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5 CUSTOMIZED BIOMATERIALS TO AUGMENT CHONDROCYTE GENE THERAPY
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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.

● Transfected Chondrocyte with Plasmid (pAAV/IGF-I)   ▼ IGF-I
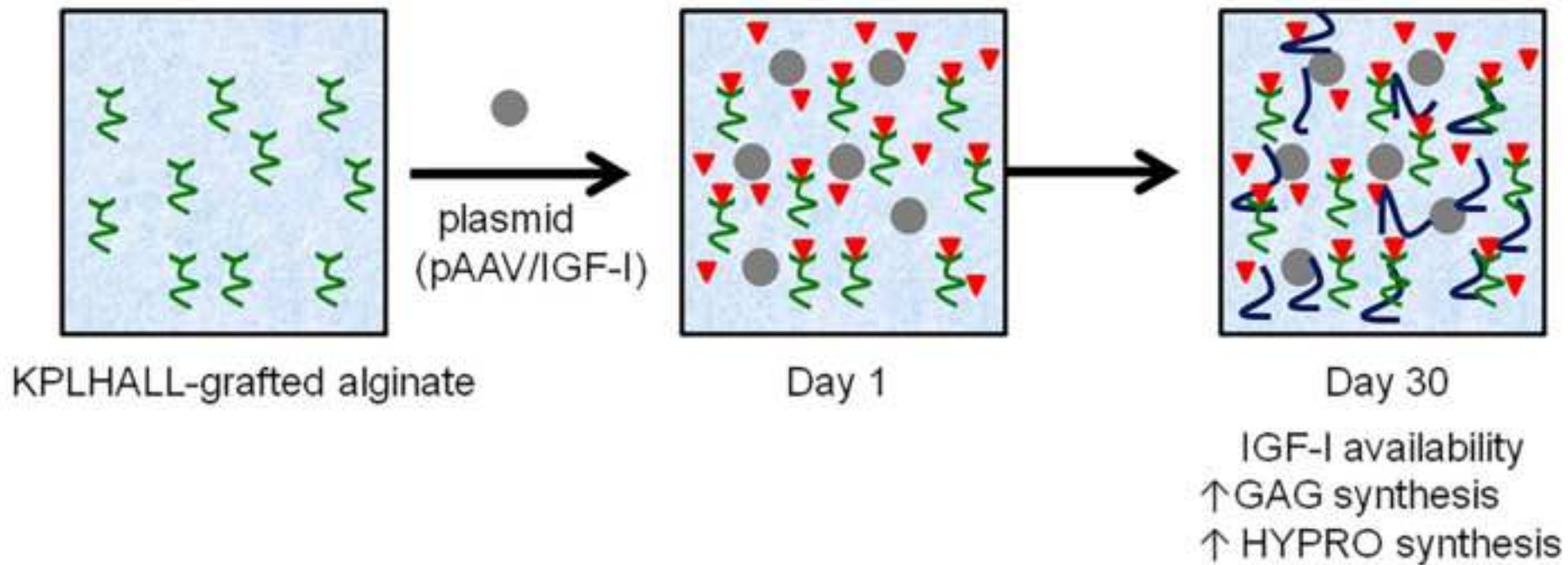
〰 KPLHALL Peptide from the IGFBP-5   〰 ECM Production

KPLHALL-grafted alginate

plasmid (pAAV/IGF-I)

Day 1

Day 30

IGF-I availability
↑GAG synthesis
↑ HYPRO synthesis

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

68   has been shown that IGFBP-5 has a specific localized small domain that is largely responsible

69   for its high affinity binding to IGF-I[17].

70   Cell-based gene therapy for articular cartilage repair requires a means of delivering cells to the

71   site in need of repair[19]. There has been increased attention to the development of biomaterials

72   that prolong release and expression of gene products. Some of those biomaterials are chitosan,

73   poly-L-lactic acid (PLLA), poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx),

74   polyethyleminine (PEI), and polyethyleneglycol (PEG). Chondrocytes have been cultured in

75   chitosan-fabricate and a plasmid DNA scaffold to study cell proliferation, adherence, and

76   transforming growth factor-ß1 (TGF-ß1) expression[20] and chitosan-pEGFP nanoparticles have

77   been used to transfer exogenous genes into primary chondrocytes for the treatment of joint

78   diseases[21]. Trimethylated chitosan (TMO) was synthesized from oligometric chitosan to

79   deliver luciferase plasmid DNA to epithelial cells[22]. PLLA has been used with mesenchymal

80   stem cells (MSCs) transfected with adenoviral SOX-9 to differentiate monolayer MSCs into

81   chondrocyte-like cells[23]. PHBHHx scaffolds have been seeded with chondrocytes which

82   employed tetracycline (Tet-on) to induce Sox9 expression[24]. Modified polyethylenimine (PEI)

83   exhibited lower toxicity and higher gene expression of plasmid DNA in COS-7 cells and

84   HepG2cells[25]. Polyethylene glycol-grafted polyethylenimine (PEG-PEI) has been used as a

85   vector to deliver gene products to adipose stem cells to differentiate them to cartilage or

86   osteoblast cells[26]. Hydrogels, including alginate, have also been shown to serve this

87   purpose[27]. There are numerous examples of short peptide sequences being grafted to materials

88   to enhance cell adhesion[17,28,29]. Similarly, materials have been modified to contain heparin-

89   like carbohydrate components which have been shown to enhance binding of growth factors such

90   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

114 hours. The additional 3 glycines at the N- terminus of this synthetic peptide sequence were added

115 in order to provide a linker layer that would more fully expose the active peptides in the

116 sequence once the peptide was bound to a surface. The lysine on this sequence was protected

117 from unwanted chemical reactions by an ivDde protecting group; this was removed using 5%

118 hydrazine, anhydrous (Sigma) in dimethylformamide (DMF). Following the deprotection step,

119 the modified alginate solution GGG-KPLALL (KPLHALL) was purified by dialysis (3500

120 MWCO) (Fisher Scientific) with DI H_2O for 24 hours. Samples were stored at -80 °F for 24

121 hours and then they were lyophilized for 24 hours.

