



Controlled release of bioactive PDGF-AA from a hydrogel/nanoparticle composite



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ABSTRACT

Polymer excipients, such as low molar mass poly(ethylene glycol) (PEG), have shown contradictory effects on protein stability when co-encapsulated in polymeric nanoparticles. To gain further insight into these effects, platelet-derived growth factor (PDGF-AA) was encapsulated in polymeric nanoparticles with vs. without PEG. PDGF-AA is a particularly compelling protein, as it has been demonstrated to promote cell survival and induce the oligodendrocyte differentiation of neural stem/progenitor cells (NSPCs) both *in vitro* and *in vivo*. Here we show, for the first time, the controlled release of bioactive PDGF-AA from an injectable nanoparticle/hydrogel drug delivery system (DDS). PDGF-AA was encapsulated, with high efficiency, in poly(lactide-co-glycolide) nanoparticles, and its release from the drug delivery system was followed over 21 d. Interestingly, the co-encapsulation of low molecular weight poly(ethylene glycol) increased the PDGF-AA loading but, unexpectedly, accelerated the aggregation of PDGF-AA, resulting in reduced activity and detection by enzyme-linked immunosorbent assay (ELISA). In the absence of PEG, released PDGF-AA remained bioactive as demonstrated with NSPC oligodendrocyte differentiation, similar to positive controls, and significantly different from untreated controls. This work presents a novel delivery method for differentiation factors, such as PDGF-AA, and provides insights into the contradictory effects reported in the literature of excipients, such as PEG, on the loading and release of proteins from polymeric nanoparticles.

Statement of Significance

Previously, the polymer poly(ethylene glycol) (PEG) has been used in many biomaterials applications, from surface coatings to the encapsulation of proteins. In this work, we demonstrate that, unexpectedly, low molecular weight PEG has a deleterious effect on the release of the encapsulated protein platelet-derived growth factor AA (PDGF-AA). We also demonstrate release of bioactive PDGF-AA (in the absence of PEG). Specifically, we demonstrate the differentiation of neural stem and progenitor cells to oligodendrocytes, similar to what is observed with the addition of fresh PDGF-AA. A differentiated oligodendrocyte population is a key strategy in central nervous system regeneration. This work is the first demonstration of controlled PDGF-AA release, and also brings new insights to the broader field of protein encapsulation.

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1. Introduction

Platelet-derived growth factor-AA (PDGF-AA) is a promising factor for both *in vitro* and *in vivo* differentiation of neural stem/progenitor cells (NSPCs). PDGF-AA can expand the NSPC-derived oligodendrocyte population, acting both as an oligodendrocyte

differentiation factor and a survival and proliferation factor for oligodendrocyte progenitor cells [1–4]. The effects of PDGF-AA have been demonstrated for multiple cell sources, including brain-derived [1] and spinal cord-derived [2] NSPCs; however, brain-derived NSPCs have demonstrated greater survival and oligodendrocyte differentiation after spinal cord transplantation [5]. In a rat model of spinal cord injury (SCI), intrathecal infusion of a combination of PDGF-AA and other growth factors for 7 d enhanced the survival and oligodendrocyte differentiation of transplanted brain-derived NSPCs [6,7]. When covalently immobilized

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to hydrogels, PDGF-AA has been shown to promote both the differentiation of NSPCs to oligodendrocytes *in vitro* [1,8] and tissue benefit *in vivo* [9,10]. PDGF-AA is a promising factor for use in conjunction with stem cell transplantation.

To be useful *in vivo*, such as delivery to the spinal cord, protein therapeutics require a minimally invasive, local, sustained release system. One previous strategy for PDGF-AA delivery relied upon a high dose delivered via a highly invasive catheter infusion system that is susceptible to infection and results in PDGF-AA being dispersed throughout the cerebrospinal fluid (CSF) [6,7]. As an alternative to intrathecal infusion, several hydrogel-based systems have been developed that employ covalent [1,8,9] or affinity-based [11,12] immobilization of PDGF-AA. Although these systems have promoted oligodendrocyte differentiation, they are limited in their control over both protein loading and release profile. Consequently, an injectable, minimally invasive hydrogel-based drug delivery system (DDS) has been developed that provides sustained and localized release to the spinal cord [13,14]. This system is composed of protein encapsulated in poly(lactide-co-glycolide) (PLGA) nanoparticles that are dispersed in a hyaluronan/methyl cellulose hydrogel (HAMC). The ability of this DDS to be adapted for a specific release period [13], as well as its biocompatibility [15], make it attractive for the release of PDGF-AA.

Polymeric nanoparticles have been widely studied for the release of small molecules and proteins [16,17]. For example, platelet-derived growth factor-BB (PDGF-BB) has previously been encapsulated and released from polymeric nanoparticles [18], yet to date there are no strategies for the controlled release of PDGF-AA. Challenges for the encapsulation of proteins, such as PDGF-AA, include poor encapsulation efficiency, incomplete release, and loss of activity [19]. The addition of protein and polymer excipients, such as low molecular weight poly(ethylene glycol) (PEG), have been shown to improve encapsulation efficiency and release [20].

The aim of this project is to determine the optimal strategy for the controlled release of bioactive PDGF-AA and the role of PEG therein. PDGF-AA was encapsulated in PLGA nanoparticles and the effect of excipients on encapsulation efficiency, release and bioactivity were studied. The PDGF-AA DDS was then tested in a transwell culture system to determine the effect of released PDGF-AA on NSPC differentiation and proliferation relative to untreated NSPCs and fetal bovine serum (FBS)-treated cells.

