

SUPPORTING INFORMATION

FOR

Tunable growth factor delivery from injectable hydrogels for tissue engineering

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Materials and Methods

2.1 Materials

3-maleimidopropionic acid was purchased from TCI America (Portland, USA). pET28 vector was purchased from Invitrogen (Burlington, Canada). Cloning of SH3-rhFGF2 fusion protein was done by GenScript (Piscataway, USA). Sodium hyaluronate of 2600 kg/mol was purchased from Lifecore (Chaska, USA). Methyl cellulose of 300 kg/mol was purchased from Shin Etsu (Tokyo, Japan). Sandwich ELISA kit for rhFGF2 was purchased from Peptotech (Rocky Hill, USA). All buffers were made with distilled and deionized water (dH₂O) prepared using a Millipore Milli-RO 10 Plus and Milli-Q UF Plus at 18 MΩ resistance (Millipore, Bedford, USA). Artificial cerebrospinal fluid (aCSF) was prepared as previously described.¹ All other solvents and reagents were purchased from Sigma-Aldrich and used as received. All FT-IR spectra were obtained using a Spectrum 1000 FT-IR spectrometer (Waltham, MA), collecting 32 scans in the 400-4000 cm⁻¹ range with a resolution of 2 cm⁻¹.

2.2 Synthesis and Characterization

Synthesis of carboxylated methyl cellulose (MC-CO₂H) **2**

Methyl cellulose (2 g, 4% w/v) was dissolved in dH₂O (50 mL) and chilled to 4 °C. Sodium hydroxide (6 g, 150 mmol, 3 M) and bromoacetic acid (6.95 g, 50 mmol, 1 M) were added and the solution stirred for 3 h at 4 °C. The reaction was stopped by adding 0.4% NaH₂PO₄ and was neutralized to pH 7 by addition of 3 M HCl. The solution was dialyzed (MWCO 12-14 kDa, Spectrum Labs) against 0.2 M NaCl for 2 d, changing the dialysis buffer frequently during the first 24 h. The product was lyophilized (Labonco, Kansas City, USA) and characterized using FT-IR spectroscopy.

Synthesis of thiolated methyl cellulose (MC-SH) **3**

MC-CO₂H **2** (1 g, 0.25% w/v) was dissolved in dH₂O, pH 4.5 (200 mL) at 4 °C. Once dissolved, the solution was warmed to room temperature and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide (EDC, 958 mg, 5 mmol) and 3,3'-dithiobis(propionic dihydrazide) (DTP, 1.19 g, 5 mmol) were added sequentially with stirring. The pH of the reaction was monitored to ensure it remained at pH 4.5. After stirring for 2 h the reaction was stopped with addition of 1 M NaOH to pH 7. Dithiothreitol (DTT) (5 g, 32.5 mmol) was added and the pH of the solution was raised to pH 8.5 by addition of 1 M NaOH. After stirring for 24 h, the solution was acidified to pH 3.5 by addition of 3M HCl and dialyzed (MWCO 12-14 kDa, Spectrum Labs) against 100 mM NaCl, pH 3.5 for 3 d, changing the dialysis buffer frequently in the first 24 h. The solution was lyophilized and the product was characterized by FT-IR spectroscopy. Thiol concentration (expressed per gram MC) was quantified using the Ellman method.

Synthesis of 3-maleimidopropionic-SH3-binding peptides (**4** and **5**).

