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Cell interaction with protein-loaded interpenetrating networks containing modified gelatin and poly(ethylene glycol) diacrylate

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Abstract

The effects of modification to an interpenetrating network (IPN) system composed of gelatin and poly(ethylene glycol) diacrylate were characterized by protein release kinetics, fibroblast adhesion, and in vivo host response. The maximum cumulative percent of parvalbumin released from various IPN formulations ranged from $17.6 \pm 3.2\%$ to $56.9 \pm 35.4\%$ over 2–96 h. Despite modifying gelatin with ethylenediaminetetraacetic dianhydride and/or monomethoxy poly(ethylene glycol) monoacetate ester or increasing the gelatin content, the largest amount of parvalbumin released occurred within 24 h, prior to material bulk degradation. Over the time period evaluated, little (i.e. $<1\%$) of the basic fibroblast growth factor (bFGF) loaded into the IPNs evaluated was released, independent of modifications made to the IPN formulation. Fibroblast adhesion to IPNs with or without loaded bFGF was quantified. The adherent fibroblast density on the IPNs was significantly lower than that of TCPS controls at all times independent of the IPN formulation tested and bFGF loading. Select IPN formulations elicited a comparable level of acute and chronic inflammatory response in vivo when compared with the gelatin and poly(ethylene glycol) diacrylate starting materials. IPNs provide a minimal cell-active surface that could be employed as delivery matrices and for further bioconjugation to mediate specific cellular response.

Keywords: Protein release kinetics; bFGF; Parvalbumin; Fibroblast; Host foreign body reaction

1. Introduction

Interpenetrating networks (IPNs) are a macromolecular system formulated by reacting a polymer around a second material to form an inter-entangled structure. IPNs can be created through photopolymerization allowing for the conversion of photoreactive solutions into gels or solids under physiologic conditions without the use of toxic crosslinkers commonly employed to create biomedical hydrogels. Additionally, IPNs have advantages such as desired amounts of bioactive agents are easily loaded into the matrix and the crosslinking density that effects the degradation and drug release rate can be controlled [1].

Gelatin, a model biopolymer, and photopolymerizable poly(ethylene glycol) (PEG) diacrylate (PEGdA) were selected as the two major components in the IPN formulation. Gelatin has been utilized in many biomedical applications ranging from drug-releasing matrices to scaffolds from tissue engineering devices [2–6]. To alter the swelling/degradation kinetics and subsequent release kinetics of model drugs/proteins from the IPN, we modified gelatin lysyl groups with ethylenediaminetetraacetic dianhydride (EDTAD) and/or monomethoxy poly(ethylene glycol) monoacetate ester (mPEGmA). Lysyl groups constitute 4–6% of all gelatin amino acids [7]. EDTAD modification imparts a polyanionic character to the gelatin, resulting in unfolding of the gelatin molecule via intramolecular electrostatic repulsion and introduces three carboxyl groups per gelatin lysyl residue modified on the gelatin backbone, providing multiple binding sites for water [8]. PEGdA improves the mechanical integrity and biocompatibility in terms of minimizing cellular interaction of gelatin-based IPNs

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as well as serves as a photopolymerizable crosslinking agent.

In previous studies, we analyzed the effects that gelatin modification, gelatin weight percent, and PEGdA molecular weight had on the physicochemical properties of IPNs [9]. Modifying gelatin with EDTAD and/or mPEGmA increased the IPN surface hydrophilicity ($135.9 \pm 1^{\circ}$ to $148.9 \pm 1^{\circ}$). Increased PEGdA molecular weight (from 2 to 8 kDa) or mPEGmA modification of gelatin, resulted in an increased IPN surface morphology. However, increasing PEGdA content decreased the elasticity of the IPNs at RT (Young's moduli of 266.1 ± 12.8 to 0.6 ± 0.2 MPa). In the presence of water and elevated temperature, degradation of the IPNs was promoted and the tensile properties of the IPNs decreased. Increasing the gelatin weight percent had a dominant effect on the swelling/degradation parameters independent of the pH level (pH 4–7.4): the maximum swelling ratio (R_{max}), the time to reach R_{max} (T_{max}), the swelling ratio just prior to failure (R_{fail}) and the time at which R_{fail} occurred all decreased. Specifically, EDTAD modification increased R_{max} and decreased T_{max} whereas modification with mPEGmA had very little effect on swelling/degradation. However, mPEGmA and EDTAD modification increased R_{max} at all gelatin weight ratios. Maximum swelling of the IPNs used in the current study ranged from $100 \pm 0\%$ to $200 \pm 0\%$ of the original IPN mass and this occurred over time periods ranging from 23 ± 14 to 691 ± 149 h depending on the IPN formulation. The time to complete degradation varied amongst the IPNs and ranged from 1288 ± 56 to >1344 h. The percent of cumulative chlorhexidine digluconate (MW ~ 900 Da) released from various IPNs ranged from $55.8 \pm 26.8\%$ to $109 \pm 3.6\%$. As the formulation was modified (i.e. changing the gelatin weight percent, EDTAD and/or mPEGmA modification), the maximum percent of drug released increased. This increase in drug release may partially be attributed to the increase in swelling observed with modification to the IPN system.

In the current study, we further investigated the effects that varying gelatin backbone modification and gelatin weight percent had on the IPN system as characterized by the release of model proteins (molecular weights ranging from 12.3 to 16.4 kDa) and fibroblast adhesion. We also quantified the level of host inflammatory response in response to IPNs.

