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Inflammatory response on injection of chitosan/GP to the brain

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Abstract Chitosan is a well-known biomaterial that, with the addition of glycerophosphate salt (GP), gels at physiological temperatures and therefore is useful for tissue engineering purposes. This study examines the procedure of injecting chitosan/GP to the brain in order to form a gel track. The gel system and surgical technique were successful in this endeavour; however, on examining the inflammatory response to the material it was found that the chitosan/GP was wholly engulfed by macrophages within 7 days. This was determined by staining for both the gel and the macrophages, an important technique for localising injected material. The chitosan/GP-containing macrophages formed a neat tract at the lesion site, but after 45 days no chitosan/GP was found. It was concluded that, although chitosan/GP is present after implantation, it is not available for direct scaffolding in the brain.

1. Introduction

Chitosan is a $(1, 4)$-linked 2-amino-2-deoxy-β-D-glucan (see Fig. 1), produced by deacetylating chitin that has wide ranging biomedical and cosmetic applications. It has interesting properties attracting interest in its use as a potential scaffold material for tissue repair, including antibacterial activity [1] and tumoricidal properties [2]. In tissue culture it is biocompatible with many cell types and, in the presence of glycerophosphate salt (GP), soluble at low temperatures while upon heating to body temperature it forms a macroporous gel [3], making it suitable for injection into tissues and to fill irregular cavities. There is now a realistic prospect of deploying stem cells to repair organs such as the brain that have been damaged by disease or trauma. However, the deployment of these cells in the brain may require scaffolds to support them in injured tissue and to assist in the guidance of axons to relevant targets. This study was directed at the question of whether chitosan could be injected into the brain and act as a useful scaffold.

The properties of chitosan are governed primarily by the degree of deacetylation (DD), determined from the relative amounts of acetyl groups and amine groups at the C2 position (labelled *R* in Fig. 1).

Chitosan is soluble in dilute acidic solutions, but phase-separates at pH > 6 to form a hydrogel. However, the pH of a chitosan solution can be raised to neutral without causing phase-separation by addition of GP [4, 5]. The system becomes thermally sensitive, forming a gel when near body temperature. This allows the solution to flow down a needle before forming a gel in-situ at the injection site, making it ideal for filling odd-shaped volumes in a non-invasive manner.

For a material to be suitable as a tissue engineering construct, it must be non toxic, facilitate tissue outgrowth, and remain stable in its implanted location for a sufficient period to allow tissue repair. This implies minimal inflammatory response and relatively slow degradation. While chitosan has been regarded a suitable scaffold material, the evidence is divided regarding its capacity to meet these requirements. Chitosan is biocompatible with many cell types [6–15]. Its

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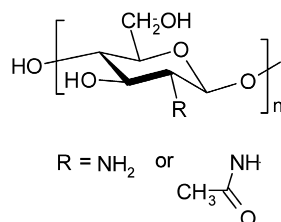
Fig. 1 Chemical structure of chitosan.



toxicity (83% deacetylated) was systematically investigated [16] and no toxic effects were found following oral, intravenous or intraperitoneal administration, and only minimal effects after intranasal administration. Chitosan also attracts and activates macrophages and elicits a brisk inflammatory response [6, 17, 18] that can be reduced by increasing the DD [19, 20]. Implants of mid-range deacetylated chitosan produce a prominent inflammatory response after implantation [19, 20] with chitosan fragments found near or within macrophages [6]. On the other hand, 100% deacetylated chitosan elicited very little inflammation, and appeared to be bioinert. The addition of GP to raise the pH of chitosan solution to neutral without causing phase-separation [4, 5] is complicated by the factor of high ionic strength, although the presence of salt does not appear to account for the severity of associated inflammatory reactions [21, 22].

Chitosan has been used for neural tissue engineering previously [22–26] with mixed results [22, 23, 25]. Polylysine has been added to chitosan [27] to improve biocompatibility, especially for use with neurons. Polylysine is well known for its high biocompatibility with neurons and its promotion of neurite outgrowth [28–31], presumably because it is a positively charged molecule [28]. Mixing and coating chitosan with polylysine improves cellular adhesion and neurite outgrowth of gliosarcoma (9L) cells and foetal mouse cerebral cortex (FMCC) cells [22, 27].

