CUSTOMIZED BIOMATERIALS TO AUGMENT CHONDROCYTE GENE THERAPY

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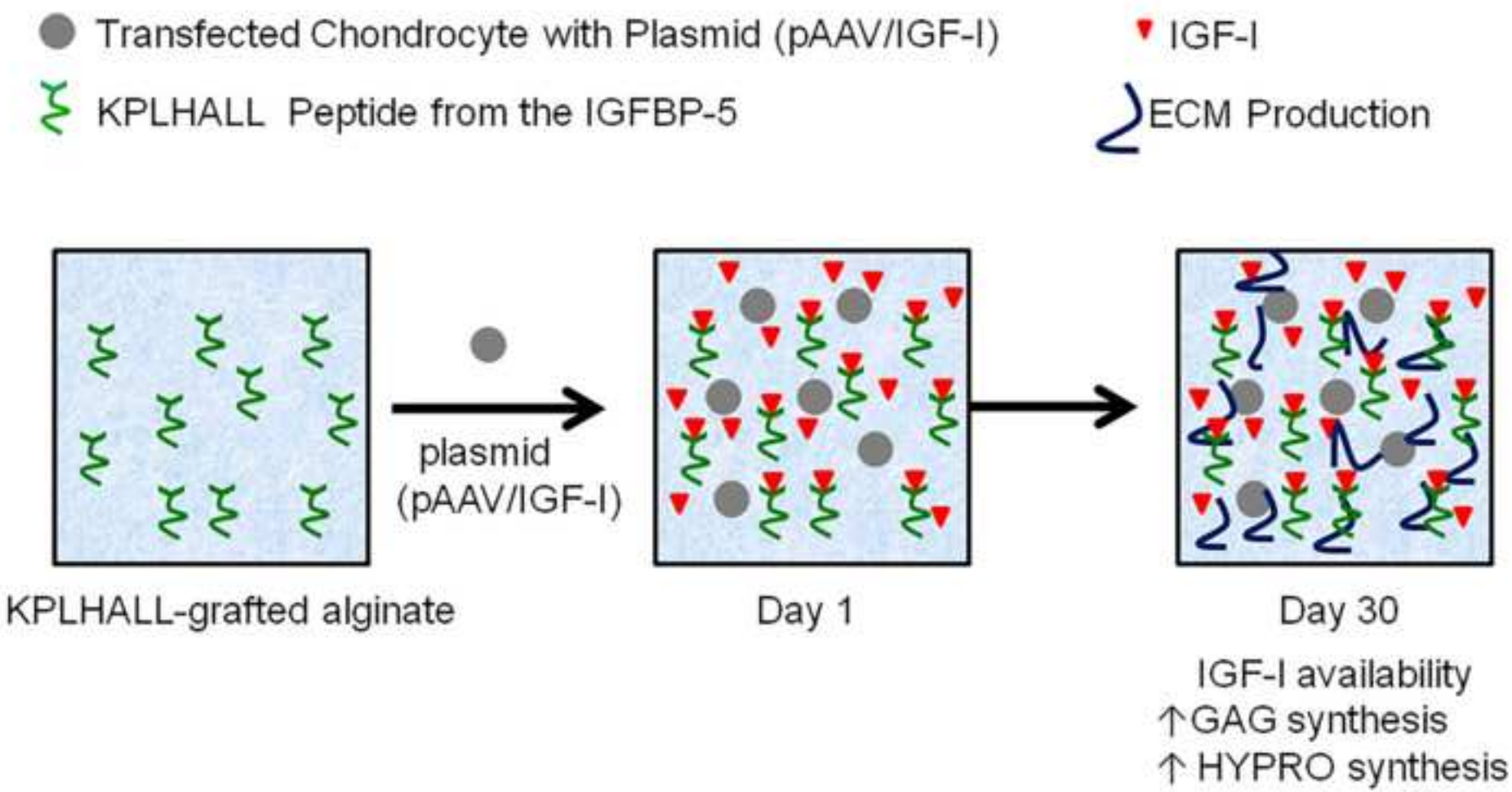
Key Words: Osteoarthritis, Growth Factor, IGF-I, Binding Peptide

Running Title: Biomaterials for Gene Therapy

ABSTRACT

A persistent challenge in enhancing gene therapy is the transient availability of the target gene product. This is particularly true in tissue engineering applications. The transient exposure of cells to the product could be insufficient to promote tissue regeneration. Here we report the development of a new material engineered to have a high affinity for a therapeutic gene product. We focus on insulin-like growth factor–I (IGF-I) for its highly anabolic effects on many tissues such as spinal cord, heart, brain and cartilage. One of the ways that tissues store IGF-I is through a group of insulin like growth factor binding proteins (IGFBPs), such as IGFBP-5. We grafted the IGF-I binding peptide sequence from IGFBP-5 onto alginate in order to retain the endogenous IGF-I produced by transfected chondrocytes. This novel material bound IGF-I and released the growth factor for at least 30 days in culture. We found that this binding enhanced the biosynthesis of transfected cells up to 19-fold. These data demonstrate the coordinated engineering of cell behavior and material chemistry to greatly enhance extracellular matrix synthesis and tissue assembly, and can serve as a template for the enhanced performance of other therapeutic proteins.

*Graphical Abstract



1. INTRODUCTION

A number of recent studies have focused on delivering therapeutic proteins using gene therapy to enhance the repair of skeletal muscle, brain, spinal cord, and cartilage[1–3]. Many of these studies have focused on a variety of membrane repair proteins, transcription factors, and growth factors to increase cellular synthesis and control paracrine cascades[1,4,5]. However, one of the main limiting factors in the efficacy of gene therapy is the transient availability of the gene product, regardless of the vector used. For example the use of gene therapy in cartilage repair has targeted increased growth factor production; however, the desired gene product is typically upregulated for up to two weeks[6,7]. Further, the highest concentrations of growth factors are in the first days after transfection, and have the potential to produce supra-therapeutic, or toxic levels of the growth factor[8].

One of the proteins commonly targeted for cartilage repair in gene therapy is insulin-like growth factor–I (IGF-I)[7–10]. Various viral and nonviral vectors have been used to transduce/transfect chondrocytes to upregulate IGF-I synthesis prior to therapeutic cell delivery[7,11–13]. Although IGF-I expression can remain elevated for a month or more[14], this expression decreases with time, and is often low by 2 weeks[7,15]. This is an extremely short time compared to the 6-12 months typically needed for effective cartilage repair[16], and may not induce optimal repair *in vivo*. As such, there is a great need for approaches to extend the availability of IGF-I for cartilage therapy.

In tissues such as cartilage, IGF-I is retained in the tissue by a family of binding proteins called insulin-like growth factor binding proteins (IGFBPs). These binding proteins are highly specific to IGF-I, with binding affinities of 1-10 nM[17]. IGFBPs also bind to cartilage enhanced cellular matrix (ECM), acting as both a sink and source for the growth factor as needed[18]. It

has been shown that IGFBP-5 has a specific localized small domain that is largely responsible for its high affinity binding to IGF-I[17].