2.2 Nuclear Magnetic Resonance Studies

123 The freeze-dried KPLHALL alginate and unmodified alginate were dissolved in D_2O at

124 0.0012% w/v. There were three groups: alginate, a mixture of alginate with free KPLHALL

125 peptide, and KPLHALL-grafted alginate. [1]H nuclear magnetic resonance (^{1}H NMR) spectra were

126 recorded on 600MHz Varian Inova NMR. ^{1}H NMR was normalized to residual solvent D_2O. The

127 final molar concentration of peptide KPLHALL grafted in alginate was obtained by integrating

128 the area under the curve with MNova NMR Software, 100 μM of binding sites of KPLHALL

129 (0.3% of degree of graft on alginate backbone). Also, diffusion ordered spectroscopy (DOSY)

130 was performed to demonstrate stable attachment of the peptide sequence to the alginate.

2.3 Surface Plasmon Resonance Studies

132 Surface Plasmon Resonance (SPR) measurements were performed with an SPR

133 Refractometer Instrument (Reichert, Inc., Depew, NY). A range of KPLHALL concentrations

134 from 0 to 100 μM was achieved by mixing the 100 μM KPLHALL with unmodified alginate in

135 varying ratios. Modified and unmodified alginate were bound to 50 nm thick gold chips (Fischer

136 Scientific, Pittsburg, PA) using carbodiimide chemistry. Samples were run at 25° C in buffer for

137   10 minutes per sample, using a constant flow rate of 25 μl /min over the surface of the SPR chip.

138   Concentrations of IGF-I ranged from 5 to 3000 nM. PBST (PBS plus 0.05% v/v Tween 20) was

139   used as both the sample and flow buffer. After each binding experiment, the surface was

140   regenerated with 40 mM HCl. The sensorgram profiles were analyzed using SigmaPlot where

141   response to equilibrium was calculated with an three parameter exponential rise to maximum

142   model and then a Langumir binding model was used to determine the binding constant (K_D) [31].

143   *2.4 Analysis*

144   The microrefractive index unit at equilibrium was measured for each concentration of IGF-I as it

145   flowed through the chip (Equation 1).

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

146   Where, R_{eq} is the refractive index unit at equilibrium, t is time, t_0 is the time after baseline, and

147   Ｔ is the exponential time constant.

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

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

149

150   R_{eq} is the response at equilibrium for each curve; [IGF-I] is the concentration of IGF-I; R_{max} is

151   the maximum equilibrium response and K_D is the binding affinity constant. The dissociation rate

152   constant (k_{off}) was calculated by fitting the dissociation phase[31] (Equation 3)

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

153   Where, R_T is the total response where it is assumed that the total complex concentration of IGF-I

154   and KPLHALL [IGF-I*KPLHALL]$_{TOTAL}$ is proportional to the total response (RT). R_{eq} is the

155   response at equilibrium for each curve and R_{off} is the equilibrium response at the end of the

156   dissociation phase. The experiment was measured at different times, t (time where dissociation

157   finished) and t_1 (time where dissociation started).

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

159   (Equation 4)

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

160

161   *2.5 Cell Culture and Matrix Synthesis:*

162   Chondrocytes were isolated as previously described[7,32]. Articular cartilage was

163   harvested from stifle (knee) condyles of 1-3 day old bovids (*Bos taurus*). Articular cartilage was

164   washed with PBS and 1% antibiotics and antimycotic. Chondrocytes were then isolated by

165   adding 0.3% type 2 collagenase (catalogue number LS004177, Worthington Biochemical,

166   Lakewood, NJ) overnight. Isolated chondrocytes were washed and suspended in DMEM

167   containing 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% fetal bovine serum (FBS)

168   and placed in T-75 flasks plates at 60% confluency at 37˚C in 5% CO_2. After 48 hours, cells

169   were transfected using two different complexes, made 30 minutes before transfection, at a ratio

170   of 3:1[7,10,33]: FuGENE®6 (Roche Applied Science, Indianapolis, IN) + pAAV/IGF-I or

171   FuGENE®6+pAAV/ MCS (Empty). After 16 hours, the transfection was stopped by replacing

172   the culture medium with 5 mL of fresh media without FBS, and 100 U/mL penicillin, 100 µg/mL

173   streptomycin. Afterwards, cells were trypsinized and mixed with 2% (w/v) modified or

174   unmodified alginate. These culture, encapsulation, and transfection methods were selected based

175   on previous work [9] demonstrating the successful formation of tissue in vivo using these

176   protocols.

177   A range of KPLHALL concentrations from 0 to 100 µM was achieved by mixing the 100

178   µM KPLHALL with unmodified alginate in varying ratios. Cells were encapsulated in beads

179   formed by extrusion through a 22-gauge needle into a 102 mM $CaCl_2$ solution. Beads were

180   incubated with DMEM without FBS for 30 days; beads were collected every six days.

181   *2.6 Biochemical Analysis*

182   Beads collected every six days for 30 days were used to measure DNA,

183   glycosaminoglycan (GAG) and hydroxyproline (HYPRO) content. DNA content was measured

184   via Hoechst dye assay[34]. GAG content was measured using the DMMB dye-binding assay[35],

185   and HYPRO content was measured using DMAB dye assay[36]. Syntheses of GAG [35] and

186   HYPRO [36] were used as the primary measure of chondrocyte metabolic activity. The kinetics

187   of GAG and HYPRO accumulation were fitted to an established model of matrix synthesis to

188   calculate steady-state GAG and HYPRO content [37] (Equation 5).

