Tunable Growth Factor Delivery from Injectable Hydrogels for Tissue Engineering

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ABSTRACT: Current sustained delivery strategies of protein therapeutics are limited by the fragility of the protein, resulting in minimal quantities of bioactive protein delivered. In order to achieve prolonged release of bioactive protein, an affinity-based approach was designed which exploits the specific binding of the Src homology 3 (SH3) domain with short proline-rich peptides. Specifically, methyl cellulose was modified with SH3-binding peptides (MC-peptide) with either a weak affinity or strong affinity for SH3. The release profile of SH3-rhFGF2 fusion protein from hyaluronan MC-SH3 peptide (HMC-peptide) hydrogels was investigated and compared to unmodified controls. SH3-rhFGF2 release from HMC-peptide was extended to 10 days using peptides with different binding affinities compared to the 48 h release from unmodified HAMC. This system is capable of delivering additional proteins with tunable rates of release, while maintaining bioactivity, and thus is broadly applicable.

Many promising therapeutics are increasingly protein-based; however, bioactive protein delivery remains a challenge. Two main approaches have emerged to control protein release: (i) encapsulation in nano/microparticles, which provides a diffusive barrier and (ii) incorporation in affinity-based drug delivery systems, which establishes a dynamic equilibrium to delay release. Although protein encapsulation is common, the harsh environments (organic solvents, aqueous/organic interfacial free energy, shear force, and lyophilization) present during the encapsulation process can diminish protein bioactivity and drug loading is generally low. Affinity-based release systems overcome these limitations by sequestering proteins, commonly growth factors, in a matrix, much like the extracellular matrix in vivo. These systems generally consist of a hydrogel that has been chemically modified to bind a growth factor with moderate or high affinity, depending on the required rate of release, to attenuate the diffusional release of the protein. For example, heparin or heparin-binding peptides have been immobilized to various matrices to deliver a variety of heparin-binding proteins; however, this approach is inherently limited to heparin-binding proteins. Recombinant human basic fibroblast growth factor (rhFGF2) binding peptide can be used to control the release of rhFGF2 from PEG hydrogels and, yet, is similarly limited to

FGF2. Collagen scaffolds have been shown to bind therapeutic fusion proteins that contain a collagen binding domain; however, this system requires collagen as a scaffold and the rate of release cannot be tuned. A system which can deliver a diversity of proteins with a tunable rate of protein release is required.

We have developed a versatile hydrogel that combines both of these properties to deliver therapeutic proteins at tunable rates of release. Importantly, our system is amenable to a variety of proteins.

An injectable, fast gelling blend of two polysaccharides, hyaluronan (HA) and methyl cellulose (MC), provides minimally invasive, localized drug delivery to the injured spinal cord and brain. Additionally, HAMC can be loaded with proteins to provide localized, diffusion-mediated release. Protein release from HAMC is complete within 1 to 2 days in vitro; however, factors must often be available for longer times to elicit functional recovery. Thus, extending the protein release profile of this therapeutic drug delivery matrix would improve administration of an exciting new class of drugs.

Here we present a platform technology that permits minimally invasive and localized delivery of therapeutic proteins with tunable and extended release profiles. We used HAMC as a drug delivery matrix and exploited peptide-protein interactions to develop a system adaptable to any protein with the ability to finely tune the rate of its release from the matrix. We are interested in the delivery of rhFGF2 in these studies because it is a neuroprotective, angiogenic factor that requires at least 5 days of continuous delivery to achieve tissue and functional benefit in rat models of spinal cord injury.

To achieve sustained release of this protein from the HAMC hydrogel, rhFGF2 was expressed in Escherichia coli (E. coli) as a fusion protein with the Src homology 3 domain (SH3) (SH3-rhFGF2, Figure 1) and MC was modified with one of two SH3-binding peptides (Scheme 1). Specifically, chemical modification of methyl cellulose, MC (1), was achieved starting with a Williamson ether synthesis using 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC), followed by disulfide reduction with dithiothreitol (DTT) to yield thiolated MC 3 (Scheme 1, Figure S1, Supporting Information (SI)). Thiolated MC was reacted with 3-maleimidopropionic-SH3-binding peptide (4,5)

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Figure 1. Controlled release of SH3-rhFGF2 from hydrogels modified with SH3-binding peptides. Transient association between SH3-binding peptides covalently bound to methyl cellulose and the SH3 protein modulate release of the fusion protein SH3-rhFGF2 from the matrix.

