ORIGINAL ARTICLE

The graft survival protection of subcutaneous allogeneic islets with hydrogel grafting and encapsulated by CTLA4Ig and IL1ra

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In this study, we examined the combined effect of two recombinant immunomodulator proteins, cytotoxic T-lymphocyte antigen-4-Ig (CTLA4Ig) and interleukin-1 receptor antagonist (IL1ra), on the survival of subcutaneous mouse islet grafts with temperature-sensitive hydrogel, poly(N-isopropylacrylamide) (pNIPAAm)-chitosan-hyaluronic acid (CPNHA), and gelatin microspheres in an allogeneic mouse model. The phase-transition temperature for the CTLA4Ig-grafted CPNHA (CPNCHA) was 28 °C. An in vitro cytotoxicity assay and in vivo tissue reactions showed that both CPNCHA and the gelatin microspheres were biocompatible, biodegradable and nontoxic. The survival of the allogeneic islets was prolonged when the grafts were transplanted into a subcutaneous matrix composed of CPNCHA and gelatin microspheres containing IL1ra relative to those implanted in CPNHA containing gelatin microspheres without an immunomodulator. In conclusion, our results suggest that the temperature-sensitive hydrogel, CPNCHA, is a suitable subcutaneous matrix for islet transplantation, and grafted CTLA4Ig and encapsulated IL1ra reduce immune rejection and prolong the survival of the functioning allogeneic islets.
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Keywords: gelatin microspheres; islet transplantation; temperature-sensitive hydrogels

INTRODUCTION

The primary cause of type 1 diabetes is the immune-mediated destruction of pancreatic β-cells. Because the exocrine cells of the pancreas are unaffected by type 1 diabetes mellitus, successfully replacing the islets or β-cells should theoretically restore glucose homeostasis.[1] The main challenge for such transplantations is the immune system's rejection of the foreign tissues. Current approaches for overcoming the immune barrier to allow successful allo- and xeno-islet transplantation include using immunosuppressive agents, immune modulators, immunoisolation and inducing an immune tolerance.[2,3] Cytotoxic T-lymphocyte antigen-4-Ig (CTLA4Ig), a soluble immunosuppressive fusion protein, blocks CD28–CD86 interactions, which leads to T-cell inactivation.[4,5] The interleukin-1 receptor antagonist (IL1ra) binds the IL-1 receptor and blocks the action of IL-1 to protect the islets from cytokine-mediated inflammatory destruction.[6,7] Because their half-life is short, continually administering high concentrations of immunomodulator proteins such as CTLA4Ig and IL1ra is clinically inconvenient. Thus, designing a method to control the local release of such immunosuppressant proteins, for example, using gelatin microspheres as drug delivery devices, is important to improve the

treatment effectiveness and reduce systemic drug-related side effects.[8] Gelatin is natural, nontoxic, inexpensive, biocompatible and biodegradable and has been used as a carrier for sustained drug release.[9,10]

Subcutaneous space is one of the many sites chosen for islet engraftment because it is easily manipulated for grafts. An ideal subcutaneous matrix for islet implantation should block direct cellular contact between the islets and the immune cells but remain permeable to low-molecular-weight nutrients, metabolites, oxygen and insulin.[11,12] We determined the temperature-sensitive hydrogel, Poly(N-isopropylacrylamide) (pNIPAAm)-chitosan-hyaluronic acid (CPNHA), to be a candidate matrix for subcutaneous islet implantation because it is biocompatible, temperature-sensitive and has immunoisolation characteristics.[13,14] At room temperature, CPNHA is a liquid, mixing easily with cells. However, after injecting into subcutaneous space, where the temperature is above its transition temperature, the CPNHA hydrogel transforms into a solid gel matrix.

This study was designed to evaluate the effect of subcutaneously implanting islets into CTLA4Ig-grafted CPNHA (CPNCHA) containing IL1ra-encapsulated gelatin microspheres on the graft survival

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using an allogeneic mouse model. Our aim was to develop a subcutaneous matrix containing a local immunosuppressive protein delivery system that could prevent graft rejection and support the long-term survival of functioning islet grafts.

EXPERIMENTAL PROCEDURE

Construction and purification of recombinant mouse IL1ra and human CTLA4Ig

We cloned and inserted mouse IL1ra cDNA (510 bp) and human CTLA4Ig cDNA (1135 bp) sequences into pGEX-KG, a bacteria expression vector, for protein synthesis as described previously.[15] After transformation, *E. coli* expressed the GST-IL1ra or GST-CTLA4Ig fusion proteins. We then used glutathione-agarose beads to purify the fusion proteins. In this study, we did not remove the GST from the fusion proteins, and we denote GST-IL1ra and GST-CTLA4Ig as IL1ra and CTLA4Ig, respectively, throughout the study.

Preparation of IL1ra-encapsulated gelatin microspheres

Gelatin (10% w/v) mixed with IL1ra (1% or 5%, w/v) was dissolved in 0.1 M 2-(*N*-morpholino)ethanesulfonic acid, and 25 or 75 mM of buffer 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was added. After injecting this solution, a 7.5-fold volume of olive oil was added to create a water-in-oil emulsion. The solution was stirred at 900 r.p.m. for 30 min at 37 °C, followed by homogenizing for 30 s. After chilling on ice, the solution was stirred at 900 r.p.m. for 20 min. The microspheres were collected via vacuum filtration and washed with acetone. Finally, the mixture was sieved to obtain suitably sized particles.

Preparation of the CTLA4Ig-grafted temperature-sensitive hydrogels

pNIPAAm was first grafted onto chitosan to form CPN, and hyaluronic acid was then grafted onto this CPN to form CPNHA as previously reported.[13] CPN (2 g) and CTLA4Ig (25 mg) were dissolved in 100 ml of 0.1 M 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 5.0) before

adding 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.46 g) and NHS (1.38 g). The resulting product was CPNCHA, which was dialyzed for 4 days (Spectra/Por, MW cut-off 100 kDa), and then freeze-dried to obtain the polymer. The reaction scheme for synthesizing CPN and CPNCHA is shown in Figure 1.

