Sustained release of multicomponent platelet-rich plasma proteins from hydrolytically degradable PEG hydrogels

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Abstract: Platelet-rich plasma (PRP), an autologous blood derived product is a concentrated mix of multiple growth factors and cytokines. Direct injections of PRP are clinically used for treatment of various musculoskeletal disorders and in wound healing. However, PRP therapy has met with limited clinical success possibly due to unpredictable and premature bolus delivery of PRP growth factors. The objective of this study was to predictably control the bioavailability of PRP growth factors using a hydrolytically degradable polyethylene glycol (PEG) hydrogel. We used a step-growth polymerization based on a Michael-type addition reaction between a 6-arm PEG-acrylate and a dithiol crosslinker, which led to the formation of a homogenous hydrogel network under mild, physiologically relevant conditions. Specifically, to model the release of multicomponent PRP through PEG hydrogels, we examined bulk diffusion of PRP as well as model proteins in a size range corresponding to that of growth factors found in PRP. Our results indicated that protein size and hydrogel degradation controlled diffusion of all proteins and that secondary structure of proteins encapsulated during gelation remained unaffected post-release. Analysis of specific PRP proteins released from the hydrogel showed sustained release until complete hydrogel degradation. PRP released from hydrogels promoted proliferation of human dermal fibroblast, indicating retained bioactivity upon encapsulation and release. The versatile hydrogel system holds clinical potential as a therapeutic drug delivery depot of multicomponent mixtures like PRP. © 2017 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 105A: 3304–3314, 2017.

Key Words: hydrogel, polyethylene glycol (PEG), growth factor, drug delivery, platelet rich plasma


INTRODUCTION
Platelet-rich plasma (PRP) is a blood-derived product containing a concentrated mixture of over 300 growth factors and cytokines extracted from activated platelets. Bioactive molecules in PRP play important roles in biological processes, including inflammation, angiogenesis, cell migration and cell metabolism. Clinical applications of PRP in orthopedics (intraarticular injections for osteoarthritis (OA)), sports medicine (acute ligamentous injury), or dentistry (periodontal surgery) utilize direct PRP injections to stimulate regeneration. Studies with PRP have shown that upon activation of platelets there is a bolus biomolecule release (~70%) within 10 min of clotting and ~100% release within 1 h. Since PRP is an assortment of growth factors which have inherently short half-life and circulation times, this bolus release fails to maximize their cell-stimulating potential as most biomolecules are cleared before they can exert a therapeutic effect. Thus, a controlled release strategy is necessitated.

Several polymer delivery systems have been developed to provide localized and sustained PRP release to improve its therapeutic utility. Hydrogels are a popular choice due to their tissue-like water content, injectability, and tenability. Primarily, natural hydrogels have been used for PRP delivery. For example, PRP encapsulated in alginate capsules showed controlled release for 14 days based on increased proliferation and alkaline phosphatase (ALP) activity in osteoblast-like cells. Intraarticular delivery of PRP from gelatin microspheres showed significantly reduced development of OA in rabbit knee compared to direct injection of PRP over 10 weeks. Similar results of superior repair have been reported upon sustained PRP delivery from other natural hydrogels, indicating the importance of sustained and localized release.

However, disadvantages associated with the use of natural polymers are batch-to-batch variability, nonspecific polymer-protein interactions, immunotoxicity, and limited control over hydrogel properties. Thus, using synthetic polymers of tightly controlled and tunable properties would be preferable. Recently, PRP-laden synthetic electrospun scaffolds of polycaprolactone and polylactic acid were shown to sustain PRP release up to 35 days, and promote the proliferation of adipose derived stem cells (ADSCs). However, electrospun materials are suitable as tissue engineering scaffolds or for surface...
wound healing applications but may not be used for localized delivery via injection.

Of the many synthetic polymers used for drug delivery, polyethylene glycol (PEG) hydrogels have been widely studied for controlled protein delivery and have shown excellent biocompatibility and preservation of protein bioactivity. Here, we designed a PEG hydrogel for sustained PRP delivery. We used step growth polymerization based on Michael-type addition reaction between an acrylate and a thiol for hydrogel formation. The reaction occurs under mild, physiological conditions with negligible nonspecific interactions. Moreover, in a stepwise network formed by a conjugate Michael addition reaction between an acrylate end-functionalized PEG and a dithiol crosslinker, a thioether-ester link is formed at every reaction site. The close proximity of the thio-ester bond to the PEG-acrylate ester accelerates the hydrolytic lability of that ester by several folds. Subsequently, the modified ester is cleaved by hydrolysis under physiological conditions or by esterases leading to slow breakdown of the entire crosslinked network on time scales relevant for many drug delivery and tissue regeneration applications.