2. Materials and methods

2.1. PEGdA synthesis

PEG diol (*Aldrich*, MW 2 kDa) was reacted with acrylolyl chloride (*Aldrich*) and triethylamine (*Aldrich*) in a respective 1:4:6 molar ratio at RT in tetrahydrofuran under Ar for 3 h, precipitated in cold hexanes, filtered and vacuum dried to produce PEGdA (Fig. 1). The final PEGdA purity was quantified with ^{1}H and ^{13}C NMR and a reverse phase HPLC system (10–100% acetonitrile at a flow rate of 1 ml/min in 60 min with Jordi 500 Å column on a Gilson system coupled to a UV–Vis and evaporated light scattering detectors). The elution time of the PEGdA was approximately 13.2 min with a purity of approximately 100 wt% PEGdA ($n = 5$).

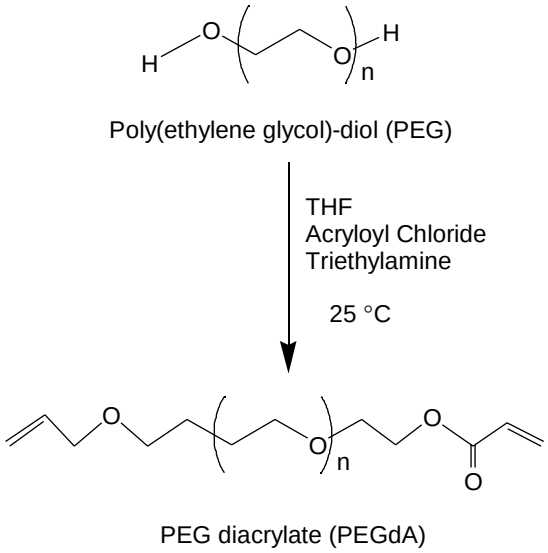

Fig. 1. Reaction scheme for PEG diacrylate (PEGdA).

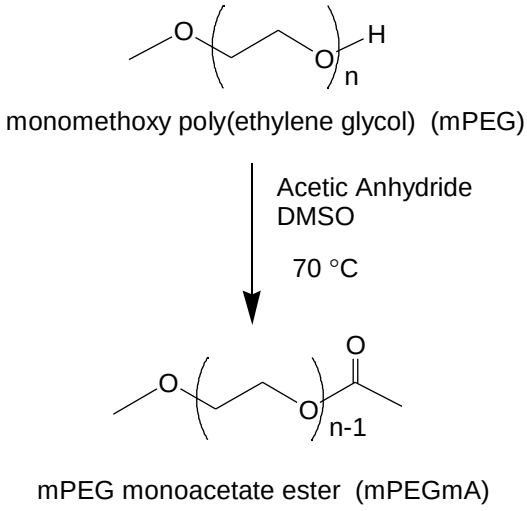

Fig. 2. Reaction scheme for mPEG monoacetate ester (mPEGmA).

2.2. mPEGmA synthesis

Monomethoxy poly(ethylene glycol) (mPEG) (*Fluka*, MW 2 kDa) was reacted with acetic anhydride (*Aldrich*) and DMSO (*Fisher*) in a respective 1:20:140 molar ratio under Ar at 70°C for 2 h, precipitated in cold diethyl

Table 1
Nomenclature and chemical structure of modified gelatin

Type of modification (nomenclature)	Structure (side groups of modified and unmodified lysyl residues indicated)
None, gelatin backbone (G)	NH_2 NH_2 NH_2
EDTAD (EG)	NH_2 $NHCOCH_2N(CH_2COOH)(CH_2CH_2N(CH_2COOH)_2)$
mPEGmA (mPmAG)	NH_2 $NHCO(OCH_2CH_2)_nOCH_3$
mPEGmA and EDTAD (mPmAEG)	NH_2 $NHCOCH_2N(CH_2COOH)(CH_2CH_2N(CH_2COOH)_2)$ $NHCO(OCH_2CH_2)_nOCH_3$

ether, filtered, dissolved with minimal amounts of methylene chloride, precipitated in cold diethyl ether, filtered, and vacuum dried to obtain mPEGmA (Fig. 2) [10]. The presence of the ketone group was determined with Schiff's reagent (*Fluka*) and with HPLC. mPEGmA had an elution time of approximately 11.7 min and purity close to 65 wt% mPEGmA with unreacted mPEG being the other main component ($n = 4$). The mPEGmA synthesized was utilized subsequently without further purification.

2.3. Gelatin backbone modifications

Gelatin (G) (*Sigma*, Type A: derived from porcine skin, 300 bloom, 50–100 kDa MW) lysyl groups were modified with EDTAD (*Aldrich*) in a respective 1:0.034 weight ratio. Briefly, gelatin was dissolved in deionized water to make a 1 wt% solution (0.1 g/ml) at 50–60°C for 30 min. The solution was then allowed to cool to 40°C and the pH was adjusted to 10. EDTAD was then added incrementally over 30 min, mixed for 3 h, dialyzed at RT for 24 h and lyophilized to form EDTAD-G (EG) (Table 1) [8]. G and EG lysyl groups were also modified with mPEGmA and sodium cyanoborohydride ($NaCNBH_3$) (*Aldrich*) in a respective 1:0.66:0.186 weight ratio. Briefly, a 20 wt% G or EG solution (0.2 g/ml) was created by dissolving G or EG in deionized water at 50–60°C for 30 min. $NaCNBH_3$ (0.33 g/ml) and mPEGmA (0.093 g/ml) solutions in deionized water were added slowly and simultaneously to the G or EG solution, mixed for 24 h at 50–60°C to form mPmAG or mPmAEG, respectively (Table 1) [10]. After 24 h, the solution was dialyzed at 50–60°C for 24 h and lyophilized.