Cell-based gene therapy for articular cartilage repair requires a means of delivering cells to the site in need of repair[19]. There has been increased attention to the development of biomaterials that prolong release and expression of gene products. Some of those biomaterials are chitosan, poly-L-lactic acid (PLLA), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx), polyethyleminine (PEI), and polyethyleneglycol (PEG). Chondrocytes have been cultured in chitosan-fabricate and a plasmid DNA scaffold to study cell proliferation, adherence, and transforming growth factor-ß1 (TGF-ß1) expression[20] and chitosan-pEGFP nanoparticles have been used to transfer exogenous genes into primary chondrocytes for the treatment of joint diseases[21]. Trimethylated chitosan (TMO) was synthesized from oligometric chitosan to deliver luciferase plasmid DNA to epithelial cells[22]. PLLA has been used with mesenchymal stem cells (MSCs) transfected with adenoviral SOX-9 to differentiate monolayer MSCs into chondrocyte-like cells[23]. PHBHHx scaffolds have been seeded with chondrocytes which employed tetracycline (Tet-on) to induce Sox9 expression[24]. Modified polyethylenimine (PEI) exhibited lower toxicity and higher gene expression of plasmid DNA in COS-7 cells and HepG2cells[25]. Polyethylene glycol-grafted polyethylenimine (PEG-PEI) has been used as a vector to deliver gene products to adipose stem cells to differentiate them to cartilage or osteoblast cells[26]. Hydrogels, including alginate, have also been shown to serve this purpose[27]. There are numerous examples of short peptide sequences being grafted to materials to enhance cell adhesion[17,28,29]. Similarly, materials have been modified to contain heparin-like carbohydrate components which have been shown to enhance binding of growth factors such as FGF-2[30]. All of the previous materials have been used to promote gene delivery and to

prolong the release and expression of the gene product, but none of these materials have interacted in any specific way with the proteins that are targeted for production by the cells. The goal of this study was to develop a material that could bind to the targeted gene product and assess whether that binding will enhance matrix production. To our knowledge, peptide-based modification of materials to localize growth factors has not been reported.

Here we demonstrate the development of a new material with high affinity for IGF-I by grafting a binding peptide sequence from IGFBP-5 onto alginate. This new material greatly extends the availability of the growth factor during chondrocyte culture and enhances cartilage matrix biosynthesis up to 19-fold.

2. MATERIALS AND METHODS

2.1 Chemistry

UP LVG alginate (from NovaMatrix®) was modified with carbodiimide chemistry[28]. 100 mg alginate (1% w/v alginate solution) was dissolved in 0.1 M of 1-(*N*-morpholino) ethanesulfonic acid (MES) buffer (Sigma-Aldrich) at pH 6.5. The reaction scheme is shown in Figure 1 where amide linkages were obtained by adding 2.5 mM of 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC) (Sigma-Aldrich) and 1.2 mM of sulfo-*N*-hydroxysuccinimide (NHS) to raise the efficiency of amide bond formation (Pierce Chemical)[28]. 1 mg of GGG-K(ivDde)PLALL peptide sequence (INNOVAGEN) was added after 5 minutes; the solution was left to react for 20 hrs. The reaction was quenched with 0.026 mM of hydroxyamine hydrochloride (Sigma-Aldrich) at 20 hrs. Alginate solution was purified by dialysis (3500 MWCO) (Fisher Scientific) with different salt concentrations varying 115 mM to 0 mM. Samples were stored at -80 °F for 24 hours and then they were lyophilized for 24

hours. The additional 3 glycines at the N- terminus of this synthetic peptide sequence were added in order to provide a linker layer that would more fully expose the active peptides in the sequence once the peptide was bound to a surface. The lysine on this sequence was protected from unwanted chemical reactions by an ivDde protecting group; this was removed using 5% hydrazine, anhydrous (Sigma) in dimethylformamide (DMF). Following the deprotection step, the modified alginate solution GGG-KPLALL (KPLHALL) was purified by dialysis (3500 MWCO) (Fisher Scientific) with DI H_2O for 24 hours. Samples were stored at -80 °F for 24 hours and then they were lyophilized for 24 hours.

2.2 Nuclear Magnetic Resonance Studies

The freeze-dried KPLHALL alginate and unmodified alginate were dissolved in D_2O at 0.0012% w/v. There were three groups: alginate, a mixture of alginate with free KPLHALL peptide, and KPLHALL-grafted alginate. ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were recorded on 600MHz Varian Inova NMR. ^{1}H NMR was normalized to residual solvent D_2O. The final molar concentration of peptide KPLHALL grafted in alginate was obtained by integrating the area under the curve with MNova NMR Software, 100 μM of binding sites of KPLHALL (0.3% of degree of graft on alginate backbone). Also, diffusion ordered spectroscopy (DOSY) was performed to demonstrate stable attachment of the peptide sequence to the alginate.

2.3 Surface Plasmon Resonance Studies

Surface Plasmon Resonance (SPR) measurements were performed with an SPR Refractometer Instrument (Reichert, Inc., Depew, NY). A range of KPLHALL concentrations from 0 to 100 μM was achieved by mixing the 100 μM KPLHALL with unmodified alginate in varying ratios. Modified and unmodified alginate were bound to 50 nm thick gold chips (Fischer Scientific, Pittsburg, PA) using carbodiimide chemistry. Samples were run at 25° C in buffer for

10 minutes per sample, using a constant flow rate of 25 µl /min over the surface of the SPR chip. Concentrations of IGF-I ranged from 5 to 3000 nM. PBST (PBS plus 0.05% v/v Tween 20) was used as both the sample and flow buffer. After each binding experiment, the surface was regenerated with 40 mM HCl. The sensorgram profiles were analyzed using SigmaPlot where response to equilibrium was calculated with an three parameter exponential rise to maximum model and then a Langumir binding model was used to determine the binding constant (K_D) [31].

2.4 Analysis

The microrefractive index unit at equilibrium was measured for each concentration of IGF-I as it flowed through the chip (Equation 1).

$$R(t) = R_{eq}\left(1 - e^{\left(\frac{(t-t_0)}{\tau}\right)}\right)$$

Where, R_{eq} is the refractive index unit at equilibrium, t is time, t_0 is the time after baseline, and Ƭ is the exponential time constant.

K_D was calculated by fitting the data to Langmuir's Affinity Kinetics Model [31] (Equation 2)

$$R_{eq} = \frac{[IGF - I] * R_{max}}{[IGF - I] + K}$$

R_{eq} is the response at equilibrium for each curve; [IGF-I] is the concentration of IGF-I; R_{max} is the maximum equilibrium response and K_D is the binding affinity constant. The dissociation rate constant (k_{off}) was calculated by fitting the dissociation phase[31] (Equation 3)

$$R_T = \left(R_{eq} - R_{off}\right)\left(1 - e^{\left(\frac{(t-t_1)}{k_{off}}\right)}\right)$$

Where, R_T is the total response where it is assumed that the total complex concentration of IGF-I and KPLHALL [IGF-I*KPLHALL]$_{TOTAL}$ is proportional to the total response (RT). R_{eq} is the response at equilibrium for each curve and R_{off} is the equilibrium response at the end of the

dissociation phase. The experiment was measured at different times, t (time where dissociation finished) and t_1 (time where dissociation started).