In-vivo chitosan is enzymatically degraded by oxidation and lysozyme [32, 33], a plentiful glycosidase in the body that acts on N-acetyl glucosamine. Therefore, the amount and distribution of the acetyl groups on the backbone strongly affect how fast the material is broken down - the rate of degradation is slower at higher deacetylation [5, 19, 32]. Tomihata and Ikada found that chitosan of 69% DD degraded by 2 weeks *in-vivo,* 85% DD chitosan lost 20% of mass after 12 weeks and 100% DD chitosan showed no appreciable mass loss after 12 weeks [19].

Taken together this suggests that chitosan is an appropriate material for long-term implantation as tissue-engineered scaffolds: it is biocompatible with neurons and, at high DD, it does not induce much inflammation [19, 20] and is only slowly degraded [19]. This study originally set out to examine the optimum chemical conformation of chitosan to use as a scaffold by testing its persistence in the brain in a form suitable for a scaffold. However, we found that chitosan was promptly engulfed by macrophages and is not suitable as a scaffold material.

2. Methodology

2.1. Animals

12 male Wistar rats weighing approximately 300 g were used in these studies. All methods conform to the Australian National Health and Medical Research Council published code of practice for the use of animals in research and were approved by the Howard Florey Institute Animal Ethics Committee.

2.2. Materials

Chitosan (Sigma) 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 hrs. Commercial chitosan (Ultrasan, BioSyntech) was used for comparison purposes, and β-glycerophosphate disodium salt (GP, Sigma) was used as received. Poly-D-lysine hydrobromide (Sigma) was dissolved in ddH_2O to a concentration of 0.6 mg/mL.

2.3. ^{13}C Cross polarisation magic angle spinning nuclear magnetic resonance (CP/MAS NMR) spectroscopy

In order to determine the degree of deacetylation, high-resolution solid state ^{13}C CP/MAS NMR was undertaken on a Varian Unity Plus spectrometer at room temperature, using previously well-dried samples. The resonance frequency used was 75 MHz, contact time 1 ms (optimised by measuring in the range of 10 μs to 10 ms), while the relaxation delay time was 2 or 5 s. The 90° pulse was of 4.5 μs and the spinning rate for MAS was 8–10 kHz. The degree of deacetylation of purified chitosan samples was found to be $85 \pm 2\%$, while for Ultrasan was found to be $92 \pm 2\%$ (manufacturer characterises as 93–97%).

2.4. Gel permeation chromatography (GPC)

To determine the molecular weight and polydispersity of chitosan used, GPC was employed. Purified chitosan was dissolved in 0.2 M acetic acid/0.1 M sodium acetate to a concentration of 0.5 w/v% (5 mg/mL) and filtered through a 0.22 μm PVDF membrane. The flow rate used was 0.5 mL/min, at 35°C, the HPLC system comprised DGU-12A degasser, LC-10AT VP pump and CTO-10A VP column oven (Shimadzu). The columns were Ultrahydrogel 2000 and 250 (Waters) in series with an Ultrahydrogel guard column, with eluent of 0.2 M acetic acid/0.1 M potassium acetate, filtered