2. Materials and methods

2.1. Nanoparticle fabrication and hydrogel preparation

PDGF-AA-loaded PLGA nanoparticles were prepared by a double emulsion-solvent evaporation process [13]. PDGF-AA (Peprotech, Rock Hill USA) with 12 mg of bovine serum albumin (BSA, Sigma-Aldrich, Oakville CAN) dissolved in aqueous buffer was added to 50/50 PLGA (7–17 kg/mol, Sigma) dissolved in dichloromethane (Caledon Labs, Georgetown CAN) and a primary emulsion formed by sonication for 10 min on ice. In a modified formulation, 5 μ L of 400 g/mol poly(ethylene glycol) (PEG400, Sigma) was added to the inner aqueous phase. In an additional formulation, brain-derived neurotrophic factor (BDNF, Peprotech) was encapsulated in PLGA nanoparticles via the same method, with and without the addition of PEG400. Upon addition of an outer aqueous phase of 2.5 w/v% poly(vinyl alcohol) (PVA, Sigma) in distilled water, and a further 10 min of sonication, a secondary emulsion was formed. After stirring at room temperature for 4 h, the particles were centrifuged and washed four times to remove surface PVA. The particles were then lyophilized and stored at -20°C .

HAMC hydrogels were prepared by dispersing methyl cellulose (300 kg/mol, Shin-Etsu, Tokyo Japan) and sodium hyaluronan (HA,

$1.4\text{--}1.8 \times 10^6$ g/mol, NovaMatrix, Sandvika Norway) in artificial cerebrospinal fluid (aCSF, pH 7.4) [11] or serum-free media using a dual asymmetric centrifugal mixer for a final composition of 0.5 wt% HA and 0.5 wt% MC. Hydrogels were incubated overnight at 4°C to ensure polymer dissolution before use.

2.2. Nanoparticle characterization

To determine the encapsulation efficiency of PDGF-AA, 2.5 mg of nanoparticles were dissolved in dichloromethane. After 1 h of incubation, the protein was extracted into aqueous buffer and the protein concentration was analyzed by an enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis USA). The encapsulation efficiency of extracted BDNF was also analyzed by ELISA (Promega, Madison USA). The loading of BSA was determined by dissolving the nanoparticles in dimethyl sulfoxide (DMSO) and adding 0.05 M sodium hydroxide (NaOH) containing 0.05 wt% sodium dodecyl sulfate (SDS). BSA concentration was determined by a micro-bicinchoninic acid (microBCA) assay (Thermo Scientific, Nepean CAN). BDNF-loaded nanoparticles were sized via dynamic light scattering (DLS, Malvern Instruments, Malvern UK) in aCSF.

2.3. Release of PDGF-AA and BSA

The release of PDGF-AA from nanoparticles was studied by dispersing 10 mg of nanoparticles in 100 μ L of HAMC. The nanoparticle/hydrogel DDS was warmed to 37°C , and 900 μ L of aCSF was added. The supernatant was sampled periodically with complete replacement over 21 d. At the end of the release study, the DDS was dissolved in dichloromethane and any remaining protein was extracted into aqueous buffer. The concentration of protein in the supernatant or the dissolved DDS was determined by ELISA (PDGF-AA, BDNF) and microBCA (BSA).

2.4. Stability of PDGF-AA in the presence of PEG400

The stability of PDGF-AA after incubation with PEG400 was examined by ELISA, differential scanning fluorimetry (DSF) and dynamic light scattering (DLS).

For the ELISA study, PDGF-AA in aCSF was incubated with 0.5 wt% PEG400 at 37°C for 1 and 7 d. At each timepoint, the concentration of PDGF-AA was measured by ELISA and compared to an untreated sample.

DSF was used to perform a fluorescence-based thermal shift assay (FTS) to assess the thermal unfolding of PDGF-AA and a reference protein, lysozyme [21]. Solutions of PDGF-AA or lysozyme (Sigma) were prepared in phosphate buffered saline (PBS) with 0, 0.1, 0.5, 1, 5, or 10 wt% PEG400. Protein was dispensed into a 96 well plate held at 4°C , and SYPRO Orange (Invitrogen, Burlington CAN, diluted 1:5000 from 5000 \times concentrate in DMSO) was added, with a final protein concentration of 0.5 mg/mL. A thermal shift assay was performed in a reverse-transcriptase polymerase chain reaction (RT-PCR) thermocycler, and fluorescence intensity (excitation wavelength 498 nm, emission wavelength 580 nm) was measured over a range of $10\text{--}85^{\circ}\text{C}$ with a linear thermal increase of $0.5^{\circ}\text{C}/\text{min}$.

Dynamic light scattering was performed to determine the aggregation of PDGF-AA with PEG400. PDGF-AA in PBS (0.5 mg/mL) was incubated with 0.5 wt% PEG400 at 37°C . In a similar study, 0.5 mg/mL of each of BSA, lysozyme, α -chymotrypsin, insulin, or ovalbumin (Sigma) was incubated at 37°C with and without PEG400. Dynamic light scattering was performed on samples and buffer controls using a DynaPro Plate Reader II (Wyatt Technology, Santa Barbara USA).

2.5. Cell culture

This study was carried out in strict accordance with the guidelines set out by the Canadian Council on Animal Care. The study protocol was approved by the University Animal Care Committee at the University of Toronto (Protocol Number 20008663). Neural stem/progenitor cells were harvested from adult male Wistar rats that were humanely sacrificed by gradual-fill carbon dioxide exposure, and all efforts were made to minimize suffering.

The bioactivity of released PDGF-AA was assayed using neural stem/progenitor cells. Cells were isolated from the subependymal region of the lateral ventricles in the forebrain of adult male Wistar rats and grown in complete serum-free media containing 20 ng/mL epidermal growth factor (EGF, Invitrogen), 20 ng/mL basic fibroblast growth factor (bFGF, Invitrogen), and 2 ng/mL heparin (Invitrogen). Neurospheres were observed after 2–3 weeks, with cells passaged weekly thereafter. Passages 3–5 were used in all cell studies. Cells were seeded on fibronectin-coated coverslips in complete media for 48 h, with a media change at 24 h, to ensure cell adherence and viability. A subset of cultured cells was fixed and stained (0 d control). The complete media was then replaced with media lacking EGF, FGF and heparin (media control). The PDGF-AA DDS was prepared by dispersing 0.3 wt% PDGF-AA-loaded nanoparticles in filter-sterilized 0.5/0.5 wt% HAMC. 100 μ L of the DDS was added to each transwell. Additional treatment groups were 1% fetal bovine serum (FBS, Invitrogen) and 40 ng/mL of soluble PDGF-AA added to the media. All groups were cultured for 7 d, with a media change after 3 d.