The percent of gelatin lysyl residues modified by EDTAD and/or mPEGmA was quantified using the established trinitrobenzene sulfonic acid (TNBS) spectrophotometric method [8,11,12]. Briefly, 0.5 ml of a 1% protein solution (i.e. G, EG, mPmAG, or mPmAEG) was added to 1 ml of 4% $NaHCO_3$, followed by the addition of 0.2 ml of 12.5 mg/ml TNBS (*Sigma-Aldrich*) (blanks were created using the same procedure without the protein solution). The resulting solution was placed in a 40°C water bath for 2 h. Hydrochloric acid (12 N) was added (3.5 ml) and the resulting solution was incubated at 110°C, cooled, and worked up to 10 ml with water. This solution was extracted twice with cold diethyl ether (*Fisher Scientific*), retaining the aqueous layer, which was placed in a 40°C water bath for approximately 30 min to remove any trace amounts of ether. The absorbance of this solution was measured against a blank with a spectrophotometer (Genesys 8 UV–Vis spectrophotometer with 2 nm spectral slit width, *Thermospectronic*) at 415 nm. Absorbance/optical density (OD) was converted to number of lysyl residues using

$$\begin{aligned} &\#\ \text{lysyl residues} \\ &= \frac{\text{mol lysyl residues}}{\text{mol gelatin}} \\ &= \frac{(\text{OD} \times \text{number of dilutions})}{(\text{molar ext. coeff} \times b \times \text{conc. of gelatin})} \end{aligned} \quad (1)$$

where the number of dilutions in the procedure was 20, the molar extinction coefficient of lysine was 1.5×10^7 ml/mol × cm, and the path length (b) was 1 cm [12]. The molar concentration of gelatin was 4.645×10^{-8} mol/ml as determined through the use of a bovine serum albumin standard curve and assuming that the average molecular weight of 300-bloom gelatin was 75 kDa. The number of lysyl residues of modified and unmodified gelatin was determined. Eq. (2) was then used to determine the percent of gelatin lysyl groups modified. EDTAD, mPEGmA, and both EDTAD/mPEGmA modification resulted in modifications of $45 \pm 6\%$, $32 \pm 6\%$, and $69 \pm 5\%$ of gelatin lysyl residues ($n = 3$–5), respectively.

Table 2
IPN nomenclature

	ABC
A	Weight percent gelatin 0 = 0 wt% 4 = 40 wt% 6 = 60 wt% 10 = 100 wt%
B	Type of gelatin G EG mPmAG mPmAEG
C	Weight percent PEGdA 0 = 0 wt% 4 = 40 wt% 6 = 60 wt% 10 = 100 wt%

$$\%\ \text{modification of gelatin lysyl groups} = 1 - \left[\frac{\#\text{lysyl residues in modified gelatin}}{\#\text{lysyl residues in unmodified gelatin}}\right] \times 100. \quad (2)$$

2.4. IPN synthesis

IPNs were synthesized with modified or unmodified gelatin, PEGdA, and initiator (2,2-dimethoxy-2-phenylacetophenone, DMPA). Specifically, modified or unmodified gelatin (i.e. G, EG, mPmAG, or mPmAEG) was dissolved in deionized water with heat (40°C) to form a 10 wt% solution (0.1 g/ml). PEGdA was dissolved in deionized water, without heat, in an aluminum foil wrapped glass vial to form a 100 wt% solution (1 g/ml). The gelatin solution was added to the PEGdA solution and vortexed thoroughly. DMPA was added to this mixture, vortexed and heated to 40°C throughout the rest of the procedure. The final gelatin/PEGdA/DMPA mixture was injected with a Pasteur pipette into a Teflon mold (approximate dimensions of 7 mm diameter and 0.8 mm thickness) that was clamped between 2 cleaned glass slides. The mold/IPN mixture assembly was then irradiated with UV light (long wavelength (365 nm) with 21,700 μΩ/cm^2 at 2″ away from the sample) from the top and bottom simultaneously for approximately 3 min while a fan circulates ambient air around the mold. IPN nomenclature was based on the type of gelatin and the weight percent of gelatin (Table 2) (i.e. 4G6P indicates IPNs containing 40 wt% gelatin and 60 wt% PEGdA). IPNs loaded with basic fibroblast growth factor (bFGF) (human, recombinant, 16.4 kDa MW, *Sigma*, at 700pg or 0.25 μg per IPN for respective release and cell adhesion studies) and parvalbumin (from codfish, with Alexa Fluor® 488 conjugate, 12.3 kDa MW, *Molecular Probes*, at 100 μg per IPN) were synthesized as described above. However, protein solutions were added to the PEGdA along with deionized water.

