

Contents lists available at ScienceDirect

Journal of Controlled Release



journal homepage: www.elsevier.com/locate/jconrel

Poly(ethylene glycol) modification enhances penetration of fibroblast growth factor 2 to injured spinal cord tissue from an intrathecal delivery system

Catherine E. Kang^{a,b}, Charles H. Tator^c, Molly S. Shoichet^{a,b,d,*}

^a Department of Chemical Engineering and Applied Chemistry, University of Toronto, 200 College Street, Toronto, ON, Canada M5S 3E5

^b Institute of Biomaterials and Biomedical Engineering, 164 College Street, Toronto, ON, Canada M5S 3G9

^c Krembil Neuroscience Centre, Toronto Western Research Institute, and Department of Surgery, University of Toronto, 399 Bathurst Street, Toronto, ON, Canada M5T 2S8

^d Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, ON, Canada M5S 3H6

ARTICLE INFO

Article history: Received 6 November 2009 Accepted 5 January 2010 Available online 28 January 2010

Keywords: Biodistribution Fibroblast growth factor 2 (FGF2 or bFGF) Poly(ethylene glycol) (PEG or PEGylation) Spinal cord injury Injectable

ABSTRACT

There is no effective treatment for spinal cord injury and clinical drug delivery techniques are limited by the blood–spinal cord barrier. Our lab has developed an injectable drug delivery system consisting of a biopolymer blend of hyaluronan and methylcellulose (HAMC) that can sustain drug release for up to 24 h in the intrathecal space. Fibroblast growth factor 2 (FGF2) has great potential for treatment of spinal cord injury due to its angiogenic and trophic effects, but previous studies showed no penetration into spinal cord tissue when delivered locally. Conjugation to poly(ethylene glycol) (PEG) is known to improve penetration of proteins into tissue by reducing clearance and providing immunogenic shielding. We investigated conjugation of PEG to FGF2 and compared its distribution relative to unmodified FGF2 in injured spinal cord tissue when delivered intrathecally from HAMC. Importantly, PEG conjugation nearly doubled the concentration of FGF2 in the injured spinal cord when delivered locally and, contrary to previous reports, we show that some FGF2 penetrated into the injured spinal cord using a more sensitive detection technique. Our results suggest that PEGylation of FGF2 enhanced tissue penetration by reducing its rate of elimination.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Spinal cord injury is a serious condition affecting nearly 300,000 people in the US. Most frequently, a spinal cord injury is caused by broken fragments of the vertebral column compressing the spinal cord and resulting in immediate and delayed cell death and blood vessel rupture. The primary injury causes limited tissue damage, but the series of events in the hours to days after injury include hemorrhage, ischemia, hypoxia, inflammation, and edema, all of which contribute to the characteristic tissue degeneration and major loss of function [1]. The current clinical treatment for this condition includes an intravenous injection of high dose methylprednisolone; however, the clinical benefit of this treatment has been highly debated [2,3] and at present there are no other proven treatments [4].

Drug delivery to the central nervous system (CNS) is particularly difficult due to the blood-spinal cord barrier and the dura and arachnoid membranes that surround the cord, both of which are effective in preventing the passage of most drug and protein therapeutics delivered systemically. This emphasizes the need for other methods than the traditional intravenous and oral delivery strategies, which often require high doses for penetration and can lead to significant and undesirable side effects. Intrathecal drug delivery strategies to circumvent these barriers have been developed, such as bolus delivery, implantable catheters, or sustained delivery from minipumps. However, these strategies are invasive and prone to infections [5] and bolus intrathecal delivery is evanescent. To achieve sustained intrathecal delivery, our lab has developed a minimally invasive, injectable drug delivery system consisting of a physical blend of hyaluronan and methylcellulose (HAMC) which has proven safe for *in vivo* use [6]. A therapeutic agent can be dispersed either alone or in polymeric nanospheres within this hydrogel [7], and the hydrogel alone has shown promise in vivo to provide short-term, localized delivery of therapeutic molecules when injected into the intrathecal cavity following spinal cord injury [8]. To relieve pressure on the spinal cord, decompressive surgery is often required to remove vertebral fragments. During this procedure, HAMC could be injected into the intrathecal space directly over the injury site (Fig. 1A) to achieve maximal therapeutic benefit from localized release.

Fibroblast growth factor 2 (FGF2) is a protein with multiple modes of action in the CNS and has been tested both in experimental models of spinal cord injury [9,10] and clinically for the treatment of stroke [11]. Aside from its well known trophic properties in promoting angiogenesis [12–14], FGF2 has also been shown to be neuroprotective [15,16]. It stimulates endogenous ependymal cells to proliferate when co-delivered with EGF [17] and can reduce permeability of the

^{*} Corresponding author. Donnelly Centre for Cellular and Biomolecular Research, 160 College Street, Room 514 Toronto, ON, Canada M5S 3E1. Tel.: +1 416 978 1460; fax: +1 416 978 4317.

E-mail address: molly.shoichet@utoronto.ca (M.S. Shoichet).