Furthermore, the association rate constant (k_{on}) of each IGF-I concentration was calculated as (Equation 4)

$$K = \frac{k_{off}}{k_{on}}$$

2.5 Cell Culture and Matrix Synthesis:

Chondrocytes were isolated as previously described[7,32]. Articular cartilage was harvested from stifle (knee) condyles of 1-3 day old bovids (*Bos taurus*). Articular cartilage was washed with PBS and 1% antibiotics and antimycotic. Chondrocytes were then isolated by adding 0.3% type 2 collagenase (catalogue number LS004177, Worthington Biochemical, Lakewood, NJ) overnight. Isolated chondrocytes were washed and suspended in DMEM containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS) and placed in T-75 flasks plates at 60% confluency at 37˚C in 5% CO_2. After 48 hours, cells were transfected using two different complexes, made 30 minutes before transfection, at a ratio of 3:1[7,10,33]: FuGENE®6 (Roche Applied Science, Indianapolis, IN) + pAAV/IGF-I or FuGENE®6+pAAV/ MCS (Empty). After 16 hours, the transfection was stopped by replacing the culture medium with 5 mL of fresh media without FBS, and 100 U/mL penicillin, 100 µg/mL streptomycin. Afterwards, cells were trypsinized and mixed with 2% (w/v) modified or unmodified alginate. These culture, encapsulation, and transfection methods were selected based on previous work [9] demonstrating the successful formation of tissue in vivo using these protocols.

A range of KPLHALL concentrations from 0 to 100 μM was achieved by mixing the 100 μM KPLHALL with unmodified alginate in varying ratios. Cells were encapsulated in beads formed by extrusion through a 22-gauge needle into a 102 mM $CaCl_2$ solution. Beads were incubated with DMEM without FBS for 30 days; beads were collected every six days.

2.6 Biochemical Analysis

Beads collected every six days for 30 days were used to measure DNA, glycosaminoglycan (GAG) and hydroxyproline (HYPRO) content. DNA content was measured via Hoechst dye assay[34]. GAG content was measured using the DMMB dye-binding assay[35], and HYPRO content was measured using DMAB dye assay[36]. Syntheses of GAG [35] and HYPRO [36] were used as the primary measure of chondrocyte metabolic activity. The kinetics of GAG and HYPRO accumulation were fitted to an established model of matrix synthesis to calculate steady-state GAG and HYPRO content [37] (Equation 5).

$$[ECM](t) = [ECM]_{SS}\left(1 - e^{\frac{-t}{\tau}}\right)$$

Where, [ECM] is the matrix synthesized by the transfected or control of chondrocytes in the different concentrations of KPLHALL. $[ECM]_{SS}$ is steady state matrix production produced by the chondrocytes at different concentrations of KPLHALL. Each steady state value was normalized to alginate (0 μM) steady state value for GAG and HYPRO. These normalized steady-state values of GAG and HYPRO synthesis were used to determine the effect of KPLHALL content on chondrocyte matrix synthesis using a generalized variable slope concentration–response model (Equation 6) [37].

$$\frac{[ECM]_{SS}}{\left[ECM_{0\mu M}\right]_{SS}} = [ECM]_{min} + \frac{[ECM]_{max} - [ECM]_{min}}{1 + \left(\frac{[KPLHALL]}{EC_{50}}\right)^{-Hillslope}}$$

Where, $[ECM]_{SS}$ is the steady state matrix production, $[ECM_{0\mu M}]_{SS}$ is the steady state matrix production, $[ECM]_{max}$ is the maximum stimulation, $[ECM]_{min}$ is the minimum stimulation,

198 [KPLHALL] is the concentration of KPLHALL- modified alginate, and EC_{50} is the dose required
199 to produce 50% response.

200 Beads from day 30 were collected for immunohistochemistry analysis. Samples were
201 treated with citrate antigen retrieval buffer for 10 minutes at 90°C. Slides were then washed with
202 TBS/TWEEN20 and PBS. Samples were placed in humidity chambers for 30 minutes with 3%
203 hydrogen peroxide. Blocking solution (goat serum) was added and incubated for 1 hour at room
204 temperature. Afterwards the primary antibody Rb pAb for IGF-I (ab40657) was applied and left
205 overnight at 4°C. Negative controls were obtained by omitting the primary antibody to a section
206 on each slide (See supplementary figure S1). Next Rabbit IgG for secondary antibody was
207 applied for 30 minutes at room temperature, followed by ABC reagent (Vectastain PK-4000,
208 Vector Labs). Slides were developed with DAB peroxidase (Vector Labs) for approximately 3
209 minutes.

210 *2.7 Statistical Analysis*

211 GAG and HYPRO data are expressed as mean ±SD. The effect of culture time and
212 transfection were analyzed by 2-way ANOVA using Tukey's t-test for post hoc analysis
213 performed in SigmaPlot 11 (SYSTAT, Chicago, IL), with significance determined with $p<0.05$.
214 The temporal patterns in GAG and HYPRO data were then fit to equation 5 using SigmaPlot 11,
215 which calculated best fit values of $[ECM]_{SS}$ and τ, with the uncertainties in these fits expressed as
216 standard error.

217 Each steady state values and standard errors were normalized to alginate (0 μM) steady
218 state value for GAG and HYPRO. The normalized values were fitted to Equation 6 using
219 software SigmaPlot 11, which enabled the calculation of best fit values for EC_{50}, $[ECM]_{max}$, and
220 $[ECM]_{min}$ values and their uncertainty, expressed as standard error. Statistical differences

between EC_{50}, $[ECM]_{max}$, and $[ECM]_{min}$ were determined by an unpaired t test using GradPad Prism (GraphPad, Inc., La Jolla, CA).

3. RESULTS

3.1 Generation and Characterization of Hydrogels with Affinity for IGF-I

The peptide sequence KPLHALL from the hydrophobic binding pocket of IGFBP-5 was chosen as a candidate for enhancing IGF-I binding based on analysis of the crystal structure of IGFBP-5 /IGF-I complex. These data showed that 6 of the 7 amino acids in this peptide sequence were within 4Å of IGF-I when the complex is formed, enabling a high level of affinity [38].

The high affinity sequence was synthesized and grafted to alginate via previously established carbodiimide/sulfo-NHS chemistry[39](Fig 1A). The modification of alginate was confirmed by ^{1}H NMR analysis, using the leucine peaks at ~0.7 ppm to both demonstrate successful grafting and measure the amount of conjugated peptide (Fig 1B). Additionally the stability of the peptide graft was demonstrated by diffusion ordered NMR (DOSY), which showed the persistence of leucine peaks in the grafted material (See supplementary figure S2).

Analysis of IGF-I binding to modified and control alginate via surface plasmon resonance (SPR) demonstrated significant enhancement of binding by the modified material (Fig1C). The dissociation constant (K_D) decreased from 513 nM to 50 nM due to modification with KPLHALL (Fig1C). This level of affinity for IGF-I was lower than that for full length of IGFBP-5 (K_D=3.7 nM), but similar to a truncated form of IGFBP-5 containing only the binding pocket (K_D=37 nM)[17].

3.2 Enhancement of IGF-I Binding and Matrix Production

The enhanced binding demonstrated by KPLHALL-modified alginate motivated the hypothesis that this material would retain IGF-I produced by encapsulated chondrocytes. To test this hypothesis, articular chondrocytes were transfected with an adeno-associated virus-based plasmid vector (pAAV/IGF-I), carrying human IGF-I cDNA (transfected chondrocytes) or with empty vector (control chondrocytes) and encapsulated as previously described[33,39] in modified and unmodified alginate and cultured for 30 days.