2.5. Glutaraldehyde crosslinked hydrogel synthesis

Glutaraldehyde crosslinked gelatin hydrogels were employed for comparisons and synthesized by dissolving gelatin (G) in deionized water (0.1 g/ml) at 70°C, pouring the gelatin solution into polystyrene Petri dishes (60 × 15 mm, *Cole-Parmer*) and allowing the gelatin to set overnight. Hydrogels were cut and crosslinked with 0.1%, 0.01% (v/v in deionized water) glutaraldehyde (EM grade, 10% (v/v) aqueous solution, Electron Microscopy Sciences) for 6 h with gentle shaking. Crosslinked hydrogels were washed with clean deionized water for 3–5 min then this washing procedure was repeated 10 times, and left overnight in deionized water to remove any residual glutaraldehyde [13]. Hydrogels containing parvalbumin were synthesized in a similar fashion but after equilibrating overnight the gels were dried at RT in ambient air for 48 h. Individual, dried hydrogels (swollen dimensions: 0.5 × 0.5 × 0.6 cm^3) were placed into individual wells in a 48-well tissue culture plate. 35 μl of parvalbumin solution or 35 μl of aqueous solution (pH 7.4) for "no protein control" was added to each well (in duplicates), and the hydrogels were allowed to completely absorb the protein solution or no protein control overnight with gentle shaking (i.e. approximately 30 rpm on a bi-directional platform shaker). Based on the maximum swelling weight ratios from previous swelling studies, each hydrogel was loaded with 35 μl of parvalbumin solution (2.86 mg/ml or 100 μg per hydrogel), a volume well below the maximum volume the hydrogel could absorb [13]. Glutaraldehyde crosslinked gelatin hydrogel nomenclature was based on the percent glutaraldehyde solution employed to crosslink the gelatin (i.e. G-0.1% indicates that 0.1% v/v glutaraldehyde was utilized).

2.6. Parvalbumin release kinetics analysis

Each IPN and glutaraldehyde-fixed hydrogel with and without loaded parvalbumin was placed in individual test tubes containing 5 ml of pH 7.4 PBS at 37°C, and covered to prevent light exposure to the parvalbumin. The mass ratio of parvalbumin released, m_t/m_o (mass of drug released at time t divided by the original mass of drug loaded) was quantified at 2 h, 24 h, 4 d, and 7 d to quantify the protein release kinetics prior to previously observed time for bulk IPN degradation (i.e. > 8 weeks) [9]. At each time point, IPNs were carefully transferred to a test tube of fresh pH 7.4 PBS, and the absorbance of

the remaining solution was measured at a wavelength of 495 nm using a spectrophotometer (Genesys 8, *Thermospectronic*). The average absorbance of the corresponding IPN formulation without loaded protein was subtracted from each absorbance of the parvalbumin loaded IPNs, which were then averaged. The resulting absorbance value was converted to concentration using a standard curve developed with the use of parvalbumin solutions that were exposed to UV light for 3 min to mimic the IPN synthesis procedure: the absorbance at 495 nm = 0.0127 × concentration of parvalbumin (μg/ml) + 0.0204 ($R^2 = 0.9992$). A similar standard was obtained without 3 min UV exposure to the parvalbumin standard. The concentration at each time point was then added to the concentration at all previous points to obtain a cumulative concentration of protein released at each time point. The resulting cumulative concentration was then multiplied by the volume (5 ml) and divided by the original mass of parvalbumin loaded ($m_o = 100$ μg/IPN), to obtain m_t. The mass ratio of parvalbumin released at each time point was calculated by dividing m_t by m_o. The maximum cumulative percent of parvalbumin released and the time to reach this maximum was determined for each IPN formulation.

2.7. bFGF release kinetics analysis

IPNs loaded with bFGF were placed in a 48 well TCPS culture plate along with 1 ml of pH 7.4 PBS in a 37°C incubator. The release of bFGF from the IPNs was not evaluated with spectrophotometric methods because bFGF's maximum absorbance occurred at ~278 nm, which is close to gelatin's maximum absorbance of ~272 nm. After 2 h, 24 h, 4 d, and 7 d of incubation the buffer from the wells was removed, centrifuged, the supernatant was removed and assayed. To determine the amount of bFGF released from the IPNs, commercially available anti-human bFGF enzyme-linked immunosorbantassay (*R&D Systems*) kits were used following manufacturer's instructions. Buffers from wells containing 4G6P IPNs without bFGF were used to determine any cross reactivity of degraded gelatin with the antibody employed in the assay. The concentration at each time point was then added to the concentration at all previous points to obtain a cumulative concentration of protein released at each time point. The resulting cumulative concentration was then multiplied by the volume (1 ml) and divided by the original mass of bFGF loaded ($m_o = 700$ pg/IPN), to obtain m_t. The mass ratio of bFGF released at each time point was calculated by dividing m_t by m_o. The maximum cumulative percent of bFGF released and the time to reach this maximum was determined for each IPN formulation.

2.8. Fibroblast adhesion assays

IPNs loaded with or without bFGF were placed in a 48 well TCPS culture plate (*Falcon*), commercially available human dermal fibroblasts (50000) (*Clonetics*, passage 3–7) were seeded with 1 ml of FBM without supplements. TCPS was employed as the surface controls. FBM supplemented with 0.5 μg/ml of bFGF was used as positive controls with TCPS. Pure gelatin hydrogels (10G0P) were not chosen as controls due to the fast degradation of 10G0P (i.e. complete degradation of 10G0P occurs within 1 h) [13]. For comparison, FBM supplemented with 0.5 μg/ml of bFGF was also used with 0G10P IPNs and G-0.1% hydrogels. 0G10P and G-0.1% hydrogels without bFGF were not evaluated, as these hydrogels were to serve as positive control conditions in promoting fibroblast adhesion. After 2 h, 24 h, 4 d, and 7 d, samples were collected, rinsed with PBS, and stained with Wright stain (*Sigma*). Adherent fibroblast density was quantified based on measurements from 2 randomly selective fields using an Olympus TE300 phase-contrast microscope.