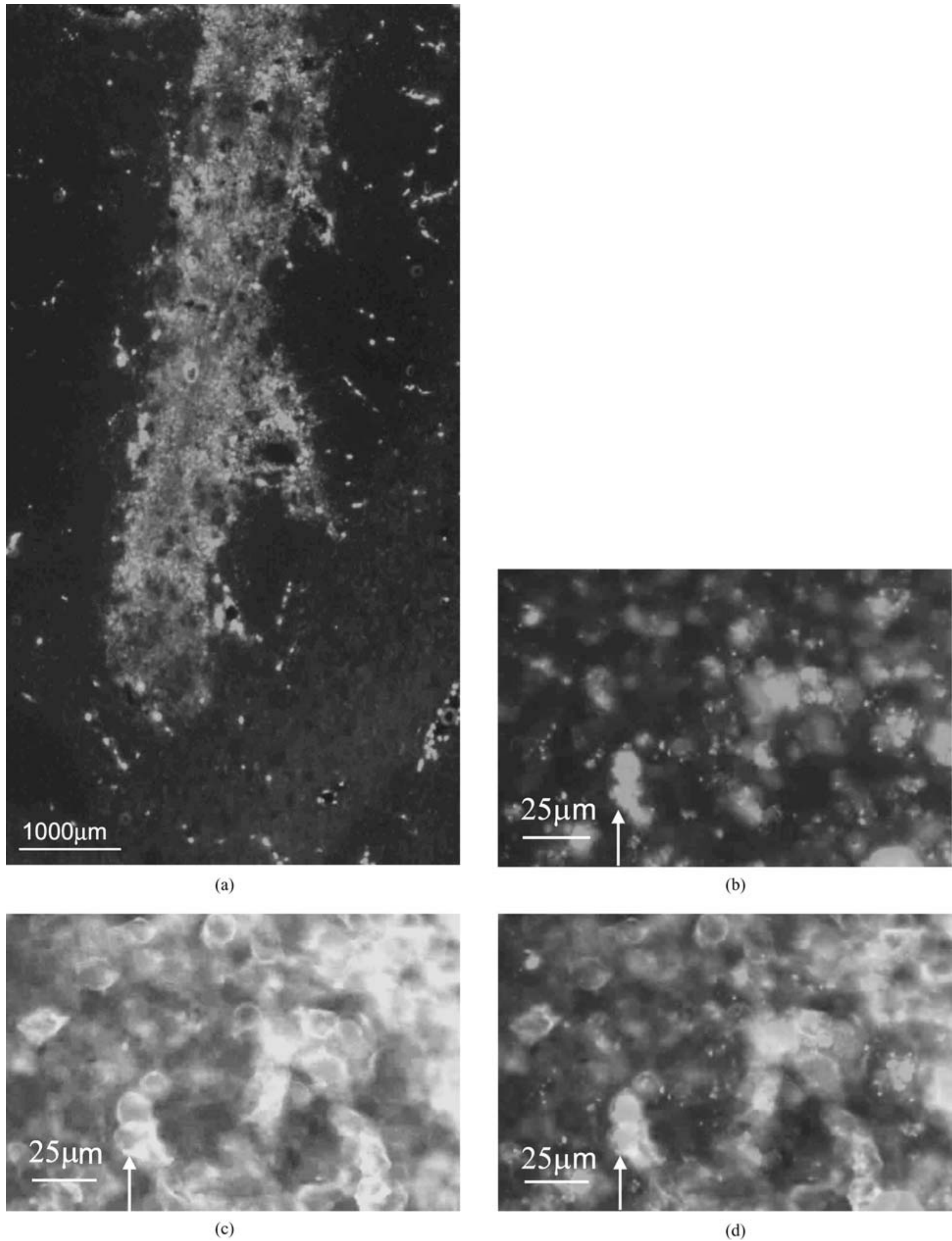


Fig. 2 A is a low power photomicrograph of a chitosan gel tract in the striatum, taken 7 days after injection. Figs. B, C and D are higher power photomicrographs of the same tract. The three figures are of the same field: A and B are with red and green filters showing chitosan (red, Texas Red) and microglia (green, OX-42) respectively. C is the subtraction of figures B and C with co-labelling showing as yellow. Note that the OX-42 labels the macrophage membrane and appears as a ring surrounding the Texas Red labelled chitosan.