^{0168-3659/\$ –} see front matter 0 2010 Elsevier B.V. All rights reserved. doi:10.1016/j.jconrel.2010.01.029



Fig. 1. A diagram of injection paradigm shows hydrogel spreading following intrathecal injection. Arrows indicate potential directions for protein flux from HAMC into the CSF or into spinal cord tissue. B Tissue sampling paradigm for observing depth of dorsoventral penetration. Spinal cord segments of 2 cm spanning the entire rostrocaudal spread of the hydrogel were removed and then sectioned sagittally into 1 mm thick sections. The most medial two sections were then sliced longitudinally into 300 µm sections. These 1 mm × 300 µm × 2 cm sections were used to measure the dorsoventral penetration of FGF2 or PEG-FGF2 from the dorsal aspect to the center of the spinal cord.

blood-brain barrier [18,19]. Additionally, FGF2 has enhanced functional recovery in rats after spinal cord injury when delivered locally from an osmotic minipump [9,10]. However, because FGF2 does not cross the blood-spinal cord barrier, a localized delivery system is necessary and this need is underscored by FGF2 being mitogenic and upregulated in cancer [14], reflecting the limitations of systemic administration [20]. Notwithstanding the interesting functional benefits ascribed to FGF2, its penetration into the spinal cord when delivered locally beneath the dura and arachnoid mater has only been studied once. This study was based on immunohistochemistry and demonstrated that FGF2 delivered intrathecally from a collagen hydrogel did not penetrate into spinal cord tissue [17]. To enhance tissue penetration and increase local concentrations, we conjugated poly(ethylene glycol) (PEG) to FGF2, using a methodology similar to PEG modification of other proteins [21,22]. We then investigated the penetration and distribution of PEG-FGF2 in the spinal cord relative to unmodified FGF2.

PEG modification has been used to improve molecular stability [23], as well as reduce immunogenicity [24] and decrease tissue clearance [25], thus enhancing its penetration into tissue. It has been shown to increase the biologic half-life of several molecules and also enhance distribution in tissues [26]. The distribution of PEGylated proteins in tissue depends on both the physico-chemical factors of the

PEG-protein conjugate and the physiological/anatomical factors of the host tissue. To better understand FGF2 biodistribution in spinal cord tissue, we developed a tissue Enzyme-Linked Immunosorbent Assay (ELISA) technique to study the penetration of FGF2 vs. PEG-FGF2 delivered locally from the injectable HAMC hydrogel. This technique was more sensitive than the previously used immunohistochemical method and demonstrated tissue penetration of both FGF2 and PEG-FGF2. Our results suggest that the greater tissue penetration observed for PEG-FGF2 than FGF2 is ascribed to reduced elimination.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich Chemical Co (Mississauga, ON) and used as received unless otherwise noted. Media and cells were purchased from ATCC (American Type Culture Collection, Rockville, MD) and reagents were sterile-filtered in a 0.22 µm Stericup filter before use.

2.1. Synthesis of PEG-FGF2

Poly(ethylene glycol) was modified with a maleimide functional group prior to conjugation with FGF2. PEG monomethyl ether (PEG, 5000 Da) was dissolved in dichloromethane and reacted with a 3× molar ratio of succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC, Pierce, Chicago, IL) in the presence of 1.5× molar ratio of triethylamine under nitrogen. After 24 h PEG-maleimide (PEG-mal) was precipitated dropwise in cold ether and filtered through a 0.42 µm glass microfiber filter. PEG-mal was dried under vacuum overnight to remove excess ether and stored at -20 °C. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, Voyager Elite Mass Spectrometer, Toronto Integrated Proteomics Lab) was performed to characterize PEG (CH₃(OCH₂ CH₂)₁₁₃OH (M+) 5000 Da, found 5088 Da) and PEG-mal (CH₃(OCH₂ CH₂)₁₁₃OC₁₁H₁₄NO₄ (M+) 5236 Da, found 5223 Da).

Recombinant human fibroblast growth factor 2 (FGF2, Biovision, Mountain View, CA) was dissolved in 5 mM Tris buffer to a final concentration of 1 mg/ml. PEG-mal was added in a 5× molar ratio to 300 μ l of FGF2 and was reacted at room temperature on a shaker at 500 rpm for 3 h. A heparin affinity binding column (GE Healthcare Biosciences AB, Uppsala, Sweden) was used to remove free PEG from the reaction mixture. Purified PEG-FGF2 was dialyzed (10,000 MWCO) overnight at 4 °C and subsequently lyophilized in conical tubes with sterile nylon 0.2 μ m filter caps prior to use.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12% acrylamide resolving gel and 5% stacking gel (Biorad, Constant Voltage = 120 V) to confirm the PEG-FGF2 product had formed and that free PEG was fully removed. A Benchmark pre-stained protein ladder (Invitrogen, Burlington, ON) was used for molecular weight classification. Proteins were stained with Simply BlueTM Safestain (Invitrogen, Burlington, ON) and PEG was stained for 20 min with sequential submersion in 2.5% barium chloride and 1.3% iodine/2% potassium iodide using a modified protocol from [27].