We analyzed the dependence of PRP and model protein diffusion through the hydrogels on protein size and hydrogel degradation rate. Further, we ascertained the sustained release of specific PRP proteins from PEG hydrogels by multiplex analysis. We also used fluorescence correlation spectroscopy (FCS) to probe whether protein-protein interactions and protein crowding affect the release of PRP proteins from PEG. Lastly, we confirmed the bioactivity of the released PRP in vitro by measuring cell proliferation in human dermal fibroblasts (HDFs). Our results show sustained release of hydrogel-encapsulated PRP components in an active form. The versatile hydrogel system holds clinical potential as a therapeutic drug delivery depot for multicomponent mixtures like PRP.

**MATERIALS AND METHODS**

**Preparation of PRP**

Fresh human whole blood (Biological Specialty Corp; Colmar, PA) from 5 donors was pooled and PRP was prepared by using the Harvest® Smart Prep® PC60 procedure pack (Multicellular Processing System; Lakewood, CO) as per the manufacturer’s instructions. The concentrated platelets in PRP were separated by a 0.5 mm silicon spacer (Grace Bio-Labs, Bend, OR). The solution was incubated at room temperature (RT) for 1 h to allow complete gelation. For protein encapsulation, the proteins of interest or PRGF were mixed with the hydrogel precursor solution prior to gelation at a final concentration of 2% w/v unless specified otherwise.

**Measurement of hydrogel gelation time, mass loss, swelling ratio, and mesh size**

Hydrogels were made as described above. Gelation time was measured by the inverted tube method. Hydrogels were weighed immediately upon gelation to obtain the initial gel mass, Mᵣ, and incubated in 1X PBS pH 7.4 at 37°C for 24 h. Postincubation, gels were quickly blotted using a KimWipe® and weighed again to obtain the swollen mass, Mₛ. The gels were then dried at 60°C for 24 h and weighed to get their dry mass, M₀. The equilibrium swelling ratio, Qₑ, was calculated as 

\[ Qₑ = \frac{Mₛ}{M₀} \]

Hydrogel degradation was obtained indirectly by measuring the change in Qₑ as a function of time until the hydrogel samples had insufficient physical integrity to handle (~28 days). The obtained Qₑ was normalized to the Qₑ of the un-swollen gel at time zero (that is, Mᵣ/M₀). Mesh size (ξ) was calculated from Qₑ using the Flory-Rhener theory as described by us previously:

\[ \xi = \frac{V}{(\mathcal{R}₂)_{1/2}} \]

**Fabrication of PEG hydrogels and protein encapsulation**

PEG hydrogels were prepared as described by us previously. Briefly, a 20% w/v stock solution of 6-arm PEG acrylate (PEGAc; MW: 15 kDa; Sunbio, Anyang-si Gyeonggi-do, South Korea) and 5% w/v stock of dithiothreitol (DTT; Promega, Madison, WI) were made in 0.3 M triethanolamine (TEA; in 1X phosphate buffer saline (PBS) pH 7). A 10% w/v gel was made by mixing the two stock solutions in a 1:1 molar ratio of acrylate:thiol in 0.3 M TEA pH 7. The precursor solution was mixed well and 50 μL aliquots were placed between two glass slides precoated with RainX® and separated by a 0.5 mm silicon spacer (Grace Bio-Labs, Bend, OR). The solution was incubated at room temperature (RT) for 1 h to allow complete gelation. For protein encapsulation, the proteins of interest or PRGF were mixed with the hydrogel precursor solution prior to gelation at a final concentration of 2% w/v unless specified otherwise.
PEG hydrogels during gelation at a final concentration of 2% w/v. The hydrogels’ mass, gel thickness and diameter were recorded immediately after gelation. The hydrogels were immersed in 15 mL of 1X PBS pH 7.4 and incubated at 37°C with constant shaking. At regular intervals, a 1 mL sample was removed from the sink solution and replaced with 1 mL of fresh 1X PBS. The samples were analyzed for protein content using a Bradford protein assay (Bio-Rad, Hercules, CA) following the manufacturer’s procedure. Modified Fick’s law for short release times for slab geometry was used for calculating the effective diffusion coefficient, $D_e$, as reported by us previously. All experiments were performed at 37°C and $D_e$ was normalized to $D_0$ (Table I), which is the free solute diffusivity in 1X PBS pH 7.4 at 37°C.

**Determination of protein secondary structure via circular dichroism**

CD spectra (CD; Jasco J715; Jasco Analytical Instruments, Easton, MD) of BSA and lysozyme prerelease and postrelease from PEG hydrogels was obtained in the far ultraviolet (UV) range (190–260 nm) at 1 nm bandwidth and 1.5 s/nm scanning speed using 1X PBS pH 7.4 as the baseline.