Protein grafting of CPNCHA

To quantitate CTLA4Ig of CPNCHA, we analyzed the protein content of the liquid using the Bradford reagent. To quantitate the amount of CTLA4Ig added, we weighed the CTLA4Ig powder before the hydrogel synthesis. The grafting ratio was calculated using the following formula:

$$\text{Grafting ratio (\%)} = \frac{\text{CTLA4 Ig amount of CPNCHA}}{\text{Amount of added CTLA4 Ig}} \times 100\% \qquad (1)$$

Lower critical solution temperature of CPNCHA

Differential scanning calorimetry was performed using a Q10 DSC calorimeter (TA Instruments, New Castle, DE, USA). Thermal analysis profiles of the aqueous polymer solutions were obtained over a temperature change from 20 to 45 °C at a rate of 2 °C min^{-1} under nitrogen.

Water content of CPNCHA

A 4% (w/w) polymer solution was allowed to gel in water at 37 °C for 1 h. One milliliter of double-distilled water was added to the samples, and any excess water was removed after 48 h. The weights of the remaining solid matrices were recorded. The hydration ratio of the test samples was calculated as follows: water content ratio $= (W_H - W_0)/W_0$, where W_H is the weight of the hydrated gel and W_0 is the weight of the dried test sample.

Surface potential (zeta potential) of CPNCHA

The zeta potential of the polymer solutions (4%, w/w) was measured using a Nano ZS 90 Zetasizer (Malvern Instruments, Malvern, Worcestershire, UK) at room temperature. These measurements were repeated three times per sample for all three samples.
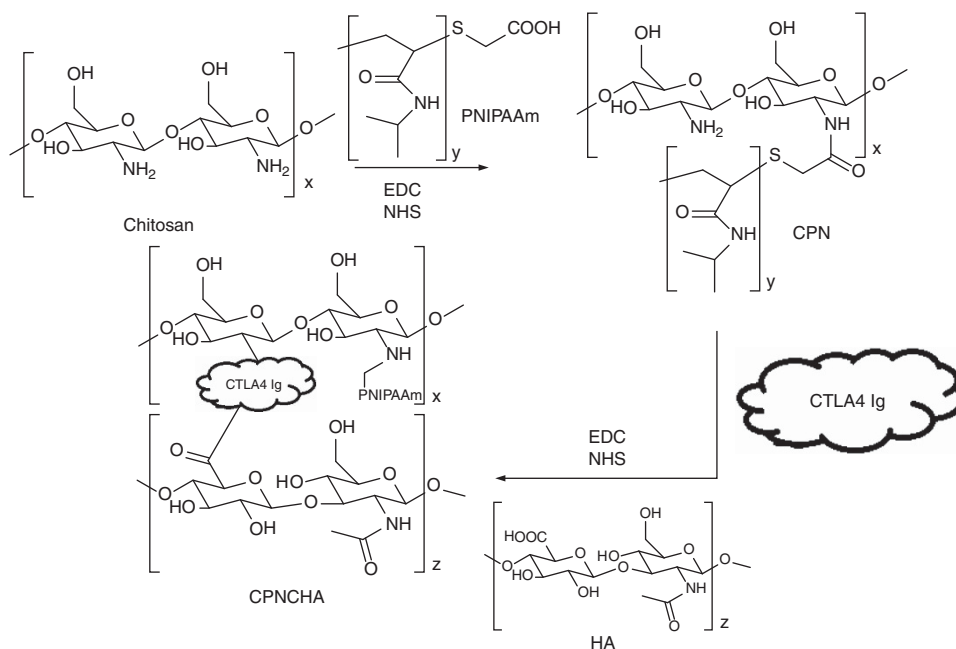

Figure 1 Chemical reaction scheme for synthesizing CPN and CPNCHA. Hydrogels, CPN and pNIPAAm-chitosan-CTLA4Ig-HA (CPNCHA) were prepared using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) as coupling agents. We used the carboxyl group and amino group in CTLA4Ig to bind the amino group of CPN and the carboxyl group of HA, respectively. The CPNHA hydrogel was prepared via the same procedure but without CTLA4Ig.

Determination of the material morphology using scanning electron microscopy

The samples were lyophilized by freeze-drying, fractured in liquid nitrogen and sputter-coated with gold. The resulting dried samples were examined using a Hitachi S-2400 scanning electron microscope (Tokyo, Japan).

Effect of protein loading on the *in vitro* release kinetics of IL1ra from gelatin microspheres

A 100-μl solution of gelatin microspheres containing either 1% or 5 IL1ra fusion protein was added to several microtubes. These microspheres were immersed in 1 ml of phosphate-buffered saline with a pH of 7.4. The microspheres and buffer were incubated at $37\,^{\circ}C$ and shaken at 200 r.p.m. After varying incubation times, the supernatant of each microtube were collected by centrifuging for 3 min at $13\,000\,g$ and replaced with $100\,\mu l$ of fresh phosphate-buffered saline buffer. The released protein assay was performed as follows. A reagent containing Coomassie Blue G-250 buffer (Bradford reagent), buffer (ethanol: phosphoric acid: water, 1:2:17) and 50 mg of Coomassie Blue G-250 was used. The absorbance was measured at 595 nm. Electrophoresis (12% PAGE) was used to confirm the molecular weight of the released protein.