2.6. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (PFA) and incubated with the following primary antibodies overnight at 4 °C: mouse anti-nestin (BD Biosciences, Mississauga CAN, 1:1000), rabbit anti-gial fibrillary acidic protein (GFAP, DAKO, 1:200), mouse anti-Rip (University of Iowa, Iowa City USA, 1:5), and rabbit anti- β -III tubulin (Abcam, Toronto CAN, 1:1000). Cells were incubated with appropriate secondary antibodies (Invitrogen, 1:500) and Hoechst (Invitrogen, 1:500) for 1 h at room temperature. Live/dead assays were performed at 2 and 7 d by incubating cultures for 20 min with Hoechst (Invitrogen, 1:500), calcein-AM (Invitrogen, 1:500), and ethidium homodimer-1 (Invitrogen, 1:250). Cultures were imaged using an Olympus IX71 inverted microscope coupled to an on-line digital camera. Additional images were taken on an Olympus FV1000 confocal microscope.

2.7. Statistical analysis

All statistical analyses were performed using GraphPad Prism 6 (GraphPad, La Jolla USA). Differences between groups were assessed by one-way ANOVA with the Bonferroni's post-hoc analysis to identify statistical differences between three or more treatments, or by unpaired *t*-test to identify differences between two groups. All data are presented as mean \pm standard deviation.

3. Results

3.1. PDGF-AA encapsulation and release

To achieve the sustained release of PDGF-AA, it was encapsulated in PLGA nanoparticles using a double-emulsion solvent evaporation process. Bovine serum albumin (BSA) was co-encapsulated at 10 wt% to protect the PDGF-AA from denaturation and to increase the release rate by the formation of interconnected pores. PEG400 at 5 wt% was also co-encapsulated and increased the

encapsulation efficiency of PDGF-AA from $49 \pm 3\%$ to $60 \pm 5\%$. However, this difference in encapsulation efficiency between formulations was not significant at 95% confidence by unpaired *t*-test ($p = 0.0602$). The loading was approximately the same for formulations with and without PEG400, at 20 ng PDGF-AA per mg of polymeric nanoparticle, because less PDGF-AA was added to the initial emulsion for the PEG400 formulation.

The release of PDGF-AA from PLGA nanoparticles dispersed in HAMC vs. PDGF-AA released from HAMC alone were compared. Unencapsulated PDGF-AA is completely released from HAMC within 48 h, as expected for diffusion-controlled release. Encapsulation of PDGF-AA within PLGA nanoparticles extends its release to at least 21 d (Fig. 1A). For both nanoparticle formulations (with and without PEG400), the release is incomplete, indicating that the PLGA does not fully degrade within the 21 d release period and that some PDGF-AA remains within the nanoparticles. Without PEG400, the release of PDGF-AA is rapid for the first 10 d, resulting in the release of approximately 8 ng/mg polymeric nanoparticle, or 40% of the total encapsulated protein, over 21 d (Fig. 1B). Unexpectedly, despite a comparable loading, the addition of PEG400 resulted in a significantly lower amount of PDGF-AA detected (Fig. 1B, $p < 0.001$).

To further investigate the effect of PEG400 on PDGF-AA, the nanoparticles were dissolved at the end of the 21 d release study to extract any remaining PDGF-AA. For the formulation without PEG400, a mass balance accounted for all encapsulated PDGF-AA by measuring the amount in both the release medium and remaining in the nanoparticles. However, for the formulation with PEG400, less than 50% of the encapsulated PDGF-AA was detected. We then tested the effect of PEG400 on the detection of PDGF-AA by ELISA at 1 h, 24 h, and 7 d. After 1 h of PDGF-AA incubation with PEG400 at room temperature, no effect on ELISA detection was observed (data not shown). After 24 h of PDGF-AA incubation with PEG400 at 37 °C, a decrease in PDGF-AA detection by ELISA was observed (Fig. 2A, $p > 0.05$). However, after 7 d of PDGF-AA incubation with PEG400 at 37 °C, only 19% of the original PDGF-AA was detected, which was significantly lower than the 60% detected in the absence of PEG400 ($p < 0.05$). Interestingly, by microBCA, PEG400 encapsulation had no effect on BSA detection from PLGA nanoparticles (Fig. 2B, $p > 0.05$). Thus, while PEG400 increased the encapsulation efficiency, it negatively affected PDGF-AA during its release.

We examined possible mechanisms for the reduced detection of PDGF-AA with both a fluorescence-based thermal shift assay (FTS) for protein unfolding and dynamic light scattering (DLS) for protein aggregation. The FTS assay demonstrated that a reference protein, lysozyme, undergoes thermal unfolding over 10–85 °C, with a melting temperature of 69 ± 1 °C and that PEG400 does not affect this melting temperature (Supplemental Fig. 1). However, PDGF-AA does not undergo thermal unfolding over this temperature range, in either the absence or presence of PEG400 over a range of concentrations. Thus, it is unlikely that PEG400 affected PDGF-AA stability via thermal unfolding. We next examined protein aggregation by DLS. Initially, the size of PDGF-AA is unaffected by the presence of PEG400. After 1 d, the hydrodynamic radius of PDGF-AA is significantly higher in the presence of PEG400, indicating increased aggregation and suggesting a mechanism for decreased detection and activity of PDGF-AA in the presence of PEG400 (Fig. 3).