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

189   Where, [ECM] is the matrix synthesized by the transfected or control of chondrocytes in

190   the different concentrations of KPLHALL. $[ECM]_{SS}$ is steady state matrix production produced

191   by the chondrocytes at different concentrations of KPLHALL. Each steady state value was

192   normalized to alginate (0 µM) steady state value for GAG and HYPRO. These normalized

193   steady-state values of GAG and HYPRO synthesis were used to determine the effect of

194   KPLHALL content on chondrocyte matrix synthesis using a generalized variable slope

195   concentration–response model (Equation 6) [37].

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

196   Where, $[ECM]_{SS}$ is the steady state matrix production, $[ECM_{0\mu M}]_{SS}$ is the steady state matrix

197   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

10

221 between EC_{50}, $[ECM]_{max}$, and $[ECM]_{min}$ were determined by an unpaired t test using GradPad

222 Prism (GraphPad, Inc., La Jolla, CA).

223

3. RESULTS

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

226   The peptide sequence KPLHALL from the hydrophobic binding pocket of IGFBP-5 was

227 chosen as a candidate for enhancing IGF-I binding based on analysis of the crystal structure of

228 IGFBP-5 /IGF-I complex. These data showed that 6 of the 7 amino acids in this peptide sequence

229 were within 4Å of IGF-I when the complex is formed, enabling a high level of affinity [38].

230   The high affinity sequence was synthesized and grafted to alginate via previously

231 established carbodiimide/sulfo-NHS chemistry[39](Fig 1A). The modification of alginate was

232 confirmed by ^{1}H NMR analysis, using the leucine peaks at ~0.7 ppm to both demonstrate

233 successful grafting and measure the amount of conjugated peptide (Fig 1B). Additionally the

234 stability of the peptide graft was demonstrated by diffusion ordered NMR (DOSY), which

235 showed the persistence of leucine peaks in the grafted material (See supplementary figure S2).

236   Analysis of IGF-I binding to modified and control alginate via surface plasmon resonance

237 (SPR) demonstrated significant enhancement of binding by the modified material (Fig1C). The

238 dissociation constant (K_D) decreased from 513 nM to 50 nM due to modification with

239 KPLHALL (Fig1C). This level of affinity for IGF-I was lower than that for full length of

240 IGFBP-5 (K_D=3.7 nM), but similar to a truncated form of IGFBP-5 containing only the binding

241 pocket (K_D=37 nM)[17].

3.2 Enhancement of IGF-I Binding and Matrix Production

243   The enhanced binding demonstrated by KPLHALL-modified alginate motivated the

244   hypothesis that this material would retain IGF-I produced by encapsulated chondrocytes. To test

245   this hypothesis, articular chondrocytes were transfected with an adeno-associated virus-based

246   plasmid vector (pAAV/IGF-I), carrying human IGF-I cDNA (transfected chondrocytes) or with

247   empty vector (control chondrocytes) and encapsulated as previously described[33,39] in

248   modified and unmodified alginate and cultured for 30 days.

249   We have previously shown that IGF-I transfection is highly efficient, but also transient,

250   with enhanced IGF-I expression subsiding within 6 days[7,33]. Consistent with this data,

251   immunohistochemistry staining of unmodified alginate (i.e. 0 µM binding peptide) cultures

252   showed minimal presence of IGF-I at 30 days (Fig.2). In contrast, control chondrocytes cultured

253   in alginate modified with KPLHALL showed enhanced retention of IGF-I at 30 days, with IGF-I

254   retention proportional to the concentration of the grafted binding peptide. This enhancement was

255   greater in chondrocytes transfected with pAAV/IGF-I, where the highest concentrations of

256   binding peptide (i.e. 33µM and 100 µM) resulted in robust staining for IGF-I at 30 days (Fig.2).

257   Both empty vector control and pAAV/IGF-I transfected chondrocytes showed

258   accumulation of the principal cartilage matrix components with time. Transfection with

259   p/AAV/IGF-I enhanced accumulation of glycosaminoglycan (GAG) ($p<0.001$ by 2-way

260   ANOVA) (Fig3A) and collagen, as indicated by hyproxyproline (HYPRO) ($p<0.001$) when

261   compared to empty vector controls (Fig.3B). These typically reached steady state after two

262   weeks in culture. The average of the time constants (τ) varied from 5.4 ± 3.6 hr to 10.0 ± 8.0 hr

263   for GAG; tau values varied from 3.0 ± 3.7 hr to 27.9 ± 7.2 hr for HYPRO. The presence of

264   peptide binding enhanced matrix synthesis in empty vector and pAAV/IGF-I transfected cultures

265   (*$p<0.001$ by 2-way ANOVA) for both GAG and HYPRO. In IGF-I transfection had a dramatic

266 effect on HYPRO production both in the presence and absence of the binding peptide (*p<0.01

267 by 2-way ANOVA).

268   To understand the effect of IGF-I retention via binding peptide sites, the steady-state

269 matrix contents were calculated using an established kinetic model [37]. For each culture

270 condition, steady state matrix contents were normalized to the unmodified alginate (0 μM),

271 which revealed that matrix synthesis was clearly dose-dependent (R^2>0.93 for all fits) on binding

272 peptide density (Fig.4A). For the control cells, enhancement of GAG and HYPRO synthesis

273 were 1.6 and 3-fold, respectively. This effect was more dramatic in transfected cells, with GAG

274 and HYPRO synthesis upregulated 6.9 (*p<0.0001 by unpaired t test) and 19.4-fold (%p<0.02 by

275 unpaired t test), respectively. For all cultures, EC_{50} for enhancement of synthesis ranged from 17

276 to 41 μM KPLHALL.