**Multiplex analysis of PRP releasate from PEG hydrogels**

Ten percent w/v PEG hydrogels containing 10% w/v PRGF (50 µL each) were prepared and incubated in 500 µL Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Sigma-Aldrich, Saint Louis, MO). The entire incubation medium containing released PRGF was collected in the far ultraviolet (UV) range (190–260 nm) at 1 nm bandwidth and 1.5 s/nm scanning speed using 1X PBS pH 7.4 as the baseline.

**Measurement of protein diffusion via fluorescent correlation spectroscopy (FCS)**

BSA and lysozyme were labeled with Atto 655 NHS-ester dye (Thermo Fisher Scientific, Waltham, MA) as per the manufacturer’s protocol. Unbound fluorophores were removed using 5 dye removal columns (Thermo Fisher Scientific, Waltham, MA) with 95% efficiency. Solutes were prepared in 1X PBS pH 7.4 and in PEG hydrogels (10% w/v) in the presence and absence of 2% w/v PRGF. The concentrations of the fluorescent solutes Atto 655, Atto 655-labelled lysozyme, and Atto 655-labelled BSA were: 20 nM, 1 µM, and 10 µM in 1X PBS (with and without 2% PRGF), respectively and 200 nM, 10 µM, and 100 µM in PEG hydrogels (with and without 2% PRGF), respectively. For FCS measurements, the respective solutions were placed in CoverWell perfusion chamber gaskets (Grace Bio-Labs, Bend, OR) and gels were hydrated in Nunc™ Lab-Tek™ II Chambered Coverglass (Thermo Fisher scientific, Waltham, MA). FCS (MicroTime 200; PicoQuant, Berlin, Germany) was used to measure solute diffusion times ($\tau_D$). FCS was calibrated using 20 nM Atto 655 in 1X PBS. A 640 nm ps pulsed laser was used at an optical power of ~3.8–7.6 µW. Six measurements at different spots (180 s each) were performed per sample. Autocorrelation function $G(\tau)$ was obtained for each measurement:

$$G(\tau)=1+\frac{1}{N}\left[1+\left(\frac{\tau}{\tau_0}\right)^\alpha\right]\left[1+p\left(\frac{\tau}{\tau_0}\right)^\beta\right]$$

where $N$ is the number of fluorescent particles, $p = r_o/z_o$ is an instrumental constant, $r_o$ is the radius and $z_o$ is the axial length of the focused laser beam spot, and $\alpha$ is the diffusion exponent, where $\alpha < 1, \alpha = 1,$ and $\alpha > 1$ indicate anomalous, normal, and super-diffusion, respectively.

**Measurement of cell proliferation in the presence of released PRP**

Human dermal fibroblasts (HDFs; ATCC; Manassas, VA) were maintained in DMEM/F12 media supplemented with 10% v/v fetal bovine serum (PBS; Biowest, Riverside, MO, USA) and 1% v/v penicillin-streptomycin (P/S; Hyclone, Pittsburgh, PA) in a humidified incubator at 5% CO2 and 37°C. Medium was refreshed every 2–3 d, cells were passaged at 80% confluency and used for experiments up to passage 5. Three experimental set-ups were assessed. First, HDFs were seeded at a density of 25,000 cells/transwell insert (0.96 cm² growth area and 3 µm pore size) and incubated at 37°C for 2 h. Then, PEG hydrogels containing 2 or 10% w/v PRGF were placed at the bottom of each transwell. Medium only and 0.1% w/v PRGF in media (a bolus dose) were used as controls. Cell proliferation was measured at 1 and 3 d. Second, using the same set-up as above, cells were cultured in the presence of PRGF-containing media (0.1% w/v) and/or PRGF-containing hydrogels (2 or 10% w/v) for 2 h. The media was then replaced with fresh media without PRGF. Cell proliferation was measured at 3 and 7 d. Third, 10% w/v PRGF-containing PEG hydrogels were incubated in 500 µL of complete medium at 37°C until the hydrogel degraded completely (~28–31 d). The incubation medium was collected at 1, 2, 10, and 24 h at degradation, and replaced each time with fresh 500 µL medium without PRGF. The collected PRGF-containing medium was stored at −80°C until further use. HDF were seeded in a 48-well plate at a density of 25,000 cells/well, incubated for 2 h and exposed to PRGF-containing medium collected as described above. Cells in medium only