To better understand the universality of the detrimental effect of PEG400 on PDGF-AA, we examined the effect of PEG400 on other proteins. The encapsulation efficiency of brain-derived neurotrophic factor (BDNF) in PLGA nanoparticles containing PEG400 was $59 \pm 8\%$, compared with $54 \pm 6\%$ in the absence of PEG400. The release profile was not significantly affected by co-encapsulation of PEG400 (Supplemental Fig. 2), indicating that

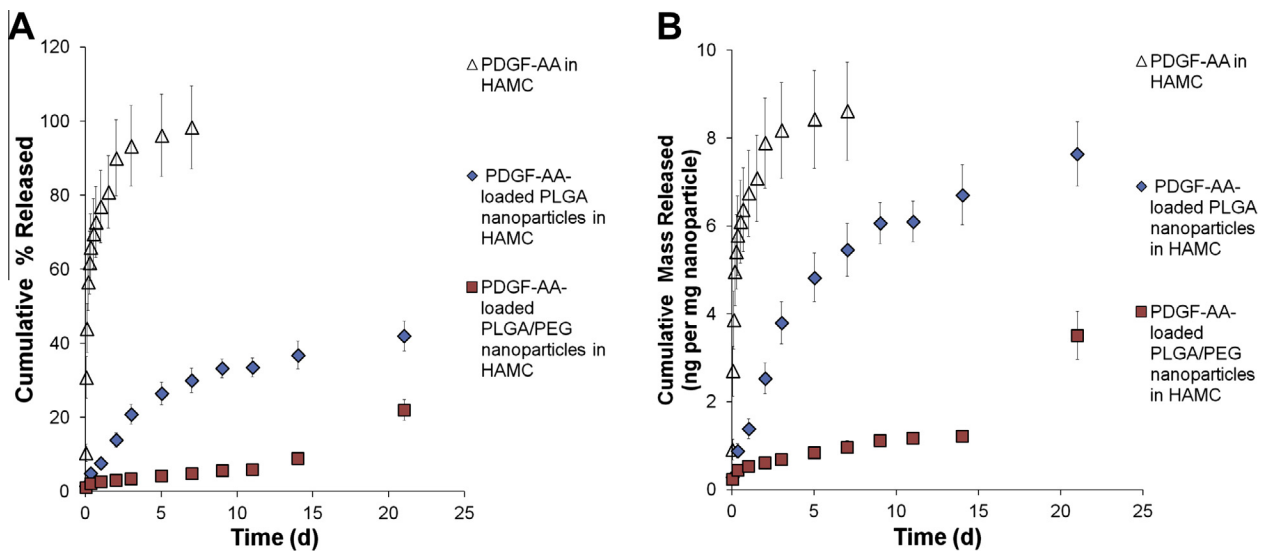


Fig. 1. PDGF-AA release is modulated by the nanoparticle/hydrogel DDS. (A) PDGF-AA release from HAMC is extended by encapsulation in PLGA nanoparticles. (B) Despite comparable loading, the amount of PDGF-AA detected from nanoparticles is reduced with the addition of PEG400 [$p < 0.001$ for all timepoints comparing PDGF-AA from PLGA/PEG nanoparticles vs. PLGA nanoparticles vs. PDGF-AA from HAMC, $n = 3$, mean \pm standard deviation plotted].

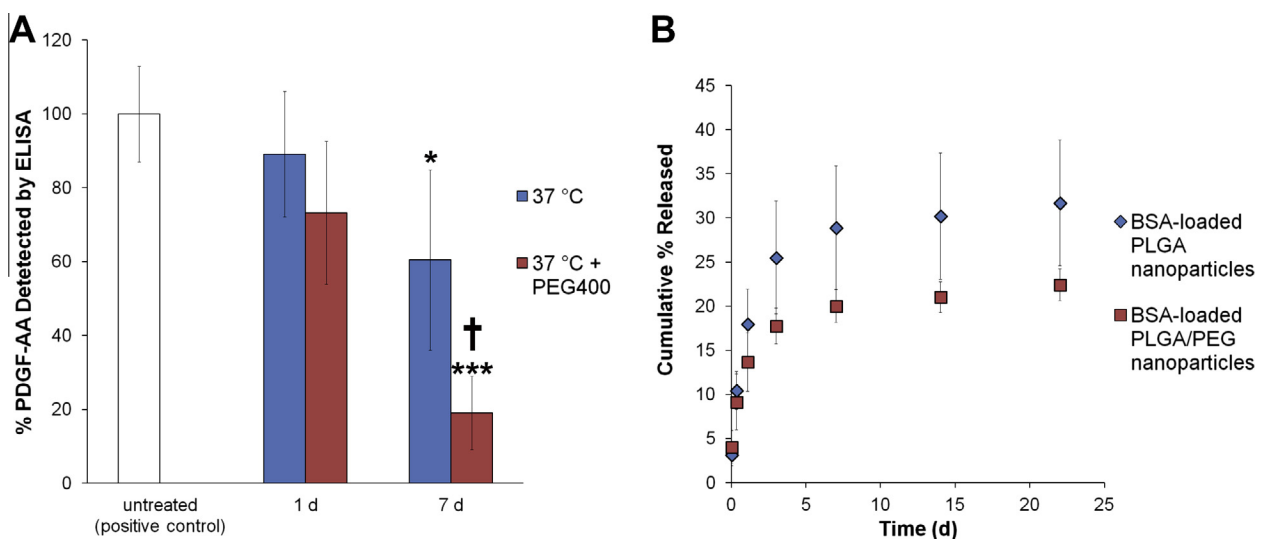


Fig. 2. PEG400 specifically affects PDGF-AA detection by ELISA. (A) Incubation of PDGF-AA at 37 °C reduces its detection by ELISA after 7 d; detection is further reduced with the addition of PEG400 [\dagger = significantly different from untreated control, \dagger = significantly different from PDGF-AA, 7 d at 37 °C, * $p < 0.05$, *** $p < 0.001$, $n = 3$, mean \pm standard deviation plotted]. (B) Unlike PDGF-AA, BSA release from PLGA nanoparticles is not significantly affected by PEG400 [$p > 0.05$, $n = 3$, mean \pm standard deviation plotted].

the stability of BDNF, in contrast with PDGF-AA, is not affected by PEG400.