2.2. Bioactivity of PEGylated FGF2

Dose dependent proliferation of a Balb/3T3 fibroblast cell line (ATCC, CCL-163) was used to investigate the bioactivity of FGF2 and PEG-FGF2 products. Cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 1% Penicillin/Streptomycin and 10% calf bovine serum. To test the activity of PEG-FGF2 relative to that of FGF2, Balb/3T3 cells were cultured in serum free media with either FGF2 or PEG-FGF2 mitogens at 10, 20, and 40 ng/ml with a 20:1 weight ratio of Heparin:FGF2. These cells were incubated for 24 h at 37 °C and 5% CO₂, and cell density then assayed with Cell Titer 96, a substrate metabolized into a colored product only by live cells. The

absorbance was measured at 490 nm with a VERSAmax tunable microplate reader and cell density was calculated relative to a standard curve of known cell density.

2.3. In vitro release of FGF2 vs. PEG-FGF2 from HAMC

Sodium hyaluronate (Novamatrix, 1.5×10⁶ Da, Drammen, Norway) was sterilized by filtering a 0.1% solution through a 0.2 µm filter and lyophilizing prior to use. Methylcellulose $(13 \times 10^3 \text{ Da})$, was sterilized similarly. Following lyophilization in conical tubes with sterile nylon 0.2 µm filter caps, sterile HAMC was produced by mixing polymer solutions in a laminar flow hood. Artificial cerebrospinal fluid (aCSF) was prepared in dH₂O with 148 mM NaCl, 3 mM KCl, 0.8 mM MgCl₂, 1.4 mM CaCl₂, 1.5 mM Na₂HPO₄, and 0.2 mM NaH₂PO₄ [28]. The MC and HA powders were sequentially dissolved in a solution of 100 µg/ml of either FGF2 or PEG-FGF2 in aCSF at 4 °C. This resulted in 100 µg/ml FGF2 or PEG-FGF2 loaded into a 2% HA and 7% MC solution. HAMC with no protein was similarly prepared as a control to ensure HAMC did not interfere with the ELISA assays. A 100 µl aliquot of this solution was injected into the bottom of centrifuge tubes containing 900 µl of aCSF at 37 °C, approximating the ratio of HAMC to CSF that is expected in vivo by injection into the intrathecal space of a rat. These samples were incubated at 37 °C on an orbital shaker and aCSF was fully removed and replaced with fresh aCSF at t = 1, 3, 6, 12, 24, and 48 h. A sandwich ELISA assay (R&D Systems, Human FGF basic Duoset) was used to determine the concentration of FGF2 or PEG-FGF2 in the aCSF that was removed at each time point (n = 4).

2.4. In vivo distribution of FGF2 in spinal cord tissue

All animal procedures were performed in accordance with the Guide to the Care and Use of Experimental Animals developed by the Canadian Council on Animal Care and approved by the Animal Care Committee at the Research Institute of the University Health Network. Fifty two adult Sprague–Dawley rats (200–250 g; Charles River, Montreal, QC) were anesthetized by inhalation of halothane, and a laminectomy performed at the T1-2 vertebral level. A modified aneurysm clip calibrated to a closing force of 26 g was applied to the spinal cord at T1 for 1 min to simulate a spinal cord injury as previously described [29]. A durotomy was performed immediately caudal to the injury site with a 30 G bent beveled needle and then a 30 G blunt, bent tipped needle was inserted into the intrathecal space (see Fig. 1A) for injection of HAMC. To allow for HAMC gelation, the needle was held in the intrathecal space for 1 min before removal, at which time a small plug of HAMC would form at the durotomy site and prevent leakage of CSF [6]. Animals received a 10 µl injection in the intrathecal cavity of HAMC loaded with either 100 µg/ml FGF2 (n = 5/timepoint) or $100 \,\mu\text{g/ml}$ PEG-FGF2 (n = 5/timepoint). To control for cross-reactivity in the ELISA of endogenous rat FGF2 with the recombinant human FGF2 injected, animals were injured but not injected (n = 3/timepoint). Following injection, the overlying muscles and fascia were sutured closed; rats were ventilated with pure oxygen and placed under a heat lamp for recovery. Buprenorphine was administered post-surgery for pain management.

At t = 0 (immediately after injection), 3, 6, and 24 h, animals were administered a lethal dose of sodium pentobarbital, and a 2 cm section of the spinal cord was removed at the T1-2 level. Removal of fresh tissue prevented loss of HAMC in the intrathecal space and FGF2 from tissue during the fixation process. Tissue was flash frozen in 2-methylbutane on ice and subsequently cut parasagittally on a tissue chopper (McIlwain, Redding, CA) into 1 mm thick slices. The most medial two slices were then cut into 300 µm longitudinal sections dorsoventrally so that FGF2 could be detected spatially through the depth of the spinal cord (See Fig. 1B). Homogenate buffer was prepared in distilled H₂O with 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid, 1 mM dithiothreitol, 1 mM phenylmethanesulphonyl fluoride. Each 1 mm× 300μ m×2 cm segment of spinal cord was digested in homogenate buffer using a Pellet Pestle (Kontes, USA). Samples were centrifuged for 15 min at 15,000 rpm at 4 °C and the supernatant collected. A sandwich ELISA assay (R&D Systems, Human FGF basic Duoset) performed in duplicate on supernatant samples was used to determine the concentration of FGF2 and PEG-FGF2 in each tissue segment. Low concentrations of rat FGF2 cross reacted with human FGF2 antibodies. These concentrations were then subtracted from concentrations detected in the cords with rhFGF2 and PEG-rhFGF2 that was delivered locally for each timepoint, respectively.