Effect of lysozyme on the *in vitro* release kinetics of IL1ra from CPNCHA-embedded gelatin microspheres

A 100-μl solution of 4% CPNCHA and 10% gelatin microspheres containing 5% IL1ra fusion protein was added to several microtubes. These microspheres were immersed in 1 ml of phosphate-buffered saline at a pH of 7.4 either with or without $1\,mg\,ml^{-1}$ of lysozyme. A control group of microtubes containing only the 5% IL1ra/gelatin microspheres was used to evaluate the effect embedding in CPNCHA had on the IL1ra release kinetics. Microtubes containing CPNCHA, the gelatin microspheres and the buffer were incubated at $37\,^{\circ}C$ and shaken at 200 r.p.m. After varying incubation times, the supernatants were collected by centrifuging for 3 min at $13\,000\,g$ and replaced with $100\,\mu l$ of phosphate-buffered saline buffer either with or without lysozyme. A protein release assay was conducted as follows. A reagent containing Coomassie Blue G-250 buffer (Bradford reagent), buffer (ethanol: phosphoric acid: water, 1:2:17) and 50 mg Coomassie Blue G-250 was used. The absorbance was measured at 595 nm. Electrophoresis (12% PAGE) was used to confirm the molecular weight of the released protein.

The immunosuppressive function of IL1ra microspheres and CPNCHA

RINm5F cells (1×10^5 cells per well) were placed in 96-well plates. After cultivating in the medium overnight, IL-1β ($1\,ng\,ml^{-1}$) and IFN-γ ($100\,U\,ml^{-1}$) were added to each well. Cells in the control group did not receive any microspheres, whereas cells in the experimental groups received IL1ra microspheres ($50\,ng\,ml^{-1}$). After 24 h, 0.1 ml of the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reagent ($5\,\mu g\,ml^{-1}$) was added to each well. The cells were then incubated at $37\,^{\circ}C$ in the dark for 4 h. The MTT solution was removed, and 0.1 ml of 100% DMSO was added at room temperature for 15 min to dissolve the blue-purple formazan product. The absorbance of the supernatant at 570 nm was measured using a spectrophotometer. To quantitate the degree of cellular infiltration into the hematoxylin and eosin-stained tissue sections of the retrieved grafts, we counted the number of mononuclear cells under a high-powered field ($\times 40^* \times 10$).

In vitro cytotoxicity test (MTT assay) of the hydrogels

RINm5F cells (1×10^4 cells per well) were placed in 48-well plates. After cultivating the medium overnight, 150 μl of the hydrogel mixture was added to each well. Cells in the control group received no hydrogel. Cells in the experimental groups received 100 μl CPNHA containing 50-μl blank gelatin microspheres or 100 μl CPNCHA containing 50 μl 5% IL1ra/gelatin microspheres. After 24 h, 0.5 ml of MTT was added to each well. The cells were incubated at $37\,^{\circ}C$ in the dark for 4 h. The MTT solution was removed, and 0.5 ml of 100% DMSO was added at room temperature for 15 min to dissolve the blue-purple formazan product. The absorbance of the supernatant at 570 nm was measured using a spectrophotometer.

Animal care and induction of diabetes

Diabetes was induced in male C57BL/6 mice (8–12 weeks old) via an intraperitoneal injection of streptozotocin ($200\,mg\,kg^{-1}$ body weight). Mice with whole-blood sugar levels exceeding $360\,mg\,dl^{-1}$ for over 2 weeks were defined as having diabetes. Three to five mice were housed in each cage and fed standard pellet food and tap water *ad libitum*. The animal room had an automatic light/dark cycle of 12 h.

Isolation of pancreatic islets

Pancreatic islets isolated from BALB/c mice via collagenase digestion were handpicked after enriching on a density gradient using Histopaque-1077 from the Sigma Chemical Company (St. Louis, MO, USA). Briefly, under sodium amobarbital anesthesia, the pancreases of non-fasted healthy mice were distended with 2.5 ml of RPMI-1640 medium containing $1.5\,mg\,ml^{-1}$ collagenase and then excised and incubated in a $37\,^{\circ}C$ water bath. The islets were purified using a density gradient and handpicked using a dissecting microscope. Isolated islets with diameters of 125–150 μm were separated into groups of 50. To minimize the batch-to-batch variations in islet function during the experimental observations, all of the islets were isolated from 8–10 mice on a single day and transplanted into an equal number of control and experimental mice.

Subcutaneous transplantation of islet cells

The streptozotocin-induced diabetic C57BL/6 mice were divided into two groups. Mice in group 1 received allogeneic islets of BALB/c mice in 0.5 ml of the 4% CPNHA hydrogel containing 4 mg of the 5% gelatin microspheres (CPNHA + Ms). Mice in group 2 received allogeneic islets in 0.5 ml of the 4% CPNCHA hydrogel containing 4 mg of the 5% IL1ra/gelatin microspheres (CPNCHA + IL1ra Ms). Each diabetic mouse received 2200 allogeneic BALB/c islets and 200 ng vascular endothelial growth factor in hydrogel. After transplantation, the body weight and glucose levels of whole-blood samples drawn from a tail vein were measured daily for 2 weeks. The grafts were removed 18 days after transplantation for histological examination.

Immunohistochemical staining of islets for insulin

For the immunohistochemical staining, tissue sections were immersed in an ethanol solution containing 0.3% H_2O_2 at room temperature for 5 min. The sections were pre-incubated for 5 min with Tris-buffered saline containing 1% bovine serum albumin to block any nonspecific binding and labeled with polyclonal anti-insulin antiserum before incubating with HRP-conjugated secondary antibody (1:100) for 70 min. The antigen localization was indicated by a brown precipitate of 3,3′-diaminobenzidine as the chromogenic substrate of the peroxidase activity. The samples were further examined under an optical microscope.

RESULTS

IL1ra/gelatin microsphere morphology

The gelatin microspheres became rougher and more irregularly shaped, and their largest diameter increased from 8.5 ± 2 to $11.6 \pm 2\,\mu m$ ($n = 30$, $P < 0.05$) when the protein load was increased from 1 to 5% (Figure 2).