277 **4. DISCUSSION**

278   These data illustrate that the biomimetic modification of alginate with a binding peptide

279 from IGFBP-5 enables high affinity binding of IGF-I. The data further demonstrate that this

280 modification extend the presence of the growth factor in cell-seeded constructs to at least one

281 month, and that this extended presence of IGF-I in turn substantially enhances cartilage matrix

282 synthesis.

283   Producing materials that specifically bind IGF-I present specific technical challenges.

284 Proteins that are known to bind IGF-I have complex 3D configurations that stabilize the

285 formation of the binding protein-IGF-I complex[38]. IGFBP-5, which has 252 residues, and

286 mini-IGFBP-5, which has 52 residues, have binding affinities of 3.7 nM and 37 nM respectively.

287 Grafting such proteins to a material to enhance IGF-I binding would be technically challenging

288 and expensive, due to the cost of producing the protein recombinantly. Additionally, stability of

13

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 ε 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

334   modification of scaffold material provides a platform where cellular production and extracellular

335   binding of IGF-I act synergistically to enhance cellular biosynthesis. IGF-I regulates many other

336   tissues including spinal cord[53], heart[54] and brain[55,56]. As such, these studies suggest the

337   possibility of using such modified biomaterial not only with chondrocytes, but with other cell

338   types. Furthermore, this approach can be extracted for use with other growth factors, such as

339   TGF-β and BMP-2, that also have known protein binding sites in extracellular matrix[57,58].

340   **5. CONCLUSION**

341   Modifying alginate with the peptide KPLHALL from the binding pocket of IGFBP-5

342   enhanced binding affinity more than 10-fold, extended IGF-I availability over 30 days and

343   increased GAG and HYPRO synthesis 7 and 20 fold respectively. The approach of controlling

344   growth factor binding, by the grafting of small peptides for biomaterials represents an important

345   new approach to drug delivery and tissue engineering.

346

347   **Figure Captions**

348   **Figure 1**

349   **1A Reaction Scheme:** EDC and Sulfo-NHS activate the alginate forming a carboxylate carbon

350   intermediate. The carboxylate carbon intermediate is attacked by the primary amine nitrogen

351   forming the amide bond to the alginate backbone.

352   **1B NMR Spectra:** First spectra shows the alginate sample with broad peaks for a

353   polysaccharide, from 4.8 to 3.2, and distinct peak for the anomeric proton[59]. Second spectrum,

354   mixture of the alginate and peptide, shows both different peaks for alginate and high leucine

355   amino acids peaks. The third spectrum shows the modified alginate with KPLHALL where the

356   chemical shift in the leucine peaks and the height of the peaks differs from the mixture.

357 **1C SPR Spectra and Langmuir's Affinity Kinetics Model:** Representative examples of curve

358 fits for the affinity kinetic analysis of control (alginate) and modified alginate (KPLHALL

359 alginate). Concentrations of IGF-I for alginate varied from 3000 nM to 0 nM. Concentrations of

360 IGF-I for KPLHALL alginate varied from 1000 nM to 0 nM.

361 Langmuir's Affinity Kinetics Model for alginate is the top one (blue) and KPLHALL alginate is

362 on the bottom (red). K_D shifted 10 times, where K_D is 50 nM sin KPLHALL alginate (K_D of 50

363 nM) when compared to alginate (K_D of 513 nM). KPLHALL alginate had a k_{on} ranging from 1 to

364 $4(10^8/M\bullet sec)$ and a k_{off} from 5/s to 19/s (R^2: 0.40 to 0.90).The parameters for alginate k_{on} and

365 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).

366 **Figure 2**

367 **IGF-I Binding**

368 Immunohistochemistry of constructs for IGF-I at Day 30. Scale bar = 100 µm. Alginate does not

369 show differences in immunolocalization staining at day 30. Immunolocalization staining for IGF-

370 I changes as the concentration of binding sites (KPLHALL) increases in the constructs. The

371 constructs with 0 µM of binding sites barely show retention of f IGF-I; and constructs with 1 and

372 3 µM of binding sites show some retention of IGF-I. The highest immunolocalization of IGF-I is

373 at 100 µM.

374 **Figure 3**

375 **3A GAG matrix accumulation kinetic profiles:** The production of GAG increases in both

376 transfected and control groups. Control chondrocytes have smaller changes in kinetic profiles

377 between the differences in KPLHALL concentrations. pAAV/IGF-I transfected chondrocytes

378 have a greater effect (*$p<0.001$ by 2-way ANOVA) between the differences in KPLHALL

379 concentrations.

380 **3B HYPRO matrix accumulation kinetic profiles:** The production of HYPRO increases in

381 both pAAV/IGF-I transfected chondrocytes and control chondrocytes. The effect in

382 chondrocytes that are transfected with pAAV/IGF-I is greater than the effect on those transfected

383 with pAAV/ MCS (Empty) ($p < 0.001$ by 2-way ANOVA). This effect is greater in concentrations

384 of 10, 33 and 100 μM binding sites ($p < 0.001$ by 2-way ANOVA). When alginate is compared

385 with the highest concentration of binding sites (100 μM) there is an increase from 0.08 μg/mg at

386 0 uM to 1.4 μg/mg at 100 μM ($p < 0.001$ by 2-way ANOVA)in pAAV/IGF-I transfected

387 chondrocytes.

388 **Figure 4**

389 **4A GAG and HYPRO Dose Response:** GAG at the steady state concentration of each

390 concentration is plotted against the different concentrations of binding sites of KPLHALL

391 covalently attached to alginate. Transfected chondrocytes with pAAV/IGF-I produce 600%

392 GAG, than the control chondrocytes transfected with pAAV/ MCS (Empty) (*$p < 0.0001$ by

393 unpaired t test). pAAV/IGF-I transfected chondrocytes required 16.9 μM of binding sites in the

394 alginate to produce an increase in GAG of 6.9 (Table 2). Control chondrocytes required almost

395 twice the amount of KPLHALL modified alginate only to produce 60% (Table 2). *$p < 0.001$

396 when compared to min. +$p < 0.001$ when compared to max empty.