2.5. Statistical analysis

One way analysis of variance (ANOVA) followed by Tukey's posthoc *t*-test was used to compare the depth of dorsoventral penetration concentrations of FGF2 and PEG-FGF2. Differences were considered statistically significant at p<0.05. All error bars shown represent standard deviations.

3. Results

3.1. PEGylation of FGF2

A maleimide functionalized PEG (Fig. 2) was conjugated to free FGF2-cysteine thiols. The crystal structure of FGF2 reveals four free cysteine residues that do not participate in disulfide bonding in the active FGF2 structure or in the well known binding sites for heparin sulfate proteoglycans [30]. These free cysteines also do not interfere with the binding sites for the FGF receptors 1 and 2 [31].

Gel electrophoresis showed 3 bands in the purified PEG-FGF2 mixture (Fig. 3, lane 3): ~17 kDa, ~25 kDa, and ~35 kDa. Although there are 4 free cysteines on FGF2, Cys 96 and Cys 78 are more readily available on the surface of the protein for conjugation [32]. Cys 34 and Cys 101 face the internal portion of the protein when the active conformation is maintained, making the conjugation of PEG-maleimide difficult on these residues [32]. For this reason, the PEG/FGF2 reaction mixture delivers 3 distinct bands: FGF2, mono-PEG-FGF2, and di-PEG-FGF2. The molecular weights expected were ~22 kDa and ~27 kDa for single and double PEGylation respectively; however, bands were observed shifted higher at ~25 and ~35 kDa. This is likely due to the complexation of SDS with PEG, which is known to slow the movement of PEGylated proteins in SDS-PAGE [33].

When the reaction was allowed to proceed for several hours longer, 5 bands were observed (data not shown), also shifted higher than the expected molecular weights, with the higher molecular weights corresponding to three and four PEG molecules bound to FGF2. Because these products with 3 and 4 PEGs would likely exhibit reduced bioactivity due to the conformational change necessary to expose the internal Cys residues, the protocol which produced only single, double, and unPEGylated FGF2 was used, and this mixed product is henceforth referred to as PEG-FGF2.

Lane 3 in Fig. 3 was loaded with products previously separated on a heparin affinity binding column and shows no PEG band at low molecular weights. Lane 4 shows the flow through of the reaction solution, with a dark band of PEG observed near ~6 kDa. These results show that free PEG was fully removed from the PEG-FGF2 solution prior to dialysis. Densitometry of the bands showed that of the products, 18% was non-PEGylated FGF2, 44% was *mono*-PEG-FGF2, and 38% *di*-PEG-FGF2. Thus 82% of the FGF2 was PEGylated.

3.2. PEG-FGF2 bioactivity

Dose dependent proliferation of Balb/3T3 cells was used to ensure that the PEG-FGF2 maintained bioactivity after PEG modification and processing steps. The cell density increased similarly with increasing



Fig. 2. FGF2 with free cysteine groups reacts with PEG-mal to produce single PEGylated FGF2 and double PEGylated FGF2 (with some unreacted, non-PEGylated FGF2).

concentrations of either FGF2 or PEG-FGF2 (Fig. 4), indicating that PEG-FGF2 maintained dose dependent activity following the PEGylation and purification steps.

3.3. FGF2 release from HAMC In vitro

The *in vitro* release profiles of PEG-FGF2 and FGF2 from HAMC were compared to understand whether PEG modification would



Fig. 3. Representative gel electrophoresis shows molecular weight ladder, control FGF2, products of PEG-FGF2 reaction isolated from heparin binding column, and flow through PEG from heparin binding column.

influence diffusion of FGF2 and to establish the duration of release. Since the HAMC hydrogel is comprised of 9% solids and 91% buffer and is highly porous, a diffusive mechanism of release was expected based on previous results with other proteins of comparable size [8]. The release profiles of PEG-FGF2 and FGF2 were similar and release was complete after 24 h (Fig. 5).

