JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY
Communication
pubs.acs.org/JACS

Tunable Growth Factor Delivery from Injectable Hydrogels for Tissue Engineering

Katarina Vulic[†] and Molly S. Shoichet*[,†,‡]

[†]Department of Chemistry, University of Toronto, 80 St. George Street, Toronto, Ontario, Canada M5S 3H6

[‡]Department of Chemical Engineering & Applied Chemistry, University of Toronto, 200 College Street, Toronto, Ontario, Canada M5S 3E5

Supporting Information

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 (HAMC-peptide) hydrogels was investigated and compared to unmodified controls. SH3-rhFGF2 release from HAMC-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;[1−3] however, bioactive protein delivery remains a challenge.[4] 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.[5,6] 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.[7,8] 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.[6] For example, heparin or heparin-binding peptides have been immobilized to various matrices to deliver a variety of heparin-binding proteins;[9−18] 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.[19] Collagen scaffolds have been shown to bind therapeutic fusion proteins that contain a collagen binding domain;[20] 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.[21,22] 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*;[23,24] however, factors must often be available for longer times to elicit functional recovery.[25,26] 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.[27]

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[28] to produce carboxylated MC **2**. This was then coupled with 3,3′-dithiobis(propionic dihydrazide)[29] 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**)

Received: November 11, 2011
Published: December 28, 2011

   dx.doi.org/10.1021/ja210638x | *J. Am. Chem.Soc.* 2012, 134, 882−885

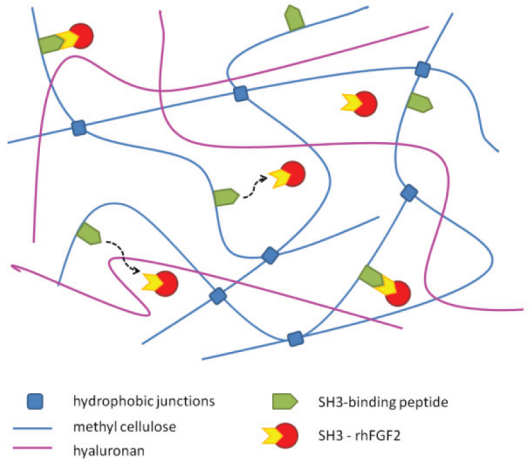
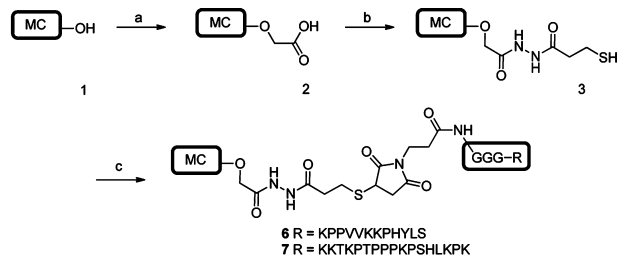


Figure 1. Controlled release of SH3-rhFGF2 from hydrogels modified with SH3-binding peptides. Transient association between SH3-binding peptides covalently bound to methyl cellulose and the SH3 protein modulate release of the fusion protein SH3-rhFGF2 from the matrix.

Scheme 1. Synthesis of Methyl Cellulose-Peptide[a]



[a]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, $N_{2(g)}$, 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 (K_d) ranging from 10^{-5} to 10^{-7} M.[30] Two different SH3-binding peptides with varying affinity to SH3 (**4**, $K_d = 2.7 \times 10^{-5}$ M or **5**, $K_d = 2.7 \times 10^{-7}$ 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: KPPVVKKPHYLS (weak binder, **4**, $K_d = 2.7 \times 10^{-5}$ M) and KKTKPTPPPKPSHLKPK (strong binder, **5**, $K_d = 2.7 \times 10^{-7}$ 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 S2, 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.[33] 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