SH3-binding peptide on resin (China Peptides, Shanghai, China) was washed into an ISOLUTE column reservoir (Biotage, Charlotte, USA) with dichloromethane (DCM) and dried. 3-maleimidopropionic acid (338 mg, 2 mmol) and N, N'-diisopropylcarbodiimide (2.0 mL, 8 mmol) were stirred in DCM for 30 min under N_{2(g)}. The mixture was filtered and the filtrate was added to dry SH3-binding peptide on resin (0.2 mmol SH3-binding peptide), and reacted for 2 h with stirring. The resin was washed with DCM, methanol and 2-propanol sequentially using an ISOLUTE column and dried under vacuum. The peptide was deprotected and cleaved from resin by reacting with 95% TFA (10 mL) with stirring for 2 h. The solution was filtered and the filtrate was collected in a round bottomed flask and TFA was evaporated. Crude peptide was precipitated in cold diethyl ether and purified by reverse-phase HPLC (Shimadzu, Japan) with a Phenomenex C18 250 x 10 mm column. Peptide purity was confirmed by ESI mass spectrometry (ABI/Sciex Qstar mass spectrometer).

Synthesis of SH3-binding peptide modified methyl cellulose (MC-peptide **6** and **7**)

MC-GGGKPPVVKKPHYLS and MC-GGGKKTPTPPKPSHLKPK were synthesized. MC-SH (300 mg, 1% w/v, 0.012 mmol thiols) was dissolved in degassed PBS (pH 6.8, 30 mL) at 4 °C. The solution was purged intermittently for 15 min with N_{2(g)}. 3-maleimidopropionic-GGGKPPVVKKPHYLS (102 mg, 0.059 mmol) was added and the reaction was stirred under N_{2(g)}, in the dark for 24 h. The reaction was dialyzed (MWCO 8 kDa, Spectrum Labs) for 48 h against PBS, pH 4 and then against dH₂O for 24 h. The product MC-GGGKPPVVKKPHYLS (**6**) was lyophilized and characterized by amino acid analysis (Pico-tag system, Waters Corporation). The identical synthesis was followed for MC-GGGKKTPTPPKPSHLKPK (**7**) with 3-maleimidopropionic-GGGKKTPTPPKPSHLKPK (133 mg, 0.059 mmol). The identical synthesis was followed for control reactions using SH3-binding peptide alone instead of 3-maleimidopropionic-SH3-binding peptide: GGGKPPVVKKPHYLS and GGGKKTPTPPKPSHLKPK. Control SH3-binding peptides were used to confirm the Michael addition between 3-maleimidopropionic-SH3-binding peptide and thiolated MC (Figure S2).

Expression and purification of SH3-rhFGF2

A plasmid was purchased from Genscript, which coded for a fusion protein with a hexahistidine tag at the N-terminus, followed by a tobacco etch virus cut site (ENLYFQ), Src homology 3 domain (SH3), a spacer (EFPKPSTPPGSSGAP)², and rhFGF2 (C78S, C96S) at the C-terminus in a pET28a(+) plasmid. The SH3-rhFGF2 plasmid was transformed into BL21 (DE3) *Escherichia coli* (*E. coli*) and expressed. A starting culture of 20 mL LB with 50 µg/mL of kanamycin was shaken overnight at 37 °C, and then transferred into a 1.8 L solution of terrific broth, 50 µg/ml of kanamycin and 6 drops of anti-foam 204. The culture was placed in a 37 °C water bath with an air bubbler until an OD₆₀₀ of 0.8 when 1.8 mL of 190 mg/mL of IPTG was added and the protein was expressed for 5 h at 37 °C. The bacterial pellet was collected by centrifugation at 12,227g (Beckman coulter centrifuge Avanti J-26 with rotor JLA-8.1000) for 10 min at 4 °C and re-suspended in binding buffer (50 mM Tris pH 7.5, 500 mM NaCl, 5 mM imidazole) to a total volume of 60 mL. The sample was divided into 2 vials of 30 mL and each was lysed by probe sonication (Misonix S-4000 Sonicator Ultrasonic Processor equipped with a Dual Horn probe) with 20% amplitude and an on/off pulse of 5 s for a total sonication time of 10 min. The cell lysates were centrifuged at 48,384g (Beckman coulter centrifuge Avanti J-26 with rotor JA-25.50) for 15 min at 4 °C, the soluble fraction was collected and mixed with 2 mL of Ni-NTA resin at 4 °C for 15 min. The resin was washed with 10 x 10 mL of wash buffer (50 mM Tris pH 7.5, 500 mM NaCl, 30 mM imidazole) and eluted with elution buffer (50 mM Tris pH 7.5, 500 mM NaCl, 250 mM imidazole) until the Bradford assay, which detects protein, indicated all protein had eluted. The protein solution was dialyzed (MWCO 8 kDa) at 4 °C against 4 L of 50 mM phosphate buffer pH 7.0 with 100mM NaCl and 2 mM DTT for 4 hours, then transferred to 4 L of 50 mM phosphate buffer pH 7.0 with 100mM NaCl and dialyzed another 24 h, changing the buffer 2 more times. 12 mg of SH3-rhFGF2 was collected. The protein was sterile-filtered (0.22 µM) and verified by ESI mass spectrometry (ABI/Sciex Qstar mass spectrometer) and denaturing gel electrophoresis (Figures S3 and S4). An extinction coefficient of 38390 M⁻¹ cm⁻¹ and MW of 27.7 kDa, respectively, were used to identify SH3-rhFGF2.