3.4. In vivo distribution of FGF2 and PEG-FGF2

Previously, our laboratory found that FGF2 did not penetrate into spinal cord tissue based on detection by immunohistochemistry where the limit of detection was 100 µg/ml [17]. This restricted delivery of FGF2 motivated the present study to enhance the diffusive penetration into the spinal cord of FGF2 by PEG modification. To overcome the limited sensitivity of immunohistochemistry, we used the more sensitive tissue ELISA technique that increased the sensitivity by four orders of magnitude to 10 ng/ml. With ELISA, both FGF2 and PEG-FGF2 were detected in spinal cord tissue when delivered locally (Fig. 6). The spinal cord was removed and frozen with the dura intact to enable measurement of the concentration of FGF2 and PEG-FGF2 in both the drug delivery system and the tissue. At 3 h, protein release from HAMC was constant since the concentration in the gel did not change from t = 0 h. At this time, a significantly greater concentration of PEG-FGF2 than FGF2 was detected in the spinal cord tissue (Fig. 6B). There was generally a greater concentration of PEG-FGF2 than FGF2 at all depths of penetration from the



Fig. 4. Dose dependent activity of FGF2 (white bars) and PEG-FGF2 (black bars) show that bioactivity based on cell density is maintained after PEGylation and subsequent purification. (n = 5, mean \pm standard deviation are shown).



Fig. 5. *In vitro* release of FGF2 (\Box) and PEG-FGF2 (\bullet) from HAMC drug delivery system shows diffusive release within 24 h in almost identical concentrations. (*n* = 5, mean \pm standard deviation are shown).

dorsal surface of the spinal cord, with significant differences at 0.6 and 1.75 mm from the dorsal surface (Fig. 6B). Concentrations within the spinal cord were highest near the dorsal surface in close contact with HAMC; however, because HAMC spread around the spinal cord tissue, diffusion of PEG-FGF2 and FGF2 was likely from both dorsal and ventral sides, thereby accounting for the increase in protein concentration in the center of the cord. Additionally, cerebrospinal fluid transport of the protein through the central canal may have contributed to these elevated protein concentrations. At 6 h, the concentration in the gel had dropped to $\sim 1 \,\mu\text{g/ml}$ and release was no longer constant due to drug depletion. Concentration profiles at 6 h show that concentrations of PEG-FGF2 tended to be higher than FGF2 throughout the cord, but not statistically significant at this time (Fig. 6C). At 24 h, no FGF2 or PEG-FGF2 could be detected in the gel, and only small amounts (10-15 ng/ml) were detected in the spinal cord (data not shown).

We used Eq. (1), based on concentrations detected in tissue, to better understand FGF2 and PEG-FGF2 diffusion *in vivo* [34,35]:

$$C = C_{\rm o} \exp\left[-\varphi\left(\frac{x}{a}-1\right)\right] \tag{1}$$

where *C* is the concentration in the tissue, C_0 is the initial concentration in the polymer delivery system, *x* is the distance of the protein from the polymer and *a* is the characteristic thickness of the polymer gel that is bound by the spinal cord and the dura. The φ is termed the diffusion/elimination modulus [34,36] and is defined as:

$$\varphi = a\sqrt{\binom{k}{D}} \tag{2}$$

where *k* is a lumped elimination parameter accounting for cellular interactions and D is the diffusion coefficient. Because this equation is applicable when drug delivery is at steady state, estimated concentration profiles were obtained for t = 3 h only, where drug release was constant. In vivo steady state model predictions using Eq. (1) above, as has previously been described [36,37] for similar systems, were fit to the data for up to 1.2 mm into the cord. These predicted concentration profiles of FGF2 and PEG-FGF2 are shown in Fig. 6B, from which the φ values were calculated using the least fit squares method. This model fits those points closest to the surface of the cord up to ~ 1.2 mm into the tissue, where release is most similar to a point source. Deviation occurs beyond this depth likely due to concentric protein flux from other surfaces of the cord (see Fig. 1B, inset) as well as from CSF transport of protein through the central canal. Importantly, this analysis provides useful insights into mechanism that accounts for the greater tissue penetration of PEG-FGF2 vs. FGF2 by providing an



Fig. 6. A Cumulative comparison shows a greater amount of PEG-FGF2 (\bullet) in the injured spinal cord than FGF2 (\Box) when delivered from the intrathecal HAMC drug delivery system. B At 3 h post-intrathecal injection, higher concentrations of PEG-FGF2 (\bullet) than FGF2 (\Box) have diffused into the cord. Lines indicate the predicted concentration profiles of PEG-FGF2 (solid) and FGF2 (dotted). C At 6 h post-intrathecal injection, drug depletion from the hydrogel results in a plateau of drug in the tissue, but elevated concentrations of PEG-FGF2 (\bullet) are still observed in the spinal cord. Asterisks indicate statistical significance at p < 0.05. (n = 5, mean \pm standard deviation are shown).

understanding of the relationship between the elimination constant (*k*) and diffusion coefficient (*D*). The φ values, based on *in vivo* concentration values of φ_{FGF2} and $\varphi_{\text{PEG-FGF2}}$, were 1.2 and 0.7, respectively. Because *a* is constant, the φ values indicate the relationship between elimination and diffusivity of FGF2 and PEG-FGF2, where $\varphi > 1$ indicates greater elimination than diffusivity.