We examined the aggregation of the proteins BSA, lysozyme, α -chymotrypsin, insulin, and ovalbumin in the presence and absence of PEG400. After 1 d of incubation, only ovalbumin was affected by PEG400, with a significant increase in hydrodynamic radius (Supplemental Fig. 3). These results further demonstrate that PEG400 may be detrimental to some, but not all, protein formulations.

3.2. Bioactivity of released PDGF-AA

The bioactivity of PDGF-AA released from our nanoparticle/hydrogel DDS was assessed by examining its effects on rat NSPC differentiation. These cells initially expressed high levels of nestin, a marker of undifferentiated neural stem cells, and low levels of differentiated progeny. After 7 d of treatment with 1% FBS, the

NSPCs lost nestin expression and differentiated into GFAP⁺ astrocytes, Rip⁺ oligodendrocytes, and β -III-tubulin⁺ neurons (Fig. 4A–E). Importantly, PDGF-AA released from the DDS remained bioactive as shown by a similar differentiation profile to that of soluble PDGF-AA (Fig. 5A). In the presence of either soluble PDGF-AA or the PDGF-AA DDS, there was a significant decrease in GFAP⁺ astrocytes as well as a significant increase in both the percentage (Fig. 5A) and number (Fig. 5B) of Rip⁺ cells (i.e., immature and mature oligodendrocytes) compared to media controls (or even 1% FBS). These results demonstrate that PDGF-AA remains bioactive after encapsulation and release from our nanoparticle/hydrogel DDS, in the absence of PEG400.

We next examined the effect of the PDGF-AA DDS on cell viability. Cultures were treated as described above, and cell viability assessed after 2 and 7 d. The cell viability, as indicated by the percentage of live cells, after treatment with the PDGF-AA DDS was maintained in comparison with 1% FBS and was similar to that of

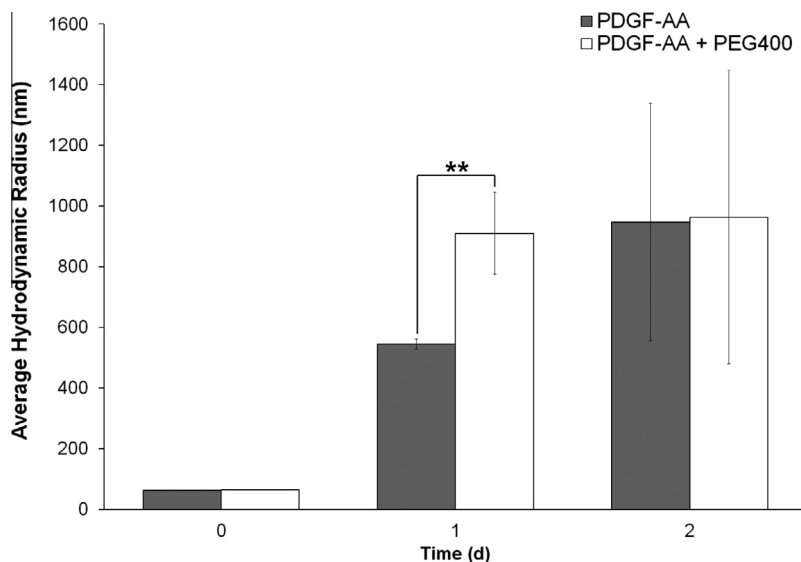


Fig. 3. PEG400 accelerates the aggregation of PDGF-AA. After 1 d of incubation at 37 °C with PEG400, the hydrodynamic radius of PDGF-AA is significantly increased relative to PDGF-AA alone [$**p < 0.01$, $n = 3$, mean \pm standard deviation plotted].