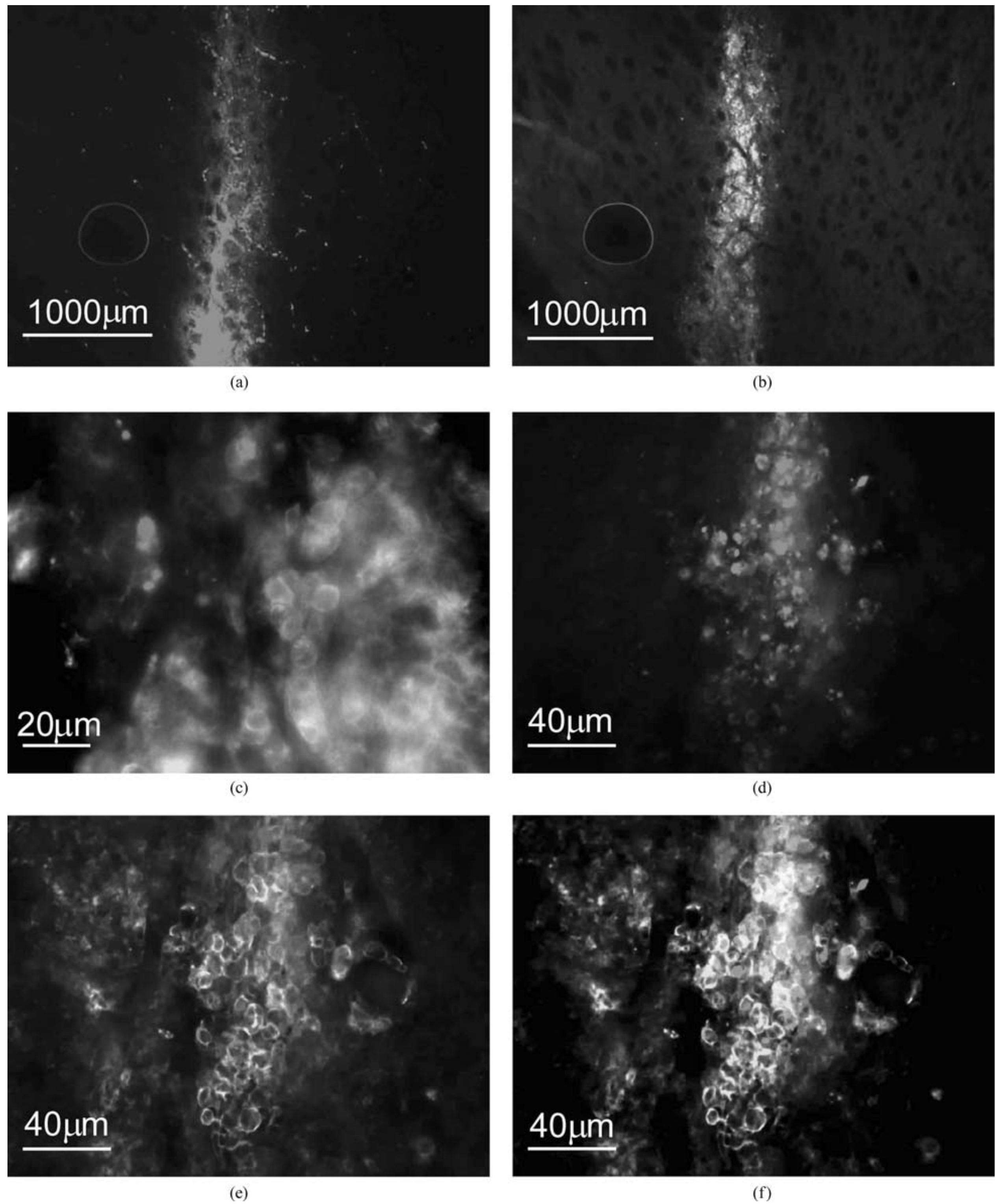


Fig. 3 A and B shows chitosan/GP-polylysine mix with the chitosan lying apparently as a gel tract that is identical in outline to the green OX-42 macrophages (B). Fig. C shows a high power subtraction showing much of the chitosan within macrophages. Figs. D, E and F show the same view of a portion of an Ultrasan tract with chitosan (D, red, Texas Red)), microglia (E, green, OX-42) respectively and F is the subtraction of figures D and E with co-labelling showing as yellow.

under vacuum and degassed. The light scattering detection was performed by multi-angle laser light scattering (MALLS, DAWN EOS, Wyatt Technology), quasi-elastic light scattering (QELS, Wyatt Technology) and differential refractometry (Optilab DSP, Wyatt Technology) to determine polymer concentration. A bovine serum albumin (BSA, 5.0 mg/ml) standard was used. Calculated values are taken from the Astra 4.90.07 control software running under Win2000.