4. Discussion

Intrathecal injection of HAMC containing either FGF2 or PEG-FGF2 allows local release of the protein at the site of spinal cord injury. The HAMC hydrogel forms a gel spatially bound by the dura and arachnoid membrane and the spinal cord from which the protein can diffuse laterally into the CSF, radially into the spinal cord tissue, as well as across the dura and arachnoid membrane into the epidural space (Fig. 1A). Since the dura and arachnoid membrane contributes to the blood-spinal cord barrier and is highly impermeable to proteins, minimal diffusion is expected in this direction [38]. The spinal cordhydrogel interface and the CSF-hydrogel interface are the main sites for drug release from the hydrogel. The gel spreads over the surface of the spinal cord and fills much of the intrathecal space at the site of injection, thereby resulting in a significant spinal cord-hydrogel interface; however, protein flux into the CSF is also expected. Penetrability of proteins into the spinal cord tissue is affected by diffusion of the molecule in the extracellular space (which is bounded by cell membranes), as well as by the elimination rate dictated by surface receptor binding or internalization of the protein, degradation, and blood-spinal cord barrier clearance. Penetrability increases with dispersivity and decreases with elimination, as is described in Eqs. (1) and (2) above.

To further probe how PEG affected penetrability, we used the φ values calculated from *in vivo* concentration values of FGF2 and PEG-FGF2, (1.2 and 0.7, respectively), which showed an overall decrease in φ due to PEGylation. While the absolute values of *k* and *D* cannot be determined for FGF2 and PEG-FGF2 within tissue, the ratios of each indicates relative differences between the two proteins, which can be used to better understand why greater concentrations of PEG-FGF2 are observed in the spinal cord. This ratio is shown as:

$$\frac{\varphi_{\text{FGF2}}}{\varphi_{\text{PEG-FGF2}}} = \sqrt{\frac{k_{\text{FGF2}}}{k_{\text{PEG-FGF2}}}} \frac{D_{\text{PEG-FGF2}}}{D_{\text{FGF2}}}$$
(3)

The ratio of $\varphi_{\text{FGF2}}/\varphi_{\text{PEG-FGF2}}$ obtained from the *in vivo* concentration profiles yields a ratio of 1.7. Given that FGF2 has a higher diffusion/elimination modulus, we then investigated how the relative differences of the elimination and diffusion constants, which define φ , were affected by PEGylation.

Absolute diffusion coefficients for FGF2 and PEG-FGF2 in tissue cannot be calculated due to the complexities of protein-tissue interaction, but the relative difference of diffusion coefficients between FGF2 and PEG-FGF2 can be estimated based on the ratio of molecular weight, where $D \propto 1/M^{1/3}$ [39]. The weighted average MW of PEG-FGF2 based on the relative amounts of the reaction products was 23.2 kDa. The ratio of diffusion coefficients, $D_{FGF2}/D_{PEG-FGF2} = 1$, indicated that diffusion should decrease due to PEGylation. Because D is inversely proportional to φ , if penetration of each molecule was only affected by diffusion then φ would be expected to increase as a result of PEGylation, and result in $\varphi_{FGF2}/\varphi_{PEG-FGF2} > 1$. However, in *vivo* measurements yield $\varphi_{FGF2}/\varphi_{PEG-FGF2} = 1.7$, suggesting that PEGylation impacted the elimination parameter. The relation of k to φ (Eq. (2)) suggests that elimination would have to decrease significantly after PEGylation, with more than a three-fold change needed to produce the nearly two-fold change in φ that was observed, where $k_{\text{FGF2}}/k_{\text{PEG-FGF2}} = 3.2$. Since k is a lumped elimination parameter, it can be impacted by several biological factors. Elimination can occur through intra- or extracellular degradation of the protein, phagocytosis by cellular components, surface receptor binding, and clearance via the bloodstream. PEG is known to enhance molecular stability of proteins [23], and conjugation to FGF2 could decrease elimination by reducing extracellular degradation of the protein. In addition, PEG conjugation is known to mask proteins via preventing phagocytosis by the reticulo-endothelial system (RES), which has been most commonly studied in renal clearance of intravenously delivered PEG conjugates [40]. In the CNS, the RES is represented by specialized immune cells called microglia, which become activated macrophages following an injury. These cells phagocytose foreign material and cellular debris at the injury site. If exogenously delivered FGF2 is detected as foreign, it will be cleared from the extracellular space as it diffuses into the tissue. In Fig. 6B, concentrations of FGF2 are highest near the surface of the spinal cord where the drug loaded HAMC is releasing protein into the tissue. As FGF2 diffuses into the tissue, macrophages that accumulate at the injury site may internalize the foreign recombinant human FGF2. The PEG-FGF2 is thought to evade phagocytosis due to the PEG chains that shield the exogenous FGF2, thus allowing greater concentrations to reach the center of the cord. The analysis described here suggests that PEGylation of FGF2 reduces elimination, further supporting previous reports of PEGylation leading to enhanced molecular stability and biological evasion of macrophage phagocytosis.