2.9. Subcutaneous cage implantation study

The effects of various IPN formulations on the host foreign body response were evaluated in vivo by following a well-established cage implant system [14–20]. Briefly, IPNs or respective material control were placed into cylindrical (2.8 cm long × 1 cm diameter) wire mesh cages made of medical grade stainless steel which were implanted subcutaneously at the backs of rats (Sprague Dawley females, age 54–64 d, weight 175–199 g), and empty cages were implanted as controls. At 4, 7, 14, or 21 d post-implantation, inflammatory exudates that collected inside the cages were withdrawn and the distribution of lymphocyte, monocyte, and PMN subpopulations in the exudates were determined by employing standard hematological methods [14–20]. The level of an acute inflammatory response, which occurs from the onset of implantation and dissipates with time, is characterized by the PMN exudate concentration. The chronic inflammatory response, which follows the acute inflammatory response and attenuates towards resolution, is characterized by the monocyte and lymphocyte exudate concentration.

2.10. Statistical analysis

Statistical analysis of parvalbumin and bFGF release data ($n = 2$–3 in duplicates and triplicates, respectively, $p < 0.05$) and fibroblast adhesion ($n = 3$ in triplicates, $p < 0.05$) was performed using one-way ANOVA and Tukey multiple comparisons test. The statistical analysis of the exudate leukocyte subpopulation concentrations was performed using paired, unequal Student's *t*-tests

($n = 3-7$, $p < 0.05$). Statistical analyses were performed with original data of appropriate significant figures; however, data presented in Tables 5 and 6 were rounded off for clarity.

3. Results and discussion

3.1. In vitro parvalbumin release kinetics

The maximum percent of parvalbumin released from various IPN and glutaraldehyde crosslinked gelatin hydrogels is shown in Table 3. Parvalbumin was selected as a model protein of comparable molecular weight to that of human bFGF. The percent of parvalbumin released ranged from $17.6 \pm 3.2\%$ to $97.3 \pm 2.2\%$ while the time to reach these release percentages ranged from 2 to 96 h. Modifying gelatin with EDTAD and/or mPEGmA had no significant effect on the amount of parvalbumin released from the IPN. The glutaraldehyde crosslinked gelatin hydrogels had much higher percent parvalbumin release compared to that of IPNs. The higher swelling ratios of the glutaraldehyde crosslinked gelatin hydrogels compared to IPNs of various formulations may partly contribute to this observation [9]. As shown in Fig. 3, the largest amount parvalbumin released from all samples occurred early with little if any further release of parvalbumin by day 7. This suggests that the parvalbumin close to the surface was released from the IPN. The study focused on diffusion of the protein through the IPN as the main driving force for protein release rather than bulk sample degradation and thus the study was only 7 d long. Previous studies determined that the maximum swelling ratio for the IPNs used in this study ranged from $100 \pm 0\%$ to $200 \pm 0\%$, the time to reach these swelling ratios ranged from 23 ± 14 to 691 ± 149 h, and these samples did not fully degrade for 1288 ± 56 to > 1344 h [9]. Parvalbumin has a molecular weight of 12.3 kDa and thus may be too large to easily diffuse through the IPN matrix. Hence, parvalbumin may not be completely released prior to the onset of IPN bulk degradation. The glutaraldehyde crosslinked gelatins employed in this study had a significantly higher maximum swelling ratio compared to the IPNs used. G-0.1% has a maximum swelling ratio of $500 \pm 100\%$ that occurs after 443 ± 234 h and G-0.01% has a maximum swelling ratio of $2400 \pm 200\%$ that occurs after 28 ± 8 h [13]. The large amount of swelling and the fast swelling rate of G-0.01% may partially contribute to the almost complete release of parvalbumin from G-0.01% after 24 h.

Table 3
Maximum cumulative percent of parvalbumin released from selected IPNs and glutaraldehyde crosslinked gelatin hydrogels

	Maximum percent released (mean ± s.e.m.)	Time to maximum percent released (h)
4G6P	19.9 ± 3.5	96
4EG6P	56.9 ± 35.4	2
4mPmAG6P	17.6 ± 3.2	24
4mPmAEG6P	21.4 ± 7.4	96
G-0.1%	48.0 ± 1.5[a]	24
G-0.01%	97.3 ± 2.2[a]	24

[a] Maximum percent release is significantly different at 95% confidence level from that of 4G6P.

3.2. In vitro bFGF release kinetics

The maximum cumulative percent of bFGF released from various IPNs is shown in Table 4. The amounts of bFGF released from the IPNs ranged from $0.154 \pm 0.106\%$ to $0.980 \pm 0.286\%$ and occurred from 2 to 96 h. Generally, modifications to the IPN system (i.e. gelatin modification or wt% gelatin) slightly increased the amount of bFGF released as well as the time to maximum release. The study focused on diffusion of the protein through the IPN as the main driving force for protein release rather than bulk sample degradation. Overall, very little bFGF was released from any of the IPN formulations over the time periods evaluated. This lack of bFGF release could be contributed to the lack of bFGF (16.4 kDa MW) diffusion out of the IPN system prior to the onset of bulk IPN degradation. Complex formation between bFGF and gelatin or PEGdA fragments may also occur, which may inhibit the immunoassay in the detection bFGF. Others have found bFGF to ionically interact with acidic gelatin and form polyion complexes [21,22].