dx.doi.org/10.1021/ja210638x | J. Am. Chem.Soc. 2012, 134, 882–885

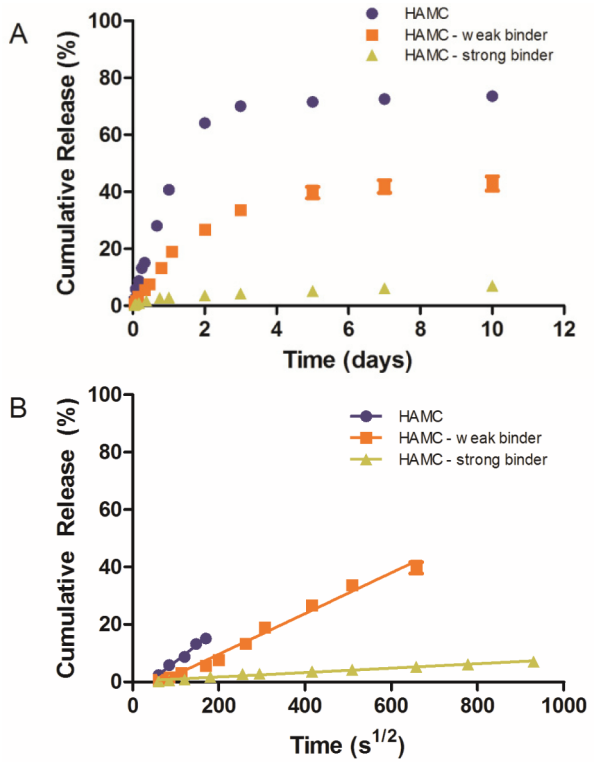


Figure 2. (A) *In vitro* release profile of SH3-rhFGF2 delivered from HAMC, HAMC-weak binder, and HAMC-strong binder hydrogels. SH3-binding peptides attenuate release such that different release profiles are achieved. $p < 0.001$ for all groups, except between HAMC-weak binder and HAMC-strong binder at $t = 1$ and 2 h where $p < 0.05$. (B) The slope of SH3-rhFGF2 release from HAMC, HAMC-weak binder and HAMC-strong binder against the square root of time is representative of Fickian diffusion coefficients for each gel ($p < 0.001$ between all groups). Furthermore, diffusion-controlled release is sustained for 5 days from HAMC-weak binder and for 10 days from HAMC-strong binder. The nonzero intercept indicates that swelling affected diffusion at the early time points. Cumulative release (%) is calculated relative to amount of protein loaded ($n = 4$, mean ± standard deviation are plotted).

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.[34] Since these HAMC hydrogels have been shown to be stable *in vitro* for over 28 days,[23] neither polymer degradation nor dissolution was expected to affect the release profile. Thus, immobilizing SH3-binding peptides to HAMC confers the ability to tune the rate of diffusion-controlled release of SH3 fusion proteins.

The release profile for a drug can have a dramatic effect on the effectiveness of the therapy. Consequently, a priority of drug delivery system design is to allow tunable release rates. Herein, we showed that the release of a therapeutic fusion protein can be controlled through physical binding interactions with a biomaterial matrix. Protein release is linear and tunable. This versatile and simple system demonstrates several improvements over current affinity-based release systems, the most important of which is its broad applicability to a variety of proteins and a diversity of materials. Several protein-peptide binding pairs can be investigated to achieve tunable release of multiple proteins and at distinct rates of release.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed materials and methods, characterization of methyl cellulose modifications, amino acid analysis, SH3-rhFGF2 characterization and confirmation of bioactivity. In vitro release profile of SH3-rhFGF2 normalized to amount of protein detected. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

molly.shoichet@utoronto.ca

■ ACKNOWLEDGMENTS

This research was funded by the Canadian Institutes of Health Research (M.S.S.) and the Ontario Graduate scholarship (K.V.). We are grateful to Professor Mark Nitz (University of Toronto) and Drs. Roger Tam and Ryan Wylie along with other members of the Shoichet lab for thoughtful discussions.