397 **4B Dose Response:** The production of HYPRO increases both in pAAV/IGF-I transfected

398 chondrocytes and control chondrocytes. The effect in chondrocytes that are transfected with

399 pAAV/IGF-I is greater than the effect in cells transfected with pAAV/ MCS (Empty).

400 pAAV/IGF-I increased HYPRO accumulation by 20 fold at the maximum effective

401 concentration of IGF-I binding sites($p < 0.02$ by unpaired t test). The parameters are summarized

18

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

403   when compared to max empty.

404   **Supplemental 1**

405   Negative Control for IGF-I IHC in 100μM KPLHALL Alginate with pAAV/IGF-I transfected

406   chondrocytes. Scale bar = 100 μm

407   **Supplemental 2**

408   Diffusion NMR was performed to see the stability of conjugation in the alginate; the NMR signal

409   of the leucines is present even as the gradient increases. In contrast to the mixture sample, the

410   leucine signals disappear as the gradient increases.

411   **Supplemental 3**

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

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

414   constructs.

415   **Supplemental 4**

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

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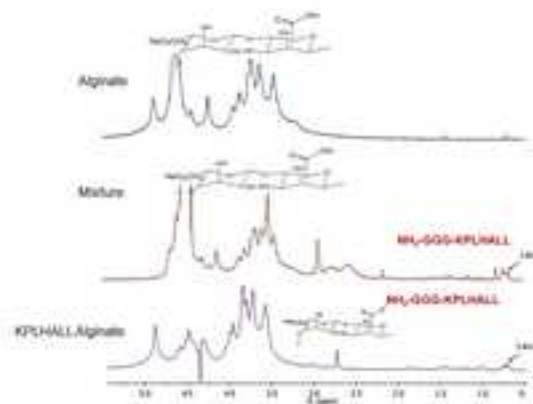
Figure 1
Click here to download high resolution image

A) Reaction Scheme

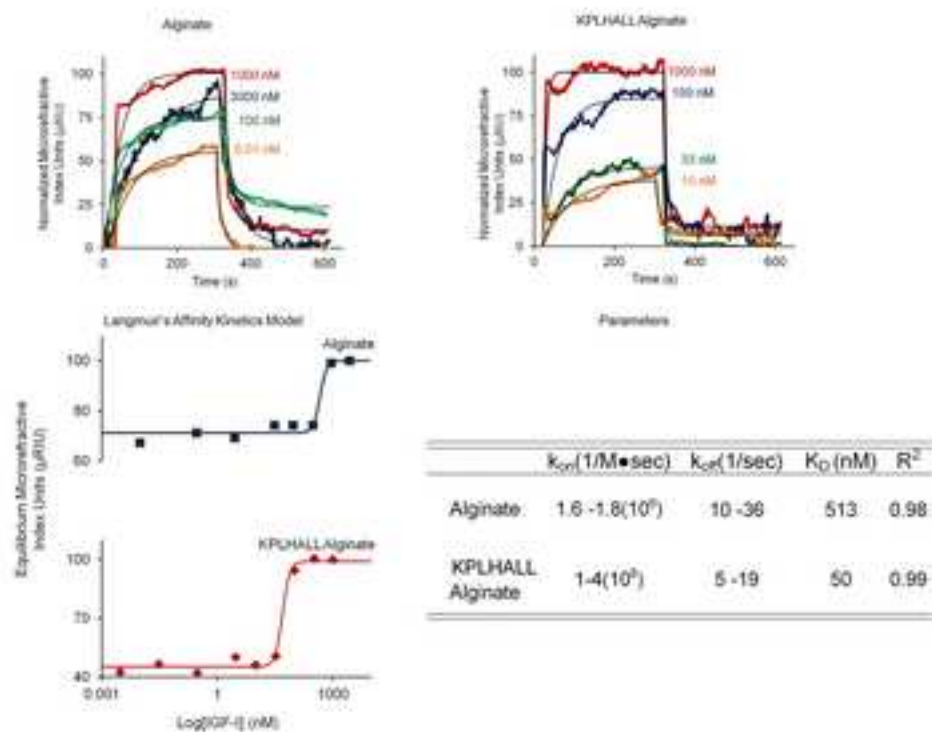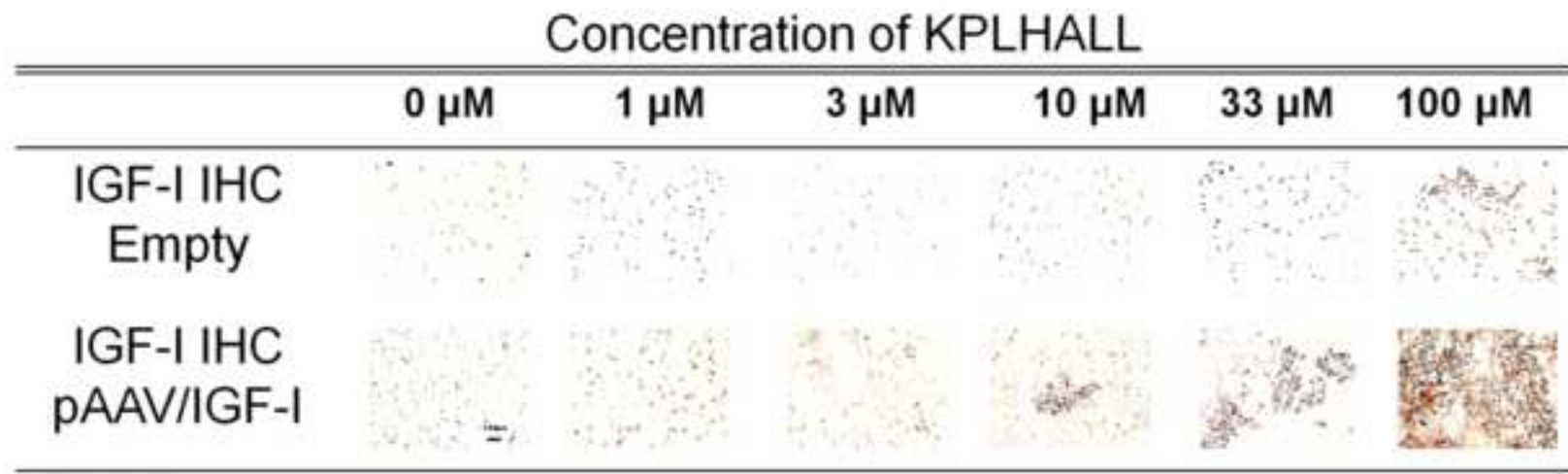B) 1H NMR Spectra