3.3. Fibroblast adhesion

The adherent fibroblast density on various IPNs was quantified at 2 h, 24 h, 4 d, and 7 d (Table 5). As controls, fibroblasts were seeded onto TCPS with or without bFGF added to the medium. The cells adhered to the TCPS control with bFGF showed a more extensive cytoplasmic spreading than cells adhered to the TCPS control without bFGF at the early time points. Cells that adhered to the IPNs were round without cytoplasmic spreading and pseudopodial extension compared to those on the TCPS. Modifying gelatin with EDTAD and/or mPmA, or altering the gelatin weight percent had little effect on adherent fibroblast density. Generally, the number of adhered fibroblasts on IPNs loaded with or without loaded bFGF was significantly lower than the number of fibroblasts on the TCPS controls (with or without bFGF in the medium) at all time points. Within each IPN formulation, the number of fibroblasts adhered to the IPN

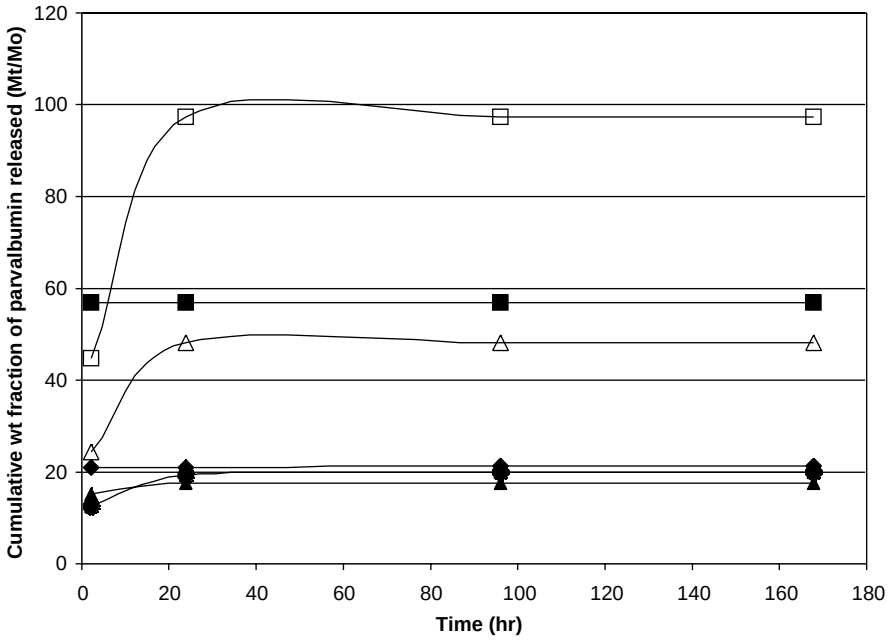

Fig. 3. In vitro cumulative parvalbumin mass ratio for various IPNs at pH 7.4. 4G6P -●-, 4EG6P -■-, 4mPmAG6P -▲-, 4mPmAEG6P -◆-, G 0.1% -△-, G 0.01% -□-; error bars were omitted for clarity.

Table 4
Maximum cumulative percent of bFGF released from IPNs of various formulations and the time to reach that maximum percent released

	Maximum % released (mean ± s.e.m.)	Time to maximum percent released (h)
4G6P	0.154 ± 0.106	2
6G4P	0.567 ± 0.138[a]	24
4EG6P	0.665 ± 0.046	24
6EG4P	0.980 ± 0.286	96
4mPmAG6P	0.399 ± 0.207	2
6mPmAG4P	0.189 ± 0.189	2
4mPmAEG6P	0.602 ± 0.419	96
6mPmAEG4P	0.735 ± 0.207	96

[a] Maximum percent release is significantly different from 4G6P at a 95% confidence level.

loaded with bFGF was in general not significantly different from the number of fibroblasts adhered to the IPN without loaded bFGF at all time points. The nominal level of fibroblast adhesion on IPNs loaded with bFGF could be contributed to: (i) the low level of bFGF release, (ii) the presence of high PEGdA concentration in the IPN formulation inhibiting cell adhesion, and/or (iii) the inactivation of bFGF via complex formation with degraded gelatin or PEGdA. When compared to 0G10P, 4G6P had a higher adherent fibroblast density after 24 h and 4 d. Whereas after 2 h, 4G6P had a lower adherent fibroblast density compared to G-0.1%.

3.4. Host inflammatory response

The host inflammatory response to the IPNs was evaluated (Table 6) [14]. 10G0P and 0G10P elicited a level of host responses similar to that of the empty cage controls. When 4G6P was present, the total leukocyte concentrations increased compared to the empty cage controls at each respective time, although only significantly at 4 d post-implantation. These concentrations also increased steadily over the 21-d implantation period, indicating an elevated chronic inflammatory response throughout the 21-d time period when 4G6P was present. The concentration of PMNs in the exudate increased significantly in the presence of 4G6P versus empty cage controls at 7 and 14 d post-implantation, indicating an increased acute inflammatory response. Conversely, the presence of 6G4P elicited a similar inflammatory response as the empty cage controls, 10G0P and 0G10P.