Bioactivity assay for SH3-rhFGF2

The bioactivity of SH3-rhFGF2 was determined *in vitro* using mice neural stem cell cultures as previously described³ with 10ng/mL rhFGF2 and 10 ng/mL (rhFGF2 equivalent) of SH3-rhFGF2 (Figure S5).

Preparation of HAMC and HAMC-peptide hydrogels

Physical hydrogel blends of hyaluronan (HA) and methyl cellulose (MC) or methylcellulose-SH3-binding peptide (MC-peptide) were prepared in the following compositions with aCSF or SH3-rhFGF2: 1 wt % 2600 kg/mol HA, 3 wt % 300 kG/mol MC or MC-peptide (1:3 HAMC or HAMC-peptide). For example, to prepare 1000 µL of 1:3 HAMC or HAMC-peptide, 10 mg HA and 30 mg MC or MC-peptide was mechanically dispersed in 960 µL cold aCSF (HAMC) or 480 µL cold aCSF and 480 µL cold 40 µM SH3-rhFGF2 solution with 0.4 mg/mL heparin using a planetary mixer (Flacktek Inc., Landrum, USA) and chilled for 1 h. At this time the gels were dispersed again using a planetary mixer and then centrifuged at 16,162g (Sigma 1-14 microcentrifuge) for 1 min at 4 °C to remove air bubbles. The solutions were left to dissolve overnight at 4 °C before use.

In vitro release of SH3-rhFGF2 from HAMC and HAMC-peptide

HAMC and HAMC-peptide hydrogels were prepared as described above with SH3-rhFGF2 (20µM) vs. controls without protein to ensure the hydrogel itself did not interfere with the ELISA detection of SH3-rhFGF2. Each hydrogel was loaded into a 250 µL Hamilton syringe (Hamilton Company USA, Reno, USA) equipped with a 30 G needle and injected into the bottom of a 2.0 mL microcentrifuge tube. The gel was allowed to settle for 1 h at 4 °C and then pre-warmed to 37 °C for 5 min to induce gel formation. 900 µL of pre-warmed aCSF with 0.2 mg/mL heparin (release buffer) was added to each tube, approximating the dilution of hydrogel to CSF of 1:10 v/v that is expected *in vivo* when injected into the intrathecal space of a rat. Samples were incubated at 37 °C on an orbital shaker and release buffer was fully removed and replaced with fresh release buffer at t = 1, 2, 4, 8, 16, 24, 48, 72, 120, 168 and 240 h. Aliquots were frozen at -20 °C until assayed for SH3-rhFGF2 detection. A sandwich ELISA (Peprotech, Human FGF-basic ELISA Development Kit) was used to determine the concentration of SH3-rhFGF2 in the aCSF removed at each timepoint (n = 4).