5. Conclusions

Using a reproducible method for PEGylation of FGF2, the penetration of FGF2 and PEG-FGF2 were compared: FGF2 and PEG-FGF2 were detected at ng/ml concentrations within the injured spinal cord. These results demonstrate that exogenous FGF2 penetrates the injured spinal cord when delivered locally and, importantly, that higher concentrations of PEG-FGF2 vs. FGF2 can be achieved. Analysis of the diffusion/elimination modulus suggests that elimination of FGF2 decreased with PEGylation, supporting biological models of PEG-mediated penetration of proteins *in vivo*. These results support the concept that therapeutic proteins can be locally delivered to the injured spinal cord with a minimally invasive injection of HAMC into the intrathecal space.

Acknowledgements

We are grateful to Peter Poon for tissue processing and Ankeeta Tadkase and Charles Liu for tissue analysis. We acknowledge funding from the Canadian Institute of Health Research (MSS, CHT).

References

- C.H. Tator, M.G. Fehlings, Review of the secondary injury theory of acute spinal cord trauma with emphasis on vascular mechanisms, J. Neurosurg. 75 (1991) 15–26.
- [2] M.G. Fehlings, Editorial: recommendations regarding the use of methylprednisolone in acute spinal cord injury: making sense out of the controversy, Spine 26 (2001) S56–S57.
- [3] R.J. Hurlbert, The role of steroids in acute spinal cord injury: an evidence-based analysis, Spine 26 (2001) S39–S46.
- [4] C.H. Tator, Review of treatment trials in human spinal cord injury: issues, difficulties, and recommendations, Neurosurgery 59 (2006) 957–982 discussion 982-7.
- [5] L.L. Jones, M.H. Tuszynski, Chronic intrathecal infusions after spinal cord injury cause scarring and compression, Microsc. Res. Tech. 54 (2001) 317–324.
- [6] D. Gupta, C.H. Tator, M.S. Shoichet, Fast-gelling injectable blend of hyaluronan and methylcellulose for intrathecal, localized delivery to the injured spinal cord, Biomaterials 27 (2006) 2370–2379.
- [7] M.D. Baumann, C.E. Kang, J.C. Stanwick, Y. Wang, H. Kim, Y. Lapitsky, M.S. Shoichet, An injectable drug delivery platform for sustained combination therapy, J. Control. Release 138 (2009) 205–213.
- [8] C.E. Kang, P.C. Poon, C.H. Tator, M.S. Shoichet, A new paradigm for local and sustained release of therapeutic molecules to the injured spinal cord for neuroprotection and tissue repair, Tissue Eng. Part A 15 (2009) 595–604.
- [9] A.G. Rabchevsky, I. Fugaccia, A. Fletcher-Turner, D.A. Blades, M.P. Mattson, S.W. Scheff, Basic fibroblast growth factor (bFGF) enhances tissue sparing and functional recovery following moderate spinal cord injury, J. Neurotrauma 16 (1999) 817–830.
- [10] A.G. Rabchevsky, I. Fugaccia, A.F. Turner, D.A. Blades, M.P. Mattson, S.W. Scheff, Basic fibroblast growth factor (bFGF) enhances functional recovery following severe spinal cord injury to the rat, Exp. Neurol. 164 (2000) 280–291.
- [11] J. Bogousslavsky, S.J. Victor, E.O. Salinas, A. Pallay, G.A. Donnan, C. Fieschi, M. Kaste, J.M. Orgogozo, A. Chamorro, A. Desmet, Fiblast (trafermin) in acute stroke: results of the European–Australian phase II/III safety and efficacy trial, Cerebrovasc. Dis. 14 (2002) 239–251.

- [12] Y. Shing, J. Folkman, C. Haudenschild, D. Lund, R. Crum, M. Klagsbrun, Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor, J. Cell. Biochem. 29 (1985) 275–287.
- [13] R. Montesano, J.D. Vassallí, A. Baird, R. Guillemin, L. Orci, Basic fibroblast growth factor induces angiogenesis *In vitro*, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 7297–7301.
- [14] M. Relf, S. LeJeune, P.A. Scott, S. Fox, K. Smith, R. Leek, A. Moghaddam, R. Whitehouse, R. Bicknell, A.L. Harris, Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis, Cancer Res. 57 (1997) 963–969.
- [15] K. Nozaki, S.P. Finklestein, M.F. Beal, Basic fibroblast growth factor protects against hypoxia-ischemia and NMDA neurotoxicity in neonatal rats, J. Cereb. Blood Flow Metab. 13 (1993) 221–228.
- [16] T.T. Lee, B.A. Green, W.D. Dietrich, R.P. Yezierski, Neuroprotective effects of basic fibroblast growth factor following spinal cord contusion injury in the rat, J. Neurotrauma 16 (1999) 347–356.
- [17] M.C. Jimenez Hamann, C.H. Tator, M.S. Shoichet, Injectable intrathecal delivery system for localized administration of EGF and FGF-2 to the injured rat spinal cord, Exp. Neurol. 194 (2005) 106–119.
- [18] K. Bendfeldt, V. Radojevic, J. Kapfhammer, C. Nitsch, Basic fibroblast growth factor modulates density of blood vessels and preserves tight junctions in organotypic cortical cultures of mice: a new in vitro model of the blood-brain barrier, J. Neurosci. 27 (2007) 3260–3267.
- [19] B. Reuss, R. Dono, K. Unsicker, Functions of fibroblast growth factor (FGF)-2 and FGF-5 in astroglial differentiation and blood-brain barrier permeability: evidence from mouse mutants, J. Neurosci. 23 (2003) 6404–6412.
- [20] S.E. Epstein, S. Fuchs, Y.F. Zhou, R. Baffour, R. Kornowski, Therapeutic interventions for enhancing collateral development by administration of growth factors: basic principles, early results and potential hazards, Cardiovasc. Res. 49 (2001) 532–542.
- [21] D.P. Ankeny, D.M. McTigue, Z. Guan, Q. Yan, O. Kinstler, B.T. Stokes, LB. Jakeman, Pegylated brain-derived neurotrophic factor shows improved distribution into the spinal cord and stimulates locomotor activity and morphological changes after injury, Exp. Neurol. 170 (2001) 85–100.
- [22] M. Stroh, W.R. Zipfel, R.M. Williams, S.C. Ma, W.W. Webb, W.M. Saltzman, Multiphoton microscopy guides neurotrophin modification with poly(ethylene glycol) to enhance interstitial diffusion, Nat. Mater. 3 (2004) 489–494.
- [23] M.J. Roberts, M.D. Bentley, J.M. Harris, Chemistry for peptide and protein PEGylation, Adv. Drug Deliv. Rev. 54 (2002) 459–476.
- [24] A. Abuchowski, T. Vanes, N.C. Palczuk, R. Chen, F.F. Davis, Effect of covalently attached polyethylene-glycol on immunogenicity of enzymes, Fed. Proc. 36 (1977) 867.