<table>
<thead>
<tr>
<th>Protein</th>
<th>Hydrodynamic Radius (nm)</th>
<th>MW (kDa)</th>
<th>$D_0$ in PBS ($x 10^{-7} \text{cm}^2/\text{s}$) at 37°C</th>
<th>$p_l$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>1.9</td>
<td>14</td>
<td>9.7</td>
<td>11.35</td>
<td>26</td>
</tr>
<tr>
<td>BSA</td>
<td>3.6</td>
<td>65</td>
<td>5.3</td>
<td>4.5</td>
<td>27</td>
</tr>
<tr>
<td>IgG</td>
<td>5.3</td>
<td>150</td>
<td>4.4</td>
<td>7.2</td>
<td>28</td>
</tr>
</tbody>
</table>

TABLE I. Properties and Diffusion Coefficients of Model Proteins as Reported in the Literature
and in 0.1% w/v PRGF-containing medium were used as controls. Cell proliferation was measured at d 3. In all three setups, HDF proliferation upon exposure to PRGF-conditioned releasate/released PRGF was measured via an MTS assay (Promega, Madison, WI). Briefly, prior to MTS measurements, culture medium in the wells was replaced with fresh medium containing the MTS reagent (10 μL/100 μL media) and the plate was incubated at 37°C for 1 h. Postincubation, 100 μL of medium from each well was transferred to a new 96-well plate and absorbance was read at 490 nm using a spectrophotometer (SpectraMax i3; Molecular Devices, Sunnyvale, CA). The absorbance was correlated to cell number based on a standard curve obtained by performing an MTS assay on a serially-diluted known number of HDF cells of the same passage number.

Cell proliferation was expressed as fold-change in cell proliferation as compared to cells cultured in medium without PRGF.

**Statistical analysis**

Each experiment was conducted using 3–4 replicate samples per condition. The results are expressed as average ± standard deviation from 2–3 independent experiments. One way Analysis of Variance (ANOVA) was used to determine statistical significance for multiple parameters followed by Tukey’s posthoc test. Student’s t test was used to compare between two groups. A value of p < 0.05 was considered significant. All statistical analysis was done in Microsoft Excel and Graph Pad-Prism.

**RESULTS**

PRP is a mixture of various growth factors, cytokines, chemokines, other proteins and lipids. Some important growth factors in PRP, which have been a focus for wound healing and musculoskeletal regeneration, are listed in Table II together with their molecular weights (MW) and hydrodynamic radii (rs). Both MW and rs vary in a wide range, namely 6–92 kDa and 1.4–3.7 nm, respectively. Thus, in this work we used PRP as well as model proteins in a range of sizes similar to PRP proteins to establish PRP diffusivity trends from PEG hydrogels and understand the factors that determined those trends.

**Gelation time, Qm, degradation kinetics and mesh size of PEG hydrogels**

Gelation time of PEG hydrogels decreased with an increase in reaction buffer pH, and was not affected by the encapsulation of 10% w/v PRGF but increased slightly (but significantly) when 10% w/v BSA was encapsulated (Table III). The longest gelation time was ~15 min (at pH 7), but hydrogels were left to gel for 1 h to assure complete gelation. There was a slight but significant increase in Qm of hydrogels from 11.41 ± 0.84 to 13.03 ± 0.91 with increase in reaction pH; no significant change in Qm was observed upon BSA or PRGF encapsulation. Mesh size for all hydrogels was in the range of 7–8.5 nm, being largest (8.42 ± 0.47 nm) for the reaction buffer pH of 8.5.

We also continuously monitored the change in Qm and mesh size to determine the rate of gel degradation. Representative data for a gel prepared at pH 7 is presented in Figure 1. Both Qm and mesh size increased ~1.9 times over a period of 28 d. All hydrogels maintained their physical integrity for ~22 d (15 d for hydrogels prepared at pH 8.5) after which it declined rapidly. The hydrogels degraded completely in ~20–31 d, where degradation time was dependent on reaction pH but independent of protein

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**TABLE II. Representative Growth Factors Found in PRP and Their Properties**

<table>
<thead>
<tr>
<th>PRP Component</th>
<th>MW (KDa)</th>
<th>Hydrodynamic Radii (nm)</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>6</td>
<td>1.4</td>
<td>30</td>
</tr>
<tr>
<td>Regulated upon activation, normal T cell expressed and secreted (RANTES)</td>
<td>8</td>
<td>1.6</td>
<td>31</td>
</tr>
<tr>
<td>Fibroblast growth factor 2 (FGF 2)</td>
<td>18</td>
<td>2.1</td>
<td>32</td>
</tr>
<tr>
<td>Platelet Derived growth factor (PDGF-1)</td>
<td>35</td>
<td>2.7</td>
<td>33</td>
</tr>
<tr>
<td>Platelet Derived growth factor (PDGF-2)</td>
<td>27</td>
<td>2.5</td>
<td>33</td>
</tr>
<tr>
<td>Vascular derived endothelial growth factor (VEGF)</td>
<td>43</td>
<td>3.0</td>
<td>34</td>
</tr>
<tr>
<td>Transforming growth factor Beta (TGF-β)</td>
<td>67</td>
<td>3.5</td>
<td>35</td>
</tr>
<tr>
<td>Hepatocyte growth factor (HGF)</td>
<td>74–92</td>
<td>3.7</td>
<td>36</td>
</tr>
</tbody>
</table>