Normalization of *in vitro* release data

In vitro release profiles have been normalized to total amount of protein detected (by ELISA) (Figure S6). After release, gels were disrupted and assayed for remaining protein content. Total protein detected from each group was highly different: HAMC (73.6 ± 1.9) %, HAMC-weak binder (44.2 ± 9.3) % and HAMC-strong binder (10.1 ± 1.1) %. Importantly, the amount of protein detected is related to the rate of protein release. That is, the faster the rate of protein release, the more total protein was detected. Nakamura *et al.* have shown that incubation of FGF-2 for less than 1 day at 37 °C reduces bioactivity to < 10 %.⁴ Since sampling during the first day of release is frequent, protein instability at 37 °C does not contribute greatly. However, after 1 day, the sampling interval decreases to 1 day or more, in which case loss of detectability due to incubation of the protein at 37 °C has a significant effect.

2.3 Statistical Analysis

All data are presented as mean (\pm standard deviation) unless otherwise noted. Comparisons of groups of means were determined using one-way analysis of variance (ANOVA) with Tukey's post hoc t-test to identify differences and pairs of means were analyzed by a student's t-test as appropriate. A p value less than 0.05 was set as the criteria for statistical significance.

SUPPLEMENTAL DATA

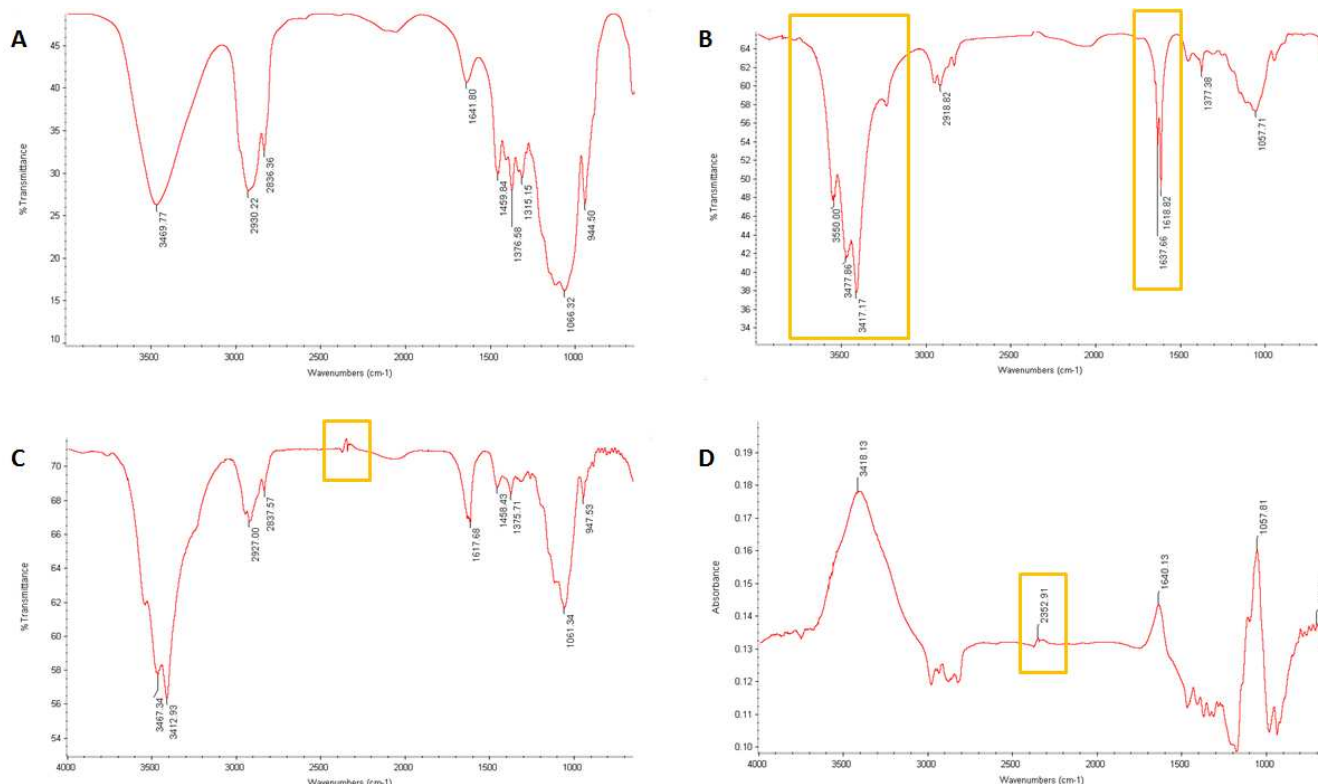


Figure S1. FT-IR characterization of A) MC, B) MC-CO₂H, and C) MC-SH. In B), new O-H stretches (3400-3600 cm⁻¹) and new C=O stretch (~1600 cm⁻¹) are highlighted. In C), the S-H stretch (~2300 cm⁻¹) is highlighted. This stretch is more evident in D) the spectral subtraction of A) MC from C) MC-SH.

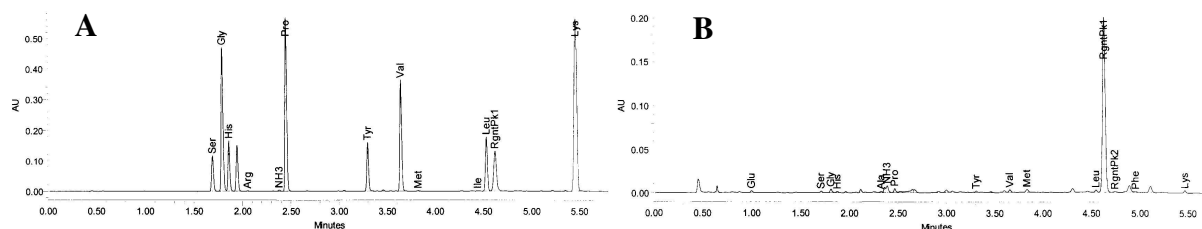


Figure S2. Amino acid analysis of the product of MC-SH **3** reacted with A) 3-maleimidopropionic-GGGKPPVVKKPHYLS and B) GGGKPPVVKKPHYLS (control SH₃-binding peptide). A known amount of SH₃-binding peptide dissolved in MC was used as a standard, peak areas were calculated for each amino acid. The concentration of SH₃-binding peptide was A) 188 μ mol/g MC and B) 0.69 μ mol/g MC, demonstrating successful immobilization of the SH₃-binding peptide to MC-SH in (A).