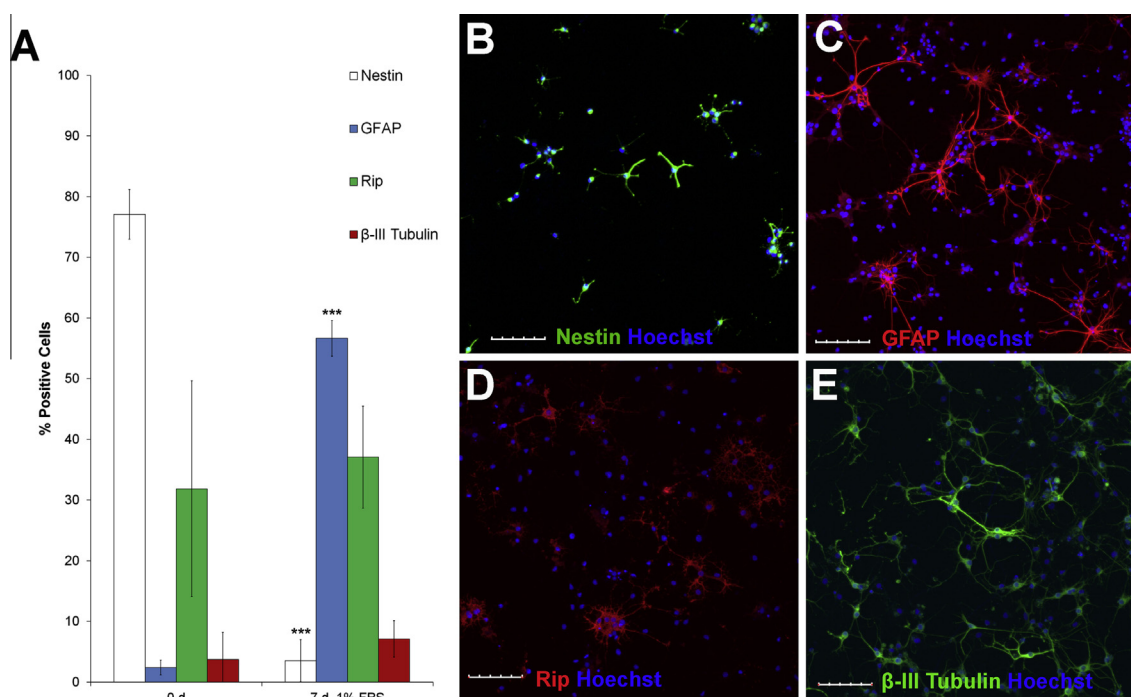


Fig. 4. NSPCs are multipotent. (A) At 0 d, NSPCs express high levels of nestin, whereas NSPCs treated with 1% FBS for 7 d lose nestin expression and differentiate into astrocytes, oligodendrocytes, and neurons [$*** =$ significantly different from 0 d, $p < 0.001$; $n = 5$; mean \pm standard deviation plotted]. Immunocytochemically labeled NSPCs and progeny at: (B) 0 d for nestin; and at 7 d, 1% FBS treatment for (C) GFAP⁺ astrocytes, (D) Rip⁺ oligodendrocytes, and (E) β -III tubulin⁺ neurons [scale bar is 100 μ m].

soluble PDGF-AA. A significant decrease in cell viability was observed for cells in media alone (Fig. 6A). At 2 d, the total number of cells increased in comparison with media controls (Fig. 6B). This indicates that the nanoparticle/hydrogel DDS is not cytotoxic, and that the PDGF-AA released from the DDS is able to promote cell proliferation and survival.

4. Discussion

A critical challenge in the development of cell therapies for SCI is cell survival after transplantation. The sustained delivery of

growth or differentiation factors has been shown to promote cell viability and induce differentiation into a desired lineage [22–25]. Oligodendrocyte differentiation, for example, requires NSPCs to be exposed to bioactive PDGF-AA for at least 7 d [6,7], making PDGF-AA a desirable molecule for controlled release. To this end, we sought to deliver bioactive PDGF-AA from a nanoparticle/hydrogel DDS. We found that the co-encapsulation of PEG400 increased the encapsulation efficiency of PDGF-AA in PLGA nanoparticles, as has been previously reported for NT-3 in PLGA nanoparticles and nerve growth factor (NGF) in PLGA microspheres [20,26]. This is likely due to an increase in viscosity during the primary emulsion, which protects the protein from shear effects.