We have previously shown that IGF-I transfection is highly efficient, but also transient, with enhanced IGF-I expression subsiding within 6 days[7,33]. Consistent with this data, immunohistochemistry staining of unmodified alginate (i.e. 0 μM binding peptide) cultures showed minimal presence of IGF-I at 30 days (Fig.2). In contrast, control chondrocytes cultured in alginate modified with KPLHALL showed enhanced retention of IGF-I at 30 days, with IGF-I retention proportional to the concentration of the grafted binding peptide. This enhancement was greater in chondrocytes transfected with pAAV/IGF-I, where the highest concentrations of binding peptide (i.e. 33μM and 100 μM) resulted in robust staining for IGF-I at 30 days (Fig.2).

Both empty vector control and pAAV/IGF-I transfected chondrocytes showed accumulation of the principal cartilage matrix components with time. Transfection with p/AAV/IGF-I enhanced accumulation of glycosaminoglycan (GAG) ($p<0.001$ by 2-way ANOVA) (Fig3A) and collagen, as indicated by hyproxyproline (HYPRO) ($p<0.001$) when compared to empty vector controls (Fig.3B). These typically reached steady state after two weeks in culture. The average of the time constants (τ) varied from 5.4 ± 3.6 hr to 10.0 ± 8.0 hr for GAG; tau values varied from 3.0 ± 3.7 hr to 27.9 ± 7.2 hr for HYPRO. The presence of peptide binding enhanced matrix synthesis in empty vector and pAAV/IGF-I transfected cultures (*$p<0.001$ by 2-way ANOVA) for both GAG and HYPRO. In IGF-I transfection had a dramatic

effect on HYPRO production both in the presence and absence of the binding peptide (*$p<0.01$ by 2-way ANOVA).

To understand the effect of IGF-I retention via binding peptide sites, the steady-state matrix contents were calculated using an established kinetic model [37]. For each culture condition, steady state matrix contents were normalized to the unmodified alginate (0 μM), which revealed that matrix synthesis was clearly dose-dependent ($R^2>0.93$ for all fits) on binding peptide density (Fig.4A). For the control cells, enhancement of GAG and HYPRO synthesis were 1.6 and 3-fold, respectively. This effect was more dramatic in transfected cells, with GAG and HYPRO synthesis upregulated 6.9 (*$p<0.0001$ by unpaired t test) and 19.4-fold (%$p<0.02$ by unpaired t test), respectively. For all cultures, EC_{50} for enhancement of synthesis ranged from 17 to 41 μM KPLHALL.

4. DISCUSSION

These data illustrate that the biomimetic modification of alginate with a binding peptide from IGFBP-5 enables high affinity binding of IGF-I. The data further demonstrate that this modification extend the presence of the growth factor in cell-seeded constructs to at least one month, and that this extended presence of IGF-I in turn substantially enhances cartilage matrix synthesis.

Producing materials that specifically bind IGF-I present specific technical challenges. Proteins that are known to bind IGF-I have complex 3D configurations that stabilize the formation of the binding protein-IGF-I complex[38]. IGFBP-5, which has 252 residues, and mini-IGFBP-5, which has 52 residues, have binding affinities of 3.7 nM and 37 nM respectively. Grafting such proteins to a material to enhance IGF-I binding would be technically challenging and expensive, due to the cost of producing the protein recombinantly. Additionally, stability of

289 the grafted material would be of great concern. Grafting shorter peptides would solve this
290 problem; however it is not clear whether shorter peptides would produce high affinity binding.
291 Here we showed that grafting a seven peptide sequence from the binding pocket of IGFBP-5 to
292 alginate produced a material with high affinity (K_D = 50 nM). This peptide sequence is short
293 enough that folding is likely absent or minimally important. However, it is important to note that
294 grafting to alginate via the Ɛ amino group in the lysine residue of the peptide rather than the
295 glycine leader sequence eliminated the beneficial effect on matrix synthesis (data not shown).
296 This data suggests that some amount of leader sequence is necessary for proper binding of IGF-I
297 to the modified alginate. Additionally, the affinity may be aided by the fact that alginate is a
298 polyanion and IGF-I is positively charged (pI = 8.5) [40], and charge based interaction may play
299 a role in aiding binding. Nevertheless the significant enhancement of IGF-I binding by the
300 addition of a short peptide may provide a template for targeted modification of biomaterials for
301 growth factor binding.

302 Other studies have explored alternative approaches for expanding the time of IGF-I
303 action through controlled delivery. For example, the interaction of IGF-I with the cartilage ECM
304 has been altered by generating a recombinant fusion protein of IGF-I with a heparin binding
305 domain[41]. This modified IGF-I bound to heparin sulfate and chondroitin sulfate with high
306 affinities (21 nM and 172 nM) and extended the retention time of the growth factor in
307 cartilage[41]. Other approaches for delivery include gene-activated matrix (GAM) [42]
308 materials, in which a material is loaded with a plasmid that is released slowly to the cells.
309 Similarly IGF-I peptide was released from degradable microparticles to achieve delivery over ~2
310 weeks[43]. The approach described in the current paper compliments these previously described

311 approaches and provides additional flexibility for tuning both cellular and matrix responses over
312 many weeks. Additionally, we can use our modified alginate with other methods of gene therapy.
313 Similarly this modified material may be helpful in enhancing other methods of gene
314 therapy. Many studies on cartilage gene therapy have focused on extending the time of
315 production and availability of IGF-I. Adeno-associated virus (AAV), a non-pathologic human
316 parvovirus, is capable of transfecting non-dividing cells for extended periods of time and can
317 transduce normal and osteoarthritic articular cartilage *in vitro*[44]. Adenoviral vectors (Ade.IGF-
318 I) have been used to transduce cells where production of IGF-I ranges from 21 days up to 150
319 days[45,46]. Also, chondrocytes transduced with recombinant adeno-associated virus
320 (rAAV)[47] have produced IGF-I for over 20 days. While our approach described here focused
321 on plasmid transfection, such an approach can also be used with any of the above vectors.
322 Tuning both IGF-I production and binding could enable even more extended availability of the
323 growth factor.
324 Both IGF-I as a target growth factor[48,49] and alginate as a delivery vehicle[50,51] have
325 been used *in vivo* to enhance the repair of cartilage. The modified alginate presented here shows
326 great promise to further improve chondrocyte matrix production and cartilage repair *in vivo*[50].
327 We noted a robust staining for type II collagen, particularly at higher concentrations of
328 KPLHALL constructs (See supplementary figure S3). Furthermore, the DNA analysis showed
329 similar amounts of DNA throughout all the groups throughout the duration of the experiments.
330 This data, in combination with the matrix synthesis data suggests that a relatively uniform
331 number of cells was maintained during culture (See supplementary figure S4). This new
332 biomaterial can be incorporated into other studies that have used alginate as a scaffold or have
333 used other vectors[48,52]. Overall, these data suggest that combining gene therapy with targeted

modification of scaffold material provides a platform where cellular production and extracellular binding of IGF-I act synergistically to enhance cellular biosynthesis. IGF-I regulates many other tissues including spinal cord[53], heart[54] and brain[55,56]. As such, these studies suggest the possibility of using such modified biomaterial not only with chondrocytes, but with other cell types. Furthermore, this approach can be extracted for use with other growth factors, such as TGF-β and BMP-2, that also have known protein binding sites in extracellular matrix[57,58].