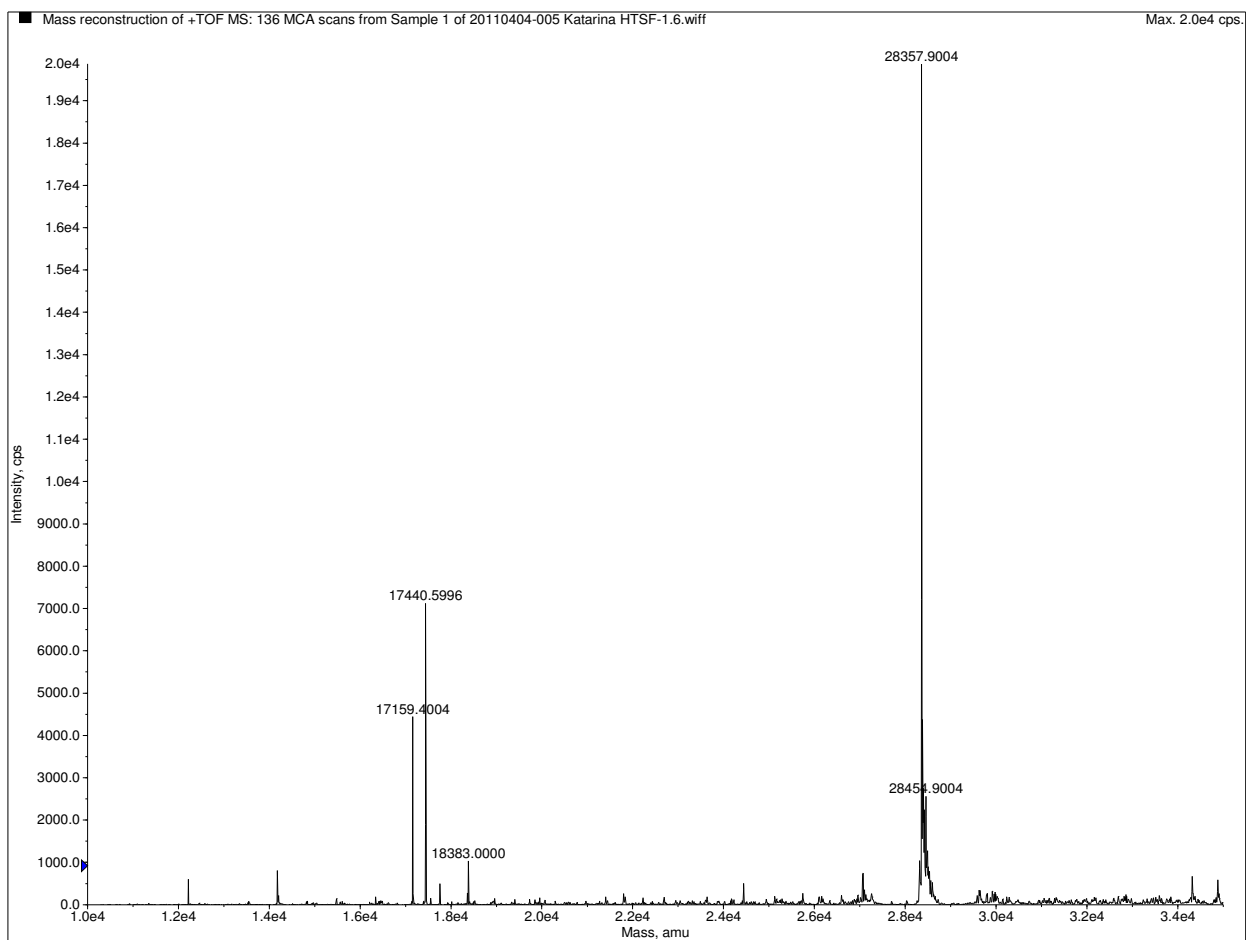


Figure S3. ESI protein mass spectrum of SH3-rhFGF2 (computer predicted mass 27.7 kg/mol). M+ found 28.4kg/mol (salts bound to M+ account for the shift in molecular weight).

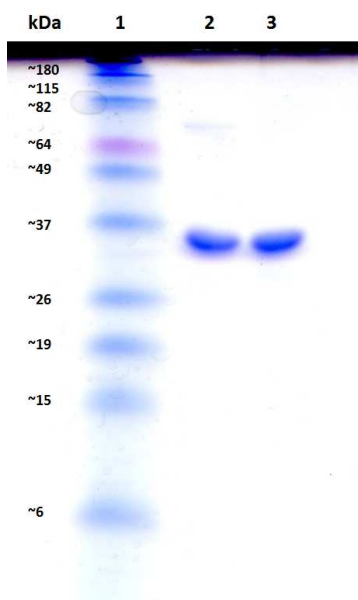


Figure S4. SDS-PAGE characterization of SH3-rhFGF2 shows that it has the predicted and stable molar mass over a 4 month storage period. Lane 1) Ladder; 2) SH3-rhFGF2 (MW27.6kDa); 3) SH3-rhFGF2 (stored for 4 months at -80 °C).