Release kinetics of IL1ra from the gelatin microspheres

The loading rates were 90.1 ± 8.57 and $46.8 \pm 0.9\%$ ($n = 3$, $P < 0.05$) for the 1 and 5% protein-loaded gelatin microspheres, respectively. The microspheres exhibited a significant burst release of 36.97 ± 4.68 and $70.76 \pm 3.72\%$ ($n = 3$, $P < 0.05$) of the total IL1ra fusion protein load during the first hour for the 1 and 5% protein-loaded gelatin microspheres, respectively. The higher the protein loading, the higher the total amount released (Figure 3a). After 145 h, 98.94 ± 1.26 and $87.85 \pm 4.15\%$ of total loaded protein had been released for the 1 and 5% protein-loaded gelatin microspheres, respectively. The average amount of IL1ra released was 2.45 ± 0.03 and $5.67 \pm 0.24\,mg\,h^{-1}$ ($n = 3$, $P < 0.01$) for the 1 and 5% microspheres,
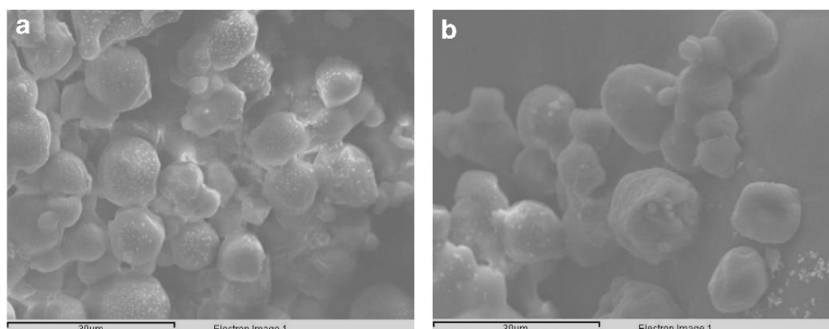
Figure 2 Gelatin/IL1ra microspheres were observed via scanning electron microscopy. (**a**) 1% IL1ra and (**b**) 5% IL1ra.
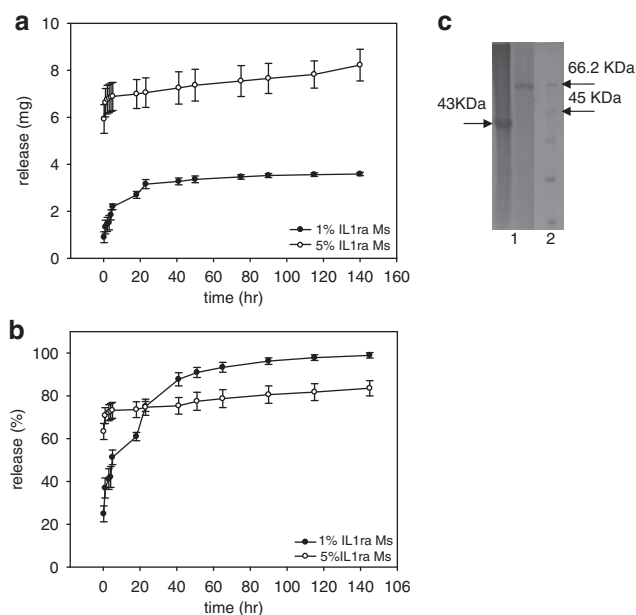

Figure 3 Effect of protein loading on the *in vitro* release kinetics of IL1ra from the gelatin microspheres. (**a**, **b**) The release kinetics of the IL1ra fusion protein from the gelatin microspheres ($n=3$). (**c**) SDS-PAGE of the released proteins: Lane 1, IL1ra fusing protein (43 kDa); Lane 2, bovine serum albumin (66 kDa). A full color version of this figure is available at *Polymer Journal* online.
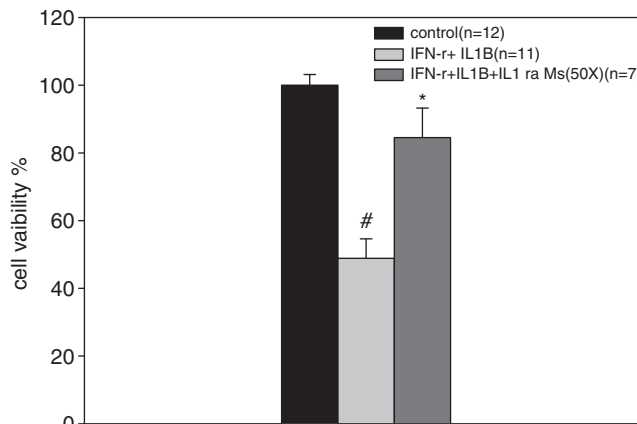

Figure 4 Prevention of cytokine-induced cell death by the IL1ra microspheres. RINm5F cells (1×10^5) were incubated with cytokines for 24 h in the presence or absence of IL1ra Ms. The cytokine concentrations used were IL-1β, 1 ng ml^{-1}, and IFN-γ, 100 U ml^{-1}. The percentage of viable cells after these treatments was determined via MTT colorimetric assays and calculated versus the A570 values of the control cells. The values are the mean ± s.e. #: The IFN + IL-1β-treated group had a significantly decreased score relative to the control group ($P<0.05$). *: The IL1ra Ms-treated group had a significantly increased score compared with IFN + 1 IL-1β-treated group ($P<0.05$).

respectively. After 145 h, the release percentage had reached almost 100% of the 1% protein-loaded gelatin microspheres, but only 80% of the 5% loaded microspheres (Figure 3b). The proteins released from the microspheres were analyzed by SDS-PAGE; both the model protein (bovine serum albumin (66 kDa)) and GST-IL1ra (43 kDa) were observed and showed no evidence of degradation (Figure 3c).

Prevention of cytokine-induced cell death by IL1ra from the microspheres

We investigated the anti-immune effect of the IL1ra released from the microspheres at the cellular level. The RINm5F cells from a rat pancreatic β-cell line were cultured to near confluence and then treated with a combined dose of 1 ng ml^{-1} IL-1β and 100 U ml^{-1} of IFN-γ for 24 h. The percentage of viable cells was measured via an MTT assay. As shown in Figure 4, the cytokine-treated cells had a viability of 48.9 ± 5.8% ($n=11$), whereas the cells treated with 50× IL1ra Ms (IL1ra, 50 ng ml^{-1}) had a viability of 84.5 ± 8.7% ($n=7$, $P<0.05$).