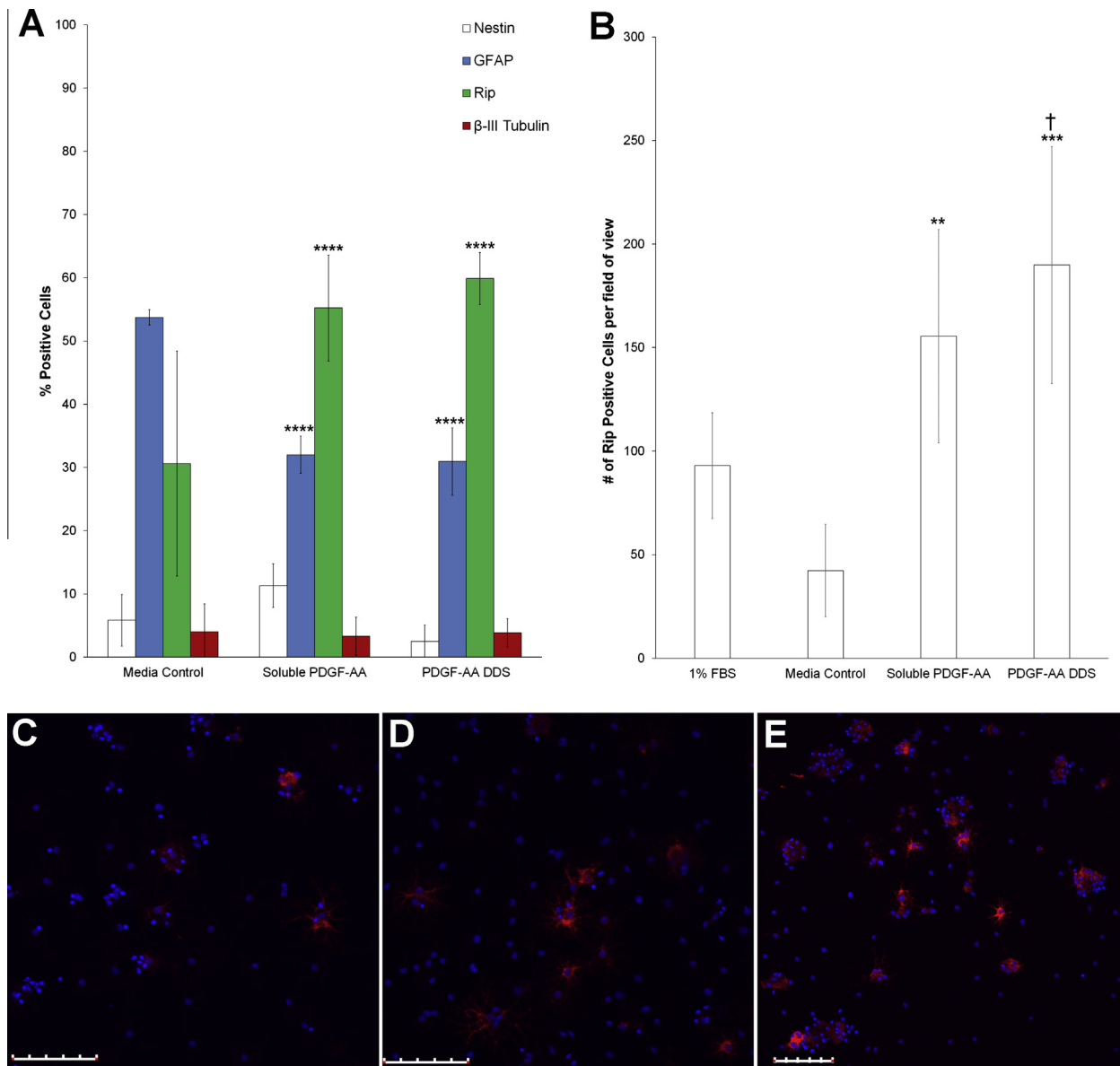


Fig. 5. Bioactive PDGF-AA is released from the DDS. (A) NSPC differentiation to Rip⁺ oligodendrocytes is increased in the presence of either fresh soluble PDGF-AA that is added to the cell culture media or a transwell containing PDGF-AA PLGA nanoparticles dispersed in HMC (PDGF-AA DDS). The percentage of Rip⁺ cells is not significantly different between soluble and released PDGF-AA, yet significantly different from untreated cells. Correspondingly, the percentage of GFAP⁺ cells is significantly decreased with PDGF-AA treatment [*** $p < 0.0001$, $n = 5$; mean \pm standard deviation plotted]. (B) The number of Rip⁺ cells also increases with PDGF-AA treatment [** $p < 0.01$, *** $p < 0.001$ against media control, [†] $p < 0.05$ against 1% FBS, $n = 5$; mean \pm standard deviation plotted]. Immunocytochemically labeled NSPCs for Rip treated with: (C) media control; (D) soluble PDGF-AA; (E) PDGF-AA DDS [red = Rip, blue = Hoechst, scale bar is 100 μ m]. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

We found that PDGF-AA was released from the DDS over a period of 21 d, with release primarily occurring in the first 7–10 d. The release profile exhibited a minimal burst release, which is similar to previous studies of NT-3 release from this DDS [26]. These studies also demonstrated that PLGA degradation over the initial 30 d is minimal [26], and we therefore suggest that the rate of release is controlled by diffusion through the wetted nanoparticles and hydrogel.

We observed that the release of PDGF-AA was reduced with PEG400, and that half of the encapsulated protein could not be accounted for at the end of the release period, after the dissolution of the DDS. Since the release of BSA was not significantly affected by the co-encapsulation of PEG400, it was unlikely that that PEG400 altered nanoparticle structure, but rather directly affected protein stability. This was supported by our stability studies, in

which PDGF-AA detection was reduced with the addition of PEG400. These results indicate that a larger fraction of the encapsulated PDGF-AA may have been released from the PEG400 formulation than is shown in Fig. 1, but was degraded or aggregated such that it was no longer detectable by ELISA. Therefore, although PEG400 improved the encapsulation efficiency of PDGF-AA, it was detrimental to the protein during release.

However, we found that PEG400 has variable effects upon protein release from nanoparticles. The release of BDNF was not affected by PEG400, whereas PDGF-AA showed significantly lower release. Previous investigations have also reported inconsistent effects related to the inclusion of PEG in PLGA nanoparticle or microparticle formulations, depending on the protein, formulation protocol, and molecular weight of PEG used. For example, the rate of release of NGF from PLGA microparticles was greater with the

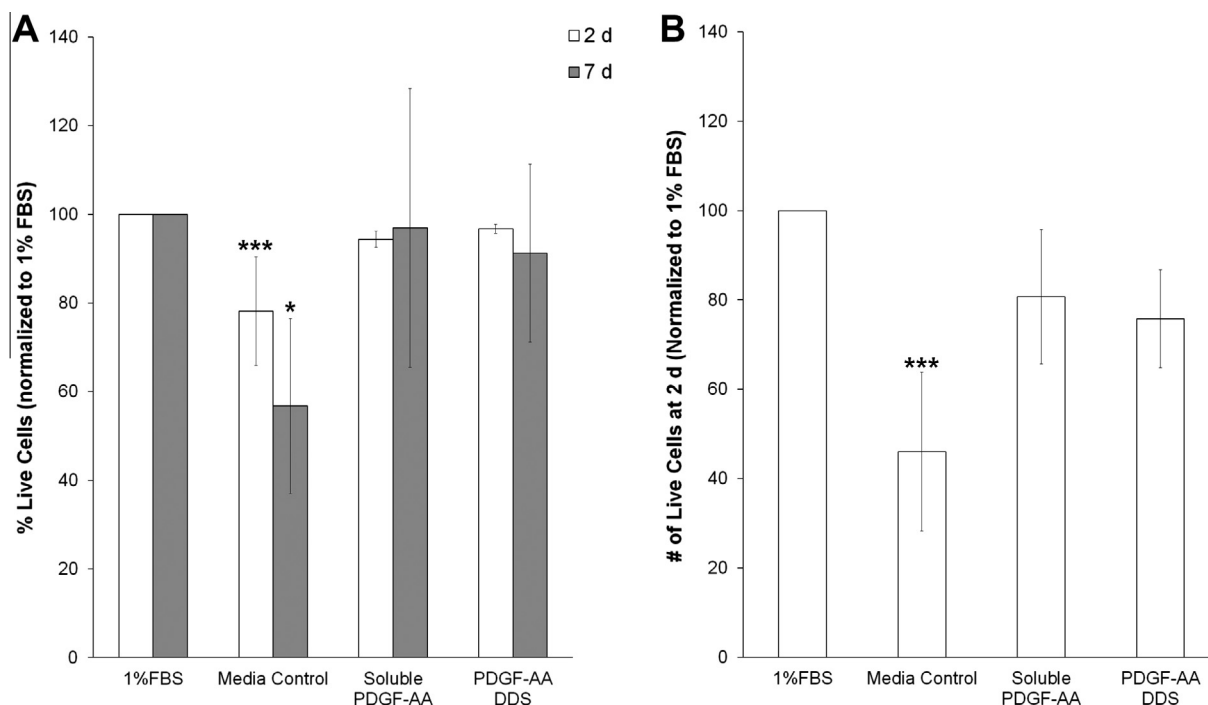


Fig. 6. PDGF-AA DDS maintains cell viability. (A) NSPCs treated with either soluble PDGF-AA or the PDGF-AA DDS maintain cell viability similarly to 1% FBS treatment at both 2 and 7 d, in contrast with media controls [$p < 0.05$, $***p < 0.001$, $n = 4$; mean standard deviation plotted]. (B) At 2 d, 1% FBS and PDGF-AA treatments also increase cell proliferation, as indicated by the number of live cells, in comparison to the media control [$***p < 0.001$, $n = 4$; mean \pm standard deviation plotted].