2.5. Sample preparation for *in-vivo* injection

Sample solutions were made by dissolving 0.8 w/v% chitosan or Ultrasan in HCl to a molar ratio of 0.9:1 with chitosan or Ultrasan 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 the amine.

Chitosan/GP was mixed with polylysine (PL) by preparing the chitosan/GP solution as above and then adding 0.6 mg/mL PL solution drop wise to an end concentration of 3 w/w% with chitosan. All solutions prepared for injection were sterilised by ultrafiltration.

2.6. *In-vivo* injection

The rat was anaesthetised (Nembutal, Merial, 0.1 mL/100 g body weight, supplemented with 0.1 mL atropine in saline) and a craniotomy was made over the right and left striatum (from bregma: anteroposterior 0.2 mm, lateral 3 mm).

The chistosan/GP solution was filtered and Dextran Texas Red (Molecular Probes) was added at a concentration of 1:100. The chilled chitosan solution was drawn up into 100 μL syringe placed in a motorised injection pump and attached by tubing to a 23G1$\frac{1}{4}$ needle held in a stereotaxic guided micromanipulator. The rat was positioned in the stereotaxic frame and the needle was inserted through the craniotomy to a depth of 5.5 mm. Injection of chitosan solution began at this depth and slow extrusion of chitosan was continued as the needle was slowly withdrawn from the brain at rate of 250 μm/min. The injection rate was 30 μL/hr, with a total injection volume of 5 μl. This process created a gel tract of about 5 mm long extending dorsally in the striatum (3 mm below the surface in order to avoid the cortex). For the purpose of a control, a needle was inserted at corresponding co-ordinates in the opposite striatum, and injected with a saline and Texas Red mix at the same volume and rate. Animals were killed by an overdose of sodium pentobarbitone (Letho-barb; 0.35 mg/g) and perfused with 30 ml of warmed (37°C) 0.1 M PBS, pH 7.4, with heparin (1 U/ml), followed by 30 ml of chilled 4% paraformaldehyde (Sigma, St. Louis, MO) and 0.2% picric acid in 0.1 M phosphate buffer (4°C), pH 7.4. A total of 12 rats were used for the experiment, with in-vivo residence times and material injected described in Table 1.

Table 1 Experimental conditions for each material

Material	Period *in-vivo*	No. animals
Chitosan/GP	7 days	5
	45 days	4
Ultrasan/GP	3 days	2
Chitosan/GP-PL blend	3 days	1

2.7. Immunohistochemistry

Brain tissue was placed in a 30% sucrose/PBS mix for 2 days. The brains were then frozen, and cut 50 μm thick on a cryostat. Cut sections were placed free-floating in wells of cryoprotectant. Sections in which an injection tract were visible were selected for immunohistochemistry. Sections were stained with a primary antibody against OX-42 (Serotec) at a dilution of 1:100 over 2 nights, followed by a secondary antibody Anti-mouse Ig biotin conjugated (Chemicon) at 1:400 for 2 hrs, and finally Streptavidin Fluorescein conjugate (Molecular probes) at 1:100 for 2 hrs. Some sections were also counterstained with Hoechst at 1:1000 for 5 min. Stained sections were then mounted onto slides with PBS, and cover slipped with Dako Fluorescent Mounting medium. Sections were then viewed and photographed using fluorescence and confocal microscopy.

3. Results and discussion

3.1. Materials characterisation

The molecular weight (MW) and DD of chitosan and Ultrasan were compared using NMR and GPC (Table 2). These showed that Ultrasan has a higher MW and DD than chitosan and thus should degrade or be removed more slowly than chitosan.