**TABLE III. Properties of PEG Hydrogels Prepared at Different Reaction pH and with Encapsulated Model BSA Protein or PRGF Mixture**

<table>
<thead>
<tr>
<th>Hydrogel</th>
<th>pH</th>
<th>Swelling Ratio ± SD</th>
<th>Gelation Time (s) ± SD</th>
<th>Mesh Size (nm) ± SD</th>
<th>Degradation Time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-arm PEGAc-DTT</td>
<td>7</td>
<td>13.45 ± 1.11</td>
<td>623 ± 36</td>
<td>9.59 ± 0.41</td>
<td>31</td>
</tr>
<tr>
<td>6-arm PEGAc-DTT</td>
<td>7.4</td>
<td>12.48 ± 0.52</td>
<td>430 ± 92</td>
<td>10.86 ± 0.21</td>
<td>28</td>
</tr>
<tr>
<td>6-arm PEGAc-DTT</td>
<td>8.5</td>
<td>13.03 ± 0.91</td>
<td>50 ± 3</td>
<td>10.52 ± 0.86</td>
<td>20</td>
</tr>
<tr>
<td>6-arm PEGAc-DTT</td>
<td>7</td>
<td>13.75 ± 0.73</td>
<td>707 ± 33*</td>
<td>9.68 ± 0.79</td>
<td>31</td>
</tr>
<tr>
<td>with 10% BSA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-arm PEGAc-DTT</td>
<td>7</td>
<td>12.35 ± 0.64*</td>
<td>567 ± 38</td>
<td>9.14 ± 0.27*</td>
<td>31</td>
</tr>
<tr>
<td>with 10% PRGF</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
encapsulation (Table III). PEG hydrogels of 10% w/v and prepared at pH 7 were used for all subsequent experiments.

**Diffusion of model proteins and PRGF from PEG hydrogels**

The release from PEG hydrogels for the three model proteins, namely lysozyme, BSA and IgG, which varied in MW and \( r_s \) (Table I), predictably decreased with an increase in protein size (Figure 2).\(^2\)\(^9\) The release of PRGF appeared similar to that of lysozyme. Fractional release (\( M_r \text{eff}/M_r \text{tot} < 0.6 \)) was found to be linear for short diffusion times of \( M_r \text{eff}/M_r \text{tot} < 0.6 \) [Figure 2(A)]. We noted \( \sim50\% \) reduction in lysozyme diffusivity in the hydrogel as opposed to in PBS (\( D_e/D_o \approx 0.51 \)), \( \sim85\% \) reduction in BSA diffusivity (\( D_e/D_o \approx 0.09 \)), and \( \sim100\% \) reduction in IgG diffusivity (\( D_e/D_o \approx 0.002 \); note the linear portion of the graph up to \( 15\) s\(^{1/2} \) in Figure 2(A) was used for this \( D_e \) calculation) [Figure 2(B)]. The \( D_e \) for PRGF release from the hydrogel was found to be \( 1.3 \times 10^{-7} \) cm\(^2\)/s (Table I). The \( D_e \) in the hydrogel could be directly linked to protein size in relation to hydrogel mesh size. The fastest diffusing protein, lysozyme, had a hydrodynamic radius (\( r_s = 1.9 \) nm), which was 4-times lower than the hydrogel mesh size (that is, \( r_s < \xi \)), BSA had a hydrodynamic radius (\( r_s = 3.7 \) nm) 2-times lower than the hydrogel mesh size (that is, BSA diameter \( \approx \xi \)), and IgG had a radius (\( r_s = 5.3 \) nm) similar to the hydrogel mesh size (that is, IgG diameter \( > \xi \)).

Lysozyme exhibited a burst release, where \( \sim25\% \) was released in the first 10 min and \( 82\% \) of the protein was released in the first 2 h [Figure 2(C)]. PRGF release was rapid and similar to lysozyme; \( \sim60\% \) of PRGF was released in the first 2 h. BSA release was more controlled (\( \sim5\% \) released in the first 10 min due to burst release) with \( \sim65\% \) released in 24 h. In the case of IgG, \( \sim10\% \) was released in the first 4 h, and \( \sim82\% \) was released at 24 d, which coincided with significant gel degradation. Further comparing IgG fractional release to gel mesh size [Figure 2(D)], we found excellent correlation between them, confirming degradation-dependent IgG release.