5. CONCLUSION

Modifying alginate with the peptide KPLHALL from the binding pocket of IGFBP-5 enhanced binding affinity more than 10-fold, extended IGF-I availability over 30 days and increased GAG and HYPRO synthesis 7 and 20 fold respectively. The approach of controlling growth factor binding, by the grafting of small peptides for biomaterials represents an important new approach to drug delivery and tissue engineering.

Figure Captions

Figure 1

1A Reaction Scheme: EDC and Sulfo-NHS activate the alginate forming a carboxylate carbon intermediate. The carboxylate carbon intermediate is attacked by the primary amine nitrogen forming the amide bond to the alginate backbone.

1B NMR Spectra: First spectra shows the alginate sample with broad peaks for a polysaccharide, from 4.8 to 3.2, and distinct peak for the anomeric proton[59]. Second spectrum, mixture of the alginate and peptide, shows both different peaks for alginate and high leucine amino acids peaks. The third spectrum shows the modified alginate with KPLHALL where the chemical shift in the leucine peaks and the height of the peaks differs from the mixture.

1C SPR Spectra and Langmuir's Affinity Kinetics Model: Representative examples of curve fits for the affinity kinetic analysis of control (alginate) and modified alginate (KPLHALL alginate). Concentrations of IGF-I for alginate varied from 3000 nM to 0 nM. Concentrations of IGF-I for KPLHALL alginate varied from 1000 nM to 0 nM.

Langmuir's Affinity Kinetics Model for alginate is the top one (blue) and KPLHALL alginate is on the bottom (red). K_D shifted 10 times, where K_D is 50 nM sin KPLHALL alginate (K_D of 50 nM) when compared to alginate (K_D of 513 nM). KPLHALL alginate had a k_{on} ranging from 1 to 4(10^8/M●sec) and a k_{off} from 5/s to 19/s (R^2: 0.40 to 0.90).The parameters for alginate k_{on} and k_{off} ranged from 1.6 to 1.8(10^8/Ms) and k_{off} 10/s to 29/s (R^2: 0.74 to 0.9).

Figure 2

IGF-I Binding

Immunohistochemistry of constructs for IGF-I at Day 30. Scale bar = 100 μm. Alginate does not show differences in immunolocalization staining at day 30. Immunolocalization staining for IGF-I changes as the concentration of binding sites (KPLHALL) increases in the constructs. The constructs with 0 μM of binding sites barely show retention of f IGF-I; and constructs with 1 and 3 μM of binding sites show some retention of IGF-I. The highest immunolocalization of IGF-I is at 100 μM.

Figure 3

3A GAG matrix accumulation kinetic profiles: The production of GAG increases in both transfected and control groups. Control chondrocytes have smaller changes in kinetic profiles between the differences in KPLHALL concentrations. pAAV/IGF-I transfected chondrocytes have a greater effect (*$p<0.001$ by 2-way ANOVA) between the differences in KPLHALL concentrations.

3B HYPRO matrix accumulation kinetic profiles: The production of HYPRO increases in both pAAV/IGF-I transfected chondrocytes and control chondrocytes. The effect in chondrocytes that are transfected with pAAV/IGF-I is greater than the effect on those transfected with pAAV/ MCS (Empty) ($p<0.001$ by 2-way ANOVA). This effect is greater in concentrations of 10, 33 and 100 µM binding sites ($p<0.001$ by 2-way ANOVA). When alginate is compared with the highest concentration of binding sites (100 µM) there is an increase from 0.08 µg/mg at 0 uM to 1.4 µg/mg at 100 µM ($p<0.001$ by 2-way ANOVA)in pAAV/IGF-I transfected chondrocytes.

Figure 4

4A GAG and HYPRO Dose Response: GAG at the steady state concentration of each concentration is plotted against the different concentrations of binding sites of KPLHALL covalently attached to alginate. Transfected chondrocytes with pAAV/IGF-I produce 600% GAG, than the control chondrocytes transfected with pAAV/ MCS (Empty) (*$p<0.0001$ by unpaired t test). pAAV/IGF-I transfected chondrocytes required 16.9 µM of binding sites in the alginate to produce an increase in GAG of 6.9 (Table 2). Control chondrocytes required almost twice the amount of KPLHALL modified alginate only to produce 60% (Table 2). *$p<0.001$ when compared to min. +$p<0.001$ when compared to max empty.

4B Dose Response: The production of HYPRO increases both in pAAV/IGF-I transfected chondrocytes and control chondrocytes. The effect in chondrocytes that are transfected with pAAV/IGF-I is greater than the effect in cells transfected with pAAV/ MCS (Empty). pAAV/IGF-I increased HYPRO accumulation by 20 fold at the maximum effective concentration of IGF-I binding sites($p<0.02$ by unpaired t test). The parameters are summarized

on table 2 where all R^2 are higher than 0.93 (Table 2). %p<0.02 when compared to min. ^p<0.02 when compared to max empty.

Supplemental 1

Negative Control for IGF-I IHC in 100μM KPLHALL Alginate with pAAV/IGF-I transfected chondrocytes. Scale bar = 100 μm

Supplemental 2

Diffusion NMR was performed to see the stability of conjugation in the alginate; the NMR signal of the leucines is present even as the gradient increases. In contrast to the mixture sample, the leucine signals disappear as the gradient increases.

Supplemental 3

Immunohistochemistry of constructs for type II collagen at Day 30. Scale bar = 100 μm

Robust staining for type II collagen, particularly at higher concentrations of KPLHALL constructs.

Supplemental 4

Amount of DNA via Hoechst DNA assay throughout the duration of the experiment.

ACKNOWLEDGMENTS

This research is supported by Alfred P. Sloan Foundation, Coleman Foundation, Award NIH/NIAMS F31AR061982, NIH Grant R01 AR047702, and the Department of Veterans Affairs. We would like to thank Dr. Keresztes for his help with the NMR, Dr. Parker for all her help with the SPR and Ms. Michele Karr for all her help.