For comparison, the host inflammatory response to glutaraldehyde crosslinked gelatin was evaluated. G-0.1% elicited comparable levels of total leukocyte concentration compared to the empty cage controls. However, large magnitudes and variations in PMN, lymphocyte, and monocyte concentrations in the presence of G-0.1% suggest that this material elicited slightly enhanced acute and chronic response from day 4–14 post-implantation. Similar to 10G0P, the presence of G-0.01% elicited comparable levels of acute inflammation to the empty cage controls. The observed lymphocyte and monocyte concentrations in the

Table 5
Adherent fibroblast density on various IPNs and glutaraldehyde crosslinked gelatins loaded with or without bFGF

	Adherent fibroblast density (cells/mm^2)			
	2 h	24 h	4 days	7 days
TCPS				
bFGF	22.09 ± 2.09[a,b]	31.73 ± 7.63[a]	549.73 ± 529.08	3408.78 ± 1330.77
No bFGF	29.03 ± 3.27[a]	550.17 ± 529.03	15.25 ± 3.57[a]	11.32 ± 2.34
4G6P				
bFGF	0.61 ± 0.31	0.25 ± 0.08	0.16 ± 0.07	0.54 ± 0.30
No bFGF	1.06 ± 0.39	0.20 ± 0.06	0.23 ± 0.09	0.04 ± 0.03
6G4P				
bFGF	1.92 ± 0.57[a]	0.20 ± 0.07	0.81 ± 0.45	0.09 ± 0.05
No bFGF	1.09 ± 0.34	0.2 ± 0.11	0.16 ± 0.06	0.09 ± 0.06
4EG6P				
bFGF	1.12 ± 0.37[a]	0.17 ± 0.08	0.29 ± 0.14	0.49 ± 0.12
No bFGF	0.82 ± 0.48	0.39 ± 0.23	1.14 ± 0.86	0.33 ± 0.12
6EG4P				
bFGF	0.33 ± 0.15	0.24 ± 0.12	0.23 ± 0.08	0.38 ± 0.12
No bFGF	0.33 ± 0.12	0.44 ± 0.16	0.07 ± 0.03	0.23 ± 0.13
4mPmAG6P				
bFGF	1.30 ± 0.58	0.20 ± 0.10	0.22 ± 0.08	0.16 ± 0.06[b]
No bFGF	0.87 ± 0.20	0.26 ± 0.14	0.04 ± 0.03	0.01 ± 0.01
6mPmAG4P				
bFGF	1.72 ± 0.47	0.32 ± 0.13	0.28 ± 0.11	0.19 ± 0.06
No bFGF	1.50 ± 0.89	0.73 ± 0.45	0.32 ± 0.08	0.22 ± 0.09
4mPmAEG6P				
bFGF	0.55 ± 0.18	0.45 ± 0.15	0.26 ± 0.09	0.07 ± 0.03
No bFGF	0.96 ± 0.44	0.09 ± 0.05	0.10 ± 0.05	0.19 ± 0.08
6mPmAEG4P				
bFGF	0.26 ± 0.12	0.35 ± 0.11	0.09 ± 0.05	0.07 ± 0.05[b]
No bFGF	0.31 ± 0.10	0.23 ± 0.08	0.15 ± 0.07	0.20 ± 0.09
0G10P				
bFGF	0.00 ± 0.00	0.00 ± 0.00[a]	0.00 ± 0.00[a]	0.00 ± 0.00
G 0.1%				
bFGF	3.29 ± 0.98[a]	1.11 ± 0.55	0.26 ± 0.10	0.26 ± 0.15

Note: All values expressed as mean ± s.e.m. Each bFGF sample was loaded with 0.25 μg bFGF.
[a] Significantly different from 4G6P at a 95% confidence level.
[b] bFGF samples are significantly different from No bFGF samples at a 95% confidence level.

presence of G-0.01% appeared to decrease between 4 and 7 d post-implantation and again between 7 and 14 d post-implantation indicating that chronic inflammation was resolved slightly later in the presence of G-0.01% as compared to the empty cages.

4. Conclusion

We have demonstrated that IPNs could be utilized as a delivery matrix for large molecular weight (~12 kDa) proteins as well as small molecular weight drugs with various release kinetics. IPNs minimized the level of fibroblast adhesion compared to TCPS positive controls. Select IPN formulations elicited a comparable level of host inflammatory response in vivo when compared to empty cage controls and the IPN starting materials. IPNs provide a minimal cell-active surface that could be employed as delivery matrices and for further bioconjugation to mediate specific cellular response.

Table 6
Total and differential leukocyte concentrations in the inflammatory exudates of cages containing IPNs and glutaraldehyde crosslinked gelatin

Sample	Day sample retrieved	Exudate cell concentration (× 100 cells/μl)			
		Total Leukocyte	Lymphocyte	Monocyte	PMN
Empty cage controls	4	18±3	17±2	2±1	0±0
	7	6±1[a]	5±1[a]	1±0	0±0
	14	6±1[a]	4±0[a]	1±0	1±1
	21	9±6	7±5	2±1	0±0
10G0P controls	4	11±5	11±5	1±0	0±0
	7	6±2	5±2	1±0	0±0
	14	4±0	3±1	1±0	0±0
	21	1±0	1±0	0±0	0±0
0G10P controls	4	20±9	18±9	1±1	0±0
	7	3±1	2±1	0±0	0±0
	14	1±0[b]	1±0[b]	0±0[b]	0±0
	21	2±2	1±1	1±1	0±0
4G6P	4	52±5[b]	39±15	4±2	10±10
	7	38±8	15±9	4±0[b]	19±1[b]
	14	127±73	73±36	39±35	16±2[b]
	21	393±254	303±215	38±21	52±30
6G4P	4	7±7	7±7	1±1	0±0
	7	113±72	93±62	7±5	13±7
	14	8±3	7±3	1±1	0±0
	21	30±20	22±14	8±6	1±1
G-0.1%	4	60±39	26±12	13±11	22±21
	7	18±13	8±4	3±1	8±7
	14	24±7	12±3	4±2	8±8
	21	10±0	8±8	2±2	0±0
G-0.01%	4	48±20	41±17	6±3	1±1
	7	18±8	16±8	2±0[b]	0±0
	14	7±4	6±3	1±1	0±0
	21	9±0	7±1	1±0	1±1

Note: All values expressed as mean±s.e.m.
[a] Significantly different from the same sample formulation at the day 4 time point at a 95% confidence level.
[b] Significantly different from empty cage control at same time point at a 95% confidence level.