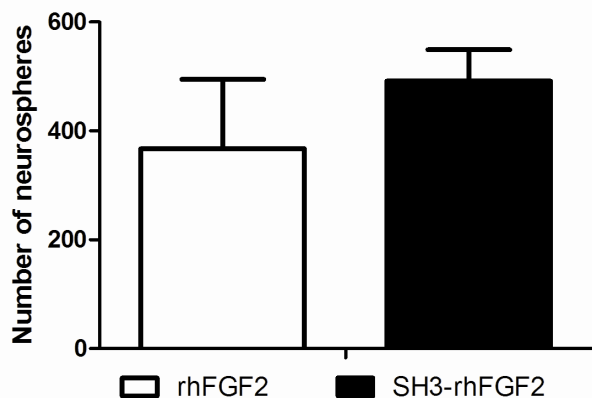


Figure S5. Bioactivity of SH3-rhFGF2 is equivalent to that of commercial rhFGF2 using a neurosphere assay and identical concentrations of rhFGF2 (10ng/mL). No statistical significance between groups ($p > 0.05$).

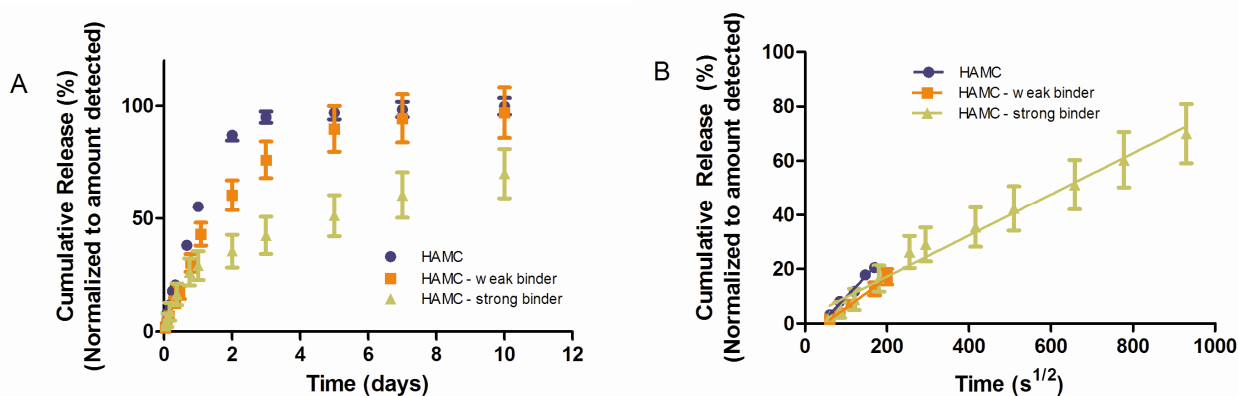


Figure S6. A) *In vitro* release profile of SH3-rhFGF2 delivered from HAMC, HAMC-weak binder and HAMC-strong binder hydrogels normalized to total amount of protein detected. Protein release from HAMC-weak binder is slower than from HAMC for the first 3 days of release ($p < 0.01$), after which there is no difference in the amount of protein released. Protein release from HAMC-strong binder is slower than HAMC ($p < 0.005$) and HAMC-weak binder ($p < 0.05$) after the first 24 h of release. 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). Diffusion-controlled release is sustained for 8 hours from HAMC, 12 hours from HAMC-weak binder and for 10 days from HAMC-strong binder. The non-zero intercept indicates that swelling affected diffusion at the early timepoints. ($n=4$, mean \pm standard deviation are plotted)

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- (4) Nakamura, S.; Kanatani, Y.; Kishimoto, S.; Nakamura, S.-i.; Ohno, C.; Horio, T.; Masanori, F.; Hattori, H.; Tanaka, Y.; Kiyosawa, T.; Maehara, T.; Ishihara, M. *Journal of Biomedical Materials Research Part A* **2009**, *91A*, 814-823.