- [25] K.A. Witt, T.J. Gillespie, J.D. Huber, R.D. Egleton, T.P. Davis, Peptide drug modifications to enhance bioavailability and blood-brain barrier permeability, Peptides 22 (2001) 2329–2343.
- [26] J.M. Harris, N.E. Martin, M. Modi, Pegylation: a novel process for modifying pharmacokinetics, Clin. Pharmacokinet. 40 (2001) 539–551.
- [27] S.B. Zimmerman, L.D. Murphy, Electrophoresis of polyethylene glycols and related materials as sodium dodecyl sulfate complexes, Anal. Biochem. 234 (1996) 190–193.
- [28] M.C. Jimenez Hamann, E.C. Tsai, C.H. Tator, M.S. Shoichet, Novel intrathecal delivery system for treatment of spinal cord injury, Exp. Neurol. 182 (2003) 300–309.
- [29] A.S. Rivlin, C.H. Tator, Effect of duration of acute spinal cord compression in a new acute cord injury model in the rat, Surg. Neurol. 10 (1978) 38–43.
- [30] S. Faham, R.E. Hileman, J.R. Fromm, R.J. Linhardt, D.C. Rees, Heparin structure and interactions with basic fibroblast growth factor, Science 271 (1996) 1116–1120.
- [31] A.N. Plotnikov, S.R. Hubbard, J. Schlessinger, M. Mohammadi, Crystal structures of two FGF-FGFR complexes reveal the determinants of ligand-receptor specificity, Cell 101 (2000) 413–424.
- [32] J.D. Zhang, L.S. Cousens, P.J. Barr, S.R. Sprang, 3-dimensional structure of human basic fibroblast growth-factor, a structural homolog of interleukin-1-beta, Proc. Natl. Acad. Sci. U.S.A. 88 (1991) 3446–3450.
- [33] Q. Yun, W.C. Xing, G.G. Mal, Z.G. Su, Preparation and characterization of mono-PEGylated consensus interferon by a novel polyethylene glycol derivative, J. Chem. Technol. Biotechnol. 81 (2006) 776–781.
- [34] M.F. Haller, W.M. Saltzman, Localized delivery of proteins in the brain: can transport be customized? Pharm. Res. 15 (1998) 377–385.
- [35] C. Nicholson, Diffusion and related transport mechanisms in brain tissue, Rep. Prog. Phys. 64 (2001) 815–884.
- [36] M.J. Mahoney, W.M. Saltzman, Controlled release of proteins to tissue transplants for the treatment of neurodegenerative disorders, J. Pharm. Sci. 85 (1996) 1276–1281.
- [37] M. Mak, L. Fung, J.F. Strasser, W.M. Saltzman, Distribution of drugs following controlled delivery to the brain interstitium, J. Neurooncol. 26 (1995) 91–102.
- [38] C.M. Bernards, H.F. Hill, Morphine and alfentanil permeability through the spinal dura, arachnoid, and pia mater of dogs and monkeys, Anesthesiology 73 (1990) 1214–1219.
- [39] M.E. Young, P.A. Carroad, R.L. Bell, Estimation of diffusion-coefficients of proteins, Biotechnol. Bioeng. 22 (1980) 947–955.
- [40] P. Caliceti, F.M. Veronese, Pharmacokinetic and biodistribution properties of poly (ethylene glycol)-protein conjugates, Adv. Drug Deliv. Rev. 55 (2003) 1261–1277.