashizume K, Hayashi T. Viscoelastic property of human brain-for the analysis of impact injury (author's transl). *Brain Nerve* 1981;33:1057–65.
- [34] Varon S. The culture of chick embryo dorsal root ganglionic cells on polylysine-coated plastic. *Neurochem Res* 1979;4: 155–73.
- [35] Franklin TJ, Snow GA. *Biochemistry of antimicrobial action*. London: Chapman & Hall; 1981.
- [36] Sun T, Jackson S, Haycock JW, Macneil S. Culture of skin cells in 3D rather than 2D improves their ability to survive exposure to cytotoxic agents. *J Biotechnol* 2006;122:372–81.
- [37] Ahmed I, Liu HY, Mamiya PC, Ponery AS, Babu AN, Weik T, et al. Three-dimensional nanofibrillar surfaces covalently modified with tenascin-C-derived peptides enhance neuronal growth in vitro. *J Biomed Mater Res A* 2006;76:851–60.
- [38] Hill IRC, Garnett MC, Bignotti F, Davis SS. In vitro cytotoxicity of poly(amidoamine)s: relevance to DNA delivery. *Biochim Biophys Acta* 1999;1427:161–74.
- [39] Kotecha B, Richardson GP. Ototoxicity in vitro: effects of neomycin, gentamicin, dihydrostreptomycin, amikacin, spectinomycin, neamine, spermine and poly-L-lysine. *Hear Res* 1994;73: 173–84.
- [40] Makohliso SA, Valentini RF, Aebischer P. Magnitude and polarity of a fluoroethylene propylene electret substrate charge influences neurite outgrowth in vitro. *J Biomed Mater Res* 1993;27: 1075–85.