The physicochemical properties of CTLA4Ig-modified thermosensitive hydrogel

Protein assays showed that 6.20 ± 2.15 mg of CTLA4Ig was cross-linked to each gram of CPNHA ($n=4$). The lower critical solution temperature was determined for temperatures ascending from 25 to 40 °C, and the gel-formation temperatures of the CPNHA and CPNCHA were 31 and 28 °C, respectively. CPNCHA contained 20% more water than CPNHA. The zeta potential of the polymers (4%, w/v) was determined to examine the electrical charge both before and after conjugation. CPNHA itself exhibited a neutral or only slightly negative charge. Upon CPNCHA formation, the net positive charge rose to + 3.53 mV (Table 1).
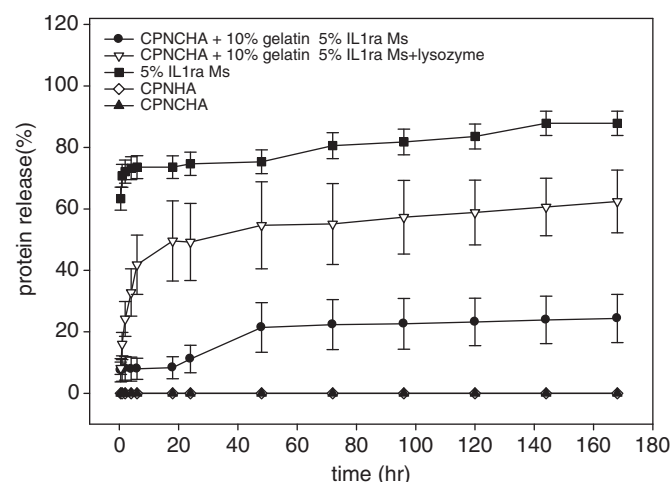
Effect of lysozyme digestion and CPNCHA hydrogel embedding on the release kinetics of IL1ra from the gelatin microspheres

A large burst release of IL1ra from 5% IL1ra-gelatin microspheres was observed, that is, 70.76 ± 3.72% of the total loading protein was released from the microspheres within the first hour (Figure 5). The burst release from the 5% IL1ra-gelatin microspheres embedded in the CPNCHA was significantly reduced (15 ± 10%, $P<0.05$).

Table 1 The physicochemical properties of CTLA4Ig-modified thermosensitive hydrogels

Hydrogel	Protein:gel ($mg\,g^{-1}$)	LCST (°C)	Water content (water weight/polymer weight)	Zeta potential (mV)
4% CPNHA	0	31	0.38	0.23 ± 0.21
4% CPNCHA	6.20 ± 2.15	28	0.47	3.53 ± 1.53

Abbreviations: CPNHA, pNIPAAm-chitosan-hyaluronic acid; CPNCHA, cytotoxic T-lymphocyte antigen-4-Ig-grafted CPNHA; LCST, lower critical solution temperature.
LCST was measured using differential scanning calorimetry using a Q10 DSC Calorimeter. The thermal analysis profiles of the aqueous polymer solutions were obtained as the temperature changed from 20 °C–45 °C at a rate of 2 °C min $^{-1}$ under nitrogen. CPNHA, chitosan-graft-PNIPAAm and HA. CPNCHA, chitosan-graft-PNIPAAm-CTLA4Ig and HA.

Figure 6 Scanning electron microscope image of the CPNCHA-blended microspheres revealing that the gelatin microspheres (arrow ↑) were homogenously embedded in the CPNCHA hydrogel matrix (arrowhead ▲).

Figure 5 Effect of lysozyme on the *in vitro* release kinetics of immuno-modulator from CPNCHA-embedded gelatin microspheres.

Figure 7 *In vitro* cytotoxicity tests (MTT assay) for mixtures ($n = 3$).
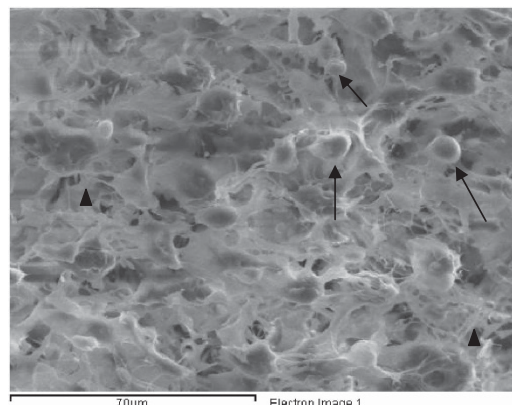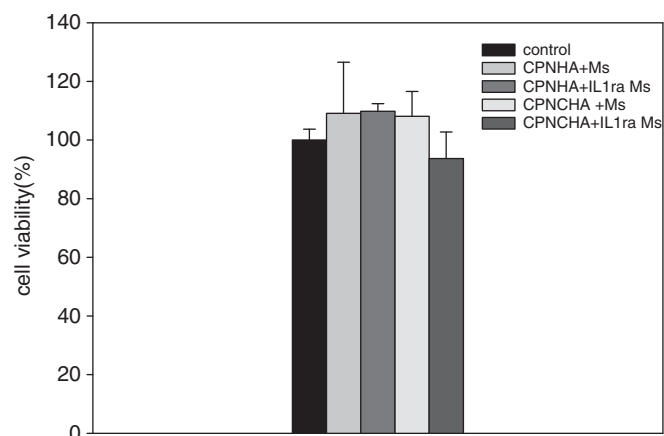
The release of 5% IL1ra-gelatin microsphere-embedded CPNCHA increased to 55 ± 13% of the total protein loading within the first hour when a lysozyme was added. No proteins were released from CPNCHA after 168 h.