Scheme 1. Synthesis of Methyl Cellulose-Peptide

“Reagents: (a) 3 M bromoacetic acid, 1 M NaOH, 3 h, 4 °C. (b) (i) EDC, 3,3′-dithiobis(propionic dihydrazide), pH 4.5, 2 h, rt. (ii) DTT, pH 8.5, 24 h, rt. (c) 4 or 5, PBS, pH 6.8, N2(g), 24 h, rt.

via a Michael addition to afford MC-SH3-binding peptide (MC-peptide) 6 or 7. The latter was then combined with unmodified HA to form HAMC-peptide. HA decreases the gelation temperature of MC, resulting in a fast gelling polymer that is also easily injectable through a fine needle due to the shear-thinning property of HA. 21

SH3 has previously been shown to bind to various proline-rich peptide sequences with different affinities (Kd) ranging from 10^{-5} to 10^{-7} M. 30 Two different SH3-binding peptides with varying affinity to SH3 (4, Kd = 2.7 × 10^{-5} M or 5, Kd = 2.7 × 10^{-7} M) were tested in our system as a way to control release. As shown in Figure 1, we hypothesized that transient interactions between the binding pairs would slow the diffusion of SH3-rhFGF2 from the matrix. Thus, the rate of release could be tuned by either changing the concentration of the binding peptide or using binding peptides with different affinities, where peptides with stronger affinities would further attenuate release.

MC was modified with one of two peptides that have different binding affinities to SH3: KPPVKKPHYS (weak binder, 4, Kd = 2.7 × 10^{-5} M) and KKTTPPPPSPHLPK (strong binder, 5, Kd = 2.7 × 10^{-7} M). 30 Three glycine residues were incorporated at the N-terminus of the SH3-binding peptide to facilitate protein–peptide recognition and binding once the peptide was covalently attached to the MC hydrogel. This spacer minimizes possible steric hindrance that may affect binding interactions of immobilized ligands with the corresponding protein. 19 A substitution rate of 1 SH3-binding peptide per 15 monomer units, or 180–200 μmol peptide/g MC (Figure 2F, SI), was consistently achieved for each peptide. MC-peptide (3 wt %) was then simply blended with HA (1 wt %) to form a physical hydrogel blend of HAMC-peptide.

A bifunctional fusion protein of SH3 and rhFGF2 was designed to include a small linker region between SH3 and rhFGF2 that acts as a hinge to ensure each protein will fold correctly and function as it does in its native state. 31 While the SH3 domain can be bound at either the N- or C-terminus of the fusion protein, it was bound at the N-terminus of rhFGF2 to maintain bioactivity. The fusion protein was expressed in BL21 E. coli and purified via a hexahistidine tag using a nickel affinity column. The fusion protein was characterized by mass spectrometry (Figure S3, SI) and denaturing gel electrophoresis (Figure S4, SI). To confirm the rhFGF2 portion of the fusion protein was still bioactive, a cell survival assay using mouse-derived neural stem progenitor cells was performed. 32 The activity of the fusion protein was identical to that of commercial rhFGF2 (p > 0.05), indicating that bioactivity was preserved in the fusion protein (Figure S5, SI).

Release of SH3-rhFGF2 (20 μM) was investigated in vitro under conditions that mimic the in vivo environment of the spinal cord. Artificial cerebrospinal fluid with 0.2 mg/mL heparin was used as a release buffer and was added to tubes containing HAMC and HAMC-peptide (188 μmol peptide/g MC) hydrogels. Tubes were placed on an oscillatory shaker at 37 °C, and release buffer was completely removed and replaced with fresh buffer at multiple time points. Release samples were frozen at −20 °C until protein was assayed by an enzyme-linked immunosorbent assay (ELISA). Data are presented as cumulative protein release (relative to initial protein loaded) as a function of time. Data normalized to the total amount of protein detected are shown in Figure S6, SI. Release of SH3-rhFGF2 from HAMC alone was nearly complete at 2 days whereas release from HAMC-peptide hydrogels (HAMC-weak binder and HAMC-strong binder) extended to more than 10 days (Figure 2A).

The fastest release was obtained from HAMC, followed by HAMC-weak binder and HAMC-strong binder, and was statistically significant between all groups (p < 0.001) except between HAMC-weak binder and HAMC-strong binder at t = 1 and 2 h (p < 0.05). This confirms the hypothesis that tunable release profiles are achieved by changing the affinity of the binding peptide.

To investigate differences in the diffusion coefficient of SH3-rhFGF2 in the three hydrogels, we plotted fractional protein release against the square root of time (t^{1/2}, Figure 2B). In this plot, a linear relationship is indicative of Fickian diffusion. 22 By comparing the slopes in the linear region for each hydrogel, we determined that the relative diffusion coefficient for SH3-rhFGF2 was significantly different for each gel (p < 0.001). For HAMC alone, the data fit linearly for the first 8 h of release, similar to published data for diffusional release of immunoglobulin G and α-chymotrypsin from HAMC. 23 Notably, for HAMC-weak binder and HAMC-strong binder the data fit linearly for 5 and 10 days of release respectively. This confirms that release from HAMC-peptide hydrogels, which is sustained...
for a 10 day period, is still mediated by Fickian diffusion. Importantly, protein release from HAMC-peptide hydrogels is linear, overcoming the burst and biphasic release often observed in encapsulated drug delivery systems. Since these HAMC hydrogels have been shown to be stable in vitro for over 28 days, neither polymer degradation nor dissolution was detected. This material is available free of charge via the Internet at http://pubs.acs.org.

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