**Multiplex analysis of released growth factor**

Here we followed the release of 4 PRGF proteins (PDGF-AA, PDGF-BB, RANTES, and EGF) until complete gel degradation \( \text{via} \) a multiplex ELISA. The proteins were of small MW and \( r_s < \xi \) (6-35 kDa; Table II) and we anticipated them to be released early. Surprisingly, all 4 proteins were released continually until gel degradation, but still exhibited a burst release with up to \( 40\% \) of protein released in 1 h (Figure 3). An additional \( \sim30-40\% \) release was observed at 10–24 h, with the remaining protein being released at complete gel degradation. Thus, we observed a sustained release from the hydrogels even for proteins with a size below the hydrogel mesh size. Note that the collection time points used here were not evenly spaced; hence, we observed a time-dependent accumulation of proteins in the medium. For example, immediately after incubation of the hydrogels in the release medium, a large protein accumulation was seen at 1 h indicative of burst release. However, for the second time point at 2 h, protein accumulation in the medium was lower indicative of a sustained release phase. As the incubation time was extended to 10 h, 24 h, and later time points, it led to accumulation of proteins by slow sustained diffusion into the medium.

**In situ diffusion of model proteins in the presence of PRGF**

We investigated diffusivities of model proteins in the PEG hydrogel in the presence and absence of PRGF to determine if crowding or interactions with PRGF contributed to the delayed protein release. The diffusion times of Atto 655, lysozyme, and BSA were measured in 4 diffusion media: PBS only, \( 2\% \) w/v PRGF in PBS, PEG gel, and \( 2\% \) w/v PRGF in PEG gel (Figure 4). The diffusion times in each medium, \( \tau_D \), were normalized to the free diffusion times in PBS, \( \tau_o \). Note that normalized diffusion time \( \tau_D/\tau_o \) is equivalent to the normalized diffusion coefficient, \( D_e/D_o \). A representative autocorrelation function for lysozyme in PBS, PEG gel and PEG gel with \( 2\% \) w/v PRGF is shown in Figure 4(A), where a shift to longer diffusion times was noted with encapsulation in gel. The residuals were minimal and the \( \chi^2 \) values (Table IV) were close to 1, indicating a good fit. As expected, the diffusivities of all solutes in PBS were significantly higher than the diffusivities in the gel, but were not significantly affected by the presence of PRGF in either PBS or in the gel. [Figure 4(B)]. Lastly, the diffusivity of the small dye Atto 655 was significantly higher in all diffusing media compared to the diffusivities of the proteins. Overall, the diffusion trends obtained via FCS correlated well with results from bulk diffusion.

Diffusion times, \( \tau \) values, and \( \chi^2 \) are reported in Table IV. We observed an anomalous diffusion for BSA (\( \alpha < 0.9 \)) in gels with and without PRGF, but not in PBS, indicating that confinement by the gel rather than crowding by and/or interactions with PRGF were responsible for the anomaly. Note that anomalous diffusion was only observed for BSA, which is similar in size to the hydrogel mesh size. It should also be noted that we only tested diffusivities in \( 2\% \) w/v PRGF since higher concentrations of PRGF led to a high level of autofluorescence that interfered with FCS measurements.
Analysis of the secondary structure of released model proteins from PEG hydrogels

We again used the model proteins lysozyme and BSA as their secondary structure is well-characterized.\textsuperscript{18–20} The CD spectra of BSA [Figure 5(A)] and lysozyme [Figure 5(B)] prior to encapsulation into and post-release from the PEG gel were nearly identical, indicating no significant structural protein changes. The CD spectra of BSA showed two
negative bands at 208 nm and 222 nm corresponding to α helices, while α helices in lysozyme were characterized by the negative bands at 205 and 210 nm.

Analysis of PRGF biological activity upon release from PEG hydrogels

HDFs were chosen to assess PRGF biological activity, as HDFs are commonly used to study wound healing responses in vitro and represent a simple model to study the effect of growth factors on cell proliferation. When HDFs were cultured continuously in the presence of PRGF, cell growth in response to PRGF released from PEG hydrogels (2% or 10% w/v PRGF) was similar to that of bolus PRGF at 0.1% w/v (control) [Figure 6(A)]. This result indicated that PRGF retained biological activity upon encapsulation and release from PEG hydrogels. The gel itself did not show a significant effect on cell growth (normalized cell growth 0.83 ± 0.40) and cells in all PRGF conditions showed ~20% increase in normalized cell growth at d 3 compared to d 1.