addition of PEG400 to the inner aqueous phase [20]. Similarly, a higher burst release of glial-derived neurotrophic factor (GDNF) was observed when the PEG400 concentration was increased from 1% to 10% in PLGA microparticles [27]. In contrast, the burst release and release rate of ovalbumin and recombinant human bone morphogenic protein-1 (rhBMP-1) from PLGA microspheres was reduced with the addition of PEG400 [28,29]. The co-encapsulation of PEG400 also substantially reduced the release of NT-3 from PLGA nanoparticles [26]. Despite these reports of the detrimental effects of PEG400 on some protein formulations, the mechanism of PEG400-induced instability has not previously been investigated.

We gained insight into the reduced detection of PDGF-AA in the presence of PEG400 by examining protein unfolding and aggregation. Previous studies have shown that low molecular weight PEG (i.e., ≤ 1000 g/mol) preferentially binds and stabilizes human serum albumin and BSA in their unfolded states [30–32]. However, using an FTS assay we found PDGF-AA does not undergo thermal unfolding and that PEG400 does not affect the melting temperature of a model protein, lysozyme.

We next examined the aggregation of PDGF-AA by DLS and found that PEG400 significantly accelerates the aggregation of PDGF-AA. We therefore suggest that the reduced detection by ELISA is due to increased aggregation. In a screen of several proteins, we found that only ovalbumin also showed significant aggregation in the presence of PEG400. As protein structure can be important for ELISA detection, the PDGF-AA aggregation we observed with PEG400 could be responsible for the lower detected release. This is consistent with both our observation of ovalbumin, which also aggregated in the presence of PEG400, and that of others who found that the release of ovalbumin was reduced with PEG400 [28]. Importantly, our findings indicate that the use of PEG400 as an excipient in protein-loaded PLGA nanoparticles can have a detrimental effect on protein release by promoting protein aggregation. Detection methods such as ELISAs, which often require the structural stability of the protein to be maintained,

are critical in assessing the amount of intact protein released. Other methods of analysis may not detect changes in protein structure and may lead to inconsistent results.

As the co-encapsulation of PEG400 decreased the total detectable release amount and the release rate of PDGF-AA from our DDS, we investigated the bioactivity of PDGF-AA released from PLGA nanoparticles in the absence of PEG400. PDGF-AA is known to expand the oligodendrocyte population derived from NSPCs by promoting oligodendrocyte differentiation and the proliferation of oligodendrocyte progenitor cells [4]. We assessed the activity of released PDGF-AA by determining its effect on the staining of rat NSPCs with Rip, an oligodendrocyte marker for immature, non-myelinating, and myelinating oligodendrocytes [33,34]. In the presence of 1% FBS, the NSPCs differentiated primarily into GFAP⁺ astrocytes, as has been previously shown [1,8]. Relative to untreated controls and controls treated with 1% FBS, a significantly higher percentage of Rip⁺ cells were observed with both fresh PDGF-AA (positive control) and our PDGF-AA DDS. Further, a significantly higher number of Rip⁺ cells was observed with our PDGF-AA DDS, in comparison with both untreated and FBS-treated cells. This is consistent with previous reports on the effect of PDGF-AA on the survival and proliferation of oligodendrocyte progenitors [4]. As PDGF-AA concentrations from 10 to 140 ng/mL have been reported to effectively direct oligodendrocyte differentiation [1,4], it was not feasible to determine a dose response for differentiation. These *in vitro* experiments demonstrate that our PDGF-AA DDS is able to release bioactive PDGF-AA.

We also examined the effect of our composite DDS on cell viability. We observed an increase in viability for cells treated with the PDGF-AA DDS in comparison with media controls, and similar to treatment with either 1% FBS or soluble PDGF-AA. The increase in cell number after 2 d indicates that PDGF-AA promotes cell proliferation. The cell viability results show that the DDS can be used to deliver growth or differentiation factors, such as PDGF-AA, to NSPCs without adverse effects and indeed with effects similar to soluble PDGF-AA. Previous research has shown that the

nanoparticle/hydrogel DDS used herein is biocompatible in spinal cord injury repair strategies [15] and permissive for cell survival [35], suggesting that this PDGF-AA nanoparticle/hydrogel DDS may be useful for cell delivery strategies in the central nervous system.

5. Conclusions

Our results demonstrate, for the first time, the sustained release of bioactive PDGF-AA from a nanoparticle/hydrogel DDS. The co-encapsulation of PEG400 increased the PDGF-AA loading, but promoted protein aggregation, thereby reducing PDGF-AA stability and ELISA detection. The contradictory results of PEG400 addition for the proteins PDGF-AA and BDNF demonstrate that PEG400 can have variable effects on protein stability. With the detection methods of ELISA and DLS, it is clear that PEG400 promoted aggregation of PDGF-AA and is thus deleterious to protein activity. In the absence of PEG400, the DDS preserved PDGF-AA bioactivity after encapsulation and release and enhanced cell viability. This DDS may be useful for the local delivery of PDGF-AA in spinal cord injury repair strategies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.actbio.2015.08.002>.

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