C) SPR Analysis

Concentration of KPLHALL
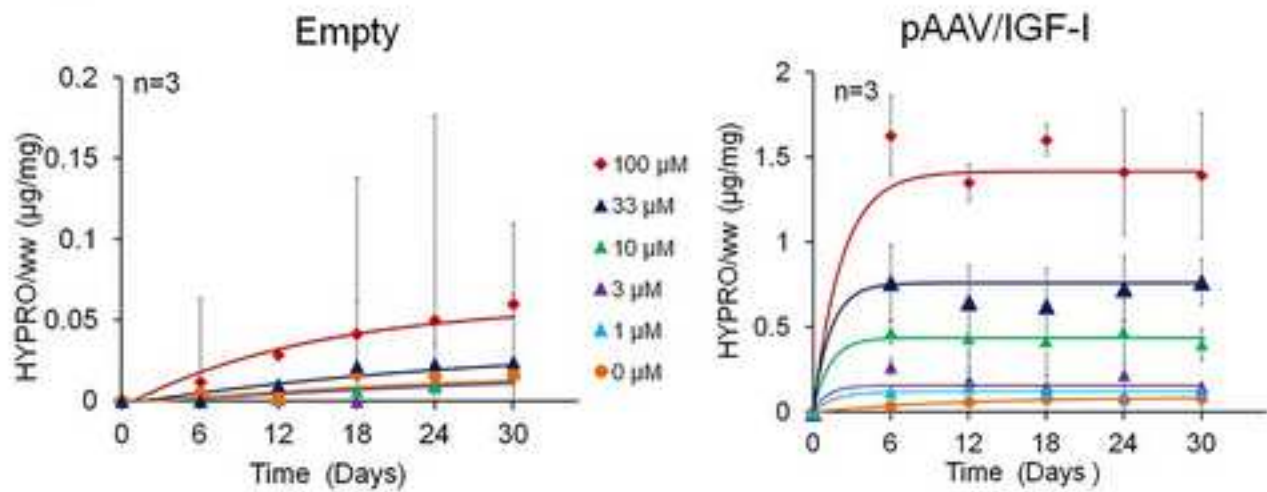
	0 μM	1 μM	3 μM	10 μM	33 μM	100 μM
IGF-I IHC Empty						
IGF-I IHC pAAV/IGF-I						

Matrix Synthesis

A) GAG

Empty — n=3, GAG/ww (μg/mg) vs Time (Days)

pAAV/IGF-I — n=3, GAG/ww (μg/mg) vs Time (Days)

Legend:
- 100 μM
- 33 μM
- 10 μM
- 3 μM
- 1 μM
- 0 μM

B) HYPRO

Empty — n=3, HYPRO/ww (μg/mg) vs Time (Days)

pAAV/IGF-I — n=3, HYPRO/ww (μg/mg) vs Time (Days)

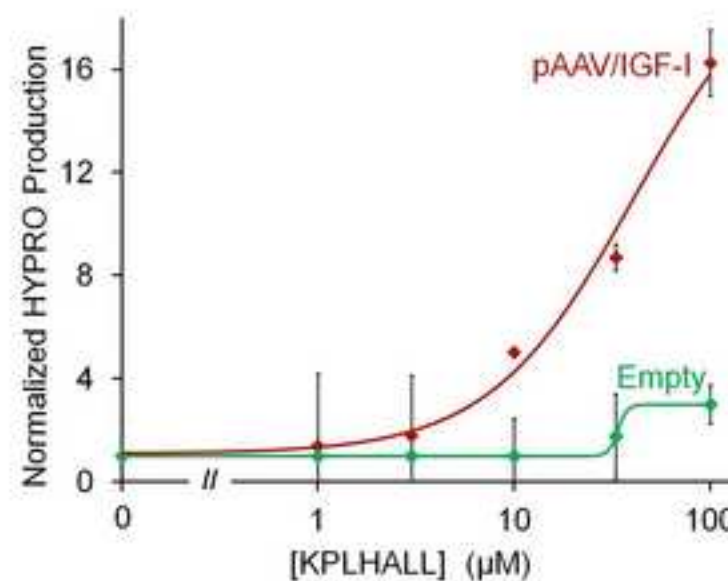Legend:
- 100 μM
- 33 μM
- 10 μM
- 3 μM
- 1 μM
- 0 μM
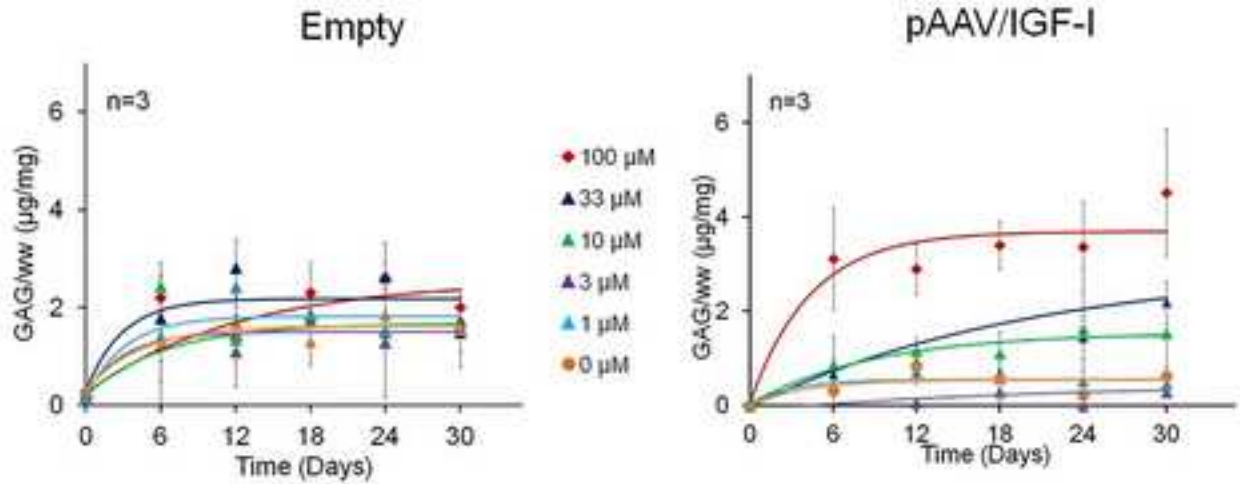
Figure 4
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A) Dose Response