We next repeated the above experiment with one difference: the media was refreshed after 2 h of culture with media containing no PRGF [Figure 6(B)]. Hence, cells were exposed to bolus PRGF for only 2 h and all PRGF components released from the gels during burst release (see Figure 2) were removed. This was done to emulate in vivo conditions where fluids at the site of injury are refreshed routinely (for example, knee capsule where intraarticularly delivered factors are lost in ~2 h due to synovial fluid exchange). Since previous studies and our own experience showed that the PRGF has a maximum cell proliferation effect on d 3 or 4, for this study we chose d 3 and 7 (a later time point) for assessment. Cell growth in response to the 10% w/v PRGF hydrogel was significantly higher than either PRGF-containing media or the 2% w/v PRGF hydrogel, specifically at d 7. Our results indicated that the PRGF proteins released from PEG hydrogels after the initial burst release were still potent enough to induce cell proliferation.

Lastly, we sought to determine whether various PRGF release fractions from the gel, collected at different time points, retained the proliferative capacity of PRGF [Figure 6(C)]. PEG gels with 10% w/v PRGF were incubated in supplemented cell culture medium and relesates were collected continually at 1, 2, 10, and 24 h from the same gel. We observed that all PRGF fractions, except for the one at 24 h, showed cell growth equivalent to bolus PRGF control.

**DISCUSSION**

The aim of this study was to characterize degradable PEG hydrogels formed via Michael addition reaction for sustained release of a multicomponent PRGF mixture. Compared to other hydrogels formed by free radical polymerization, the hydrogels formed via step-growth polymerization have more defined and homogenous structures, with increased control over mesh size, degradation and solute release.

To control PRGF release kinetics, we physically entrapped a lyophilized PRGF powder in PEG hydrogels prior to

**TABLE IV. Diffusion Times and α Values (Refer to Eq. 1) of Solutes in Various Diffusion Media**

<table>
<thead>
<tr>
<th>Diffusion Medium</th>
<th>Atto 655 Dye</th>
<th>Labeled Lysozyme</th>
<th>Labeled BSA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \tau_0 (\text{ms}) )</td>
<td>( \alpha )</td>
<td>( \chi^2 ) Range</td>
</tr>
<tr>
<td>PBS</td>
<td>0.089 ± 0.001</td>
<td>1.000 ± 1.263</td>
<td>0.199 ± 0.055</td>
</tr>
<tr>
<td>2% PRGF in PBS</td>
<td>0.090 ± 0.005</td>
<td>0.922 ± 1.581</td>
<td>0.247 ± 0.008</td>
</tr>
<tr>
<td>Hydrogel</td>
<td>0.127 ± 0.007</td>
<td>0.985 ± 0.614</td>
<td>1.735 ± 0.008</td>
</tr>
<tr>
<td>2% PRGF in Hydrogel</td>
<td>0.127 ± 0.007</td>
<td>0.973 ± 0.689</td>
<td>1.735 ± 0.008</td>
</tr>
</tbody>
</table>
gelation. Compared to other methods of protein encapsulation, physical entrapment is more simplistic as no protein modification is required, thus, ensuring the protein activity and stability.\textsuperscript{17,19,20,42} Sustained release of a protein mixture such as PRGF (Table II), would depend on the diffusivities of the individual PRGF components. The protein diffusivities, on the other hand, would depend on protein size (MW and $r_s$), hydrogel mesh size ($\xi$) and degradation rate as well as protein-protein and protein-hydrogel interactions.\textsuperscript{20,42}

To test the PEG hydrogel system for sustaining release of protein mixtures, we first studied the diffusion of 3 model proteins: lysozyme, BSA and IgG. The chosen proteins not only had well-defined physical properties,\textsuperscript{18–20} but a wide range of MW and $r_s$ (Table I), which corresponded to the MW ($6$-$70$ kDa) and $r_s$ ($1.4$-$4.0$ nm) range of proteins in PRGF (Table III). For PRGF we observed a release pattern similar to lysozyme: a burst release at early times (<120 min) followed by a sustained release until 200 min [Figure 2(C)]. Comparing the release of lysozyme (14 kDa) to the release of PRGF proteins, it is expected that small MW proteins would be released at early times (that is, burst phase). Factors, which contribute to burst release, are protein size (proteins with $r_s \ll \xi$ exhibit free diffusion in the hydrogel), easy escape of proteins on the gel periphery, and faster protein release through larger gel pores, compared to a slower release from pores obstructed by polymer entanglements.\textsuperscript{19,20,42}