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References

[1] Okino H, Nakayama Y, Tanaka M, Matsuda T. In situ hydrogelation of photocurable gelatin and drug release. J Biomed Mater Res 2002;59:233–45.
[2] Bozzy J, David A, Denes M, Rusznak I. Production of granules with controlled drug release rate. Pharmazie 1979;34:172–4.
[3] Yamamoto M, Tabata Y, Hong L, Miyamoto S, Hashimoto N, Ikada Y. Bone regeneration by transforming growth factor beta1 released from a biodegradable hydrogel. J Controlled Release 2000;64:133–42.
[4] McDonald JA, Kelley DG, Broekelmann TJ. Role of fibronectin in collagen deposition: fab' to the gelatin-binding domain of fibronectin inhibits both fibronectin and collagen organization in fibroblast extracellular matrix. J Cell Biol 1982;92:485–92.
[5] Thomson RC, Yaszemski MJ, Powers JM, Mikos AG. Fabrication of biodegradable polymer scaffolds to engineering trabecular bone. J Biomater Sci: Polym Ed 1995;7:23–38.
[6] Choi YS, Hong SR, Lee YM, Song KW, Park MH, Nam YS. Studies on gelatin-containing artificial skin: II. Preparation and characterization of cross-linked gelatin-hyaluronate sponge. J Biomed Mater Res 1999;48:631–9.
[7] Budavari S, editor. Merck index, 12th ed. no. 4388. Whitehouse Station, NJ: Merck Research Laboratories Division of Merck & Co., 1996. p. 742.
[8] Hwang DC, Damodaran S. Chemical modification strategies for synthesis of protein-based hydrogel. J Agric Food Chem 1996;44:751–8.
[9] Burmania JA, Stevens KR, Kao WJ. Synthesis and physicochemical analysis of interpenetrating networks containing modified

gelatin and poly(ethylene glycol) diacrylate. J Biomed Mater Res 2003, in press.

[10] Harris JM, Struck EC, Case MG, Paley MS. Synthesis and characterization of poly(ethylene glycol) derivatives. J Polym Sci: Polym Chem Ed 1984;22:341–52.

[11] Hall J, Trinder N, Givens DI. Observation on the use of 2,4,6-trinitrobenzenesulphonic acid for the determination of available lysine in animal protein concentrates. Analyst 1973;98: 673–86.

[12] Offner CM, Bubnis WA. Chemical and swelling evaluations of amino group crosslinking in gelatin and modified gelatin matrices. Pharma Res 1996;13:1821–7.

[13] Einerson NJ, Stevens KR, Kao WJ. Synthesis and physicochemical analysis of gelatin-based hydrogels for drug carrier matrices. Biomaterials 2003;24:509–23.

[14] Stevens KR, Einerson NJ, Burmania JA, Kao WJ. In vivo biocompatibility of gelatin-based hydrogels and interpenetrating networks. J Biomater Sci: Polym Ed 2003;13:1353–66.

[15] Kao WJ, Anderson JM. The cage implant testing system. In: Von Recum AF, editor. Handbook of biomaterials evaluation. 2nd ed.. Philadelphia, PA: Taylor & Frances; 1999. p. 659–71.

[16] Kao WJ, Zhao QH, Hiltner A, Anderson JM. Theoretical analysis of in vivo macrophage adhesion and foreign body giant cell formation on poly dimethylsiloxane, low density polyethylene, and polyetherurethanes. J Biomed Mater Res 1994;28: 70–3.

[17] Zhao Q, Topham N, Anderson JM, Hiltner A, Lodoen G, Payet CR. Foreign-body giant cells and polyurethane biostability: in vivo correlation of cell adhesion and surface cracking. J Biomed Mater Res 1991;25:177–83.

[18] Kao WJ, McNally AK, Hiltner PA, Anderson JM. Role for interleukin-4 in foreign-body giant cell formation on a poly(etherurethane urea) in vivo. J Biomed Mater Res 1995;29:1267–75.

[19] Kao WJ, Hiltner A, Anderson JM, Lodoen GA. Theoretical analysis of in vivo macrophage adhesion and foreign body giant cell formation on strained poly(etherurethane urea) elastomers. J Biomed Mater Res 1994;2:819–29.

[20] Suggs LJ, Shive MS, Garcia CA, Anderson JM, Mikos AG. In vitro cytotoxicity and in vivo biocompatibility of poly(propylene fumarate-*co*-ethylene glycol) hydrogels. J Biomed Mater Res 1999;46:22–32.

[21] Tabata Y, Ishii T, Muniruzzaman MD, Hirano Y, Ikada Y. Influence of gelatin complexation on cell proliferation activity and proteolytic resistance of basic fibroblast growth factor. J Biomater Sci: Polym Ed 2000;11:571–82.

[22] Muniruzzaman MD, Tabata Y, Ikada Y. Complexation of basic fibroblast growth factor with gelatin. J Biomater Sci: Polym Ed 1998;9:459–73.