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Figure 1

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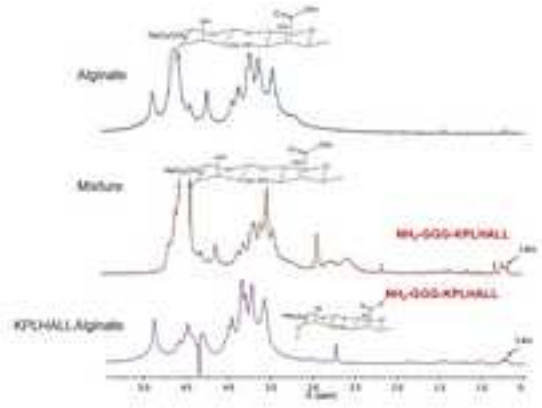
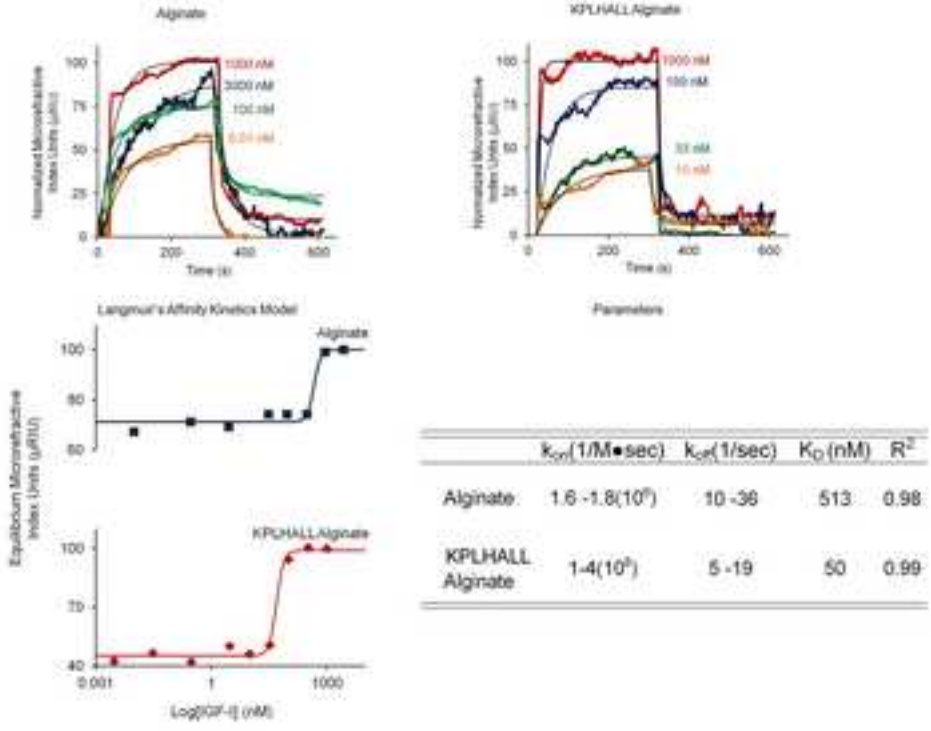
A) Reaction Scheme

B) 1H NMR Spectra



C) SPR Analysis



	k_{on}(1/M•sec)	k_{off}(1/sec)	K_D (nM)	R^2
Alginate	1.6 -1.8(10^5)	10 -36	513	0.98
KPLHALL Alginate	1-4(10^5)	5 -19	50	0.99

Figure 2

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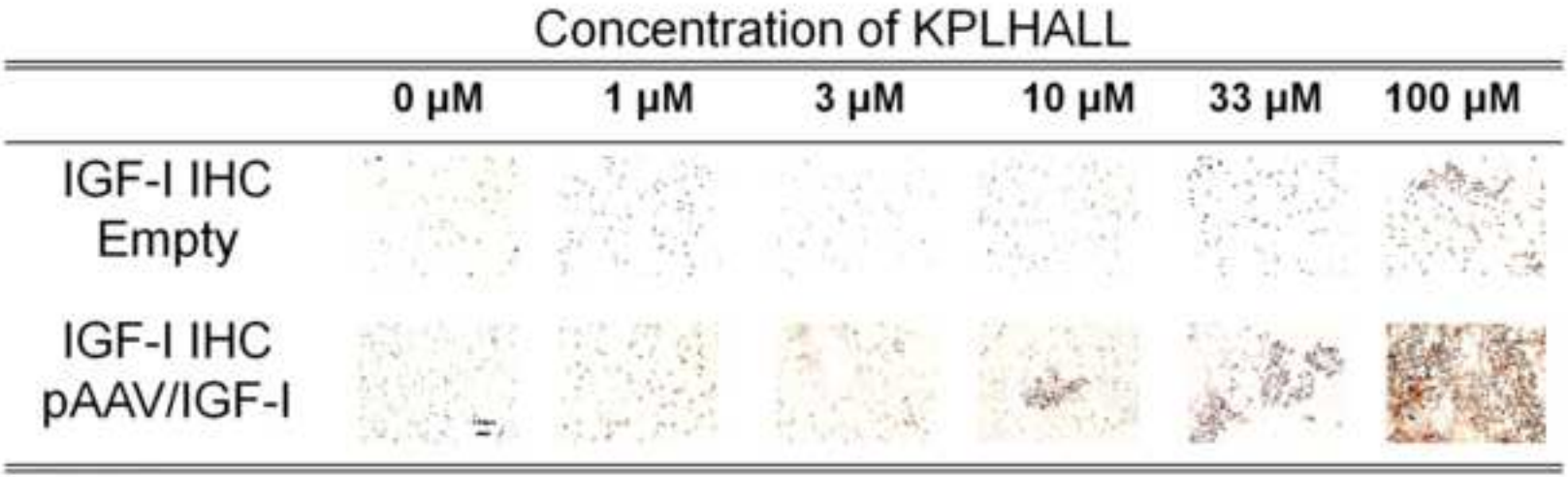

Figure 3

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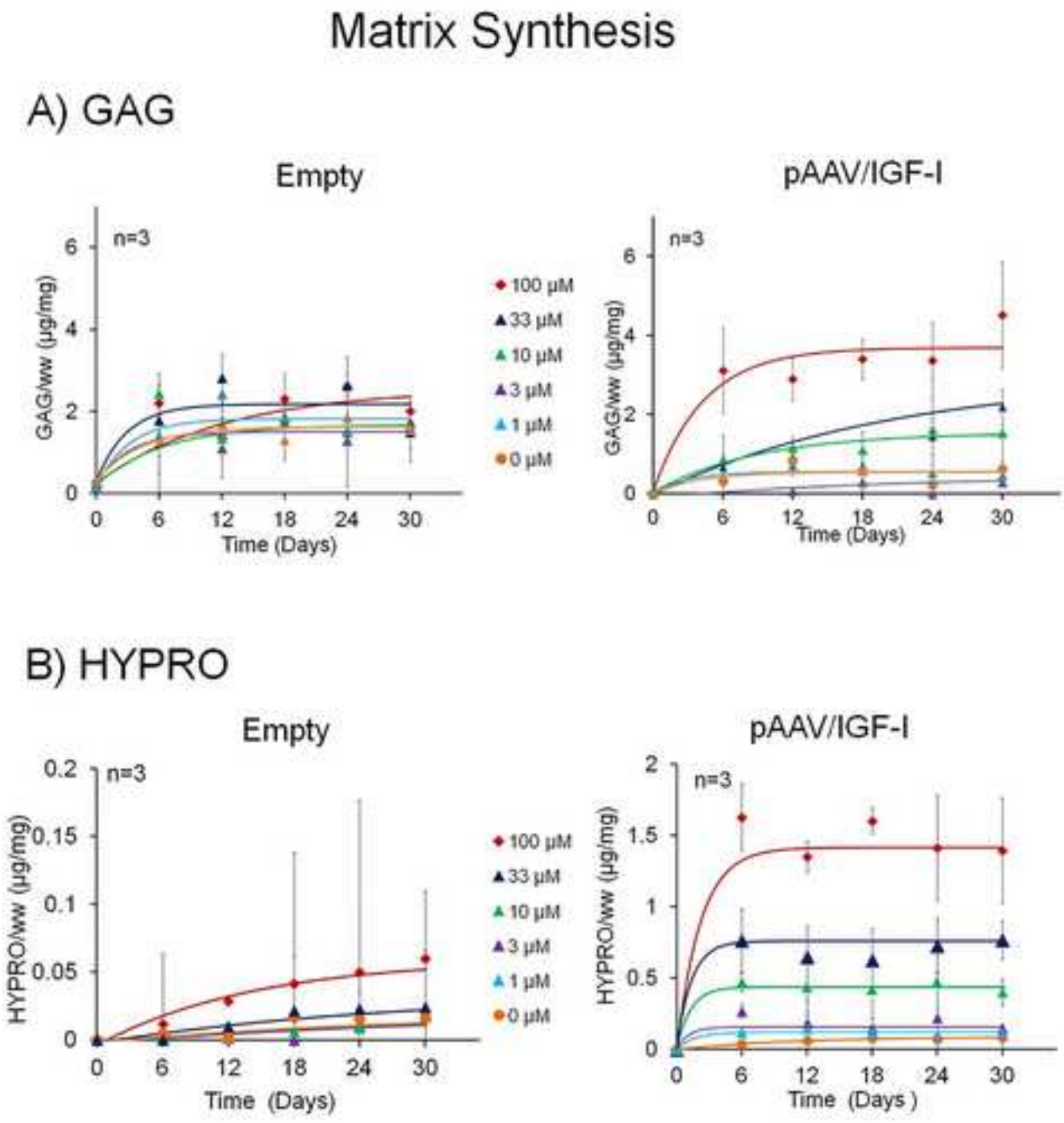