Morphological examination and cytotoxicity test for the hydrogel mixtures

Scanning electron microscopy revealed that the gelatin microspheres were homogenously embedded in the CPNCHA hydrogel matrix (Figure 6). We examined the cytotoxicity of the CPNHA with gelatin microspheres, CPNCHA with gelatin microspheres, CPNHA with IL1ra-encapsulated gelatin microspheres and CPNCHA with IL1ra-encapsulated gelatin microspheres for RINm5F insulinoma cells. The results suggested that neither CPNHA with gelatin microspheres nor CPNCHA with IL1ra-gelatin microspheres interfered with the cell growth (Figure 7).

Histological examination of the retrieved hydrogel complex

A histological examination of the implanted hydrogel samples from group 1 (CPNHA and gelatin microspheres, without CTLA4Ig or IL1ra) showed that the islets were disrupted and destroyed with infiltrated inflammatory cells that were obvious 18 days after the transplantation (Figures 8a and c). There were a few lightly stained cells for the anti-insulin antibodies in the hydrogel, with an obvious infiltration of polymorphonuclear cells around the gelatin microspheres as noted (Figures 8b and d). In contrast, the immunomodulators grafted/encapsulated onto the hydrogel reduced the infiltration of the

mononuclear and polymorphonuclear cells both around and within the implanted CPNCHA-blended IL1ra-gelatin microspheres (Figures 9a and c), and many viable islet cells were found to contain heavily stained anti-insulin antibodies in the retrieved grafts (Figures 9b and d). On day 18, the total number of mononuclear cells for the CPNHA and CPNCHA grafts were 107.0 ± 5.1 and 98.1 ± 4.9, respectively ($n = 20$, $P > 0.05$).

Body weight and blood glucose level of the transplanted B6 mice

The body weight of mice in group 1 gradually decreased after the islet transplantation (Figure 10a). In contrast, the body weight of mice in group 2, who had received islets in the CPNCHA and IL1ra-gelatin microspheres, increased slightly 2 weeks post transplantation. However, the blood sugar levels did not differ between the two groups during the 2-week observation period (Figure 10b).

DISCUSSION

The immunosuppressive protein CTLA4 used in this study is similar in sequence to CD28, including the six-amino acid sequence (MYPPPY) that forms the CD80/CD86-binding site. The affinity of CTLA4 for CD80 and CD86 is higher than that of CD28 for CD80 and CD86; thus, the soluble CTLA4Ig fusion protein became a candidate for blocking CD28-B7 interactions.[16] Recent reports provide evidence that IL1β causes β-cell destruction in type 2
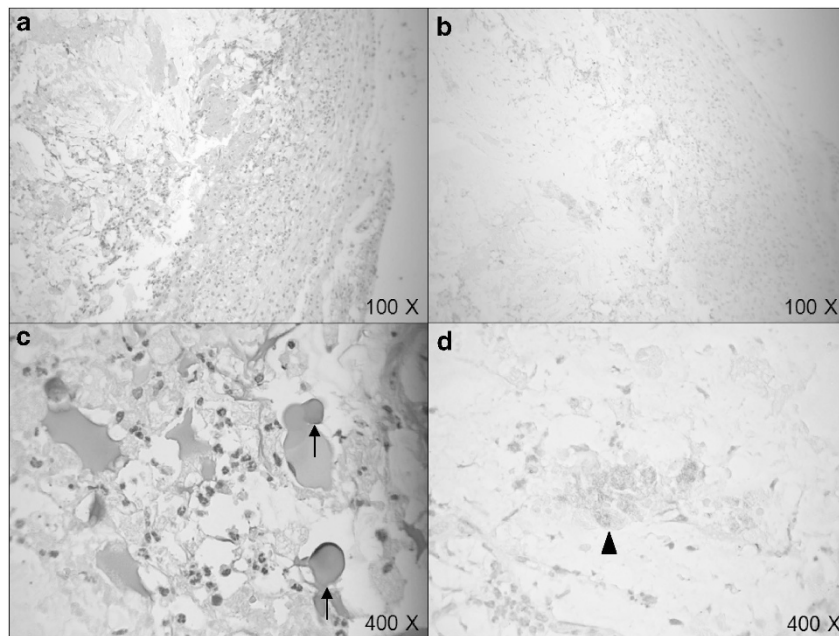
Figure 8 Histological examination of a subcutaneous graft comprising CPNHA mixed with blank microspheres and islet cells retrieved 18 days post transplantation. (**a**) Hematoxylin and eosin (HE) staining, ×100. (**b**) Immunostaining using anti-insulin antibodies, ×100. (**c**) HE staining, ×400. (**d**) Immunostaining using anti-insulin antibodies, ×400. Arrow (↑) indicates a gelatin microsphere; arrowhead (▲) indicates positive insulin-stained islets. A full color version of this figure is available at *Polymer Journal* online.
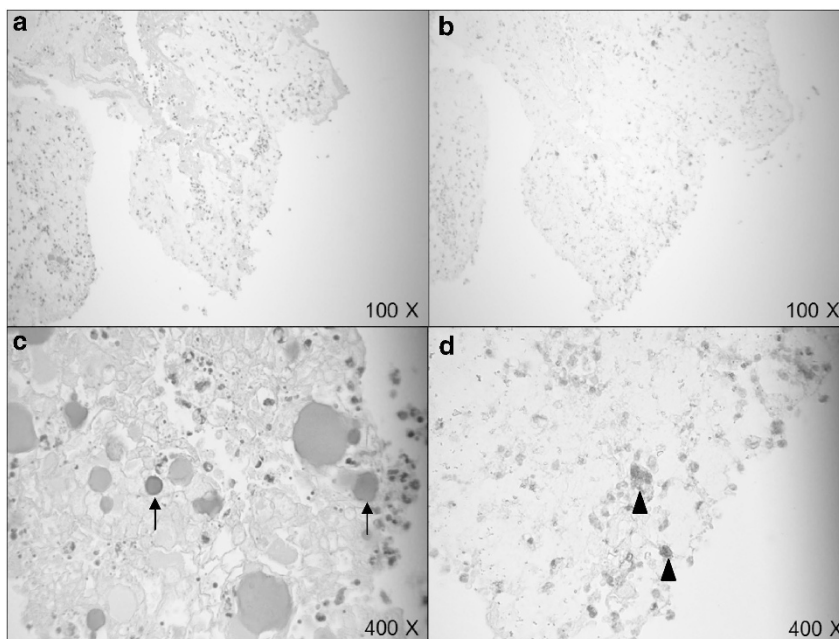