(Left graph: Normalized GAG Production vs [KPLHALL] (µM), showing pAAV/IGF-I and Empty curves)

(Right graph: Normalized HYPRO Production vs [KPLHALL] (µM), showing pAAV/IGF-I and Empty curves)
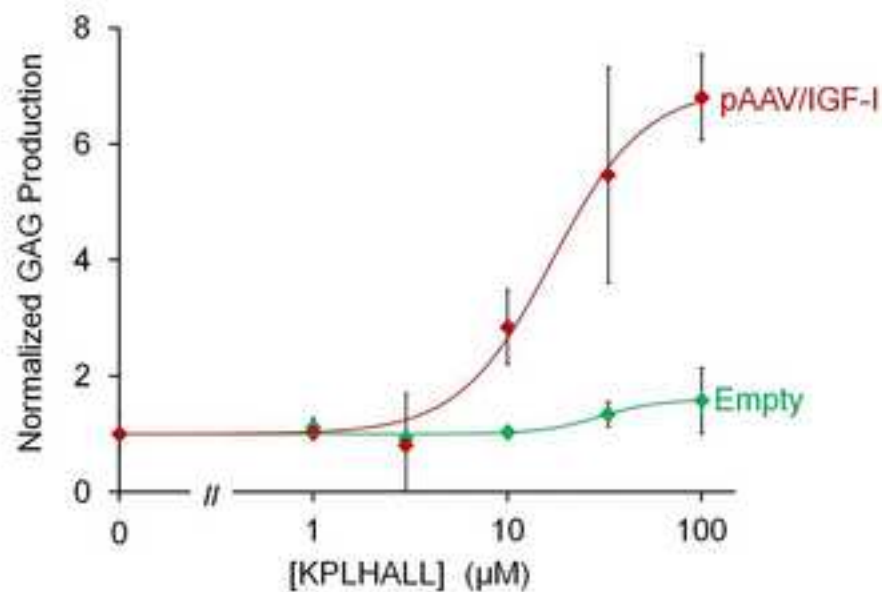
B) Dose Response Parameters

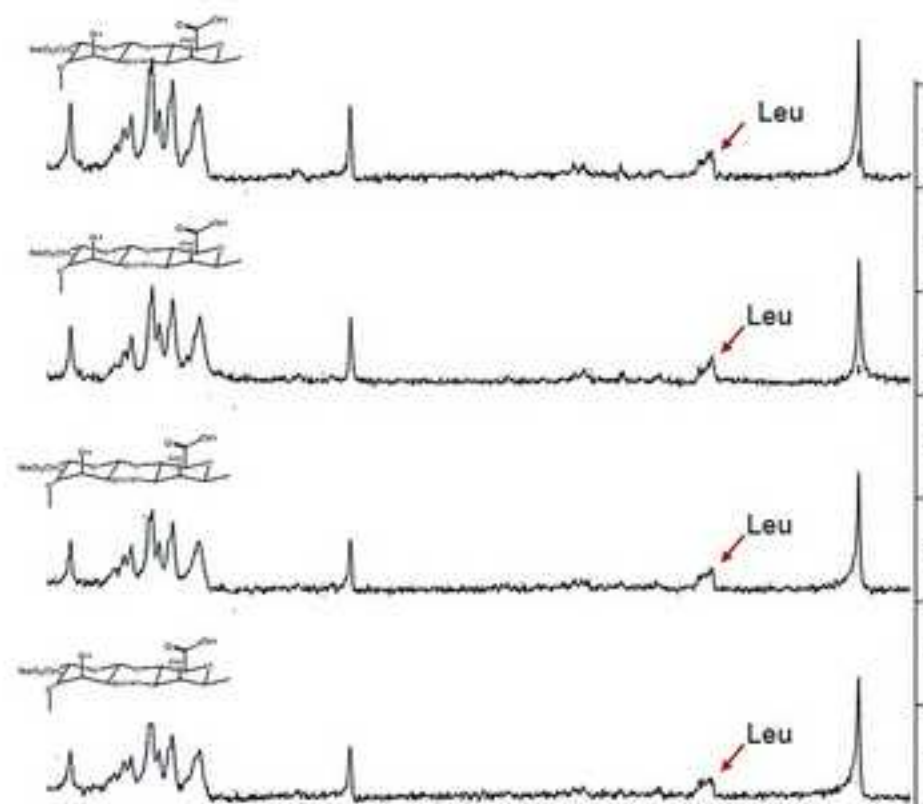|	GAG		HYPRO	
	pAAV/IGF-I	**Empty**	**pAAV/IGF-I**	**Empty**
EC_{50}(µM)	16.9±3.3	29.7± 9.2	41.0 ± 29.4	33.8±0.03
Max/Min	6.9±0.5 [*+]	1.6±0.1	19.4± 6.7[%^]	3.0 ± 0.1
R^2	0.99	0.93	0.99	0.99