Figure 4

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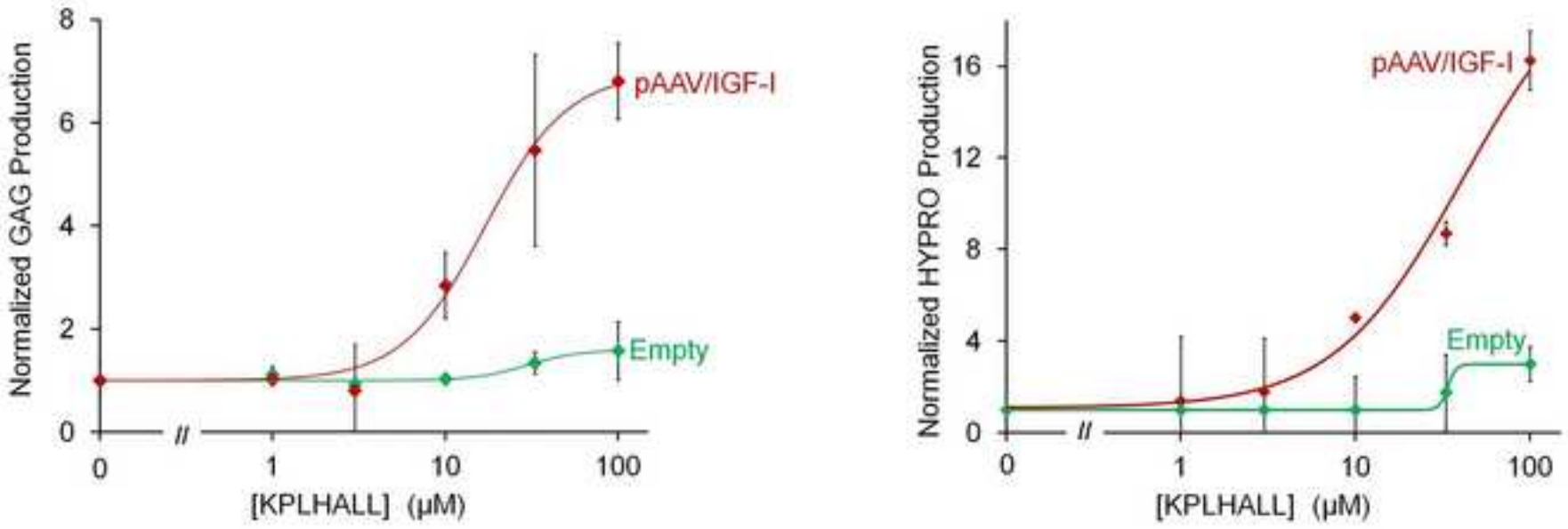
A)Dose Response



B)Dose Response Parameters

	GAG		HYPRO	
	pAAV/IGF-I	Empty	pAAV/IGF-I	Empty
EC_{50}(μM)	16.9±3.3	29.7± 9.2	41.0 ± 29.4	33.8±0.03
Max/Min	6.9±0.5 *+	1.6±0.1	19.4± 6.7%^	3.0 ± 0.1
R^2	0.99	0.93	0.99	0.99

Supplementary S1

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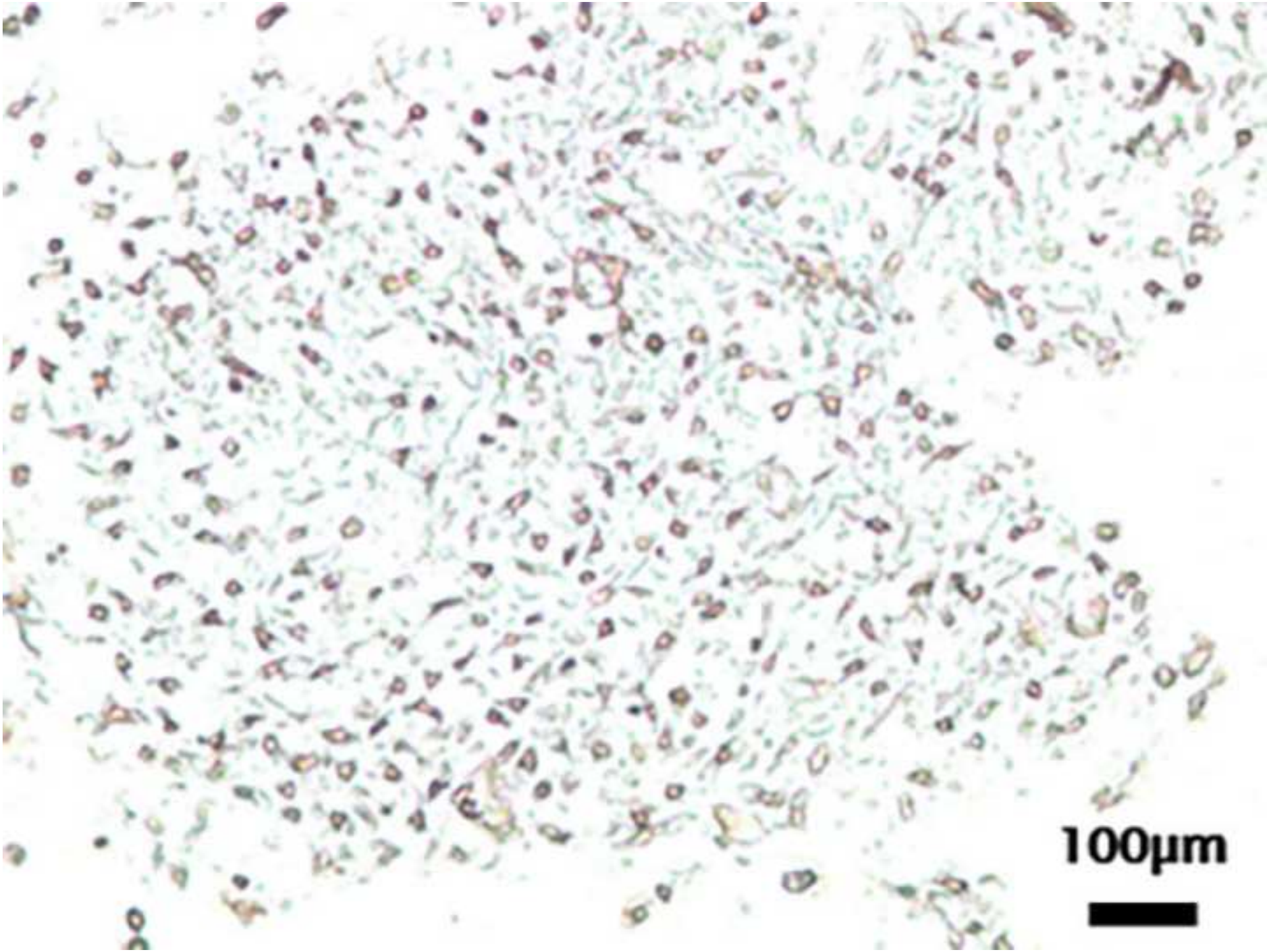