Figure 9 Histological examination of the subcutaneous grafts. CPNCHA-mixed gelatin/IL1ra microspheres and islet cells were retrieved 18 days post transplantation. (**a**) Hematoxylin and eosin (HE) staining, ×100. (**b**) Immunostaining using anti-insulin antibodies, ×100. (**c**) HE staining, ×400. (**d**) Immunostaining using anti-insulin antibodies, ×400. Arrow (↑) indicates a gelatin microsphere; arrowhead (▲) indicates a positive insulin-stained islet. A full color version of this figure is available at *Polymer Journal* online.

diabetes. *In vitro*, IL1β-mediated inflammatory processes have been shown to lead to β-cell death. Reducing the IL1β concentration *in vivo* may therefore inhibit IL1β-mediated inflammatory responses, thereby maintaining islet regeneration. IL1ra inhibits the activity of IL1α and IL1β and modulates a variety of IL-1-mediated immune and inflammatory responses.[17]

In this study, we examined the protective effect of allogeneic islets on the graft survival of two immune modulators, CTLA4Ig and IL1ra, grafted and encapsulated onto a temperature-sensitive hydrogel, CPNHA and gelatin, respectively, in subcutaneous space. pNIPAAm has been shown to undergo a temperature-induced sol–gel transition;[18] however, the clinical applications of pNIPAAm hydrogels are
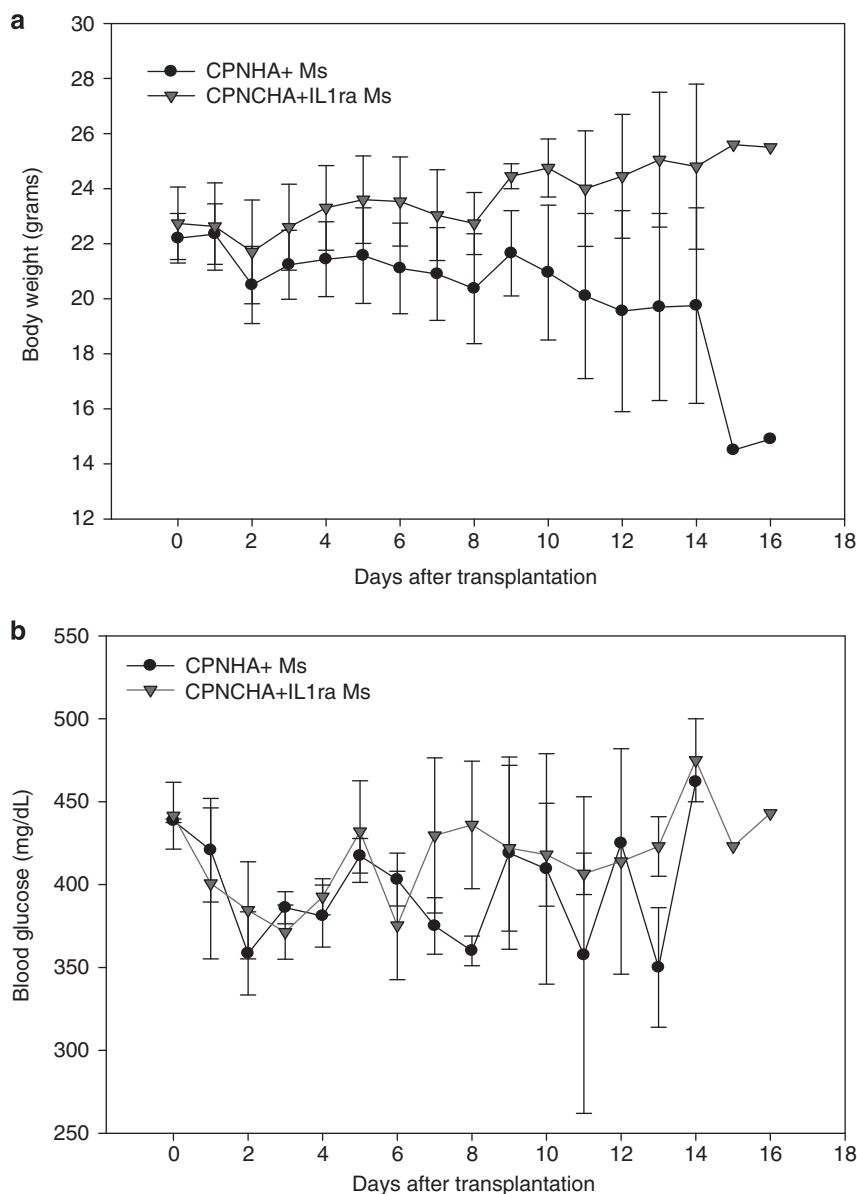
Figure 10 Profile of the body weight (**a**) and blood glucose concentration (**b**) after transplantation. A full color version of this figure is available at *Polymer Journal* online.

limited because of the carcinogenic and teratogenic toxicity of monomeric acrylamide.[19,20] The toxicity of pNIPAAm can be reduced with chitosan, which increases the biocompatibility. Many studies have reported that chitosan can cause allergies and other immune problems,[21] whereas other studies have reported that chitosan reduces inflammatory cytokines IL-6 and TNF-α and decreases the expressions of CD44 and TLR4 receptors.[22] Overall, the immune effects of chitosan and its derivatives are not clear. The hydrogels used in this study, CPNHA and CPNCHA, are non-cytotoxic; however, more in-depth studies are needed to identify any allergies or other immune-related problems associated with their use.