100μm
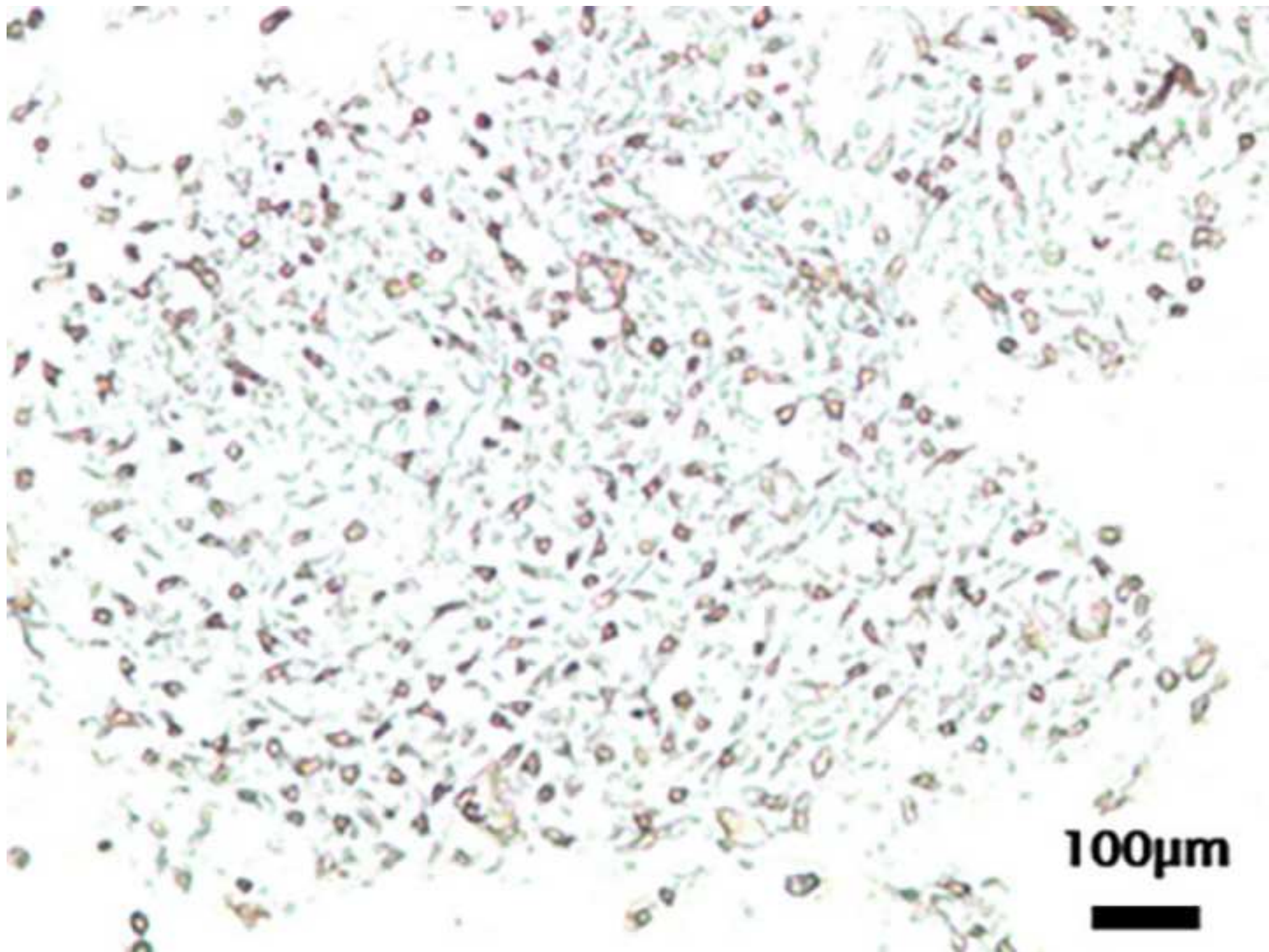
Diffusion Ordered Spectroscopy (DOSY)
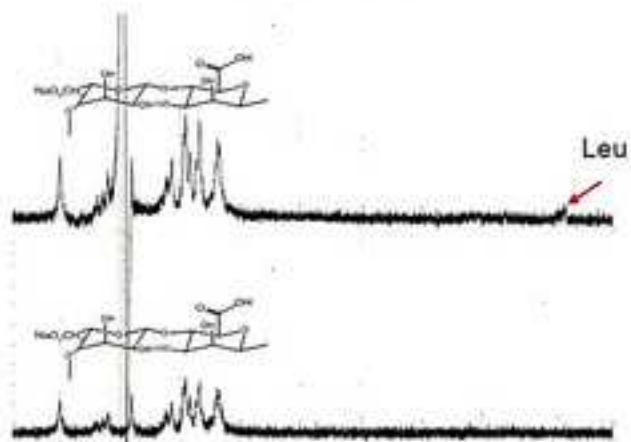
Mixture   KPLHALL Alginate

Concentration of KPLHALL

	0 μM	1 μM	3 μM	10 μM	33 μM	100 μM
Col II IHC pAAV/IGF-I						