3.2. *In-vivo* injection of chitosan/GP

Chitosan was inserted into the cortex and the surrounding inflammatory response was compared to a control stab wound. Based on the number of activated microglia and macrophages, the inflammatory response surrounding chitosan was far greater than the response to the control stab wound. Seven days after the lesion, the extruded chitosan impregnated with Texas Red could be easily seen as a tract (Fig. 2).

Table 2 Materials properties of the two chitosans used

Material	MW (Da)	DD (%)
Chitosan	9.8×10^4	85 ± 2
Ultrasan	3.75×10^5	95 ± 2

From these figures it is obvious that the method of injection allows the chitosan to be laid down as a tract of gel. First appearance suggested that the chitosan remained well preserved in its original form within the substrate of the brain despite a brisk inflammatory response. However, closer inspection showed that the chitosan was actually present as small globules almost entirely within macrophages. No chitosan could be seen when the brain was examined 45 days post-injection suggesting that the material had been entirely removed by that time.

3.3. *In-vivo* injection of Ultrasan/GP

Chitosan/GP gel may have been phagocytosed because it was readily broken apart into ingestible globules. To test this possibility, Ultrasan/GP was injected. Although Ultrasan should be more difficult to degrade than chitosan because of the higher DD and MW, the macrophage response was identical for both polymers and Ultrasan was found to be almost entirely within macrophages by three days.

3.4. *In-vivo* injection of chitosan/GP-polylysine blend

Polylysine is commonly used in-vitro to promote adhesion and neurite outgrowth, however it has rarely been used in-vivo. Because there are positive effects of mixing PL with chitosan in-vitro, it was thought that the addition of PL may reduce the inflammatory response and increase longevity of chitosan within the brain. A chitosan/GP-PL blend was injected but once more the material was almost entirely ingested by macrophages by three days post injection.

4. Conclusion

The main finding of this study is that chitosan, whether as chitosan/GP, Ultrasan/GP or chitosan/GP-polylysine blend is not suitable as a brain scaffold material because it is readily ingested by macrophages as part of a foreign body response. This will present a major obstacle for its future use as a scaffold material in the brain.

Previous reports have emphasised the marked inflammatory response, noting that its severity decreased with increasing degree of deacetylation [5, 19, 20]. Hidaka and colleagues [20] implanted chitosan membranes sub-periosteally over rat calvaria, and found that when DD was 80% or less osteogenesis and inflammation was present. In this study, DD of chitosan and its additives was well above the range reported by others to diminish the inflammatory response. It therefore appears that our findings are at odds with previous reports in the literature. However we found that the distribution of chitosan in the brain maintained the shape of the tract of gel initially extruded into the brain and at least initially, it appeared that the chitosan/GP was present and seemingly unaffected by the macrophage response. It was only on closer inspection made possible with the double fluorescence of chitosan and macrophages that it was apparent that the chitosan/GP was entirely within and engulfed by macrophages.

While the extent of inflammation elicited by chitosan has been commented on, studies have reported that chitosan attracts and activates macrophages in-vitro [6, 17, 18] and chitosan fragments have been observed within macrophages 14 days after sub-cutaneous implantation [6]. The extent to which chitosan was taken up by macrophages only came to our notice because Texas Red was added to the chitosan to make it more visible within the brain. There were concerns that the high ionic strength resulting from the addition of GP to chitosan might exacerbate inflammation [21], however GP solution injected alone produced only a limited inflammatory reaction compared to the chitosan/GP, suggesting that chitosan is the major inflammatory trigger.

A second finding is that extrudable materials such as chitosan can be injected into the brain as a track rather than as a droplet. This technique provides a useful way of injecting scaffolding materials into the brain.

Our results suggest that chitosan persistence as a scaffold in the brain is ephemeral and inadequate for practical use for that purpose. Further research is currently being carried out to investigate whether conjugates minimise the inflammatory response.

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