Supplementary S2

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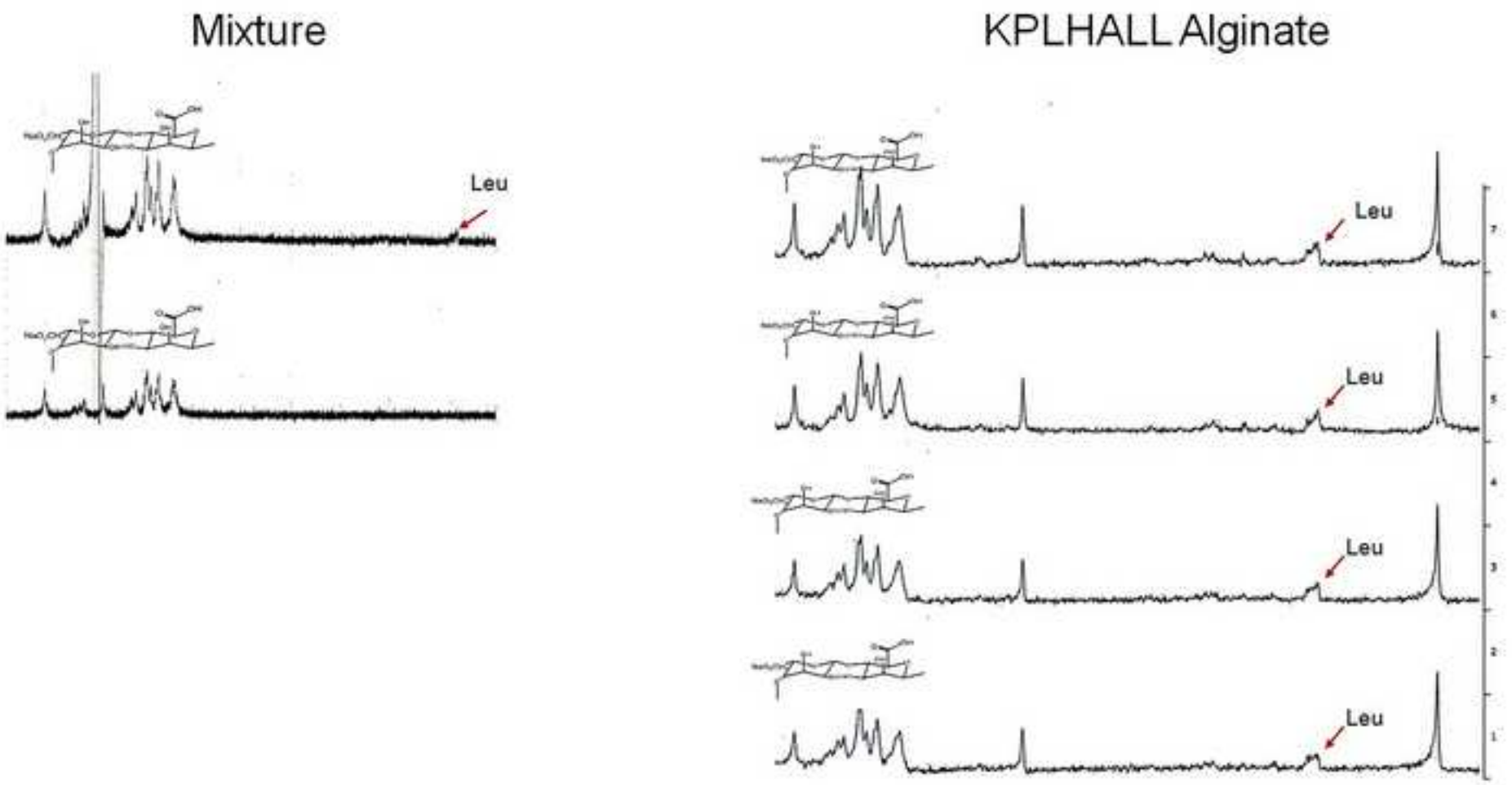
Diffusion Ordered Spectroscopy (DOSY)



Supplementary S3

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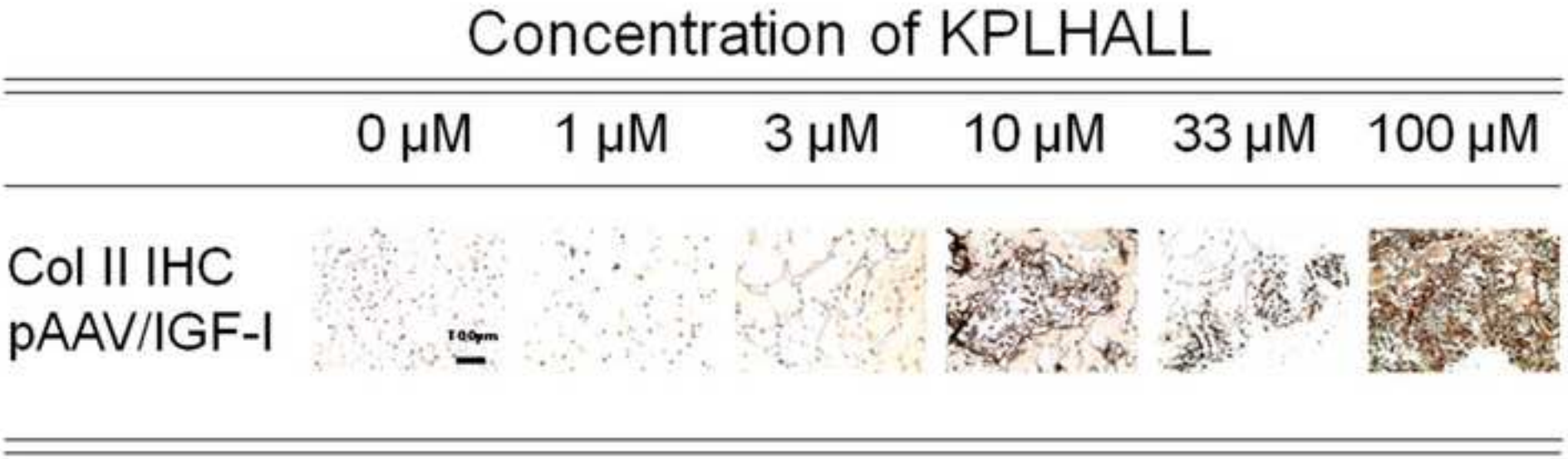

Supplementary S4

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