■ REFERENCES

(1) Bradbury, E. J.; Moon, L. D. F.; Popat, R. J.; King, V. R.; Bennett, G. S.; Patel, P. N.; Fawcett, J. W.; McMahon, S. B. *Nature* **2002**, *416*, 636−640.
(2) Schnell, L.; Schneider, R.; Kolbeck, R.; Barde, Y.-A.; Schwab, M. E. *Nature* **1994**, *367*, 170−173.
(3) Moon, L. D. F.; Asher, R. A.; Rhodes, K. E.; Fawcett, J. W. *Nat. Neurosci.* **2001**, *4*, 465−466.
(4) Putney, S. D.; Burke, P. A. *Nat. Biotechnol.* **1998**, *16*, 153−157.
(5) LaVan, D. A.; McGuire, T.; Langer, R. *Nat. Biotechnol.* **2003**, *21*, 1184−1191.
(6) Wang, N. X.; von Recum, H. A. *Macromol. Biosci.* **2011**, *11*, 321−332.
(7) Perez, C.; Castellanos, I. J.; Costantino, H. R.; Al-Azzam, W.; Griebenow, K. *J. Pharm. Pharmacol.* **2002**, *54*, 301−313.
(8) Kumar, V.; Prud'homme, R. K. *J. Pharm. Sci.* **2008**, *97*, 4904−4914.
(9) Ho, Y.-C.; Mi, F.-L.; Sung, H.-W.; Kuo, P.-L. *Int. J. Pharm.* **2009**, *376*, 69−75.
(10) Lee, J. S.; Go, D. H.; Bae, J. W.; Lee, S. J.; Park, K. D. *J. Controlled Release* **2007**, *117*, 204−209.
(11) Tae, G.; Scatena, M.; Stayton, P. S.; Hoffman, A. S. *J. Biomater. Sci., Polym. Ed.* **2006**, *17*, 187−197.
(12) Yoon, J. J.; Chung, H. J.; Lee, H. J.; Park, T. G. *J. Biomed. Mater. Res., Part A* **2006**, *79A*, 934−942.
(13) Maxwell, D. J.; Hicks, B. C.; Parsons, S.; Sakiyama-Elbert, S. E. *Acta Biomaterialia* **2005**, *1*, 101−113.
(14) Sakiyama-Elbert, S. E.; Hubbell, J. A. *J. Controlled Release* **2000**, *69*, 149−158.
(15) Sakiyama-Elbert, S. E.; Hubbell, J. A. *J. Controlled Release* **2000**, *65*, 389−402.
(16) Wood, M. D.; Borschel, G. H.; Sakiyama-Elbert, S. E. *J. Biomed. Mater. Res., Part A* **2009**, *89A*, 909−918.
(17) Wood, M. D.; Sakiyama-Elbert, S. E. *J. Biomed. Mater. Res., Part A* **2008**, *84A*, 300−312.
(18) Nie, T.; Baldwin, A.; Yamaguchi, N.; Kiick, K. L. *J. Controlled Release* **2007**, *122*, 287−296.
(19) Lin, C.-C.; Anseth, K. S. *Adv. Funct. Mater.* **2009**, *19*, 2325−2331.
(20) Fan, J. A.; Xiao, Z. F.; Zhang, H. T.; Chen, B.; Tang, G. Q.; Hou, X. L.; Ding, W. Y.; Wang, B.; Zhang, P.; Dai, J. W.; Xu, R. X. *Journal of Neurotrauma* **2010**, *27*, 1671−1683.
(21) Gupta, D.; Tator, C. H.; Shoichet, M. S. *Biomaterials* **2006**, *27*, 2370−2379.
(22) Cooke, M. J.; Wang, Y.; Morshead, C. M.; Shoichet, M. S. *Biomaterials* **2011**, *32*, 5688−5697.

(23) Baumann, M. D.; Kang, C. E.; Stanwick, J. C.; Wang, Y.; Kim, H.; Lapitsky, Y.; Shoichet, M. S. *J. Controlled Release* **2009**, *138*, 205–213.
(24) Kang, C. E.; Tator, C. H.; Shoichet, M. S. *J. Controlled Release* **2010**, *144*, 25–31.
(25) Thuret, S.; Moon, L. D. F.; Gage, F. H. *Nat. Rev. Neurosci.* **2006**, *7*, 628–643.
(26) Ramer, M. S.; Priestley, J. V.; McMahon, S. B. *Nature* **2000**, *403*, 312–316.
(27) Rabchevsky, A. G.; Fugaccia, I.; Turner, A. F.; Blades, D. A.; Mattson, M. P.; Scheff, S. W. *Exp. Neurol.* **2000**, *164*, 280–291.
(28) Brunswick, M.; Finkelman, F.; Highet, P.; Inman, J.; Dintzis, H.; Mond, J. *J. Immunol.* **1988**, *140*, 3364–3372.
(29) Shu, X. Z.; Liu, Y.; Luo, Y.; Roberts, M. C.; Prestwich, G. D. *Biomacromolecules* **2002**, *3*, 1304–1311.
(30) Stollar, E. J.; Garcia, B.; Chong, P. A.; Rath, A.; Lin, H.; Forman-Kay, J. D.; Davidson, A. R. *J. Biol. Chem.* **2009**, *284*, 26918–26927.
(31) Deyev, S. M.; Waibel, R.; Lebedenko, E. N.; Schubiger, A. P.; Pluckthun, A. *Nat. Biotechnol.* **2003**, *21*, 1486–1492.
(32) Wylie, R. G.; Shoichet, M. S. *Biomacromolecules* **2011**, *12*, 3789–3796.
(33) Ritger, P. L.; Peppas, N. A. *J. Controlled Release* **1987**, *5*, 23–36.
(34) Huang, X.; Brazel, C. S. *J. Controlled Release* **2001**, *73*, 121–136.