We combined two different hydrogels, CPNHA and gelatin, to control the drug release and achieve immune protection for allogeneic islet transplantation. Essentially, the temperature-sensitive *in situ* gelation of the CPNCHA and IL1ra-gelatin microsphere system extends the release time of the immune modulators, CTLA4Ig and IL1ra, and vascular endothelial growth factor, which prolongs the protective effect

for the islets and maintains persistent angiogenesis. After the CTLA4Ig graft, the transition temperature of the CPNCHA hydrogel is 28 °C, hence it remains as liquid at room temperature but solidifies at body temperature. The water content of CPNCHA is greater than that of CPNHA, partly because the grafting protein, CTLA4Ig, is water soluble. Moreover, the amide group, which is rich in CTLA4Ig and CPNCHA, is regarded as highly hydrated.[23] Therefore, CPNCHA is a good *in situ* gelation formulation that provides cell growth with a high water content. We used CPNCHA to embed the IL1ra-gelatin microspheres, which further reduced the burst release of IL1ra from the gelatin/IL1ra microspheres.

Previous studies usually used adsorption or embedding to load drugs; however, these methods have fast release times and poor encapsulation efficiencies.[24,25] In this study, we used cross-linked materials and drugs in process, and the results showed that the release time was extended without removing the IL1ra functionality. Although the total number of mononuclear cells was not

significantly different between CPNHA and CPNCHA, CPNCHA containing Ms-IL1ra demonstrated a protective effect on the islets during the insulin staining. We suggest that CPNCHA with Ms-IL1ra inhibits the cytokine-mediated cytotoxicity rather than suppressing mononuclear gathering within the hydrogel.

Previous studies have demonstrated the effects of CTLA4Ig via primary human mixed-lymphocyte response assays, where T-cell proliferation inhibition was first observed at 0.1 µg ml^{-1} and maximized at 10 µg ml^{-1}.[26] Based on an allogeneic mouse model, diabetic recipients receiving a continuous infusion of murine IL1ra via subcutaneous osmotic pumps (1 mg kg^{-1} per day) experienced a protective effect for islet allografts that resembled 8 mg kg^{-1} per day over an 11-day period following the transplantation.[27] The immunomodulator proteins in this study were sufficient because the complex gel included 3.1 mg of CTLA4Ig and 200 µg of IL1ra per mouse.

However, it is known that lysozyme is secreted by phagocytes, which suggests that investigating its effects on the inflammatory cells themselves might be profitable. Monocytes continuously secrete lysozyme and migrate relatively late to areas of inflammation.[28] This study used lysozyme to mimic the matrix destruction and protein release of a real situation, and confirmed the protective function of CPNCHA via the hydrolysis of the peptidoglycan linkages.

The histology of the implants showed that the CPNCHA and IL1ra-conjugated gelatin microsphere blends experienced fewer immune reactions and slower degradation than the CPNHA and bare gelatin microsphere. On day 18 after the transplantation, more viable allogeneic islets stained positive using anti-insulin antibodies for those grafts implanted in CPNCHA and IL1ra-gelatin microspheres, whereas fewer viable islet cells were seen in the CPNHA and gelatin microsphere grafts. This suggests that both grafting CTLA4Ig onto CPNCHA and encapsulating IL1ra into gelatin microspheres help protect the allogeneic islets, whereas islets with no protection were rejected and died 2 weeks post transplantation.

The blood glucose levels of the mice in group 1, which received islets in a matrix containing CPNHA and gelatin microspheres, remained high and their body weight gradually decreased after islet transplantation. In contrast, the body weight of mice in group 2, who received CPNCHA and IL1ra-gelatin microspheres, increased slightly. However, the blood sugar levels of the mice in group 2 did not decrease significantly after the islet transplantation. This suggests that the amount of insulin secreted from the viable islet cells in mice from group 2 was sufficient to maintain the body weight but insufficient to restore normoglycemia.

It has been demonstrated that 10–20% of the islet mass of a normal pancreas is required to maintain normal blood glucose and glycated hemoglobin levels.[29] For mouse islet transplantation, transplantation of 1000 islets into the subcutaneous hollow tube was found to significantly decrease the blood sugar content.[30] Because the subcapsular space of the kidney and hepatic portal vein has better access to blood circulation than the subcutaneous space, a lower number of islets was required to regain normoglycemia in diabetic mice.[31] However, the number of islets used in our study (up to 2200 allogeneic islets per mouse) should have been sufficient. Reasons for these differences in the viability and function of the islet beta cells between the hydrogels and hollow tubes used for implantation may include issues such as oxygen supply, metabolite disposal, nutrients supplementation and insulin diffusion. Hollow fibers provide islet cells with a larger interactive surface for oxygen and nutrient exchange. However, islet cells in a hydrogel are far from capillaries and have less interaction with circulating blood, metabolites, nutrients, oxygen and insulin, which cannot easily enter and leave the hydrogel matrix.

In conclusion, IL1ra-conjugated gelatin microspheres 10 µm in diameter with rough surfaces can be made using the W/O method. Cross-linking these microspheres containing IL1ra sustained this protein's release. After grafting with CTLA4Ig, the phase-change temperature of the CPNCHA was 28 °C, which is still lower than body temperature. The water content and electrical properties increased slightly. The action of lysozyme released the conjugated IL1ra protein from the IL1ra-gelatin microspheres as rapidly as the unconjugated IL1ra in the hydrogel. Microspheres mixed with the temperature-sensitive gel reduced the burst release of IL1ra from the IL1ra-gelatin microspheres to achieve a sustained release. The animal studies using CPNCHA and gelatin/IL1ra microspheres for allogeneic islet implantation demonstrated that CTLA4Ig and IL1ra improved graft survival in subcutaneous space. To prolong the protection of the islet grafts, the amount of grafted/encapsulated CTLA4Ig and IL1ra can be increased by increasing the graft ratio and amount of encapsulated immune modulators.

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