Interestingly, contrary to rapid bulk PRGF diffusion, multiplex analysis of individual PRGF components, such as EGF, PDGF and RANTES, showed sustained release until gel degradation (Figure 3). The reason these proteins were retained in the gel until degradation was unclear. There is evidence that certain proteins within PRGF interact with each other,\textsuperscript{32,43} which would result in impeded and anomalous diffusion.\textsuperscript{44} For example, PRGF is rich in BSA ($42.5 \pm 0.6$ mg/ml) and fibrinogen ($2.82 \pm 0.34$ mg/ml),\textsuperscript{45} both negatively charged proteins at physiological pH with pl values of 4.7 and 5.2, respectively. VEGF, a positively charged protein of pl $8.5$ at physiological pH, is known to interact with fibrinogen.\textsuperscript{42} Most growth factors, including EGF and PDGF carry a charge (pl $4.6$ and $9.8$, respectively) at physiological pH.\textsuperscript{46,47} Positively charged proteins would be able to electrostatically interact with the negatively charged BSA and fibrinogen,\textsuperscript{32} which could be a reason for sustained release.

To understand this, we next used FCS to test whether individual protein diffusion (BSA and lysozyme model proteins) in the PRGF-loaded hydrogel was impeded due to crowding from or interactions with PRGF (Figure 4). Molecular crowding is a known phenomenon which can obstruct release and cause anomalous diffusion ($\chi < 1$).\textsuperscript{48} Diffusion of even small proteins like lysozyme ($r_s = 1.9$ nm) was obstructed when encapsulated in the gel, which resulted in ~10 times increase in diffusion time. Extrapolating these results to some of the smaller proteins in PRGF, a similar retarded diffusion could be expected. Note that the initial concentration of proteins inside the gels was 2% w/v, hence protein crowding could slow down the Brownian motion of solutes in the gel.\textsuperscript{20,37} Surprisingly, we did not see significant changes in diffusion due to PRGF presence, but we noted anomalous diffusion for BSA due to entrapment in the hydrogel.

Other possible explanations for retarded diffusion of small proteins in the hydrogel could be gel inhomogeneity and physical entanglements of the polymer network, which lead to a lower $\xi$ or nonspecific protein-polymer and protein-protein interactions, which contribute to decreased diffusivity.\textsuperscript{20} Protein-protein interactions could lead to the formation of aggregates (90–100 nm at neutral pH)\textsuperscript{49} or the presence of different surface residues on proteins.\textsuperscript{50} Lastly, while it was possible to encapsulate PRGF as high as 10% w/v in the gels, it retained its native clotting ability which may have led to proteins being trapped in the clot and eluted slowly.

We next assessed the effect of hydrogel encapsulation and release on protein activity. A possible concern in using thiol-based reaction chemistry for gel formation during protein encapsulation is the thiol disulfide exchange above neutral pH, which could lead to protein aggregation and secondary structure disruption.\textsuperscript{51,52} We ascertained that the gelation process did not affect protein secondary structure by examining the CD spectra of the released BSA and lysozyme (Figure 5). Our data is corroborated by earlier studies by our group and others, who have confirmed the reaction specificity and negligible effect on encapsulated proteins.\textsuperscript{19,20}

Lastly, we confirmed the bioactivity of the released PRGF from PEG gels by testing its effect on HDF cell growth under three different experimental conditions (Figure 6). In
all conditions, PRGF released from PEG hydrogels showed similar or better bioactivity than bolus PRGF dose as measured indirectly by HDF cell growth. Interestingly, we observed that released PRGF induced cell proliferation even when PRGF components released up to 2 h (that is, burst phase) (Figure 2) were removed. We concluded that the process of PRGF encapsulation and release did not significantly affect its bioactivity. Similar results illustrating the beneficial effects of sustained PRP release from alginate capsules on osteoblast proliferation have been shown in an earlier study. Another study demonstrated that controlled PRP release from gelatin gel was more effective than bolus PRP in inducing angiogenesis for critical ischemia.

CONCLUSIONS
In this study, we designed and tested a synthetic PEG hydrogel delivery system for the encapsulation and sustained delivery of PRGF. The PEG hydrogel sustained the release of different PRGF growth factors until gel degradation. The released PRGF was biologically active as indicated by the improved proliferation of HDF cells in vitro as compared to no PRGF. Moreover, released PRGF induced cell proliferation equivalent to bolus PRGF and maintained its bioactivity up to 7 d. The PEG hydrogel holds high clinical translation potential for various applications that require sustained PRGF release, such as wound healing and osteoarthritis.

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CONFLICT OF INTEREST
Authors declare no competing interest.

REFERENCES


